International Journal of Current Research and Review
DOI: http://dx.doi.org/10.31782/IJCRR.2020.12209

Scopus

Research Article

IJCRR
Section: Healthcare
Sci. Journal Impact Factor: 6.1 (2018)
ICV: 90.90 (2018)



# Development of Cost Effective Regeneration Protocol for *Aerides multiflora* Roxb. and *Rhynchostylis retusa* (L.) Blume

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## ABSTRACT

**Objective:** Tissue culture technology is firmly footed from the stage of 'concept' to 'commercialization' and plays a pivotal role in the global trade of orchids. The large scale use ofthis technology is limited due to the high production cost of regenerants and efforts are needed to develop cost-effective protocols without compromising the quality of propagules.

**Methods:** More than 80 % of the production cost is attributed to the expensive medium components (carbon source, gelling agents, etc.). The present study reports a system for effective low-cost micropropagation of *Aerides multiflora* and *Rhynchostylis retusa* on medium formulated with alternatives to gelling agents, carbon and water source.

**Results:** Medium formulated with table sugar and isabgol as carbon source and gelling agent, and autoclaved tap water gave optimal results without compromising the plantlet quality and total cost of medium preparation declined from INR 87.24 to INR 26.02. Under field conditions, the survival rate of the *in vitro* raised plantlets in a potting mix of moss, charcoal and mango bark shavings (2:1:2) was ~80%.

**Conclusion:** 70% reduction in the total medium-cost was achieved by using low-cost substitutes which can easily replace sucrose and agar in the medium.

**Key Words:** Low cost, *in vitro*, Micropropagation, Isabgol, Table sugar, Acclimatization

## INTRODUCTION

Orchids are prized for their floral exquisiteness. The unique assemblage and superior shelf life makes them best suited for trade as cut flowers and potted plants, and they contribute to about 8% of the global floriculture industry[20]. Some orchids also possess therapeutic potential and are used in traditional medicine systems[23].

Difficulties in conventional propagation methods and unrestrained commercial exploitation have led to a rapid deterioration in the natural population of orchids. About 70% of the orchids are epiphytic and therefore are more exposed to the risk of extinction due to forest fires, clearing, etc.

In this scenario, tissue culture technology serves to conserve the natural populations, producing true to type clonal varieties[15] while aiding mass propagation. However, high production cost has limited its large scale application. Attempts have been made to overcome this challenge by employing low-cost alternatives to expensive medium components[9, 11, 21].

The present study aims at developing a cost-effective system for the mass propagation of two commercially valuable epiphytic orchid species endemic to Uttarakhand; *Aerides multiflora* and *Rhynchostylis retusa*. Both the species are used in the traditional system of medicine for the treatment of cuts and wounds, rheumatism, asthma, blood dysentery and malaria. There are published reports on mass multiplication protocols of both the species using different explants[14, 19].However, this is the first study where attempts have been directed to develop low-cost micropropagation protocol for these two orchid species.

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**ISSN:** 2231-2196 (Print) **ISSN:** 0975-5241 (Online)

**Received:** 25.06.2020 **Revised:** 24.08.2020 **Accepted:** 22.09.2020 **Published:** 27.10.2020

## MATERIALS AND METHODS

### Culture establishment

Immature capsules of *A. multiflora* and *R. retusa* were surface disinfected and seeds excised out for inoculation onto basal MS medium[1]. Protocorm-like bodies (PLBs) initiated within 10 weeks of culture. PLBs were multiplied on full strength MS medium containing coconut water (10%).

### Plantlet regeneration

PLBs of *A. multiflora* and *R. retusa* were utilized to assess the efficacy of cost-effective alternatives (carbon source, gelling agents and water source) for complete plantlet regeneration. Mitra medium[2] supplemented with BAP (4.44 µM) and NAA (5. 38 µM) for *A. multiflora*, whereas BAP (4.44 µM) alone was used in case of *R. retusa*. In the first set of experiments, different carbon sources like table sugar (local vendor), sugar cubes (Trust Classic), maple syrup (American Garden) and jaggery (Trust Sunehra) were incorporated in the regeneration medium (1% - 3%), and analytical grade sucrose (HiMedia) [2%] served as control. For screening of optimal low-cost gelling agents, the second set of experiments were conducted with Isabgol (Sat Isabgol) [1.5% - 3%], corn starch (Weikfield) [4 % - 6 %], and sago (Varalakshmi Sabudana) [4 % - 6%], and nutrient medium containing table sugar (concentration standardized in the first set of experiments) and gelled with agar (0.65%) was used as control. In the third set of experiments, alternate water sources *viz*., tap water, R.O. water (Kent RO system), packaged mineral water (Bisleri), and distilled water were used for medium preparation. Here, Mitra medium prepared with autoclaved millipore water, and fortified with table sugar and Isabgol (concentrations standardized in the previous experiments) acted as control.

In all experiments, pH of the media was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl and autoclaved at 1.05 kg $cm^{-2}$ pressure and at 121°C for 15 min. The cultures were maintained at 25±2°C under cool white fluorescent light at 40 µmol $m^{-2}$ $s^{-1}$ and 16/8 hr (light/dark) photoperiod and subcultured regularly.

### Hardening and acclimatization of plantlets

*In vitro* regenerated plantlets were hardened *in vitro* in hormone-free liquid MS medium. The plantlets were then transferred to plastic cups with different potting mix combinations and were subsequently shifted to polyhouse in clay pots with a potting mixture of moss, charcoal and bark shavings (2:1:2) and maintained under controlled conditions (temperature~25–30°C and RH~60–70%). Plant growth was monitored and the incidence of an infestation was checked periodically.

### Data analysis

All the treatments were performed in ten replicates and repeated thrice. Completely Randomized Design method was used for data collection which was analyzed using Microsoft Excel ver. 2007 © Microsoft Technologies, USA. One-way Analysis of Variance (ANOVA) was used for statistical analysis of mean number and length of shoot and root. Degree of variation was shown by Standard Error (SE), Critical Difference (CD) at 5%. The significance level was determined at 5% ($p <= 0.05$), 1% ($p <= 0.01$) and 0.1% ($p <= 0.001$). The significance of the data as ascertained by F-test and the CD values computed were used for comparing differences in means of various treatments.

## RESULTS AND DISCUSSIONS

The use of PLBs as explants for micropropagation of orchids is well established[18]. PLBs serve as somatic embryos with storage proteins and germinate efficiently under *in vitro* conditions leading to complete plantlet regeneration[17].

Tissue culture technology is effective to cater to the escalating demand of orchids. However, the high production cost is a limitation for its commercial exploitation[4]. Hence there is a pressing need to search and standardize cost-effective alternatives to improve its commercial viability. In the present study, it was estimated that analytical grade (HiMedia) agar (~INR 8000/kg) and sucrose (~INR 1000/kg) collectively contribute to more than 85% of the total media preparation cost (Fig II). The market price of growth medium (HiMedia) needed per litre is approximately INR 10.00, which amounts to only 13% of the total cost. In the present study, attempts were made to standardize a low-cost medium formulation, for mass propagation of two orchid species, *A. multiflora* and *R. retusa*.

In the first step, trials were focused on the selection of carbon source. The external supply of sugars is crucial for the development of *in vitro* cultures as they are not completely autotrophic. They serve as carbon source, energy source and osmotic agents. The role of sucrose in the tissue culture medium is well established[6]. Sucrose alone contributes to approximately 20% of the total media cost[7]. In our study, it was observed that inclusion of jaggery (2%) into the medium resulted in a mean number of 4.33±0.33 healthy leaves which were comparable to the results yielded with 2% sucrose. But root development in this medium was slow and stunted. Similar results, with the low rooting percentage in jaggery incorporated medium, have been reported earlier[12] . Least number of leaves (2.00±0.00) and roots (1.00±0.58) were observed in the medium with sugar cubes. These observations were in contrast to findings of studies which suggested sugar cubes as the most viable alternative to sucrose in tissue culture medium[8]. In our study, slow rate of plantlet development was

recorded on medium with maple syrup while addition of table sugar at the concentration of 2% in the medium resulted in well developed leaves and roots (Table I). Similar results were reported in *Dendrobium* where using local sugars the cost of production was reduced[11]. The results were in tandem with several previous reports that suggest the use of table sugar[8]. This could be attributed to the fact that table sugar is extracted from sugar cane which is known to possess natural sugar (15%). It also acts as a source of elements like calcium, magnesium and potassium which are essential for cell growth and development[10]. In our study, ~25% cost reduction was obtained by only replacing sucrose with table sugar.

Agar, the commonly used gelling agent, is widely accepted due to its inert and stable nature but accounts for 25% of the total media cost[16]. In the present study for both the species, medium gelled with isabgol (2%) proved to be the most efficient (Fig Ia,b,c,d). On this medium an average of 4.33±0.33 leaves were recorded, which was comparable to the results in agar gelled medium with no discernable difference in the quality of plantlets (Table II). The medium gelled with sago (5%) resulted in 3.33±0.33 leaves in both the species. In our study, medium gelled with corn starch was not found suitable due to improper gelling, and retarded growth rate. Our findings were similar to the previous studies[5] where isabgol resulted in superior plantlet quality in comparison to corn starch based medium. The *in vitro* growth may be affected by non-inert nature of starch and that it exerts osmotic stress by yielding sugars (on autoclaving). The use of Isabgol as an effective low-cost gelling agent has been reported in previous studies[22]. Factors like resistance to enzymatic activity, reasonable clarity, and reasonable price makes isabgol as an effective alternative.

In attempts to cut down the medium preparation cost by using water from cheaper sources, all the alternatives tried gave positive results, and the optimal growth was observed in autoclaved tap water (Table III). Tap water, free from any contaminants (heavy metals), can be easily substituted for distilled water bringing down the cost incurred in equipment purchase and maintenance. The successful use of tap water has been reported previously[3]. Stunted growth was observed in medium with bottled water as its mineral content leads to concentration levels that can be toxic for plant growth.

Overall, using Isabgol and table sugar in the medium resulted in cost reduction from INR 87.24 to INR 26.02. This amounted to a remarkable 70% reduction in the total cost of production (Fig III).

Plantlets with the well-developed shoot and root system were hardened under *in vitro* conditions. Proper hardening of the *in vitro* plantlets is a key factor for improving *ex vitro* establishment and overall survival rate[13]. The selected plantlets were successively shifted to liquid MS medium, with low nutrient concentration (½ X and ¼ X) and lacking any carbon source or plant growth regulator. Subsequently, after 4- 6 weeks, the plantlets were transferred into the potting mix of peat moss, charcoal and mango bark shavings (2:1:2) and maintained in cool and shady areas (Fig Ie,f,g). The pots were covered with perforated polybags and periodically watered to avoid dehydration. Approximately 80% of the transplants survived to form fully developed plants in both the species

## CONCLUSION

The multipurpose benefits and large scale application of orchids have rendered most species of the Orchidaceae family under Appendix II of the International Trade in Endangered Species of Fauna and Flora (CITES). This necessitates the development of technologies that can fulfil the market demand without hampering the natural populations in the wild. In this scenario, tissue culture technology plays an important role. This method facilitates the symbiotic seed germination further aiding complete plantlet regeneration and hybridization at a faster rate. Despite the advantages, tissue culture technology faces the problem of being capital intensive, which hampers its optimal and large scale usage. Efforts are therefore needed for the development of cost-effective protocols without affecting the quality of the regenerated plantlets. In the present study, a method was standardized for low-cost mass production of orchids. The study performed on two orchid species, *A. multiflora* and *R. retusa* confirmed that low-cost alternatives to carbon source and gelling agent in the tissue culture medium can significantly reduce the production cost by 70 %. Furthermore, investment cost can be lowered by using alternate water sources. This proves that the appropriate choice of medium components and culture conditions plays a massive role in the production cost of individual plantlets. The quality of regenerants produced on the low-cost medium used in this study was equivalent to the plant regenerated on the control medium using analytical grade sucrose and agar. Further attempts can be made towards reducing the cost of *ex vitro* establishment (hardening) of the *in vitro* regenerated plantlets.

As the orchid industry around the globe is majorly dependent on tissue culture generated plantlets, the package formulated and proposed in this study can be successfully utilized for commercial tissue culture production of orchid plants and help in controlling the price of end products for the buyers.

## ACKNOWLEDGEMENT

The authors would like to express their gratitude towards the Dean, Wildlife Institute of India, Dehradun for the identification of all samples and the Department of Biotechnology, Graphic Era (Deemed to be University) for providing the facilities.

**Conflict of Interest:** The authors have no conflicts to declare

**Funding Information:** NA

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**Table I: Effect of alternative carbon source on plantlet regeneration**

| Treatment | *Rhynchostylis retusa* | | | | *Aerides multiflora* | | | |
|---|---|---|---|---|---|---|---|---|
| Medium + agar + carbon source | Leaf Number | Leaf Length | Root Number | Root Length | Leaf Number | Leaf Length | Root Number | Root Length |
| Control (Sucrose, 2.0%) | 4.33±0.33 | 2.10±0.15 | 3.33±0.33 | 2.03±0.04 | 3.33±0.33 | 2.45±0.05 | 3.67±0.33 | 2.47±0.04 |
| Table sugar (1.0%) | 2.00± 0.00 | 0.84±0.05 | 1.33±0.33 | 0.30±0.02 | 2.00± 0.00 | 0.84±0.05 | 1.33±0.33 | 0.30±0.02 |
| Table sugar (2.0%) | 4.67±0.33 | 2.34±0.14 | 3.67±0.33 | 2.02±0.07 | 4.33±0.33 | 2.54±0.02 | 3.67±0.33 | 2.52±0.02 |
| Table sugar (3.0%) | 3.00± 0.00 | 1.07±0.06 | 1.67±0.33 | 0.58±0.29 | 2.67±0.33 | 1.80±0.03 | 1.67±0.33 | 1.17±0.06 |
| Sugar cubes (1.0%) | 1.67±0.33 | 0.91±0.04 | 1.67±0.33 | 0.38±0.02 | 2.00± 0.00 | 0.91±0.03 | 1.33±0.67 | 0.39±0.20 |
| Sugar cubes (2.0%) | 2.00± 0.00 | 0.95±0.03 | 1.00±0.58 | 0.56±0.03 | 2.33±0.33 | 0.88±0.02 | 1.67±0.33 | 0.64±0.04 |
| Sugar cubes (3.0%) | 2.67±0.33 | 0.93±0.04 | 2.00± 0.00 | 0.53±0.03 | 2.33±0.33 | 0.86±0.07 | 2.33±0.33 | 0.40±0.03 |
| Maple Syrup (1.0%) | 3.67±0.33 | 1.55±0.05 | 3.00± 0.00 | 1.45±0.06 | 3.33±0.33 | 1.59±0.01 | 2.67±0.33 | 1.48±0.02 |

**Table I: *(Continued)***

| Treatment | *Rhynchostylis retusa* | | | | *Aerides multiflora* | | | |
|---|---|---|---|---|---|---|---|---|
| Maple Syrup (2.0%) | 3.33±0.33 | 1.40±0.03 | 2.67±0.33 | 0.79±0.06 | 3.00± 0.00 | 1.17±0.08 | 1.33±0.67 | 0.39±0.20 |
| Maple Syrup (3.0%) | 3.00± 0.00 | 0.64±0.05 | 2.00± 0.00 | 0.94±0.02 | 2.33±0.33 | 0.48±0.01 | 1.67±0.33 | 0.57±0.02 |
| Jaggery (1.0%) | 2.33±0.33 | 0.78±0.04 | 1.00± 0.00 | 0.78±0.05 | 2.00± 0.00 | 0.72±0.02 | 1.33±0.33 | 0.50±0.03 |
| Jaggery (2.0%) | 4.33±0.33 | 1.98±0.05 | 2.67±0.33 | 1.63±0.02 | 4.00± 0.00 | 1.91±0.02 | 3.00 ± 0.00 | 1.68±0.02 |
| Jaggery (3.0%) | 3.00± 0.00 | 1.02±0.04 | 1.67±0.33 | 0.88±0.01 | 2.33±0.33 | 0.93±0.03 | 2.00 ± 0.00 | 0.79±0.02 |
| Significance | *** | *** | *** | *** | *** | *** | *** | *** |
| CD (5%) | 0.37 | 0.1 | 0.43 | 0.13 | 0.37 | 0.06 | 0.54 | 0.12 |

**Table II: Effect of alternative gelling agents on plantlet regeneration**

| Treatment | Rhychostylis retusa | | | | Aerides multiflora | | | |
|---|---|---|---|---|---|---|---|---|
| Medium+ table sugar + gelling agent | Leaf Number | Leaf Length | Root Number | Root Length | Leaf Number | Leaf Length | Root Number | Root Length |
| Control (Agar, 0.65%) | 4.67±0.33 | 2.34±0.14 | 3.67±0.33 | 2.02±0.07 | 4.33±0.33 | 2.54±0.02 | 3.67±0.33 | 2.52±0.02 |
| Isabgol (1.5 %) | 2.33±0.33 | 0.98±0.06 | 1.67±0.33 | 0.48±0.02 | 2.67±0.33 | 1.18±0.01 | 2.00 ± 0.00 | 0.67±0.02 |
| Isabgol (2.0 %) | 4.67±0.33 | 2.55±0.03 | 4.00± 0.00 | 2.20±0.05 | 4.67±0.33 | 2.68±0.02 | 4.00 ± 0.00 | 2.65±0.02 |
| Isabgol (3.0 %) | 1.67±0.33 | 0.74±0.03 | 0.67±0.33 | 0.18±0.09 | 2.67±0.33 | 0.88±0.02 | 2.00 ± 0.00 | 0.48±0.01 |
| CS (4.0 %) | 1.33±0.33 | 0.91±0.04 | 1.67±0.33 | 0.38±0.02 | 2.33±0.33 | 0.96±0.01 | 1.33±0.67 | 0.41±0.2 |
| CS (5.0 %) | 1.67±0.33 | 0.62±0.02 | 2.00± 0.00 | 0.64±0.02 | 2.67±0.33 | 0.85±0.02 | 2.33±0.33 | 0.77±0.02 |
| CS (6.0 %) | 3.00± 0.00 | 1.33±0.03 | 2.67±0.33 | 0.85±0.02 | 2.67±0.33 | 0.98±0.01 | 2.33±0.33 | 0.64±0.01 |
| Sago (4.0 %) | 2.00± 0.00 | 0.88±0.01 | 1.33±0.33 | 0.56±0.01 | 2.00± 0.00 | 0.50±0.01 | 1.33±0.67 | 0.25±0.13 |
| Sago (5.0 %) | 3.33±0.33 | 1.10±0.05 | 2.33±0.33 | 0.85±0.03 | 3.33±0.33 | 1.89±0.02 | 2.67±0.33 | 1.18±0.01 |
| Sago (6.0 %) | 1.33±0.33 | 0.38±0.02 | 1.00±0.58 | 0.25±0.13 | 2.00± 0.00 | 0.68±0.01 | 1.67±0.33 | 0.54±0.01 |
| Significance | *** | *** | *** | *** | *** | *** | *** | *** |
| CD (5%) | 0.43 | 0.08 | 0.45 | 0.08 | 0.43 | 0.02 | 0.56 | 0.13 |

**Table III: Effect of alternate water sources on plantlet regeneration**

| Treatment | Rhychostylis retusa | | | | Aerides multiflora | | | |
|---|---|---|---|---|---|---|---|---|
| Medium+table sugar + isabgol | Leaf Number | Leaf Length | Root Number | Root Length | Leaf Number | Leaf Length | Root Number | Root Length |
| Control (Millipore) | 4.67±0.33 | 2.55±0.03 | 4.00 ± 0.00 | 2.20±0.05 | 4.67±0.33 | 2.68±0.02 | 4.00 ± 0.00 | 2.65±0.02 |
| Distilled Water | 4.00±0.00 | 2.00±0.01 | 3.67±0.33 | 2.05±0.03 | 3.67±0.33 | 2.23±0.03 | 3.00 ± 0.00 | 1.98±0.01 |
| Tap Water | 4.67±0.33 | 2.65±0.01 | 4.33±0.33 | 2.26±0.04 | 5.00 ± 0.00 | 3.01±0.05 | 4.33±0.33 | 3.03±0.03 |
| RO Water | 3.33±0.33 | 1.65±0.03 | 2.67±0.33 | 1.06±0.03 | 3.67±0.33 | 1.98±0.01 | 2.67±0.33 | 1.30±0.01 |
| Mineral Water | 3.67±0.33 | 1.97±0.02 | 2.67±0.33 | 1.58±0.02 | 3.33±0.33 | 1.69±0.01 | 2.33±0.33 | 1.30±0.01 |
| Significance | *** | *** | *** | *** | *** | *** | *** | *** |
| CD (5%) | 0.42 | 0.03 | 0.42 | 0.05 | 0.42 | 0.04 | 0.37 | 0.03 |


**Figure I:** Low cost micropropagation of *Aerides multiflora* and *Rhyncostylis retusa* (a) A. multiflora regeneration on medium containing sucrose and agar (b) *A. multiflora* regeneration on medium contraining table sugar and isabgol (c) *R. retusa* regeneration on medium containing sucrose and agar (d) *R. retusa* regeneration on medium containing table sugar and isabgol (e,f,g) Hardening of *in vitro* raised plantlets.

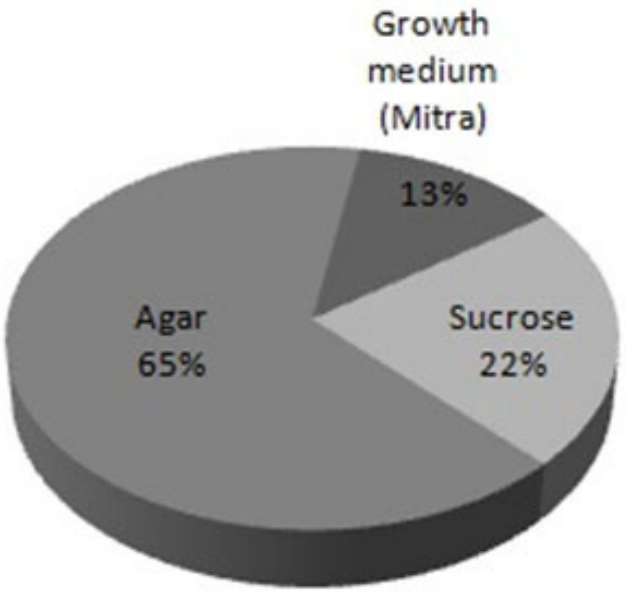


**Figure II:** Cost contribution (per cent) of different media components on total cost (per litre medium).

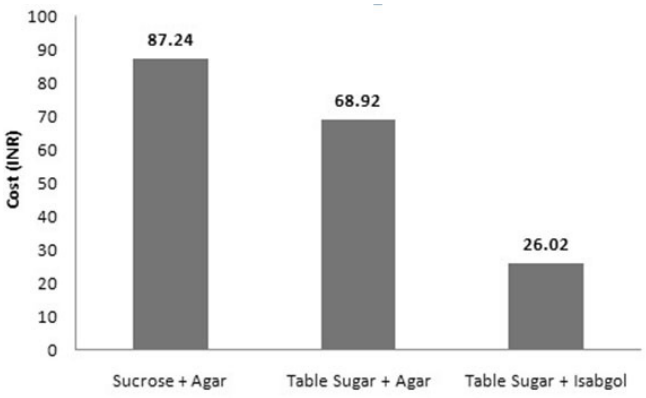


**Figure III**: Effect of carbon source and gelling agent on cost (per litre) of Mitra medium.