RESEARCH ARTICLE Indian Journal of Agricultural Research, Volume 59 Issue 11: 1656-1661 (November 2025)

Effect of Plant Growth Regulators and Growth Additives on the *In vitro* Micropropagation of *Aerides odorata* Var. Alba from Immature Pod Seeds

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10.18805/IJARe.A-6326

ABSTRACT

Background: The propagation of *Aerides odorata* through sexual means is a very slow process as its seeds lack endosperm and propagation through *in vitro* culture techniques from vegetative parts used as explants may results in the loss of diversity and variation in the species, therefore it is important to take the initiative for its mass propagation from seeds, followed by their subsequent establishment in its natural habitat.

Methods: The investigation was conducted at Central Agriculture University, Imphal, Manipur. The seeds were inoculated on ½ MS (Murashige and Skoog) media augmented with different concentrations of BAP and coconut water to enhance seed germination. Different concentrations of NAA (2.0 mg/L) and BAP (0.1-4.0 mg/L) were added to ½ MS medium for shoot induction. The regenerated multiple shoots were then excised and single shoots were cultured individually on ½ MS media supplemented with various growth regulators *viz*., NAA, IBA, IAA and growth additive, banana powder (50 g/L).

Result: The earliest seed initiation (25.5 days) and maximum seed germination rate (72.42%) were recorded with ½ MS media supplemented with 2.0 mg/L BAP and 15% Coconut water. Whereas, shortest duration taken for shoot initiation (17.17 days) was observed with ½ MS treated with 4.0 mg/L BAP and 2.0 mg/L NAA. Highest number of leaves (10) and highest number of shoots (6.58) were observed with ½ MS supplied with 4.0 mg/L BAP and 2.0 mg/L NAA. The treatment combinations of ½ MS supplemented with 0.5 mg/L NAA and 50 g/L Banana powder recorded the lowest number of days taken for root initiation (31.17 days), highest number of roots (5) and maximum root length (2.64 cm).

Key words: Growth additives, Micropropagation, Orchid, PGR.

INTRODUCTION

North-eastern Indian state of Manipur is renowned for having the fourth-highest orchid diversity in the world (Medhi and Chakrabarti, 2009). Among the 251 species of wild orchids identified in Manipur, *Aerides* is a valuable commercial medicinal orchid (Paraste *et al*., 2023). *A. odorata* is a wild species of Thailand, an epiphytic orchid with monopodial growth habit. It is collectively referred to as a Fox-tail orchid because of its flowered bush-like inflorescence (Hongthongkham and Bunnag, 2014). The flowers of *A. odorata* Lour. starts blooming usually in May-July (Sangma *et al*., 2021). It has a pleasant odour and a white blossom with a pinkish tinge (Al-Faruque *et al*., 2015). Their scent has made them a valuable source for the scent extraction and production of numerous artificial hybrids and cultivars. They may be used in the perfumery and pharmaceutical industries. The leaves of *A. odorata* Lour. also has antibacterial properties. It has been shown to possess alkaloids and other naturally occurring chemicals with various antibacterial, antioxidant and anticancer activities (Paraste *et al*., 2023). The fruit of this orchid is mainly use for the treating of wounds by traditional healers of Manipur, while the juice from the fleshy leaves of this orchid is used for healing of boils in nose and ear. Due to its various medicinal uses, there is an indiscriminate wild collection and illegal trade by the people which has led to the loss of its biodiversity and urges to initiate various conservation methods to prevent this orchid from becoming extinct. But one of the challenges in conserving this species is their prolonged and complex propagation process. The propagation of this species through sexual means is a very slow process as its seeds lack endosperm. The size of orchid seeds can vary significantly (Akhila *et al*., 2024)

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How to cite this article: Thiyam, S., Uchoi, A., Singh, S.R., Sharma, G.R., Raman, T., Stina, K. and Khangjarakpam, G. (2025). Effect of Plant Growth Regulators and Growth Additives on the *In vitro* Micropropagation of *Aerides odorata* Var. Alba from Immature Pod Seeds. *Indian Journal of Agricultural Research*. **59(11):** 1656-1661. doi: 10.18805/IJARe.A-6326.

Submitted: 22-10-2024 **Accepted:** 08-04-2025 **Online:** 26-05-2025

and need fungal stimulant for germination in nature to provide the necessary physiochemical stimuli for seed germination and development (Lal *et al*., 2020). The fungus is believed to augment the carbohydrate, auxin and vitamin transport in the orchid (Arditti *et al*., 1982). Hence, the aims of the present study were to investigate the suitable medium for seed germination and the suitable concentration of plant growth regulators to establish plantlets from *A. odorata* seeds *via in vitro* culture.

MATERIALS AND METHODS

Preparation of explant

The immature and un-dehisced pods of *A. odorata* var. Alba was acquired from M/s Kwaklei and Khonggunmelei Orchids Private Limited, Imphal, Manipur, during July 2021. The research investigation was conducted in the Tissue Culture laboratory at the Department of Genetics and Plant Breeding, Central Agriculture University (CAU), Imphal, Manipur. The orchid pods were soaked with Tween 20 (0.01%) in a shaker for 30 to 40 min and washed vigorously for 15 to 20 min under running water. Inside the Laminar airflow hood, they were dipped in 70% ethyl alcohol for 30 sec, flamed and sterilized for 8 min within a solution containing Mercuric chloride ($HgCl_2$) (0.1%) and 1 to 2 drops of tween 20. They were rinsed with sterilized distilled water and the pods were treated with Streptomycin (0.02%) and Bavistin (0.01%) solutions for 6 to 7 min each. After that, they were repeatedly washed 5 to 6 times with sterilized distilled water to eliminate all sterilizing agent remnants. Lastly, the sterilized capsules were sliced open longitudinally with a sterilized blade in an aseptic setting inside a laminar airflow cabinet to expose the immature seeds.

Seed viability test

Tetrazolium chloride was used to determine whether the seeds were viable. After soaking in sterile water for 24 h, the seeds were treated with a final application of 1% (w/v) solution of 2, 3 and 5 triphenyl tetrazolium chloride (TTC) (HiMedia, Mumbai, India) before being immersed in a solution of Calcium oxy-chloride [$Ca(OCl_2$], 5% (W/V) and Tween 20, 1% (V/V). The Petri dish was immediately placed in a dark room for around 12 hours and the seeds were examined in a microscopic field. The non-viable embryos remained colourless, whereas the viable ones became red.

In vitro seed germination

The seeds were inoculated on ½ MS media augmented with different concentrations of BAP *viz*., 0.1, 0.5, 1.0 mg/L (HiMedia, Mumbai, India) and 15% coconut water (HiMedia, Mumbai, India) to enhance seed germination and subsequent development. Then, 3% sucrose (HiMedia, Mumbai, India) was added and gelled by using 0.8% (w/v) agar. The pH of the medium was adjusted to 5.8±0.2 either with 0.1 N NaOH or HCl before autoclaving at 121°C and 151 psi for 15 to 20 min. The cultures were kept at a temperature of 25±2°C and exposed to 16 h, using fluorescent tubes (40 w Philips India Ltd., Mumbai) by illuminating 3500 lux intensity of light. In each treatment, 100 seeds were cultured in culture phytajars (200 ml, HiMedia, Mumbai, India) filled with 50 ml of the medium. All treatments were triplicated with 4 culture phytajars in each replication.

The rate of germination was estimated using the formula:

Rate of germination (%) =

$$\frac{\text{Number of seeds forming spherule}}{\text{Total number of seeda inoculated}} \times 100$$

The observations were recorded accordingly.

In vitro shoot formation from seed using different plant growth regulators (PGRs)

Different concentrations of NAA (2.0 mg/L) and BAP (0.1-4.0 mg/L) were added to ½ MS medium for shoot induction. After autoclaving, the media was maintained at 25±2°C temperature and exposed to 16 hours of normal light followed by 8 hours of dark. The observations were recorded for 15 days until the 60th day after inoculation.

In vitro rooting of regenerated shoots using growth regulator and additives

The shoots obtained from the seed germination were used for inducing roots. The regenerated multiple shoots were excised and single shoots particularly longer than 1 cm with 1-2 leaves were cultured individually on ½ MS media supplemented with various growth regulators *viz.,* NAA, IBA, IAA (0.5 mg/L) and growth additive, banana powder (50 g/L). The cultures were maintained in the culture room under the same conditions as done above. The observation was recorded at regular intervals accordingly.

Statistical analysis

The experiment was conducted in completely randomized design (CRD). The significance and non-significance of the variance due to different treatments were determined by calculating the respective 'F' values as the method described by Gomez and Gomez (2010).

RESULTS AND DISCUSSION

In vitro seed germination

The effects of different concentrations of PGRs and growth additives on orchid seed germination indicated a positive response. Out of the eight treatment combinations, higher seed germination was observed with T_7 which involved a medium of ½ MS augmented with 2.0 mg/L BAP and 15% Coconut water. The swelling of the seed was the first visible change observed in the explants followed by the development of a swollen spherical green corm-like embryo *i.e.,* Protocorm-like body (PLB) (Fig 1) which are structurally similar to protocorms and arise from the explants (Gantait *et al*., 2020). PLBs initially broke through the fine membranous seed covering and developed a fine layer of rhizoids during the typical germination process which eventually developed into a seedling. Seed germination was noticed within just

25.50 days of inoculation in T_7. Meanwhile, a delay in seed germination was observed in T_1 (43.42 days). These differences in the rate of germination clearly illustrate the efficiency of the different treatment combinations. These findings corroborate several prior studies such as those of Prarab and Krishnan (2012) in *Aerides maculosa* Lindl. and *Rhynchostylis retusa* (L.) Bl; Vijayakumar *et al*. (2012) in *Dendrobium aggregatum* Roxb. and Piri *et al*. (2013) in *Acampe papillosa* (Lindl.) Lindl where they also observed accelerated germination rates in response to specific growth additives like 15% coconut water. Coconut water is renowned for being a rich source of essential minerals, amino acids, sugars, antioxidants, organic acids and most importantly, growth regulators like cytokinins which significantly spur *in vitro* orchid growth (Yong *et al*., 2009). Their role in seed germination is crucial as they enable critical processes such as cell division, thereby expediting the onset of germination.

Furthermore, the highest rate of germination was spotted in T_7 (Table 1), where 72.42% of the seeds germinated after the 60th day of inoculation. It was substantially larger than the control group, where only 20.50% of the seeds had achieved germination. Introducing PGRs to the media improves orchid seed germination and seedling growth. According to Dohling *et al*. (2010), BAP additions increased the frequency of *Dendrobium formosum* Roxb. ex Lndl germination. The stimulatory impact of BAP on protocorm multiplication and shoot formation in *Cymbidium pendulum* (Roxb.) was also documented in the investigations carried out by Pathak *et al*. (2001). Growth may be boosted by combining PGR and growth additive, however, the effects of these mixtures depend on the type

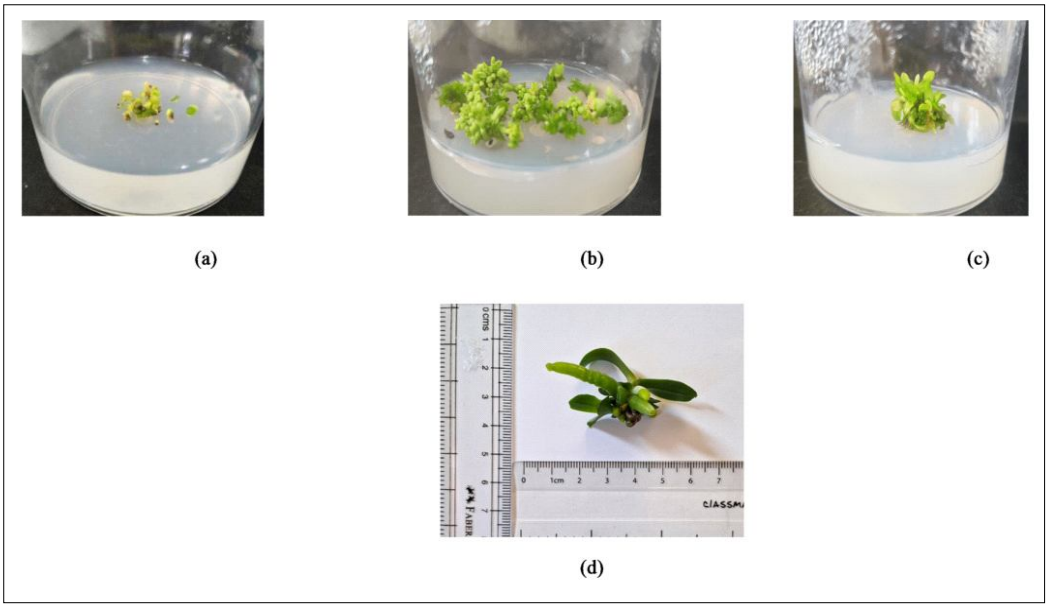


Fig 1: (a) Seed germination from immature pod, (b) PLBs formation from germinated seed, (c) Initiation of shoots from PLB after 17 days of inoculation, (d) Root proliferation from shoot after 31 days of inoculation.

Table 1: Effect of PGRs and growth additives on seed germination of spores under 16 h normal light and 8 h dark.

Treatment	Days taken for germination	Rate of germination			
		15 DAI	30 DAI*	45 DAI*	60 DAI*
T_1: ½ MS (CONTROL)	43.42	0.00	0.00 (0.29)	14.50 (22.38)	20.50 (26.92)
T_2 : ½ MS + 15% coconut water	39.08	0.00	0.00 (0.29)	25.33 (30.22)	33.08 (35.11)
T_3: ½ MS + 1.0 mg/L BAP	40.50	0.00	0.00 (0.29)	17.25 (24.51)	24.17 (29.42)
T_4: ½ MS + 2.0 mg/L BAP	27.42	0.00	4.25 (11.89)	39.00 (38.64)	58.67 (50.00)
T_5: ½ MS + 4.0 mg/L BAP	39.00	0.00	0.00 (0.29)	30.25 (33.37)	45.25 (42.27)
T_6: ½ MS + 1.0 mg/L BAP + 15% coconut water	29.50	0.00	2.50 (9.07)	44.17 (41.65)	54.67 (47.68)
T_7: ½ MS + 2.0 mg/L BAP + 15% coconut water	25.50	0.00	5.17 (13.12)	60.58 (51.11)	72.42 (58.35)
T_8: ½ MS + 4.0 mg/L BAP + 15% coconut water	29.42	0.00	0.83 (4.35)	52.83 (46.62)	64.58 (53.49)
C.D. at 5%	2.55	0.00	2.35	1.59	2.81

BAP: Benzyl amino purine, MS: Murashige and skoog, DAI: Days after inoculation.

*The figures are in arc sine transformation.

Table 2: Effect of PGRs on shooting under 16 h normal light and 8 h dark.

Treatment	Days taken for shoot initiation	Number of shoots per explant				Shoot length (cm)				Number of leaves per explant			
		15 DAI	30 DAI*	45 DAI	60 DAI	15 DAI	30 DAI	45 DAI	60 DAI	15 DAI	30 DAI*	45 DAI	60 DAI
T_1: ½ MS (CONTROL)	28.83	0.00	0.67 (0.22)	1.58	2.58	0.00	0.32	0.67	0.88	0.00	0.10 (0.03)	2.00	4.17
T_2 : ½ MS + 0.1 mg/L BAP + 2.0 mg/L NAA	28.25	0.00	0.83 (0.25)	1.75	2.75	0.00	0.38	0.74	1.07	0.00	0.20 (0.06)	2.33	5.25
T_3: ½ MS + 0.5 mg/L BAP + 2.0 mg/L NAA	27.92	0.00	1.08 (0.32)	2.00	3.00	0.00	0.43	0.81	1.18	0.00	0.80 (0.26)	2.50	6.67
T_4: ½ MS + 1.0 mg/L BAP + 2.0 mg/L NAA	26.50	0.00	1.25 (0.34)	2.33	3.67	0.00	0.50	1.10	1.38	0.00	1.10 (0.32)	3.00	7.50
T_5: ½ MS + 2.0 mg/L BAP + 2.0 mg/L NAA	24.25	0.00	1.33 (0.36)	3.08	4.75	0.00	0.83	1.24	1.65	0.00	1.40 (0.37)	3.50	8.17
T_6: ½ MS + 3.0 mg/L BAP + 2.0 mg/L NAA	21.33	0.00	1.75 (0.43)	4.00	5.08	0.00	0.69	1.31	1.70	0.00	2.00 (0.47)	4.42	9.25
T_7: ½ MS + 4.0 mg/L BAP + 2.0 mg/L NAA	17.17	0.00	2.25 (0.51)	5.00	6.58	0.00	0.97	1.72	2.18	0.00	3.10 (0.61)	6.67	10.00
C.D. at 5%	1.69	0.00	0.14	0.91	0.85	0.00	0.22	0.30	0.33	0.00	0.12	0.79	0.76

*The figures are $\log_{10}$ (x+1) transformation.

of orchid and the growth regulators that are employed, as well as their combination and ratios.

In vitro shoot formation using different PGRs

In the present study, different concentrations of PGRs *viz.,* BAP and NAA in six combinations and a Control used for studying shoot initiation revealed that with increasing BAP and NAA concentrations, the frequency of shoot regeneration and the total number of shoots per explant increased (Table 2). Earliest shoot initiation (17.17 days), highest number of shoots (6.58), longest shoot (2.18 cm) and highest number of leaves per explant (10.00) were observed at ½ MS media supplemented with high concentrations of BAP (4.0 mg/L) and NAA (2.0 mg/L). PGRs play a vital role in regenerating cell differentiation, the combined treatment of BAP and NAA is found to have a positive effect on shoot growth and multiplication as reported in *A. multiflora* Roxb. by Bhowmik and Rahman (2020) and Devi *et al.* (2013) in *A. odorata* Lour. The physiological process of interaction between both hormones might have a synergistic effect on the shoot initiation and growth, cytokinin was found to proliferate embryogenesis but NAA inhibits the process Regmi *et al*. (2017) in *Cymbidium aliofolium*. Thus, the above findings suggest that the combination of high cytokinin: auxin ratio, has proved more effective for shooting.

In vitro rooting using different rooting hormones and growth additives from derived shoots

The quantity of roots and the length of the roots after four weeks of culture varied across all the treatments. The interaction between PGRs and growth additive (banana powder) showed significant results in root development (Table 3) irrespective of treatments. The root initiation was recorded earliest (31.17 days) in T_3 (½ MS + 0.5 mg/L NAA + 50 g/L Banana powder). While, the maximum days taken for root initiation (43.83 days) were observed with T_1. Moreover, the maximum number of roots (5.00) and length of root (2.64 cm) were also recorded in the same treatment *viz*., T_3 and the least was recorded with T_1. The findings are in line with Devi *et al.* (2013) who found that NAA (auxin) additions improved the rooting of *A. odorata* Lour. Moreover, the interaction of PGRs (auxin) with Growth additives like banana powder might have given a synergistic effect to form roots in the current study. The usage of banana powder might have helped to strengthen plantlets and develop roots. According to the variety, the chemical makeup of banana flour, banana powder, banana extract, or banana homogenate varies between 61-76.5% starch, 19-23% amylose, 2.5-3.3% protein and 0.3-0.8% lipids (Mota *et al*., 2000). The banana powder contains maltodextrose, which offers extra carbohydrates without having osmotic effects which boost root development. The findings were in agreement with Decruse *et al*. (2003) in *Vanda spathulata* and Alam *et al.* (2010) in *V. teres* where they concluded that a combination of auxin and banana powder obtained maximum rooting and plantlet formation. Further,

Table 3: Effect of PGRs on rooting of derived shoots under 16 h normal light and 8 h dark.

Treatment	Days takan for root initiation	Number of roots per explant				Root length (cm)			
		15 DAI	15 DAI	15 DAI	15 DAI	15 DAI	15 DAI	15 DAI	15 DAI
T_1: ½ MS (CONTROL)	43.83	0.00	0.00	0.67 (0.21)	1.25	0.00	0.00	0.22	0.52
T_2: ½ MS + 0.5 mg/L NAA	35.08	0.00	0.00	2.17 (0.50)	4.25	0.00	0.00	1.05	2.10
T_3: ½ MS + 0.5 mg/L NAA + 50 g/L Banana powder	31.17	0.00	0.00	3.00 (0.60)	5.00	0.00	0.00	1.54	2.64
T_4: ½ MS + 0.5 mg/L IBA	41.58	0.00	0.00	2.08 (0.49)	2.83	0.00	0.00	0.68	1.42
T_5: ½ MS + 0.5 mg/L IBA + 50 g/L Banana powder	37.08	0.00	0.00	2.33 (0.52)	3.67	0.00	0.00	0.87	1.72
T_6: ½ MS + 0.5 mg/L IAA	42.67	0.00	0.00	1.00 (0.30)	1.83	0.00	0.00	1.08	1.84
T_7: ½ MS + 0.5 mg/L IAA + 50 g/L Banana powder	39.33	0.00	0.00	1.92 (0.46)	3.00	0.00	0.00	1.31	2.32
C.D. at 5%	3.34	0.00	0.00	0.10	0.60	0.00	0.00	0.24	0.75

*The figures are $\log_{10}$ (x+1) transformation.

Naing *et al*. (2011) in *Coelogyne cristata* also reported maximum rooting in ½ MS supplemented with auxin and Banana Powder.

CONCLUSION

Aerides odorata is one of the important and endemic orchids with great medicinal significance in Manipur. This developed *in vitro* immature seed culture protocol will offer a beneficial method for large-scale production of this orchid. The present study will hold significant potential for both commercial applications in the cut-flower industry and conservation endeavours aimed at restoring this orchid. Additional studies could examine the potential for scaling up this technology to produce large quantities of high-quality, disease-free planting material and to use it with more commercially significant orchid species.

ACKNOWLEDGEMENT

The authors appreciate the facilities presented by the Central Agriculture University, Imphal, Manipur, India and are also very thankful to M/s Kwaklei and Khonggunmelei Orchids Private Limited, Imphal, Manipur for providing the un-dehisced pods of *Aerides odorata* var. Alba.

Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript. Informed consent all animal procedures for experiments were approved by the Committee of Experimental Animal Care and Handling Techniques were approved by the University of Animal Care Committee.

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