Research Article

In vitro Regeneration Responses of Dendrobium Orchids to Different Plant Growth Regulators

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Abstract | Plant growth and development are regulated by growth regulators. Cytokinin and auxin are crucial for *in vitro* propagation and act either synergistically or antagonistically to regulate several key developmental processes, such as callogenesis, embryogenesis, and organogenesis in plants. This study highlights an efficient protocol for rapid *in vitro* multiplication of Dendrobium orchids using leaf disc culture with different auxin–cytokinin interactions. Young growing leaves of Dendrobium orchids were inoculated on a modified Murashige and Skoog (1962) medium (MS-medium) supplemented with different auxins: Naphthalene acetic acid (NAA), indole-3-butyric acid (IBA), and cytokinin 6-Benzyleaminopurine (BAP) in varying concentrations. Early protocorm-like bodies (PLBs) formation and more callus growth were observed in MS medium modified with 2.0+2.0 (mg/L) NAA+BAP. Better shoot multiplication (10.6) and somatic embryo formation (21.6) were observed in MS-medium supplemented with 2.0+1.0 and 2.0+1.5 (mg/L) of NAA+BAP. Similarly, greater shoot length (4.64cm), number of roots (2.22), and a higher rate of embryo development were observed in MS-medium modified with 1.5+2.0 (mg/L) of NAA+BAP. The optimized media will be useful for efficient regeneration and micropropagation of Orchids.

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Introduction

Orchids are ecologically well adapted to various habitats on the earth (Ramya *et al*., 2020). Orchidaceae is one of the largest and most diverse families of flowering plants with more than 28,000 species classified around the different climatic zones of the world (Christenhusz *et al*., 2016). Although orchids are absent in polar and desert regions, they are abundant in the wet tropical regions, which divide them into two major categories, i.e., terrestrial and epiphytic orchids (Ogliore, 2024; Pérez-Escobar *et al*., 2024). Many of the orchid species are only locally distributed, whereas many wild species have

become endangered (Ogliore, 2024). Associated with a large number of Orchidaceae species, orchids have extraordinary floral diversity representing a highly advanced and terminal floral evolution (Pérez-Escobar *et al*., 2024). Orchids are highly valued because of their long floral lifespan and exquisite flowers of high diversity in floral size, variable colors, pleasant fragrance, aroma, floral form, and texture (Ramya *et al*., 2020). The unique interaction between pollinators and orchid flower contribute to the richness of orchid species (Kirillova *et al*., 2023).

Thailand dominates the global orchid industry around the globe. In 2023, the top exporters of orchids were Thailand ($69.8M), Netherlands ($59.5M), Chinese Taipei ($35.3M), Vietnam ($7.46M), and Malaysia ($6.16M). The top importers of Orchids were Japan ($56.3M), United States ($24.7M), Vietnam ($14.4M), Italy ($13M), and Germany ($13M) (OEC, 2025). Between 2022 and 2023, the exports of Orchids grew the fastest in Netherlands ($3.19M), Slovenia ($2.62M), China ($606k), Chinese Taipei ($580k), and Denmark ($102k). Germany ($2.62M), Belgium ($1.4M), Zimbabwe ($1.26M), Macau ($382k), and Bulgaria ($322k) are the fastest-growing importers of orchids, as this data is published by OEC (2025).

Orchids are also used to treat medical complications, such as in the West Indies; residual water of boiled *Bletia pupurea* is used to treat food poisoning from fish and seafood (Kumaraswamy *et al*., 2022). Orchids are also part of the food in various regions of the world, such as in Malaysia; the leaves of *Dendrobium salaccense*are cooked as a seasoning with rice (Tiwari *et al*., 2024). In certain parts of the Asian tropics, the tubers of some species of *Gasrtrodia* are eaten like potatoes *Vanila planifolia* Andr is an orchid, commercially cultivated for pods (beans) from which the popular flavoring substance called vanillin is extracted. Vanillin is mainly used in flavoring ice cream, soft drinks, condiments and oleoresins (Tiwari *et al*., 2024).

In vitro plant growth and development are mainly regulated by auxins and cytokinins (Kimoto, 2003; Hou *et al*., 2025). The interaction between two plant growth regulators is important to control the plant developmental processes. The meristems' development is an important step to establish the whole plant body (Mohammadi *et al*., 2019). For instance, shoot meristems produce aboveground parts and root meristems produce underground plant parts (Zhang *et al*., 2023). Recent studies provide a better understanding of the molecular mechanisms of auxin and cytokinin interaction in the regulation of plant growth and meristem development, as described (Zhang *et al*., 2020). Similarly, auxin supplementation of the growth medium shows a synergistic effect for improving growth and root proliferation in Dendrobium orchids (Tini *et al*., 2025). Similarly, exogenous kinetin and auxin have a synergistic effect on root formation, while BAP and auxin have a synergistic effect on shoot formation (Manokari *et al*., 2021). Moreover, plant hormones such as auxin and cytokinin are critical for *in vitro* plant regeneration, with cytokinin playing an instrumental role in shoot organogenesis (Lee *et al*., 2022). The cytokinin signaling pathway represents a potential target for manipulating de novo shoot organogenesis and *in vitro* plant regeneration (Pokimica *et al*., 2024).

Plant tissue culture and micropropagation techniques play an important role in conservation programs and management of botanical collections (Oseni *et al*., 2018). Micro-propagation is a mass vegetative plant propagation system on an artificial nutrient medium under a controlled sterile environment to ensure pathogen-free, true-to-type, and rapid production (Rafique *et al*., 2012, 2013; Somaya *et al*., 2025). The production of these commodities through seeds further leads to variations and undesirable plant traits. Tissue culture has revolutionized the orchid industry due to the rapid and efficient multiplication rate under *in vitro* growth conditions. It is a significantly important alternative to conventional vegetative propagation of orchids through division (Lal *et al*., 2023). Vegetative propagation technique in orchids through division contains different hurdles, such as a high rate of mortality, a low rate of adventitious root formation, and high plant infectivity with nematodes and fungi. The plants produced through tissue culture provide much export potential as they are shipped internationally with limited quarantine restrictions (Rafique *et al*., 2013).

Although some previous studies have focused on micropropagation of orchids, there is still a need for an improved *in vitro* regeneration method. Orchid propagation, considering the effect of plant growth regulators, can help with large-scale multiplication, which can reduce the import of this plant. Therefore,

the present research was carried out to develop an efficient protocol for mass multiplication of orchids by adjusting the auxin and cytokinin ratio in growth media.

Materials and Methods

Growth conditions and PGR treatments:

The experiments on standardization of protocols were conducted in the Plant Tissue Culture Cell at the Institute of Horticultural Sciences, University of Agriculture, Faisalabad. *In vitro* leaf disc cultures were used as an explant for the experiment. Uniform-sized leaf segments were cultured in test tubes containing 10 ml growth medium of pH 5.8. Sucrose 3% was added as a carbohydrate source, along with 0.8% agar was as a gelling agent for callus induction. The details on regeneration protocols for orchids were presented (Rafique *et al.*, 2012, 2013). MS-Medium was supplemented with different auxins and cytokinins in variable concentrations, i.e. IBA+BAP and NAA+BAP (0.0+0.0, 0.5+2.0, 1.0+2.0, 1.5+2.0, 2.0+2.0, 2.0+0.5, 2.0+1.0 and 2.0+1.5 mg/L). The cultures were sub-cultured after four weeks on the same nutrient medium, and after 2 weeks, protocorm-like bodies (PBLs) were sub-cultured for organogenesis, in a growth room with temperature adjusted at 25 ± 2 °C, 2000 lux light intensity, and a 16-hour photoperiod was maintained during the experiment.

Acclimatization of plants

Plantlets with four expanded leaves and roots were hardened on 1/2 strength basal MS medium for 4-6 weeks in glass jars and were transferred to small plastic pots containing 1:1 (w/w) mixture of peat moss and compost (autoclaved at 121°C for 30 min). Transplanted plants in pots were covered with polyethylene bags to maintain high humidity and partially cut after 2 weeks and fully opened after another 2 weeks for better acclimatization and transferred to the greenhouse. Potted plants were kept at 25 ± 1°C temperature and artificial light in a growth room 4–6 weeks. During this period, plantlets were moistened using a diluted 1/2 MS macro nutrient solution for better growth.

Growth parameters

Orchid cultures were observed daily for the whole experimental period days and changes in growth and development were observed. Data for traits: callus induction probability on visual observation, number of days to induce callus formation, Callus growth (mg), days to callus induction, fresh weight of callus (mg) after 20, 40, and 60 days, number of days for embryogenesis and embryogenesis percentage. Similarly, the number of emerged shoots, shoot length (cm) and number of roots were also observed.

Statistical analysis

All experiments were conducted in controlled conditions and laid out in a Completely Randomized Design (CRD) with four replications and six tubes per replication. Statistical analysis of experiments was performed using analysis of variance (ANOVA), and differences among treatment means were compared using the Least Significant Difference (LSD) test at the 5 % probability (P) level, with Statistix 8.1 software.

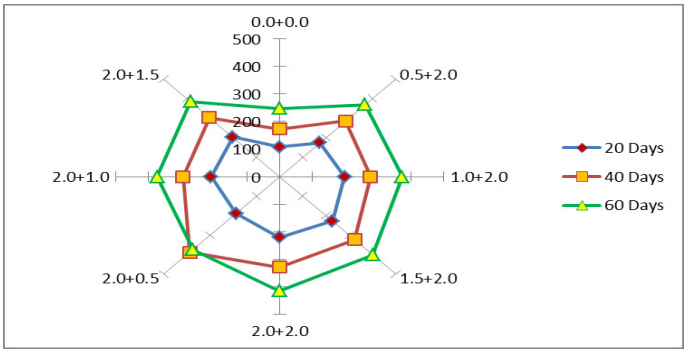

Figure 1: *Fresh weight of Callus (gm) after 20, 40, and 60 days on MS medium supplemented with different NAA+BAP concentrations*

Results and Discussions

Effect of Media modification (MS-Medium + (NAA+BAP mg/L)) for better in vitro response of Dendrobium orchid

Callus induction from leaf disc culture

The interactive radar graph in Figure 1 indicates the callus fresh weight growth after 20, 40, and 60 days on MS-supplemented medium with different NAA and BAP concentrations, along with control, i.e., no auxin or cytokinins added. The results after twenty (20) days of culturing showed significantly higher callus weight (228 mg) in MS-medium modified with Naphthalene acetic acid and 6-Benzyleaminopurine (NAA+BAP) @1.5+2.0 mg/L compared to control (107.67 mg), i.e., unmodified MS-Medium. After 60 days of inoculation, higher calli weight was observed in MS medium modified with 1.5 + 2.0 mg/L (401.00 mg) and 2.0 + 2.0 mg/L (413.67 mg) compared with

Table 1: *Effect of Modified MS Media + [NAA+BAP (mg/L)] for in vitro response of Dendrobium orchid (Cymbidium sp.)*

MS-Medium+(-NAA+BAP) (mg/L)	Days to callus induction	Callus induction probability @ PGRs level	Growth of callus (g)			Days to somatic embroids formation	Number of shoots	Length of shoots (cm)	Number of roots	Embryo formation percentage
			Days-20	Days-40	Days-60					
0.0+0.0	23.333a	+	107.67^{i}	171.67^{g}	246.17^{g}	22.50^{ab}	3.00^{h}	2.83 ^{g}	0.35 ^{j}	70
0.5+2.0	17.500b	++	173.50^{h}	283.83^{de}	368.00^{bef}	18.33^{de}	6.16^{g}	3.60^{de}	0.66^{i}	87
1.0+2.0	16.667b	++	198.67^{efg}	277.50^{e}	374.00^{de}	18.33^{de}	15.13^{bc}	3.70^{de}	1.80^{ef}	95
1.5+2.0	12.500de	+++	228.00^{a}	323.33^{a}	401.00^{ab}	17.50^{ef}	14.33^{c}	4.63^{a}	2.22^{bc}	100
2.0+2.0	10.833e	+++	219.67^{abc}	327.67^{a}	413.67^{a}	15.00^{fg}	16.16^{b}	3.43^{e}	0.96^{gh}	87
2.0+0.5	15.833bc	++	187.67^{g}	388.33^{cde}	373.17^{de}	20.00^{bcde}	6.16^{g}	2.13^{g}	1.83^{ef}	91
2.0+1.0	16.667b	++	208.33^{cde}	294.50^{cd}	373.83^{de}	20.8^{b}	10.16^{e}	2.68^{f}	2.12^{bcd}	87
2.0+1.5	15.000bcd	++	201.83^{def}	302.67^{bc}	383.50^{cd}	21.66^{abc}	12.66^{d}	3.66^{de}	1.72^{f}	83.5
Mean	16.042a		190.67^{a}	283.69^{a}	366.76^{a}	19.217^{a}	10.50^{b}	3.26^{a}	1.46 ^{b}	87.6

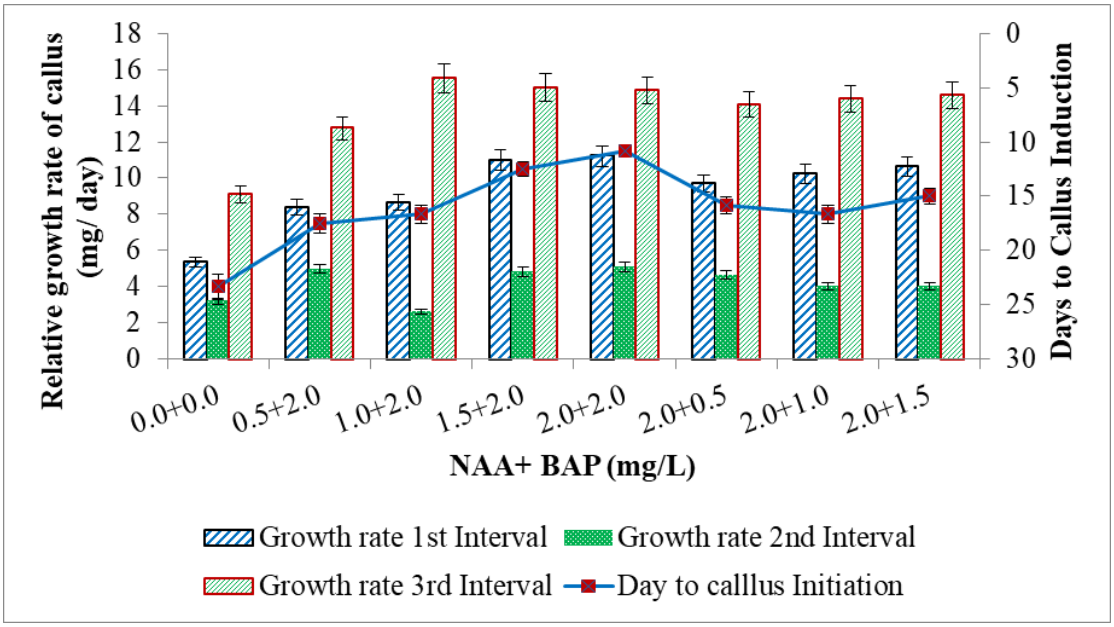

Figure 2: *Relative callus growth rate (gm/day) for 1st interval, 2nd interval, and 3rd interval of 20 days each and days to induce callus on different NAA+BAP concentrations*

the control (246.17 mg) (Table 1).

Days to callus initiation and callus growth rate

The results of our experiment also indicate that Naphthalene acetic acid and 6-Benzyleaminopurine (NAA+BAP) in modified MS-Medium at 2.0 + 2.0 mg/L showed early callus induction in (10.83 days), 1.5+ 2.0 mg/L (12.50 days) followed by (NAA+BAP), 2.0 + 1.5 mg/L (17.5 days) and 2.0 + 0.5 (16.66 days) mg/L compared with unmodified MS-medium (23.33 days). Callus induction was significantly earlier in MS-Medium 2.0 + 2.0 mg/L (Figure 3). Similarly, the growth rate of callus per day was higher for the 1st growth interval, i.e., initial 20 days of culture, than the growth rate for the 2nd growth interval (40 days). Rapid cell multiplication and callus growth rate per day have been recorded for the 3rd growth interval (60 days) as indicated in Figure 2. Moreover, callus induction probability was higher at 2.0 + 2.0 mg/L and 1.5+2.0 mg/L NAA+BAP treatments in comparison with other treatments and control.

Embryogenesis in callus cultures

Modified MS-medium supplemented with NAA+BAP (1.5 + 2.0 mg/L) showed 100% embryogenesis, which was found to be the best treatment, followed by 1.0 + 2.0 mg/L with 95% embryogenesis (Figure 3). Lower response of embryogenesis was recorded at simple MS-medium as greater number of days, i.e., 22.5, were taken for embryo formation compared with modified

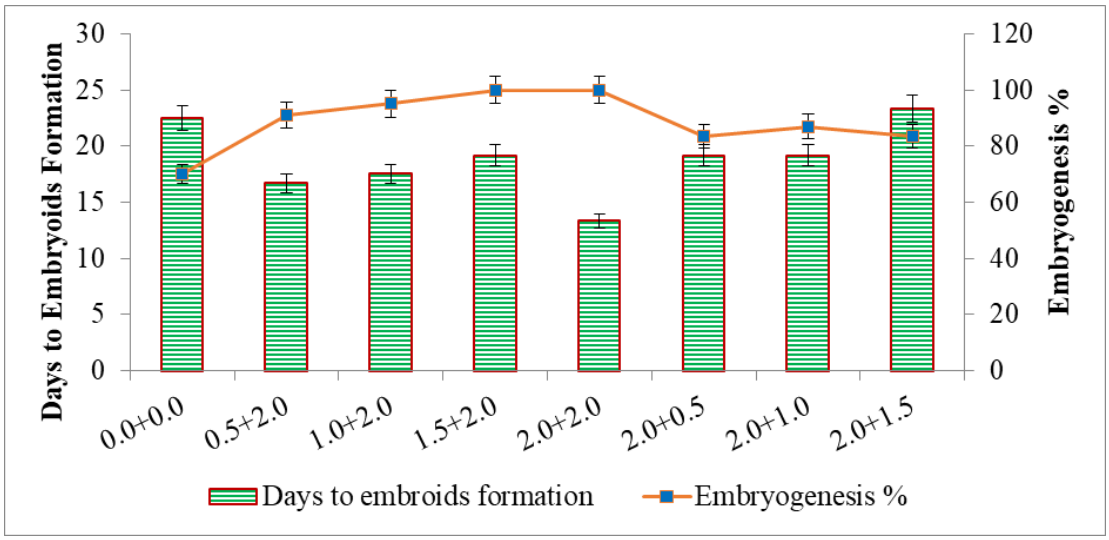

Figure 3: *Number of days to embryoid formation and embryogenesis % on different NAA+BAP concentrations*

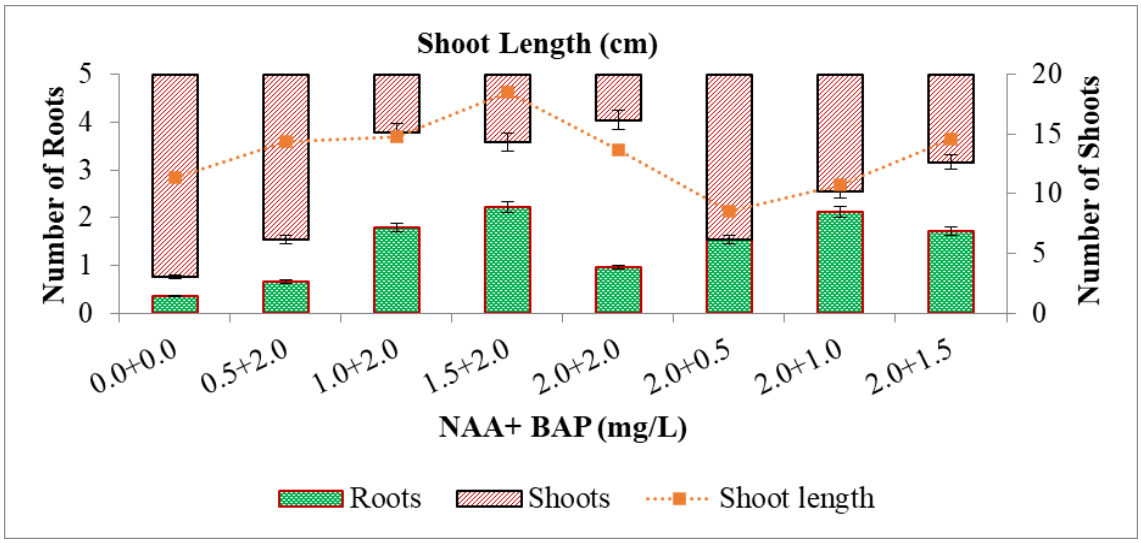

Figure 4: *Shoot length (cm), Number of Shoots and number of roots per plantlet on different NAA+BAP concentrations*

MS media supplemented with 2.0 + 1.0 mg/L, i.e., 20 days, 2.0 + 0.5 mg/L, i.e., 19.6 days, and 1.5+2.0 mg/L, i.e.,18.3 days, as shown in Figure 3.

Organogenesis from PLBs

The organogenesis showed interesting results at varying NAA and BAP concentrations (Figure 4). Significantly higher shoot length (cm) was observed in MS-medium modified with 1.5 + 2.0 mg/L (4.43 cm) compared with simple MS-medium (2.83 cm) followed by 1.0 + 2.0 mg/L (3.70 cm), 2.0 + 1.5 mg/L (3.66 cm) and 0.5 + 2.0 mg/L (3.60cm). Number of shoots significantly increased compared to control, and a greater number of shoots (16.16) were recorded in MS-medium modified with 2.0+2.0 mg/L NAA+BAP followed by 1.0 + 2.0 mg/L (15.13), 1.5+2.0 mg/L (14.33), 2.0+0.5 mg/L (6.58), and 0.5 +2.0 mg/L (7.08) compared to simple MS-medium. More roots were observed in MS-medium with 1.5 +2.0 mg/L, i.e., 2.2 per plantlet, followed by 2.0+1.0 mg/L, i.e., 2.1 per plantlet, compared with unmodified MS-medium, i.e., 0.35 per plantlet.

Effect of media modification (MS-Medium + IBA+BAP (mg/L)) for better vitro response in dendrobium orchid

Callus induction from leaf disc culture

The interactive radar graph indicates varying callus fresh weight gain after 20, 40, and 60 days on MS-supplemented medium with different NAA and BAP concentrations in comparison with the control (Figure 5). The results after twenty days of inoculation showed (<0.05) significantly higher callus weight in MS-medium modified with 2.0+ 2.0 mg/L IBA+BAP (224 mg), followed by 1.5 +2.0 mg/L IBA+BAP (220 mg). After forty days of inoculation, fresh callus formation was significantly high in MS-medium modified with 1.5 + 2.0 mg/L IBA+BAP (326 mg) and 2.0 + 2.0 mg/L IBA+BAP (316 mg).

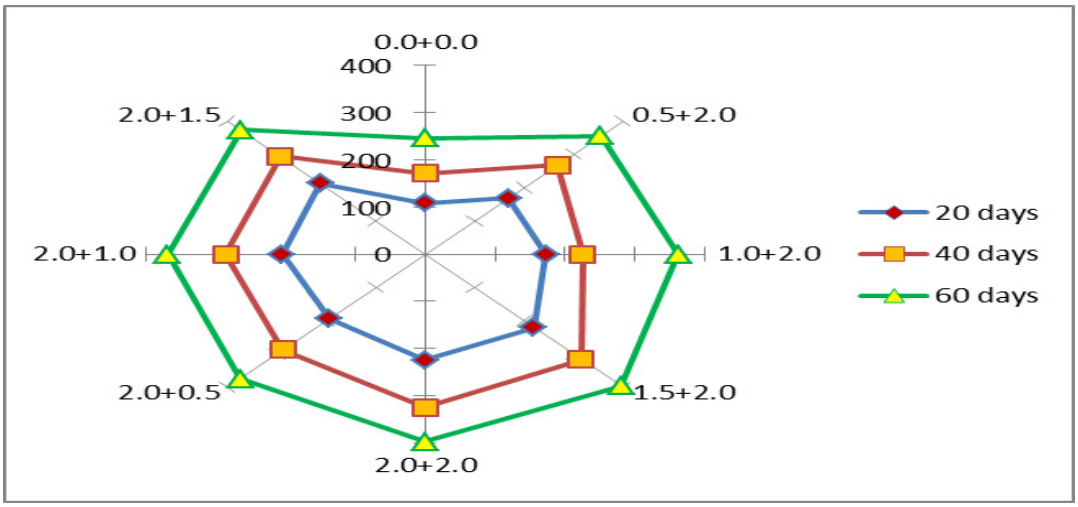

Figure 5: *Fresh weight of Callus (gm) after 20, 40 and 60 days on MS medium supplemented with different IAA+BAP concentrations*

Table 2: *Effect of Media modification (MS-Medium + (IBA+BAP (mg/L)) for better in vitro response in Dendrobium orchid (Cymbidium sp.)*

MS-Medium+(IBA+BAP) (mg/L)	Days @ Callus induction	Callus induction probability @ PGRs level	Growth of callus (g)			Days to somatic embroids formation	Number of Shoots	Length of shoots (cm)	Number of roots	Embryogenesis %
			Days-20	Days-40	Days-60					
0.0+0.0	23.333^{a}	+	107.67^{i}	171.67^{g}	246.17^{g}	22.50^{ab}	3.00^{h}	2.83^{g}	0.35^{j}	70
0.5+2.0	16.667^{b}	++	167.67^{h}	267.17^{e}	354.83^{f}	16.667^{d}	8.00^{f}	3.60^{de}	0.82^{hi}	91
1.0+2.0	17.500^{b}	++	172.83^{h}	225.00^{f}	362.33^{ef}	17.50^{ef}	16.00^{b}	3.86^{cd}	1.72^{f}	95
1.5+2.0	10.833^{e}	+++	220.00^{ab}	316.33^{ab}	396.33^{bc}	19.16^{cde}	15.66^{bc}	4.30^{a}	2.83^{a}	100
2.0+2.0	12.500^{de}	+++	224.50^{a}	326.00^{a}	398.50^{abc}	13.33^{g}	17.66^{a}	3.62^{be}	1.13^{g}	100
2.0+0.5	15.000^{bcd}	++	194.17^{fj}	287.00^{de}	373.83^{de}	19.16^{cde}	7.00^{fg}	2.23^{g}	1.98^{cde}	83.5
2.0+1.0	15.833^{bc}	++	204.50^{def}	285.17^{de}	368.33^{def}	19.16^{cde}	12.66^{d}	2.72^{f}	2.25^{b}	87
2.0+1.5	13.333^{cde}	++	212.33^{bcd}	293.17^{cd}	372.50^{de}	23.33^{a}	12.66^{d}	4.10^{bc}	1.90^{def}	83.5
Mean	15.625^{a}		180.21^{a}	275.69^{b}	359.10^{b}	18.96^{a}	11.58^{a}	3.13^{a}	1.63^{a}	88.75

After 60 days, significantly high callus fresh weight was recorded in MS-medium modified with 1.5 +2.0 mg/L IBA+BAP (396 mg) and 2.0+2.0 mg/L IBA+BAP (399 mg) compared with the control.

Number of days to callus initiation and callus growth rate:

The results of our experiment indicate that Indole acetic acid and 6-Benzyleaminopurine (IAA+BAP) in modified MS-Medium with 1.5+ 2.0 mg/L IAA+BAP showed early callus induction i.e., 10.8 days, for 2.0 + 2.0 mg/L 12.5 days, followed by IAA+BAP @ 2.0 + 0.5 mg/L 15 days, and for 2.0 + 1.0 mg/L 15.8 days compared with unmodified MS-medium, i.e., 23 days. Callus induction is significantly earlier in MS-Medium supplemented with 1.5 + 2.0 mg/L IAA+BAP (Figure 6). Rapid cell multiplication and callus growth rate per day have been recorded for the 3rd growth interval (60 days) as indicated in Figure 6 and Table 2. Furthermore, callus induction probability is higher for 2.0 + 2.0 mg/L and 1.5+2.0 mg/L IAA+BAP treatments in comparison with other treatments and the control.

Embryogenesis in callus cultures

Modified MS-medium with IAA+BAP (1.5 + 2.0 mg/L and 2.0 + 2.0 mg/L) with 100% embryogenesis was found to be the best followed by 1.0 + 2.0 mg/L with 95% embryogenesis, compared with various levels of Media modification in addition to control (Figure 7). Lower response of embryogenesis was recorded at unmodified MS-medium as a greater number of days, i.e., 22.5 days, were taken for embryo formation compared with MS-medium modified with 2.0 + 1.0 mg/L, i.e., 13.3 days, 2.0 + 1.0 mg/L, i.e., 19.1 days, 2.0 + 0.5 mg/L, i.e., 19.2 days and 1.5+2.0 mg/L, i.e.,

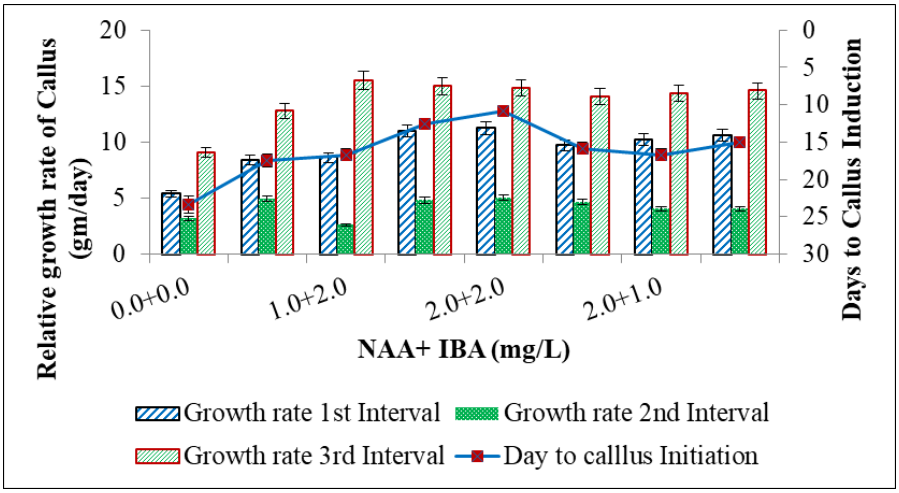

Figure 6: *Relative callus growth rate (gm/day) for 1st interval, 2nd interval, and 3rd interval of 20 days each and days to induce callus on different IAA+BAP concentrations*

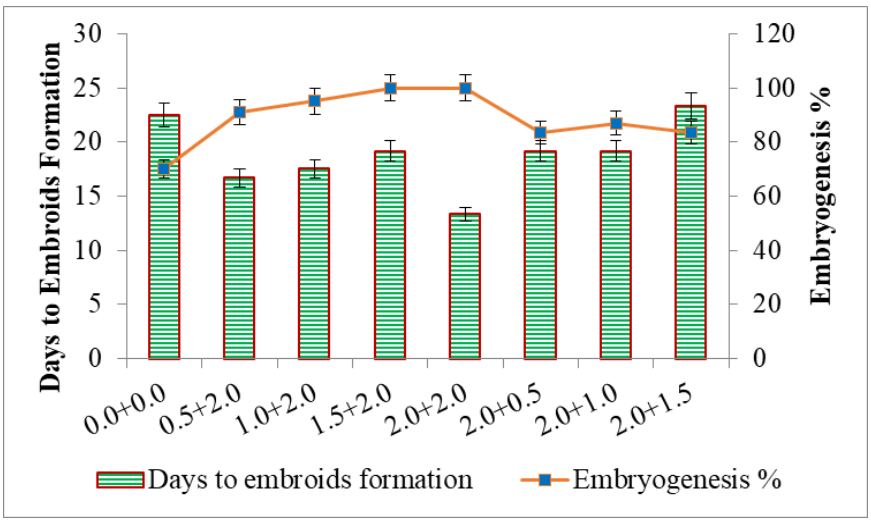

Figure 7: *Number of days to embryoids formation and embryogenesis % on different IAA+BAP concentrations*

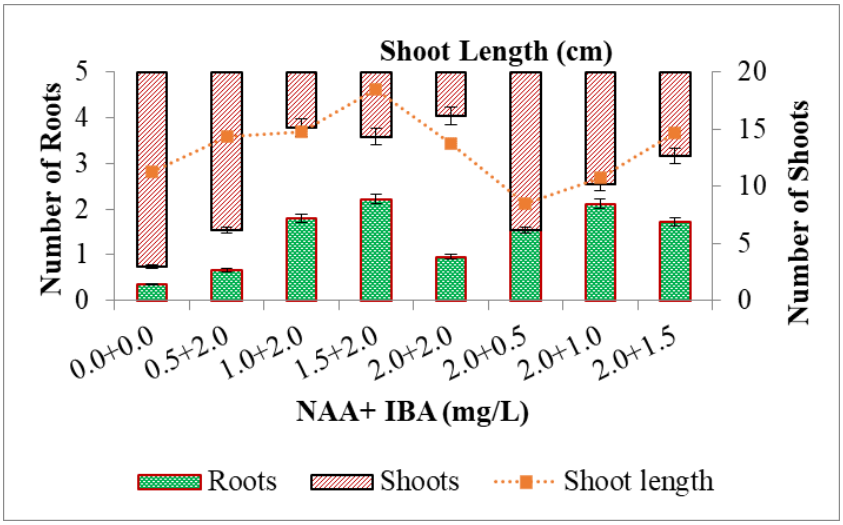

Figure 8: *Shoot length (cm), Number of Shoots, and number of roots per plantlet on different IAA+BAP concentrations*

19.2 days as indicated in Figure 7. 2.0 mg/L IAA+BAP (17.66). The maximum number of roots was produced in MS-medium modified with IBA+BAP at 1.5 +2.0 mg/L, i.e., 2.3 per plantlet, compared to the control, as shown in Figure 8.

Organogenesis from PLBs

Organogenesis of the Dendrobium orchids indicates

very fascinating results on varying IAA and BAP concentrations (Figure 8). Significantly more shoot length was observed in MS-medium modified with 1.5 + 2.0 mg/L IAA+BAP, i.e., 4.30 cm compared with simple MS-medium, i.e., 2.83 cm, followed by 1.0 + 2.0 mg/L, i.e., 3.86 cm, 2.0 + 2.0 mg/L, i.e., 3.62 cm, and 0.5 + 2.0 mg/L, i.e., 3.60cm. Results show that the maximum number of shoots was observed at 2.0 + 4.3

Discussion

Dendrobium orchid is used for direct and indirect somatic embryogenesis for mass multiplication of orchids (Deng *et al*., 2024). *Cymbidium forrestii sps*. have a significant response against 6-Benzyleadenine for cytoplasmic zone induction from apical meristem in direct and early leaf formation (Mosoh *et al*., 2024). Direct shoot induction is possible from leaf tips, callus driven from the shoot tip in orchids in MS-medium supplemented with NAA, BA, and IBA and this method is used for multiple shoot induction in present research. In orchids, direct shoot induction is the most significant method of plant regeneration. In direct regeneration, the shoots that first appeared are small, green protuberances at the leaf bases and develop into shoots without any callus or PLB formation, as shown in Figure 9. These PLBs are normally confused by callus formation, as described by Ahlawat *et al*. (2022). The PBLs are, in fact, *in vitro* seeds that can be further used for multiplication and mass regeneration of plants. The PLBs started rapid growth in 3–4 weeks after being placed on culture media, as shown in Figure 9.

During the present research, the least days, i.e., 10.8, were observed in MS-Medium modified with 2.0+2.0 (mg/L) of NAA+BAP and IBA+BAP for PLBs and calli formation. These results are consistent with Salem *et al*. (2022), who showed a similar response of growth regulators during *in vitro* propagation. The observations in the present research showed that the callus mass (413.67g) for 60 days after inoculation was increased two-fold higher 20 days after inoculation in MS-Medium modified with NAA+BAP with 2.0+2.0 mg/L. Embryos or PBLs in general, germinate freely comparatively better than the mature botanical nutrient flow, which is obstructed by the seed coat and seed dormancy (Lee *et al*., 2023). Moreover, the nutritional requirements of embryos during the initial stages of development are quite simple, facilitating their germination (Jolman *et al*., 2022). The present research showed excellent embryo formation and germination in MS-medium modified with NAA+BAP (21.6) and IBA+BAP (23.3) at the level of 2.0+1.5. The tendency of germination, development and protocorm proliferation using for growth regulators has been reported in some other orchid species, including *Cymbidium aloifolium, C. giganteum C. pendulum*, *Dendrobium aphyllum* and *Epidendrum ibaguense* as discussed by Guo *et al*. (2024) and Balilashaki *et al*. (2023). Previous studies show

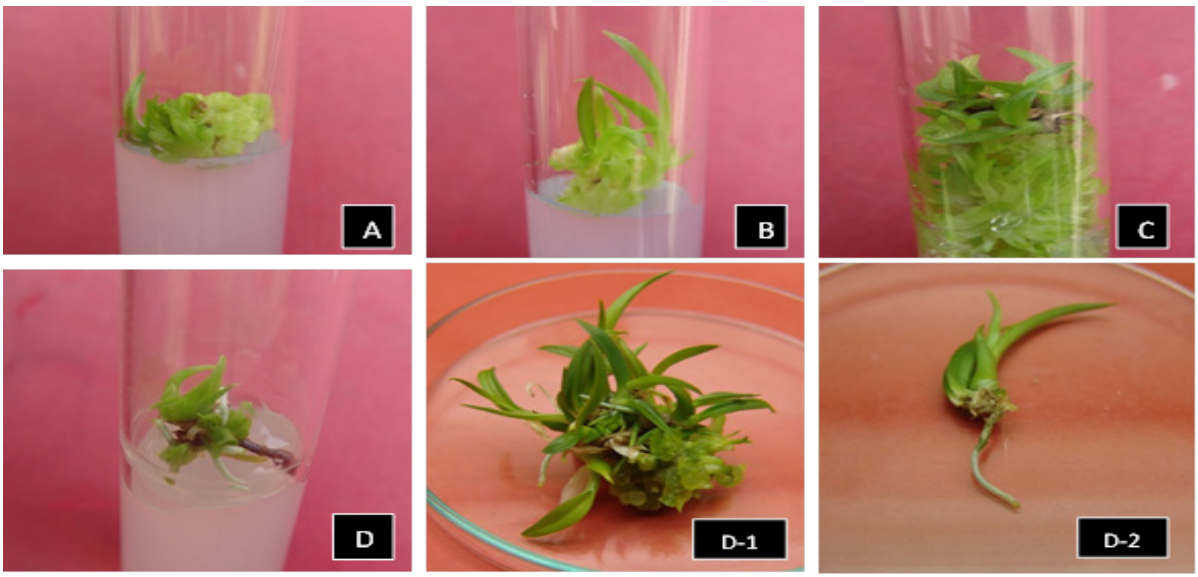

Figure 9: *Stages from in vitro Protocorm-like body (PLB) formation from leaf disc of terminal leaves and initiation of shoot proliferation (A), Shoot induction in orchids (B), Multiple shoot regeneration from protocorm like bodies (C) Root initiation after sub culturing from emerging shoots (D) organogenesis in orchids (D-1) plantlet with emerged root and 3-4 leaves (D-2)*

fewer weak roots from multiplied adventitious shoots in MS-Medium modified with IAA, as described by Hossain *et al*. (2012) and Mastuti *et al*. (2017). The same rooting behavior was observed in orchids during the present research, as a few roots were developed in MS-medium modified with NAA+BAP, while the rooting response was slightly better in IBA+BAP (2.25) compared with NAA+BAP (2.22).

Conclusions and Recommendations

The present study standardizes an efficient *in vitro* protocol from leaf disc cultures through promoting PLB formation in supplemented MS-medium that would help the private sector in minimizing orchid imports. It is concluded that MS-media modification with BAP + IBA is better in terms of plantlet development and growth in the *in vitro* environment compared with different levels of NAA+BAP. MS-medium supplemented with 1.5+2.0 mg/L NAA+BAP or IAA+BAP performed well. Rooting was successfully induced on MS-medium supplemented with 1.5+2.0 mg/L of NAA+BAP or IAA+BAP. The optimized protocol will enable local production of orchid plant material for growers and other stakeholders at a lower cost and with less threat of invasive diseases, and would contribute towards a sustainable floriculture industry.

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Novelty Statement

The study provides a simple but novel and efficient *in vitro* propagation protocol for orchids that can effectively be used at a commercial scale.

Author Contribution

Rizwan Rafique: The research work was conducted.
Bilquees Fatima: Supervised
Muhammad Usman, Monis Hussain Shah, Tanzila Rafique and Muhammad Rafique Sajjad: Have contributed to analyzing results, drafting, and reviewing the paper.

Generative AI and AI-assisted technology statement
The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Declaration of competing interest
There exists no conflict of interest, and all co-authors agree with this submission. Authors adhere to and follow the publishing policy of the Journal.

References

Ahlawat, J., A.R. Sehrawat, R. Chaudhary and D. Pandey. 2022. *Ascophyllum nodosum*: A potential substitute for synthetic hormones for tissue culture propagation of *Capparis decidua* (Forsk) Edgew. Regen. Eng. Transl. Med., 8: 145-151. https://doi.org/10.1007/S40883-021-00215-0

Balilashaki, K., M.E. Martinez-Montero, M. Vahedi, J.C. Cardoso, C.L. Silva Agurto, M. Leiva-Mora, F. Feizi and M.M. Hossain. 2023. Medicinal use, flower trade, preservation and mass propagation techniques of cymbidium orchids—An overview. Horticulture, 9(6): 690. https://doi.org/10.3390/horticulturae9060690

Christenhusz, M.J.M. and J.W. Byng. 2016. The number of known plant species in the world and its annual increase. Phytotaxa., 261: 201–217.

Deng, J., W. Li., X. Li., D.Liu and G. Liu. 2024. A fast: efficient, and tissue-culture-independent genetic transformation method for *Panax notoginseng* and *Lilium regale*. Plants: 13:2509. Available from: https://doi.org/10.3390/plants13172509

Guo, B., H. Chen, Y. Yin, W. Wang and S. Zeng. 2024. Tissue culture via protocorm-like bodies in an orchids hybrids paphiopedilum SCBG Huihuang-90. Plants 13: 197. https://doi.org/10.3390/plants13020197.

Hossain, M.M., Sharma, M. and P. Pathak. 2012. *In vitro* propagation of *Dendrobium aphyllum* (Orchidaceae) seed germination to flowering. J. Plant Biochem. Biotechnol., 22: 157-167.

Hou, M., Y. Zhang, X. Xu and H. Ali. 2025. Advances in auxin synthesis, transport, and signaling in rice: implications for stress resilience and crop improvement. Front. Plant Sci., 15: 1516884. https://doi.org/10.3389/fpls.2024.1516884

Kimoto, T. 2003. Perception and signal transduction

of cytokinins. Annu. Rev. Plant Biol., 54: 605–627.

Jolman D., M.I. Batalla, A. Hungerford, P. Norwood, N. Tait and L.E. Wallace. 2022. The challenges of growing orchids from seeds for conservation: An assessment of asymbiotic techniques. Appl. Plant Sci.. 10(5): e11496. https://doi.org/10.1002/aps3.11496.

Kirillova, I.A., Y.A. Dubrovskiy, S.V. Degteva and A.B. Novakovskiy. 2023. Ecological and habitat ranges of orchids in the northernmost regions of their distribution areas: A case study from Ural Mountains, Russia. Plant Divers., 2023, 45: 211-218.

Kumaraswamy, K.P., N. Nallaperumal, S. Karimulla, S. Devarajan, P. Jagannathan, V. Thangavel, I. Yusuff and B. Boddu. 2022. Formulation and evaluation of capsule of ethanolic extract of *Cnidoscolus chayamansa* Mc Vaugh leaves for the treatment of diabetes. Ann. Phytomed., 11(2): 339-343. http://dx.doi.org/10.54085/ap.2022.11.2.40.

Lal, N. and S. Madhulika. 2023. Prospects of plant tissue culture in orchid propagation: A Review. Ind. J. Biol., 7: 103-110. https://doi.org/10.21088/ijb.2394.1391.7220.15.

Lee, H.J., I.S. Cho and R.D. Jeong. 2022. Nanopore metagenomics sequencing for rapid diagnosis and characterization of lily viruses. Plant Pathol. J., 38: 503-512. https://doi.org/10.5423/PPJ.OA.06.2022.0084.

Lee, Y.I. and E.C. Yeung. 2023. The orchid seed coat: a developmental and functional perspective. Bot. Stud., 64: 27 https://doi.org/10.1186/s40529-023-00400-0

Manokari, M., S. Priyadharshini, P. Jogam, A. Dey and M.S. Shekhawat. 2021. Meta-topolin and liquid medium mediated enhanced micropropagation via ex vitro rooting in Vanilla planifolia Jacks. Ex-Andrews Plant Cell Tissue Organ. Cult., 146: 69-82.

Mastuti, R., A. Munawarti and E.R. Firdiana. 2017. The combination effect of auxin and cytokinin on *in vitro* callus formation of *Physalis angulata* L. A medicinal plant. In AIP Conference Proceedings.

Mohammadi, M., B. Kaviani and S.H. Sedaghathoor. 2019. Micropropagation of two near threatened orchid. Part 2: Phalaenopsis amabilis Blume var. Grandiflora. Adv. Hort. Sci., 33(4): 485–493.

Mosoh, D.A., A.K. Khandel, S.K. Verma and W.A. Vendrame. 2024. Optimizing callus induction and indirect organogenesis in nondormant corm explants of *Gloriosa superba* (L.) Via media priming. Front. Hortic., 3: 1378098. https://doi.org/10.3389/fhort.2024.1378098.

OEC. 2025. Orchids. Available at: https://oec.world/en/profile/hs/orchids

Ogliore, T. 2024. The Record: New insight into orchid origins. Available at: https://source.washu.edu/2024/02/new-insight-into-orchid-origins/

Oseni, O.M., V. Pande and T. K. Nailwal. 2018. A review on plant tissue culture, a technique for propagation and conservation of endangered plant species. Intl. J. Curr. Microbiol. Appl. Sci., 7(7): 3778–3786. https://doi.org/10.20546/ijcmas.2018.707.438.

Pérez-Escobar, O.A., D. Bogarín, N.A. Przelomska, J.D. Ackerman, J.A. Balbuena, S. Bellot, R.P. Bühlmann, B. Cabrera, J.A. Cano and M. Charitonidou., 2024. The origin and speciation of orchids. New Phytol. 242:700–716.

Pokimica, N., T. Cosic, B. Uzelac, S. Ninkovic and M. Raspor. 2024. Dissecting the Roles of the Cytokinin Signaling Network: The Case of De Novo Shoot Apical Meristem Formation. Biomolecules.m 14: 381. https://doi.org/103390/biom14030381

Rafique, R., B. Fatima, S. Mushtaq, M.S. Iqbal, M. Rasheed, M. Ali and S.U. Hasan. 2012. Effect of indole-3-butyric acid (IBA) on *in vitro* root induction in dendrobium orchid (*Dendrobium sabin* H.). Afr. J. Biotechnol., 11: 4673-4675.

Rafique, R., B. Fatima, M. Usman, M.S. Iqbal, S.U. Hasan, K. Shabbir and M. Rasheed. 2013. Effect of sucrose on *in vitro* callogenesis, embryogenesis and organogenesis of *Dendrobium sabin* H. Int. J. Mod. Agric., 2: 43-47.

Ramya, M., S. Jang, H.R. An, S.Y. Lee, P.M. Park and P.H. Park. 2020. Volatile organic compounds from orchids: From synthesis and function to gene regulation. Int. J. Mol. Sci., 21: 1160.

Salem, J., A. Hassanein, D.A. El-Wakil and N. Loutfy. 2022. Interaction between growth regulators controls *in vitro* shoot multiplication in paulownia and selection of NaCl-tolerant variants. Plants (Basel). 11(4):498. https://doi.org/10.3390/plants11040498.

Somaya, S., M.H. Shah, R.U. Rehman, Z. Ullah

and S. Ikram. 2025. Multivariate analysis, morphological characterization and *in vitro* organogenesis in elite varieties of carnation (*Dianthus caryophyllus*). Pak. J. Bot., 57(6): http://dx.doi.org/10.30848/PJB2025-6(25)

Tini, E.W., Z. Ulinnuha, R.R. Kurniawan, P.Widodo, R. Prasetyo, A.R. Milan and G. Sugiyono. 2025. Optimizing auxins applications for *in vitro* plantlet development of a new dendrobium hybrid. Planta Tropika, 13(2): 182-191.

Tiwari. P., A. Sharma, S.K. Bose and K.I. Park. 2024. Advances in orchid biology: Biotechnological achievements, translational success, and commercial outcomes. Hortic., 10(2): 2, https://doi.org/10.3390/horticulturae10020152.

Zhang, H., Y. Mu, H. Zhang and C. Yu. 2023. Maintenance of stem cell activity in plant development and stress responses. Front. Plant Sci., 14: 1302046. https://doi.org/10.3389/fpls.2023.1302046.

Zhang, H., L. Li, Y. He, Q. Qin, C. Chen and Z. Wei, *et al.* 2020. Distinct modes of manipulation of rice auxin response factor OsARF17 by different plant RNA viruses for infection. Proc. Natl. Acad. Sci. U. S. A. 117: 9112-9121. https://doi.org/10.1073/pnas.1918254117.