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Original Research Paper

Royal jelly as a novel bio-stimulant for asymbiotic seed germination and protocorm development in *Phalaenopsis*

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ABSTRACT

Orchid micropropagation is often limited by slow seed germination and early seedling development. This study evaluated the potential of royal jelly (RJ), a nutrient-rich organic substance, as a growth-promoting additive in the asymbiotic culture of five *Phalaenopsis* varieties viz., *Nottingham, Dubrovnik, Andorra, Memphis, and Bucharest*. A factorial experiment was conducted using two peptone concentrations (1/ gL{ ¹ and 2/ gL{ ¹) combined with four RJ levels (0, 150, 300, and 450/ ppm) in half-strength Murashige and Skoog (MS) medium. Results demonstrated that RJ significantly enhanced germination and seedling development in a concentration-dependent manner. The variety Nottingham cultured in 2/ gL{ ¹ peptone medium supplemented with 300/ ppm RJ achieved the highest germination rate of 96.33%, reduced the time to protocorm formation by 2–3/ days, and increased leaf and root primordia numbers by 15–20% compared with the control. Higher RJ concentrations (450/ ppm) occasionally reduced germination and delayed early growth, indicating an optimal range for supplementation. These findings suggest that RJ can serve as an effective organic bio-stimulant for *Phalaenopsis* micropropagation, offering a sustainable alternative to conventional synthetic growth regulators. Future studies should isolate active compounds in RJ and assess its scalability across different orchid species.

Keywords: Micropropagation, organic bio-stimulant, *Phalaenopsis*, protocorm development, royal jelly, seed germination

INTRODUCTION

Phalaenopsis orchids are among the most economically important floricultural crops worldwide. Hybridization and cross-pollination generate cultivars with diverse flower colors, capsule lengths, and seed traits (Bidarnamani et al., 2023 & 2024). However, large-scale *in vitro* propagation remains constrained by slow plantlet growth, low multiplication rates, poor rooting, and somaclonal variation (Paek et al., 2011).

Organic additives enhance growth and development by supplying vitamins, amino acids, sugars, and phytohormone-like compounds, promoting protocorm formation, protocorm-like body (PLB) enlargement, and plantlet regeneration (Islam et al., 2003; Chew et al., 2018; Heriansyah & Indrawanis, 2020). Royal jelly (RJ), secreted by worker honeybees, is rich in proteins (12–15%), sugars (10–12%), lipids (3–7%), amino acids, vitamins, and minerals. Queens fed exclusively on RJ exhibit extended lifespans and fully developed reproductive organs compared with infertile workers (Ramadoss et al., 2015). RJ, along with other bee products such as honey, propolis, and pollen, shows antioxidant and antimicrobial activities, with major royal jelly proteins (MRJPs) and royalisin inhibiting Gram-positive bacteria, including *Staphylococcus aureus* (Altun & Aydemir, 2022; Fujiwara et al., 1990; Boukraa et al., 2008). RJ and honey act synergistically to reduce *S. aureus* growth more effectively than when used individually (Boukraâ et al., 2008).

Tissue culture outcomes are highly dependent on media composition (Setiani et al., 2018), and organic additives improve PLB proliferation, shoot multiplication, and somatic embryo regeneration (Gnasekaran et al., 2010; Wahyuni et al., 2021; Mose et al., 2020; Isda et al., 2019). RJ thus represents a novel biostimulant for orchid tissue culture. To our knowledge, this study is the first to evaluate RJ for enhancing germination, protocorm development, and seedling growth of *Phalaenopsis in vitro*. Moreover, supplementation with activated charcoal in Murashige and Skoog (MS) medium has been shown to enhance *in vitro* growth of orchids (Wasiati et al., 2021), suggesting that optimization of basal media with natural additives like RJ could further improve propagation efficiency.

Table 1 : Analysis of variance (ANOVA) for germination-related traits of *Phalaenopsis* seeds under different varieties, tissue culture methods, and royal jelly concentrations

Source of variation	df	Number of germinated seeds	Leaf primordia	Root primordia	Protocorm	Number of leaves	Number of roots
Variety	4	783.70**	120.24**	368.77**	40.40**	7.33**	16.99**
Tissue culture	1	371.01**	28.03*	32.03**	20.00**	0.83ns	6.07**
Royal jelly concentration	3	914.83**	56.40**	822.86**	94.23**	0.28ns	13.16**
Variety × Tissue culture	4	44.61**	16.10ns	0.93ns	6.86**	2.75**	2.49*
Variety × Royal jelly concentration	12	15.82**	3.00ns	15.99**	11.43**	1.28**	2.94**
Tissue culture × Royal jelly concentration	3	16.65**	163.77**	36.90**	1.03ns	0.90ns	3.39**
Variety × Tissue culture × Royal jelly concentration	12	14.34**	11.50ns	7.05*	2.02ns	0.48ns	0.55**
Error	78	5.90	9.43	4.06	1.25	0.54	0.97
CV (%)	–	2.95	2.41	1.31	2.42	18.69	12.87

*, ** indicate significance at P d" 0.05 and P d" 0.01, respectively, according to DMRT

MATERIALS AND METHODS

Seed capsules of five *Phalaenopsis* cultivars (Nottingham, Dubrovnik, Andorra, Memphis, Bucharest) were harvested at full maturity from greenhouse-grown plants at the Research Institute of Zabol, Iran. Capsules were collected prior to dehiscence, rinsed with tap water and detergent, surface-sterilized in 5% sodium hypochlorite with Tween-20 for 10 min, immersed in 70% ethanol for 30 s, and rinsed three times with sterile distilled water under laminar flow. Seeds were cultured on half-strength Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) containing 30 gL{ ¹ sucrose, 7 gL{ ¹ agar, and peptone (1 or 2 gL{ ¹). Freeze-dried royal jelly (RJ; Sigma-Aldrich, Lot No. XXX) was dissolved in sterile triple-distilled water, filter-sterilized (0.22 µm), and incorporated into cooled medium at 0, 150, 300, or 450 ppm.

A factorial experiment (5 cultivars × 2 peptone levels × 4 RJ concentrations) was arranged in a completely randomized design with three replications of 25 seeds per petri dish. Data recorded included germination percentage, time to protocorm formation, initiation of leaf and root primordia, number of leaves and roots per explant, and root and leaf lengths. Data were subjected to ANOVA using SAS (v. X; SAS Institute, Cary, NC, USA), and means were separated by Duncan's multiple range test at *p* d" 0.05.

RESULTS AND DISCUSSION

Germination percentage

Analysis of variance showed that variety significantly affected all traits, while, tissue culture medium and royal jelly (RJ) concentration significantly influenced all traits except the number of leaves (Table 1). Interactions among variety, medium, and RJ concentration were significant for germination percentage (P d" 0.01), indicating that the effect of RJ depends on both cultivar and culture conditions.

Among the varieties, Nottingham exhibited the highest germination percentage, followed by Andorra, Memphis, Bucharest, and Dubrovnik, irrespective of the culture medium. Between the two micropropagation media, ½ MS + 2 gL{ ¹ peptone supported higher germination (84.3%) than ½ MS + 1 gL{ ¹ peptone. Incorporation of RJ enhanced germination from 74.8% to 88%, with 150–300 ppm being optimal. Higher concentrations (450 ppm) slightly reduced germination, indicating a dose-dependent effect (Table 2, Fig. 4).

Table 2 : Germination percentage of *Phalaenopsis* seeds under different culture media and royal jelly concentrations

Culture media	Royal jelly concentration	Nottingham	Andorra	Memphis	Bucharest	Dubrovnik
½ MS + 1 g/L peptone	Control	83.667^{ghijkl}	76.333^{opq}	78.000^{nop}	74.667^{pqr}	71.667^{rs}
½ MS + 2 g/L peptone	Control	86.333^{fgh}	78.000^{nop}	71.000^{rs}	60.333^{t}	69.667^{s}
½ MS + 1 g/L peptone	150 ppm	91.33 ^{bcd}	84.000^{ghijk}	81.667^{ijklmn}	79.000^{mnop}	72.333^{qrs}
½ MS + 2 g/L peptone	150 ppm	91.000^{bcde}	86.333^{fgh}	85.667^{fghi}	87.667^{defg}	78.333^{mnop}
½ MS + 1 g/L peptone	300 ppm	93.667^{ab}	89.333^{bcdef}	86.667^{efgh}	79.333^{lmno}	78.000^{nop}
½ MS + 2 g/L peptone	300 ppm	96.333^{a}	92.667^{abc}	91.000^{bcde}	89.000cdef	84.000^{ghijk}
½ MS + 1 g/L peptone	450 ppm	93.667^{ab}	84.667^{fghij}	87.000^{defgh}	80.000^{klmno}	82.667^{hijklm}
½ MS + 2 g/L peptone	450 ppm	86.667^{efgh}	87.667^{defg}	84.66 ^{fghij}	82.667^{hijklm}	80.667^{jklmno}

Means followed by the same letter(s) are not significantly different according to Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$

Fig. 4 : Germination response of Phalaenopsis seeds under different royal jelly treatments

The interaction between varieties and media (Fig. 1) showed that Nottingham maintained superior germination in both media, while Dubrovnik performed worst in ½ MS + 1 gL{ ¹ peptone. Interactions between varieties and RJ (Fig. 2) confirmed that 150–300 ppm RJ consistently improved germination in all cultivars, with Nottingham at 300 ppm RJ achieving the highest germination (96.33%) and Dubrovnik without RJ the lowest (60.33%). The interaction of RJ and media (Fig. 3) revealed that ½ MS + 2 gL{ ¹ peptone combined with 300 ppm RJ produced optimal germination, while, 450 ppm was inhibitory in both media. These findings suggest that RJ can effectively enhance seed germination, likely due to its rich content of proteins, amino acids, vitamins, and bioactive compounds that support early seedling development (Obaid et al., 2020; Salsabila et al., 2022).

Leaf primordia formation

Leaf primordia formation time was significantly affected by variety, RJ concentration, and the interaction of RJ with culture medium (P d" 0.01).

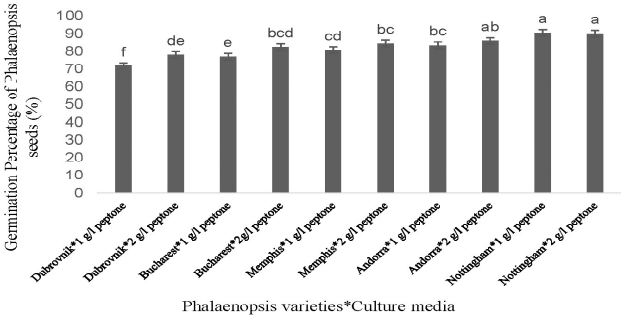

Fig. 1 : Interaction effect of *Phalaenopsis* varieties and culture media on seed germination percentage

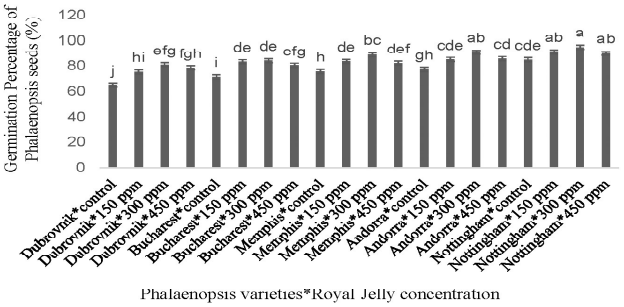

Fig. 2 : Interaction effect of *Phalaenopsis* varieties and royal jelly concentrations on seed germination percentage

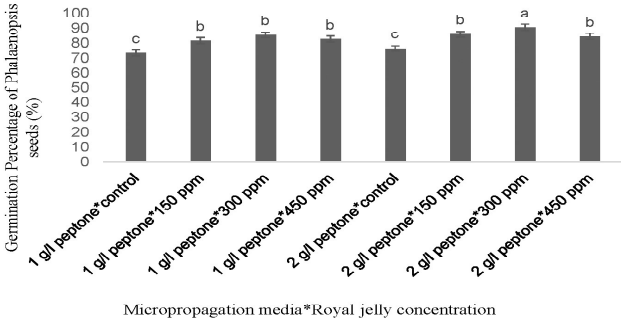

Fig. 3 : Interaction effect of royal jelly concentrations and culture media on seed germination percentage of *Phalaenopsis*

Nottingham showed the shortest time to leaf primordia, while Andorra exhibited the longest (Fig. 5). RJ supplementation (150–350 ppm) reduced the time to primordia formation compared with the control, but the highest concentration (450 ppm) accelerated leaf formation the most, suggesting that RJ may promote cell division and morphogenesis due to its nutrient-rich composition (Fig. 6 & 7).

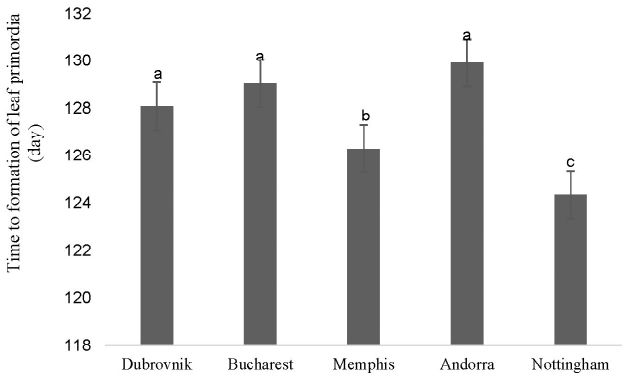

Fig. 5 : Time to leaf primordia formation in different *Phalaenopsis* varieties

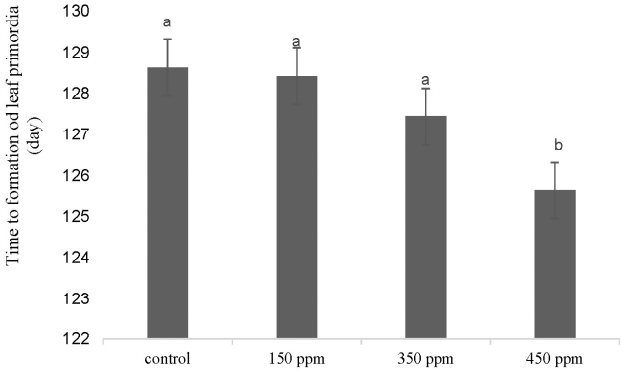

Fig. 6 : Effect of different royal jelly concentrations on the time to leaf primordia formation in *Phalaenopsis* seeds

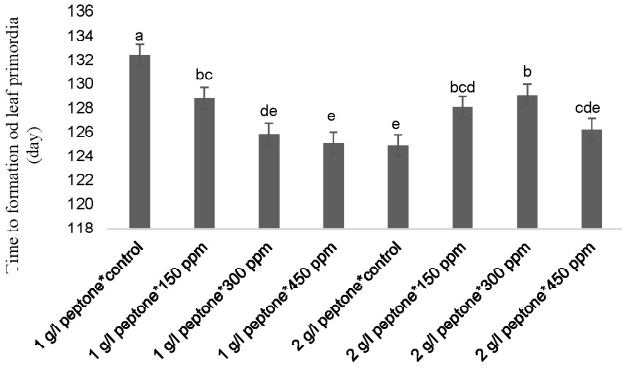

Fig. 7 : Interaction effect of culture media and royal jelly concentrations on the time to leaf primordia formation in *Phalaenopsis* seeds

Root primordia formation

Time to root primordia formation varied significantly among varieties, media, and RJ concentrations (P d" 0.01). Varieties with faster leaf primordia formation (e.g., Nottingham) required slightly longer for root primordia, indicating a developmental trade-off (Fig. 8). Media with ½ MS + 2 gL{ ¹ peptone delayed root primordia compared with 1 gL{ ¹ peptone, opposite to the trend observed for leaf primordia, highlighting differential nutrient requirements for shoot versus root initiation.

RJ supplementation decreased root primordia formation time across all varieties, with 450 ppm RJ yielding the fastest response (Fig. 8 & 9). The effect of RJ may be due to the presence of growth-promoting amino acids and vitamins, and possibly auxin-like activity, accelerating root initiation (Obaid et al., 2020).

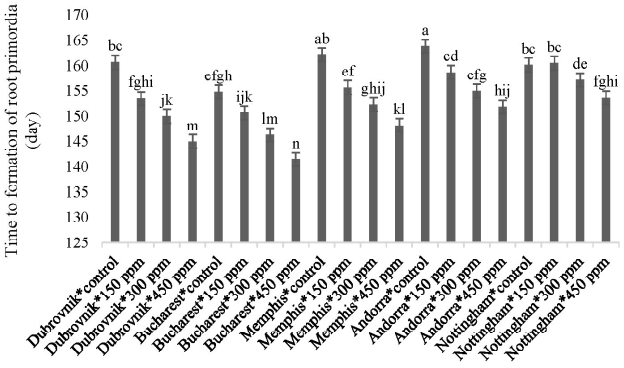

Fig. 8 : Effect of royal jelly concentrations on the time to root primordia formation in different *Phalaenopsis* varieties

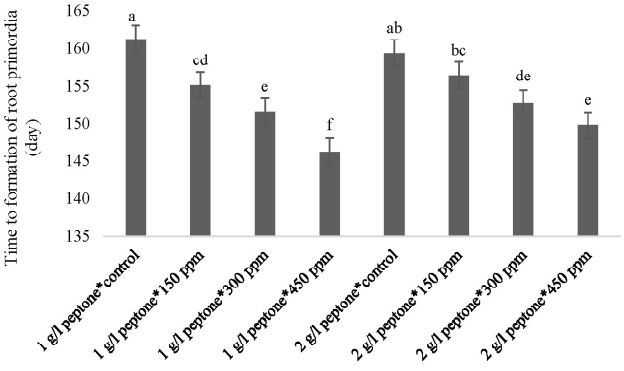

Fig. 9 : Effect of different royal jelly concentrations in two culture media on the time to root primordia formation in *Phalaenopsis* seeds

Interaction of RJ and media showed that in 1 gL{ ¹ peptone, addition of RJ significantly decreased the time to leaf primordia formation, whereas in 2 gL{ ¹ peptone, the effect of RJ was less pronounced. This likely reflects the protein contribution from both peptone and RJ, where higher total nitrogen content in 2 gL{ ¹ peptone may have diminished the marginal effect of RJ.

Leaf number

The number of leaves per explant was significantly affected by variety, and interactions of variety with media and RJ (P d" 0.01). Dubrovnik produced the most leaves, followed by Andorra and Bucharest, with Nottingham and Memphis lower (Fig. 10–12). RJ at 300 ppm enhanced leaf number in most varieties, while 450 ppm caused a reduction in some cases, indicating a threshold beyond which excess RJ may be inhibitory. Media effects were also evident; ½ MS + 2 gL{ ¹ peptone increased leaf number in some cultivars, demonstrating the combined importance of protein supplementation and RJ nutrients in shoot proliferation (Salsabila et al., 2022).

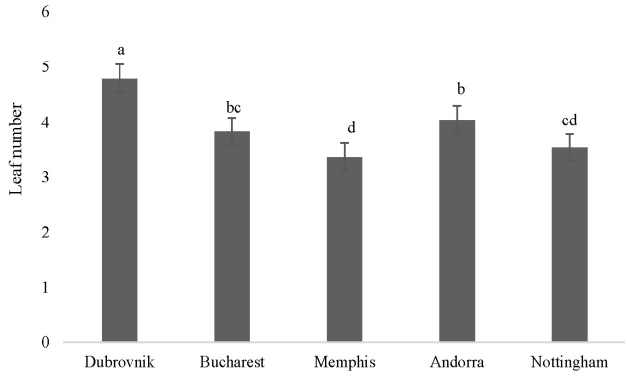

Fig. 10 : Number of leaves per explant in different *Phalaenopsis* varieties

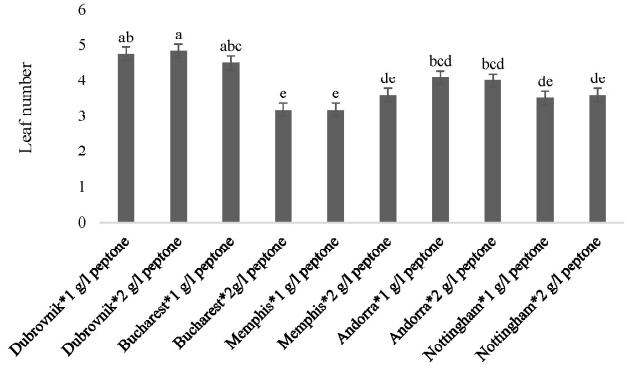

Fig. 11 : Interaction effect of culture media and *Phalaenopsis* varieties on leaf number per explant

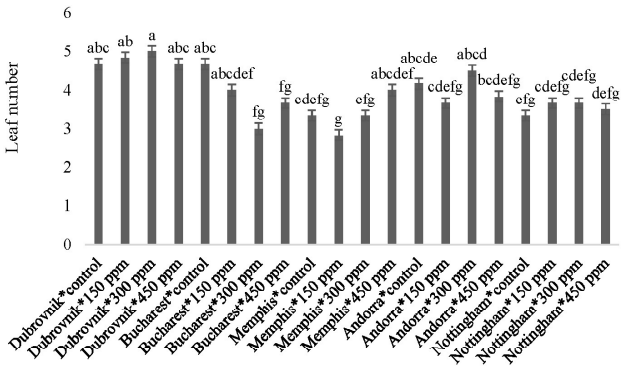

Fig. 12 : Effect of different royal jelly concentrations and *Phalaenopsis* varieties on leaf number per explant

Root number

Root number per explant was significantly influenced by variety, medium, RJ concentration, and their interactions (P d" 0.01). Dubrovnik consistently produced the highest root numbers, while Bucharest was lowest (Fig. 13–15). RJ enhanced root number across varieties, with 450 ppm producing the greatest effect in Dubrovnik (10.33 roots per explant) versus 6.17 in Bucharest without RJ. Interestingly, media with ½ MS + 1 gL{ ¹ peptone combined with RJ produced more roots than 2 gL{ ¹ peptone, possibly due to a balance between protein content and other nutrients provided by RJ. These findings align with previous reports where honey or coconut water enhanced rooting, indicating that organic additives rich in amino acids and vitamins stimulate root initiation and proliferation (Obaid et al., 2020; Salsabila et al., 2022).

The study demonstrates that RJ is an effective organic additive for *Phalaenopsis* micropropagation. Optimal germination and seedling development were achieved with 150–300 ppm RJ, depending on cultivar and

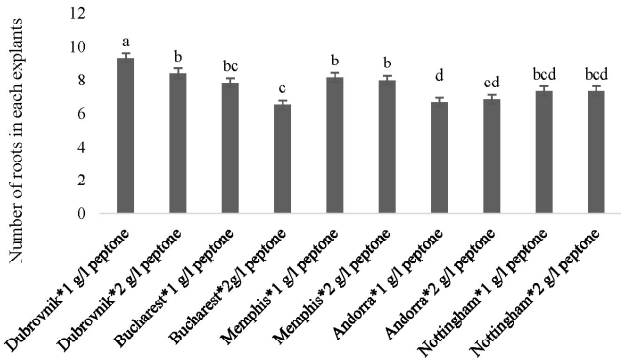

Fig. 13 : Interaction effect of two culture media and *Phalaenopsis* varieties on root number per explant

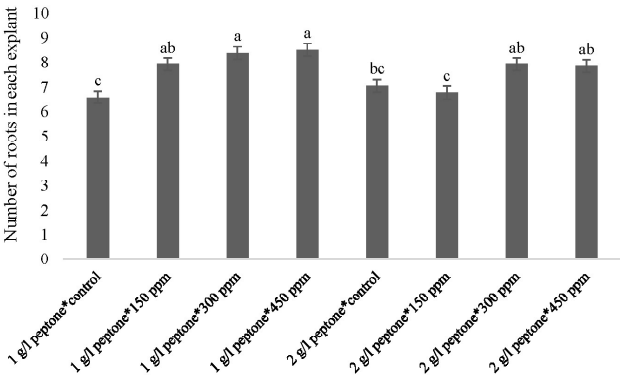

Fig. 14 : Effect of royal jelly concentrations on root number per explant in different *Phalaenopsis* varieties

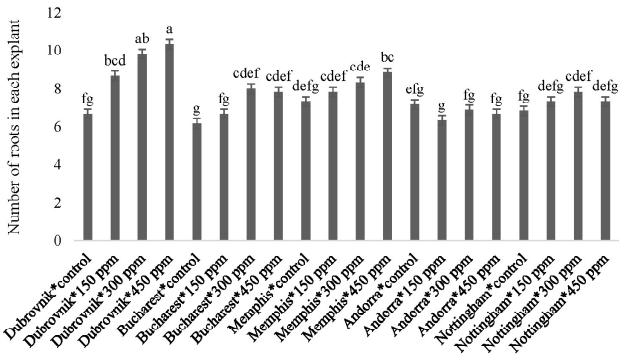

Fig. 15 : Effect of royal jelly treatments in two culture media on root number per explant in *Phalaenopsis*

media composition. Higher concentrations (450 ppm) occasionally reduced growth, highlighting the need for dose optimization. RJ likely promotes morphogenesis through its content of proteins, amino acids, vitamins, and bioactive compounds, which may complement or substitute traditional peptone or synthetic growth regulators. These results support the potential of RJ as a cost-effective, sustainable alternative for commercial orchid propagation, and indicate that both genotype and culture conditions must be considered when designing tissue culture protocols.

CONCLUSION

Royal jelly is an effective organic supplement for *Phalaenopsis* micropropagation. Among the five varieties studied, Nottingham consistently showed the highest germination (96.33% at 300 ppm RJ in ½ MS + 2 gL{ ¹ peptone) and fastest leaf primordia formation, while Dubrovnik exhibited the highest leaf and root numbers per explant. RJ concentrations of 150–300 ppm generally enhanced germination, shoot, and root development, whereas 450 ppm occasionally reduced these traits, indicating a dose-dependent effect. Media composition also influenced outcomes, with ½ MS + 2 gL{ ¹ peptone favoring germination and leaf formation, and ½ MS + 1 gL{ ¹ peptone promoting root proliferation when combined with RJ.

These findings suggest that royal jelly can serve as a cost-effective, sustainable alternative to synthetic growth regulators in orchid tissue culture. Further studies are recommended to isolate the bioactive components of RJ responsible for these effects and to test the scalability and reproducibility of RJ supplementation across additional *Phalaenopsis* cultivars and other orchid species.

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REFERENCES

Altun, S. K., & Aydemir, M. E. (2022). Determination of antioxidant capacities with the phenolic flavonoid contents of royal jelly mixtures. *Bee Studies*, *14*(1), 9–15. https://doi.org/10.51458/BSTD.2022.22

Bidarnamani, F., Mohkami, Z., & Karimian, Z. (2023). Cluster analysis helps to select appropriate pollination states by crossing of *Phalaenopsis* varieties. *Agroteqnique in Industrial Crops*, *3*(4), 223–229. https://doi.org/10.22126/atic.2023.9725.1117

Bidarnamani, F., Mohkami, Z., & Karimian, Z. (2024). Pollination phenotypes in *Phalaenopsis* crosses: Guiding selection for optimal breeding. *Journal of Horticulture Science*, *19*(2). https://doi.org/10.24154/jhs.v19i2.340

Boukraâ, L., Niar, A., Benbarek, H., & Benhanifia, M. (2008). Additive action of royal jelly and honey against *Staphylococcus aureus*. *Journal of Medicinal Food, 11*(1), 190–192. https://doi.org/10.1089/jmf.2007.567

Chew, Y.-C., Halim, M. H. A., Abdullah, W. M. A. N. W., Abdullah, J. O., & Lai, K. S. (2018). Highly efficient proliferation and regeneration of protocorm-like bodies (PLBs) of the threatened orchid, *Phalaenopsis bellina*. *Sains Malaysiana*, *47*, 1093–1099. https://doi.org/10.17576/jsm-2018-4706-03

Fujiwara, S., Imai, J., Fujiwara, M., Yaeshima, T., Kawashima, T., & Kobayashi, K. (1990). A potent antibacterial protein in royal jelly: Purification and determination of the primary structure of royalisin. *Journal of Biological Chemistry*, *265*(19), 11333–11337. https://doi.org/10.1016/S0021-9258(19)38596-5

Gnasekaran, P., Rathinam, X., Sinniah, U. R., & Subramaniam, S. (2010). A study on the use of organic additives on the protocorm-like bodies (PLBs) growth of *Phalaenopsis violacea* Orchid. *Journal of Phytology*, *2*, 29–33.

Heriansyah, P., & Indrawanis, E. (2020). Uji tingkat kontaminasi eksplan anggrek Bromheadia. *Jurnal Agroqua*, *18*(2), 223–232. https://doi.org/10.32663/ja.v18i2.1502

Isda, M. N., Amin, N. A., & Fatonah, S. (2019). Effect of 6-benzylaminopurine and honey for *in vitro* shoot initiation of mangosteen seed explant from Riau, Indonesia. *Journal of Physics*. https://doi.org/10.1088/1742-6596/1351/1/012029

Islam, M. O., Rahman, A. R. M., Matsui, S. H., & Prodhan, A. D. (2003). Effect of complex organic extracts on callus growth and PLB regeneration through embryogenesis in the Doritaenopsis orchid. *Japan Agricultural Research Quarterly*, *37*(4), 229–235. https://doi.org/10.6090/jarq.37.229

Mose, W., Daryono, B. S., Indrianto, A., Purwantoro, A., & Semiarti, E. (2020). Direct somatic embryogenesis and regeneration of an Indonesian orchid *Phalaenopsis amabilis* (L.) Blume under a variety of plant growth regulators, light regime, and organic substances. *Jordan Journal of Biological Sciences*, *13*(4), 509–518. https://doi.org/10.3390/jjbs.v13i4.509

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, *15*(3), 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Obaid, O. H., Al-juhashi, H. A. N., & Al-masoody, M. M. (2020). Effect of honey in the sterilization of explant *in vitro*. *Plant Archives*, *20*(2), 2031–2035.

Paek, K. Y., Hahn, E. J., & Park, S. Y. (2011). Micropropagation of *Phalaenopsis* via protocorms and protocorm-like bodies. In *Methods in Molecular Biology* (Vol. 710, pp. 293–306). https://doi.org/10.1007/978-1-61737-988-8_20

Ramadoss, P., Rangarajan, R., & Pavani, K. (2015). Investigation of royal jelly as a promising substitute to fetal bovine serum in cell culture. *Annals of Biological Research*, *6*(6), 60–64.

Salsabila, S. N., Fatimah, K., Noorhazira, S., Halimatun, T. S. T. A. B., Aurifullah, M., & Suhana, Z. (2022). Effect of coconut water and peptone in micropropagation of *Phalaenopsis amabilis* (L.) Blume orchid. *4th International Conference on Tropical Resources and Sustainable Sciences.* https://doi.org/10.1088/1755-1315/1102/1/012002

Setiani, N. A., Nurwinda, F., & Astriany, D. (2018). Pengaruh desinfektan dan lama perendaman pada sterilisasi eksplan daun sukun (*Artocarpus altilis* (Parkinson ex. F.A. Zorn) Fosberg). *Biotropika: Journal of Tropical Biology*, *6*(3), 78–82. https://doi.org/10.21776/ub.biotropika.2018.006.03.01

Wahyuni, F. D., Moda, K. F., Irvanash, D. R., & Sari, N. (2021). Substitution of synthetic hormones with organic materials on the growth of orchid plants (*Phalaenopsis amabilis*) as a growth regulatory substance *in vitro*. *Bioedukasi*, *19*(2), 110–117. https://doi.org/10.19184/bioedu.v19i2.27141

Wasiati, A. R., Nugraheni, I. A., & Setiawati, Y. (2021). The combination of Murashige and Skoog (MS) media and activated charcoal on the growth of the *Vanda helvola* orchid plant *in vitro*. *International Journal of Health Science and Technology*, *3*(1), 159–170. https://doi.org/10.31101/ijhst.v3i1.2247

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