Asian Journal of Plant Sciences

ISSN 1682-3974

science alert

ANSInet
an open access publisher
http://ansinet.com

OPEN ACCESS Asian Journal of Plant Sciences

ISSN 1682-3974
DOI: 10.3923/ajps.2026.62.69

Research Article
Cytokinin-Specific Modulation of Protocorm-Like Bodies (PLB) Formation and Shoot Organogenesis in *Habenaria radiata* L.

[1]Rifa Fadhilah Munifah Hasibuan, [2]Kazuhiko Takahashi, [1]Deng Tingrong, [2]Sakura Takahashi and [2]Sakae Suzuki

[1]United Graduated School of Agriculture, Tokyo University of Agriculture and Technology, Saiwaicho 3-5-8, Fuchu, Tokyo 183-8509, Japan
[2]Faculty of Agriculture, Tokyo University of Agriculture and Technology, Saiwaicho 3-5-8, Fuchu, Tokyo 183-8509, Japan

Abstract

Background and Objective: *Habenaria radiata* L. (white egret orchid) is a Near-Threatened terrestrial orchid which conservation cultivation depends on efficient *in vitro* propagation. Protocorm-like body (PLB) proliferation offers rapid clonal multiplication, whereas direct shoot organogenesis yields transplant-ready plantlets; both pathways are strongly regulated by cytokinin. We compared the effects of three cytokinin: 6-benzylaminopurine (BA); kinetin; thidiazuron (TDZ) on PLB and shoot induction in seed derived protocorms of *H. radiata.* **Materials and Methods:** The study evaluated cytokinin-mediated protocorm-like body (PLB) formation and shoot organogenesis in *H. radiata* 'Aoba wild type' under *in vitro* conditions. Seeds were germinated on half-strength MS medium and seedlings were treated with different cytokinins (BA, kinetin, TDZ). The PLB and shoot responses were assessed after 32 weeks and data were analyzed using one-way ANOVA at $p<0.05$ to identify the most effective hormonal treatment for large-scale propagation and conservation. **Results:** The TDZ (90% PLB) were the strongest inducers of PLB. By contrast, BA favored direct shoot formation. The results show that the nature of cytokinin influences the regeneration outcome in *H. radiata* showed that BA promote the growth of organogenesis shoots, while TDZ and kinetin explants promote toward somatic embryogenic PLB path. **Conclusions:** Selecting an appropriate cytokinin therefore allows propagation protocols to be tailored either to mass-produce PLB for scale-up or to generate shoot-bearing plantlets for rapid acclimatization. These insights advance the micropropagation for *H. radiata* and contribute to ex-situ conservation strategies for this orchid.

Key words: *Habenaria radiata,* cytokinin, protocorm-like bodies, shoot organogenesis

Citation: Hasibuan, R.F.M., K. Takahashi, D. Tingrong, S. Takahashi and S. Suzuki, 2026. Cytokinin-specific modulation of protocorm-like bodies (PLB) formation and shoot organogenesis in *Habenaria radiata* L. Asian J. Plant Sci., 25: 62-69.

Corresponding Author: Sakae Suzuki, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Saiwaicho 3-5-8, Fuchu, Tokyo 183-8509, Japan

Funding: Research Project was partially sponsored by Setagaya City, Tokyo, Japan [project number 5160114101] with grant number 300,000 JPY.

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Habenaria radiata L. (Thunb.) Spreng. (syn. *Pecteilis radiata*), commonly known as the white egret flower, is a terrestrial orchid native to East Asia. It bears striking white flowers resembling a flying egret. Wild populations of *H. radiata* have declined due to habitat loss and overcollection and it is listed as Near Threatened in Japan[1]. One of the most effective strategies for orchid micropropagation is the induction of protocorm-like bodies (PLB) *in vitro*[2]. PLB are meristematic structures that can regenerate into new plantlets and they often form readily from various explants in culture, essentially acting as somatic embryos for clonal Cardoso *et al*.[2]. Micropropagation via PLB is more efficient than plantlet development from seeds or adventitious shoots. This efficiency arises from the rapid proliferation of PLB on solid or liquid culture media, allowing for the production of a large quantity of PLB in a short period[3]. *H. radiata* is notably harder to re-programmed into PLB than many epiphytic orchids because its temperate, tuber-forming biology increases several culture bottlenecks: Wounding releases abundant phenolics that oxidized rapidly, darken the medium and arrest cell division[4]. The species enters a prolonged winter dormancy in nature and without a preceding cold-moist stratification or short day chill, protocorms remain in a quiescent, storage-oriented state that is poorly responsive to cytokinin-induced meristem formation[5]. Tuberous orchids accumulate abscisic and jasmonic acid during dormancy, yielding a low endogenous cytokinin:auxin ratio that requires unusually high or potent exogenous cytokinin to trigger PLB genes[6]. It is hypothesized that fungal partners may supply signaling molecules (e.g., peptides or cytokinin like compounds) that trigger meristem formation in protocorms, absence of these cues in sugar based media may delay or block shoot identity acquisition[7,8]. Viable meristematic explants are season-limited. Mature leaves senescence and tuber tissues oxidized rapidly, so only shoot apices, florets or early protocorms collected at precise developmental windows give reasonable PLB yields[9]. Together, these physiological and technical hurdles make *H. radiata* markedly more "recalcitrant" for PLB induction than the tropical epiphytes on which most standard orchid micropropagation protocols were developed.

Cytokinin play a crucial role in these processes by modulating cell division, differentiation and organogenesis. Among the most widely used cytokinin in orchid tissue culture are kinetin, 6-benzyladenine (BA) and thidiazuron (TDZ), each demonstrating unique effects on morphogenesis, particularly in terrestrial orchids such as those in the *Habenaria* genus[10]. Cytokinin are known to play a central role in inducing PLB formation and shoot organogenesis in many orchid species[11]. *In vitro* studies have shown that adding cytokinin to the medium promotes cell division and adventitious bud or PLB initiation, whereas explants grown without plant growth regulators typically exhibit poor growth or browning. However, the optimal type and concentration of cytokinin can vary widely among species and even by explant type[12]. The TDZ is one of the most potent cytokinin for de novo regeneration in plant tissue culture and it has been widely used to induce organogenesis and somatic embryogenesis across hundreds of plants species. Notably, TDZ often triggers high frequencies of shoot or embryogenic structure formation even at low concentrations[13]. The BA is a stable synthetic cytokinin known to stimulate shoot bud formation and PLB proliferation in numerous orchids[2], while kinetin proven promotes shoot differentiation in some species[14]. Given these varied effects, a comparative evaluation of different cytokinin on *H. radiata* development is needed.

In *H. radiata* prior research on tissue culture is limited. Aseptic seed germination protocols have been established, but efficient regeneration of shoots or tubers remains challenging[9]. This study aims to determine how different cytokinin (BA, kinetin and TDZ) influence two regeneration pathways in *H. radiata* PLB formation versus direct shoot induction.

MATERIALS AND METHODS

Study area: The study was conducted from October 2023 to July 2025.

Plant material and culture conditions seeds of *H. radiate*: Mature seed pods of *H. Radiata 'Aoba wild type'* cultivar were obtained from greenhouse-grown plants from Tsukuba prefecture, Japan. Seeds were surface sterilized by immersion in 1% sodium hypochlorite solution for 15 min, followed by three rinses in sterile distilled water. The sterilized seeds were sown on culture medium within 2 hrs of sterilization. Sown on Murashige and Skoog[15] basal medium. Following germination, protocorm development was closely monitored under aseptic conditions.

The basal medium was half-strength Murashige and Skoog (1/2MS) medium supplemented with 30 g/L sucrose and solidified with 8 g/L agar. The pH was adjusted to 5.8 before autoclaving (121°C for 15 min). No organic additives or auxins were included, to isolate the effects of cytokinin. Five different hormone treatments were compared: (1) Control (no plant growth regulator), (2) 1.0 mg/L 6-benzylaminopurine (BA), (3) 1.0 mg/L kinetin, (4) 1.0 mg/L TDZ.

The media were dispensed in 100 mL aliquots into sterile culture tube. Approximately 10-20 seeds were inoculated per jar for germination. For each treatment, n = 20 explants (defined as germinated protocorms or very young seedlings) were eventually used to assess PLB per shoot formation. Cultures were maintained in a growth chamber at 25±2°C under a 16 hrs photoperiod with white-LED light.

Experimental design for PLB optimization to enhance PLB production: Seedlings were transferred into various liquid culture media containing different concentrations and combinations of cytokinin: kinetin; BA; and TDZ. Formulated on a basal MS medium. The experiment was arranged in a completely randomized design with the five cytokinin treatments. After 32 weeks of incubation, each explant was evaluated for formation of PLB, production of shoots (development of one or more true leafy shoots) and overall morphology. An explant could exhibit one or both types of regeneration. However, in our observations these results were usually distinct (PLB often formed from swollen protocorms or callus, whereas shoots arose as elongated structures directly). Explants showing at least one PLB were counted as PLB-positive and those with one or more distinct shoots were counted as shoot-positive.

Evaluation of PLB response: This methodology aimed to determine the most effective hormonal combination, explant type and growth stage to facilitate large-scale *in vitro* propagation, supporting the *ex situ* conservation and restoration efforts of *H. radiata*. The percentage of explants forming PLB and the percentage producing shoots were calculated for each treatment. A One-Way Analysis of Variance (ANOVA) was used to compare the means of treatments at a significance level of $p<0.05$. All statistical analyses were performed using software from R Core Team[16].

Statistical analysis: The data were statistically analyzed using software from R Core Team[16]. This methodology aimed to determine the most effective hormonal combination, explant type and growth stage to facilitate large-scale *in vitro* propagation, supporting the *ex situ* conservation and restoration efforts of *H. radiata*.

RESULTS AND DISCUSSION

Explant germination: Across all treatments, *H. radiata* seeds began swelling and germinating into protocorms with in 16 weeks. By 32 weeks, the majority of viable seeds had formed protocorms (green spherical structures 2-3 mm in diameter) on all media, though their subsequent development differed markedly by treatment (Fig. 1a-d). In the hormone-free control, protocorms remained small and mostly dormant or slowly converting into tiny plantlets. In cytokinin-supplemented media, protocorms often showed active organogenesis (either PLB proliferation or shoot formation, depending on the cytokinin type).

Morphological observations: Figure 1 provides a visual comparison of cultures with and without different type of cytokinin. In the absence of cytokinin (Fig. 2a), explants remained in vegetative state, with minimal organogenic response. In the presence of an effective cytokinin like TDZ (Fig. 2b), explants proliferated numerous green PLB structures. BA (Fig. 1b) showed elongation of organ both in leaves and roots with no PLB formation. Shoots induced by kinetin (Fig. 1d) were maintained small size (no elongation), emerging from protocorms without intervening PLB. Notably, TDZ at 1.0 mg/L (Fig. 1c) caused slight callusing at protocorm bases, but this callus was organogenic, giving rise to PLB. No obvious hyper-hydricity was seen in any treatment; all shoots and PLB appeared morphologically normal. The overall health of explants was best on cytokinin-supplemented media.

PLB induction: The regenerative response of *H. radiata* was cytokinin-specific and dominated by thidiazuron (TDZ) (Table 1). It outperformed all other treatments in PLB formation, producing 3.2 per explant, whereas kinetin induced only 1.0 and both BA and the control failed to induce any. In contrast, BA was the most effective treatment for root induction, yielding 4.6 roots per explant. Shoot production was significantly enhanced by all cytokinins compared with the control (1.0), with TDZ (2.8), kinetin (2.6) and BA (2.2) producing statistically comparable numbers of shoots, yet all markedly exceeding the untreated explants.

Biomass accumulation further distinguished treatments: BA generated the greatest fresh weight (4.361 g), surpassing TDZ (3.735 g) and far exceeding the control (1.027 g) and kinetin (0.780 g). Leaf production followed a similar pattern, with TDZ (3.8 leaves) and BA (3.2 leaves) significantly outperforming the control (1.6), whereas kinetin completely failed to induce leaf formation. Notably, tuber production was dramatically higher under TDZ (3.8 per bulb), while kinetin produced none and both BA (1.4) and the control (1.0) remained comparatively low. Collectively, TDZ was distinctly better for PLB and tuber induction, BA was most effective for root proliferation and biomass gain and kinetin consistently exhibited the weakest overall regenerative performance.

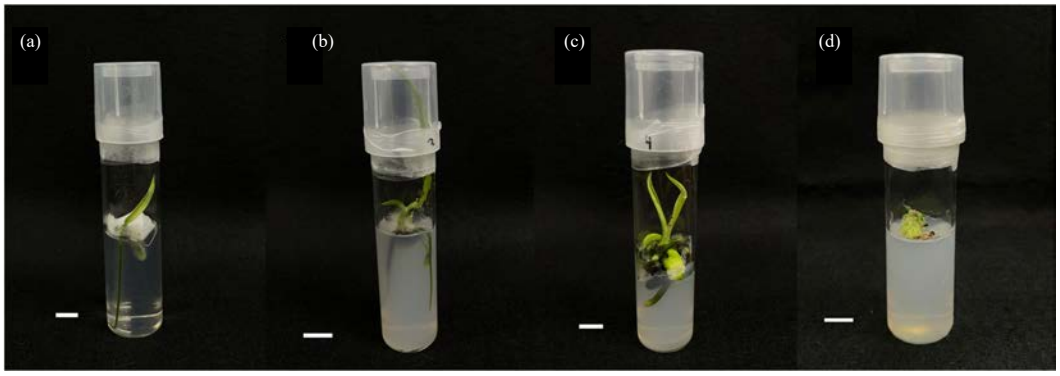

Fig. 1(a-d): Cytokinin effects on protocorm-like body (PLB) and shoot induction in *Habenaria radiata* after 32 weeks in culture (scale bar 1 cm), (a) Control (no plant hormone), (b) 1 ppm BA: 6-Benzylaminopurine, (c) 1ppm TDZ: Thidiazuron and (d) 1 ppm Kinetin

Fig. 2(a-b): Morphology of cytokinin effects on in *Habenaria radiata* after 32 weeks in culture, (a) Control and (b) TDZ treatment (scale bar 1 cm)

Figure 1 shows that TDZ (Fig. 1c) drove the highest proportion of explants to form PLB (90%), whereas kinetin (Fig. 1d) induced a moderate response (60%); BA (Fig. 1b) and the hormone-free control (Fig. 1a) all showed no visible PLB. The reciprocal trend is apparent for direct shoot organogenesis. Morphological panels confirmed the quantitative data. The TDZ explants in Fig. 3a exhibit with bright-green nodular PLB, while no cytokinin explants in Fig. 3b display smooth, uniformly green tuber with no sign of PLB.

Shoot induction: Shoot organogenesis followed an opposite trend. The TDZ yielded the greatest shoot formation followed by kinetin and BA statisctically higher than control. Shoots on TDZ, BA and control media were well-defined, bearing juvenile leaves and incipient roots, whereas the few shoots that arose on kinetin media were shorter and often developed from PLB rather than directly from the protocorm surface. Altogether, the figure highlights a clear dichotomy: TDZ and BA channel regeneration toward prolific PLB production, whereas kinetin and favour direct shoot organogenesis.

Control treatment: In the absence of cytokinin (control), *H. radiata* protocorms showed limited morphogenesis (Fig. 3a-b). Control protocorms mostly either remained as single protocorms or slowly developed a couple of tiny leaves without forming distinct shoots or additional PLB. All five

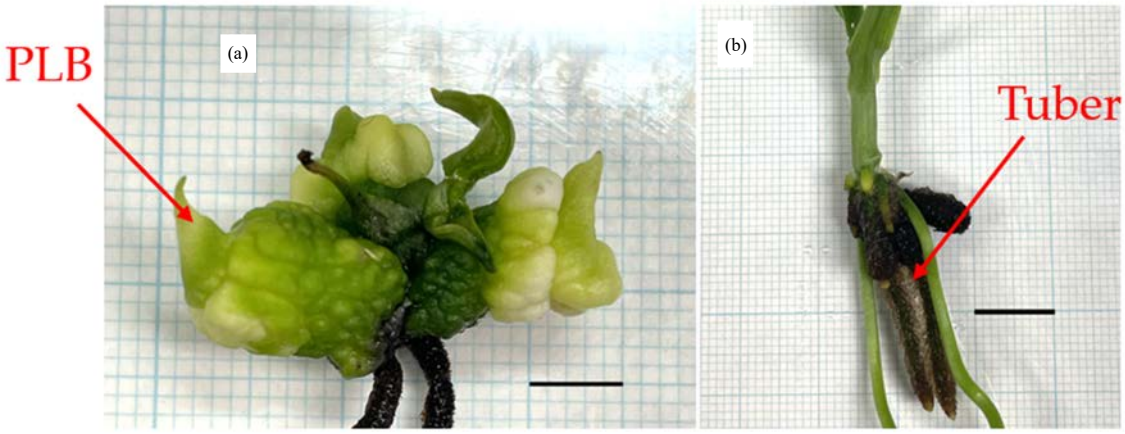

Fig. 3(a-b): Morphology of cytokinin effects on in *Habenaria radiata* after 32 weeks in culture (a) PLB and (b) Natural tuber (scale bar 1 cm)

Table 1: The different cytokinin effects on *Habenaria radiata* after 32 weeks in culture (PLB: Protocorm Like Body)

Treatment	Number of PLB	Number of root	Number of shoot	Weight (g)	Number of leaves	Number of tuber per bulb
Control	0.0^{c}	1.4^{b}	1.0^{b}	1.027^{b}	1.6^{b}	1.0^{b}
BA	0.0^{c}	4.6^{a}	2.2^{a}	4.361^{a}	3.2^{a}	1.4^{b}
TDZ	3.2^{a}	2.4^{b}	2.8^{a}	3.735^{a}	3.8^{a}	3.8^{a}
Kinetin	1.0^{b}	1.0^{c}	2.6^{a}	0.78^{b}	0.0^{c}	0.0^{c}

Values in the same column followed by different superscript letters (a, b, c) indicate significant differences at p>0.05, PLB: Propagule-like bodies, BA: Benzyladenine and TDZ: Thidiazuron

control explants exhibited browning at their bases over time and appeared less vigorous, consistent with the known requirement of growth regulators for sustained orchid explant development[12].

This study demonstrates that the type of cytokinin present in the culture medium has a significant influence on whether *H. radiata* regenerates via PLB or through direct shoot organogenesis. All cytokinin we assessed improved regenerative response compared to no growth regulator, but they differed in the pathway they promoted. The TDZ and kinetin strongly enhanced PLB formation, while BA was more effective in stimulating the outgrowth of shoots.

The promotive effect of cytokinin on orchid regeneration is well documented. Cytokinin drive cell division and meristem formation, which is essential for organogenesis *in vitro*[12]. In this experiment control (no cytokinin) culture exhibited little development, a common outcome as orchid tissues often requires exogenous growth regulators to avoid stagnation or browning. By contrast, all cytokinin-supplemented treatments produced morphogenic responses, underscoring that cytokinin were the key drivers of regeneration in these experiments.

The TDZ was the most potent inducer of PLB in *H. radiata*. At 1 mg/L, TDZ yielded 90% PLB formation. TDZ's strong morphogenic activity aligns with its known role as a powerful cytokinin-like regulator in tissue culture. TDZ has been widely used to induce de novo shoots or somatic embryos in recalcitrant species[13]. In orchids specifically, several studies have found TDZ to outperform adenine-type cytokinin for inducing PLB or embryogenic callus. For example Gantait *et al.*[17] reported that in a hybrid orchid (*Mokara*× *Ascocenda*), TDZ yielded the highest PLB induction frequency among cytokinin assessed Nongdam *et al.*[6]. The TDZ has mode of action may involve not only cytokinin receptor stimulation but also modulation of endogenous auxin levels and other hormonal crosstalk[13]. Interestingly, we observed that TDZ in *H. radiata* led to prolific PLB but almost no direct shoots. This suggests that TDZ channels the developmental program towards an embryogenic route (PLB), possibly by inducing a broad meristematic competence in cells, rather than triggering organized shoot meristems. Similar phenomena have been noted in African violet which showed that low TDZ induces shoots, whereas higher TDZ induces somatic embryo-like structures[13]. In current case, 1 mg/L TDZ have been effectively a high concentration for *H. radiata* maximizing PLB (somatic embryo) formation.

The BA failed to induce any detectable PLB formation in *H. radiata* under the condition. Although BA is widely regarded as a reliable inducer of organogenesis in many orchids, its benzyl substituent confers metabolic stability

that often enhances meristem initiation. Our data indicate that, for *H. radiata* BA alone does not re-programme protocorm tissue toward the embryogenic PLB pathway. This contrasts with reports in *Phalaenopsis violacea*, where 0.8 µM BA produced PLB in 70% of explants while an equimolar TDZ treatment yielded only 40% Cardoso *et al*.[2], emphasizing the pronounced species-specificity of cytokinin responses. The limited but consistent shoot production we observed on BA suggests that, in *H. radiata* BA favours direct shoot meristem activation rather than indirect PLB formation, possibly by sustaining chlorophyll content and leaf primordium expansion rather than driving cells into a pluripotent embryogenic state.

Kinetin behaved differently from TDZ/BA; in that they favored direct callus organogenesis over PLB formation in *H. radiata*. Kinetin produced the highest callus formation and shoot induction (25%). Kinetin is one of the first discovered cytokinin, less potent in many systems but sometimes effective for shoot regeneration. Our findings suggest kinetin can trigger the development of apical shoots from protocorm tissue, possibly by promoting organized meristem development rather than unorganized proliferation. The fact that kinetin alone yielded statistically the same results shoot number as TDZ and BA in *H. radiata* implies that this cytokinin might mimic or induce the hormonal conditions conducive to apical dominance and shoot growth. Kinetin at 1 mg/L may be a relatively mild stimulus, allowing the intrinsic developmental program of the protocorm to proceed to leafy shoot stage rather than reprogramming cells into PLB.

This result have practical implications for optimizing *H. radiata* propagation. If the goal is clonal multiplication via PLB (which can then be subdivided or encapsulated for mass propagation), TDZ would be the cytokinin of choice. These treatments produced a carpet of PLB that could likely be sub-cultured to generate numerous plantlets. On the other hand, Kinetin can be use if the goal is to produce fully formed plantlets in a single step (avoiding the need to differentiate PLB), as they yielded higher rates of direct shoot formation. It is noteworthy that the absence of cytokinin led to negligible shoot production, underscoring that an exogenous cytokinin is necessary to obtain shoots in *H. radiata* tissue culture. This is similar to many other orchids where a cytokinin is required to break protocorm dormancy and initiate shoot development[12].

Mose *et al*.[18] demonstrated that TDZ effectively induces PLB and direct somatic embryogenesis in *Phalaenopsis* by stimulating intense cellular reprogramming at low concentrations. This aligns with our finding in *H. radiata* where TDZ produced the highest PLB induction, suggesting that its strong cytokinin-like activity promotes embryogenic rather than organogenic differentiation. The study further explained that TDZ's unique urea-type structure enhances endogenous cytokinin accumulation and alters auxin metabolism, favoring dedifferentiation and meristematic competence. Similar hormonal modulation likely occurred in *H. radiata*, shifting developmental fate toward PLB formation.

For conservation purposes, a high multiplication rate is often desired. The PLB route (as achieved by TDZ) can offer exponential propagation, since each PLB can be subdivided or can give rise to secondary PLB[2]. The trade-off is that an extra step is needed to convert PLB into plantlets (usually by transferring to a medium that promotes PLB germination or shoot development, often with reduced cytokinin or added auxin). In contrast, kinetin yielding direct shoots might shorten the production cycle by producing rooted shoots directly, but the number of shoots per explant is limited. An efficient strategy might be to first use TDZ to amplify the material as PLB, then switch to a shoot-induction medium (there is possibility containing kinetin or a lower cytokinin concentration) to convert those PLB to plantlets. Similar two-stage protocols have been successful in other species. For example, Singh and Dwivedi[14] first induced multiple shoots with TDZ and then elongated them on a lower TDZ medium, analogous approaches could be tried for *H. radiata*.

Prior work on related orchids can explained more on our findings. In a study on *H. radiata* L. seed germination, Kim *et al*.[4] found that adding TDZ (along with activated charcoal) greatly enhanced protocorm development and subsequent seedling growth compared to hormone-free medium. This aligns with our result that TDZ is highly beneficial for *H. radiata* development. However, their work primarily emphasized germination percentage without assessing the effects of different cytokinins. In contrast, our study broadens this understanding by directly comparing the responses of *H. radiata* to multiple cytokinin types. Another relevant report is by Gantait *et al*.[17] and colleagues, who noted that in *Mokara* orchid leaf cultures, TDZ at all tested levels outperformed BA for PLB induction. This supports our finding of TDZ has superior compound for PLB in *H. radiata*. This work provides a foundation for refining *H. radiata* micropropagation. Future experiments could explore cytokinin concentration effects (e.g., using lower TDZ doses to see if shoots increase at the expense of PLB) or combinations of cytokinin with a low level of auxin. Now that this research has identified TDZ as strong inducers, one could evaluate if repeating subculture on those media further multiplies the PLB. Eventually, acclimatization of regenerated plantlets will be necessary; prior studies on *H. radiata* suggest that plantlets derived from tissue culture can be successfully established in soil after a hardening period[9].

CONCLUSION

In summary, our findings underscore the importance of cytokinin selection in orchid tissue culture. By choosing a cytokinin that aligns with the desired outcome (mass PLB compared with direct shoots) will tailoring the micropropagation protocol. For *H. radiata*, TDZ are excellent for generating PLB in higher numbers, while kinetin can be used when the development of shoots (and ultimately whole plantlets) is preferred. Given the conservation concerns for this species, the ability to produce dozens of clones from seed-derived protocorms using TDZ-induced PLB, could help augment nursery stocks and reduce pressure on wild populations. This study contributes practical knowledge for *H. radiata* propagation and adds to the broader understanding that different cytokinin can distinctly modulate the morphogenic route in orchid cultures.

SIGNIFICANCE STATEMENT

The white egret orchid is a rare and increasingly threatened plant species, making its conservation a priority. Efficient propagation techniques are critical for preserving this species and supporting biodiversity. This study demonstrates how specific plant hormones can be strategically used to control the development of orchid seedlings, either by producing large numbers of embryo-like structures for mass propagation or by promoting the direct formation of shoots ready for transplantation. These findings provide practical guidance for laboratory-based propagation, offering a valuable tool for conserving the white egret orchid and enhancing sustainable horticultural practices for rare and endangered plant species.

ACKNOWLEDGMENTS

The authors would especially like to thank the Ministry of Education, Culture, Sports, Science and Technology (MEXT) for the scholarship to H.R.F.M.

REFERENCES

1. Takeda, S., Y. Nishikawa, T. Tachibana, T. Higaki, T. Sakamoto and S. Kimura, 2025. Morphological and transcriptome analysis of the near-threatened orchid *Habenaria radiata* with petals shaped like a flying white bird. Plants, Vol. 14. 10.3390/plants14030393.
2. Cardoso, J.C., C.A. Zanello and J.T. Chen, 2020. An overview of orchid protocorm-like bodies: Mass propagation, biotechnology, molecular aspects, and breeding. Int. J. Mol. Sci., Vol. 21. 10.3390/ijms21030985.
3. Wei, M., S.T. Jiang and J.P. Luo, 2007. Enhancement of growth and polysaccharide production in suspension cultures of protocorm-like bodies from *Dendrobium huoshanense* by the addition of putrescine. Biotechnol. Lett., 29: 495-499.
4. Kim, D.H., K.W. Kang, G. Enkhtaivan, U. Jan and I. Sivanesan, 2019. Impact of activated charcoal, culture medium strength and thidiazuron on non-symbiotic *in vitro* seed germination of *Pecteilis radiata* (Thunb.) Raf. S. Afr. J. Bot., 124: 144-150.
5. Poff, K.E., J. Sharma and M. Richards, 2016. Cold-moist stratification improves germination in a temperate terrestrial orchid. Castanea, 81: 292-301.
6. Nongdam, P., D.G. Beleski, L. Tikendra, A. Dey, V. Varte, S. EL-Merzougui, V.M. Pereira, P.R. Barros and W.A. Vendrame, 2023. Orchid micropropagation using conventional semi-solid and temporary immersion systems: A review. Plants, Vol. 12. 10.3390/plants12051136.
7. Cowden, C.C. and R.P. Shefferson, 2013. Diversity of root-associated fungi of mature *Habenaria radiata* and *Epipactis thunbergii* colonizing manmade wetlands in Hiroshima Prefecture, Japan. Mycoscience, 54: 327-334.
8. Li, T., S. Wu, W. Yang, M.A. Selosse and J. Gao, 2021. How mycorrhizal associations influence orchid distribution and population dynamics. Front. Plant Sci., Vol. 12. 10.3389/fpls.2021.647114.
9. Mitsukuri, K., T. Arita, M. Johkan, S. Yamasaki, K.I. Mishiba and M. Oda, 2009. Effects of type of explant and dark preconditioning on bud formation in *Habenaria radiata* (Thunb.) *in vitro*. HortScience, 44: 523-525.
10. Giri, C.C. and M. Praveena, 2015. *In vitro* regeneration, somatic hybridization and genetic transformation studies: An appraisal on biotechnological interventions in grasses. Plant Cell Tissue Organ Cult., 120: 843-860.
11. Fritsche, Y., F. Deola, D.A. da Silva, D.F. Holderbaum and M.P. Guerra, 2022. *Cattleya tigrina* (Orchidaceae) *in vitro* regeneration: Main factors for optimal protocorm-like body induction and multiplication, plantlet regeneration, and cytogenetic stability. S. Afr. J. Bot., 149: 96-108.
12. Media, Z.A. Noli and M. Idris, 2023. An overview: Effect of plant growth regulatory on orchid propagation through the thin cell layer technique. Int. J. Progressive Sci. Technol., 39: 340-345.
13. Erland, L.A.E., R.T. Giebelhaus, J.M.R. Victor, S.J. Murch and P.K. Saxena, 2020. The morphoregulatory role of thidiazuron: Metabolomics-guided hypothesis generation for mechanisms of activity. Biomolecules, Vol. 10. 10.3390/biom10091253.

14. Singh, P. and P. Dwivedi, 2013. Two-stage culture procedure using thidiazuron for efficient micropropagation of *Stevia rebaudiana*, an anti-diabetic medicinal herb. 3 Biotech, 4: 431-437.
15. Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant., 15: 473-497.
16. Grunsky, E.C., 2002. R: A data analysis and statistical programming environment-an emerging tool for the geosciences. Comput. Geosci., 28: 1219-1222.
17. Gantait, S., T. Subrahmanyeswari and U.R. Sinniah, 2022. Leaf-based induction of protocorm-like bodies, their encapsulation, storage and post-storage germination with genetic fidelity in *Mokara* Sayan × *Ascocenda* Wangsa gold. S. Afr. J. Bot., 150: 893-902.
18. Mose, W., A. Indrianto, A. Purwantoro and E. Semiarti, 2017. The influence of thidiazuron on direct somatic embryo formation from various types of explant in *Phalaenopsis amabilis* (L.) Blume orchid. HAYATI J. Biosci., 24: 201-205.