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Evaluating constraints and prospects of black soldier fly frass for sustainable *Coelogyne pandurata* acclimatization

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Abstract. *Kusuma R, Wulandari TM, Lariman, Samsurianto, Nugroho RA, Manurung H, Aryani R, Rudianto. 2025. Evaluating constraints and prospects of black soldier fly frass for sustainable* Coelogyne pandurata *acclimatization. Asian J Agric 9: 316-325.* The black orchid (*Coelogyne pandurata* Lindl), a critically endangered species endemic to Borneo, faces significant threats from habitat destruction and over collection. In vitro propagation provides an effective conservation strategy; however, the acclimatization phase remains a major hurdle. This study assessed the potential of Black Soldier Fly Larvae (BSFL) frass as an organic amendment for the ex-vitro acclimatization of *C. pandurata*, in comparison with traditional substrates (husk charcoal, cocopeat, and fern roots), with and without supplemental fertilizer. Over 56 days, growth parameters including survival rate, plant height, leaf production, and shoot emergence were evaluated. While husk charcoal showed the highest height gain (3.23 ± 0.31 cm), cocopeat with 2 g L^{-1} of fertilizer resulted in superior leaf (3.47 ± 2.06) and shoot (2.49 ± 0.49) development. Despite its high nitrogen and micronutrient content, BSFL frass underperforms when used alone because of its poor structure and rapid nutrient release but may be beneficial as a supplement. These findings highlight the importance of the media types and nutrient balance in successful orchid acclimatization. This research not only supports sustainable conservation practices for *C. pandurata* but also promotes the upcycling of organic waste via BSFL frass, contributing to environmentally responsible horticultural applications.

Keywords: Acclimatization, black orchid, black soldier fly frass, conservation, plant tissue culture

Abbreviations: BSFL: Black Soldier Fly Larvae, CITES: Convention on International Trade in Endangered Species, C/N ratio: Carbon-nitrogen ratio, DAP: Day After Planting, EM4: Effective Microorganisms 4, NPK: Nitrogen Phosphorus Potassium, PKM: Palm Kernel Meal

INTRODUCTION

Orchids (Orchidaceae) are among the most diverse flowering plants, with over 700 genera (Wong and Peakall 2022) and 28,000 species globally (Awasthi et al. 2023; Awasthi et al. 2024; Chachad et al. 2024). Their unique floral morphology and ecological relationships have intrigued botanists and horticulturists for decades. One particularly captivating species is the black orchid (*Coelogyne pandurata* Lindl), which is notable for its vibrant green petals and distinctive black lip (Hartini 2019). Endemic to Borneo, especially East Kalimantan and parts of Malaysia, *C. pandurata* faces near extinction due to deforestation, land conversion, and overcollection (Lestari and Deswiniyanti 2015; Puspitaningtyas 2020; Widiarsih and Dwimahyani 2023). Its short flowering period (5-6 days) (Sabran et al. 2003), and limited cross-pollination success (Hartati et al. 2019) further complicate conservation efforts.

As per the Convention on International Trade in Endangered Species (CITES) Appendix II-listed species (Wahyudiningsih et al. 2018), black orchid conservation is imperative, representing both species protection and broader biodiversity goals. In addition to their ecological importance, orchids have cultural and economic value. In vitro propagation, through tissue culture, provides a viable conservation approach (Semiarti 2018), allowing mass production of disease-free, genetically stable plantlets in controlled environments (Lai and Lai 2019; Lengkong et al. 2023; Manohar et al. 2024).

However, the acclimatization phase, transitioning plantlets from in vitro to ex-vitro conditions, remains a critical bottleneck (Ziraluo 2021; Nimavat and Parikh 2024). During this stage, plantlets encounter fluctuating light, temperature, humidity, and nutrient availability, leading to water stress, pathogen exposure, and nutrient imbalances (Phillips and Garda 2019; Baby et al. 2024; Eisa et al. 2025). Therefore, substrate selection is vital and requires optimal water retention, aeration, and nutrient supply. Common orchid substrates, namely: charcoal, fern roots, coconut fiber, and moss, may lack sustainability or sufficient nutrients (Haniva 2020; dos Santos and Smozinski 2021; Nuammee et al. 2024).

To address this, researchers have turned to nutrient-enriched organic amendments such as frass from Black Soldier Fly Larvae (BSFL) (*Hermetia illucens* (Linnaeus, 1758)) (Triwijayani et al. 2023; Odongo et al. 2024; Siddiqui et al. 2024). The BSFL frass, a byproduct of larvae fed on organic waste, is rich in nitrogen, phosphorus, potassium, and micronutrients with biostimulant properties (Addo et al. 2022; Abd Manan et al. 2024). Its effectiveness has been demonstrated in various crops, including *Brassica juncea* (L.) Czern. (Kesumaningwati et al. 2023), *Brassica rapa* L. (Agustiyani et al. 2021), *Solanum lycopersicum* L. (Salomon et al. 2025), *Triticum turgidum* L. (Boudabbous et al. 2023), *Glycine max* (L.) Merr. (Yudistira et al. 2025), and *Lactuca sativa* L. (Dzepe et al. 2022), enhancing growth, nutrient uptake, and resilience against pathogens.

Despite its documented benefits in agriculture, the BSFL frass remains underutilized in orchid propagation, particularly in the acclimatization of endangered species such as *C. pandurata*. Existing studies on black orchid acclimatization have shown promising results using moss, ferns, and mixtures of wood charcoal and coconut fiber (Adi et al. 2014; Zakiah and Turnip 2023). Optimal outcomes have been reported for combinations of moss, charcoal, coconut fiber, ferns, and humus (Astarini et al. 2015; Zakiah et al. 2023). Nutrient analyses of BSFL frass indicate pH 4-9, organic C > 15%, C/N ratio < 25, NPK > 2%, and Fe < 500 mg/kg (Agustin et al. 2023; Barrantes-Sandoval et al. 2024), supporting its potential as a biofertilizer.

Nevertheless, orchid-specific studies assessing the physiological and morphological effects of BSFL frass are scarce. Literature largely overlooks its use in ornamental or endangered plant species, thereby creating a gap in conservation-oriented horticultural research. While the BSFL frass provides nutrients, its properties and nutrient release dynamics in orchid substrates are not well understood.

This study aimed to evaluate the use of BSFL frass as an organic amendment in the acclimatization of *C. pandurata*, comparing its effectiveness with that of conventional substrates such as husk charcoal, fern roots, and cocopeat, combined with different fertilization levels. By measuring the survival rate, growth performance, and plant development, this study investigated whether BSFL frass can serve as a sustainable medium component.

The novelty of this study lies in applying insect-derived organic waste to conserve endangered orchids, offering dual benefits: improved ex situ acclimatization and waste valorization. These findings will support the sustainable use of insect-based biofertilizers in horticulture while addressing biodiversity conservation.

MATERIALS AND METHODS

Materials

The materials used in this study were BSFL (CV Ahasa Larva Group, Samarinda, East Kalimantan, Indonesia), black orchid, fungicides and bactericides (Zephyr+ 80WP, CV Javamas Agrophos, Yogyakarta, Indonesia), water, red plastic, chicken pellets (PT Japfa Comfeed, Tbk, Indonesia), palm kernel meal (PT REA Kaltim Plantation, East Kalimantan, Indonesia), EM4 (PT. Songgo Langit Persada, Indonesia), cocopeat, husk charcoal, fern roots, Gandasil® (PT Kalatham Corporation, Bekasi, West Java) fertilizer, distilled water, tissue, 70% alcohol, and masks.

Population and research variable

The plant population used in this study was the black orchid population, which was obtained from Kersik Luway National Park, Sekolaq Darat Sub-district, West Kutai District, East Kalimantan, whereas the sample used was 48 black orchid individuals to represent the black orchid population. Two dependent and independent variables were used in this study. The dependent variable was black orchid plants, whereas the independent variables consisted of various media (M): BSFL frass (M1), husk charcoal (M2), fern root (M3), cocopeat (M4), and three concentrations of Gandasil ® fertilizer (F). 0 g L^{-1} (F0) and 1 g L^{-1}. (F1), and 2 g L^{-1} (F2). Current research was conducted with a Completely Randomized Design (CRD) with 2 factors. The first factor was the type of media, while the second factor was the concentration of Gandasil® D Fertilizer (three levels). Each treatment was repeated four times.

Procedures

BSFL frass production

The BSFL frass was obtained from two months of BSFL rearing fed fermented Palm Kernel Meal (PKM) and chicken pellets. PKM fermentation was performed by mixing PKM with EM4, water, and sugar in containers. The ratio used was that every 1 kg of PKM was mixed with 65 mL of EM4, 32 g of sugar, and 1 L of water. The ingredients were homogenized in a container and covered with plastic until no air space was left. The fermentation process lasted for 2 weeks before the meal was ready for use. The fermented meal was mixed with chicken pellets in a 1:1 ratio and provided as BSFL feed (Nugroho et al. 2024). The BSFL rearing was carried out for approximately two months to obtain good-quality frass. The resulting frass was collected weekly and air dried. The dried frass was then sterilized using an autoclave 121°C, 15 psi for 30 min, and ready to be used for a planting medium nutrient content.

Planting media preparation

A planting container was used to plant the orchids. The planting media to be used (BSFL frass, cocopeat, husk charcoal, and fern roots) were dried in the sun until dry, and sterilized by autoclaving at 121°C, 15 psi for 30 min (Elguera et al. 2025). The sterilized media were then cooled and dried. Fifty grams of each medium was placed into the planting container. To support the evaluation data, BSFL frass, cocopeat, husk charcoal, and fern roots were analyzed for C-organic, total N, C/N ratio, P_2O_2, K_2O, Mg, and Mn percentage.

Acclimatization process

During the acclimatization stage, the black orchid that was ready (good overall health, free from contamination, discoloration, or deformities) for acclimatization was removed from the culture bottle using sterilized long tweezers. The planlets were placed on a tray, prepared and filled with water. The planlets were cleaned of the remaining agar media, soaked with fungicide and bactericide solutions, rinsed with water until clean, and then drained. The drained planlets were then initially measured and acclimatized to the prepared media.

The planlets that were planted were then watered and covered with plastic for five days. During the time the planlets were in the hood, the orchid planlets were watered every two days in the morning or evening using a hand sprayer. After five days, the plastic lid was opened slightly after the planlets appeared healthy, and the plastic lid was opened completely. The application of gandasil® fertilizer according to the treatment (no fertilizer, 1%, and 2%) and measurement of test parameters were performed 14, 28, 42, and 56 Days After Planting (DAP).

Growth parameters

The percentage of life, plant height gains, number of leaves, and shoot growth were measured at 14, 28, 42, and 56 DAP. The percentage of life was calculated by counting the number of individuals that live and grow using the following formula (Putri et al. 2022):

$$\text{Percentage of life} = \frac{\text{Number of living orchids}}{\text{Number of orchids planted}} \times 100\%$$

Plant height gain was measured using a ruler from the base of the plant to the tallest leaf (monocots) (Mubarok et al. 2024), and the number of leaves was counted on each plant, starting from the bud leaves to the leaves that had opened (Zhang et al. 2017). For shoot growth, the number of buds was determined for each plant using a previously described method (An et al. 2021).

Data analysis

The data obtained from the measurement of the parameters were analyzed using SPSS version 24 (SPSS, Inc. USA). All data were statistically analyzed using Two-Way Analysis of Variance. Any significant differences were further evaluated using the Duncan Multiple Range Test at a significant level of $P < 0.05$ to determine differences in plant growth parameter data.

RESULTS AND DISCUSSION

Nutrient analysis of the growing media used in this study revealed significant variations in organic matter, macronutrients, and micronutrients, which could influence the acclimatization of *C. pandurata* (Table 1).

The current analysis found that BSFL frass exhibited a high total nitrogen content (5.00%) but a relatively low C/N ratio (4.03), indicating rapid nitrogen availability. However, its phosphorus (4.19%) and potassium (1.27%) contents were lower than those typically required for optimal plant growth. In contrast, husk charcoal showed the highest C-organic content (25.93%), but a lower nitrogen level (0.70%) and an extremely high C/N ratio (37.04), which may limit nitrogen availability for plant uptake.

Furthermore, cocopeat showed the highest organic carbon content (38.55%), with a moderate nitrogen concentration (1.19%) and a C/N ratio of 32.40. These characteristics suggest that cocopeat can serve as a stable organic amendment with slow nutrient release. Similarly, fern roots contained a relatively balanced C-organic content (24.14%) and nitrogen (1.11%), with a moderate C/N ratio (21.82), potentially making them suitable for supporting gradual nutrient supply and microbial activity.

Micronutrient analysis further indicated that BSFL frass contained the highest levels of magnesium (1.28%) and manganese (8.45%), which are essential for enzymatic functions and photosynthesis. Although husk charcoal and cocopeat are rich in organic carbon, they have relatively low micronutrient concentrations. The fern root contained the highest Mn levels (9.24%), which could enhance plant stress tolerance.

Furthermore, the results in Table 2 illustrate the height gain (cm) of *C. pandurata* over 56 Days After Planting (DAP) under different medium compositions, with and without fertilizer application. A key observation is that the treatment group M2F0 (Husk charcoal without fertilizer) exhibited a significantly higher height gain at 28 DAP (1.83 ± 0.13 cm) and at 56 DAP (3.23 ± 0.31 cm) than to other groups. Similarly, M2F1 and M2F2 (husk charcoal with fertilizers) showed a consistent increase in height gain, suggesting that husk charcoal may provide optimal aeration and nutrient retention for root establishment.

In contrast, M1F0 (frass without fertilizer) displayed one of the lowest height gains throughout the study, indicating that frass alone may not be sufficient to support growth. However, fertilization improved height gain across all groups. The M4 groups (cocopeat) also showed improved height gain when supplemented with fertilizers, particularly M4F1 (1 g L^{-1} fertilizer), which achieved 2.60 ± 0.46 cm at 56 DAP (Figure 1). This suggests that the water-holding capacity of cocopeat, coupled with fertilization, positively influences growth.

Table 1. Nutrient composition of different acclimatization media for *Coelogyne pandurata*

Samples	**Total (%)**						
	C-organic	**N total**	**C/N ratio**	**P_2O_2**	**K_2O**	**Mg**	**Mn**
BSFL frass	20.14	5.00	4.03	4.19	1.27	1.28	8.45
Husk charcoal	25.93	0.70	37.04	0.23	0.42	0.50	7.91
Cocopeat	38.55	1.19	32.40	0.09	0.22	0.28	8.28
Fern root	24.14	1.11	21.82	0.10	0.31	0.36	9.24

Table 2. Mean ± Standard Error (SE) of the height gain of *Coelogyne pandurata* acclimated with various medium compositions 56 Days After Planting (DAP)

Groups	Height gain (cm)			
	14 DAP	**28 DAP**	**42 DAP**	**56 DAP**
M1F0	1.46 ± 0.76^{a}	1.35 ± 0.00^{a}	1.55 ± 0.55^{a}	0.91 ± 0.52^{a}
M1F1	1.73 ± 0.60^{a}	1.55 ± 0.13^{a}	2.80 ± 0.33^{a}	1.45 ± 0.45^{a}
M1F2	1.55 ± 0.55^{a}	1.41 ± 0.64^{a}	2.19 ± 0.22^{a}	1.36 ± 0.45^{a}
M2F0	0.79 ± 0.79^{a}	1.83 ± 0.13^{b}	2.27 ± 0.45^{a}	3.23 ± 0.31^{c}
M2F1	2.00 ± 0.76^{a}	1.52 ± 0.10^{b}	1.45 ± 0.45^{a}	2.90 ± 0.47^{c}
M2F2	2.00 ± 0.76^{a}	1.71 ± 0.16^{b}	2.19 ± 0.22^{a}	2.48 ± 0.38^{c}
M3F0	1.88 ± 0.68^{a}	1.76 ± 0.06^{ab}	2.33 ± 0.32^{a}	2.19 ± 0.22^{bc}
M3F1	1.88 ± 0.68^{a}	1.45 ± 0.11^{ab}	2.37 ± 0.56^{a}	2.79 ± 0.39^{bc}
M3F2	2.58 ± 0.89^{a}	1.41 ± 0.06^{ab}	1.55 ± 0.54^{a}	2.52 ± 0.27^{bc}
M4F0	1.88 ± 0.68^{a}	1.69 ± 0.05^{b}	2.45 ± 0.43^{a}	2.00 ± 0.19^{b}
M4F1	2.48 ± 0.38^{a}	1.61 ± 0.11^{b}	1.55 ± 0.55^{a}	2.60 ± 0.46^{b}
M4F2	2.56 ± 0.53^{a}	1.58 ± 0.09^{b}	2.66 ± 0.32^{a}	2.00 ± 0.19^{b}

Note: Mean ± Standard Error (SE) followed by different superscripts (a, b) in the same column indicate significant difference at $p < 0.05$. M1: Frass, M2: Husk charcoal, M3: Fern root, M4: Cocopeat, F0: without fertilizer, F1 and F2: with fertilizer at 1 g L^{-1} and 2 g L^{-1}

A sharp decrease in height gain between 14 and 28 DAP was observed in treatments involving BSFL frass (M1 groups). Specifically, the M1F0 (BSFL frass without fertilizer) group showed a decline in height gain from 1.46 ± 0.76 cm at 14 DAP to 1.35 ± 0.00 cm at 28 DAP, while M1F1 (BSFL frass with 1 g L^{-1} fertilizer) exhibited a reduction from 1.73 ± 0.60 cm to 1.55 ± 0.13 cm over the same period. This trend revealed that BSFL frass alone, despite its high nitrogen content (5.00%), may not sustain consistent growth due to rapid nutrient release and poor structural support for root development. In contrast, husk charcoal (M2 groups) demonstrated more stable height gains, with M2F0 (without fertilizer) increasing from 0.79 ± 0.79 cm at 14 DAP to 1.83 ± 0.13 cm at 28 DAP, indicating better nutrient retention and aeration. The sharp decrease in height gain for BSFL frass treatments between 14 and 28 DAP can be attributed to the low C/N ratio (4.03) of BSFL's frass, resulting in quick nitrogen mineralization, which may initially boost growth but fails to sustain it over time (Table 1). The BSFL's frass may lack the physical properties (e.g., aeration, water retention) that are required for stable root development, hindering long-term growth.

The overall trend suggests that husk charcoal, particularly without fertilizer, facilitated the highest growth rates compared with the other substrates. Additionally, cocopeat also demonstrated effectiveness when combined with fertilization. These findings highlight the importance of substrate selection and fertilization strategies for optimizing *C. pandurata* acclimatization.

The number of new foliage formed by *C. pandurata* under different medium compositions and fertilization treatments over 56 Days After Planting (DAP) is shown in Table 3. A notable trend was observed for M2F2 (husk charcoal with 2 g L^{-1} fertilizer), M3F2 (fern root with 2 g L^{-1} fertilizer), and M4F2 (cocopeat with 2 g L^{-1} fertilizer), which displayed significantly higher foliage formation at 56 DAP. Specifically, M4F2 achieved the highest foliage production (3.47 ± 2.06), followed closely by M3F2 (1.43 ± 1.43) and M2F2 (2.87 ± 1.66). This suggests that a higher fertilizer concentration (2 g L^{-1}) positively influenced foliage development across different substrates.

Nevertheless, several treatment groups, including M1F0 (frass without fertilizer), M1F1 (frass with 1 g L^{-1} fertilizer), and M1F2 (frass with 2 g L^{-1} fertilizer), exhibited limited foliage formation, particularly at 56 DAP, when the number of new foliage remained at 0.00 ± 0.00. This indicates that frass alone may not sufficiently support foliage growth without appropriate substrate or additional supplementation.

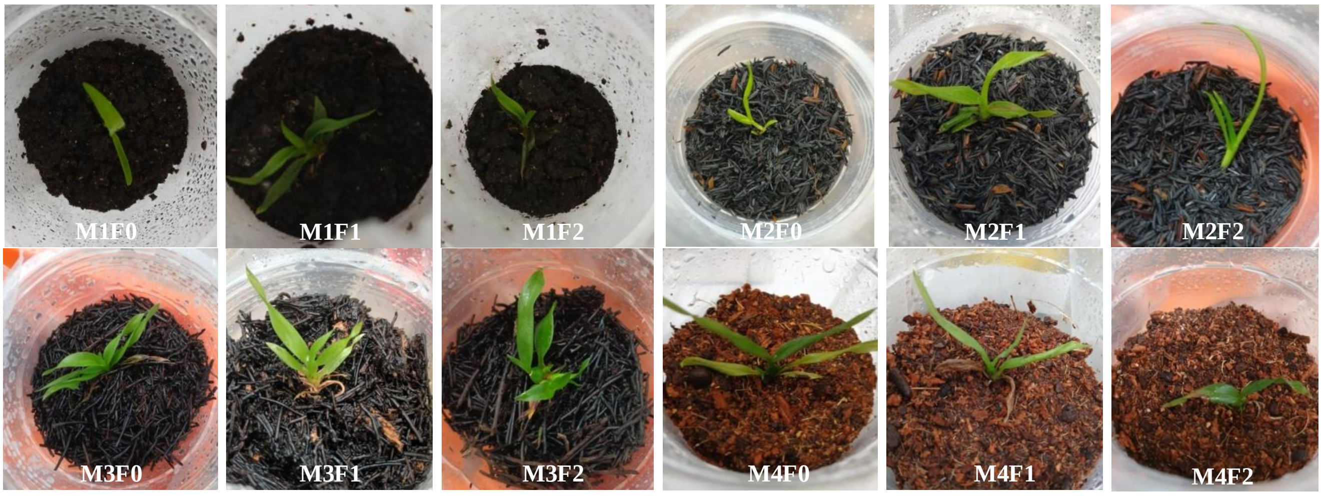

Figure 1. The *Coelogyne pandurata* acclimatized using various media and concentrations of Gandazil® fertilizer at 56 days after Planting. Note: M1: Frass, M2: Husk charcoal, M3: Fern root, M4: Cocopeat, F0: without fertilizer, F1 and F2: with fertilizer at 1 g L^{-1} and 2 g L^{-1}

Table 4. Mean ± Standard Error (SE) of the number of new shoots of *Coelogyne pandurata* acclimated with various medium compositions 56 Days After Planting (DAP)

Groups	**Number of new shoots**				
	0 DAP	**14 DAP**	**28 DAP**	**42 DAP**	**56 DAP**
M1F0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
M1F1	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
M1F2	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
M2F0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
M2F1	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
M2F2	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
M3F0	1.43 ± 0.43^{a}	2.03 ± 0.03^{a}	2.49 ± 0.49^{a}	2.49 ± 0.49^{a}	2.49 ± 0.49^{a}
M3F1	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	1.43 ± 0.43^{a}	1.43 ± 0.43^{a}
M3F2	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
M4F0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	1.43 ± 0.43^{a}	1.43 ± 0.43^{a}	1.43 ± 0.43^{a}
M4F1	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	1.43 ± 0.43^{a}	1.43 ± 0.43^{a}	1.43 ± 0.43^{a}
M4F2	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	1.43 ± 0.43^{a}	2.03 ± 0.03^{a}	2.49 ± 0.49^{a}

Note**:** Mean ± Standard Error (SE) followed by different superscripts (a, b) in the same column indicate significant difference at $p<0.05$. M1: Frass, M2: Husk charcoal, M3: Fern root, M4: Cocopeat, F0: without fertilizer, F1 and F2: with fertilizer at 1 g L^{-1} and 2 g L^{-1}

Table 3. Mean ± Standard Error (SE) of the number of new foliage of *Coelogyne pandurata* acclimated with various medium compositions 56 Days After Planting (DAP)

Groups	**Number of the new foliage**			
	14 DAP	**28 DAP**	**42 DAP**	**56 DAP**
M1F0	4.30 ± 1.43^{a}	2.87 ± 1.66^{a}	2.87 ± 1.66^{a}	0.00 ± 0.00^{a}
M1F1	3.47 ± 2.06^{a}	1.43 ± 1.43^{a}	1.43 ± 0.43^{a}	0.00 ± 0.00^{a}
M1F2	2.87 ± 1.66^{ab}	4.07 ± 2.35^{a}	2.03 ± 2.03^{a}	0.00 ± 0.00^{a}
M2F0	0.00 ± 0.00^{a}	1.43 ± 0.43^{a}	3.47 ± 2.06^{a}	1.43 ± 0.43^{b}
M2F1	1.43 ± 0.43^{a}	1.43 ± 1.43^{a}	2.87 ± 1.66^{a}	2.87 ± 1.66^{b}
M2F2	0.00 ± 0.00^{ab}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	2.87 ± 1.66^{b}
M3F0	1.43 ± 0.43^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	3.47 ± 2.06^{b}
M3F1	2.87 ± 1.66^{a}	2.87 ± 1.66^{a}	0.00 ± 0.00^{a}	1.43 ± 0.43^{b}
M3F2	0.00 ± 0.00^{ab}	2.87 ± 1.66^{a}	1.43 ± 0.43^{a}	1.43 ± 0.43^{b}
M4F0	0.00 ± 0.00^{ab}	1.43 ± 0.43^{a}	0.00 ± 0.00^{a}	3.47 ± 2.06^{b}
M4F1	2.87 ± 1.66^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	1.43 ± 0.43^{b}
M4F2	0.00 ± 0.00^{ab}	0.00 ± 0.00^{a}	3.47 ± 2.06^{a}	3.47 ± 2.06^{b}

Note**:** Mean ± Standard Error (SE) followed by different superscripts (a, b) in the same column indicate significant difference at $p<0.05$. M1: Frass, M2: Husk charcoal, M3: Fern root, M4: Cocopeat, F0: without fertilizer, F1 and F2: with fertilizer at 1 g L^{-1} and 2 g L^{-1}

In addition, the study observed a decline in the number of new foliage acclimated during 14, 28, 42, and 56 Days After Planting (DAP) in *C. pandurata*, particularly in treatments involving Black Soldier Fly Larvae (BSFL) frass (M1 groups). This trend can be attributed to several factors related to substrate properties, nutrient dynamics, and plant physiological responses such as nutrient depletion and substrate limitations. The BSFL frass, despite its high nitrogen content (5.00%), exhibited a low carbon-to-nitrogen (C/N) ratio (4.03), indicating rapid nitrogen mineralization. While this initially supported early growth, the quick depletion of nutrients likely led to a subsequent decline in foliage production. The lack of structural stability in frass further exacerbated this issue, as it failed to provide sustained aeration and moisture retention necessary for root and shoot development. In contrast, substrates like husk charcoal and cocopeat, with higher C/N ratios (37.04 and 32.40, respectively), demonstrated slower nutrient release, supporting more consistent foliage growth over time.

Moreover, the absence of new foliage in certain groups at 28 and 42 DAP, particularly in those without fertilization, suggests that the initial establishment phase may require both structural support from the substrate and nutrient availability. Consistent foliage production in husk charcoal, fern roots, and cocopeat, particularly with higher fertilizer concentrations, reinforces the importance of balanced substrate selection and fertilization strategies for successful acclimatization.

The number of new shoots produced by *C. pandurata* acclimatized under different medium compositions and fertilization treatments over 56 DAP is shown in Table 4. The majority of treatments exhibited no shoot formation throughout the observation period, except a few specific treatments that showed significant responses at later stages. Among the treatments, M3F0 (fern root without fertilizer) exhibited the earliest shoot emergence at 14 DAP (1.43 ± 1.43), which remained consistent throughout the acclimatization period. At 28 DAP, M3F0 demonstrated an increase in shoot formation (2.49 ± 2.49), maintaining the highest shoot number among all treatments. Other notable responses were observed in M3F1 (fern root with 1 g L^{-1} fertilizer) and M3F2 (fern root with 2 g L^{-1} fertilizer), which showed significant shoot emergence at 42 and 56 DAP, reaching 1.43 ± 1.43 and 2.49 ± 2.49, respectively. Interestingly, M4F2 (cocopeat with 2 g L^{-1} fertilizer) also demonstrated a gradual increase in shoot production, reaching 2.49 ± 2.49 at 56 DAP, similar to M3F2. This suggests that both fern roots and cocopeat, when supplemented with appropriate fertilization, may enhance shoot initiation during acclimatization. However, treatment with BSFL frass (M1F0, M1F1, M1F2) did not induce shoot formation at any time point, indicating that frass alone may not be suitable for promoting shoot growth in *C. pandurata* under the tested conditions.

Discussion

The selection of appropriate acclimatization media significantly affected the growth and survival of *C. pandurata*. Each tested medium exhibited distinct nutrient profiles that influenced plant development. The high nitrogen content in BSFL frass supports early vegetative growth, but its low C/N ratio may lead to rapid nutrient depletion. This suggests that while BSFL frass can enhance initial growth, they may require supplementation with structurally supportive media to sustain long-term development. Current findings are in line with past studies that mentioned that substrates with low C/N ratios initially promote rapid vegetative growth due to high nitrogen availability but lead to nutrient depletion over time. Structurally supportive media (e.g., husk charcoal, cocopeat) with higher C/N ratios provide sustained nutrient release, improving long-term acclimatization success (De Stefano et al. 2022; Gebremikael et al. 2022; Chia et al. 2024).

On the other hand, husk charcoal, with its high organic carbon content and excellent aeration properties, supports root development but lacks sufficient nitrogen and phosphorus. This limitation may necessitate the addition of nitrogen-rich amendments to optimize plant growth. Cocopeat, with its high water-holding capacity, provides stable moisture conditions and benefits acclimatization. However, its low phosphorus and potassium levels indicate the need for external supplementation to sustain its metabolic function. Cocopeat is widely used in soilless cultivation systems for its high water-holding capacity and ability to maintain stable moisture conditions, which are beneficial for seedling acclimatization. Despite these advantages, cocopeat is low in essential nutrients such as phosphorus and potassium, necessitating the addition of external fertilizers to support healthy metabolic function and plant growth (Mariotti et al. 2020).

The fern roots demonstrated a balanced nutrient composition with moderate nitrogen availability and the highest Mn concentration, which can enhance stress tolerance. Its structure supports both moisture retention and aeration, making it a promising substrate for long-term acclimatization. The varying micronutrient content across media highlights the importance of selecting substrates that not only provide essential macronutrients but also support enzymatic functions and physiological stability.

Integrating nutrient-rich amendments with structurally supportive substrates can optimize acclimatization outcomes. Combining BSFL frass with cocopeat or fern roots may improve nutrient availability while maintaining adequate aeration and moisture retention. These findings emphasize the need for strategic substrate selection and supplementation to enhance the survival and growth of *C. pandurata* under ex-vitro conditions.

The significant performance of husk charcoal as a growth medium aligns with previous findings that husk charcoal provides optimal conditions for root aeration and moisture retention, thereby enhancing seedling establishment (Asadi et al. 2021; Karam et al. 2022). The porosity and structure of the husk charcoal likely contributed to improved water drainage and aeration, which are critical factors in orchid root development. Husk charcoal or biochar, is recognized for its high organic carbon content and excellent aeration properties, which support root development and improve soil structure. However, due to its inherently low nitrogen and phosphorus levels, it often requires supplementation with nutrient-rich amendments to optimize plant growth. Studies have shown that combining biochar with additives such as plant ash or effective microorganisms significantly enhances soil fertility (Luo et al. 2025; Sun et al. 2025). Additionally, the ability of husk charcoal to retain nutrients may support plant growth, especially in the absence of fertilizer.

Compared to cocopeat, which demonstrated moderate growth improvements when combined with fertilizers, husk charcoal appears to be a more efficient medium for orchid acclimatization. This contrasts with previous findings suggesting that cocopeat, when combined with organic amendments such as compost, could outperform husk charcoal owing to improved microbial activity (Adekiya et al. 2022; Younis et al. 2022; Melo et al. 2024). The discrepancies in the findings could be due to differences in the orchid species, environmental conditions, and fertilization rates used in each study.

The relatively low performance of frass alone as a substrate was unexpected, considering its high organic matter content. However, the absence of a significant height gain in M1F0 suggests that while frass may provide essential nutrients, they may lack the necessary structural properties for optimal root development. This is consistent with previous studies that highlight the importance of combining organic amendments with structurally supportive substrates to enhance plant growth (Wei et al. 2016; Li et al. 2021; Singh et al. 2022). When combined with husk charcoal or cocopeat, frass may function as an organic enhancer rather than as a primary substrate.

Another important finding of the present study was the role of fertilization in improving growth outcomes. The M4F1 (cocopeat with 1 g L^{-1} fertilizer) exhibited a significantly higher growth rate than its unfertilized counterpart, indicating that nutrient supplementation is crucial for cocopeat-based substrates. This supports the findings of previous studies on the benefits of balanced fertilization during orchid acclimatization (Hariyanto et al. 2019; Rineksane et al. 2023). These results suggest that BSFL frass alone may not be the most effective organic amendment for in vivo acclimatization, but when combined with husk charcoal or cocopeat and supplemented with fertilizer, it may enhance plant growth. Future studies should explore microbial interactions in different substrates and optimize fertilizer composition for black orchid acclimatization.

The findings of this study also suggest that fertilization plays a crucial role in stimulating foliage production in *C. pandurata* during acclimatization. The significantly higher foliage formation in M4F2 (cocopeat with 2 g L^{-1} fertilizer) than in the other treatments highlights the potential of cocopeat as a favorable substrate when supplemented with adequate nutrients. Cocopeat is known for its high water-holding capacity and its ability to retain nutrients, which may contribute to improved foliage development. This result aligns with a previous study that found that cocopeat, when combined with appropriate organic amendments and fertilization, enhanced foliage growth in orchid species because of its ability to support microbial activity and maintain adequate moisture levels (Treder 2008; Kaushal and Kumari 2020; Sachin et al. 2020).

A similar trend was observed for M3F2 (fern root with 2 g L^{-1} fertilizer) and M2F2 (husk charcoal with 2 g L^{-1} fertilizer), in which foliage production was significantly improved. The effectiveness of husk charcoal in supporting foliage development has been reported by Onggo (2013); Fadillatunnisa et al. (2023), who emphasized its role in enhancing aeration and drainage, thereby reducing the risk of root rot, while maintaining sufficient moisture for plant growth. In contrast, fern roots have traditionally been used as an orchid substrate because of their natural affinity for epiphytic species, providing both physical support and essential micronutrients (Sari et al. 2018; Tini et al. 2019).

However, the poor performance of frass-based treatments, particularly M1F0, M1F1, and M1F2, raises concerns regarding the suitability of frass as a standalone substrate for *C. pandurata* acclimatization. BSFL frass are rich in organic matter and essential nutrients, and its limited structural support and potential rapid nutrient leaching may hinder effective plant growth. This observation contrasts with previous findings by Tan et al. (2021) and Antoniadis et al. (2023), who demonstrated that frass application enhanced vegetative growth in leafy greens and fruiting plants. The difference in response could be attributed to the specific growth requirements of orchids, which typically rely on substrates that provide optimal aeration and slow nutrient release, rather than high initial nutrient availability.

One possible explanation for the lower foliage formation in the frass-treated groups is microbial activity associated with frass decomposition. Previous studies have suggested that high microbial activity in organic substrates can lead to temporary nitrogen immobilization, making it less available for plant uptake (Gannett et al. 2024; He et al. 2024; Zhang et al. 2024). This phenomenon might have contributed to the reduced foliage growth in the frass-based treatments, as nitrogen is essential for leaf expansion and photosynthetic activity. Future research should investigate microbial interactions in frass-amended substrates to determine optimal application methods for orchid acclimatization.

The significant effects of fertilization on foliage development also supports the findings of previous studies on nutrient requirements during orchid acclimatization. According to Tang (2025), balanced fertilization is necessary to support leaf initiation and overall plant vigor during the transition from in vitro to ex-vitro conditions. The results of the present study confirmed that fertilization at 2 g L^{-1} is more effective than fertilization at 1 g L^{-1}, suggesting that orchids require higher nutrient availability during the early acclimatization stages. Further studies are required to determine the long-term effects of high fertilizer concentrations on plant health and flowering potential.

Our results also suggest that fern roots and cocopeat, particularly in combination with fertilization, positively influenced the production of new shoots in *C. pandurata* during acclimatization. The superior performance of M3F0 (fern root without fertilizer) at early stages, followed by an increasing trend in M3F1, M3F2, and M4F2, highlights the potential of these substrates to provide suitable conditions for shoot development. These findings align with those of previous studies that emphasized the importance of organic substrates with good aeration properties in promoting shoot formation in orchids (Utami and Hariyanto 2020; Agarwal et al. 2023). Fern root has been widely recognized as an effective medium for orchid cultivation because of its natural ability to retain moisture while maintaining proper aeration, which is essential for root and shoot development (Hariyanto et al. 2019; Arthagama et al. 2021).

Furthermore, the role of cocopeat in enhancing shoot formation, as observed in M4F2, is consistent with previous studies that demonstrated that cocopeat improves the microenvironment for root establishment, leading to enhanced shoot initiation (Atzori et al. 2021; Wu et al. 2025). The high water-holding capacity and organic matter content of cocopeat may have contributed to the steady shoot emergence observed in this study. In addition, the increased shoot production with higher fertilizer concentrations in the fern root and cocopeat treatments is in line with a previous study, which reported that nutrient supplementation, particularly nitrogen, is crucial for shoot differentiation and meristematic activity in orchids during acclimatization (Zhang et al. 2017; Zhang et al. 2018).

In contrast, black soldier fly frass-based treatments (M1F0, M1F1, and M1F2) failed to induce shoot formation. Although frass is known for its high nutrient content, the inability to support shoot growth in this study suggests that it may not provide the necessary structural or microbial environment for effective orchid shoot initiation. BSFL frass application improves vegetative growth in leafy crops (Agustiyani et al. 2021), but its effect on orchids remains uncertain. One possible explanation is that the decomposition process of the BSFL frass leads to temporary nitrogen immobilization, making nitrogen unavailable for plant uptake during critical growth phases (Gebremikael et al. 2022). This might have limited shoot formation in *C. pandurata*, which relies on steady nutrient availability for successful acclimatization.

Moreover, the absence of new shoots in most treatments until later stages suggests that *C. pandurata* requires an extended acclimatization period for shoot differentiation. Certain orchid species may exhibit delayed shoot emergence when transitioning from in vitro to ex-vitro conditions because of physiological adjustments and environmental stress responses (Vaz and Kerbauy 2008; Teixeira da Silva et al. 2014; Kaur 2022). The delayed response in fertilized treatments (M3F1, M3F2, and M4F2) further reinforces the idea that nutrient availability plays a significant role in stimulating shoot growth, but only after the initial root establishment.

Another factor influencing shoot emergence could be microbial interactions within different substrates. While fern roots and cocopeat may support beneficial microbial communities that enhance nutrient availability and hormone regulation, frass-based treatments may have promoted microbial competition, potentially suppressing shoot development. Microbial-driven nutrient cycling in organic substrates can influence plant growth dynamics, particularly in slow-growing species, such as orchids. Future investigations should explore the microbial composition of frass-amended substrates to determine their potential impact on orchid acclimatization (Schleuss et al. 2021; Koch and Sessitsch 2024).

Additionally, the variation in shoot number among the treatments underscores the importance of optimizing substrate selection based on specific orchid growth requirements. Although husk charcoal (M2 treatments) has been widely used in orchid cultivation, its performance in this study did not yield significant shoot formation. This could be attributed to its relatively low water retention capacity, which might not be ideal for *C. pandurata*. Further research is needed to assess the combination of husk charcoal with moisture-retaining amendments to improve its efficacy as an orchid growing medium. Overall, this study underscores the importance of selecting appropriate substrates and fertilization strategies to optimize foliage development during *C. pandurata* acclimatization. Although cocopeat, fern root, and husk charcoal demonstrated promising results, frass alone appeared to be insufficient to support vigorous foliage growth. Future studies should explore the potential of combining frass with structurally supportive substrates to enhance their efficacy as organic amendments. Additionally, investigations into microbial interactions and nutrient dynamics in frass-amended substrates could provide valuable insights into optimizing organic fertilization strategies for orchid cultivation.

This study evaluated the effectiveness of Black Soldier Fly Larvae (BSFL) frass as an organic amendment for in vivo acclimatization of black orchids. The results showed that husk charcoal was the most effective growth medium, whereas cocopeat, when supplemented with fertilizer, enhanced leaf and shoot development. BSFL frass exhibited high nitrogen content but was insufficient to support optimal growth when used alone. However, combining frass with structurally supportive media, such as husk charcoal or cocopeat, has improved its effectiveness. These findings highlight the importance of selecting appropriate growing media and fertilization strategies to enhance the acclimatization success of black orchids. This study contributes to orchid conservation efforts through a sustainable approach that utilize organic waste as a nutrient source for plants.

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