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Original Research Paper

In vitro regeneration competence of a Sumbawa indigenous plant, *Begonia bimaensis* Undaharta & Ardaka, using leaf explants

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Abstract: *Begonia bimaensis* Undaharta & Ardaka, an indigenous plant to Sumbawa, Indonesia, is classified as vulnerable by the IUCN Red List criteria. Thus, an appropriate strategy is necessary to ensure the plant's survival, including tissue culture. In this study, *B. bimaensis* leaves were used as a source of explants for callus induction in MS media containing 2 mg L^{-1} IAA, 2 mg L^{-1} NAA, 3 mg L^{-1} BA, and 0.5 mg L^{-1} kinetin. After the callus had formed, it was moved to MS medium without growth regulators for two weeks. The calli were then transferred to a new MS medium containing different amounts of NAA and BA for regeneration. As a result, *B. bimaensis* leaf explants swelled after 13 weeks on callus induction media. Three media (NAA-BA 0-0, 1-0, 2-0 mg L^{-1}) successfully regenerated all explants into shoots and roots. NAA-BA 0-0 mg L^{-1} resulted in the fastest shoot formation, whereas the NAA-BA 1-0 mg L^{-1} treatment resulted in the quickest root emergence. Media with NAA-BA 1-0 mg L^{-1} produced the greatest number of shoots (83.2±12.1). NAA-BA 1-0 mg L^{-1} and 2-0 mg L^{-1} were the treatments that resulted in the longest and most abundant roots. We conclude that NAA and BA effectively regenerate shoots and roots in *B. bimaensis* explants. Small amounts of auxin may benefit in *in vitro* regeneration of this species.

Keywords: Auxin-cytokinin interactions; conservation; callus regeneration; endemic; indirect organogenesis.

1. Introduction

Begonia bimaensis Undaharta & Ardaka is an attractive plant from the Begoniaceae family. It is an endemic plant of Mount Muria, Bima District, Sumbawa, West Nusa Tenggara, Indonesia, and belongs to the Reichenheimea section. This species was discovered in the shadow of high rocky cliffs at an elevation of 525 m. It is a small, monoecious plant with tubers less than 10 cm in height and pink blooms (Undaharta et al., 2015). In addition to its aesthetic appeal, studies have shown that this genus has significant functional benefits. *B. malabarica* possesses significant hypoglycemic and antifungal activity (Suresh et al., 2016), while *B. medicinalis* is a scientifically validated herbal medication with strong

immunostimulant activity (Zubair et al., 2021; Zubair et al., 2022; Syamsidi et al., 2023). Other species, such as *B. fischeri* var. *palustris* and *B. grandis* subsp. *grandis* were reported to have antimicrobial properties (Karpova et al., 2019a; Karpova et al., 2019b). *B. bimaensis* is a vulnerable species, according to The International Union for Conservation of Nature criteria, as only one known population exists (Undaharta et al., 2015). As a result, an adequate approach is required to ensure the plant's survival.

Tissue culture is frequently used in plant propagation for mass production and conservation (Bourrain, 2018; Jang et al., 2019). This approach employs only small plant components, making it suitable for plants with a vulnerable or threatened status. *In vitro* propagation technologies offer an option to meet market demand by allowing for the quick multiplication of disease-free and true-to-type chosen clones in a relatively short time (Almemary, 2020; Chavhan et al., 2023). According to Jahanshir and Mohammad (2019), *Begonia* may be mass-produced quickly using every part of various species, including leaf, petiole, peduncle, shoot, and meristem micro-samples. Callus formation plays an important role in *in vitro Begonia* propagation. Callus can be obtained from a limited explant and easily regenerated onto a planlet. Some *Begonia* species successfully formed callus by *in vitro* propagation, i.e.: *B. lucerna* Hort. (Jahanshir and Mohammad, 2019), *B. malabarica* Lam. (Aswathy and Murugan, 2019), and *B. semperflorens* cv. 'Ambassador' White (Romocea, 2011) . However, several studies show that the effectiveness of this approach is determined by various parameters, including plant and explant type, growth regulators, and environmental conditions such as light and temperature.

Growth regulators serve a vital role in plant growth. Studies show that two of the most important growth regulators are cytokinins and auxins. Moreover, plant development is influenced by single growth regulators or mixtures of multiple regulators. In *B. lucerna* micropropagation, a combination of 0.2 mg L^{-1} naphthalene acetic acid (NAA) and 0.6 mg L^{-1} benzyl adenine (BA) significantly increased shoot production. The optimal treatment for rooting was 0.6 mg L^{-1} NAA and 0.1 mg L^{-1} BA (Jahanshir and Mohammad, 2019). Shoot production of *B. montaniformis* × *B. ningmingensis* var. *bella* was greatly increased by the supplementation of NAA and BA (Lai et al., 2018). Accordingly, Kumari et al., (2017) discovered that MS medium with gibberellic acid (GA_3) and BA produced the highest number of *B. homonyma* shoots *in vitro*. At the same time, rooting was effective in MS medium with indole-3-butyric acid (IBA) and NAA.

Studies on the *in vitro* culture of *B. bimaensis* are limited. Hendriyani et al., (2020) stated that the use of the leaf as an explant for callus induction gave the best results. However, the regeneration of the calli has not been carried out. As previously stated, *Begonia* studies have employed a combination of NAA and BA to regenerate calli. Therefore, this study explored combinations of NAA and BA for callus regeneration in *B. bimaensis.* We hope that this study can contribute to conservation strategies by regenerating *B. bimaensis in vitro*.

2. Materials and Methods

2.1. Materials

Begonia bimaensis leaves were taken from a plant collection at Eka Karya Bali Botanic Garden, National Research and Innovation Agency, Indonesia.

2.2. Explant disinfection and initial growth

To remove the surface debris, the leaves were washed with detergent and rinsed in running water for 15 minutes. The cleaned explants were then disinfected with 70% alcohol for 1 minute, followed by 15% (v/v) NaClO mixed with three drops of Tween-20 for 15 minutes, and washed three times with sterile distilled water.

The disinfected whole leaf explants were cultured on full-strength MS medium containing 20 g L^{-1} sucrose and 4 g L^{-1} gelzan, without the addition of plant growth regulators for two weeks to evaluate explant contamination. The sterile leaf explants were then used for callus induction.

2.3. Callus induction

The callus induction medium consisted of Murashige and Skoog (MS) with vitamins, 20 g of sucrose, solidified with 4 g of gelzan, and supplemented with 2 mg L^{-1} IAA, 2 mg L^{-1} NAA, 3 mg L^{-1} BA, and 0.5 mg L^{-1} kinetin. This combination of growth regulators was selected based on a preliminary study we conducted (unpublished). Four leaf explants were cultured on each callus induction medium. All cultures were incubated at 20–25°C and a 12-hour photoperiod with cool white fluorescent lighting (900-1200 lux) for 112 days, and observations of callus formation were made weekly. Once the callus had developed, it was transferred to MS medium without growth regulators (as described in section 2.2) to mitigate any residual hormonal effects from the previous medium.

2.4. Callus regeneration

After two weeks in the MS medium without growth regulators, the calli were transferred to a new fresh MS medium supplemented with varying concentrations of NAA and BA for regeneration. Each growth regulator contained three levels: 0, 1, or 2 mg L^{-1}. The cultures were maintained at 20–25°C under a 12-hour photoperiod with cool white fluorescent lighting (900-1200 lux) for 91 days. Data were collected weekly regarding the time of first callus regeneration, callus regeneration rate, number of shoots, and roots. To find the best medium, it was important to record the first time of callus regeneration. At the end of the observation, the root length and number of emerged shoots and roots were collected.

2.5. Experimental setup and statistical analysis

The experiment followed a completely randomized design. Each treatment was performed five times. The data were analyzed using analysis of variance, followed by Duncan's Multiple Range Test with a 5% significant threshold.

3. Results

3.1. Callus induction

Begonia bimaensis leaf explants swelled after 91 days on a callus induction medium (Figure 1a). During the process, the explants were maintained in the same medium and were not subcultured. The callus formed on the wounded areas of the leaf and along its edges. Thickening occurred in these areas, producing bright yellowish-green spheres (Figure 1b).

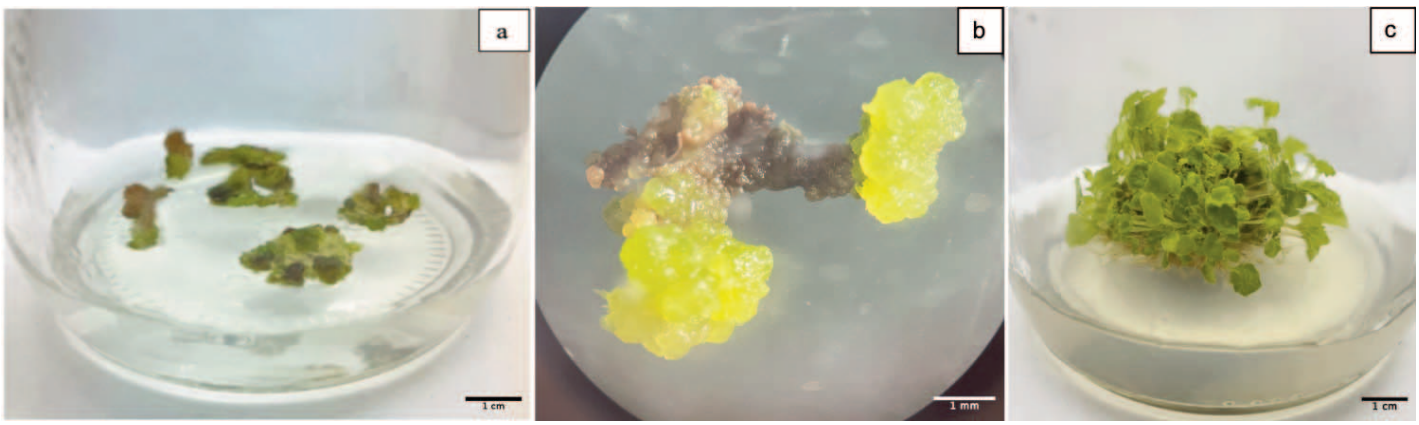

Figure 1. *In vitro* plant regeneration from *Begonia bimaensis* leaf explants: (a) callus formation at 91 days, (b) thickened cells forming green spheres (callus), (c) regenerated callus into shoots and roots.

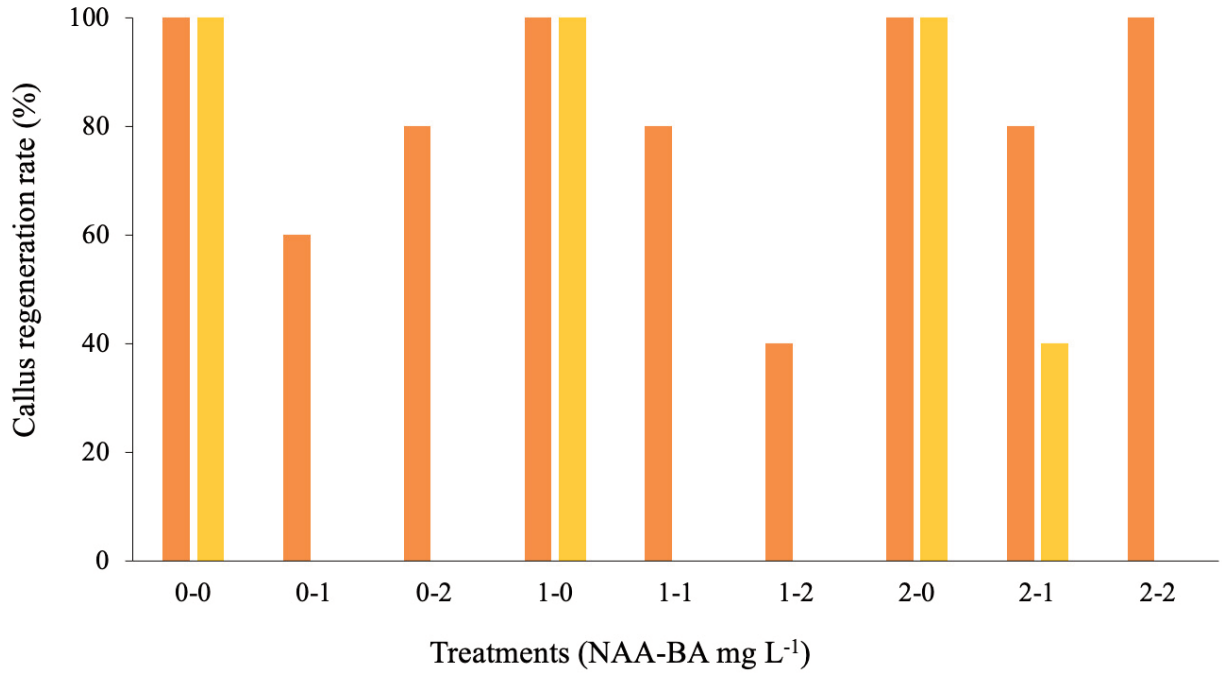

Figure 2. The final regeneration rate of *Begonia bimaensis* callus was documented at 91 days after planting. The orange bar indicates shoot and the yellow bar indicates root development.

3.2 Callus regeneration

The result showed that shoots formed in all media combinations, while roots developed in only four (Figure 2). Three media could regenerate all explants into shoots and roots (Figure 1c). These three effective media are NAA-BA 1-0 mg L^{-1}, 2-0 mg L^{-1}, and medium without growth regulators. The medium without growth regulators led to the fastest shoot emergence (Figure 2).

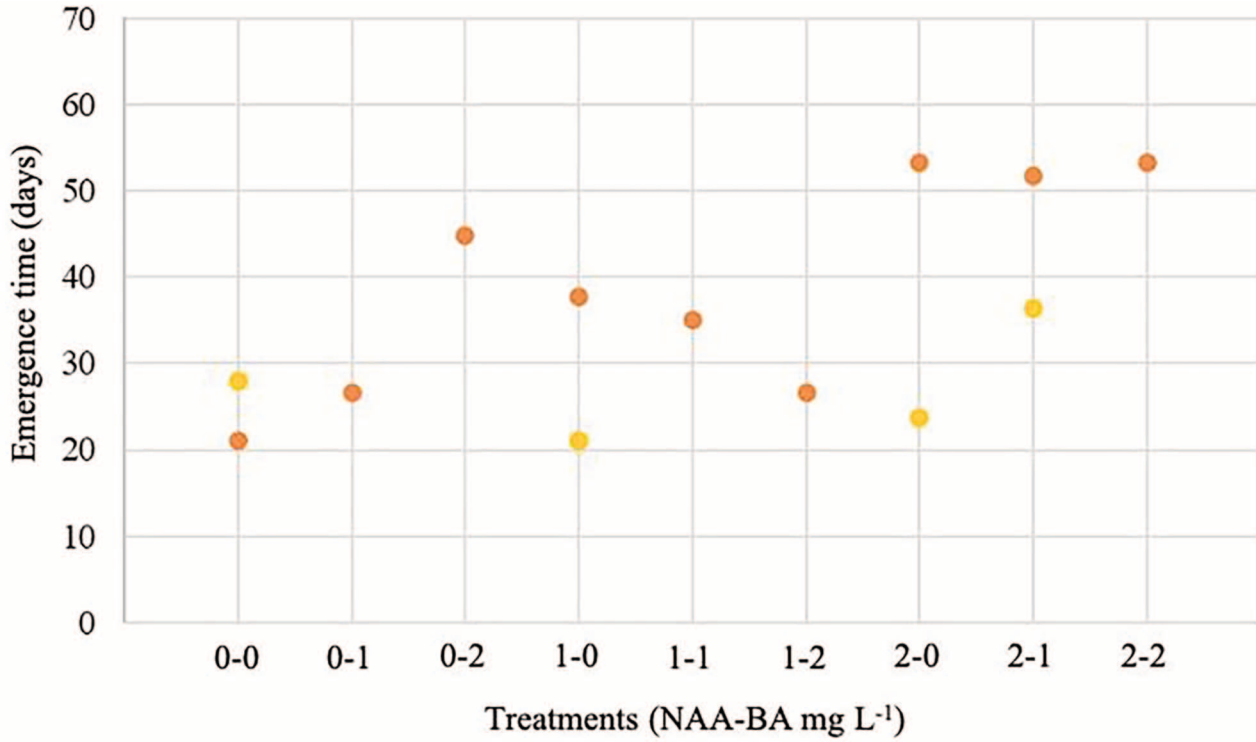

Figure 3. First day of the shoot and root emergence of *Begonia bimaensis* callus. Orange dots indicate shoot and yellow dots indicate root.

Our findings indicate that untreated callus initiated shoot formation 21 days after planting (DAP). In contrast, the slowest shoot emergence (53 DAP) was observed in the treatment containing a higher concentration of NAA (2 mg L^{-1}) and no BA (0 mg L^{-1}), and also in a balanced NAA-BA combination (2-2 mg L^{-1}) (Figure 3). Endogenous growth regulators have the potential to encourage shoot production until 63 DAP, and then they gradually diminish until the last days of observation. The average number of shoots in untreated callus (37.0±10.5) at 63 DAP was comparable to the NAA-BA 1-0 mg L^{-1} treatment (37.2±9.0). Moreover, from 84 DAP, the average number of shoots generated in the NAA-BA 1-0 mg L^{-1} treatment (78.2±11.3) was considerably greater than that in other treatments and continued to rise until 91 DAP (83.2±12.1) (Figure 4).

Our data demonstrated that supplementing with NAA alone considerably boosted the growth of *B. bimaensis* roots (Figure 2). The treatment with the quickest root initiation was NAA-BA 1-0 mg L^{-1} (21 DAP), followed by NAA-BA 2-0 mg L^{-1} (24 DAP) and NAA-BA 0-0 mg L^{-1} (28 DAP) (Figure 3). The fourth treatment that induced root formation was a combination of 2 mg L^{-1} NAA and 1 mg L^{-1} BAP, and the addition of BAP extended the time required for root formation, which took 36 DAP.

In our study, we classified root production into four categories: extremely low (0–10 roots per callus), low (11–50 roots per callus), sufficient (51–100 roots per callus), and abundant (more than 100 roots per callus) (Figure 5).

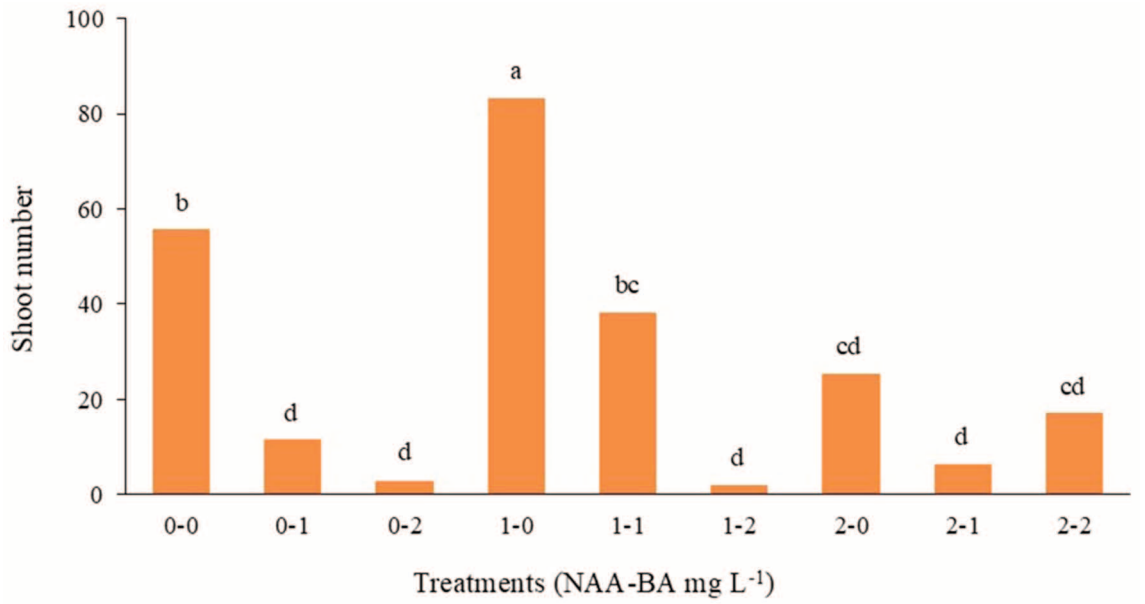

Figure 4. The average number of shoots produced at 91 days after planting for various NAA-BA treatments. The bar indicates the standard error of the mean. Different letters above the bars indicate significant differences based on Duncan's Multiple Range Test ($p < 0.05$).

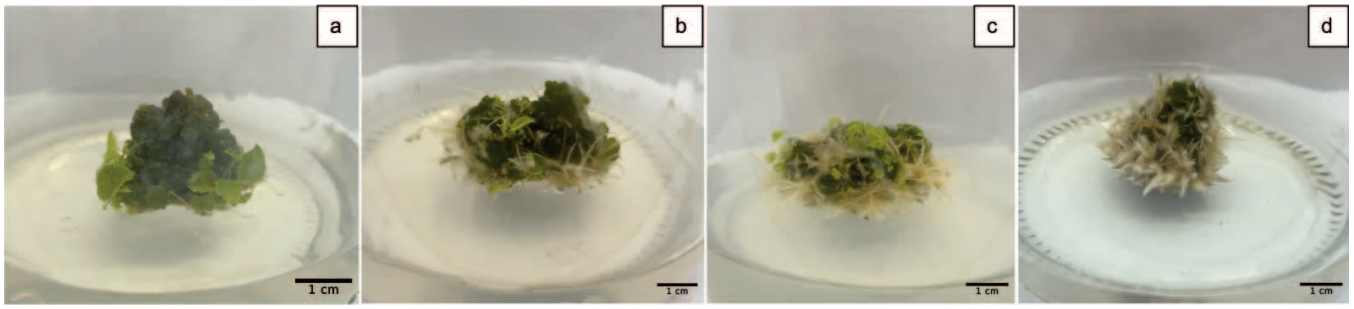

Figure 5. The root categories of *Begonia biogenesis*: (a) extremely low (0-10 roots per callus), (b) low (11-50 roots per callus), (c) sufficient (51-100 roots per callus), (d) abundant (more than 100 roots per callus).

Based on those criteria, at week 13, NAA-BA 1-0 mg L^{-1} and NAA-BA 2-0 mg L^{-1} were considered abundant, whereas NAA-BA 0-0 mg L^{-1} was considered low, while NAA-BA 2-1 mg L^{-1} was extremely low. The number of roots developed was directly related to the root length, with NAA-BA 1-0 mg L^{-1} and NAA-BA 2-0 mg L^{-1} producing the maximum number of roots and much longer roots than the other treatments, respectively, 1.17 cm and 1.24 cm (Figure 6).

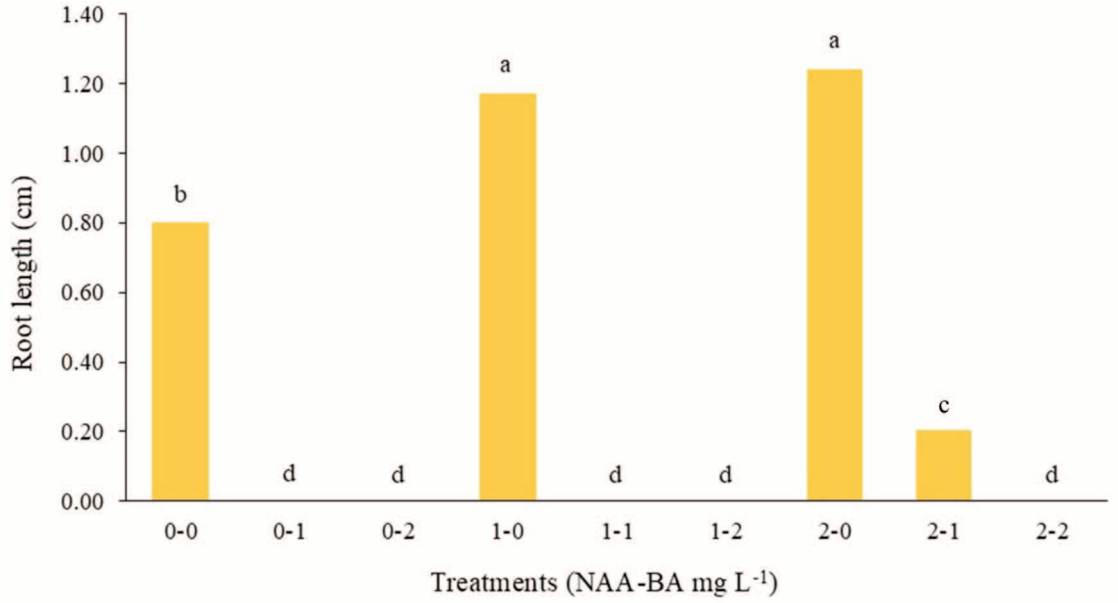

Figure 6. The average root length at 91 days after planting. The bar indicates the standard error of the mean. Different letters above the bars indicate significant differences based on Duncan's Multiple Range Test ($p < 0.05$).

4. Discussion

The callus is a temporary tissue made up of parenchyma cells with an irregular form, typically appearing at the wound or cut sites on plants (Almemary, 2020; Zhang et al., 2023). Kruglova et al., (2023) observed that the callus began to form around leaf incisions due to thickening. Furthermore, the callus thickens and forms little spheres, eventually developing into shoots or other organs. Callus is commonly used to reproduce plants by differentiating tissues into organogenesis, which involves nurturing this unspecialized tissue on culture media while regulating endogenous and exogenous growth regulator levels (Fehér, 2019; Almemary, 2020). In this investigation, callus induction in *B. bimaensis* was carried out in MS medium containing 2 mg L^{-1} IAA, 2 mg L^{-1} NAA, 3 mg L^{-1} BA, and 0.5 mg L^{-1} kinetin. The combination of auxin and cytokinin indicated that the explant required an exogenous growth regulator to enable indirect organogenesis, as studies reported that various kinds of auxin and cytokinin may be combined to induce callus in a variety of plant species (Aswathy and Murugan, 2019; Nivetha et al., 2020; Wahyuni et al., 2020). The damaged cells from the explant were encouraged to form undifferentiated tissues known as callus. Callus production is an important intermediary step that allows shoots, roots, and other plant organs to grow, therefore it is known as indirect organogenesis.

The choice of explant plays a critical role in the success of callus induction and subsequent micropropagation. Previously, Hendriyani et al., (2020) found that at the callus induction stage of *B. bimaensis*, leaf bases outperformed petioles and leaf blades, with the quickest callus developing on the 20 DAP on an MS medium containing 1 ppm of cytokinin. In this study, we found that using the whole leaves as an explant can generate callus after 13 weeks on supplemented MS media. This demonstrates that *B. bimaensis* micropropagation can provide diverse outcomes even within the same species, likely due to various biotic and abiotic variables. Biotic factors such as explant age, type, and cellular composition, coupled with abiotic factors, including medium composition and environmental conditions, collectively

influence the efficiency of callus induction (Pasternak and Steinmacher, 2024). The fact that leaf blades may outperform leaf bases under specific conditions shows that *B. bimaensis* has innate flexibility and plasticity. This adaptability is crucial for optimizing micropropagation protocols, as it provides flexibility in selecting explants based on availability and practical considerations.

Shoot development in *in vitro* culture affects the ability to mass-produce uniform plants in a short period. The more shoots are generated, the more plants grow (Thiem et al., 2023). The immature tissues employed as explants are responsible for the production of shoots. Young tissues have meristematic features, including actively dividing cells and endogenous growth regulators (Wahyuni et al. 2020). In this study, the concentration of endogenous growth regulators in explants was shown to be ideal for cell growth, division, and proliferation, resulting in faster shoot production. Correspondingly, Naïtchédé et al., (2023) reported that a medium without growth regulators was the most effective for inducing shoot and root formation from *Momordica charantia* calli. The appearance of shoots in the treatment with the addition of growth regulators was delayed, presumably due to the high concentration of growth regulators. A small number of growth regulators can affect or regulate physiological processes, increasing plant output and quality (Bons and Kaur, 2020; Sabagh et al., 2021).

The interplay of endogenous growth regulators in explants and exogenous growth regulators added to the medium altered shoot development (Jazib et al., 2020; Wahyuni et al., 2020). Exogenous growth regulators, such as cytokinin, are commonly utilized to promote growth and development. They often increase cell proliferation, especially when combined with an auxin (Jazib et al., 2020; Wati et al., 2020; Dar et al., 2021; Quamruzzaman et al., 2021). Our findings show that cytokinin (BA) promotes the development of *B. bimaensis* shoots but can be blocked by excessive auxin levels. A drop in NAA concentration to 1 mg L^{-1}, paired with BA, could regenerate callus 10-20 days faster.

The addition of exogenous auxin to the culture medium stimulated vascular tissue development and increased tissue quantity and size (Wang et al., 2022). Our recent study discovered that applying NAA alone was more effective in increasing shoot multiplication than treating with NAA and BA concurrently. This is a peculiar result, considering that pioneering tissue culture research established that auxin promotes root growth and cytokinin promotes shoot growth. Thus, we concluded that the endogenous cytokinin levels in explants were likely sufficient, hence, exogenous cytokinin did not appear to be necessary. According to Schaller et al. (2015), auxin induces cellular differentiation and organ outgrowth, while cytokinin promotes the proliferation of undifferentiated cells in the shoot apical meristem. Auxin regulates cell polarity, cell elongation, vascular tissue development, and promotes differentiation of certain cell types, ensuring the organized growth of organs such as roots and shoots (Jamil et al., 2021; Konstantinova et al., 2021). The auxin mechanism involves activating gene expression pathways that regulate cell wall remodeling and cytoskeletal organization, which are crucial for cell expansion and specialization (Zhang et al., 2022). In addition to encouraging cell division, cytokinin interacts with auxin gradients to produce zones of proliferation and differentiation in cell spatial organization (Wong et al., 2023). The interplay between auxin and cytokinin is essential for maintaining the dynamic equilibrium in plant growth. For instance, auxin accumulates at sites of organogenesis to initiate differentiation, while cytokinin ensures sufficient cell proliferation to support new growth. This hormonal crosstalk orchestrates the formation of complex plant structures, such as leaves, flowers, and lateral roots, by tightly regulating cellular activities and developmental cues (Raspor et al., 2021).

Aside from the capacity to produce shoots, callus grown on MS media with a combination of NAA and BA effectively produced roots. Since 21 DAP, the addition of 1 mg L^{-1} NAA resulted in the greatest root development. Several studies demonstrated that a single application of NAA on MS medium successfully boosted root formation in bananas, bamboo, and *B. homonyma* (Hossain et al. 2016; Kumari et al. 2017; Khare et al. 2021). Higher NAA concentrations led to more root formation, but more than 4 mg L^{-1} of NAA inhibits root production (Hossain et al. 2016; Khare et al. 2021). Similarly, although we found that NAA successfully increased the root production of *B. bimaensis*, root formation was reduced when NAA concentrations exceeded 1 mg L^{-1}. By contrast, Kumari et al. (2017) discovered that the

rooting stage of *B. homonyma* was effectively improved in MS medium supplemented with 2 µM IBA and 0.5 µM of NAA while adding 2 µM IBA in MS medium significantly improved its root elongation. Along with root formation, we found that the combination of 1-0 or 2-0 mg L^{-1} of NAA-BA yielded longer roots than the other treatments. Thus, we infer that 1 mg L^{-1} of NAA is sufficient for serial roots of *B. bimaensis*.

A callus typically regenerates into a shoot, which is followed by the root formation. To encourage roots, shoots are transplanted to other media with the addition of an auxin such as NAA, IAA, or IBA (Kumari et al. 2017; Lai et al. 2018). In a previous study on *Chlorophytum borivilianum*, it was shown that if the roots develop first, it will be difficult for the callus to produce shoots because shoot morphogenesis stops with the formation of roots (Nakasha et al., 2016). In contrast, our study demonstrated that the callus that generates roots initially was able to form shoots, as documented in the treatment NAA-BA 1-0, 2-0, and 2-1 mg L^{-1}. Meanwhile, the callus in other supplemented media that formerly generated shoots failed to establish roots. Several plant species have been reported to create shoots from callus, followed by the formation of roots, which can subsequently grow and develop into plantlets (Kshirsagar et al., 2021; Nabieva and Fershalova, 2023; Nivetha et al., 2020; Prusty et al., 2020; Rahman et al., 2018; Thorat et al., 2018; Yang et al., 2022). Studies demonstrate the ability to regenerate varies across species. Preserving the unique traits of each species is crucial, and one effective approach is through *in vitro* regeneration studies. The results of this study can provide valuable guidance for propagating endangered species, particularly *Begonia*, thus contributing to the conservation of genetic diversity.

5. Conclusions

The addition of NAA-BA was successful in regenerating shoots and roots from the *Begonia bimaensis* explants. MS medium containing NAA-BA 1-0 mg L^{-1} produced the most shoots. A single auxin (1 mg L^{-1} NAA) treatment has also resulted in the most abundant and longest roots. Thus, we discovered that adding small doses of auxin can improve *in vitro* regeneration of *B. bimaensis*. However, while a large number of shoots were generated, both the shoots and roots remained small in size. Further study is required to stimulate shoot and root elongation.

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Conflicts of Interest: The authors declare no conflict of interest.

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