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Organogenesis and morphogenesis of *Kaempferia parviflora in vitro*

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Abstract

In vitro propagation of *Kaempferia parviflora* is not yet widely adopted in commercial production, as conventional propagation methods remain more commonly utilised. However, to meet future market demands, plant tissue culture technology offers a promising alternative for large-scale propagation. A major challenge in developing an efficient in vitro protocol for *K. parviflora* is determining the optimal plant growth regulator (PGR) concentrations for successful morphogenesis. This research aimed to analyse and determine the optimum or ideal hormone combination between 6-Benzylaminopurine (BAP) and 2,4-Dichlorophenoxyacetic acid (2,4-D on organogenesis and somatic embryogenesis of *K. parviflora*. This study can provide or enhance knowledge for further research on propagating *K. parviflora*. Explants were cultured on Murashige and Skoog (MS) medium supplemented with varying levels of BAP and 2,4-D (0.5 mg/L, 1.0 mg/L, 2.0 mg/L, and 4.0 mg/L). The cultures were maintained at 25±2°C under a 16-hour photoperiod. Organogenic and somatic embryogenic responses were recorded and analysed using analysis of variance (ANOVA) in SPSS software. The combination of 4.0 mg/L BAP with 1.0 mg/L 2,4-D resulted in the highest explant regeneration rate, with mean organogenesis and somatic embryogenesis responses of 0.50±0.19 and 0.38±0.52, respectively.

1. Introduction

Kaempferia parviflora, commonly known as Thai black ginger or 'halia hitam Thailand', is a perennial rhizomatous herb that typically reaches a height of approximately 30 cm. The plant is characterised by its dark green, glossy leaves and small, white to purple flowers. Its aromatic rhizomes have long been valued in traditional medicine, particularly for the treatment of ailments such as digestive disorders, fever, and muscle pain. Recognised as one of the most valuable medicinal herbs in Southeast Asia and beyond, *K. parviflora* has a long history of use as a folk remedy among local populations in Thailand.

In Malaysia, *K. parviflora* is moderately popular due to its limited availability compared to Thailand, where large-scale cultivation is well established (Jalil, 2018). Nonetheless, several research institutions, including the Malaysian Agricultural Research and Development Institute (MARDI), have undertaken studies to explore its potential applications. According to Jalil (2018), Othman Saidon - a supplier and distributor of fruit tree and herbal seedlings - reported that MARDI's findings revealed that *K. parviflora* rhizomes possess high antioxidant content, which may enhance human energy levels and serve as a valuable resource for cosmetic formulations and therapeutic products targeting cardiovascular health.

The increasing commercial demand for black ginger (*K. parviflora*) poses a significant challenge for growers and industry stakeholders in maintaining a consistent

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supply. Tissue culture technology is considered one of the most promising approaches for large-scale propagation of this valuable medicinal plant. Given the anticipated rise in global demand, optimising micropropagation protocols is crucial to ensure sustainable production and market availability. Propagation through seeds is limited due to the plant's low seed set despite its flowering ability, while conventional rhizome-based propagation is constrained by the limited supply of high-quality planting materials. These factors contribute to the low cultivation rate of *K. parviflora* in Malaysia, highlighting the urgent need for alternative propagation strategies, particularly advanced in vitro culture techniques, to enhance its availability and support commercial expansion.

Tissue culture provides an essential alternative for the propagation of *K. parviflora*, overcoming the limitations associated with low seed viability and restricted availability of rhizome planting material. Successful in vitro regeneration, however, is highly dependent on the selection and optimisation of plant growth regulators (PGRs), particularly their type and concentration, to promote efficient shoot and root induction. The proper balance of hormones is critical for achieving rapid, healthy tissue development and ensuring a reliable micropropagation protocol. Therefore, this study aimed to (i) determine the optimal combination of 6-Benzylaminopurine (BAP) and 2,4-Dichlorophenoxyacetic acid (2,4-D) for inducing in vitro organogenesis and morphogenesis in *K. parviflora*, and (ii) characterise the morphogenic responses under varying hormonal treatments.

2. Materials and methods

2.1 Planting material

Healthy, disease-free rhizomes of *K. parviflora* were obtained from the UiTM Puncak Alam greenhouse, Malaysia. Bud induction was carried out using the wet tissue method, and after one week, the buds were ready for use. The rhizomes were treated with a fungicide to eliminate fungal growth during the bud induction process. Subsequently, the buds were trimmed and thoroughly washed to remove any adhering dirt.

2.2 Preparation of hormone stock solutions

The hormone dilution ratio was prepared based on the principle that 1 mL = 1 mg. To prepare 1 mg/mL stock solutions of BAP and 2,4-D, 0.1 g of each hormone powder was weighed using an electronic balance. Each hormone was placed in a separate beaker and dissolved in a few drops of 0.1 N NaOH. The powders were gently crushed with a spatula until they formed a clear solution. The solutions were then transferred to separate 100 mL measuring cylinders, and the volumes were topped up to 100 mL with distilled water. The stock solutions were stored in airtight glass bottles and kept in a refrigerator at 4°C until use in the preparation of Murashige and Skoog (MS) medium. No heating step was used during dilution, as high temperatures can denature hormones and reduce their biological activity (McClements, 2018). NaOH was chosen as the solvent because it is highly effective for dissolving BAP and 2,4-D.

2.3 Preparation of MS basal medium

In this experiment, 17 media samples were prepared. One of the samples serves as a hormone-free control, while the remaining 16 samples contain different combinations of the hormones BAP and 2,4-D. Firstly, MS media were prepared by using 22.00g/L of MS powder and 30.00 g/L of sucrose in a 1.0 L conical flask (Smith, 2013). Approximately 700 mL of distilled water is added to the medium, and the medium is stirred.

After the MS powder and sucrose have been added, hormones will be the next. A total of 16 media that will be prepared contain different amounts of BAP and 2,4-D, according to Table 1. In order to make medium A, 0.5 ml/L of BAP and 2,4-D were measured and added to the solution. For other media, the volume for BAP and 2,4-D was changed according to Table 1 for different solutions.

After hormones were added, the pH solution was adjusted to 5.8. While the solution was being stirred, an electronic pH metre was put into the solution, and NaOH was added drop by drop into the solution gently. The value of the pH metre was read after the reading stopped. This procedure will be continued until the pH metre shows the desired value. Then, 8.0 g of agar will be added to the medium. Finally, distilled water will be added until it reaches 1000 mL. After a medium has been prepared, the next medium will be prepared by using the same procedure according to the different amounts of hormone combinations based on Table 1.

After the MS media have been prepared, they will be autoclaved at 121°C for 20 min. After the autoclave is done, the media will be poured into 10 different containers. This procedure will be done in a laminar air flow. The medium will be allowed to cool and become solid in the laminar flow. The container will be labelled based on Table 1 according to its respective values.

2.4 Preparation of bleach for explant sterilisation

To prepare the bleach solution for explant sterilisation, commercial Clorox was diluted to 15% (v/v) with distilled water. Specifically, 150 mL of Clorox was measured and transferred into a 1000 mL glass bottle, followed by the addition of 850 mL of distilled

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water to reach a final volume of 1000 mL. The prepared bleach solution was stored for use during explant sterilisation.

Table 1. Amount of BAP and 2,4-D.

Treatment	Amount of BAP (mL/L)	Amount of 2,4-D (mL/L)
1	0.5	0.5
2	0.5	1.0
3	0.5	2.0
4	0.5	4.0
5	1.0	0.5
6	1.0	1.0
7	1.0	2.0
8	1.0	4.0
9	2.0	0.5
10	2.0	1.0
11	2.0	2.0
12	2.0	4.0
13	4.0	0.5
14	4.0	1.0
15	4.0	2.0
16	4.0	4.0
control	0.0	0.0

2.5 Preparation of bleach for explant sterilisation

Rhizomes were rinsed under running tap water for 1 h, followed by immersion in 15% (v/v) Clorox for 15 min. The ends of the buds were then trimmed to a length of 5–10 mm and placed on sterile filter paper in a Petri dish. Explants were immersed in 70% (v/v) ethanol, then rinsed three times with sterile distilled water, using a fresh portion of water for each rinse. The explants were immediately transferred to the culture medium. All procedures were conducted under a laminar airflow cabinet, following standard operating procedures, to minimise contamination and prevent damage to the explants.

2.6 Data collection and observation

Culture growth was monitored weekly to record morphological changes in the explants. Data were collected at week 8 of culture. The response variables measured were organogenesis and somatic embryogenesis. A binary scoring system was used, where a value of 1 indicated a positive response to the parameter, and 0 indicated no response. Data were analysed using analysis of variance (ANOVA) in SPSS software. Mean values with different letters within a column (subject to explant type) differ significantly at $p < 0.05$, by one-way ANOVA and Duncan's multiple range test.

3. Results and discussion

3.1 Induction of callus

3.1.1 Induction of callus response

In this research, the buds from rhizomes were used to stimulate the growth of callus in order to determine the optimal concentration of the hormone combination of BAP (6-benzylaminopurine) and 2,4-D (2,4-dichlorophenoxyacetic acid), in conjunction with studying organogenesis and morphogenesis by assessing the organogenesis response and somatic embryogenesis response. To evaluate the success of the experiment, the number and length of shoots, roots, and leaves produced after 8 weeks of culture were used as the main parameters. Unfortunately, the culture did not grow as anticipated, so the expected outcome of shoot, root, and leaf formation directly from the explant or through the callus could not be measured.

The outcome of the culture only demonstrated the growth of callus and a limited number of shoots from only a few cultures. Despite the limited growth, the callus formation can still provide valuable information on the effects of the hormone combination on the plant tissue. Thus, the parameters used in the study were the organogenesis response and the somatic embryogenesis response, which were recorded as either "yes" or "no." "Yes" indicated the presence of callus and direct growth from explant or somatic embryo formation, while "no" indicated the absence of these growths.

Out of 136 cultures supplemented with different concentrations of hormone, 22 of them show the formation of a callus or the growth of an explant directly after 8 weeks of culture (Figure 1). These growths illustrate the success of performing plant tissue culture according to the chosen method. The rest of the cultures were contaminated and did not show growth.

Figure 1. The callus induction of tissue culture explant into media supplemented with a hormone combination of 1.0 mg/L BAP and 0.5 mg/L 2,4-D.

3.1.2 Observation of callus

After two weeks of culture, several explants have shown the formation of calluses. At this stage, the callus

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is generally in a colourless mass, and the texture of the callus is moist. After several days, the colourless mass turned into a white mass. From the white mass, the callus turned a light green and slowly became darker (Figure 2).

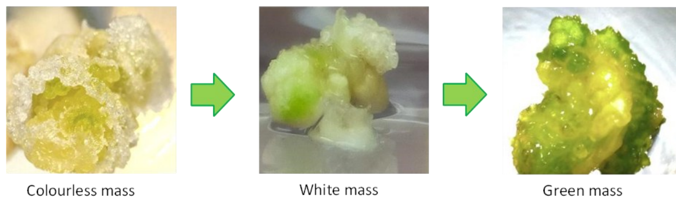

Figure 2. Colour changing of the callus.

3.2 Organogenesis response

3.2.1 Mean of organogenesis response

The values of the mean for each treatment can be seen in Table 2. The highest mean of organogenesis is treatment 4.0 mg/L BAP + 1.0 mg/L 2,4-D, which is 0.50±0.189. The lowest mean for treatments 0.5 mg/L BAP + 0.5 mg/L 2,4-D, 1.0 mg/L BAP + 4.0 mg/L 2,4-D, and 4.0 mg/L BAP + 4.0 mg/L 2,4-D is 0.00±0.000. This result occurs because there is no sign of the formation of a callus in of the 8 replications. Explants for both treatments do grow and become stunted.

3.2.2 Observation of organogenesis response

Table 2. Percentage mean of organogenesis response.

Treatment	Mean (%)*
0.0 mg/L BAP + 0.0 mg/L 2,4-D	0.25±0.164[ab]
0.5 mg/L BAP + 0.5 mg/L 2,4-D	0.00±0.000[b]
0.5 mg/L BAP + 1.0 mg/L 2,4-D	0.13±0.125[ab]
0.5 mg/L BAP + 2.0 mg/L 2,4-D	0.25±0.164[ab]
0.5 mg/L BAP + 4.0 mg/L 2,4-D	0.13±0.125[ab]
1.0 mg/L BAP + 0.5 mg/L 2,4-D	0.13±0.125[ab]
1.0 mg/L BAP + 1.0 mg/L 2,4-D	0.13±0.125[ab]
1.0 mg/L BAP + 2.0 mg/L 2,4-D	0.25±0.164[ab]
1.0 mg/L BAP + 4.0 mg/L 2,4-D	0.00±0.000[b]
2.0 mg/L BAP + 0.5 mg/L 2,4-D	0.13±0.125[ab]
2.0 mg/L BAP + 1.0 mg/L 2,4-D	0.25±0.164[ab]
2.0 mg/L BAP + 2.0 mg/L 2,4-D	0.13±0.125[ab]
2.0 mg/L BAP + 4.0 mg/L 2,4-D	0.13±0.125[ab]
4.0 mg/L BAP + 0.5 mg/L 2,4-D	0.25±0.463[ab]
4.0 mg/L BAP + 1.0 mg/L 2,4-D	0.50±0.189[a]
4.0 mg/L BAP + 2.0 mg/L 2,4-D	0.13±0.125[ab]
4.0 mg/L BAP + 4.0 mg/L 2,4-D	0.00±0.000[b]

Values are presented as mean±SD. Values with different superscripts in the same column (subject to explant type) are statistically significantly different ($p<0.05$) by one-way ANOVA and Duncan's multiple range test.

From the experiment, there are two ways of explant growth: direct organogenesis and indirect organogenesis. Direct organogenesis took place when the explant grew directly without the formation of a callus (Figure 3). This case mostly occurs when a large explant is used.

Direct organogenesis proves superior in efficiency, bypassing the callus phase for faster organ growth and plant regeneration compared to indirect methods. This process fosters genetic stability by skipping the callus stage, minimising genetic variations that might occur. Additionally, it enhances uniformity in plants as organs or tissues form directly, decreasing the likelihood of somaclonal variation (Halder *et al.,* 2021). For instance, African violets demonstrate direct organogenesis, propagating efficiently from leaf explants to generate uniform plants without the intermediary callus phase, showcasing the advantages of this method in producing consistent and genetically stable ornamental varieties (Missaghi *et al.,* 2023).

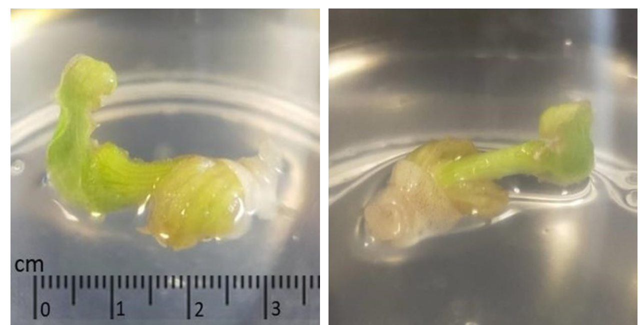

Figure 3. The growth of the shoot directly from explant without the formation of callus in the MS medium supplemented with 0.5 mg/L BAP + 4.0 mg/L 2,4-D.

In this research, most of the culture undergoes indirect organogenesis, which is the formation of the callus first before differentiation into organs. This is because most of the cultured explants were small in size. This led to the formation of a callus. However, callus also forms for big explants, as shown in Figure 4. Indirect organogenesis is more prone to somaclonal variation compared with direct organogenesis. Molecular investigations conducted on regenerated plantlets of vanilla through indirect organogenesis have identified a genetic polymorphism of 71.66% (Ramírez-Mosqueda and Iglesias Andreu, 2015).

Figure 4. Induction of callus in MS media supplemented with 1.0 mg/L BAP + 0.5 mg/L 2,4-D.

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3.2.3 Mean graph of organogenesis response

From the mean graph of organogenesis response in Figure 5, the highest mean is 0.50 mg/L, which is treatment 4.0 mg/L BAP + 1.0 mg/L 2,4-D. Most of the means were higher in the treatment with a higher amount of BAP. The pattern of the graph is hard to describe as the hormone combination plays a different role. As the BAP concentration increases from the first treatment to the fourth treatment, there is an increment in the mean as the amount of BAP increases with a constant value of 2,4 -D.

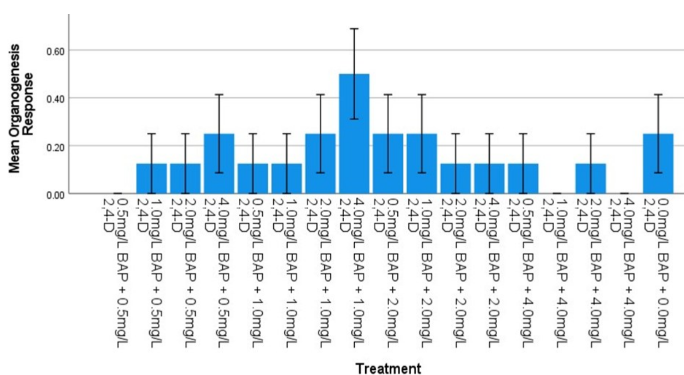

Figure 5. Graph mean of organogenesis response.

Then, from the fifth value to the eighth value, the pattern of the graph also experiences an increase when the value of BAP changes with the constant of 2,4-D at 2.0 mg/L. This case shows that the hormone for the shoot is higher than the hormone for the root, resulting in more growth of the explant and shoot. That is why there is no root to be seen in all cultures (Pascual and Marin, 2005).

3.3 Somatic embryogenesis response

3.3.1 Mean of somatic embryogenesis response

The value of the highest mean of embryogenesis response is 0.38±0.518 for the treatment 4.0 mg/L BAP + 1.0 mg/L 2,4-D. The mean value for each treatment is always lower than the mean of the organogenesis response. This is due to a smaller number of explants that respond to the organogenesis response. Meanwhile, most of the somatic embryos grow when the concentration of BAP is high (Table 3).

3.3.2 Observation of somatic embryogenesis response

The results in Figures 6 and 7 show that only indirect somatic embryogenesis occurs in the cultures from the callus. From the observation, some calli form globular shapes and turn into heart-shaped masses. This shows the formation of a somatic embryo from the embryogenic cells of the explants. Meanwhile, another way to form a somatic embryo is through direct somatic embryogenesis. Somatic embryos grew directly from the callus in this path.

Table 3. Percentage mean of somatic embryogenesis response.

Treatment	Mean (%)
0.0 mg/L BAP + 0.0 mg/L 2,4-D	0.13±0.354^{ab}
0.5 mg/L BAP + 0.5 mg/L 2,4-D	0.00±0.000^{b}
0.5 mg/L BAP + 1.0 mg/L 2,4-D	0.00±0.000^{b}
0.5 mg/L BAP + 2.0 mg/L 2,4-D	0.25±0.463^{ab}
0.5 mg/L BAP + 4.0 mg/L 2,4-D	0.00±0.000^{b}
1.0 mg/L BAP + 0.5 mg/L 2,4-D	0.00±0.000^{b}
1.0 mg/L BAP + 1.0 mg/L 2,4-D	0.13±0.354^{ab}
1.0 mg/L BAP + 2.0 mg/L 2,4-D	0.13±0.354^{ab}
1.0 mg/L BAP + 4.0 mg/L 2,4-D	0.00±0.000^{b}
2.0 mg/L BAP + 0.5 mg/L 2,4-D	0.13±0.354^{ab}
2.0 mg/L BAP + 1.0 mg/L 2,4-D	0.25±0.463^{ab}
2.0 mg/L BAP + 2.0 mg/L 2,4-D	0.00±0.000^{b}
2.0 mg/L BAP + 4.0 mg/L 2,4-D	0.00±0.000^{b}
4.0 mg/L BAP + 0.5 mg/L 2,4-D	0.25±0.463^{ab}
4.0 mg/L BAP + 1.0 mg/L 2,4-D	0.38±0.518^{a}
4.0 mg/L BAP + 2.0 mg/L 2,4-D	0.13±0.354^{ab}
4.0 mg/L BAP + 4.0 mg/L 2,4-D	0.00±0.000^{b}

Values are presented as mean±SD. Values with different superscripts in the same column (subject to explant type) are statistically significantly different ($p<0.05$) by one-way ANOVA and Duncan's multiple range test.

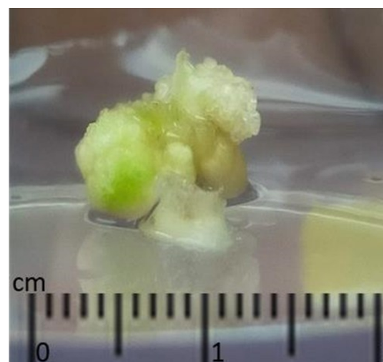

Figure 6. Somatic embryo induction from the callus on MS media added with 4.0 mg/L BAP + 1.0 mg/L 2,4-D.

Figure 7. Somatic embryo induction from the callus on MC medium added with 2.0 mg/L BAP + 1.0 mg/L 2,4-D.

Indirect somatic embryogenesis, despite its more complex process than direct embryogenesis, presents various advantages. It allows for enhanced regeneration of embryos from diverse plant tissues or cell types, broadening the scope of viable material for embryo production (Bekalu *et al.,* 2023). Moreover, the callus phase in indirect somatic embryogenesis introduces

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genetic and epigenetic variations, potentially advantageous for selecting desired traits or fostering genetic diversity in regenerated plants (Stelmach and Grzebelus, 2023). Particularly beneficial for species challenging to regenerate through direct methods, this approach serves as an alternative means of propagation. It also excels in mass production, generating numerous embryos from minimal starting materials, crucial for commercial or research applications (Delgado-Aceves *et al.,* 2021). Additionally, the callus phase enables long-term culture maintenance, ensuring a continuous source for plant regeneration and genetic resource preservation. Furthermore, indirect somatic embryogenesis proves useful in genetic transformation studies, offering a platform to introduce and explore genetic modifications in plants, thus contributing significantly to plant tissue culture and biotechnology (Abiri *et al*., 2020).

3.3.3 Mean graph of somatic embryogenesis response

Based on the graph in Figure 8, the highest peak of somatic embryogenesis is in the culture with treatment 4.0 mg/L BAP + 1.0 mg/L 2,4-D. The pattern of the graph is unpredictable because there is no formation of a valid pattern. The graph increases as the concentration of BAP increases for control 0.5 mg/L and 1.0 mg/L 2,4-D. At a concentration of 4.0 mg/L 2,4-D, there is almost no formation of a somatic embryo. This is because 2,4-D is higher than BAP. There will be no formation of shoots (Pascual *et al*., 2005).

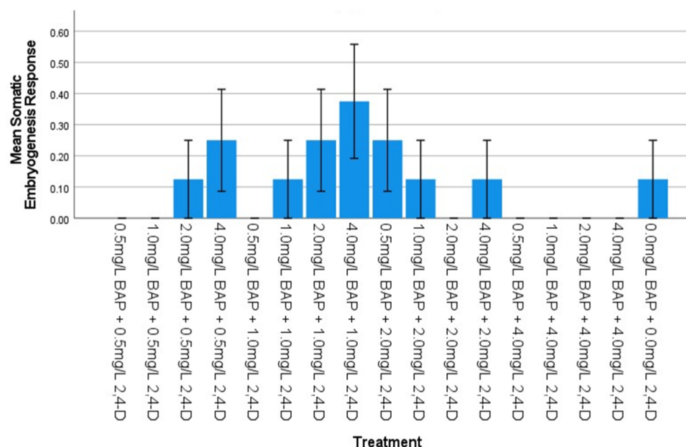

Figure 8. Graph of mean of somatic embryogenesis response.

4. Conclusion

The research was conducted to study the organogenesis and morphogenesis of *K. parviflora* and the effect of different hormone combinations of BAP and 2,4-D on organogenesis and somatic embryogenesis responses. Most of the explants grew up forming a callus with several morphological changes. Hormone combinations of 4.0 mg/L BAP + 1.0 mg/L 2,4-D regenerate the highest percentage of explant growth with a number of organogenesis and somatic embryogenesis responses of 0.50±0.189 and 0.38±0.518, respectively.

For the future study of *K. parviflora*, it is important to find a better technique of surface sterilisation to avoid contamination, which is very challenging to avoid. The characteristic of the bud needs more attention, as it is small in size compared to other plant buds. It is important to follow established protocols and guidelines when conducting experiments on *K. parviflora* to ensure the safety of the plant species and the environment and to adhere to ethical considerations. It is advisable to carefully consider the potential impacts of the experiment on the species and the environment and to take appropriate measures to minimise or mitigate these impacts.

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