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Research article

Promotive Effect of Ginger Rhizome Extract Supplement on *In Vitro* Rooting of Persian Violet Shoot Tip Culture

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Abstract

Ginger is recognized for its economic value, which is derived from its rhizome that is often utilized as a spice and a herb. In plant tissue culture, various natural supplements are often added into culture media. Ginger rhizome extract has not been added to plant tissue culture media before, although it is anticipated to have plant hormones needed by plant tissue culture. This study was aimed to investigate if the indole-3-acetic acid (IAA) contents in the extracts from young and old ginger rhizomes might be related to their effects on Persian violet shoot tips cultured on media supplemented with the extracts. Spectrophotometric analysis revealed distinct IAA concentrations of 97.04 and 95.57 µg/mL in the extracts from young and old ginger rhizomes, respectively. Basal MS medium was then supplemented with these extracts at concentrations of 0, 5, 10, and 20 mL L^{-1} for culturing Persian violet shoot tips over 4 weeks. Remarkably, the medium supplemented with 5 mL L^{-1} of old ginger rhizome extract was the most effective in inducing root formation from Persian violet shoot tips under *in vitro* conditions, resulting in an average of 29 roots formed per shoot tip explant. In this treatment, the average root length was 6.7 cm, and maximum shoot height (3.6 cm) was also promoted. Therefore, the extract of old ginger rhizome distinctly stimulated both root and shoot development of Persian violet *in vitro*. This investigation supports the use of ginger rhizome extract (containing auxin) in plant tissue culture, particularly for enhancing propagation and development of Persian violet.

Keywords: *Exacum affine*; micropropagation; old ginger extract; root organogenesis; young ginger extract

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1. Introduction

The underground stem or rhizome of ginger (*Zingiber officinale* Roscoe), a member of the Zingiberaceae family, is one of the most widely utilized dietary condiments globally. The name “ginger” is derived from the Sanskrit term "srngaveram," meaning "horn root," attributed to its characteristic appearance. The ginger rhizome is notably rich in dietary fiber, vitamin E, vitamin B6, iron, magnesium, manganese, potassium, and selenium. Traditional Indian and Chinese medicinal practices spanning over 5,000 years have extensively employed ginger rhizome for treating diverse ailments such as colds, headaches, toothaches, asthma, gingivitis, nausea, migraines, and hypertension. Also, during the Coronavirus Disease 2019 (COVID-19) pandemic, ginger supplements showed significant promise in reducing hospital stays for infected individuals. This therapeutic versatility and pharmacological efficacy of ginger rhizome are attributable to its diverse phytocompounds, with gingerols emerging as particularly notable biologically active constituents (Bode & Dong, 2011; Li et al., 2022; Verma & Bisen, 2022; Malik et al., 2023).

One pivotal application within the field of plant tissue culture is micropropagation. This technique addresses the significant challenges associated with conventional propagation methods, namely prolonged propagation durations, limitations in plant yield, susceptibility to diseases and pests, and seasonal constraints. In micropropagation, these plant production issues can be approached through the induction of axillary bud and adventitious shoot proliferation *via* caulogenesis, and subsequent rhizogenesis, or the generation of embryos from somatic cells *via* somatic embryogenesis. This approach culminates in the successful *de novo* formation of plantlets (Singh et al., 2019; Ashrafzadeh & Leung, 2021; Gusain et al., 2021).

To complete the micropropagation cycle successfully, the rooting of microcuttings is imperative to achieve the development of a fully functional whole plant. Following shoot regeneration in organogenesis, the subsequent phase necessitates the induction of root formation prior to acclimatization. In this process, various methods exist for initiating roots. However, the use of plant growth regulators, particularly auxins, stands out as a preferred approach. Auxins play a crucial role in promoting adventitious root differentiation in tissue culture. For this purpose, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and α-naphthalene acetic acid (NAA) are commonly used, and each is employed at different concentrations to facilitate this specific developmental process (Rahman et al., 2018; Yunita & Nugraha, 2021; Long et al., 2022).

Persian violet, an ornamental plant characterized by its small purple flowers with yellow pollen at the center, belongs to the family Gentianaceae and is indigenous to Socotra, Yemen. This species is commonly used as a small potted plant in both indoor and outdoor settings. Traditionally, Persian violet is propagated by seed, producing fragrant blooms. However, the diminished fertility observed in the cultivars with composite flowers has amplified the demand for effective vegetative propagation techniques (Limtiyayotin et al., 2018; Srichuay et al., 2018). In the prior studies on using plant tissue culture for propagation of Persian violet, it was found that under *in vitro* conditions root formation in the microshoot explants on hormone-free medium and speed of rooting were in need of further improvement (Sukthavornthum et al., 2018; Phanomchai et al., 2022).

Efforts to find alternatives to auxin supplementation in tissue culture media for root induction are of continued interest. Hence, this study was aimed to assess the IAA content in young and old ginger rhizomes. Subsequently, extracts from both types of ginger rhizomes were incorporated into culture media to evaluate their potential to induce roots from shoot tips of Persian violet.

2. Materials and Methods

2.1 Plant materials

The *in vitro*-cultured plantlets of Persian violet (*Exacum affine* Balf.f. ex Regel) obtained in the prior study (Noichinda & Bodhipadma, 2023) were utilized to initiate the *in vitro* mother plants. A 10 mm shoot tip explant was excised and placed onto a semi-solid basal MS medium (Murashige & Skoog, 1962). The cultures were maintained in a growth room under controlled light conditions (20.87 μmol/m^2/s) for 16 h per day, alternating with 8 h of darkness, at a constant temperature of 25±2°C for 6 weeks.

2.2 Salkowski reagent preparation

Salkowski reagent was prepared following the method outlined by Gang et al. (2019). Initially, a 0.5 M ferric chloride ($FeCl_3$) solution was prepared by dissolving 8.125 g of $FeCl_3$ in 100 mL of distilled water. Subsequently, 24.5 mL of distilled water was added to a measuring cylinder, followed by the addition of 24.5 mL of 70% (w/v) perchloric acid. To this mixture, 1 mL of the 0.5 M $FeCl_3$ solution was added to 49 mL of 35% (w/v) perchloric acid. The resulting solution was thoroughly mixed and stored in an opaque bottle at room temperature prior to its use.

2.3 Preparation of ginger juice

Young (approximately 6-8 months old) and old (approximately 9-11 months old) ginger rhizomes of the same weight (1 kg) were procured from Mahachai Fresh Market, located in the Mahachai Subdistrict, Mueang Samut Sakhon District, Samut Sakhon Province, Thailand. The rhizomes were initially washed with clean tap water to remove any dirt. Subsequently, the outer skins were carefully peeled off, and the rhizomes were rinsed again with clean tap water. The cleaned young and old ginger rhizomes at the same weight (671.5 g) were then sliced into smaller pieces in preparation for extraction of ginger juice using a CUIZIMATE Juice Extractor (model: RBSJUICEXPRESS, ROBINSON Public Company Limited, Bangkok, Thailand). The extracted ginger juice was collected in an opaque container and stored at 4°C until further analysis in subsequent steps.

2.4 IAA assay

The preparation of an IAA standard curve was carried out following a modified protocol based on Ahmad et al. (2008).

2.4.1 IAA standard curve

Initially, a 1,000 μg mL^{-1} stock solution of IAA was prepared and subsequently diluted to concentrations of 0, 20, 40, 60, 80, 100, and 120 μg mL^{-1}. For each concentration of IAA, 1 mL was pipetted into individual test tubes and 2 mL of Salkowski reagent was added to each tube. After incubating in darkness for 30 min, the absorbance of the mixture was then measured using a spectrophotometer (model SP-830 Plus, Metertech Inc., Taipei, Taiwan, R.O.C.) at a wavelength of 530 nm.

2.4.2 IAA content from young and old ginger extracts

In the quantitative analysis of IAA from young and old ginger extracts (as detailed in Section 2.3), the extracted ginger juice was centrifuged at 10,000 rpm for 10 min at 4°C. Following centrifugation, 1 mL of the extract was combined with 2 mL of Salkowski reagent. The mixture was incubated in darkness for 30 min before the absorbance was measured at a wavelength of 530 nm using a spectrophotometer.

2.5 Effect of young and old ginger extracts on in vitro Persian violet shoot tip culture

A Persian violet mother plant maintained in a sterile environment was cultured on semi-solid basal MS medium for 6 weeks, as outlined in Section 2.1 (Figure 1). Subsequently, a shoot tip (10 mm) was excised and then placed in a jar with semi-solid basal MS medium supplemented with extracts of either young or old ginger, at concentrations of 0, 5, 10, and 20 mL L^{-1}. The cultures were kept in a growth room under identical conditions as described in Section 2.1 for 4 weeks. At the end of the experiments, data on root number, root length, and shoot height were recorded.

Figure 1. *In vitro* Persian violet mother plant

2.6 Statistical analysis

The means obtained from 12 replications of indole-3-acetic acid (IAA) quantification in extracts from both young and old ginger were subjected to statistical comparison using an Independent Samples t-test ($p < 0.05$). Similarly, means derived from 10 replications each of root number, root length, and shoot height measurements were processed in statistical analysis using Duncan's Multiple Range Test (DMRT) ($p < 0.05$).

3. Results and Discussion

3.1 IAA contents in young and old ginger extracts

After mixing young and old ginger extracts with Salkowski reagent for 30 min, a pink colored complex was developed following the reaction between the reagent and IAA in the extracts. Subsequent quantification of IAA levels in these extracts using a spectrophotometer measuring absorbance at 530 nm revealed markedly higher concentrations of IAA in young ginger rhizome extract compared to old ginger rhizome extract, 97.04 and 95.57 μg mL^{-1}, respectively.

Auxin is the generic name of a major plant hormone derived from the Greek word "auxein," meaning to grow or increase, and IAA was the first auxin discovered. IAA is primarily synthesized via the indole-3-pyruvic acid (IPyA) pathway using tryptophan as its precursor (Enders & Strader, 2015; Fu et al., 2015; Olatunji et al., 2017). A common method to measure IAA production is Van Urk Salkowski's reagent test, known for its simplicity and effectiveness in colorimetric analysis. This assay involves the reaction of IAA with $FeCl_3$ in the reagent, leading to the reduction of Fe^{3+} and the formation of a pink-colored complex (De-Bashan et al., 2008; Mohite, 2013; Gang et al., 2019).

In this study, the development of a pink color after mixing young and old ginger extracts with Salkowski's reagent for 30 min indicated the presence of IAA in both types of rhizomes. Using spectrophotometric quantification, it was found that old ginger rhizomes had significantly less IAA (95.57 μg mL^{-1}) than young ginger rhizomes (97.04 μg mL^{-1}), suggesting that the developmental stage of the ginger rhizome affects IAA accumulation. This has also been observed in tomato roots, shoots, leaves, and fruit at different stages. Interestingly, while IAA concentration increased with the age of tomato organs, it decreased in older ginger rhizomes (Ahmed et al., 2022). This difference in IAA production is likely related to the different organs and developmental stages in these two plants.

3.2 Effect of young and old ginger extracts on Persian violet shoot tips cultured *in vitro*

3.2.1 Root number

In the experiment involving the culture of 10 mm long Persian violet shoot tip explants on semi-solid basal MS medium supplemented with various concentrations of young and old ginger extracts for 4 weeks, it was observed that the addition of 5 mL L^{-1} of old ginger extract resulted in the highest number of roots (28.8). This was followed by the formation of 8.0 roots on basal MS medium supplemented with 10 mL L^{-1} of old ginger extract. Conversely, the inclusion of young ginger extract resulted in a lower root count compared to old ginger extract at the equivalent concentrations. Notably, no roots were formed when the medium was supplemented with 20 mL $L^{-1}$of young ginger extract (Table 1 and Figure 2).

Table 1. Root number of Persian violet shoot tips grown on semi-solid MS medium supplemented with various concentrations of young ginger extract (YGE) or old ginger extract (OGE) for 4 weeks

Medium	Number of Roots
Basal MS	5.5 ± 0.8^{c}
MS+5 mL L^{-1} YGE	3.2 ± 0.7^{cd}
MS+10mL L^{-1} YGE	1.3 ± 0.8^{de}
MS+20mL L^{-1} YGE	0.0 ± 0.0^{e}
MS+5 mL L^{-1} OGE	28.8 ± 1.3^{a}
MS+10mL L^{-1} OGE	8.0 ± 1.1^{b}
MS+20mL L^{-1} OGE	3.7 ± 0.4^{cd}

Data are mean±SE from 10 replications, and the means within the same column followed by different letters are significantly different ($p<0.05$).

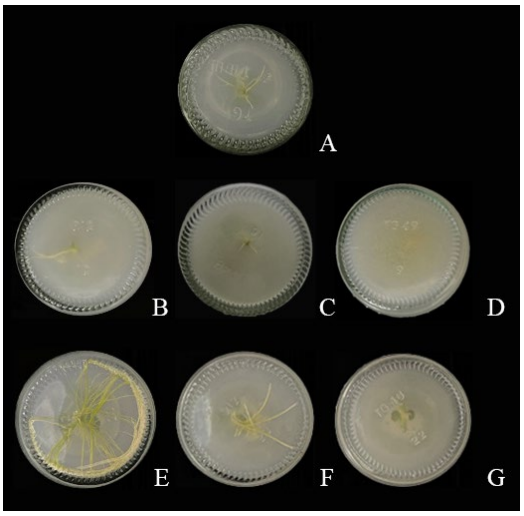

Figure 2. Root development from Persian violet shoot tips cultured on semi-solid MS medium supplemented with various concentrations of young ginger extract (YGE) or old ginger extract (OGE) at week 4 (A = control; B, C, and D = 5, 10, and 20 mL L^{-1} YGE, respectively; E, F, and G = 5, 10, and 20 mL L^{-1} OGE, respectively).

3.2.2 Root length

The impact of young and old ginger extracts on root length of Persian violet shoot tips cultured *in vitro* mirrored the results obtained in root formation. Specifically, the longest roots (6.67 cm) were observed when the shoot tips were cultured on MS medium supplemented with 5 mL L^{-1} of old ginger extract. The next longest roots (0.91 cm) were observed in shoot tip cultures supplemented with MS basal medium containing 10 mL L^{-1} of old ginger extract. In contrast, roots were consistently shorter when cultured on medium supplemented with the equivalent concentrations of young ginger extract. Remarkably, in the shoot tip cultures on medium supplemented with 20 mL L^{-1} of young ginger extract, root growth was completely inhibited (Table 2 and Figure 2).

Table 2. Root length of Persian violet shoot tips grown on semi-solid MS medium supplemented with various concentrations of young ginger extract (YGE) or old ginger extract (OGE) for 4 weeks

Medium	Root Length (cm)
Basal MS	0.62 ± 0.08^{bc}
MS+5 mL L^{-1} YGE	0.81 ± 0.20^{bc}
MS+10mL L^{-1} YGE	0.35 ± 0.23^{cd}
MS+20mL L^{-1} YGE	0.00 ± 0.00^{d}
MS+5 mL L^{-1} OGE	6.67 ± 0.27^{a}
MS+10mL L^{-1} OGE	0.91 ± 0.08^{b}
MS+20mL L^{-1} OGE	0.52 ± 0.05^{bc}

Data are mean±SE from 10 replications, and the means within the same column followed by different letters are significantly different ($p<0.05$).

3.2.3 Shoot height

During *in vitro* root development in the Persian violet shoot tips, shoot elongation then followed. The supplementation of 5 mL L^{-1} old ginger extract notably promoted both root formation (the highest number and length of roots) and shoot elongation (3.60 cm). In contrast, higher concentrations of old ginger extract and all treatments involving young ginger extract led to diminished shoot lengths compared to the control (Table 3).

Table 3. Shoot height of Persian violet shoot tips grown on semi-solid MS medium supplemented with various concentrations of young ginger extract (YGE) or old ginger extract (OGE) for 4 weeks

Medium	Shoot Height (cm)
Basal MS	2.34 ± 0.23^{b}
MS+5 mL L^{-1} YGE	1.54 ± 0.07^{cd}
MS+10mL L^{-1} YGE	1.30 ± 0.08^{d}
MS+20mL L^{-1} YGE	1.17 ± 0.07^{d}
MS+5 mL L^{-1} OGE	3.60 ± 0.16^{a}
MS+10mL L^{-1} OGE	1.80 ± 0.12^{c}
MS+20mL L^{-1} OGE	1.27 ± 0.09^{d}

Data are mean±SE from 10 replications, and the means within the same column followed by different letters are significantly different ($p<0.05$).

In vitro propagation represents a practical technology for mass production, crucial for ensuring the sustainability of the industry. Excised shoot tips, which encompass the apical meristem along with other apical tissues, have been found to be an effective explant source for successful micropropagation across various plant groups, including annual shrubs, perennial herbs, and woody trees (Kozlowski & Pallardy, 1997; Shen et al., 2010; Babaei et al., 2014; Panwar & Joshi, 2020; Klaocheed et al., 2024).

In the micropropagation process, rooting constitutes the third stage (pre-transplant) and occurs subsequent to stock plant preparation, culture initiation, and multiplication. The development of roots is critical for the successful formation of plantlets and significantly influences subsequent stages of growth (Monthony et al., 2021). During *in vitro* clonal propagation, various methods can induce *de novo* root organogenesis, with

the application of plant hormones being the most prevalent. Among these, IAA, IBA, and NAA are the most frequently used auxins in plant tissue cultures (Rahman et al., 2018; Yunita & Nugraha, 2021; Long et al., 2022).

In addition to plant growth regulators, natural supplements or complex organic compounds are occasionally incorporated into the culture medium. These complex organics encompass a diverse array of undefined supplements, including banana homogenate, casein hydrolysate, coconut water, malt extract, potato extract, yeast extract, orange juice, and tomato juice. When fully defined plant culture media fail to yield the desired results, the utilization of these natural substances can often exert a beneficial influence on *in vitro* plant cell and tissue cultures (Molnár et al., 2011; Bhatia et al., 2015).

Ginger rhizome has been recognized for its therapeutic properties since ancient times and is regarded as a potential chemopreventive agent. In addition to polysaccharides, lipids, organic acids, and raw fibers, the ginger rhizome contains a variety of biologically active compounds, including phenolics and terpenes. The primary phenolic compounds present in ginger are gingerols, shogaols, and paradols, while the main terpene constituents include β-bisabolene, α-curcumene, zingiberene,α-farnesene, and β-sesquiphellandrene. These active constituents endow the ginger rhizome with multiple biological activities, such as antioxidant, anti-inflammatory, anti-tumor, antimicrobial, and anti-diabetic properties (Rahmani et al., 2014; Mao et al., 2019).

In the initial phase of this study, IAA production was identified in both young and old ginger rhizomes. Subsequently, ginger juice extracted from these rhizomes underwent detailed investigation for its potential as a complex organic component in plant tissue culture media. Following a culture period of 4 weeks, shoot tip explants of Persian violet exhibited varied responses to different concentrations of both young and old rhizome extracts, influencing both the number and length of roots formed. The most significant root formation and growth occurred on basal MS medium comprised 5 mL L^{-1} of old ginger extract. This medium also resulted in the greatest shoot height observed among all the treatments. Taken together, these promotive effects of old ginger rhizome extract, compared to young ginger rhizome extract on root initiation and growth correlated with the lower IAA concentration in the old ginger rhizome. However, increasing the concentrations of extracts from both types of rhizomes further did not enhance more Persian violet root initiation and growth. This suggests that the IAA contents in these extracts might have surpassed the optimal conditions required for effective root induction and growth and could exert inhibitory effects on root development. The findings here are consistent with well-known physiological effects of auxin on root development (Overvoorde et al., 2010).

When comparing the effects of extracts from young and old ginger rhizomes at equivalent concentrations on root initiation and growth, it was observed that extracts derived from old ginger rhizomes were more effective than those from young ginger rhizomes. This difference may be attributed to the higher IAA concentrations found in the extracts from young ginger rhizomes, which seem to be associated with a greater inhibitory effect on root formation in the Persian violet shoot tip at equivalent concentrations compared to those from older rhizomes.

Furthermore, regarding the speed of rooting, it was noted that a culture medium supplemented with 5 mL $L^{-1}$of old ginger extract induced roots more rapidly. Root formation was observed as early as the 2nd week of culture, whereas with other concentrations, root initiation at some concentrations was delayed until week 3 (Figure 3). Thus, the concentration of 5 mL L^{-1} of old ginger extract was considered optimal for promoting root initiation in Persian violet shoot tips.

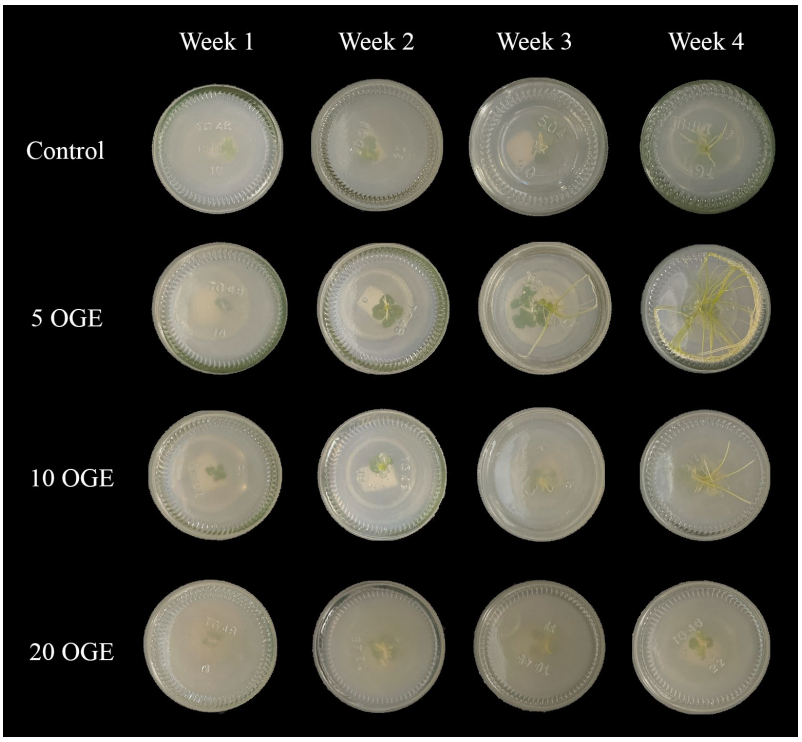

Figure 3. Comparison of root development between Persian violet shoot tips grown on semi-solid MS medium supplemented with various concentrations of old ginger extract (OGE) and control at week 1 to week 4 (5 OGE, 10 OGE, and 20 OGE are 5, 10, and 20 mL L^{-1} old ginger extract, respectively).

Conversely, when 20 mL L^{-1} of young ginger extract was added, no root formation was observed, suggesting that this IAA concentration exceeded the optimal threshold for inducing roots. Nonetheless, the concentration of 20 mL L^{-1} of young ginger extract may be useful for inducing callus formation in other plant species or explants used as a high concentration of auxin can stimulate callus induction (Ma et al., 2022).

Based on the overall results, it is evident that, in addition to the roots, shoots, leaves, fruit, and seeds of plants, the ginger rhizomes have been shown to produce IAA. Remarkably, young ginger rhizomes generate higher levels of IAA compared to older ginger rhizomes, consistent with the established understanding that younger plant tissues typically produce more IAA than mature or older tissues. Furthermore, it is plausible that not only the quantity but also the active form of IAA in the ginger rhizome influences the root formation and development.

In plant cells, IAA exists in two forms: active or free IAA, and inactive or bound/conjugated IAA. The levels of endogenous IAA within plants are regulated through various mechanisms, including *de novo* biosynthesis, transport, irreversible degradation, and reversible inactivation mediated by IAA conjugation. Free IAA levels are maintained at low concentrations, and these active IAA levels can be modulated by conversion into IBA or by conjugation to sugars or sugar alcohols (commonly D-glucose or myo-inositol) *via* an ester linkage or to amino acids (commonly aspartic acid, glutamine, and alanine) *via* an amide linkage. In many dicotyledons, IAA amides are the predominant conjugates, with

their concentrations significantly surpassing those of IAA esters (Srivastava, 2002; Rosquete et al., 2012; Méndez-Hernández et al., 2021). However, it was also found that some monocots contain IAA-amides (Ostrowski et al., 2015).

To elucidate the specific types and quantities of IAA responsible for root induction and growth in Persian violet shoot tips, it is essential to distinguish between the amounts and forms of IAA present in young and old ginger rhizomes. This understanding would provide clearer insights into the mechanisms underlying root induction and development, making it a compelling area for future research.

4. Conclusions

Ginger rhizome extract, which is classified as consisting of complex organic substances, serves as a natural source of IAA. It was found here that young ginger rhizomes exhibited a higher IAA concentration compared to older rhizomes. The incorporation of ginger rhizome extract into plant tissue culture media has been demonstrated to induce root formation in Persian violet shoot tips without the need for exogenous application of synthetic auxin or IAA. Noticeably, adventitious rooting of Persian violet shoot tips can be significantly promoted using low concentrations of old ginger rhizome extract. This might be a viable alternative for applications in plant tissue culture of other plant species, warranting further investigations.

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6. Conflicts of Interest

The authors declare no conflict of interest.

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