

# Rhizome induction and proliferation in *Anoectochilus lylei* for biomass and bioactive compounds accumulation

**Thanh-Tam Ho**

hothanhtam2@duytan.edu.vn

Duy Tan University: Dai Hoc Duy Tan <https://orcid.org/0000-0001-7516-1181>

**So-Young Park**

Chungbuk National University

**Thi Hong Trang Pham**

Duy Tan University: Dai Hoc Duy Tan

**Tolulope Joshua Ashaolu**

Duy Tan University: Dai Hoc Duy Tan

**Thanh-Do Le**

Duy Tan University: Dai Hoc Duy Tan

**Gia Huy Ngo**

Duy Tan University: Dai Hoc Duy Tan

**Tran Kim Long Nguyen**

Duy Tan University: Dai Hoc Duy Tan

**Trung Hieu Le**

Hue University of Sciences: Hue University University of Sciences

**Van Ngo Thai Bich**

Da Nang University of Technology: Danang University of Science and Technology

**Hoang Tan Quang**

Hue University

**Hoang Thi Kim Hong**

Duy Tan University: Dai Hoc Duy Tan

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## Research Article

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# Abstract

*Anoectochilus sp.* is a valuable medicinal plant with a long history of traditional uses. This study aimed to induce and optimize culture conditions for rhizome biomass cultivation in *Anoectochilus lylei*, providing a sustainable method for biomass and bioactive compound production. The highest rhizome induction rate (98.9%) with fresh weight (FW) of 12.64 g and dry weight (DW) of 1.14 g was achieved using MS medium supplemented with 2 g/L hyponex I, 2 g/L hyponex II, 2 g/L peptone, and 1.0 g/L activated charcoal. To optimize the culture condition, different salt strengths (MS, SH, B5) were tested, and  $\frac{3}{4}$ MS medium with 2 mg/L BA, 0.5 mg/L NAA, 0.2 mg/L Kin, and 35 g/L sucrose was most effective for rhizome proliferation (127.33 g/L FW and 20.40 g/L DW). The highest total phenolic content (4.9 mg/g DW) was observed in  $\frac{1}{2}$ MS medium, while the highest flavonoid (1.05 mg/g DW) and polysaccharide content (150.66 mg/g DW) was recorded in control. Optimal bioactive productivity was observed in  $\frac{3}{4}$ MS medium. In sucrose concentrations trials, 35 g/L sucrose yielded the highest rhizome biomass (157.17 g/L FW; 20.67 g/L DW). The highest phenolic (4.44 mg/g DW), and flavonoid (1.15 mg/g DW) were recorded at 10g/L sucrose, while polysaccharide (115.87 mg/g DW) content was observed at 35 g/L sucrose concentrations. Additionally, rhizome cultures exhibited higher kinsenoside (2.94 mg/g DW) and polysaccharide content than both ex vitro and in vitro plants. Furthermore, the rhizome extract show suppresses inhibited the growth of *Staphylococcus aureus* and *Escherichia coli*, demonstrating potential for antibacterial applications. These results highlight the potential for large-scale bioreactor cultivation of *A. lylei* rhizomes for enhanced biomass and bioactive compound production.

## Introduction

The genus *Anoectochilus* comprises 40 species, of which 12 were discovered in Viet Nam. These plants are known as the "King of Medicinal Plants" for their beautiful flowers and medicinal properties, such as liver-protective, anti-cancer, anti-diabetes, and anti-inflammatory effects, and treatment of cardiovascular diseases (Ket et al. 2004; Gutierrez et al. 2010). Research on *Anoectochilus* species worldwide has been quite diverse, ranging from asexual propagation to studies on rhizome biomass culture for producing bioactive compounds. For example, Gangaprasad et al. (2000) propagated *A. sikkimensis* and *A. regalis* through stem nodal segments while Ket et al. (2004) successfully micropropagated *A. formosanus* on MS medium supplemented with BA, TDZ, and activated charcoal, resulting in 100% plantlet survival and growth at ex vitro conditions.

In addition to propagation studies, research on the biological activities and medicinal properties of *Anoectochilus* species has been conducted. Numerous studies have demonstrated that *Anoectochilus* species produce a range of bioactive compounds, including polysaccharides and an alkaloid called kinsenoside used in the treatment of diabetes, hyperlipidemia, and breast cancer (Wu et al. 2007; Zou et al. 2019). Indeed, an extract of *A. formosanus* reportedly stimulates immune function and exhibits hepatoprotective activity (Wu et al. 2007). Zhang et al. (2007) tested different doses of kinsenoside from *A. roxburghii* orally to investigate its biological activity and pharmacological mechanisms related to its

hypoglycemic effect on diabetic mice. The vascular protective properties of kinsenoside extracted from *A. roxburghii* in hyperglycemic humans have also been reported (Liu et al. 2013).

Over the past decade, the application of organ and plant cell cultures has unlocked new potentials in biomass production from valuable medicinal plants, providing an alternative to natural raw materials and achieving significant milestones (Murthy et al. 2014, 2016). Adventitious root cultures of ginseng, *Echinacea* species, *Morinda citrifolia*, and *Polygonum multiflorum* have been successfully established and studied (Wu et al. 2006; Baque et al. 2010; Ho et al. 2018, 2019). In the case of *Anoectochilus* genus, rhizome culture has emerged as a focus for biomass production and the extraction of kinsenosides, polysaccharides, and other bioactive compounds. Notably, in *A. roxburghii*, several studies have optimized culture media and evaluated the biological activities of the rhizome biomass (Jin et al. 2017, 2018; Luo et al. 2018; Fan et al. 2022). The present study aimed to induce and rapidly multiply rhizomes in *Anoectochilus lylei*, an important medicinal herb, as a biomass source of biologically active compounds.

## Materials and Methods

### Rhizome induction and proliferation

In vitro plants were cut into nodal segments of 1.0–1.5 cm lengths and inoculated in the medium for rhizome induction. The induction medium was MS (Murashige and Skoog 1962) supplemented with 2 g/L peptone (Xilong Scientific Co., Ltd., China), 7 g/L agar (Hai Long Co., Ltd., Viet Nam), 1 g/L AC combined with different concentrations of hyponex I (N:P:K = 6.5:6:19; Hyponex Japan Co., Ltd, Japan) and hyponex II (N:P:K = 20:20:20; (Hyponex Japan Co., Ltd, Japan): Control (0 g/L) Rh05 (0.5 g/L), Rh1 (1.0 g/L), Rh2 (2.0 g/L), Rh3 (3.0 g/L), and Rh4 (4 g/L) of hyponex I and hyponex II, respectively. The medium pH was adjusted to 5.8 before autoclaving at 121°C, 1 atm for 20 min.

The induced rhizomes were cut to approximately 1.0 cm long and inoculated in the bottle containing 80 mL of three-quarter MS medium (Murashige and Skoog, 1962) supplemented with 0.2 mg/L kinetin, 0.5 mg/L NAA, 35 g/L sucrose, and 7 g/L agar combined with different BA concentrations (0, 0.5, 1.0, 2.0, 3.0 mg/L). The chemicals and phytohormones were purchased from Duchefa Co., Ltd. (The Netherlands). All cultures were maintained at  $25 \pm 2^\circ\text{C}$  under  $30 \mu\text{mol}/\text{m}^2/\text{s}$  of LED light (Rang Dong, Viet Nam) with a 16h photoperiod.

### Optimal liquid cultures for rhizomes production

To optimize the culture conditions, 3 g FW rhizomes of *A. lylei* from previous experiments were cultivated for 6 weeks in a 250 mL Erlenmayer Flask containing 80 mL liquid medium. Different salt strengths of three media - MS, SH (Schenk & Hildebrandt, 1972), and B5 (Gamborg, 1968) and different sucrose concentrations (0, 10, 20, 35, 50, and 70 g/L) were applied for the experiments. All experiments were performed sequentially in flasks on a shaker (Zenith Lab, Jiangsu, China) at 100 rpm.

## Determination of root biomass

The rhizomes were harvested and washed with tap water to remove the medium. The fresh weight (FW) of rhizomes was measured after removing excess surface water with tissue paper, and the dry weight (DW) of roots was recorded after drying the roots to a constant weight at 50°C for 72 h. The growth index (GI) was calculated as follows:  $GI = (Final\ FW - Initial\ FW) / Initial\ FW$

## Determination of total phenolic and flavonoid contents

### Preparation of root extract

Dried samples (0.5 g) were sonicated (Ultrasonic Cleaner UCP 20, Lab Companion, Korea) for 1 h with 30 mL of 80% methanol to ensure complete extraction of phenolic compounds. The extracts were filtered using filter paper (Advantec 110 mm, Japan).

### Determination of total phenolic content (TPC)

The total phenolic content (TPC) in the methanolic extract was evaluated through a spectrophotometric method using the Folin-Ciocalteu reagent, as described by Wu et al. (2006). Specifically, a 0.05 mL aliquot of the methanolic extracts was combined with 2.55 mL of distilled water. Next, 0.1 mL of 2 N Folin-Ciocalteu reagent was introduced to the mixture. After a 5-minute interval, 0.5 mL of 20%  $Na_2CO_3$  was added and mixed thoroughly. The reactions were kept in the dark at room temperature for 30 minutes. Subsequently, the absorbance at 760 nm was measured using a spectrophotometer (Cary 60 Conc, Agilent Technologies, USA). A standard curve for gallic acid (Sigma Chemical Co., St. Louis, MO, USA) was used to quantify the results, which were expressed as mg gallic acid equivalent (GAE) per gram of dry weight (DW) of samples.

### Determination of total flavonoid content (TFC)

The total flavonoid content (TFC) was also determined colorimetrically according to the method of Wu et al. (2006). A 0.25 mL of methanolic extracts and/or (+)-catechin (Sigma Chemical Co.) standard were mixed with 1.25 mL of distilled water. Afterward, 0.075 mL of 5%  $NaNO_2$  was added to each sample and shaken vigorously. After 6 min, 0.15 mL of 10%  $AlCl_3$  was introduced, and the sample was incubated at room temperature for 5 min. The absorbance was then recorded at 510 nm using a spectrophotometer (Cary 60 Conc, Agilent Technologies, USA). The results were reported as milligrams of (+)-catechin equivalents per gram of dry weight (DW) of samples

### Determination of total polysaccharide determination

The total polysaccharide content of the samples was extracted and analyzed using the phenol–sulfuric acid assay, as described by Jin et al. (2017). A 0.1 g ground dry sample was soaked in 80% ethanol for 6

h at 25°C to eliminate interfering substances. The sample was collected after vaporizing the ethanol and added to 50 mL distilled water, then heated at 60°C for 3 h. The resulting mixture was filtered, and 1 mL of the filtrate (sample solution) was combined with 1.0 mL of 5% phenol and 5 mL of H<sub>2</sub>SO<sub>4</sub> (100%). After 30 min of reaction at room temperature, the absorbance was measured at 490 nm using a spectrophotometer (Cary 60 Conc, Agilent Technologies, USA), with glucose (Duchefa, The Netherlands) referred to as the standard.

## Determination of kinsenoside content

The extraction and analysis of kinsenoside from the rhizome and plant samples were carried out following the method outlined by Jin et al. (2017). Specifically, 2 g of finely ground dry sample was soaked in 10 mL of 100% methanol and subjected to ultrasonic treatment for 1 hour, followed by incubation at room temperature for 12 hours. The mixture was then filtered, and the filtrate was evaporated under reduced pressure using a vacuum rotary evaporator. The concentrated extract was dried at 45°C and dissolved in 5 mL of deionized water, followed by defatting with chloroform. The sample was stored at 4°C prior to kinsenoside measurement. A high-performance liquid chromatography (HPLC) system equipped with a C8 reverse-phase column (5µm, 250 mm x 4,6 mm) and a UV detector set at 215 nm was employed for the analysis. The mobile phase consisted of a 4:96 water-methanol mixture, with a flow rate of 1 mL/min. The injection volume was 0.8 µL, and the column temperature was maintained at 30°C. The retention time was 4 minutes. The concentration of kinsenoside was determined using kinsenoside standard curve.

## Antioxidant and antibacterial activity

The antioxidant capacity of rhizome was measured using the 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma Chemical Co.) method (Hatano et al.1998). A 0.8 mL volume of 200 µM DPPH radical solution was added to 0.2 mL methanolic root extract; 40% methanol served as the control. The solutions were incubated for 5 min at room temperature, and the absorbance was measured at 517 nm using a spectrophotometer (Cary 60 Conc, Agilent Technologies, USA).

Antibacterial assay was carried out using agar well diffusion methods. This method is widely used to evaluate the antimicrobial activity of plants or microbial extracts. Briefly, *Staphylococcus aureus* (gram-positive) and *Escherichia coli* (gram-negative) colonies were diluted in Muller-Hinton broth to adjust their turbidity to 0.5 McFarland standard. 100 µL of diluted bacterial suspensions were surface inoculated on Mueller-Hinton agar plate (MHA) (90 mm in diameter), then 50 µL of extracts from ex vitro and in vitro plants, and 100 mg/mL rhizomes were loaded into 6-mm punched wells. DMSO (10%) and Rifampicin were used as negative and positive controls, respectively, in this assay. The plate was left for 2 hours at 4°C for the substances to diffuse into the agar. After overnight incubation at 37°C, the antibacterial activity of extracts was evaluated according to the inhibition zones using the following formula:

$$\text{Zone of inhibition (ZOI - mm)} = D - d$$

Where:

D is the diameter of the inhibition zone;

d is the diameter of the punched well

## Statistical analysis

A complete randomized design was used as the experimental design of this study. All results are presented as means  $\pm$  standard errors, based on at least three independent replicates. Statistical assessments of the difference between mean values were assessed by Duncan's multiple range testing at  $p < 0.05$  level using SPSS version 16.0 (IBM Corp., USA).

## Results and Discussion

### Rhizome induction and proliferation

In the present study, different combinations of MS medium strengths and hyponex I and hyponex II positively affected rhizome induction in *A. lylei*. Specifically, the Rh2 treatment, consisting of MS medium combined with 2 g/L of hyponex I (N:P:K = 6.5:6:19), 2 g/L hyponex II (N:P:K = 20:20:20), and 2 g/L peptone shows the best results. The rhizome induction rate reached 98.9%, with a fresh weight (FW) of 16.64 g and dry weight (DW) of 1.14 g (Table 1; Fig. 1A). Compared to other treatments, the rhizome induced from Rh2 treatment was not only uniform but also had many root hairs; and showing superiority in growth and development. When increasing the concentration of hyponex I and hyponex II to 4 g/L, the node segment did not grow and gradually died (Fig. 1A). Rhizome culture for biomass and bioactive compounds such as polysaccharide and kinsenoside production in *A. roxburghii* was carried out in several studies (Jin et al. 2017, 2018; Luo et al. 2018). Based on those results, we optimized the culture conditions for rhizome culture in *A. lylei*, an important medicinal plant. The suitable medium for rhizome induction in *A. lylei* is MS medium supplemented with 2 g/L hyponex I (N:P:K = 6.5:6:19), 2 g/L hyponex II (N:P:K = 20:20:20), and 2 g/L peptone; while the MS medium added with 1 g/L hyponex I, 1 g/L hyponex 2, and 2 g/L peptone were found to be the suitable medium for *A. roxburghii* rhizome formation (Jin et al. 2017, 2018). The observed difference may be attributed to varying nutritional requirements among different species of *Anoectochillus* genus.

Table 1  
Rhizome induction in *Anoectochilus lylei*.

| Medium                                                                                                                            | % induction rate <sup>z</sup> | FW (g)                    | DW (g)                    |
|-----------------------------------------------------------------------------------------------------------------------------------|-------------------------------|---------------------------|---------------------------|
| Control                                                                                                                           | 4.43 <sup>d</sup> ± 2.22      | 6.96 <sup>c</sup> ± 0.61  | 0.63 <sup>cd</sup> ± 0.06 |
| Rh05                                                                                                                              | 54.43 <sup>c</sup> ± 2.94     | 5.98 <sup>c</sup> ± 0.63  | 0.58 <sup>cd</sup> ± 0.07 |
| Rh1                                                                                                                               | 86.67 <sup>b</sup> ± 1.92     | 9.76 <sup>b</sup> ± 1.39  | 0.90 <sup>b</sup> ± 0.13  |
| Rh2                                                                                                                               | 98.90 <sup>a</sup> ± 1.11     | 12.64 <sup>a</sup> ± 1.25 | 1.14 <sup>a</sup> ± 0.12  |
| Rh3                                                                                                                               | 84.43 <sup>b</sup> ± 1.11     | 8.84 <sup>b</sup> ± 0.69  | 0.80 <sup>bc</sup> ± 0.07 |
| Rh4                                                                                                                               | 57.77 <sup>c</sup> ± 4.01     | 3.39 <sup>c</sup> ± 0.16  | 0.36 <sup>d</sup> ± 0.02  |
| <sup>z</sup> Mean with different letters within a column differs significantly by Duncan's multiple-range test ( $p \leq 0.05$ ). |                               |                           |                           |

The greatest challenge in rhizome proliferation is avoiding shoot formation due to the presence of several nodes in the rhizomes. In the proliferation process, lower BA concentrations (less than 2 mg/L) stimulated shoot induction unsuitable for rhizome multiplication. Supplementation with 2 mg/L of BA combined with NAA and Kin demonstrated the highest rhizome proliferation rate on a solid medium; FW of 11.81 g/bottle and DW of 0.96 g/bottle with high uniformity and no shoot induction. Whereas, high concentrations as high as 3.0 mg/L inhibited rhizome growth (Table 2; Fig. 1B, C). Previously, Jin et al. (2018) conducted two experiments using the uniform design method to explore the impact of various medium components on the accumulation of polysaccharides and kinsenoside in rhizomes culture of *A. roxburghii*. This study successfully appropriated culture medium for the suspension culture of *A. roxburghii* rhizomes. The optimal rhizome suspension culture medium was  $\frac{3}{4} \times$  MS medium supplemented with 2.0 mg/L BA, 0.5 mg/L NAA, 0.2 mg/L kinetin, and 35 g/L sucrose. Fortunately, the present study shows similar results on rhizome proliferation in *A. lylei* rhizome culture.

Table 2  
Effect of BA concentration on rhizome proliferation in *Anoectochilus lylei*.

| BA concentration (mg/L) <sup>z</sup>                                                                                                                                                                                                                                                                 | FW (g) <sup>y</sup>                   | DW (g)                    |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------|---------------------------|
| 0.0                                                                                                                                                                                                                                                                                                  | 4.31 <sup>c</sup> ± 0.74 <sup>z</sup> | 0.35 <sup>c</sup> ± 0.16  |
| 0.5                                                                                                                                                                                                                                                                                                  | 6.55 <sup>bc</sup> ± 0.96             | 0.53 <sup>bc</sup> ± 0.08 |
| 1.0                                                                                                                                                                                                                                                                                                  | 5.36 <sup>c</sup> ± 0.65              | 0.43 <sup>c</sup> ± 0.05  |
| 2.0                                                                                                                                                                                                                                                                                                  | 11.81 <sup>a</sup> ± 1.08             | 0.96 <sup>a</sup> ± 0.08  |
| 3.0                                                                                                                                                                                                                                                                                                  | 7.88 <sup>b</sup> ± 0.45              | 0.64 <sup>b</sup> ± 0.04  |
| <sup>z</sup> The treatment was carried out with ¾×MS medium supplemented with 2.0 mg/L BA, 0.5 mg/L NAA, 0.2 mg/L Kinetin, and different BA concentrations.<br><br><sup>y</sup> Mean with different letters within a column differs significantly by Duncan's multiple-range test ( $p \leq 0.05$ ). |                                       |                           |

## Effect of medium salt strength on rhizome biomass production and bioactive compounds accumulation

Numerous previous studies have demonstrated the role of different medium salt strengths, such as MS, SH, B5, and LS (Linsmaier and Skoog, 1965), and WPM (McCown and Lloyd, 1981) on biomass and secondary metabolite production in plant cell and organ cultures (Baque et al. 2013; Murthy et al. 2016; Ho et al. 2021). The strength of the medium significantly influences both rhizome biomass and bioactive compound accumulation in the rhizome culture of *A. lylei*, depending on the type and concentration of the medium (SH, B5, MS). Among the ten treatments tested, the ¾-strength MS medium was the most effective for rhizome production (Table 3, Fig. 2), yielding 127.33 g/L FW, 20.4 g/L DW, and a growth index (GI) of 4.09. This was followed by the ¾-strength B5 medium, with 99.78 g/L FW, 15.08 g/L DW, and a GI of 2.99, and the ¾-strength SH medium, which produced 91.18 g/L FW, 15.28 g/L DW, and a GI of 2.65. In contrast, the control treatment exhibited the poorest rhizome growth.

Table 3  
Biomass production of *A. lylei* rhizome culture in different medium salt strengths <sup>z</sup>Mean with different letters within a column differs significantly by Duncan's multiple-range test ( $p \leq 0.05$ ).

| Medium                          | FW (g/L) <sup>z</sup>       | DW (g/L)                   | Growth Index              |
|---------------------------------|-----------------------------|----------------------------|---------------------------|
| Control                         | 38.53 <sup>c</sup> ± 0.50   | 5.50 <sup>c</sup> ± 0.30   | 0.54 <sup>c</sup> ± 0.01  |
| MS                              | 70.00 <sup>bc</sup> ± 13.77 | 9.63 <sup>bc</sup> ± 1.57  | 1.80 <sup>bc</sup> ± 0.37 |
| <sup>3</sup> / <sub>4</sub> ×MS | 127.33 <sup>a</sup> ± 26.38 | 20.40 <sup>a</sup> ± 4.85  | 4.09 <sup>a</sup> ± 0.44  |
| <sup>1</sup> / <sub>2</sub> ×MS | 61.63 <sup>bc</sup> ± 5.47  | 9.53 <sup>bc</sup> ± 0.84  | 1.47 <sup>bc</sup> ± 1.05 |
| B5                              | 77.35 <sup>bc</sup> ± 15.31 | 11.60 <sup>bc</sup> ± 2.40 | 2.09 <sup>bc</sup> ± 0.41 |
| <sup>3</sup> / <sub>4</sub> ×B5 | 99.78 <sup>ab</sup> ± 12.83 | 15.08 <sup>ab</sup> ± 1.52 | 2.99 <sup>ab</sup> ± 0.34 |
| <sup>1</sup> / <sub>2</sub> ×B5 | 80.43 <sup>bc</sup> ± 7.02  | 12.63 <sup>b</sup> ± 1.09  | 2.22 <sup>bc</sup> ± 0.19 |
| SH                              | 79.53 <sup>bc</sup> ± 17.70 | 12.93 <sup>b</sup> ± 2.91  | 2.18 <sup>bc</sup> ± 0.47 |
| <sup>3</sup> / <sub>4</sub> ×SH | 91.18 <sup>ab</sup> ± 7.94  | 15.28 <sup>ab</sup> ± 1.25 | 2.65 <sup>ab</sup> ± 0.21 |
| <sup>1</sup> / <sub>2</sub> ×SH | 69.28 <sup>bc</sup> ± 8.77  | 11.05 <sup>bc</sup> ± 1.43 | 1.77 <sup>bc</sup> ± 0.23 |

Table 4  
Bioactive compound accumulation of *A. lylei* rhizome culture in different medium salt strengths

| Treatment                                                                                                                  | <i>Total Phenolic contents</i><br>(mg/g DW) <sup>z</sup> | <i>Flavonoid contents</i><br>(mg/g DW) | <i>Polysaccharides contents</i><br>(mg/g DW) |
|----------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------|----------------------------------------|----------------------------------------------|
| Control                                                                                                                    | 3.71 <sup>b</sup> ± 0.33                                 | 1.05 <sup>a</sup> ± 0.10               | 150.66 <sup>a</sup> ± 1.35                   |
| MS                                                                                                                         | 1.60 <sup>cd</sup> ± 0.14                                | 0.76 <sup>b</sup> ± 0.05               | 88.52 <sup>c</sup> ± 5.76                    |
| ¾MS                                                                                                                        | 4.03 <sup>b</sup> ± 0.06                                 | 0.67 <sup>bc</sup> ± 0.04              | 102.55 <sup>b</sup> ± 1.72                   |
| ½MS                                                                                                                        | 4.90 <sup>a</sup> ± 0.07                                 | 0.56 <sup>cd</sup> ± 0.11              | 91.36 <sup>c</sup> ± 3.37                    |
| B5                                                                                                                         | 1.43 <sup>d</sup> ± 0.20                                 | 0.47 <sup>de</sup> ± 0.02              | 102.13 <sup>b</sup> ± 2.39                   |
| ¾B5                                                                                                                        | 2.10 <sup>c</sup> ± 0.10                                 | 0.42 <sup>de</sup> ± 0.06              | 86.26 <sup>c</sup> ± 4.00                    |
| ½B5                                                                                                                        | 3.96 <sup>b</sup> ± 0.20                                 | 0.58 <sup>bcd</sup> ± 0.04             | 75.26 <sup>d</sup> ± 1.87                    |
| SH                                                                                                                         | 0.77 <sup>e</sup> ± 0.13                                 | 0.37 <sup>e</sup> ± 0.03               | 86.89 <sup>c</sup> ± 3.33                    |
| ¾SH                                                                                                                        | 0.82 <sup>e</sup> ± 0.15                                 | 0.35 <sup>e</sup> ± 0.02               | 101.07 <sup>b</sup> ± 1.68                   |
| ½SH                                                                                                                        | 1.10 <sup>de</sup> ± 0.17                                | 0.42 <sup>de</sup> ± 0.03              | 84.89 <sup>c</sup> ± 3.55                    |
| <sup>z</sup> Mean with different letters within a column differs significantly by Duncan's multiple-range test (P ≤ 0.05). |                                                          |                                        |                                              |

The TPC values were highest at the lowest concentrations of medium salt and tended to decrease with increasing salt strength in all medium types (MS, SH, B5). In this study, the highest TPC was 4.9 mg/g DW which was recorded at ½×MS medium, and approximately 4.5-fold higher compared to SH medium. In contrast, the highest TFC and polysaccharide contents were investigated based on the control treatment (1.05 mg/g DW, 150.66 mg/g DW, respectively).

The results of Ho et al. (2021) indicated that MS salt strength medium significantly affected biomass and bioactive compound production in *P. multiflorum* adventitious root cultures. Adventitious root growth increased with rising MS salt concentrations, reaching a maximum at full-strength MS, while TPC and TFC content were highest at the lowest concentration (¼×MS). Compared to other salt mediums, MS salt strength is commonly used for adventitious cultures in various medicinal plants, such as *Panax ginseng* (Yu et al. 2000; Le et al. 2019), *Morinda citrifolia* (Baque et al. 2010), *Oplopanax elatus* (Jiang et al. 2015), and *Tripterygium wilfordii* (Zhang et al. 2020). SH medium is used for the adventitious culture of *Panax vietnamensis* (Tam et al. 2015; Linh et al. 2019). In the present study, MS medium concentration showed superiority in both biomass and bioactive compound production in rhizome cultures of *Anoectochilus lylei* compared to SH or B5 medium. The suitable medium was found at ¾ MS for the highest productivity of TPC, TFC, and polysaccharide in rhizome cultures of *A. lylei*. The

differences in mineral composition between various media directly affect growth and bioactive compound accumulation in cultures. In addition, the salt concentration of the medium influences the water potential, which affects water and nutrient uptake in plant cultures. At low concentrations, there is insufficient nutrition for growth, while at high concentrations, water and mineral nutrient absorption from the culture medium was inhibited (Murthy et al. 2016; Ho et al. 2021). Similarly, the  $\frac{3}{4}$ MS combined with phytohormone was the optimal condition for rhizome culture in *A. roxburghii* (Jin et al. 2018; Luo et al. 2018).

## **Effect of sucrose concentration on rhizome biomass production and bioactive compounds accumulation**

In the present study, the effect of various sucrose concentrations (0 to 70 g/L) was tested on rhizome biomass and bioactive compound accumulation in *A. lylei*. Sucrose concentrations up to 35 g/L showed a positive effect on rhizome biomass (Table 5, Fig. 3). However, biomass dramatically decreased when sucrose concentrations reached 50 and 70 g/L. At these high concentrations, rhizome growth was inhibited, and some even died. The highest FW (157.17 g/L), DW (20.67 g/L) and GI (5.29) were investigated at 35 g/L sucrose concentration, respectively. Sucrose is the most effective carbohydrate source for the growth of adventitious root and organ cultures compared to glucose, fructose, or maltose (Murthy et al. 2014, 2016; Silpha et al., 2015). Vigorous root growth is associated with higher sucrose consumption. However, an increase in sucrose concentration leads to higher osmolarity in the medium, which inhibits root growth (Jiao et al. 2015; Ho et al. 2021). Our results show that 35 g/L of sucrose was the most suitable for rhizome growth of *A. lylei*, whereas rhizomes did not grow and eventually died in the culture medium when sucrose concentration reached 7 g/L. A similar result was also observed in *Polygonum multiflorum* adventitious root cultures, which inhibited root growth at over 70g/L sucrose concentration (Ho et al. 2021).

Table 5  
Biomass production of *A. lylei* rhizome culture in different sucrose concentrations

| Sucrose (g/L) | FW (g/L) <sup>z</sup>       | DW (g/L)                  | Growth Index             |
|---------------|-----------------------------|---------------------------|--------------------------|
| 0             | 41.88 <sup>c</sup> ± 3.26   | 4.29 <sup>c</sup> ± 0.22  | 0.76 <sup>c</sup> ± 0.14 |
| 10            | 112.88 <sup>b</sup> ± 11.70 | 9.75 <sup>b</sup> ± 1.08  | 3.52 <sup>b</sup> ± 0.47 |
| 20            | 109.42 <sup>b</sup> ± 16.31 | 11.88 <sup>b</sup> ± 0.78 | 3.38 <sup>b</sup> ± 0.65 |
| 35            | 157.17 <sup>a</sup> ± 26.92 | 20.67 <sup>a</sup> ± 1.88 | 5.29 <sup>a</sup> ± 1.08 |
| 50            | 32.33 <sup>c</sup> ± 3.22   | 3.83 <sup>c</sup> ± 0.47  | 0.29 <sup>c</sup> ± 0.13 |
| 70            | 27.08 <sup>c</sup> ± 1.10   | 3.25 <sup>c</sup> ± 0.26  | 0.08 <sup>c</sup> ± 0.04 |

<sup>z</sup>Mean with different letters within a column differs significantly by Duncan's multiple-range test ( $p \leq 0.05$ ).

**Table 6.** Bioactive compound accumulation of *A. lylei* rhizome culture in different sucrose concentrations

| Sucrose (g/L) | Total Phenolic content (mg/g DW) <sup>z</sup> | Flavonoid content (mg/g DW) | Polysaccharides content (mg/g DW) |
|---------------|-----------------------------------------------|-----------------------------|-----------------------------------|
| 0             | 2.90 <sup>b</sup> ± .021                      | 0.95 <sup>b</sup> ± 0.22    | 62.80 <sup>c</sup> ± 2.19         |
| 10            | 4.44 <sup>a</sup> ± 0.07                      | 1.15 <sup>a</sup> ± 0.06    | 64.91 <sup>c</sup> ± 3.04         |
| 20            | 2.43 <sup>c</sup> ± 0.18                      | 0.31 <sup>c</sup> ± 0.10    | 84.24 <sup>b</sup> ± 2.10         |
| 35            | 2.37 <sup>c</sup> ± 0.20                      | 0.32 <sup>c</sup> ± 0.08    | 115.87 <sup>a</sup> ± 0.49        |
| 50            | 1,10 <sup>d</sup> ± 0,23                      | 0,14 <sup>d</sup> ± 0,05    | 85.27 <sup>b</sup> ± 1.24         |
| 70            | 0,48 <sup>e</sup> ± 0,12                      | 0,08 <sup>e</sup> ± 0,01    | 51.49 <sup>d</sup> ± 1.09         |

<sup>z</sup>Mean with different letters within a column differs significantly by Duncan's multiple-range test ( $p \leq 0.05$ ).

On the other hand, sucrose also plays a key role in controlling various developmental and metabolic processes in plants (Yoon et al. 2021). Sucrose has been found to act as a signalling molecule, influencing the biosynthesis of various bioactive compounds such as anthocyanins, phenolics, flavonoids, saponins, etc (Baque et al. 2012; Yoon et al. 2021; Chang et al. 2022). In this study, the low concentration at 10 g/L sucrose show the best result on TPC and TFC (4.44 g/L and 1.15 g/L); while the highest polysaccharide content was observed at 35 g/L sucrose concentration in rhizome culture of *A. lylei*. (Table 6, Fig. 3). A sucrose concentration of 30 g/L is commonly used for plant tissue and organ culture (Murthy et al. 2014, 2016); however, the accumulation of bioactive compounds is observed at

higher sucrose concentrations due to increased osmotic stress at these levels (Baque et al. 2012). A sucrose concentration of 5% is suitable for caffeic acid derivative accumulation in adventitious roots of *Echinacea angustifolia* (Wu et al. 2006) and ginsenoside production in *Panax ginseng* (Paek et al. 2009), phenolic compound in *Polygonum multiflorum* (Ho et al. 2021). In contrast, a lower concentration (10 g/L) showed the highest total phenolic content (TPC) and total flavonoid content (TFC) in the rhizome culture of *A. lylei* in the present study, while the highest polysaccharide content was recorded at 35 g/L sucrose. Overall, 35 g/L sucrose shows the highest productivity in accumulating bioactive compounds and should be used for the rhizome suspension culture of *A. lylei*.

## **Comparison of bioactive compounds accumulation and their antioxidants and antibacterial activity in different explants**

The total phenolic, flavonoid, polysaccharide, and kinsenoside contents of ex-vitro plants, in vitro plants, and rhizomes were investigated (Fig. 4). Among the four bioactive compounds, the polysaccharides content was higher than any other compound in the extracts from all explants. There was no significant difference in the total phenolic and flavonoid contents between ex-vitro and in vitro plants, and both showed higher levels compared to the rhizomes (Fig. 4A, B). In contrast, the highest contents of kinsenoside (2.94 mg/g DW) and polysaccharides (349.04 mg/g DW) were found in the rhizome extract (Fig. 4C, D). Specifically, the polysaccharides content in the rhizome extract was approximately 3-fold higher than in ex-vitro plants (118.56 mg/g DW) and 1.6-fold higher than in in vitro plants (213.12 mg/g DW).

A comparative analysis between ex vitro plants, in vitro plants, and rhizome cultures revealed significant differences in the trends of bioactive compounds. These variations were accompanied by distinct metabolic profiles, which is expected due to the vastly different growth environments and the unique roles of each plant organ. The accumulation of bioactive compounds in plants is influenced by environmental factors, developmental stages, and the specific functions of these compounds throughout the plant's life cycle (Kim et al. 2014; Ho et al. 2018). In *P. multiflorum*, for instance, the bioactive compound levels were reported to be higher in tuberous roots compared to adventitious root cultures (Ho et al., 2018, 2019). For *A. roxburghii*, the kinsenoside content in the rhizome and ex vitro plant was not significantly different, while the total polysaccharides content was lower in the plant. Interestingly, in the present study, both kinsenoside and polysaccharide levels were higher in rhizome cultures, suggesting the potential of using rhizomes as an alternative material source to field-grown *A. lylei* plants.

Antioxidant activity was investigated to determine the effect of three explant sources (ex vitro plant, in vitro plant, and rhizome). In DPPH radical scavenging results, there were no significant differences among the three extracts (Fig. 5A). This implies that rhizomes had similar antioxidant activity as the parental plant. Besides, we investigated the antibacterial activity of three extracts against *S. aureus* and *E. coli* using the agar well diffusion method. The inhibitory effect on bacterial growth was assessed by

measuring the diameter of the sterile zone around the well, with the agar pore size subtracted (Fig. 5B, C, D). As shown in Fig. 5B, C, and D, the rhizome extract suppressed the growth of *E. coli* (ZOI = 7 mm), whereas ex vitro and in vitro plants have a slight effect on both *S. aureus* and *E. coli* at the concentration of 100 mg/mL. Previous studies also showed antibacterial effects from cell and/or organ culture extracts. An extract from *Oplopanax elatus* adventitious root inhibited the growth of bacteria including *E. coli*, *S. aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* (Jin et al. 2019). Fan et al. (2020) reported that rhizome extract showed a higher inhibitory zone (18.6 mm) than plant extract (13.8 mm) against *B. subtilis*.

## Conclusion

The protocol for rhizome biomass cultivation of *Anoetochilus lylei* to accumulate bioactive compounds was optimized in this study. Rhizomal growth induction was mostly favored on the MS medium supplemented with 2g/L hyponex I, 2g/L hyponex II, 2g/L peptone, and 1g/L activated charcoal. The rhizomes also had the highest proliferation on the  $\frac{3}{4}$ MS medium supplemented with 2 mg/L BA, 0.2 mg/L Kin, and 35 g/L sucrose. In addition, rhizome cultures exhibited higher kinsenoside and polysaccharide content than both ex vitro and in vitro plants. The rhizome extract show inhibited the growth of *Staphylococcus aureus* and *Escherichia coli*, which shows potential for antibacterial applications. This is one of the first reports on *Anoetochilus lylei* rhizome culture for biomass and bioactive compound production.

## Declarations

### Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

### Conflict of interests

The authors have no relevant financial or non-financial interests to disclose.

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## Author contributions

TTH acquired data and wrote the manuscript. TTH, THTP, TJA, TDL, GHN, TKLN, THL, VNTB, and HTQ participated in performing the experiments, interpretation of data, and revision for intellectual content.

TTH, SYP, and HTKH conceptualized and designed the study. All authors discussed and approved the final manuscript.

## Data availability

All data generated or analyzed during this study are included in this article.

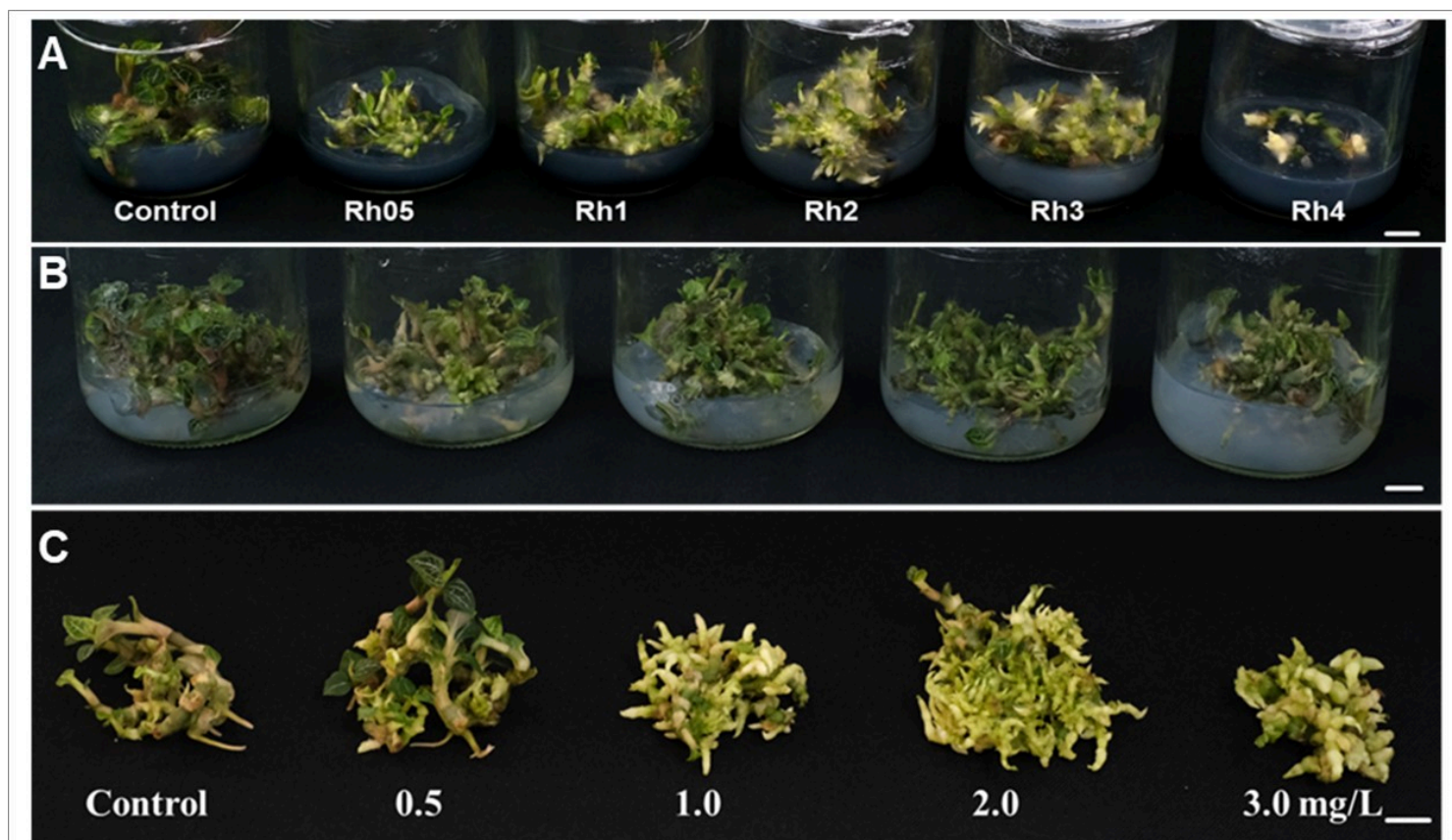
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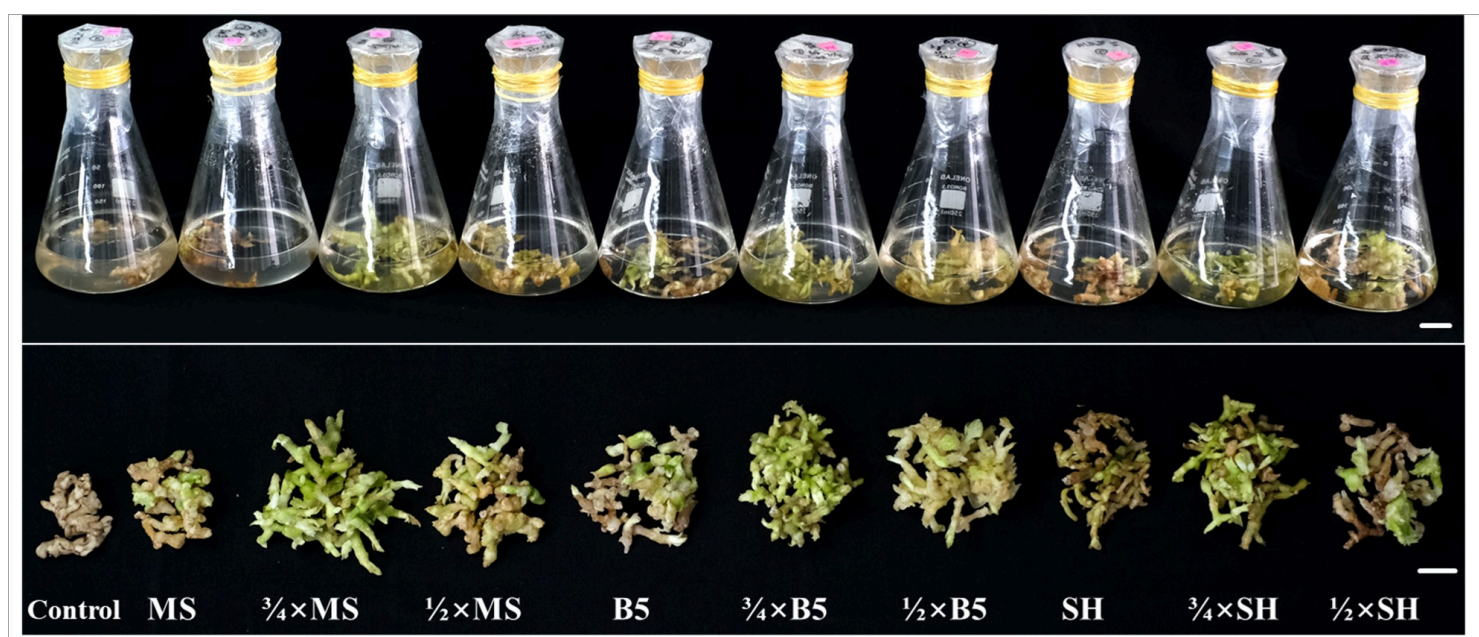
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## Figures



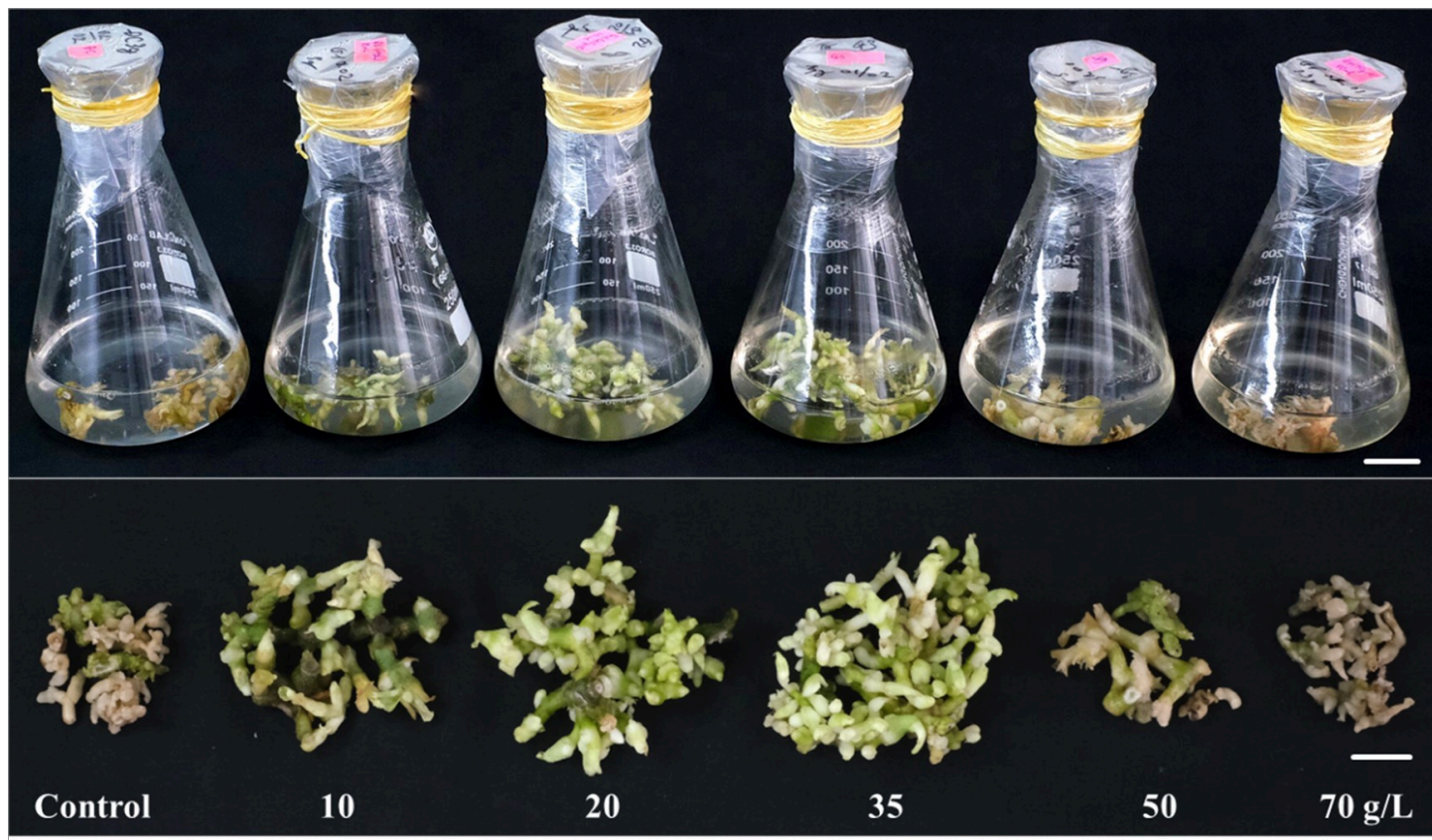
**Figure 1**

Rhizome induction (A) and proliferation in the medium supplemented with different BA concentrations (B, C). Bar: 2 cm.



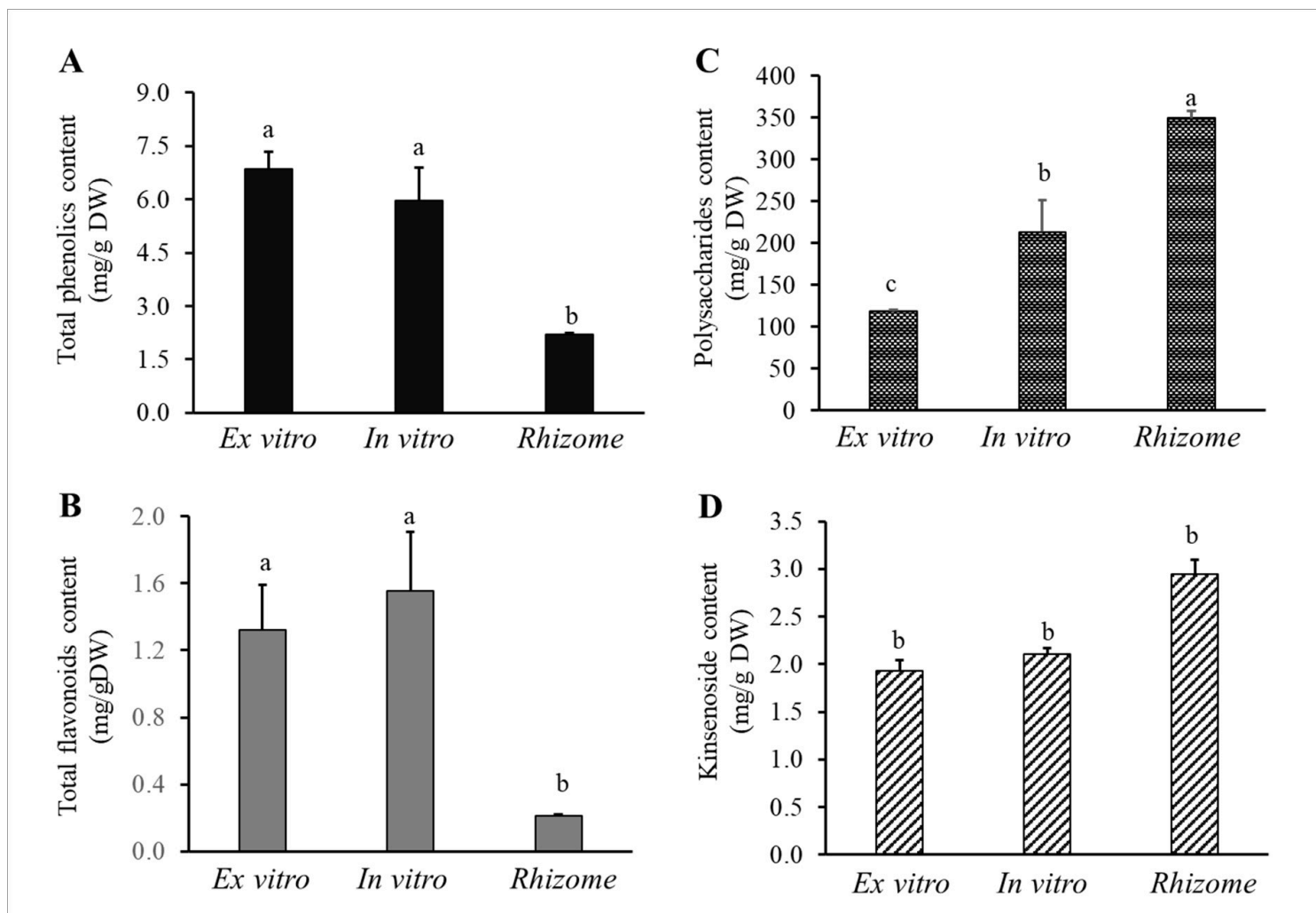
**Figure 2**

Effect of different medium salt strengths on rhizome production. Bar: 2cm



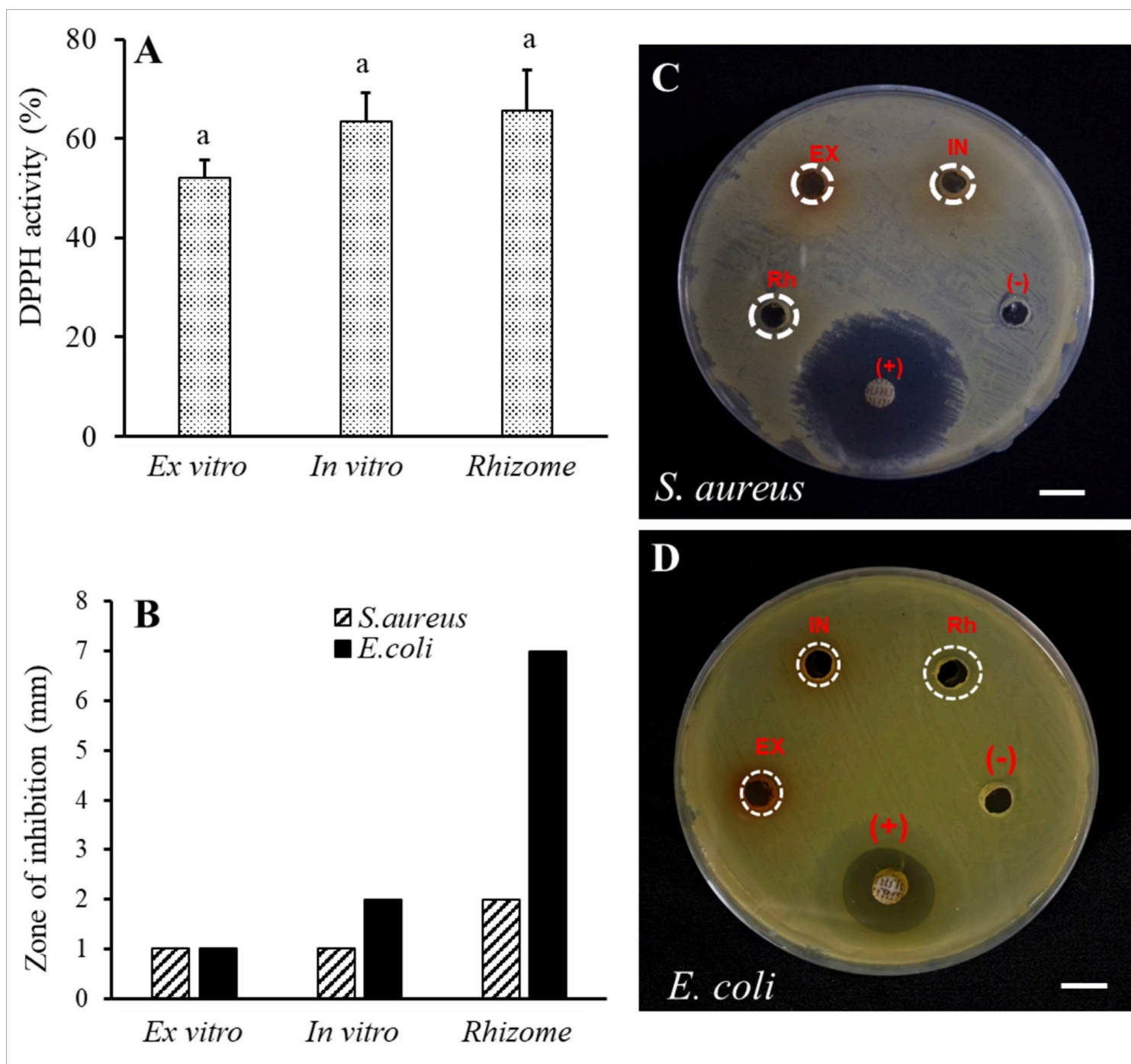
**Figure 3**

Effect of different sucrose concentrations on rhizome production. *Bar: 2 cm*



**Figure 4**

Comparison of bioactive compounds of different explant sources in *A. lylei*.



**Figure 5**

Comparison of antioxidant and antibacterial activity of different explant sources in *A. lylei*.

## Supplementary Files

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