

## Sarhad Journal of Agriculture



Official Journal of The University of Agriculture, Peshawar, Pakistan



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#### History and Introduction

Sarhad Journal of Agriculture (Print ISSN 1016-4383; Online ISSN 2224-5383) is the official journal of The University of Agriculture, Peshawar, Pakistan. It was founded and established by Dr. Naseer Hussain in 1985 and has taken roles as inaugural Editor of the journal. SJA has been publishing regularly since 1985 and is fully online since 2006. In 2014, SJA decided to join the Smith and Franklin publishers for its content management and publications, while still independently regulated under the banner of The University of Agriculture, Peshawar, Pakistan.

SJA is an open-access and peer-reviewed journal that publishes research articles on quarterly basis in every discipline of agriculture including Plant Production Sciences, Protection Sciences, Nutrition Sciences and Social Sciences. The research articles are received from national and international agricultural scientists, which are referred to highly qualified, capable and experienced subject specialists for evaluation. The journal is managed by highly qualified Editorial Board under the supervision of Chief Editor and Patron (Vice Chancellor).

More than forty organizations including foreign missions in Pakistan and some foreign countries including India, Kenya, Japan, UK and USA receive the journal regularly on exchange basis. It is also subscribed by various national and international organizations including Netherlands, UK, Germany and some Agricultural Research Stations/Institutes in the province. CAB International of UK is the permanent subscriber of SJA, which disseminates its abstracts.

#### Aims and Scope

Sarhad Journal of Agriculture is an open access and peer-reviewed journal which publishes most recent and informative research and reviews in all areas of agricultural sciences. The aim of the journal is to provide a platform for scientists, students and researchers to promote and share new trends and developments in different areas of agricultural sciences. The journal is fully committed to provide free access to all articles as soon as they are published.

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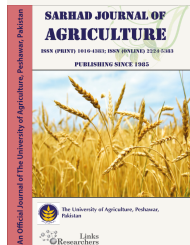
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3	MH20210303140312-R1	Sarhad Journal of Agriculture	Potential of Aloe vera for Treatment of Infection with <i>Aeromonas hydrophila</i> Bacteria on Koi Fry	Dr Rosidah Rosidah	21 Jun 2021	Accepted	<a href="#">Download PDF</a>
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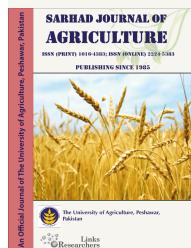
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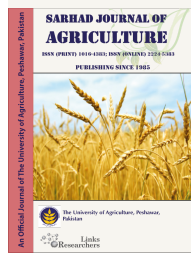
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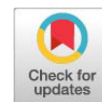
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Special Issue: Agricultural Productivity and Sustainability Improvement in Tropical Region

## Mass Production of *Panax ginseng* C.A. Mey. Root Cultures in Indonesia

Stefan Pratama Chandra<sup>1</sup>, Yoanes Maria Vianney<sup>1</sup>, Theresia Liliani Christie<sup>2</sup>, Merlyn Wongso<sup>2</sup>, Melisa Widjaja<sup>2</sup>, Deok-Chun Yang<sup>3</sup>, Se Chan Kang<sup>4</sup>, Manar Fayiz Mousa Atoum<sup>5,6</sup> and Johan Sukweenadhi<sup>1\*</sup>

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**Abstract** | *Panax ginseng* C.A. Mey. is one of the most well-known plants in traditional medicine that contains bioactive compounds called ginsenosides. It is widely used as raw material in many pharmaceutical industries in Indonesia. However, to supply for this purpose, they still rely on imports. PT. Kalbe Farma (through its subsidiary, PT. Bintang Toedjoe), University of Surabaya (Ubaya), and Hanbang-Bio Laboratory (holding company of Kyung Hee University) established the Kalbe Ubaya Hanbang-Bio Laboratory (KUH Lab), a collaboration initiated to achieve the independence of raw materials. This laboratory is devoted to developing the plant tissue culture protocol for the mass production of *P. ginseng* root cultures. This paper is the first report of *in vitro* *P. ginseng* culture in Indonesia. The initial stage of the mass production focused on optimizing the culture conditions: inoculum weight (100 g, 150 g, 200 g), medium volume (12 L, 13 L, 14 L), medium formulation (“A”, “B”), and incubation temperature (15 °C to 20 °C and 21 °C to 25 °C). Based on the biomass yield and ginsenosides content, it was concluded that the optimum growth condition was 150 g of the initial inoculum grown in 13 L of media using formulation B and incubation at 21 °C to 25 °C. In the long-term, KUH Lab aims to produce *P. ginseng* on an industrial scale to sufficiently supply the demands for *P. ginseng* in the country. Furthermore, this laboratory also intends to make standardized Indonesian herbal materials by using plant tissue culture.

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**Keywords** | Ginseng, Ginsenosides, *In vitro*, Medicine plant, Tissue culture

### Introduction

*Panax ginseng* C.A. Mey. is one of the most common ginseng species used in the community. *P. ginseng* can be found in several countries, such as Korea (common name: *Insam*), China (common

name: *Rénshēn*), and Japan (common name *Ninjin*). It has gained importance in traditional medicine because it can restore fatigue (Mahady *et al.*, 2000). *Panax* means “all-healing”, which comes from the belief that ginseng can cure all diseases in humans. Its benefits include modulating various ion channels

and signal transduction, increasing blood circulation, antioxidants, blood pressure, *etc* and tumor suppression (Kim, 2018). For these reasons, the market demand for product variants of *P. ginseng* is increasing. Still, cultivated ginseng roots are considerably high-priced because they can take 4 yr to 6 yr to be ready for harvest.

The main bioactive compounds found in *P. ginseng* are ginsenosides (Yu *et al.*, 2019). Based on chemical structure, ginsenosides can be divided into two major groups, namely protopanaxadiol (PD) and protopanaxatriol (PT) (Sengupta *et al.*, 2004). Ginsenosides reportedly have antimicrobial, anti-fungal, and even anti-cancer properties. In addition, they can cure cardiovascular (Kim, 2018) and neurological diseases (Gantait *et al.*, 2020). To date, more than 200 ginsenosides have been identified, including the major (e.g., Rb1, Rb2, Rc, Rd, Re, Rg1) and minor ginsenosides (e.g., Rg3, Rh1, Rh2) (Christensen *et al.*, 2006; Qi *et al.*, 2011). Ginsenosides are widely used for pharmaceuticals in European countries and retail products in American countries (Kim *et al.*, 2013).

Plant tissue culture is an alternative method of producing high-value secondary metabolites. It is a technique of growing a plant organ *in vitro* on a particular medium that involves incubation in a sterile and controlled condition. Usually, plants grown with tissue culture have high functional values, difficult conventional cultivation, and low yield percentages (Melviana *et al.*, 2021a). However, plant tissue culture offers several advantages compared with traditional cultivation techniques. They are not dependent on geographic location, and climate can provide a system to produce bioactive plants or compounds with consistent quality and yield and can eliminate factors that potentially reduce the production of bioactive compounds (such as pathogens and other stress factors) (Melviana *et al.* 2021b; Smetanska, 2008). Another advantage is that the plants can be produced free from harmful chemicals, such as herbicides, pesticides, insecticides, and microbial infections. As a result, they will have a higher yield than the plants grown in nature and contaminated with microorganisms (Gulzar *et al.*, 2020).

Several *in vitro* approaches that can be used to produce ginsenoside are shoot culture, callus culture, cell suspension culture, protoplast culture, bioreactor scale propagation, *in vitro* mutagenesis, polyploidization

induction, hairy roots, and root culture (Young *et al.*, 2001). Root culture has many advantages over other *in vitro* methods: it enables a stable growth phase, is genetically stable, and can produce secondary metabolites in the same or even higher level than the organ that is not transformed (Gantait *et al.*, 2020).

The main ginsenoside production organ in *P. ginseng* is the roots. Currently, some ginsenosides have been successfully obtained from other organs of *P. ginseng*, such as the Ginsenosides AP (arabinopyranoside) derived from flower buds. To overcome the long cultivation period and its risks, propagation methods are used in plant tissue cultures, including root cultures (Gantait *et al.*, 2020). *P. ginseng* is widely used in the pharmaceutical industry in Indonesia. However, 90 % of this raw material relies on imports. Therefore, PT. Kalbe Farma, through its subsidiary, PT. Bintang Toedjoe, collaborated with the University of Surabaya (Ubaya) and Hanbang-Bio Laboratory from Kyung Hee University, South Korea, in initiating efforts to reduce imports of the raw material by establishing the Kalbe Ubaya Hanbang-Bio Laboratory (KUH Lab). This laboratory is devoted to optimizing the *P. ginseng* root culture on a bioreactor scale in the country. The initial stage of the production is to optimize several factors affecting growth and ginsenoside accumulation, namely inoculum weight, growth medium volume, incubation period, and incubation temperature.

These four factors were optimized in the KUH Lab by experimenting with a few variations: 100 g, 150 g, 200 g of inoculum; 12 L, 13 L, 14 L of the growth medium; "A" and "B" formulations; and incubation at 15 °C to 20 °C and 21 °C to 25 °C. The effect of each variation on several parameters was observed, such as fresh weight, dry weight, yield, and water content of the root cultures. Based on the statistical test results, the optimal procedure for large-scale production of *P. ginseng* root cultures involves inoculating 150 g of fresh roots into 12 L of growth medium prepared with formulation "B" and incubating the inoculum at 21 °C to 25 °C for 7 wk (weeks). In the long-term, this laboratory plans to produce *P. ginseng* on an industrial scale to sufficiently meet the demands for this plant in Indonesia and manufacture many standardized Indonesian herbal materials using plant tissue culture.

## Materials and Methods

The root cultures used in this study were obtained

from the Hanbang-Bio Laboratory, Kyung Hee University, in South Korea.

#### *Procedures of large-scale production of Panax ginseng root cultures*

Large-scale production was carried out in an 18 L bioreactor (BR-BIO180, Hanbang-Bio Inc., South Korea) made of polycarbonate with a maximum content capacity of 15 L. It was specifically molded to connect the main body of the bioreactor to a sparger (bottom) and vent system (upper part). Meanwhile, the air supply was infused and discharged through a silicon tube ( $\Phi$  7 mm) with a clean filter (0.2  $\mu$ m). The bioreactor was connected to a separate air pump system. It was filled with 12 L of Reverse Osmosis (RO) water using Pure RO II. The media concentrate with formulation "A" was added to the water-filled bioreactor (final volume 13 L). The bioreactor was sterilized in an autoclave (Hankuk HK-AC200P) at 121 °C for 70 min at a pressure of 1.5 atm. The bioreactor was kept for at least 4 d before use to cool down and confirm the absence of contaminants. A total of 150 g of roots were inoculated into the bioreactor. Then, the bioreactor was connected to an air compressor to provide continuous aeration and agitation until harvest. The root culture was incubated at 21 °C to 25 °C for 7 wk at 40 % to 50 % humidity.

#### *Procedure optimization for bioreactor-scale production of Panax ginseng root cultures*

The optimized variables were inoculum weight, medium volume, medium formulation, and incubation temperature. The inoculum weights were made with three-part variation: 100 g, 150 g, and 200 g fresh weight roots; each was inoculated into a 13 L medium prepared with formulation "A". Then, the roots were incubated at 21 °C to 25 °C for seven weeks. The variation in the medium volume refers to the volume of water (11 L, 12 L, 13 L) used to dilute the concentrate with the same volume, 1 L. Afterward, each was inoculated with 150 g of roots. The roots were then incubated at 21 °C to 25 °C for 7 wk. There were two types of medium formulations: "A" and "B"; each was inoculated with 150 g of roots. Basically, both formulations were the same liquid medium, i.e., modified *Schenk* and *Hildebrandt* (SH) medium, with the letters "A" and "B" marking the different types of auxin used. The roots were incubated at 21 °C to 25 °C for 7 wk. The incubation temperature was also tested using two-part variation: 15 °C to 20 °C and 21 °C to 25 °C. A total of 150 g of roots were inoculated

into a 13L medium with formulation "A" and then incubated at 15 °C to 20 °C and 21 °C to 25 °C for 7 wk. For all variable analyses, the air humidity of the culture room was uniform, namely 40 % to 50 %.

#### *Harvest and drying of Panax ginseng root cultures*

After the incubation period was over, the roots were harvested. The medium was first removed, then the root culture was collected and placed in a container. After rinsing with tap water three times, the roots were rinsed again using RO water once. Then, they were drained in a perforated container for 1 h to minimize the amount of water on the roots and then put into a storage container. The roots were dried in a universal oven (Memmert UF750) set at 60 °C, 60 % fan, and 60 % air flap, and the duration was adjusted to the fresh weight of the cultured roots. Afterward, the dried roots were weighed using Mettler Toledo MS16001L. The dry weight data were used to calculate the percentage yield using the Formula (1):

$$\% \text{ Yield} = \frac{\text{Dry Weight}}{\text{Fresh Weight}} \dots (1)$$

Afterward, 3.7 g of the dried roots were inserted into a moisture analyzer (150 Sartorius) and then left to stand at 105 °C for 30 min to measure their water content. The water content was read three times ([Vogl and Ostermann, 2006](#)).

#### *Extraction and sample preparation of Panax ginseng root cultures*

The dried roots were extracted using the Soxhlet method. Five g of dried roots were extracted in 300 mL of 80 % methanol for 2 h. Then, the extract of the roots was dried in a rotary evaporator (Rotavapor R-300, Buchi, German). The dry extract was dissolved in 20 mL of RO water then extracted again in water-saturated butanol in a ratio of 1:1. This mixture was centrifuged at 8 000 rpm (1 rpm = 1/60 Hz) for 15 min, and then the organic phase was collected and put into a 250 mL flask. Extraction with water-saturated butanol continued until the organic phase became clear. The butanol extract was evaporated again in a rotary evaporator (Rotavapor R-300, Buchi, German) to produce a dry extract without butanol scent.

#### *Ginsenosides analysis*

Samples were analyzed with a high-performance liquid chromatography instrument (HPLC, Agilent 1260 Infinity II). The dry extract was dissolved in 50 mL

of HPLC-grade methanol then filtered with a 0.2  $\mu\text{m}$  PTFE filter. The stationary phase was Kinetex C18 with the dimension 50 mm  $\times$  4.6 mm, while the mobile phase was an eluent with acetonitrile (A)/water gradient. In the gradient elution, the composition of the mobile phase was set as follows: 7 min at 19 % A (isocratic); 7 min to 11 min from 19 % to 29 % A (isocratic), 11min to 14 min at 29 % A (isocratic); 30 min to 31.5 min from 70 % to 90 % A; 31.5 min to 34 min at 90 % A (isocratic); 34 min to 34.5 min from 90 % to 19 % A; 34.5 min to 45 min at 19 % A (isocratic). Amount 5  $\mu\text{L}$  of the sample was injected at a flow rate of 0.6 mL  $\text{min}^{-1}$ . Then, the sample absorbance was read using a Diode Array Detector (DAD) at a wavelength of 203 nm. The results were analyzed quantitatively by comparing the peak area of the sample with that of the reference standard.

### Statistical analysis

Experiments of the growth factors were conducted separately in this order: inoculum weight, medium volume, medium formulation, and then incubation temperature. All data were analyzed statistically with IBM SPSS v. 25 software. One-Way ANOVA was used if the data were normal and homogeneous (parametric test). In contrast, the Kruskal–Wallis test was used if they were not normal or homogeneous (non-parametric test). A significant difference in the data was indicated by a  $p$ -value  $< 0.05$  % (Adinurani, 2016).

## Results and Discussion

### Effect of various inoculum weights on *Panax ginseng* root cultures

In large-scale production of root cultures, several important factors can affect biomass growth and accumulation of bioactive compounds, namely the root's genetic factors (Khan *et al.*, 2015), growth media, other physical factors like incubation temperature (Wang *et al.*, 2019), and inoculum weight (inoculum density) (Manuhara *et al.*, 2015; Than *et al.*, 2004). Because of differences in climatic and topographic conditions between Indonesia and South Korea and increasing biomass and ginsenoside accumulation, it is necessary to re-optimize several production procedures, including inoculum weight, media volume, media composition, and incubation temperature.

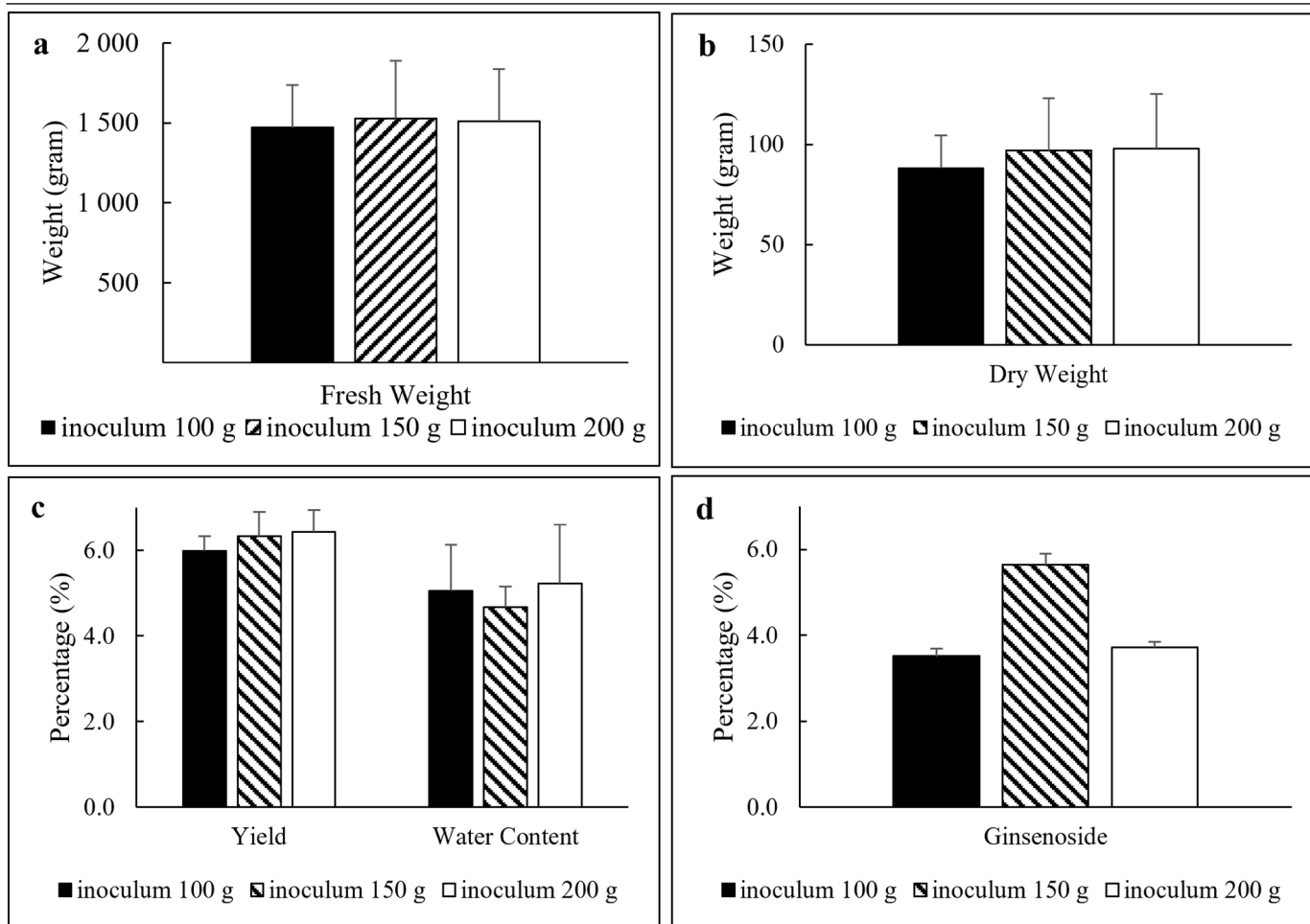
Inoculum weight is one of the factors that can influence the development of *Panax ginseng* root

cultures. If the inoculum is light, the cell growth will be slow; conversely, the cell culture will grow relatively fast without experiencing a lag phase (Than *et al.*, 2004). In this experiment, the inoculum weights were 100 g, 150 g, and 200 g, with 150 g acting as a control experiment. Figure 1 shows the effects of these weights on the properties of the root cultures and extracts: fresh weight, dry weight, yield, water content, and ginsenoside content.

The fresh weights of the root cultures produced by 100 g, 150 g, and 200 g of inoculum were 1 472.21 g  $\pm$  263.749 g, 1 528.77 g  $\pm$  361.539 g, 1 508.92 g  $\pm$  326.245 g, respectively. Based on the statistical analysis results of this parameter, the roots produced with 150 g of inoculum had the heaviest fresh weight. Still, it was not significantly different from the effects of other inoculum weights (100 g and 200 g). This result contradicts the study conducted by Srivastava and Srivastava (2007), which confirmed that a heavy inoculum could increase cell growth. However, it corresponds to Lee *et al.* (2006), who found that 60 g  $\text{L}^{-1}$  of inoculum could significantly increase the growth of *Gymnema sylvestre* R. Br. culture. Still, inoculum heavier than 60 g  $\text{L}^{-1}$  led to a less significant increase in growth. Another factor that increases the fresh weight is the cutting process before inoculum is sub-cultured into media. Some areas are injured when the roots are cut, creating space for new branches to grow during the culture.

The dry weights of the root cultures produced by 100 g, 150 g, and 200 g of inoculum were 88.12g  $\pm$  16.454 g, 97.02 g  $\pm$  26.046 g, and 97.95 g  $\pm$  27.100 g, respectively. Based on the statistical analysis results, the roots produced by 200 g of inoculum had the heaviest dry weight. However, it was not significantly different from the effects of other inoculum weights. Compared with 200 g of inoculum, the dry weight yielded by 150 g of inoculum was slightly lower. This result is common in dried roots, in which the drying process can affect the resultant dry weight depending on the duration; the duration of drying itself is determined by fresh weight and water content. Prolonged drying can remove more water from the roots, resulting in low dry weight, evidenced by the yield percentage and water content.

The yield percentage produced by 100 g, 150 g, and 200 g of inoculum were 5.99 %  $\pm$  0.337 %, 6.33 %  $\pm$  0.562 %, and 6.42 %  $\pm$  0.509 %, respectively, while the



**Figure 1:** Bar chart of the effect of inoculum weight on a) fresh weight, b) dry weight, c) yield percentage and water content, and d) ginsenoside content. Values are means  $\pm$  SD ( $n > 3$ ). Based on the One-Way ANOVA, there was no significant difference in each parameter ( $P < 0.05$  %).

water contents of the roots were  $5.05 \% \pm 1.081 \%$ ,  $4.67 \% \pm 0.490 \%$ , and  $5.22 \% \pm 1.387 \%$ . These yield percentages and water contents were not significantly different among the inoculum weights. The water content of dried roots with 150 g of inoculum was lower than that of 100 g and 200 g. Low water content can increase the material's shelf life because it inhibits microbial growth and enzyme activity (Lech *et al.*, 2017). According to Arifin *et al.* (2006), the water content in dry materials should not be more than 10%.

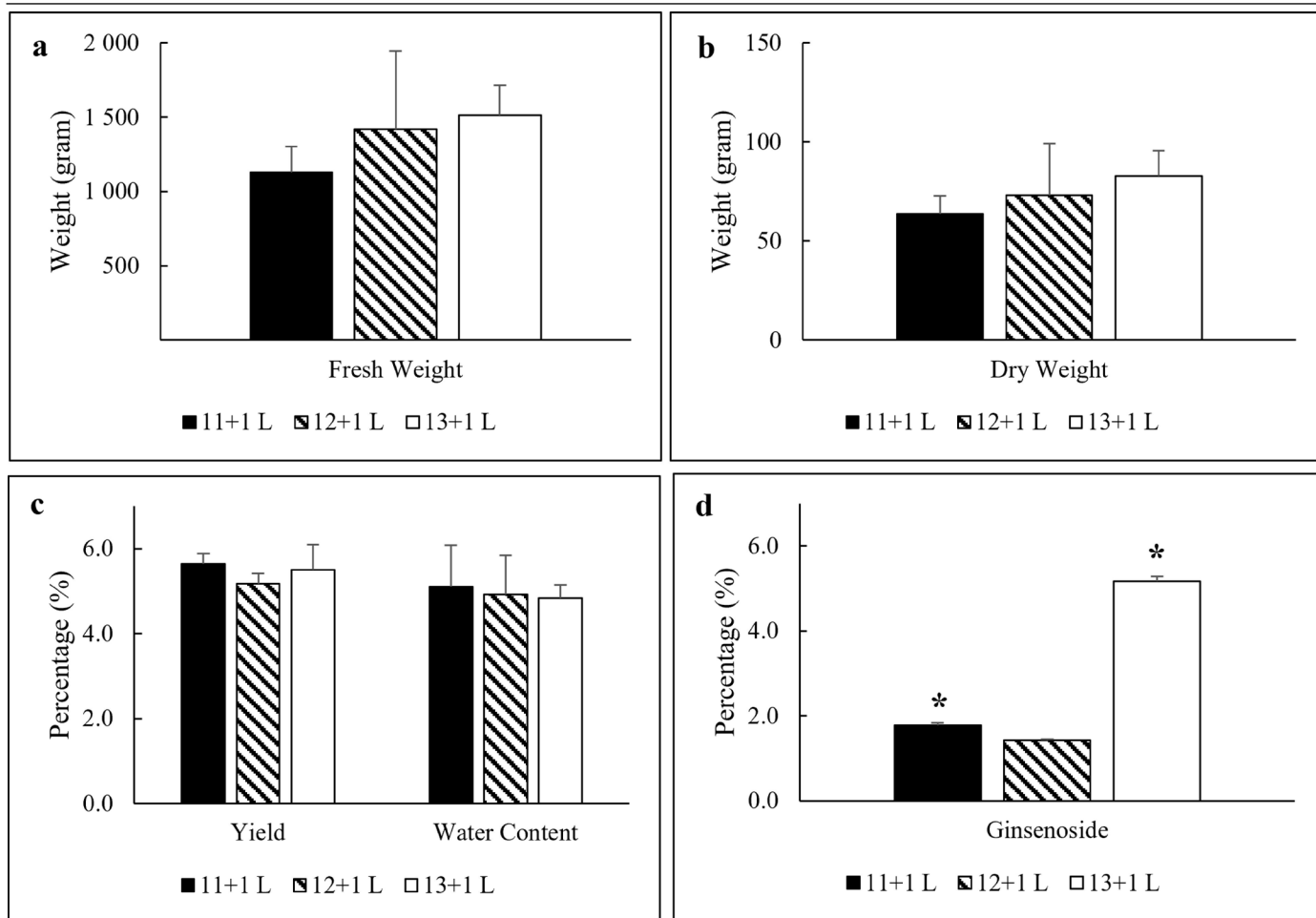
The ginsenoside contents of the root extracts made of 100 g, 150 g, and 200 g of inoculum were  $3.51 \% \pm 0.170 \%$ ,  $5.64 \% \pm 0.252 \%$ , and  $3.72 \% \pm 0.126 \%$ , respectively. In a study conducted on *Morinda citrifolia* L. root cultures, low inoculum weight can increase levels of bioactive compounds, such as anthraquinones, phenolics, and flavonoids (Baque *et al.*, 2013). Based on the analysis results of each parameter, it can be concluded that the inoculum produces root cultures and extracts with favorable properties when added to the production at the weight of 150 g.

*Effects of various growth medium volumes on Panax ginseng root cultures*

The availability of macronutrients and micronutrients in growth media can affect plant growth and development and the accumulation of bioactive compounds. In this experiment, variations were made on the volume of water used for the liquid medium, namely 11 L, 12 L, and 13 L. Meanwhile, the concentration and volume of the medium concentrate added for each variation were the same. Therefore, the total medium volumes were 12 L (11 + 1 L), 13 L (12 + 1 L), and 14 L (13 + 1 L), with the medium volume of 13 L being the control experiment. Figure 2 depicts the effects of variation in the medium volume on the parameters, such as fresh weight, dry weight, yield, water content, and ginsenoside content.

Based on the One-Way ANOVA, the fresh weights, dry weights, yield percentages, and water contents of the root cultures from the three medium volumes were not significantly different. However, the ginsenoside contents of the root cultures made in 12 L and 14 L media were significantly different from control.





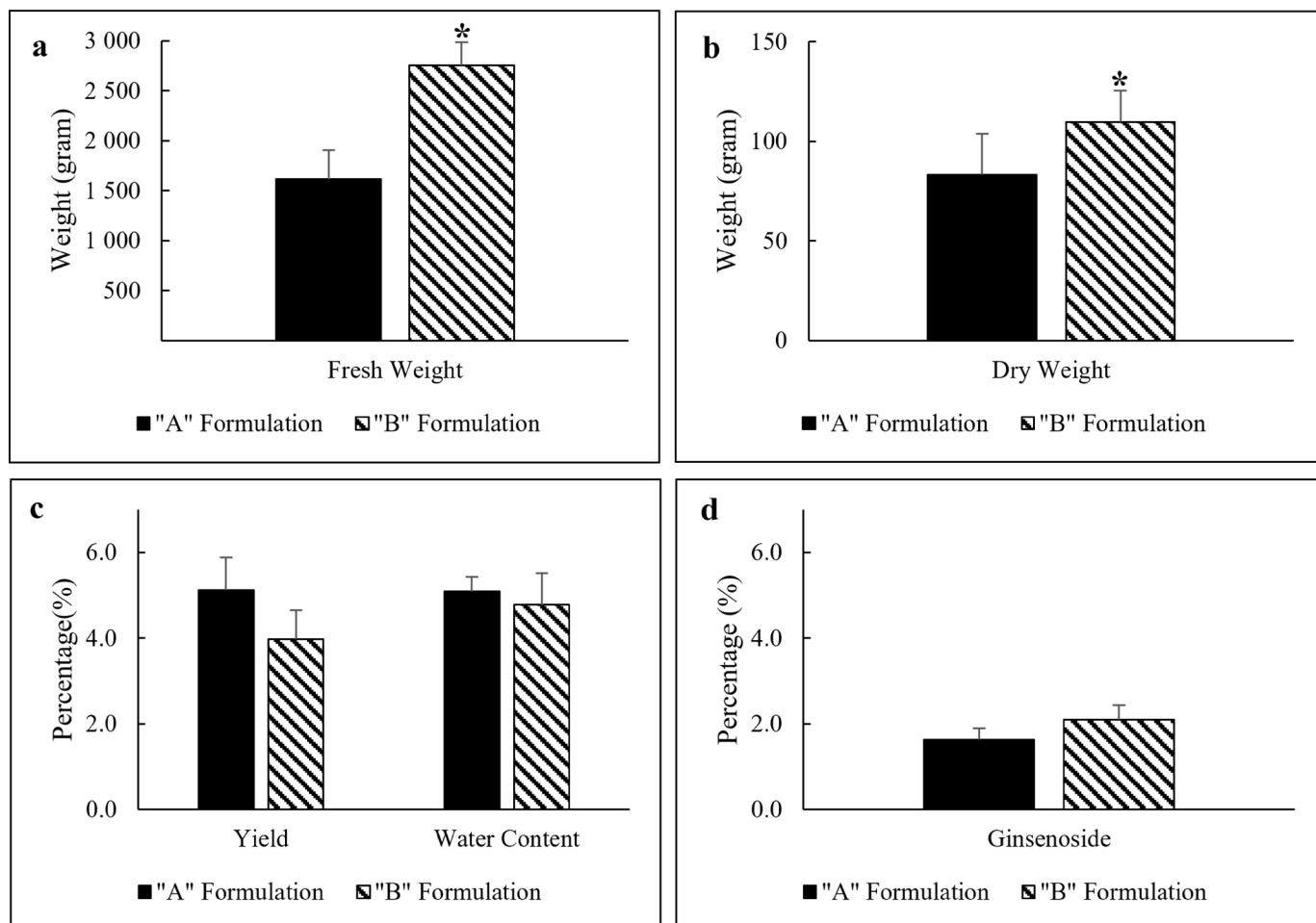
**Figure 2:** Bar chart of the effect of medium volume on a) fresh weight, b) dry weight, c) yield percentage and water content, and d) ginsenoside content. Values are means  $\pm$  SD ( $n = 3$ ). Significant differences are marked with (\*) and (\*\*) ( $P < 0.05$  % and  $P < 0.01$  %, respectively).

The fresh weights of the roots grown in the medium volumes of 12 L, 13 L, and 14 L were  $1\,128\text{ g} \pm 172.218\text{ g}$ ,  $1\,420.433\text{ g} \pm 525.689\text{ g}$ , and  $1\,513.433\text{ g} \pm 201.479\text{ g}$ , respectively. The statistical tests of this parameter revealed that the fresh weights produced by each volume were not significantly different from one another even though the 14 L medium made the heaviest fresh weight. Reducing the volume to 12 L negatively affected root growth, which can be attributed to osmotic stress because the growth media is more concentrated than the other two variations. Osmotic stress decelerates root growth but can accumulate more bioactive compounds (Akula and Ravishankar, 2011). A decrease in the fresh weight of the roots cultured in a 12 L medium is expected to be followed by an increase in ginsenoside accumulation. The 14 L medium produced roots with a slightly heavier fresh weight than 13 L. This result, however, contradicts the theory in that dilution depletes the nutrient contents in growth media; thus, there should be a decrease in the growth rate due to fewer macronutrients and micronutrients (Prinzenberg *et al.*, 2010). On the other hand, a nutritional deficiency can increase the

accumulation of bioactive compounds, meaning that the 14 L medium will produce high ginsenoside levels.

The dry weights produced in the medium volumes of 12 L, 13 L, and 14 L were  $63.63\text{ g} \pm 9.333\text{ g}$ ,  $73.03\text{ g} \pm 26.019\text{ g}$ , and  $82.93\text{ g} \pm 12.606\text{ g}$ , respectively. Based on the statistical analysis results, the medium volume variation did not lead to significantly different dry weights. The 14 L medium produced roots with the heaviest dry weight and fresh weight.

The yield percentages of root cultures in the medium volumes of 12 L, 13 L, and 14 L were  $5.65\% \pm 0.237\%$ ,  $5.18\% \pm 0.241\%$ , and  $5.50\% \pm 0.595\%$ , respectively, while the water contents were  $5.10\% \pm 0.978\%$ ,  $4.92\% \pm 0.925\%$ , and  $4.83\% \pm 0.313\%$ . The statistical analysis discovered that the yield percentages and water contents did not significantly differ among the variations. The ginsenoside contents of the root extracts from the medium volumes of 12 L, 13 L, and 14 L were  $1.78\% \pm 0.049\%$ ,  $1.42\% \pm 0.025\%$ , and  $5.17\% \pm 0.114\%$ , respectively. Based on the statistical



**Figure 3:** Bar chart of the effect of medium formulation on a) fresh weight, b) dry weight, c) yield percentage and water content, and d) ginsenoside content. Values are means ± SD ( $n > 3$ ).

analysis results, the ginsenoside levels produced by each medium volume differed significantly from one another. The 12 L medium produced root extracts with significantly higher ginsenoside content than 13 L, and the 14 L medium produced those containing significantly higher ginsenosides than 12 L and 13 L. It can be concluded that osmotic stress and nutritional deficiency can increase the accumulation of ginsenosides. The analysis of each parameter revealed that 14 L of growth medium results in favorable properties for large-scale production of root cultures (optimal procedure).

*Effects of various plant growth regulators on Panax ginseng root cultures*

Medium formulations also play an essential role in the growth and accumulation of bioactive compounds in a plant. Using different auxin hormones, such as IAA, IBA, and NAA can cause differences in the rate of root formation (Sevik and Guney, 2013; Khan et al., 2021). In this experiment, formulations "A" and "B" were the same liquid medium but different

hormones. "A" acted as a control, while "B" had variations in its formulation. The effects of varying medium formulations on the parameters, i.e., fresh weight, dry weight, %yield, moisture content, and ginsenoside content, are presented in Figure 3.

Based on the One-way ANOVA, there were significant differences in the fresh weight, dry weight, and yield percentage between the medium formulations, as marked with (\*) ( $P < 0.05$  %). However, there was no significant difference in the water and ginsenoside contents for each medium formulation.

The fresh weights of the roots grown with the medium formulations "A" and "B" were 1 614.97 g ± 292.223 g and 2 753.30 g ± 231.343 g. The statistical analysis results showed that the roots grown with the medium formulation "B" had significantly higher fresh weight than those cultured with "A". From these results, it can be concluded that "B" is more suitable for roots production. The dry weights produced using the medium formulations "A" and "B" were 83.07 g ±

20.645 g and 109.76 g ± 15.599 g, respectively. Based on the statistical analysis results, the medium formulated with “B” produced roots with significantly higher dry weights than “A”. This was because roots cultured with “B” had a higher fresh weight than “A”.

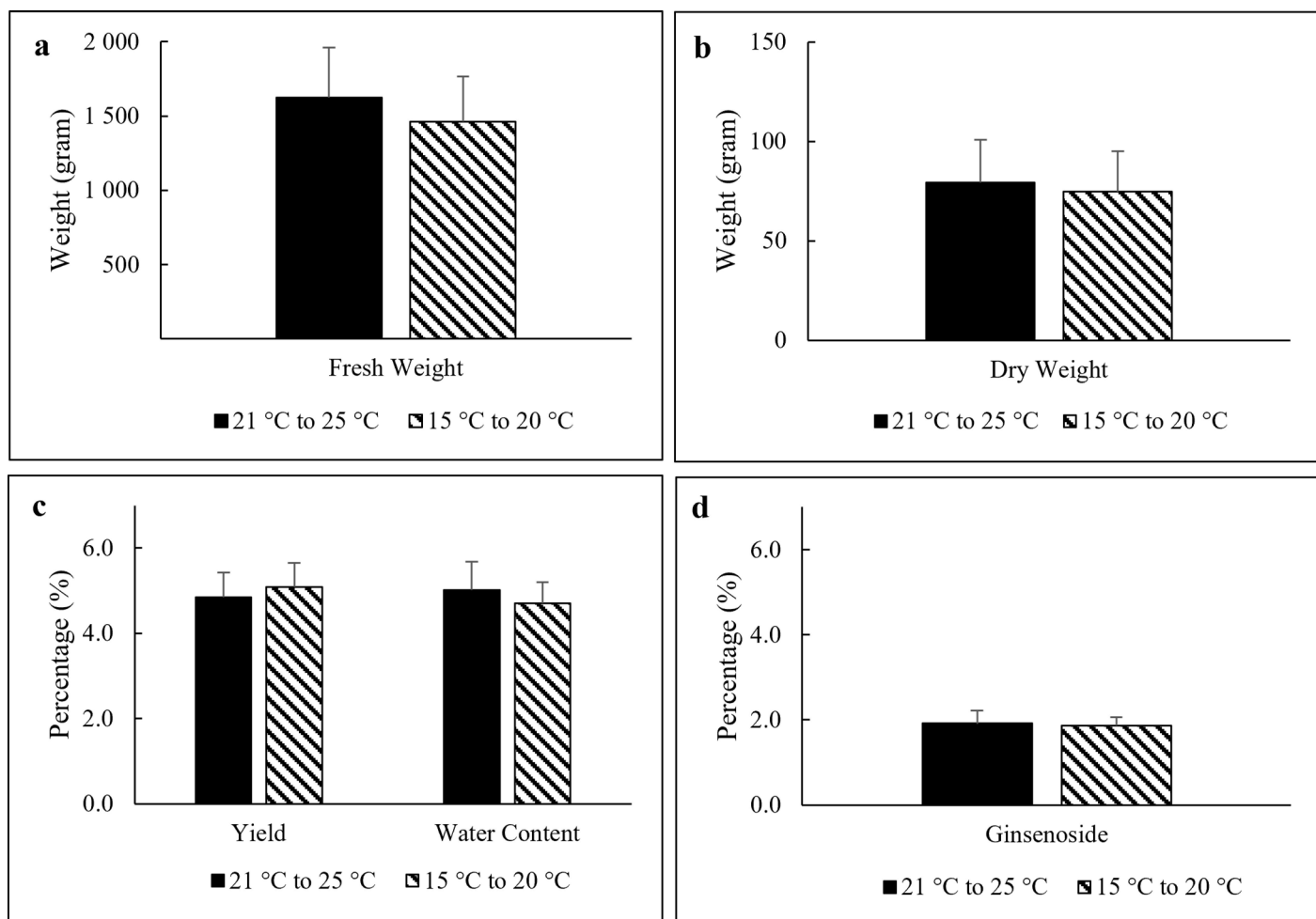
The yield percentages produced using formulations “A” and “B” were 5.12 % ± 0.766 % and 3.98 % ± 0.345 %, while the water contents were 5.09 % ± 0.670 % and 4.77 % ± 0.745 %. The statistical analysis results showed that the yield percentage produced using “B” was significantly lower than “A”, which is interesting because with “B” producing a higher dry weight, it can be expected to have a lower yield percentage. This result is thought to be caused by the hormones contained in “B” that cause the roots to have less water content than “A”, although their water contents did not differ significantly. Low water content makes the roots dry more quickly, thus affecting the yield percentage.

The extracts of the roots grown with formulations “A”

and “B” had ginsenoside contents of 1.63 % ± 0.264 % and 2.10 % ± 0.331 %, respectively. Based on the statistical analysis, culturing roots with the medium formulation “A” or “B” did not produce extracts with significantly different ginsenoside levels. Overall, the analysis results of each parameter revealed that “B” provides the optimal formulation for the large-scale production of *P. ginseng* root cultures.

*Effect of incubation temperature on Panax ginseng root cultures*

External environmental factors during the incubation (e.g., irradiation, temperature, and humidity) can affect the development and accumulation of bioactive compounds. Temperature partially causes abiotic stress that can hinder plant growth (Amir et al., 2017). In this experiment, the incubation temperature was set at two different ranges: 21 °C to 25 °C and 15 °C to 20 °C, with the former acting as a control. Figure 4 shows the effect of incubation temperature on the properties of root cultures and extracts.



**Figure 4:** Bar chart of the effect of incubation temperature on a) fresh weight, b) dry weight, c) yield percentage and water content, and d) ginsenoside content. Values are means ± SD (n > 3). Based on the One-Way ANOVA, the parameters showed no significantly different values for each variation (P < 0.05 %).

The fresh weights of the roots incubated at the temperatures of 21°C to 25 °C and 15 °C to 20 °C, were 1 625.98 g ± 333.591 g and 1 461.65 g ± 304.163 g. The statistical analysis revealed that although the roots had higher fresh weight when incubated at 21°C to 25 °C than 15 °C to 20 °C, the difference between the two was not significant. This result corresponds to the study conducted by Wang *et al.* (2019), *P. ginseng* root growth and ginsenoside accumulation were optimal when incubated at 20 °C to 25 °C.

The dry weights of the roots incubated at the temperatures of 21°C to 25 °C and 15 °C to 20 °C were 79.31 g ± 21.442 g and 74.88 g ± 20.154 g. Based on the statistical analysis results, the root cultures had higher dry weight when incubated at 21 °C to 25 °C than 15 °C to 20 °C, but the difference between the two was not statistically significant. Nevertheless, this result was better than the previous studies that used a smaller bioreactor scale, which resulted in 0.150 g to 0.250 g dry weight after five weeks of culture on ½ MS media (Hwang *et al.*, 1997; Yang *et al.*, 1996).

The yield percentages produced by the incubation temperatures of 21 °C to 25 °C and 15 °C to 20 °C were 4.85 % ± 0.578 % and 5.09 % ± 0.563 %, while the water contents were 5.01 % ± 0.668 % and 4.71 % ± 0.490 %. The statistical analysis results showed that the yield percentage and water content between the incubation temperature ranges were not significantly different. The extracts of the roots incubated at 21 °C to 25 °C and 15 °C to 20 °C contained 1.87 % ± 0.030 % and 1.91 % ± 0.019 % ginsenosides. Based on the statistical analysis results, variation in the incubation temperature did not cause a significant difference in ginsenoside levels even though the roots contained more ginsenosides when incubated at 15 °C to 20 °C than 21 °C to 25 °C. These results indicate that the incubation temperature of 15 °C to 20 °C can increase the accumulation of ginsenosides in the root extract. This can be associated with cold temperatures that potentially cause stress to the roots, which trigger them to produce bioactive compounds at higher levels (Wang *et al.*, 2019). The analysis results of each parameter suggest that 21 °C to 25 °C was the optimal range of incubation temperature.

*Optimum growth condition of Panax ginseng root cultures*

The roots were produced in three production batches according to the identified optimal conditions for

roots production. The conditions were as follows: 150 g fresh weight of roots as the inoculum, medium volume of 13 L, the medium formulation “B”, and 7 wk incubation at the temperature of 21 °C to 25 °C. Although using 14 L of medium growth effectively produces the desired results, this volume was not used because it required a different formulation. The results showed that the roots cultured in 14 L of medium prepared with formulation “A” had the highest weight among the combination of parameter values, but those grown in 13 L of medium with formulation “B” had a higher weight. The effects of applying optimum procedures to roots production on the fresh weight, dry weight, yield percentage, water content, and ginsenoside content are summarized in Table 1.

**Table 1:** *Panax ginseng* root culture profiles under optimum growth conditions.

Parameter	Mass Production Batch		
	Batch 1	Batch 2	Batch 3
Fresh Weight (g)	2 954.12 ± 95.034	2 628.11 ± 97.609	2 870.32 ± 105.483
Dry Weight (g)	124.68 ± 4.515	118.76 ± 3.107	122.52 ± 6.677
Yield (%)	4.22 ± 0.130	4.29 ± 0.241	4.27 ± 0.108
Water Content (%)	4.88 ± 0.473	5.15 ± 0.505	5.09 ± 0.867
Ginsenoside (%)	2.64 ± 0.122	3.01 ± 0.234	2.83 ± 0.241

As seen in Table 1, the procedures used for root cultures successfully produced consistent fresh weight, dry weight, yield percentage, water content, and ginsenoside content. However, they still have some weaknesses leading to ginsenoside levels that are not as high as expected. The large-scale production needs to adopt other strategies, such as adding elicitors or applying stress conditions to increase the ginsenoside concentrations in the root cultures. Therefore, it is recommended that future research focuses on increasing ginsenoside levels while maintaining a high yield of roots biomass.

**Conclusions and Recommendations**

The optimal procedure for large-scale production of *Panax ginseng* root cultures is to inoculate as much as 150 g of roots into 13 L of growth medium with the formulation “B”, followed by incubation at 21 °C to

25 °C for 7 wk. These procedures have successfully produced consistent data (root culture and extract properties) in three independent production batches.

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## Novelty Statement

Among numerous plant tissue cultures, the root culture offers many advantages as it enables stable growth, is genetically stable, and can produce higher secondary metabolites. This is the first *in vitro* *Panax ginseng* root culture report in Indonesia. The initial stage of mass production focuses on optimizing the culture growth using variations in the factors, *i.e.*, inoculum weight (100 g, 150 g, and 200 g), the media volume (12 L, 13 L, and 14 L), medium formulation (“A” and “B”), and incubation temperature (15 °C to 20 °C and 21 °C to 25 °C). Based on the biomass yield and ginsenoside contents, it can be concluded that the optimum growth conditions include the use of 150 g of initial inoculum, 13 L of growth medium prepared with the formulation “B”, and incubation at a temperature of 21 °C to 25 °C.

## Author’s Contribution

**SPC:** Performed the literature search, data acquisition, statistical analysis, and manuscript preparation.

**YMV:** Carried out experimental studies, performed literature search and data acquisition.

**TLC:** Performed data analysis and manuscript review.

**M Wongso and M Widjaja:** Carried out experimental studies, data acquisition, and data analysis.

**D-CY:** Performed data analysis and manuscript review.

**SCK:** Elaborated the intellectual content and manuscript review.

**MFMA:** Elaborated the intellectual content and manuscript review.

**JS:** Conceptualized and designed the study, elaborated the intellectual content, performed literature search, manuscript review, manuscript revision and guarantor. All authors have read and approved the final manuscript.

## Conflict of interest

The authors have declared no conflict of interest.

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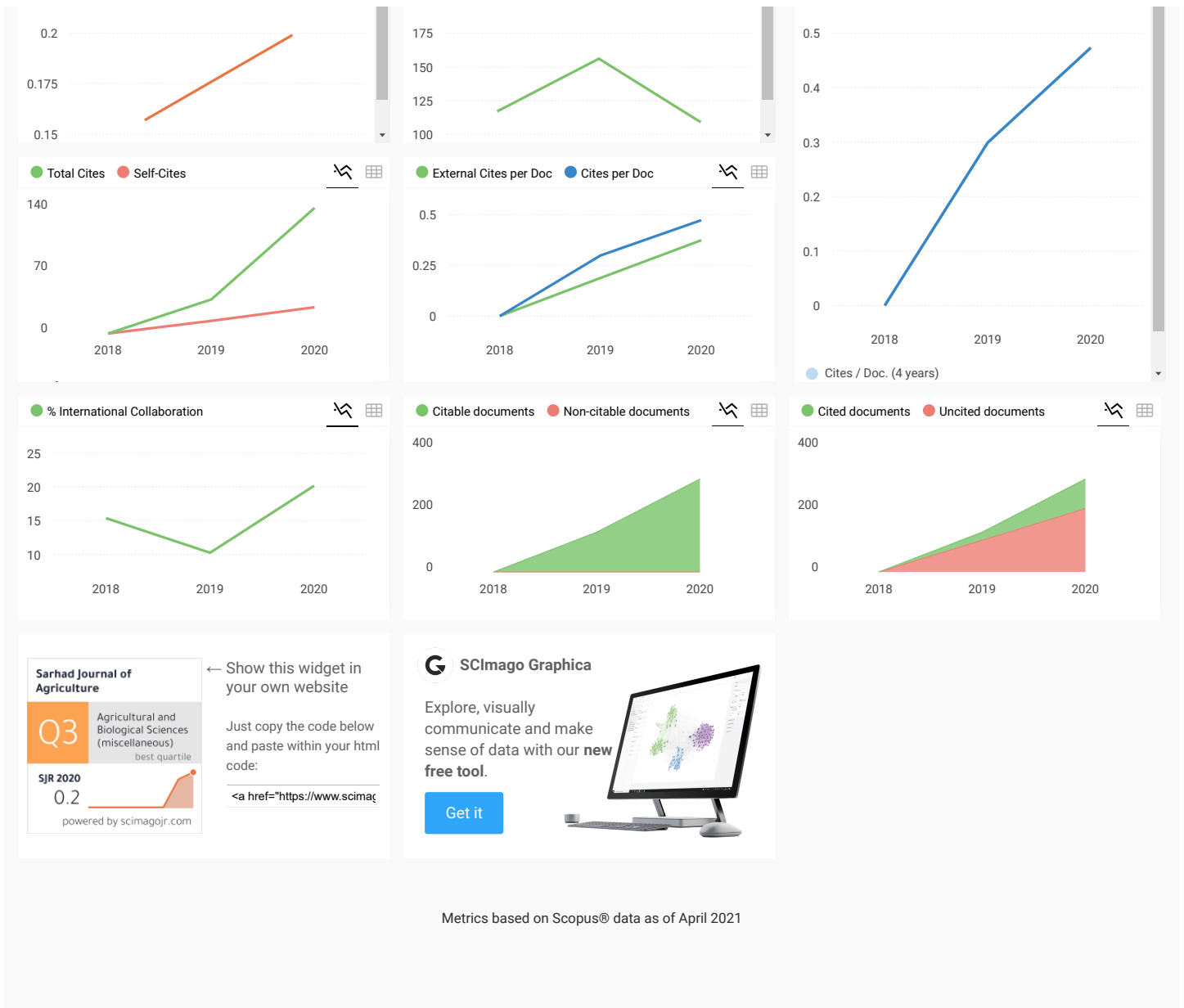
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