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OPTIMIZING ISOFLAVONES PRODUCTION ON CALLUS (*Pachyrhyzus erosus* Linn) THROUGH VARIATIONS IN SOURCES AND AMOUNT OF PLANTING MATERIAL

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Abstract
In vitro biotechnology has shown to be an effective method for producing secondary metabolites. Isoflavones possess antioxidant and estrogenic properties and are among these plant metabolites. Tissue culture technology enables the growth of plantlets or callus in small containers, addressing challenges in metabolite production. Different sources and quantities of planting materials should be tested within a single container to improve callus production efficiency. The Bengkoang plant/Jicama (*Pachyrhizus erosus*) naturally produces isoflavones. This study investigates the impact of variations in sources and amount of planting material on the production of isoflavones in Bengkoang callus using in vitro tissue culture techniques. The research was conducted at the Tissue Culture Laboratory, employing a Completely Randomized Design (CRD) factorial method. Factor I involved the explant source: C1: young leaves; C2: callus. Factor II involved the amount of explants per container: T1: 1 explant; T2: 2 explants; T3: 3 explants. The results show an interaction between source variations and the amount of planting material used for anthocyanin production successfully extracted from the callus. Planting three explants per container using subcultured callus material produced higher isoflavone content at 0.16% than other treatments.
Keywords: Invitro, Secondary Metabolites, Isoflavon, *Pachyrhizus erosus*

INTRODUCTION

Isoflavones are essential secondary metabolites found in plants known for their antioxidant and estrogenic properties, contributing to various human health benefits. As natural compounds, they play a crucial role in promoting health by combating oxidative stress and regulating hormonal balance. The Bengkoang plant (*Pachyrhizus erosus* Linn), a species native to Central and South America but primarily cultivated in Southeast Asia, has been identified as a natural source of isoflavones. Inspite of its potential, optimizing the production of these valuable compounds from Bengkoang remains a significant challenge.(Moon-Hee et al. 2023) state that Bengkoang tubers, or Jicama, have long been used as a skin lightener from generations to generation. Today, jicama is commonly used to enhance both health and beauty.

Innovative biotechnological approaches such as in vitro tissue culture have emerged as promising solutions to optimize isoflavone production (Twaij, Jazar, and Hasan 2020). Tissue culture techniques allow for the controlled growth of plant tissues, including callus formation, under sterile

conditions. These techniques can boost secondary metabolite production by manipulating environmental factors and nutrient composition, such as isoflavones. Moreover, In vitro tissue culture allows year-round production, independent of external conditions, providing a reliable method for producing bioactive compounds.

However, Several factors can affect the efficiency of isoflavone production in vitro, such as the type of plant material used (like young leaves or callus) and the number of explants in each container. Understanding these factors is essential for developing a sustainable, efficient method of producing isoflavones from *Pachyrhizus erosus*. Tissue culture is crucial for advancing agricultural science and modern farming (Twaij, Jazar, and Hasan, 2020).

This study aims to explore the effect of variations in sources and amount of planting material on isoflavone production in Bengkoang callus using in vitro tissue culture techniques. The findings provide valuable insights into optimizing explant selection and cultivation conditions to enhance the production of isoflavones, contributing to a more effective and scalable approach for obtaining these beneficial compounds from *Pachyrhizus erosus.* This study could lead to large-scale production of natural isoflavones, benefiting both the nutraceutical industry and consumers looking for natural health products.

LITERATURE REVIEW

Most plants have many organic sensory organs that, while not directly involved in growth and development, play significant roles in plant interactions with other plants, the environment, and defence. These are referred to as "metabolite sekunder." (Fazili et al., 2022). Secondary metabolites, especially from plants, have been used by humans since ancient times as medicinal ingredients and industrial ingredients. As human health problems become more complex, the demand for medicinal plants rises because the pharmaceutical industry depends on these plants and their extracts. The complex structure of organic compounds in medicinal plants makes them difficult to synthesize through chemical or conventional methods.

Plant cells, tissues, and cultured organs can produce valuable chemical compounds, as occurs in native plants in nature. Therefore, in vitro methods in the production of plants producing secondary metabolites (SMs) have become a sustainable way to meet market demand. In addition, plant tissue culture techniques offer an efficient and optimal alternative solution to the challenges faced by the phytopharmaceutical industry, especially in terms of mass propagation, germplasm conservation, research and production of biologically active compounds, and genetic improvement.

Isoflavone bioactive compounds in jicama plants can be produced from callus, using in vitro techniques. The type of explant, environmental conditions/media greatly influences callus culture) (Habibah et al., 2021) According to (Ningsih and Bagian, 2014) Isovlafon is a plant secondary metabolic compound with antioxidant and estrogenic activity in the body. Secondary metabolites from plants can be produced efficiently through tissue culture methods. The use of this technique has

several advantages, such as not depending on climatic conditions, soil, or geographic location, increasing productivity while reducing production costs

In-vitro tissue culture techniques have become a very useful tool in the genetic manipulation of plants and the production of valuable secondary metabolites. Applying this technique allows researchers to isolate and multiply plant cells in a controlled manner in a laboratory environment. This allows researchers to manipulate environmental factors, such as the type and amount of explant sources, to optimize the production of bioactive compounds, including isoflavones in jicama (Bengkoang) callus.

Plant cell culture technology was developed to produce high-value metabolites without using all parts of the plant (Priyanka et al., 2021), (Abdulhafiz et al., 2022). In-vitro studies regarding the isoflavone content in jicama callus with variations in the number and types of explant sources is an interesting and relevant research effort in plant biotechnology. In vitro plant material is a valuable source for producing secondary metabolites and provides an ideal environment for in-depth studies of biochemical and metabolic pathways.

The ability to meet the high demand for raw materials for traditional medicine makes using tissue culture techniques increasingly attractive. It is difficult to produce high-quality, uniform seeds in large quantities and quickly using conventional methods (Chauhan et al., 2015) However, tissue culture technology that produces plantlets or callus in small bottles can overcome this challenge. various plantlets from explant sources should be tested in a single planting container to improve callus production efficiency,

Pachyrhizus contains isoflavones, phytochemical compounds with significant potential for human health and agricultural use. In health, isoflavones offer benefits such as anti-inflammatory properties and potential anti-cancer effects. Isoflavones are also well known for their antioxidant properties and antimicrobial abilities and often used as cholesterol-lowering agents. The increased use of natural antioxidants is supported by epidemiological studies showing that consumption of natural antioxidants can reduce the risk of cardiovascular disease and cancer. Antioxidant activity in plants is due to various metabolites, including flavonoids, isoflavones, anthocyanins, coumarins, and carotenoids (Lukitaningsih, 2014).

This research is important due to the potential applications of isoflavones in the pharmaceutical, food, and cosmetic industries and their health benefits, including antioxidant properties and reducing the risk of chronic diseases (Sujono et al., 2021). Understanding the factors that affect isoflavone production in jicama callus can greatly develop biotechnology technology for producing plant-based bioactive compounds(Fazili et al., 2022). In the future, these things will provide a valuable method to sustain the feasibility of medicinal plants as renewable sources of medicinally important compounds, and these methods will provide successful production of desired, important, valuable, and unknown compounds.

METHOD

Place and time

The research was conducted at the Tissue Culture Laboratory of the Surabaya Food Crop Agriculture Service from early May to October 2024.

Materials and tools :

Material

The materials used were explants of young leaves and friable callus of Pachyrhizus esosus L from the results of the first year of research, MS glucose basic media, 70% and 90% alcohol, Clorox, Betadine, aluminum foil and plastic wrap.

Tool

The tools required for the research include a Sartorius scale, autoclave, oven, LAF (Laminar Air Flow), pH meter, tweezers, scalpel, Erlenmeyer flask, measuring cup, measuring pipette, dropper, petri dish, spatula, culture tube, and magnetic stirrer.

Research methods

The research used a factorial completely randomized design with two factors: the first factor was the explant source with 2 levels, and the second factor was the number of explants with 3 levels. Each treatment was repeated 3 times, with 10 samples per replication. The treatment is as follows:

Factor I: Explant source: C1: Young leaves; C2: Callus

Factor II: Number of explants, T1:1 ex /container ; T2: 2 ex/container; T3 : 3 ex/container

Implementation

Sterilization of tools

The tool is wrapped in brown paper and then sterilized in the oven at 121°C for 30 minutes. While the culture tubes were sterilized in an autoclave at 17 psi for 30 minutes

Media Creation

The basic media used is MS media with the composition as in table 1.

Table 1. Komposisi Media MS (Indarwati, 2022)

1. MS Media Composition	
materials	needs (mg / l)
KNO_3	1900
H_4NO_3	1650
$CaCl_2$ $2H_2O$	44
$MgSO_4$ $7H_2O$	370
KH_2PO_4	170
2. Micronutrients	
$MnSO_4$ $7H_2O$	22.3

$ZnSo_4$ $7H_2O$	8.6
H_3BO	6.2
Kl	0.83
$CuSO_4$. $5H_2O$	0.025
$NaMoO_4$. $2H_2O$	0.25
$CaCl_2$. $6H_2O$	0.025
$FeSO_4$. $7H_2O$	27.8
NaEDTA . $2H_2O$	37.3
3. Vitamin	
Mio-inositol	8100
Thamin HCl	0.1
Nikotinik acid	0.5
Piridoksin HCl	0.5
Glisin	2.0
4. Glucose	30000

Planting

Leaf explants were sterilized using 5%, 10% and 15% Clorox solutions added with one drop of Tween 20 each, then the explants were cut into + 1 cm pieces and planted in culture tubes according to the treatment.

Incubation

After planting, the samples are placed on the incubation rack, progressing through various incubation stages, and the callus development is then observed.

Variables:

Observations were conducted on several callus growth parameters

a. **Callus Quality:** Visually observed once a week and evaluated using a scoring system.

1 = no callus has formed

2= compact callus

3= friable callus

b. **Callus quantity:** Visually observed at 1-week intervals with scoring:

1 = Callus has not yet formed

2 = slight callus (< 1 times the size of the explant)

3 = medium callus (1-2 times the size of the explant)

4 = lots of callus (> 2 times the size of the explant)

c. Secondary metabolite content: Observed after the callus was 8 and 12 weeks old destructively through Gas

Chromatography analysis.

Secondary Metabolite Analysis: The material is extracted using absolute alcohol and then analyzed by gas chromatography

Data processing

The observation results are analyzed using analysis of variance (ANOVA). If significant differences are found, the analysis is followed by the Least Significant Difference (LSD) test at a 5% significance level.

RESULTS AND DISCUSSION

1. Observation Of Callus Quality

The callus quality score analysis (Table 1.) shows no interaction between the use of source treatment and the amount of planting material at all ages of observation, except at week 6. The interaction between the type of callus treatment and the number of callus was seen only in the callus quality parameters observed at 6 WAP. The results of observations of the growth of Pachyrisus callus quality from 1 to 10 weeks after planting can be seen in Table 1.

Table1

The mean results of callus quality observations on variations in sources and amounts of planting material from 4 to 10 weeks after planting (WAP).

Treatment	Age (Week After Planting)						
	4	5	6	7	8	9	10
C1T1	1.30	1.40	1.50	1.70	1.90	1.30	2.60
C1T2	1.30	1.40	1.50	1.70	1.90	2.30	2.60
C1T3	1.30	1.40	1.50	1.70	1.90	2.30	2.60
C2T1	1.30	1.40	1.60	1,70	2.00	2.60	2.90
C2T2	1.30	1.50	1.60	1.70	2.23	2.60	2.90
C2T3	1.30	1.56	1.66	1.70	2.23	2.60	2.90
BNT 5 %	NS	NS	0.04	NS	NS	NS	NS

Note: Number followed by the same letter in the same column are no different

NS : Not Significant; LSD (Least Significant Difference)

Table 1 shows that at the end of the observation period (10 weeks after planting), all planting materials formed compact callus (score > 2). While the differences were not statistically significant, the quality values indicate that older callus generally produced better quality. These results suggest that the treatments in this study did not significantly impact the quality of Bengkoang callus.

The quality of callus growth is indicated by its texture and color. Callus, a plant cell collection that grows irregularly, forms when explants are cultured in suitable media. Based on the results, the callus texture from the treatment appears dense and compact. A dense texture suggests that the callus contains more secondary metabolites than a crumbly texture (Indah and Ermavitalini 2013) A dense callus texture is considered good because it can accumulate more secondary metabolites in plants. A dense callus texture generally has a small cell size with dense cytoplasm, a large nucleus, a lot of carbohydrate content, and a nodule-like structure. Nodules are proembryonic masses and can be used as inoculum for somatic embryo induction. Many factors influence the formation of callus quality,

including the type of plant used, the nutritional composition of the media, growth regulators, and environmental conditions (Hariyati, Bachtiar, and Sedijani, 2016).

2. Quantity of Callus

The results of the variance analysis in callus quantity showed that callus formation at 4–5 weeks and 7.8–9 weeks after planting did not significantly affect the number of callus produced. Significant interactions in treatment were found at 6 and 10 weeks after planting (Table 2). Based on these data, callus formation at three months of age showed differences in average values for each treatment. The highest average callus quantity results were achieved by 10-week-old callus with score 2.86. Data from the callus quantity analysis results are presented in table 2.

Table 2

The mean results of the observation of the callus quantity formed in various treatments

(Week After Planting/WAP)

Treatment	Scor of Quantity Callus on several weeks Age (WAP)						
	4	5	6	7	8	9	10
C1T1	1.05	1.22	1.32e	1.46	1.68	1.04	2.43b
C1T2	1.13	1.26	1.46c	1.61	1.86	2.13	2.33c
C1T3	1.10	1.25	1.38d	1.57	1.78	2.19	2.11d
C2T1	1.30	1.50	1.81b	2.00	2.15	2.48	2.81a
C2T2	1.32	1.53	1.84ab	2.00	2.21	2.43	2.81a
C2T3	1.33	1.58	1.86a	2.02	2.26	2.53	2.86a
LSD 5 %	NS	NS	0.04	NS	NS	NS	0.05

Note ：Numbers followed by the same letter in the same column are no different.

NS : Not Significant LSD (Least Significant Difference)

Observations showed a positive correlation between the number of callus and the age after planting, meaning that longer observation periods lead to larger callus sizes in all treatments, even with some variations. This increase in size indicates growth in response to the treatments, defined as a lasting increase in the size of a plant or its parts due to more and larger cells.

Table 2 shows that at the end of the observation period (10 WAP), there was an interaction between treatments involving the type and amount of planting material regarding callus quantity. The C2T3 treatment, which used 3 planting materials per container, achieved a score of 2.86 (indicating medium-quality callus) but was not significantly different from C2T2 and C2T. The growth of callus cultures is heavily influenced by the addition of complementary compounds, growth regulators, the type of explant, and environmental conditions. An increase in callus weight indicates growth and is important in monitoring callus development (Dyshlyuk et al., 2021). The increase in callus quantity score depends on the speed of cell division.

The results of this research show ideal callus growth. Furthermore, the age of the callus also influences its growth. When it has reached optimal conditions, this will be followed by physical

changes in the callus depending on the availability of nutrients in the media. When the nutrients in the culture medium are low, the cell regeneration process will slow, affecting the color and texture of the callus that forms. This is characterized by callus cells becoming looser and changing color from yellowish white to brownish yellow to brown (Lutfiah and Habibah, 2022)

3**. Isoflavone content**

The results of the analysis of isovlavone content in Jicama callus with several treatments are presented in Table 3.

Table 3

Average Isoflavone Content Formed in Callus variations in sources and amount of planting material

Treatment	Isoflavone Content Formed in Callus (%)	
Source & number Kalus	56 DAP	84 DAP
C1T1	0.08 de	0.10 b
C1T2	0.11 abc	0.12 ab
C1T3	0.12 ab	0.14 a
C2T1	0.10 bcd	0.12 b
C2T2	0.11 abc	0.14 ab
C2T3	0.12 ab	0.16 a
LSD :5%	0.029	0.044

Note : Numbers followed by the same letter in the same column are Not differen

LSD :(Least Significant Difference); DAP : Day After Planting

The results of Gas Chromatography Analysis showed that there was secondary metabolite content (Antocyanin) in the callus, the primary secondary metabolite in Pachyrhizus callus. Callus is a collection of plant cells that grow irregularly and is formed when explants are cultured in media. Various types of treatment show variations in the compound content obtained from each treatment.

The research showed the highest levels of secondary metabolites, namely the callus treatment observed 84 days after planting. The highest results were seen in the treatment with 3 callus/container numbers at 0.16%. As stated by (Pan et al., 2020) stated, Secondary metabolites have complex chemical compositions in response to stress to perform various physiological roles in plants. Medicinal plants produce phytoconstituents like alkaloids, flavonoids, and pterocarpan. In vitro micropropagation methods are valuable for producing these secondary metabolites (Sharma et al., 2021). certain physical and environmental conditions are required for different types of planting material to produce secondary metabolites from specific plants (Fazili et al., 2022). It needs to be analyzed in depth to increase the production of secondary metabolites in callus suspension culture (Pan et al., 2020).

The gas chromatography analysis showed that some treatments did not increase anthocyanin levels due to insufficient quantities. Callus growth and secondary metabolite production are affected by environmental conditions, as well as the type of plant and its specific genetic traits. Light also

plays a crucial role in plant development, both in vivo and in vitro, with culture conditions influenced by exposure duration, light color, and intensity. These factors can regulate the production of primary and secondary metabolites.

This research aimed to provide valuable insights into optimizing isoflavone production in jicama callus, leading to the developing jicama-based products rich in these bioactive compounds. This study could significantly benefit the pharmaceutical, food, and cosmetics industries, improving human health and welfare. Furthermore, it is expected to demonstrate that secondary metabolites, such as isoflavones, can be produced in vitro. This is in line with an opinion (Chandran et al., 2020) that plant tissue culture (PTC) techniques that do not depend on climatic and geographical conditions enable the production of secondary metabolites in a sustainable, economical and efficient manner, an opportunity for sustainable biotechnology development.

CONCLUSION

Based on the description in the discussion, the researcher draws the following conclusions:

1. The amount of planting material per culture container influences the production of secondary metabolites in vitro. Treatment of 3 explants/container produced the highest isoflavones (0.16%)
2. There is an interaction between source variations and the amount of planting material on anthocyanin production. which was successfully extracted from the callus. Planting 3 explants in a container with subculture material from callus produced the highest (0.16%) isoflavones compared to other treatments.

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