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Effect of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin on callus induction and growth of *Physalis angulata* L. leaf explants

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Abstract

Physalis angulata L. is an annual herbaceous plant used as traditional medicine. The roots, stems and leaves are used to treat diabetes, hypertension, fever and headaches. The fruit contains lots of provitamin A and has antitumor, antihyperglycemic, anti-inflammatory, antidiabetic and immunomodulatory activities. *P. angulata* leaves also contain secondary metabolites of alkaloids, flavonoids, saponins, steroids, tannins, physalin A, physalin B, glycosides and stearic acid. The use of plants as a source of medicine is not only very dangerous for the survival of plants, but also not standardized. Secondary metabolites can be produced in callus by in vitro culture induced with appropriate growth regulators. Through callus culture, secondary metabolites or chemical substances from plants can be produced. This study aims to determine the effect of growth regulators 2,4-D and kinetin on callus induction time, the percentage of explants forming callus, fresh weight, dry weight, and callus morphology. This study was a laboratory experimental with a completely randomized design and 5 treatments (0.0 mg/L 2,4-D + 2.0 mg/L kinetin; 0.5 mg/L 2,4-D + 1.5 mg/L kinetin; 1.0 mg/L 2,4-D + 1.0 mg/L kinetin; 1.5 mg/L 2,4-D + 0.5 mg/L kinetin; 2.0 mg/L 2,4-D + 0.0 mg/L kinetin), each treatment consisted of five replications. The data obtained has been analyzed qualitatively and quantitatively. Qualitative data were obtained from callus morphological (color and texture) descriptions. Quantitative data obtained from callus induction time, the percentage of explants forming callus, fresh weight and dry weight of callus were analyzed using SPSS 24 with a significance value of 0,05. Data analysis on the effect of 2,4-D and kinetin concentrations used the ANOVA test. Difference between treatments were tested using the Duncan´s multiple range test. The results showed that the suitable growth regulator for inducing callus growth in leaf explants of *P. angulata L* was 2.0 mg/L 2.4D + 0.0 mg/L kinetin. Growth regulator 2.0 mg/L 2,4-D + 0.0 mg/L kinetin induced callus in a short time i. e. 10.60 days after culture, resulting in the highest fresh and dry weight of callus i.e., 0.35 g and 0.03 g, as well as the percentage of explants forming callus 100%. Callus has a compact texture with a variety of colours.

Keywords: *P angulata*; Callus induction; Callus growth; 2;4-D; Kinetin

1. Introduction

Physalis angulata L. is a traditional medicinal plant that can be used to treat diabetes, hypertension, fever and headaches. The plant parts commonly used are roots, stems, leaves, shoots, flowers and fruit (1). The fruit of *P. angulata* also has several biological activities such as antitumor, immunomodulatory, anti-hyperglycemic, anti-inflammatory and antidiabetic effects (2). Hidayat (3) reported that the ethanol extract of *P. angulata* had cytotoxic activity against blood cancer and ovarian cancer. *Physalis angulata* leaves also contain alkaloid secondary metabolites, flavonoids, saponins, steroids, tannins, physalin A, physalin B, glycosides, palmitic acid and stearic acid (1, 4, 5, 6). The use of plants (roots, stems, leaves, shoots, flowers and fruit) as a source of medicine is not only very dangerous for plant survival but also not standardized.

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Callus culture method has high potential to obtain secondary metabolites *in vitro* which is used as an alternative to isolating secondary metabolites from *P. angulata*. Through callus culture, secondary metabolites will be obtained in large quantities, quickly, not depending on the season and standardized. Callus formation *in vitro* was influenced by several factors, i.e. the type of media, the type of explants, the environment, and the addition of growth regulators. Growth regulators that are generally added are from the auxin group (IAA, NAA, IBA, 2.4D) and the cytokinin group (Kinetin, BAP, Thidiazuron). Kinetin is a cytokinin that is often used in tissue culture techniques and functions in cell division and differentiation of adventitious shoots from callus. 2,4-Dichlorophenoxyacetic acid (2,4-D) is a strong synthetic auxin which has the function of triggering callus formation (7). This study aims to determine the effect of growth regulators 2,4-D and kinetin on callus induction and growth of *P. angulata*. So that, it can be used as a basis for the development further research in the isolation of secondary metabolites.

2. Material and methods

2.1. Explant source and process sterilization

The research was conducted at the Plant Physiology Laboratory, Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Surabaya for six months. In this study *P angulata* was used and the second leaf from the shoot was used as a source of explants. The leaves were washed and soaked in detergent solution for six minutes to eliminate the dust, and then washed using sterile-distilled water. Furthermore, the leaves were sterilized in a 20% sodium hypochlorite solution for 10 minutes, then rinsed three times using sterile distilled water.

2.2. Callus induction and growth

The leaves were sliced with a sterile scalpel in a sterile Petri dish ± 1 cm2. To evaluate the effect of 2,4-Dichlorophenoxyacetic acid (2,4-D) and Kinetin on callus induction and growth of explants *P. angulata*, the explants were put on Murashige and Skoog (8) supplemented with sucrose 30 g/L and 0.0 mg/L 2,4-D + 2.0 mg/L kinetin; 0.5 mg/L 2,4-D + 1.5 mg/L kinetin; 1.0 mg/L 2,4-D + 1.0 mg/L kinetin; 1.5 mg/L 2,4-D + 0.5 mg/L kinetin; 2.0 mg/L 2,4-D + 0.0 mg/L kinetin. All media were set into pH about 5.6 and were solidified with 2 g/L gellan gum (Phytagel: Sigma Chemical Co., St. Louis, MO), followed it was sterilized at 121^0C for 15 min. For each treatment, about five explants were cultured in culture tube filled with about 25 mL of medium. Each treatment with five replications. All the cultures were maintained under 16/8 h day/night at (23 ± 2)^{0}C. The callus induction time was observed every day for four weeks of culture. After the four week of the culture period, callus growth and development observations were carried out by calculating the percentage of callus forming explants, callus fresh and dry weight, and callus morphology (texture and color). After five weeks, the fresh callus was placed on aluminum foil and dried in an incubator oven at 60-70 °C to obtain dry callus biomass. The percentage of explants forming callus in each treatment was obtained by comparing the number of explants forming callus with the number of explants cultured multiplied by 100%.

2.3. Statistical analysis

The experimental units were set up in a completely randomized design (CRD). The data were analyzed with SPSS (Version 24) using ANOVA. The mean values were separated using Duncan´s multiple range test (DMRT) with level of significance at $P \leq 0.05$ (9).

3. Results

The mean time for *P. angulata* L. explants to form callus and the percentage of explants to form callus are presented in Table 1.

In table 1 can be seen that there were different responses in each treatment on the time of callus formation. The best response occurred in the 2.0 mg/L 2,4-D + 0.0 mg/L kinetin treatment with the fastest mean time for explants to form callus, i.e., 10.60 days after culture, while the longest mean time for explants to form callus occurred in the 0.5 mg/L 2,4-D + 1.5 mg kinetin i.e., 16.60 days after culture.

Observation of the percentage of explants forming callus (Table 1) until the four weeks, at 0.0 mg/L 2,4-D + 2.0 mg/L kinetin was not able to induce callus formation. Treatments of 0.5 mg/L 2,4-D + 1.5 mg/L kinetin; 1.0 mg/L 2,4-D + 1.0 mg/L kinetin; 1.5 mg/L 2,4-D + 0.5 mg/L kinetin; and 2.0 mg/L 2,4-D + 0.0 mg/L kinetin was able to induce callus 100%.

Table 1 The mean time for *P. angulata* L. explants to form callus and the percentage of explants to form callus at different concentrations of 2,4-D and kinetin

Plant Growth Regulator Concentration (mg/L)		The mean time explants to form callus	Percentage of explants to form callus (%).
2,4-D	Kinetin		
0.0	2.0	No callus formed	0
0.5	1.5	$16.60^{a} \pm 0.54$	100
1.0	1.0	$13.00^{b} \pm 0.70$	100
1.5	0.5	$14.40^{c} \pm 0.89$	100
2.0	0.0	$10.60^{d} \pm 0.54$	100

Mean ± SD followed by the same letter is not significantly different at the P 5% by Duncan´s multiple range test.

The results of the mean fresh and dry weight of callus after being cultured for four weeks are presented in Table 2.

Table 2 The mean fresh and dry weight of *P. angulata* callus at different concentrations of 2,4-D and kinetin at four weeks after culture

Growth Regulator Concentration (mg/L)		Mean callus weight (g)	
2,4-D	**Kinetin**	**Fresh weight**	**Dry weight**
0.0	2.0	No callus formed	No callus formed
0.5	1.5	$0.08^{a} \pm 0.05$	$0.02^{a} \pm 0.00$
1.0	1.0	$0.11^{a} \pm 0.04$	$0.01^{a} \pm 0.00$
1.5	0.5	$0.30^{b} \pm 0.16$	$0.02^{a} \pm 0.01$
2.0	0.0	$0.35^{b} \pm 0.14$	$0.03^{b} \pm 0.00$

Mean ± SD followed by the same letter is not significantly different at the P 5% by Duncan´s multiple range test.

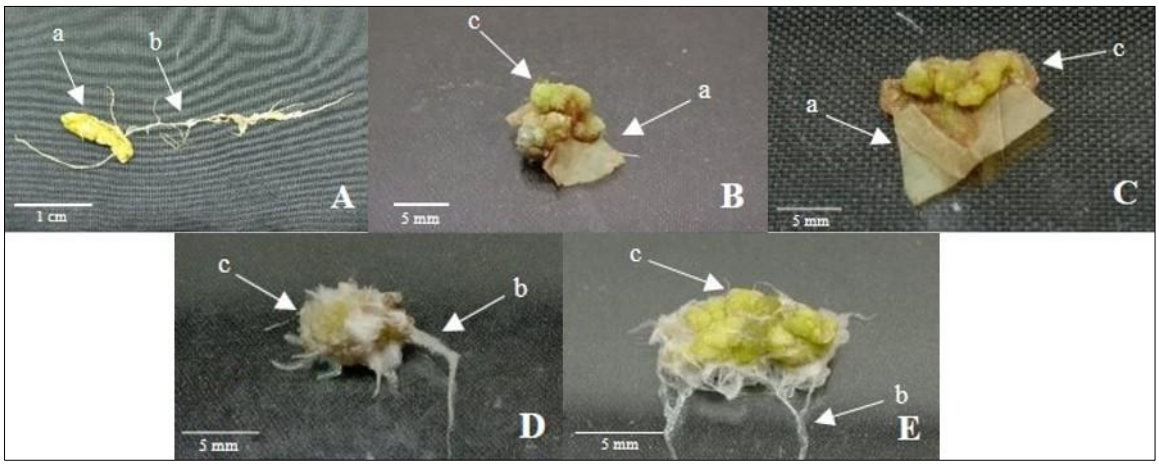

Figure 1 Callus morphology of *P. angulata* at four weeks after culture in various treatments. A. 0.0 mg/L 2,4-D + 2.0 mg/L kinetin; B. 0.5 mg/L 2,4-D + 1.5 mg/L kinetin; C. 1.0 mg/L 2,4-D + 1.0 mg/L kinetin; D. 1.5 mg/L 2,4-D + 0.5 mg/L kinetin; E. 2.0 mg/L 2,4-D + 0.0 mg/L kinetin. a= explant; b=root; c=callus

Observation of the fresh and dry weight of callus in the four weeks after culture (Table 2) the highest fresh weight of callus (0.35 g) was obtained in the treatment of 2.0 mg/L 2,4-D + 0.0 mg/L kinetin as well as callus dry weight the highest (0.03 g) was found in the 2.0 mg/L 2,4-D + 0.0 mg/L kinetin. Based on the observations in Table 2 the increasing concentration of 2.4-D is followed by an increase in the fresh weight of callus. The lowest mean fresh weight of callus was found in the 0.5 mg/L 2,4-D + 1.5 mg/L kinetin (0.08 g), then it increased (0.11 g) in the 1.0 mg/L 2,4-D + 1.0 mg/L

kinetin, increased again (0.30 g) in the 1.5 mg/L 2,4-D + 0.5 mg/L kinetin and reached the highest fresh weight found in the 2.0 mg/L 2,4-D + 0.0 mg/L kinetin (0.35g).

The morphology of *P. angulata* callus at different concentrations of 2,4-D and kinetin in the four weeks after culture is presented in Figure 1.

The response of the explants to the addition of growth regulator 2.4-D and kinetin was the formation of callus in almost all treatments except for the 0.0 mg/L 2,4-D + 2.0 mg/L kinetin, until the four weeks after culture no callus was formed. The results of observations on callus morphology, obtained callus with a compact texture, smooth surface with various colors, but the majority is dominated by green. Another response was the formation of roots in the explants (Figure 1A) and the formation of roots emerging from the callus (Figure 1D and 1E).

4. Discussion

4.1. Effect of 2,4-D and kinetin on callus induction time and percentage of explants forming callus

The speed at which explants form callus is an indicator to determine the effect of growth regulator 2,4-D and kinetin on callus induction and development of *P. angulata*. Callus appeared in the cut area on the explant (Figure 1C), due to the response of the injured explant to the hormone produced by the plant itself and given externally as an effort to cover the wound (10). Based on the results of Duncan´s significant difference test (Table 1) there is a significant difference in the mean of time explants form callus in all treatments. The fastest mean time for explants to be induced to form callus was 10.60 days after culture, which occurred at 2.0 mg/L 2,4-D + 0.0 mg/L kinetin, while the longest mean time of callus induction occurred at 0.5 mg/L 2,4-D + 1.5 mg/L kinetin, which was 16.60 days after culture.

In general, the addition of 2.4-D with higher concentration accelerated callus formation. Observation of the percentage of explants forming callus (Table 1) until the four weeks, all explants were cultured in the 0.5 mg/L 2,4-D + 1.5 mg/L kinetin; 1.0 mg/L 2,4-D + 1.0 mg/L kinetin; 1.5 mg/L 2,4-D + 0.5 mg/L kinetin and 2.0 mg/L 2,4-D + 0.0 mg/L kinetin induced 100% callus formation, but explants cultured in kinetin alone without 2,4-D (0.0 mg/L 2,4-D + 2.0 mg/L kinetin) for up to four weeks, no callus formed on the explants. This indicates that for callus induction in *P. angulata*, it is necessary to add exogenous auxins, especially 2,4-D.

4.2. Effect of 2,4-D and kinetin on fresh weight and dry weight of callus

Callus growth can be observed by measuring the cells and weighing the weights. In this study, growth data were obtained by weighing the fresh weight and dry weight of callus in the four weeks after culture. The induced callus is an indicator that a growth process has taken place where initially only a few calluses appear, then they become numerous. The fresh weight of callus can be affected by the water content present in the cells, cell metabolism and humidity conditions during the culture period. While the dry weight is influenced by the process of photosynthesis and cellular respiration. This is because in the process of photosynthesis there is a process of taking carbon dioxide and adding protoplasts which causes the size and number of cells to increase (11). Based on the results of the ANOVA test, there was an effect of giving a combination of growth regulators 2,4-D and kinetin on the mean fresh weight and dry weight of callus, then tested with Duncan's test to determine the difference between treatments. Based on the results of Duncan´s significant difference test (Table 2) there is a significant difference in the mean fresh weight of callus. The fresh weight of callus in the 0.5 mg/L 2,4-D + 1.5 mg/L kinetin and 1.0 mg/L 2,4-D + 1.0 mg/L kinetin was significantly different from the 1.5 mg/L 2,4-D + 0.5 mg/L kinetin and 2.0 mg/L 2,4-D + 0.0 mg/L kinetin. However, the fresh weight of callus in the 0.5 mg/L 2,4-D + 1.5 mg/L kinetin and 1.0 mg/L 2,4-D + 1.0 mg/L kinetin was not significantly different, as well as the fresh weight of callus in the 1.5 mg /L 2,4-D + 0.5 mg/L kinetin not significantly different from the 2.0 mg/L 2,4-D + 0.0 mg/L kinetin. Observation of the fresh weight and dry weight of callus in the four weeks after culture (Table 2), the highest fresh weight of callus (0.35 g) was obtained in the treatment of 2.0 mg/L 2,4-D + 0.0 mg/L kinetin, as well as the highest callus dry weight (0.03 g) was also found in the 2.0 mg/L 2,4-D + 0.0 mg/L kinetin. Based on the observations in Table 1 appears that the increasing concentration of 2.4-D is followed by an increase in the fresh weight of callus. The lowest mean fresh weight of callus was found in the 0.5 mg/L 2,4-D + 1.5 mg/L kinetin (0.08 g), then it increased (0.11 g) in the 1.0 mg/L 2,4-D + 1.0 mg/L kinetin, increased again (0.30 g) in the 1.5 mg/L 2,4-D + 0.5 mg/L kinetin and reached the highest fresh weight found in the 2.0 mg/L 2,4-D + 0.0 mg/L kinetin (0.35 g).

4.3. Effect of 2,4-D and kinetin on callus morphology

The results of observations on callus texture, from research obtained callus with a compact texture (Figure 1E). Compact type callus is composed of dense cells, that are difficult to separate and has large vacuoles. The compact type callus is suitable as a source of secondary metabolites because the compact type callus can accumulate more secondary

metabolites than the crumb type callus. This was also supported by Ramawat (12) who said secondary metabolites were produced from compact type callus when cells experienced a decrease in proliferative activity.

The results of observations on callus color, obtained callus with various colours, but dominated by green. In the treatment of 0.5 mg/L 2,4-D + 1.5 mg/L kinetin; 1.5 mg/L 2,4-D + 0.5 mg/L kinetin and 2.0 mg/L 2,4-D + 0.0 mg/L kinetin found brownish yellow callus. This phenomenon is caused by the oxidation of phenol produced by explants. Phenol is toxic to cells causing explant death. This is a physiological setback of explant.

The response of the explants with the addition of 2,4-D and kinetin not only formed callus but also formed roots (organogenesis). Organogenesis occurred in the treatment of 0.0 mg/L 2,4-D + 2.0 mg/L kinetin; 1.5 mg/L 2,4-D + 0.5 mg/L kinetin and 2.0 mg/L 2,4-D + 0.0 mg/L kinetin. Based on the results of the research, treatment suitable for inducing and developing callus in leaf explants of *P. angulata* was 2.0 mg/L 2,4-D + 0.0 mg/L kinetin, because in this treatment the explants formed callus (100%) and the fastest time to form callus (10.60 days) compared to other treatments.

Based on the results of fresh weight and dry weight of callus obtained in the treatment of 2.0 mg/L 2,4-D + 0.0 mg/L kinetin can produce the highest fresh weight and dry weight. This indicates that the media conditions in the treatment of 2.0 mg/L 2,4-D + 0.0 mg/L kinetin cells can grow well because during the growth period the callus cells can carry out metabolism optimally which can increase the fresh weight and dry weight of the callus.

5. Conclusion

The addition of growth regulators 2,4-D and kinetin had an effect on callus formation and growth. A single growth regulator (2.0 mg/L 2,4-D + 0.0 mg/L kinetin) increased callus most significantly in comparison than another treatment.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declared that there is no conflict of interest.

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