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THE GROWTH OF *PIMPINELLA ALPINA HOST* CALLUS AT VARIOUS TREATMENTS OF PLANT GROWTH REGULATOR CONCENTRATIONS OF NAA. 2,4 D AND ITS COMBINATION WITH BAP

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ABSTRACT

Purwaceng (Pimpinella alpina) is an herbal plant that has aphrodisiac, diuretic, and tonic properties. The imbalance between the growth and the exploitation of this plant makes it included in Appendix I (endangered), so its conservation will be crucial. One method of the conservation of this plant is tissue culture. This method is considered one way to induct the callus. The research objective is to find the effect of various treatments of plant growth regulator concentrations of NAA. 2,4 D and its combination with BAP on the growth of purwoceng callus. Concentration of plant growth regulator NAA (0.5 mg L^{-1}, 1 mg L^{-1}, 2 mg L^{-1}), 2,4-D (0.4 mg L^{-1}, 0.8 mg L^{-1}, 1.2 mg L^{-1}), a combination of NAA with BAP (0.5 mg L^{-1} NAA: 1 mg L^{-1} BAP, 1 mg L^{-1} NAA: 2 mg L^{-1} BAP, mg L^{-1} NAA: 3 mg L^{-1} BAP) and a combination of 2, 4- D with BAP (1 mg LL 2,4-D : 0.5 mg L BAP, 1 mg L^{-1} 2,4-D : 1 mg L^{-1} BAP, 2 mg L^{-1} 2,4-D : 1 mg L^{-1} BAP) into Murashige Skoog medium. Then purwaceng leaf explants were planted on the medium until the growth was seen. This research shows that the plant growth regulator concentration has a different influence on the growth of callus that is recognized by the day of callus induction, colour and callus texture. MS media with growth regulator 1.2 mg L^{-1} 2,4-D can grow callus faster (17 days) with friable texture.

Keywords: Purwaceng, Pimpinella alpina, Callus Induction, Tissue Culture, Murashige Skoog

INTRODUCTION

Purwaceng plant (*Pimpinella alpina*) is a commercial herb plant whose roots are reported to have medicinal properties as an aphrodisiac (increases sexual arousal and erection), diuretic (smooths urine channel), and tonic (can increase stamina) (Darwati and Roostika, 2016; Meiny 2017; Wahyuning-rum, 2016; Rusmin 2017). Currently, the population of Purwaceng is rare due to massive genetic erosion. Even the population in Mount Pangrango, West Java and mountainous areas in East Java have decreased (Darwati and Roostika, 2016). One of the cultivation efforts carried out in his research on *in vitro* propagation through tissue culture has been carried out to overcome this problem, but the results have not been satisfactory (Widodo et al., 2018).

The principle of tissue culture is that all plant parts, whether in the form of cells, tissues, or plant organs, can become new plants under aseptic conditions. The advantage of plant tissue culture is as a basis to produce secondary metabolites *in vitro* and to produce *in vitro* cultured plants that have good quality compared to the original plant (Bhatia, 2015) (Faramayuda et al., 2021a; Faramayuda et al., 2021b; Faramayuda et al., 2021c). *in vitro* and Efforts to induce callus in purwaceng plant tissue culture have been carried out, including basic media (1/2 MS) combined with the addition of 2, 4-D with several combinations of treatments can induce callus with the best concentration of 0.75 mg L^{-1} (Intias, 2011; Mariani, 2017). Based on the results of previous studies, it is necessary to study the concentration of 2,4-D combined with concentrations of other types of cytokinins to grow callus (Sukweenadhi et al., 2019). It is also necessary to study other types of explants from the purwaceng plant to induce callus of the purwaceng plant because the previous researcher used the purwaceng leaf stalk. Therefore, research will be conducted to determine the effect of variations in the concentration of growth regulators on callus growth of purwaceng plants (*Pimpinella alpina*).

RESEARCH METHODS

Research Material

The plant material used in this study was the leaf part of the *Pimpinella alpina* plant. The age of the plants used as explants is 6 months.

The basic media used were Murashige Skoog (MS; ;Phytotechlab) with the addition of plant growth regulators (PGR) Naphthaleneacetic Acid (NAA; Sigma-Aldrich), 2,4-D (Dichlorophenoxyaetic Acid; Sigma-Aldrich) and 6-Benzyl Amino Purine (BAP; Sigma-Aldrich).

Chemicals for sterilization include 70% alcohol (Merck), 10% Bayclin® (Sodium hypochlorite), fungicides, and sterile aqua dest.

Pimpinella alpina Host samples was obtained from Jopa Green Sleman, Yogyakarta. The material was determined to determine the correctness of its species at the Plant Taxonomy Laboratory, Department of Biology, FMIPA, Padjadjaran University.

Research design

Plant tissue culture technique on *Pimpinella alpina* Host aimed to callus induction with MS (Murashige and Skoog) with the addition of a single plant growth regulator (PGR) naphthaleneacetic acid (NAA), 2,4-D

(dichlorophenoxyaetic acid) and a combination with 6-benzyl amino purines (BAP). The research design included sampling, planting explants on tissue culture media and observing callus growth. research design follows a completely randomized design experiment. The experiment was repeated on each medium three times.

Preparation of basic media was carried out with 12 variations of PGR with MS base media, to each medium added by PGR single NAA, 2,4-D and a combination with BAP (Table 1). The making of each media was made three times repetition. The pH of the solution was measured using a pH meter up to a pH range of 5.6-5.8. Agar was added, heated on a hot plate, and stirred using a magnetic stirrer. These media were poured into culture bottles and then sterilized. Media sterilization was carried out using an autoclave at 121 ^{O}C for 15 minutes.

Equipment and Media Sterilization

The tools used are sterilized by washing them thoroughly and drying them. Placed in an autoclave at 121°C for 15 minutes.. Tools such as tweezers and scalpels can be re-sterilized by heating over a spark flame after being immersed in 70% alcohol before using the tool. The growing media was sterilized with the tool in an autoclave at 121°C for 15 minutes(Arifin, 2020).

Sterilization and implantation of explants on tissue culture media

The explants used were *Pimpinella alpina* Host young leaves. The explants were sterilized, at this stage, the explants were carried out by washing the leaves in running water and washed in a 2% fungicide solution; sterilization was continued in LAF by soaking the explants successively, in 70% alcohol, Bayclin® 10% (Sodium hypochlorite).

Rinse using sterile distilled water for three repetitions. Then the leaves were cut to get explants (1 x 2 cm). The explants were planted on the prepared tissue culture media. The explants were rinsed with water three times. The explants were planted on the prepared tissue culture media.

Callus Growth Observation

Observations were made by noting the development of the cultured explants starting to form a callus, callus colour, and callus texture. The observed callus form was friable or solid. Callus color ranges from brown, black, white or white-brown.

Table 1 The treatments of plant growth regulator concentrations of NAA. 2,4 D and its combination with BAP in MS-base media.

Treatment	MS media		Treatment	MS media	
	NAA	BAP		2,4-D	BAP
A1	0.5 mg L^{-1}	-	A2	0.4 mg L^{-1}	-
B1	1 mg L^{-1}	-	B2	0.8 mg L^{-1}	-
C1	2 mg L^{-1}	-	C2	1.2 mg L^{-1}	-
D1	0.5 mg L^{-1}	1 mg L^{-1}	D2	1 mg L^{-1}	0.5 mg L^{-1}
E1	1 mg L^{-1}	2 mg L^{-1}	E2	1 mg L^{-1}	1 mg L^{-1}
F1	2 mg L^{-1}	3 mg L^{-1}	F2	2 mg L^{-1}	1 mg L^{-1}

Data Analysis

The experiment was repeated on each medium three times.

RESULTS AND DISCUSSION

In tissue culture techniques, it is known that the ability of plant tissue to form callus is strongly influenced by, among others, the components of the media, the concentration of growth regulators and light intensity (Nurwahyuni and Tjondronegoro, 1994). The explant source used was the leaf part of the purwaceng plant in this study. The first stage in this study was determining the purwaceng plant (*Pimpinella alpina*). The purpose of the determination is to find out the truth of the identity of the plants that will be used in the research. The determination of this plant was carried out at the Plant Taxonomy Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Padjadjaran University. The determination results identified that the plant to be used in the study was a purwaceng plant with the type of *Pimpinella alpina* Host (Fig. 1).

Figure 1 Purwaceng plant (private collection)

*in vitro*In this study, there were 12 variations of growth regulators in Murashige-Skoog (MS) media with various concentrations, either single auxins (NAA and 2,4-D) or in combination with cytokinins (BAP). After explants were induced on solid media and observed and developed for approximately 35 days after planting (DAT), various callus results were obtained from each variation in the concentration of growth regulators (Table 1) with parameters ranging from callus growth duration, callus texture and callus colour (Table 1; Figure 2; Figure 3).

In this study, Murashige and Skoog (MS) media were used, which added plant growth regulators (PGR), namely auxins (NAA and 2,4-D) and cytokinins (BAP) with varying concentrations. Auxin at low concentrations can stimulate shoot development, while high concentrations can stimulate callus growth (Maggon and Singh, 1995). Thus, the regulation of growth regulators in the media will determine the success of the growth and development of tissue culture. In-plant tissue culture, it is necessary to select the right combination of auxins, cytokinins and supplements, because these combinations determine the success of callus or shoot formation (Sukweenadhi et al., 2019) .

Auxin induces callus formation, suspension culture, and roots, promoting cell elongation and division in the cambium tissue (Pierik, 1997). To stimulate the formation of embryogenic callus and somatic embryo structure, auxin is often required in relatively high concentrations. While cytokinins are organic compounds that cause cell division (cytokinesis), cytokinins affect several physiological processes in plants, especially encouraging cell division. 6-Benzyl Amino Purine (BAP) is an adenine derivative substituted at position 6, which has the most active chemical activity in stimulating shoot formation. (Zaerr and Mapes, 1982).

Figure 2 The growth of explants on MS media with various concentration of plant growth regulators NAA and its combination with BAP. A1 = MS + 0.5 mg L^{-1} NAA, B1 = MS + 1 mg L^{-1} NAA, C1 = MS + 2 mg L^{-1} NAA, D1 = MS + 0.5 mg L^{-1} NAA + 1 mg L^{-1} BAP, E1 = MS +1 mg L^{-1} NAA + 2 mg L^{-1} BAP, F1 = MS + 2 mg L^{-1} NAA + 3 mg L^{-1} BAP

Figure 3 Growth of explants on MS media with various variations of growth regulators 2,4-D and BAP. A2 = MS + 0.4 mg L^{-1} 2,4-D, B2 = MS + 0.8 mg L^{-1} 2,4-D, C2 = MS + 1.2 mg L^{-1} 2,4-D, D2 = MS + 1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP, E2 = MS + 1 mg L^{-1} 2,4-D + 1 mg L^{-1} BAP, F2 = MS + 2 mg L^{-1} 2,4-D + 1 mg L^{-1} BAP.

Table 2 The colour and texture of purwoceng callus at various concentrations of plant growth regulator NAA, 2,4D and its combination with BAP

Treatment	Day of callus formation	Callus colour	Callus texture
A1	21	Brownish-yellow	Friable
B1	21	White	Friable
C1	19	White	Compact
D1	29	Brownish-yellow	Friable
E1	29	Brownish-yellow	Friable
F1	35	Brownish-yellow	Friable
A2	21	Yellow	Friable
B2	19	Yellow	Friable
C2	17	Yellow	Friable
D2	32	Brownish-yellow	Friable
E2	32	Brownish-yellow	Friable
F2	35	Brownish-yellow	Friable

The results showed that the C2 treatment was the fastest callus induction time in leaf explants on day 17 and the late induction time in F2 treatment occurred on day 35, with an average callus growing on day 26 (Figure 3). The appearance of callus on explants is one indicator of growth in plant tissue culture. According to Gunawan (1995) Callus is a collection of amorphous substances from tissue cells that divide continuously.

The appearance of callus is characterized by swelling of the explants and the appearance of white patches. According to Suryowinoto (1996), the cells in contact with the media are encouraged to become meristematic and then actively divide, such as wound covering tissue.

The ability of a plant organ to produce callus is largely determined by the media used and the composition of growth regulators in the media. The concentration of BAP influenced the difference in callus growth rate in explants added to the media. According to research Sari (2013) in stem explants, the higher the concentration of BAP, the faster the callus growth. Auxins induce cell division, cell elongation, apical dominance, adventitious root formation, and somatic embryogenesis. Cytokinins can modify apical dominance by promoting axillary bud formation (Bhatia, 2015). However, on the contrary, leaf explants showed a different response if the higher BAP was added. The callus would grow slower. This is incorporated previous research (Intias, 2011), using stem explants with a combination of growth regulators 2,4-D 0.75 mg L^{-1} and BAP 1.5 mg L^{-1} could induce callus at 19th days.

Based on previous research (Sari, 2013), the callus emergence time in leaf explants was slower because inducing callus emergence did not require high BAP. The callus emergence time was fast to induce callus on *Anredera cordifolia* leaf explants with 2,4-D without BAP. This corresponds to Sumiati and Lestiana (2014) reported that in binahong leaf explants with the addition of 2,4-D 2 mg L^{-1}, the callus emergence time occurred on day 17. In addition to growth regulators, the speed of callus formation was also influenced by the size, age, physiology, source and genotype of explants (Katuuk, 1989).

The NAA treatment without BAP showed an average white callus based on the study results. Naphthaleneacetic acid (NAA) is an

auxin hormone that induces cell elongation (Lestari, 2019). However, the callus experienced proliferation again after that, causing the callus to change colour to brownish-yellow. At the same time, the single 2,4-D treatment showed yellow results and did not change in contrast to NAA and 2,4-D combined with BAP callus. The difference in callus colour showed the level of callus development. According to Fatmawati (2008), Callus colour indicates the presence of chlorophyll in the tissue. The greener the callus colour, the more chlorophyll content. A light or white colour can indicate that the callus condition is still quite good.

The callus colour difference indicates the level of callus development, and good quality callus is white where growth or division is faster. Callus that is increasingly brown in color, indicates that the callus cells are getting old. A light or white colour can indicate that the callus is still good (Fatmawati, 2008). Thus, the colour of the callus can also be known as to whether a callus still has cells that are actively dividing or still alive or dead.

A callus will show a yellow colour and turn brown as the callus grows. Brown colour generally indicates a callus state whose cells have died (Dwiyono, 2009). This change in callus colour was caused because the callus was getting older, and the nutrients were getting less and less so that there was competition for nutrients. Usually, fast growth and callus colour that tends to be bright indicated that the health condition of the culture is quite good. Meanwhile, brown to black colours generally indicate a callus condition whose cells have died(Ariningsih and Endang, 2003). The longer the callus was induced on the media, the darker brown colour even tended to be blackish. According to Kresnawati (2006), the formation of callus colour is influenced by growth regulators. The various callus colours are caused by light pigmentation and the origin of the explants. Pigmentation can be evenly distributed throughout the callus surface or only partially. There are differences in colour in one callus, namely white, green, brown, brownish-white, and greenish-white. The greenish-white colour allows for the brightest colours with the least chlorophyll content. The green colour of callus is due to the effect of cytokinins in the formation of chlorophyll (Widyawati, 2010). In tissue culture activity, auxin is very well known as a hormone that inhibits the action of cytokinins to form chlorophyll in callus, while cytokinin hormones function to encourage the formation of chlorophyll in callus. (Santoso and Nursandi, 2004).

Besides affecting callus proliferation, callus colour also affects the production of secondary metabolites. The darker the callus colour, the higher the secondary metabolites produced. Browning of the tissues is associated with excessive accumulation of phenol. According to Wickremisihe and Arteea 1993), the change in callus colour to brown is most likely because the growth and development of the callus have entered a stationary phase. In general, secondary metabolites begin to be formed at the beginning of the stationary phase, so the darker the callus colour, the higher the secondary metabolite level produced. According to previous research Turhan (2004) Callus texture can be divided into three types, namely compact (non-friable), intermediate and friable. A callus with a friable structure (friable) is formed

from a collection of easily detached cells, while a compact callus consists of a group of healthy cells. The experiments carried out showed that, in general, it gave a friable callus texture, except in the C1 treatment, it had a compact callus texture. According to Purwianingsih (2007), compact callus structure and the occurrence of yellowish or greenish discoloration indicate cell differentiation. This compact callus occurs through a growth process that leads to dense and dense cells.

The type of explant used in plant tissue culture is strongly influenced by callus texture. The explants used in this study were leaves. The formation of callus a with a friable structure is influenced by endogenous auxin hormone, produced internally by explants that have arisen to form the callus. Widyawati (2010). Auxin in the media will stimulate cell division and enlargement in explants, thereby inducing callus formation and growth (Wetter and Constable, 1991). The formation of friables callus is also influenced by cytokinin (BAP) in media containing auxin. According to Syahid (2010), the presence of cytokinin can increase cell division in the cytokinesis process, especially when RNA and protein synthesis will induce auxin activity in cell division to form callus. Thus, the formation of friable callus results from increased cell division activity (Pierik, 1997).

The colour of the white and yellow pigments in the callus indicated that the callus growth was good (Table 2). The green colour is callus containing chlorophyll, the interaction of 2,4-D and BAP (cytokinins), which play a role in the formation of chlorophyll in callus. According to Wardani (2004), the callus colour changes to greenish-white, and the callus cells have started to form chlorophyll as a lighting reaction so that the chloroplasts carry out photosynthesis.

The callus is the starting material for the manufacture of cell suspension cultures. Produce secondary metabolites. The cell suspension culture method has been the method of choice until now. For callus to be used as a suspension starting material, callus cells have the characteristics of callus friable texture and are easy to decompose, callus colour is white to yellow, it is not easy to oxidize phenolic substances, and the cells are easy to multiply. To get an embryogenic callus, we can do subcultures repeatedly to get a callus that has a more friablel structure.

The addition of auxin (NAA and 2,4-D) in the media effectively increased the growth rate of purwaceng callus. The results showed that adding a single 2,4-D auxin gave optimum conditions with C2 treatment producing the fastest induction time on day 17. Callus was a yellow and friable texture. For the addition of the concentration of growth regulators, the best combination was in treatment D1 with callus growing time of 29 days, brownish yellow colour and friable callus texture. However the callus formation time was still faster on C1 and C2 media.

The explants used in this study were purwaceng leaf shoots. Furthermore, the purwaceng leaf explants were sterilized. Of the several optimation sterilization methods tried, the best results were obtained with 2% fungicide, 70% alcohol and Bayclin® 10% for leaf explants, with a soaking time of 1 minute each. Sterilization is the initial stage of *in vitro* culture development. If the surface of the explant is not sterilized properly, it will

cause contamination.

According to Setiani et al (2018). The duration of immersion affected the decrease in contamination and the appearance of the colour of the explants. The shorter the immersion time with sodium hypochlorite, the more susceptible the explants were to pathogens. However, if the longer immersion with sodium hypochlorite, the development of the explant tissue will be hampered, which is marked by browning (brown colour) (Rismayanti, 2010).

CONCLUSION

Explant of purwaceng leaf inoculated on MS media with growth regulator 1.2 mg L^{-1} 2,4-D can grow callus faster (17 days) with a friable texture. The media and callus formed can be used as the basis for to further develop the cell suspension culture stage for producing active substances in purwaceng by *in vitro* culture.

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