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The Application of BAP and 2,4-D on the Growth of *Euchresta horsfieldii* (Lesch.) Benn. through *in vitro* Culture

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ABSTRACT

Euchresta horsfieldii (Lesch.) is one of the medicinal plants having many benefits and in high demand. This study was aimed at obtaining the proper concentrations of BAP and 2,4-D for increasing the production of psychic *in vitro*. The study used a completely randomized design (CRD) which was arranged in a factorial design consisting of two treatment factors with 25 treatment combinations repeated three times. The addition of 2 ppm BAP and 2,4-D 0 ppm showed the best response on shoot height, number of shoots and number of leaves. Most of the emerging calluses were green with a compact callus texture. Callus appeared in all treatments, while shoots appeared mostly on media with a concentration of 2,4-D 0 ppm. Further research can be done by using embryos as explants, using different growth regulators.

Key words: Auxins, cytokinins, plant tissue, secondary metabolites

INTRODUCTION

Euchresta horsfieldii (Lesch.) Benn. known as pronojiwo in Indonesia is a medicinal plant that grows in Java and Bali. *E. horsfieldii* has been widely used as traditional medicine by the Balinese ethnic community for rheumatism, lack of stamina, itchy skin, vomiting, low blood pressure, gout, typhoid and headaches (Hasan *et al.*, 2022). Moreover, *E. horsfieldii* acts as an antidote, expectorant and tonic that can neutralize snake venom. The roots stem, leaves and seeds of *E. horsfieldii* contain phytochemical compounds such as alkaloids which are dominant compounds, and other compounds like flavonoids, phenolate, tannin, saponin, steroids and terpenoid (Prihantini *et al.*, 2018). The raw material for traditional medicine from *E. horsfieldii*is is directly taken from their natural habitat, and is concerned with the impact of interfering with its sustainability. The efforts to comply with the increasing demand for *E. horsfieldii* are expected to cultivate *E. horsfieldii* outside their sustainable habitat (Hakim and Yuliah, 2018).

Tissue culture is one method for producing large quantities of *E. horsfieldii* plants within a short period. One of the most important steps in tissue culture is choosing a suitable growing medium for *in vitro* cultivation. MS media is a formula used for almost all plant species in plant tissue isolation techniques. MS medium contains high amounts of mineral salts and N compounds in the form of NO_3- and NH_4+. This tissue culture technique is also able to produce seeds that are free of pathogens, identical to the parent and are not affected by season.

The use of growth regulators in tissue culture media can support the growth and development of explants. Growth regulators are organic compounds that play a role in supporting plant growth. Auxin and cytokinins include Plant Growth Regulator (PGR) that can be supporting the process of root and shoot formation (Samanhudi *et al.*, 2022). 2,4-Dichlorophenoxyacetate is one of the growth promotors belonging to the auxin group. According to Zaman *et al.* (2020), 2,4-Dichlorophenoxyacetate *in vitro* culture plays a role in triggering differentiation that causes callus growth. Another growth regulator that influences callus growth is Benzyl Amino Purine (BAP). BAP is a growth regulator belonging to the cytokinin group. According to Rosa *et al.* (2018), BAP *in vitro* culture plays a role in the formation of leaves and stems

through its activity in the photosynthesis system. This research was conducted to obtain the right concentration of 2,4-D and BAP to support optimal growth of *E. horsfieldii* so that the provision of seeds can be more easily produced through tissue culture.

MATERIALS AND METHODS

The research was carried out from May to December 2021 at the Laboratory of Plant Physiology and Biotechnology, Faculty of Agriculture, Universitas Sebelas Maret. The study began with the sterile germination of *E. horsfieldii* seeds, followed by medium preparation, initiation and observation. Explants were prepared by germinating *E. horsfieldii* seeds in a sterile environment. The seeds were planted in a cotton medium and germinated in an aseptic environment. The second shoot of the germinated seeds was used as an explant in this study. Explants of *E. horsfieldii* shoots were ready to be used after one month.

Sterilization of explants was carried out by washing the explants using detergent and then soaking them in fungicides and bactericides. Sterilization was also carried out on the equipment to be used, such as culture bottles, dissection equipment (big tweezers, small tweezers and scalpel knives), and petridish. Sterilization of the tool was done by using an autoclave for 30 min at 121°C and 1 atm pressure then stored in the oven before use. Explant initiation was done in a Laminar Air Flow (LAF). The sterilized parts of the explant were cut into small sizes and then planted in a culture bottle containing the medium using sterilized tweezers. In one bottle, only one explant can be planted. The culture medium was produced by adding varying concentrations of BAP and 2,4-D to Murashige and Skoog (MS) based medium.

This study used a completely randomized design (CRD) with a factorial design consisting of two treatment components and 25 treatment combinations. The experiment was carried out three times which created 75 experimental units. The first factor was growth regulator 2,4-D (0, 0.5, 1.0, 1.5 and 2.0 ppm) and the second factor was growth regulator BAP (0, 0.5, 1.0, 1.5 and 2.00 ppm). The variables measured in the study were; the emergence time of shoots, percentage of appearing shoots, shoot height, number of shoots, time of callus emergence, percentage of callus emergence, callus colour, callus texture and number of leaves. The collected data were examined using analysis of variance at 5%, and if there was a significant difference, the Duncan Multiple Range Test (DMRT) at 5% was used.

RESULTS AND DISCUSSION

The speed at which shoots appeared varied for each type of plant. This can be influenced by various factors, such as growth regulators. According to Damayanti *et al.* (2021), the formation of shoots, as indicated by the appearance of green protrusion on the explants, is one of the successful elements in the multiplication of the tissue culture. Observations of the emergence of shoots were carried out every day after planting.

The fastest shoot emergence time was taken by the treatment by 1 ppm BAP and 0 ppm 2,4-D (6 DAP), while the longest shoot emergence time was obtained by 1 ppm BAP and 1 ppm 2,4-D of treatment (Fig. 1). Darmawati *et al.* (2019) stated that the time of shoot emergence was not significantly different from the response of exogenous hormones because the endogenous auxin and cytokinin hormones possessed by plants were in a balanced condition.

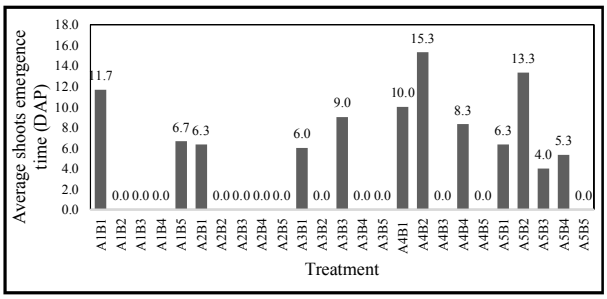

Fig. 1. Histogram of the shoot emergence of *E. horsfieldii* at the age of 4 WAP.

The percentage of shoots that emerged was calculated to determine the average shoots that could be emerged during one month of observation. The addition of BAP and 2,4-D affected the process of shoot formation after four weeks after the planting (WAP). The results showed that the percentage of callus in the BAP treatments 0, 0.5, 1.0, 1.5 and 2.0 ppm and 2,4-D 0 ppm were able to produce shoots up to 100% (Fig. 2). This indicated that the addition of BAP without 2,4-D produced the

optimal shoots in *E. horsfieldii* explants. It could be suspected that 2,4-D was an auxin that played a important role in callus formation. Based on the histogram, it can be concluded that the addition of BAP without the addition of 2,4-D was able to form shoots optimally (Fig. 2). However, the addition of 2,4-D to the media tended to inhibit shoot formation.

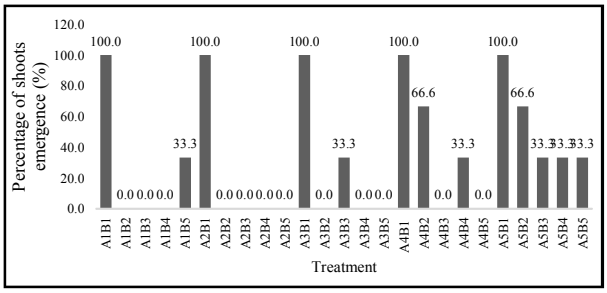

Fig. 2. Histogram of the emergence time percentage of *E. horsfieldii* shoots at the age of 4 WAP.

Shoot height was measured at the end of the observation by measuring from the base of shoot growth to the tip of the longest leaf. According to Rezaldi *et al.* (2022), shoot height can be an indicator of the physiological response of shoot growth to the composition of the given medium. Table 1 reveals that the addition of a single treatment of BAP and 2,4-D had a significant effect on shoot heights 1.63 and 2.07 cm, respectively.

Table 1. Effect of single PGR treatment of BAP and 2,4-D on shoot height, number of shoots and number of *E. horsfieldii* leaves at 4 WAP

Concentration (ppm)		Shoot height (cm)	No. of shoots	No. of leaves
BAP	0	0.43a	0.27a	0.80a
	0.5	0.43a	0.27a	0.53a
	1.0	0.63a	0.40ab	0.93a
	1.5	0.63a	0.60b	2.67b
	2.0	1.63b	1.00c	3.47c
2,4-D	0	2.07b	1.40b	4.53c
	0.5	0.53a	0.40a	1.60b
	1.0	0.53a	0.33a	0.80ab
	1.5	0.40a	0.20a	0.67ab
	2.0	0.23a	0.20a	0.40a

Numbers followed by the same letter in the same column are not significantly different at 5% level DMRT.

Shoot height at a single treatment of 2 ppm of BAP concentration was significantly different from other BAP concentrations (0, 0.5, 1.0 and 1.5 ppm), while shoot height at a single treatment of 2,4-D 0 ppm was significantly different from other concentrations (0.5, 1.0, 1.5 and 2.0 ppm). An increasing concentration of BAP was able to increase shoot height, but the increasing concentration of 2,4-D decreased shoot height. This is in accordance with Ling *et al.* (2018) that the higher concentration of BAP gave the higher proliferation process that occured in plant cells. Based on the results of data analysis, the best treatment that increased shoot height optimally was BAP 2 ppm and 2,4-D 0 ppm (without 2,4-D). According to Akbar *et al.* (2017), the shoot height of each explant was different due to differences in nutrient absorption for regeneration in each explants, such as shoot growth and development.

The formation of shoots was strongly influenced by the presence of cytokinins (Table 1). BAP supported shoot formation by stimulating cell division and expansion. According to Aworunse *et al.* (2019), the rate of adventitious and shoot growth under the influence of BAP was the basic factor that determined the efficiency of the micro propagation method. Observations were made by counting the number of shoots that emerged at the age of 4 WAP. The criterion for shoots was approximately 0.5 cm in size.

The results showed that the addition of single PGRs treatment of BAP and 2,4-D gave a significant effect on the number of shoots by 1.00 and 1.40, respectively. However, the interaction between BAP and 2,4-D did not have a significant effect. The number of shoots at a concentration of BAP 2 ppm was significantly different from other concentrations (BAP 0, 0.5, 1.0 and 1.5 ppm). Moreover, the number of shoots at BAP 1.5 ppm was significantly different from other concentrations (BAP 0 and 0.5 ppm).

Furthermore, the addition of 2,4-D 0 ppm was significantly different from 2,4-D 0.5, 1.0, 1.5 and 2.0 ppm. An increase of 2,4-D decreased the number of shoots of *E. horsfieldii*. Therefore, it was concluded that the best treatment that increased the number of shoots was 2 ppm BAP without the addition of 2,4-D. The interaction of BAP with a high concentration of 2,4-D also inhibited the emergence of shoots. Rosniawaty *et al.* (2018) explained that in absorption of exogenous cytokinins responded to the addition of shoots because plants had sufficient endogenous cytokinin content.

Observations were recorded every day after planting to determine the time required to form callus in each treatment. Explants of *E. horsfieldii* shoots grown on Murashige and

Skoog (MS) media with 25 combination treatments of concentrations of growth regulators BAP and 2,4-D gave varied responses to the duration of callus formation. The results of the analysis showed that the addition of BAP and 2,4-D had no significant effect on the emerging time of *E. horsfieldii* callus.

The time for callus to appear on *E. horsfieldii* ranged from 5-11 days after planting (Fig. 3). The longest callus emerging time was on treatment by BAP 2.0 ppm and 2,4-D 0.5 ppm. Almost all treatments could initiate callus except the control treatment which did not receive any PGR application. It indicated that the non-fulfilment of the supporting substances needed for callus formation. Moreover, the callus formation time was also influenced by the amount of auxin present in the explants and culture media. This was supported by the statement of Marissa *et al.* (2016) that as an auxin, 2,4-D increased osmotic pressure and cell permeability, decreased cell wall pressure and increased protein synthesis, plasticity and cell wall growth. Callus growth was also influenced by the concentration of cytokinins.

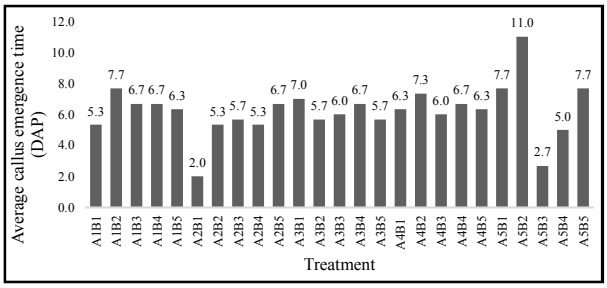

Fig. 3. Histogram of the emergence time of callus at the age of 4 WAP.

The percentage of callus emergence demonstrated the effect of adding BAP and 2,4-D on callus occurrence (Fig. 4). Almost all treatments were able to optimally grow the callus until the end of the observation. Explants meristematic tissue is known to contain endogenous hormones that help in callus formation. When endogenous hormones are combined with BAP and 2,4-D, cells differentiate to proliferate. This is in line with Han *et al.* (2018) who found that young explants were faster and more likely to produce callus than old explants.

The formation of 100% callus was found in almost all treatments. A percentage of 66.6% was found in the BAP treatment of 2.0 ppm and 2,4-D 1.5 ppm. The treatment of 0.5 ppm BAP without 2,4-D and 2.0 ppm BAP combined with

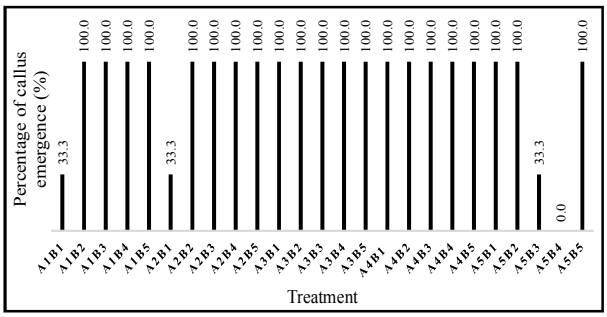

Fig. 4. Histogram of the percentage of callus emergence at the age of 4 WAP.

0.5 ppm 2,4-D resulted in the lowest percentage of 33.3% of callus formation. In addition, there was only a control treatment that did not produce any callus. It was due to lack of supporting hormones that induce callus development.

Callus colour was a parameter that can be an indicator of the compounds contained in the callus. According to Rasud and Bustaman (2020), callus colour changes are indicator of the growth of cells in callus tissue. The callus colours that emerged in *E. horsfieldii* were green, white, yellowish green and brownish green.

The results showed that the most common callus colour found was green. The green colour of the callus is an indication of the presence of chloroplasts in the callus tissue. The brownish-green colour was also common. This was allegedly caused by observations made when the callus was 8 WAP so that browning had occurred in several calluses (Table 2). Cai *et al.* (2020) explained that a callus with brown colour was a green callus that had undergone a browning process, indicating the presence of toxic phenolic chemicals in the callus. White callus indicated the lack of pigment in the callus cells.

The various callus textures can reflect the quality of the callus. Callus texture can vary from friable to compact depending on the type of explants, media, growth regulators and biotic and abiotic nutrients used. Friable and compact calluses are two types of callus texture. The compact callus is the type of callus texture that dominates among the calluses that emerged. The results showed that all treatments emerged a compact callus texture (Table 3). When a callus is lignified, it consolidates and emerges a compact callus. The addition of cytokinins that work as nutrient transporters has an impact on this

Table 2. The effect of BAP and 2,4-D on the colour of *E. horsfieldii* callus at the age of 4 WAP

2,4-D (ppm)	BAP (ppm)				
	0	0.5	1.0	1.5	2.0
0	-	Brownish green	Whitish green	White	White
0.5	Green	Green	Green	Green	Yellowish green
1	Green	Green	Brownish green	Brownish green	Brownish green
1.5	Green	Brownish green	Brownish green	Brownish green	Brownish green
2	Green	Green	Green	Yellowish green	Brownish green

Table 3. The effect of giving BAP and 2,4-D on the texture of *E. horsfieldii* callus at the age of 4 WAP

2,4-D	BAP				
	0	0.5	1.0	1.5	2.0
0	Compact	Compact	Compact	Compact	Compact
0.5	Compact	Compact	Compact	Compact	Compact
1.0	Compact	Compact	Compact	Compact	Compact
1.5	Compact	Compact	Compact	Compact	Compact
2.0	Compact	Compact	Compact	Compact	Compact

event. Castro *et al.* (2016) found that in the medium with a higher concentration of BAP, compact calluses have emerged.

The result of single PGR treatment BAP and 2,4-D had a significant effect on the number of leaves 3.47 and 4.53, respectively (Table 1). The application of BAP 2.0 ppm was different compared to BAP 0, 0.5, 1.0 and 1.5 ppm. Yuniastuti *et al.* (2018) stated that the addition of PGRs such as auxins and cytokinins affected the growth of leaves. Besides that, the treatment of 2,4-D 0 ppm was significantly different from the addition of 2,4-D 0.5, 1.0, 1.5 and 2.0 ppm. This research showed that the increasing concentration of BAP increased the number of leaves but an increase of 2,4-D decreased the number of leaves in *E. horsfieldii*. Batti *et al.* (2020) stated that the addition of auxins did not affect the growth of leaves because the endogenous auxins inside of the plant were enough to trigger the formation of leaves. The formation of shoots and leaves in plants generally requires cytokinins, while auxin can inhibit if the concentration is too high.

CONCLUSION

The best plantlet response was obtained on media with concentrations of 2.0 ppm BAP and 2,4-D 0 ppm which produced optimal shoot height, number of shoots and number of leaves. Neither BAP nor 2,4-D had a significant effect on callus and shoot emergence time. Callus appeared in all treatments with most of the percentages being 100%, while shoots could appear mostly on media with a concentration of 2,4-D 0 ppm. Further research can be done by using embryos as explants, using different growth regulators and acclimatizing *E. horsfieldii* plantlets.

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