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Effects of 2,4-dichlorophenoxy acetic acid and 6-benzylaminopurine on callus induction and secondary metabolites of *Elephantopus scaber* L.

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ABSTRACT

This study was aimed to investigate the effect of growth regulators combination 2,4-dichlorophenoxy acetic acid (2,4-D) and 6-benzylaminopurine (BAP) on induction time, percentage, fresh weight, dry weight, morphology, and secondary metabolites profile of callus from *Elephantopus scaber* L. leaves. This study was an experimental laboratory study with a completely randomaized design (9 treatments combined concentration with each 3 replications, there was $D_{0.0}B_{0.0}$(control); $D_{0.5}B_{1.5}$; $D_{0.5}B_{2.0}$; $D_{0.5}B_{2.5}$; $D_{1.0}B_{0.5}$; $D_{1.0}B_{1.5}$; $D_{1.0}B_{2.0}$; $D_{1.0}B_{2.5}$ and $D_{1.5}B_{1.5}$). *E. scaber* L. leaf explants were cultured on solid Murashige and Skoog (MS) medium supplemented with growth regulators 2,4-D and BAP. Qualitative data were obtained from leaf callus morphology and analysis of secondary metabolites profile from callus and leaf extract of *E. scaber* L. Quantitative data were obtained from a percentage of callus formed by explant, observation time of callus induction, callus fresh weight, and callus dry weight, then it were statically analyzed. The result of this study showed 2,4-D and BAP had effect explant growth on leaf of *E. scaber* L. Combination of concentration 0.5 mg/L 2,4-D and 2.0 mg/L BAP ($D_{0.5}B_{2.0}$) showed the fastest induction at that 5.67 days. $D_{0.5}B_{1.5}$ treatment produced the highest of fresh weight at 0.3176 grams, meanwhile $D_{1.0}B_{1.5}$ treatment produced the highest of dry weight at 0.0440 g. Callus morphology in $D_{0.5}B_{2.5}$ and $D_{1.0}B_{2.5}$ treatments had friable texture, while the other treatments had compact texture, and commonly callus of *E. scaber* L. has light green colors. The compound of secondary metabolites in the callus and leaf extract of *E. scaber* L. was very diverse including flavonoid, alkaloid, terpenoid, and saponin.

Key words: *2,4-D, BAP, Elephantopus scaber L., Secondary metabolite, Phytochemical screening.*

Introduction

Tropical diseases are commonly found in tropical countries, one of which is Indonesia (Wibawa and Satoto, 2016). Tropical disease caused by several bacterial, fungal and virus (Samosir *et al.*, 2018; Achmad *et al.*, 2019). Some diseases caused by microorganisms are still a national health problem (Pujara *et al.*, 2016). Until now, the prevention of tropical diseases have been dealt using antibiotics, but it has various impacts, for examples are immune disorders and microbial resistance (Alavijeh *et al.*, 2012; Cai *et al.*, 2017; Tillasman *et al.*, 2018; Rieuwpassa *et al.*, 2019; Asditya *et al.*, 2019). Exploration of plants as natural medicine is needed as an effort to overcome these problems (Ramadani *et al.*, 2018).

The compound of secondary metabolites found in Asteraceae are terpenoid, steroid, saponin, alkaloid, flavonoid, and tannin (Burlec *et al.*, 2017;

Haque *et al.,* 2012; Sülsen *et al.,* 2017). Which has potential as antimicrobial and antioxidant properties (Sapci *et al.*, 2017; Vadhana *et al.,* 2015; Souza *et al.,* 2015; Sharif *et al.,* 2016; Ugur *et al.*, 2009). *E. scaber* L. (Asteraceae) is an endemic plant species from Indonesia that has high potential for development. There is not much information that reveals bioactive compound and secondary metabolites that are responsible as antimicrobial and antioxidants in *E. scaber* L. leaves, as well as the lack of information regarding the production of bioactive compounds and secondary metabolites by the callus culture method.

Callus culture is an effective method in biotechnology that can suppress plant exploitation from native habitats and also includes appropriate methods for large-scale biomass production as well as for the accumulation of secondary metabolite components (Sharma *et al.*, 2011; Khalil *et al.*, 2015; Junairiah *et al.,* 2019). Biomass and accumulation of secondary metabolites in callus culture can be increased using growth hormone regulator auxin and cytokinin (Alfarisi *et al.,* 2019). The purpose on this study was to investigate the effect of growth regulators combination 2,4-D and BAP on induction time, percentage, fresh weight, dry weight, morphology, and secondary metabolites profile of callus from *Elephantopus scaber* L. leaves.

Materials and Methods

Plant and chemical materials

Elephantopus scaber L. were collected from Bratang flower market, Surabaya. Plant identification was carried out at the plant physiolgy laboratory, Faculty of Science and Technology, Universitas Airlangga, Surabaya. Explants are used from derived of *E. scaber* L. leaves which are still meristematic.

The chemicals needed were chemical compounds of Murashige and Skoog (MS) medium (Uddin *et al.*, 2006). Growth hormone plant regulator 2,4-D and BAP, HCl 1 N, and KOH 1 N. Then there were some other chemicals: spirtus, sterile aquades, chlorox, liquid detergent, alcohol 70%. Lastly, the materials used for screening secondary metabolites were methanol, Mayer reagents (KI and HgCl), concentrated of HCl, Mg, chloroform, Liberman Burchard reagents (anhydrous acetic acid and concentrated sulfuric acid).

Preparation of MS culture medium

The process of making MS culture medium (1 Liter), begin with process of weighing macronutrient materials and then, it has dissolved into 500 mL of aquadest. Then the solution was supplemented with 5 mL iron stock solution, 1 mL of micronutreint stock solution, 4 mL of vitamin stock solution, 100 mg of myo-inositol and 30 grams of sucrose. The growth regulators 2,4-D and BAP were added to the medium according to predetermined concentrations. Next step, the solution of medium was measured the range of pH (5.6-5.8) and after that was added of aquadest until the volume becomes 1000 mL and 8 grams of agar (Uddin *et al.*, 2006). Last, the solution was heated and immediately divided into the culture bottles (± 10 mL/culture bottle) then labeled.

E. scaber L. explants planting

E. scaber L. leaves were washed with liquid deter-

Table 1. The average duration of induction time and percentage of callus formed by explants of *E. scaber* L. leaves on MS medium with various combination of concentrations 2,4-D and BAP.

Treatments	The average time of callus induction (days)	Percentage of explants forming callus
2,4-D 0.0 mg/L + BAP 0.0 mg/L	0.00 ± 0.000^{a}	0%
2,4-D 0.5 mg/L + BAP 1.5 mg/L	6.33 ± 0.577^{bcd}	100%
2,4-D 0.5 mg/L + BAP 2.0 mg/L	**5.67 ± 0.577^{bc}**	100%
2,4-D 0.5 mg/L + BAP 2.5 mg/L	7.33 ± 1.155^{cde}	100%
2,4-D 1.0 mg/L + BAP 0.5 mg/L	6.67 ± 0.577^{bcd}	100%
2,4-D 1.0 mg/L + BAP 1.5 mg/L	7.67 ± 0.577^{de}	100%
2,4-D 1.0 mg/L + BAP 2.0 mg/L	6.33 ± 0.577^{bcd}	100%
2,4-D 1.0 mg/L + BAP 2.5 mg/L	7.33 ± 0.577^{de}	100%
2,4-D 1.5 mg/L + BAP 1.5 mg/L	8.00 ± 0.000^{e}	100%

*) Different letters indicate significant difference based on result of *Mann-Whitney* test (á=0.05).

gent for five minutes and rinsed three times using tap water, before being submerged in 70% alcohol for 6 minutes. Explants were rewashed three times using sterile distilled water, then soaked in 20% chlorox for 7 minutes. Leaves were then cut into ±1 cm^2 pieces and planted on MS medium, then cultured for six weeks in temperature of 25±2°C, lighting of 3000-3500 lux for 24 hours.

Extraction of callus and leaves of *E. scaber* L.

The leaves of *E. scaber* L. was washed with tap water, then dried for ± 7 days. After being brownish dry, the leaves are crushed using mortar to form fine powder. Then the powder was weighed as much as 0.1 g then extracted with 10 mL of methanol which was allowed to stand for 24 hours. Dried callus also crushed into powder using mortar, then weighed as much as 0.1 g then extracted with 10 mL of methanol which was allowed to stand for 24 hours. After that, the extract solution was filtered using filter paper into the vials. Then, the filter results extract were carried out an analysis of secondary metabolites by phytochemical screening methods (Bandiola, 2018).

Results

Induction time and percentage of callus formed by explant of *Elephantopus scaber* L. leaves on MS medium with the combination of concentration 2,4-D and BAP.

The average callus induction time (Table 1) of *E. scaber* L. leaves explant grown on MS medium in 9 treatments combined of concentration of 2,4-D growth regulators and BAP has varied responses. The combination of concentration 2,4-D 0.5 mg/L + BAP 2.0 mg/L showed the fastest callus induction time at 5.67 days.

Fresh weight and dry weight of *Elephantopus scaber* L. callus with combination concentration of 2,4-D and BAP.

The results of the average fresh weight and dry weight of callus in various combinations of 2,4-D and BAP can be seen in Table 2. The combination of concentration 2,4-D and BAP growth regulators for callus fresh weight and dry weight showed the different values. The combination of concentration 2,4-D 0.5 mg/L + BAP 1.5 mg/L showed the highest fresh weight (0.3176 g), while the highest dry weight of *E. scaber* L. callus was indicated by the combination of concentration 2,4-D 1.0 mg/L + BAP 1.5 mg/L (0.0440 g).

Callus morphology of *Elephantopus scaber* L. leaves with the combination of concentration 2,4-D and BAP.

Callus morphology of *E. scaber* L. leaves in the sixth week after culture can be seen in Table 3. Based on Table 3 the callus of *E. scaber* L. leaves has a compact and friable textures, also have a light green and brownish green.

Extraction of callus and leaves of *E. scaber* L. followed by analysis of secondary metabolite profiles using phytochemical screening method.

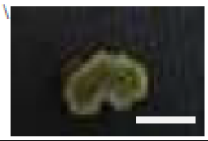
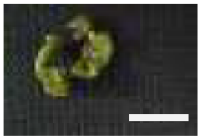
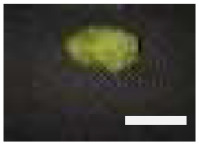
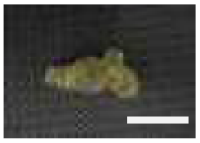
The result of phytochemical screening from methanol extract of *E. scaber* L. callus and leaves showed in Table 4. In Table 4. shows that all callus from treatments and leaves of *E. scaber* L. in this study has positive test result on flavonoid, alkaloid, terpenoid, and saponin compounds, and negative on steroid compound.

Table 2. The average fresh weight and dry weight of *E. scaber* L. callus for six weeks of culture.

Treatments	The average of fresh weight (grams)	The average of dry weight (grams)
2,4-D 0.0 mg/L + BAP 0.0 mg/L	0.0000 ± 0.0000^{a}	0.0000 ± 0.0000^{a}
2,4-D 0.5 mg/L + BAP 1.5 mg/L	**0.3176 ± 0.0022^{f}**	0.0425 ± 0.0032^{f}
2,4-D 0.5 mg/L + BAP 2.0 mg/L	0.1924 ± 0.0432^{cde}	0.0269 ± 0.0063^{cd}
2,4-D 0.5 mg/L + BAP 2.5 mg/L	0.2996 ± 0.0804^{cdef}	0.0302 ± 0.0045^{de}
2,4-D 1.0 mg/L + BAP 0.5 mg/L	0.1888 ± 0.0074^{d}	0.0351 ± 0.0061^{e}
2,4-D 1.0 mg/L + BAP 1.5 mg/L	0.2339 ± 0.0197^{e}	**0.0440 ± 0.0038^{f}**
2,4-D 1.0 mg/L + BAP 2.0 mg/L	0.2197 ± 0.0215^{de}	0.0256 ± 0.0030^{cd}
2,4-D 1.0 mg/L + BAP 2.5 mg/L	0.0878 ± 0.0089^{b}	0.0144 ± 0.0011^{b}
2,4-D 1.5 mg/L + BAP 1.5 mg/L	0.1048 ± 0.0051^{c}	0.0233 ± 0.0017^{c}

*) Different letters indicate significant difference based on result of *Mann-Whitney* test (á=0.05).

Table 3. Morphology of callus leaves of *Elephantopus scaber* L. at the sixth week after culture.

No.	Treatments	Pictures	Morphological description of callus
1.	2,4-D 0.0 mg/L + BAP 0.0 mg/L		Brown explant, no callus (dead)
2.	2,4-D 0.5 mg/L + BAP 1.5 mg/L		Light green callus and intermediate texture
3.	2,4-D 0.5 mg/L + BAP 2.0 mg/L		Light green callus and compact texture
4.	2,4-D 0.5 mg/L + BAP 2.5 mg/L		Light green callus and friable texture
5.	2,4-D 1.0 mg/L + BAP 0.5 mg/L		Brownish green callus and compact texture
6.	2,4-D 1.0 mg/L + BAP 1.5 mg/L		Light green callus and compact texture
7.	2,4-D 1.0 mg/L + BAP 2.0 mg/L		Light green callus and compact texture
8.	2,4-D 1.0 mg/L + BAP 2.5 mg/L		Light green callus and friable texture
9.	2,4-D 1.5 mg/L + BAP 1.5 mg/L		Brownish green callus and compact texture

*) scale bar = 1 cm.

Table 4. Phytochemical screening results of callus in various treatment and leaves of *E. scaber* L.

Treatments	The results of compound tests by phytochemical screening				
	Flavonoid	Alkaloid	Steroid	Terpenoid	Saponin
E. scaber L. leaves	+	+	-	+	+
2,4-D 0.0 mg/L + BAP 0.0 mg/L	-	-	-	-	-
2,4-D 0.5 mg/L + BAP 1.5 mg/L	+	+	-	+	+
2,4-D 0.5 mg/L + BAP 2.0 mg/L	+	+	-	+	+
2,4-D 0.5 mg/L + BAP 2.5 mg/L	+	+	-	+	+
2,4-D 1.0 mg/L + BAP 0.5 mg/L	+	+	-	+	+
2,4-D 1.0 mg/L + BAP 1.5 mg/L	+	+	-	+	+
2,4-D 1.0 mg/L + BAP 2.0 mg/L	+	+	-	+	+
2,4-D 1.0 mg/L + BAP 2.5 mg/L	+	+	-	+	+
2,4-D 1.5 mg/L + BAP 1.5 mg/L	+	+	-	+	+

Notes: (+) : positive test result, (-) : negative test result.

Discussion

The concentration of 2,4-D 0.5 mg/L and BAP 2.0 mg/L showed the fastest callus induction time at 5.67 ± 0.577 days after planting. The percentage of explants forming a callus that is equal to 100% in most treatments except control because it can not induce callus formation. In many previous studies have also stated that the combination of 2,4-D and BAP is able to induce callus quickly and optimum, this is because there is a balance between the combination of auxin and cytokinin with phytohormone from the explant (Wani *et al.*, 2010; Junairiah *et al.*, 2017; Keshvari *et al.*, 2018). The optimum combination of 2,4-D and BAP concentrations for fresh weight and dry weight areconcentration 2,4-D 0.5 mg/L and BAP 1.5 mg/L; and concentration of 2,4-D 1.0 mg/L and BAP 1.5 mg/L, with the average weight at 0.3176 ± 0.0032 grams and 0.0440 ± 0.0038 grams, respectively. Plant growth regulator (PGR) play important role in the induction of callus and biomass production. It rate giving varying results at different concentrations. For successful callus induction, factors such as type of explants, PGR, culture medium and cultural conditions are very important (Mohammad *et al.*, 2014; Sharma *et al.*, 2017).

The combination of growth regulator concentration 2,4-D 0.5 mg/L and BAP 2.5 mg/L; 2,4-D 1.0 mg/L and BAP 2.5 mg/L which produce friable callus and light green color, but the other treatment showed compact texture and brownish green color. It is also happened in previous study, the combination of concentration plant growth regulator also can produced variety texture (compact, friable and intermediate) and color of callus, but the combination concentration between 2,4-D and BAP commonly produced green, cream, brownish green, creamish green, light brown, etc. If callus colored green that indicated the cells contained high amount of chlorophyll, while if callus showed white color that indicated starch content in the cells with chlorophyll had yet to be developed (Mohammad *et al.*, 2014; Junairiah *et al.*, 2020). The results of secondary metabolite test callus and leaves of *E. scaber* L. using the phytochemical screening method show the same results, which contain flavonoid, alkaloid, terpenoid and saponin. Plants produce a wide diversity of secondary metabolites which serve them as defense compounds against herbivores, and other plants and microbes, but also as signal compounds. This various phytochemical compound like saponins, terpenoids, steroids, anthocyanins, coumarins, fatty acids, tannins, leucoanthocyanins and emodins can used as antioxidant. These natural products are an important source of drug candidates in pharmaceutical industry (Savithramma *et al.*, 2011; Kabera *et al.*, 2014; Wink, 2015). From Table 4 can also be seen that the combination of concentration 2,4-D and BAP can affect the production of secondary metabolite compounds.

Conclusion

The combination concentration of growth regulators 2,4-D and BAP influenced the induction of callus and secondary metabolites from *E. scaber* L. leaves. The concentration of 0.5 mg/L 2,4-D and 2.0 mg/L BAP showed the fastest callus induction (5.67 ± 0.577 days). The percentage of explants forming callus was 100% in all treatments except control. The

optimum combination of 2,4-D and BAP concentrations for fresh weight and dry weight of callus were at the concentration of 0.5 mg/L 2,4-D and 1.5 mg/L BAP (0.3176 ± 0.0032 grams); and a concentration of 1.0 mg/L 2,4-D and 1.5 mg/L BAP (0.0440 ± 0.0038 grams), respectively. Light green callus with friable texture resulted from a combination of the concentration of 0.5 mg/L 2,4-D and 2.5 mg/L BAP; 1.0 mg/L 2,4-D and 2.5 mg/L BAP. The secondary metabolites identified in the callus of *E. scaber* L. leaves are flavonoids, alkaloids, terpenoids and saponins.

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