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The Reserpine Production and Callus Growth of Indian Snake Root (*Rauvolfia serpentina* (L.) Benth. Ex Kurz) Culture by Addition of Cu^{2+}

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ABSTRACT

The objectives of this research were to study the effects of Cu^{2+} addition on the reserpine production and callus growth from in vitro culture indian snake root (*Rauvolfia serpentina* (L.) Benth. Ex Kurz). This research frame work was based on the potency of snake root which was many exploited as anti-hypertension. The addition of elicitor Cu^{2+} in the form of $CuCl_2$ would influence the ion transport of cell and changed of cytoplasm pH, and also has effects on synthesis and activity of enzymes which role in reserpine production and callus growth. The research was conducted in two steps, using Completely Randomized Design. The first step was the callus initiation to promote callus growth. Second step was the treatment to induce reserpine production. The callus was divided into five groups: 0; 5; 10; 20; 40; and 80 µM. Morphology, wet weight, dry weight, growth rate, and reserpine content of callus were determined after 15 treatment day. Data were analyzed using ANOVA and continued by DMRT 5%. The result showed that reserpine production increased in addition of 5 µM and 10 µM Cu^{2+} in callus culture of *R. serpentina* and reduced in addition of Cu^{2+} more than 10 µM. The callus growth significantly decreased by increasing concentration of Cu^{2+}.

Key words: *Rauvolfia serpentina*, reserpine, elicitor, Cu2+, callus growth.

INTRODUCTION

Pule pandak or Indian snake root (*Rauvolfia serpentine* (L.) Benth. ex Kurz) has been used medically since 2000 BC. The plant extract and alkaloids are used mostly in treating high blood pressure, sedative, aphrodisiac, and mental disorders (Ramawat, 1999). A number of alkaloids as reserpine, reserpinin, serpentine, ajmalin, and isoajmalin could be produced from this plant. Reserpine was used to cure the high blood pressure or hypertension and its complications, stroke, and the diseases related with nervous system (Achmad, 1987).

In this time, pule pandak in Indonesia included in groups of plant were endangered (Mulliken and Crofton, 2008). Requirement of raw material of pule pandak for the jamu industry and pharmacy progressively mount, whereas most raw material (more than 80%) still have to be harvested from natural habitat (Supriyadi, 2001). To get pule pandak in high amounts of secondary metabolites, the plants have to reach the certain age (years), so that exploitation from nature can menace its species, and also difficult to be done (Ramawat, 1999). Therefore require an effort to be able to lessen the pressure to population of pule pandak in nature; at the same time fulfill the request of compound reserpine in gross. This matter can be gone through with the technique of culture in vitro.

Some technique of in vitro culture has been used to improve the accumulation of secondary metabolites, one of them called elicitation technique. Elicitation technique is process of elicitor addition at plant culture to induce or improve the product of secondary metabolites. Elicitors could be biotic or abiotic factors. Numerous investigations have reported that addition of abiotic elicitor including Cu^{2+} increased alkaloid production. Sato *et al.* (1997) reporting Cu^{2+} remarkable enhanced both the growth and the alkaloid yield in hairy root cultures of *Hyoscyamus albus.* Existence Cu^{2+} at plant can also influence the transport ion from and to cytoplasm and competitively displacement of Ca^{2+} from the membrane biding site (Polle and Schutzendubel, 2002). Addition of a component that able to pursue the internal and external transportation Ca^{2+} reported can induce the accumulation and production of the indol alkaloid at hairy root culture of *Catharanthus roseus* (Valenzuela *et al*., 2003). Cu^{2+} at 5 µM caused a 50% block in Ca^{2+} transportation (Demidchik *et al*., 1997). Ions Cu^{2+} that is tied at protein can improve transportation of electron in photosynthesis and respiration and also improve the activity of enzyme catalyze (Gardner *et al*., 1991).

MATERIAL AND METHODS

The research was conducted in two steps. The first was the callus initiation to promote callus growth. The second was the treatment of elicitor addition to induce the reserpine production on callus culture.

Plant tissue culture

Young leaves (second or third leaf from sprout) of *R. serpentine* were collected from Tekil mount, Wonogiri. Leaves were surface sterilized for 10 min in liquid

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detergent, soaked in 45% sodium hypochlorite (5.25%), 10 min in sterile aquadest, 5 min in 70% ethanol and then they were washed two times with sterile aquadest in laminar air flow hood.

The callus induction media was composed of MS (Murashige and Skoog) basal medium and supplemented with 30 g/L sucrose, NAA 2 mg/L, and kinetin 2 mg/L. After growing for 30 day, the callus were elicited with 0 µM, 5 µM, 10 µM, 20 µM, 40 µM, and 80 µM Cu^{2+} as $CuCl_2$ in basal medium without added NH_4NO_3 and NAA, the amount of KH_2PO_4 reduced to 100 mg/L, and supplemented with 80 g/L sucrose and kinetin 5 mg/L (Aryanti, 2005).

Cultures were harvested on day 15 of subculture in a treatment medium. The dry weight of callus was determined after drying the sample at 38^oC to constant weight. Dried callus were crushed to a fine powder with a mortar and pestle. Approximately 0.1 g of dried callus was placed in a tube react and extracted with 10 mL ethanol p.a. and diluted to the volume with double-distilled water in a 100 mL standard flask. To each this tube was added with 1 mL of freshly prepared 0.3% (w/v) solution of sodium nitrite, then mixed and heated in a water bath at 55^oC for 30 min. After cooling, 0.5 mL of freshly prepared solution of sulfamic acid 5% (w/v) was added and diluted up to the mark with ethanol. The absorbance of solution was measured at 399 nm against a reagent blank by using spectrophotometer of UV-VIS Shimadzu (Singh *et al.,* 2004 with modification).

Data were analyzed statistically by analysis of variance (ANOVA) followed by DMRT 5%. Data of callus morphology covering color and texture presented descriptively.

RESULT AND DISCUSSION

Culture growth

The explants were incubated on basal MS media with addition 2 mg/L NAA and 2 mg/L kinetin demonstrated callus formation after 7 days incubation. Explants formed callus at the cut surface and its color is white chromatic lay. Shared explants which initiation to form callus caused by a cell which contact with the medium incited to become meristematic and here in after perform the division tissues of wound cover. Its have been explained by Santoso and Nursandi (2002) whereas callus formed as attempt of plant protection as a result of the stress or expression of wound.

Callus color at initiation medium changed from white to become yellow greenness until young green color. Existence of light can enhance chlorophyll production. Texture callus at initiation medium is compact callus with solid cell and difficult to be dissociated. Callus texture at treatment medium have no difference with texture callus at initiation medium that is compact until the end of treatment periods (Tables 1). The high of kinetin concentration at treatment medium (5 mg/L) caused the compact callus. Kinetin in high concentration can enhanced cell division and formed the compact callus.

Rauvolfia serpentina callus which elicited by addition of Cu^{2+} did not change the callus color to brown (browning) compared by color of control callus. This is possibility caused by height of concentration of kinetin in medium. Mentioned by Lemenager *et al*. (2004), that sitokinin in high concentration were known as an antioxidant agent. Antioxidant were used in medium also enhanced the chlorophyll biosynthesis at callus cells.

Callus with Cu^{2+} at 10-80 M had significantly lower of dry weight than control (Table 1). Cu^{2+} required by callus cell in a small amount. Cu^{2+} at high levels becomes strongly phytotoxic cell and causes inhibition of plant growth or even cell death (Wang *et al*., 2004). The inhibition of dry weight (DW) accumulation in callus suggested that copper (Cu) excess result in membrane damage and ion K^+ leakage. Potassium plays an important role in vacuole where it contributes largely to the osmotic pressure and thus to the turgor pressure. Cell which lost of turgor pressure leaded to decrease in cell elongation (Alaoui-Sosse *et al*., 2004). Potassium also plays to activate the enzyme which is needed to form starch. In cell which deficiency of potassium element will be accumulation of carbohydrate and decreased of starch rate, so that cause inhibition on photosynthesis (Salisbury and Ross, 1995; Alaoui-Sosse *et al.*, 2004). Inhibition of resistance photosynthesis process will cause the callus growth become pursued and also smaller dry weight yielded.

Cu is antagonist with a few ion among other are Fe, Mg, and Ca; so that the higher of Cu absorption the lower absorption of another ions (Srivastava and Gupta, 1996). Calcium (Ca) include of essential macro element in cell growth, that is to function of ion Ca^{2+} forming Ca-pektat as leasing of cell wall and also existence of ion Ca^{2+} in early anaphase follow the initiative process the division of mitosis cell phase especially anaphase (Reksoatmojo, 1993). Addition of Cu^{2+} abundant in media caused the absorption of Ca^{2+} decreased and influenced the division of cell culture.

Fe and Mg elements play important role in chlorophyll forming. Fe influenced the concentration of glycine and succinil-CoA forming ∂-amino laevulinat acid (Srivastava and Gupta, 1996), while Mg assist the forming proto-porfirin become proto-chlorofilid (Salisbury and Ross, 1995). Prassad (1998) reported that heavy metal can pursue chlorophyll forming by pursuing activity of ∂-amino laevulinat and proto-chlorofilid reductase. This is caused the degradation of amount of photosynthesis pigment which finally will influence the callus growth.

Reserpine production

Reserpine production increased in Cu^{2+} concentration of 5 µM added in

Table 1. The accumulation of dry weight (DW) of callus cultures in treatment media. The data shown are the means of five replicates experiments. Means labeled with identical letters are not significantly different at 95% of confidence level.

	Cu^{2+} concentration (µM)					
Dry weight of callus (g)	**Cu_0**	**Cu_1**	**Cu_2**	**Cu_3**	**Cu_4**	**Cu_5**
	0,2690^{d}	0,2302^{cd}	0,2182^{bc}	0,2134^{abc}	0,1814^{ab}	0,1748^{a}

Cu: concentration of $CuCl_2$. Cu_0: 0 µM; Cu_1:5 µM; Cu_2: 10 µM; Cu_3: 20 µM; Cu_4: 40 µM; and Cu_5:80 µM.

Table 2. The reserpine production of callus cultures in treatment media. The data shown are the means of five replicates experiments. Means labeled with identical letters are not significantly different at the 95% of confidence level.

	Cu^{2+} concentration (µM)					
Reserpine production (mg/g)	**Cu_0**	**Cu_1**	**Cu_2**	**Cu_3**	**Cu_4**	**Cu_5**
	0,6226^{ab}	0,8786^{a}	0,8351^{a}	0,6238^{ab}	0,6678^{ab}	0,3712^{b}

Cu: concentration of $CuCl_2$. Cu_0: 0 µM; Cu_1:5 µM; Cu_2: 10 µM; Cu_3: 20 µM; Cu_4: 40 µM; and Cu_5:80 µM.

callus culture of pule pandak (Table 2.). The addition of Cu^{2+} as a biotic elicitor in medium will cause H^+-ATP-ase inactivation (Hall, 2002; Demidchik *et al.,* 1991) and degradation of pH cytoplasm. The cytoplasm acidity will induce enzyme synthesis needed in reserpine biosynthesis. According to Hagendoorn *et al*. (1996) whereas the addition of elicitor will influence the transport of ion H^+ pass the cell membrane and will influence the degradation of pH cytoplasm. This condition will induce activation of enzymes which playing apart in secondary metabolism synthesis.

Reactive oxygen species (ROS) production increased as a response to Cu^{2+} metal ion stress; this is also suggested have an effect on reserpine accumulation. According to Mithofer *et al*. (2004) which explained that ROS are involved in the oxidation of polyunsaturated fatty acids (PUFA) to PUFA hydro-peroxide (PUFA-OOH), which are converted to oxylipin. Jasmonat represent one of linolenic acid-derived oxylipins. Synthesis and accumulation of methyl jasmonat (Me-JA) during elicitor addition plays important role to induce the defense gene and also improve the arrangement of secondary metabolites synthesis. Vom-Endt *et al*. (2002) reported that the Me-JA accumulation resulted in over expression Str biosynthesis gene (strictosidin synthase) and increased in TIA (terpenoid indole alkaloid) production. Reserpine as main secondary metabolites collected from pule pandak includes in terpenoid indole alkaloid compounds.

Callus with Cu^{2+} at 80 µM had lower reserpine production than control. This result suggested excess of Cu^{2+} elicitor in the concentration caused plasma membrane leakage so that reserpine in cell secrete into the surrounding culture medium. Mentioned by Sevon and Oksman-Caldentey (2002) those biotic and abiotic elicitors were able to release the product of secondary metabolites from hairy culture into medium without any loss of viability and production capacity of the hairy roots.

CONCLUSION

The addition of elicitor Cu^{2+} at 5 µM enhanced the reserpine production, while addition above 10 µM decreased the reserpine production of callus culture of *Rauvolfia serpentina*. Callus growth significantly decreased with addition of Cu^{2+} elicitor in MS medium.

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