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Effect of elicitors on the production of naringin and rutin in leech lime *(Citrus hystrix)* callus

(Kesan beberapa jenis pengelisit terhadap penghasilan naringin dan rutin pada kalus limau purut)

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Key words: *Citrus hystrix,* elicitors, naringin and rutin contents

Abstrak

Rawatan kalus limau purut *(Citrus hystrix)* menggunakan pelbagai jenis elisitor telah merencatkan pertumbuhan kalus dengan bertambahnya kepekatan elisitor yang digunakan. Walau bagaimanapun, pertumbuhannya malar pada 0.05% (b/i) alginat dan terdapat sedikit peningkatan (1.30 ± 0.04 g bk./kultur) pada 0.1% (b/i) alginat. Analisis bahan flavonoid menggunakan alat kromatografi turus berprestasi tinggi (HPLC) menunjukkan bahan naringin dan rutin telah dihasilkan di dalam kalus. Penghasilan maksimum naringin (12.13 ± 0.07 mg/g bk.) dan rutin (3.09 ± 0.05 mg/g bk.) ($p \leq 0.05$) telah didapati pada kalus limau purut yang dirawat menggunakan 0.5% (b/i) agarosa.

Abstract

Treatment of *Citrus hystrix* callus with various types of elicitors decreased the callus growth as the concentration of elicitors increased. However, callus growth remained relatively constant at 0.05% (w/v) alginate and slightly increased to 1.30 ± 0.04 g dwt./culture at 0.1% (w/v) alginate. Analysis of the flavonoids using high performance liquid chromatography (HPLC) showed that only naringin and rutin were produced. Maximum production of naringin (12.13 ± 0.07 mg/g dwt.) and rutin (3.09 ± 0.05 mg/g dwt.) ($p \leq 0.05$) was found in callus treated with 0.5% (w/v) agarose.

Introduction

Citrus is a rich source of flavonoids (Kanes et al. 1993). More than 60 flavonoids have been identified. Four types of flavonoids namely flavanones, flavones, flavonols and anthocyanins are commonly found in citrus. Usually, flavonols and flavones occur as O-glycosides and are found mainly in the outer parts of plants, while only trace quantities are found in roots.

Plant cell culture technique has a good potential to the biosynthesis of flavonoids in *Citrus.* However, most of the untreated callus do not accumulate high flavonoids. For example, young *Citrus* calli which had been cultured less than one year tend to

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accumulate higher flavonoids than those of mature fruits. Calli incubation in light condition resulted in losing the ability to synthesise flavonoids (Berhow et al. 1994). Therefore, studies on the effect of various types of elicitors need to be carried out.

Addition of elicitors to the culture medium may increase the production of plant secondary metabolites e.g flavonoids in cultured cells. For instance, Hahlbrock et al. (1981) reported that treatment of callus with elicitors will induce rapid transient, transcription and translation of enzymes in the general phenylpropanoid pathway such as phenylalanine ammonium lyase (PAL) and CoA ligase. The synthesis of PAL enzyme is possibly associated with the production of flavonoids (Ebel et al. 1984).

The main objective of this experiment is to study the effect of various types of elicitors on callus growth and the production of naringin and rutin in *Citrus hystrix* callus.

Materials and methods

Callus induction and subculture

Callus was induced from stem using the method developed by Suri et al. (2001). Then, callus was sub-cultured 3 times on the same fresh media for inducement before sub-culturing on the same media with different concentrations of elicitors. Four types of elicitors namely yeast extract (Sigma, USA), alginate (Sigma, USA), agarose (Sigma, USA) and salicylic acid (Sigma, USA) were used in this experiment. Fresh and dried weight and the quantity of flavonoids in *C. hystrix* were determined after 6 weeks of culture.

Preparation of standard flavonoids

Flavonoids standards such as naringin, rutin, hesperidin, quercetin and kaempferol were obtained from Sigma, St. Louis, USA. Two mg of each standard was dissolved in 2 mL of 60% methanol (v/v) and kept at 4 ºC. Then, 1 mL of standard solution was diluted with 20 mL of 68% (v/v) methanol (BDH, Germany) containing 20 mM sodium diethyl dithiocarbamate (Na-DEDTC) and 5 mL of 6 M HCL solution (AR grade) and the solution was diluted with 50 mL of 60% methanol (v/v) (Hertog et al. 1992; Siti Mahyuni 1999).

Flavonoids extraction

After lipolysation the *C. hystrix* callus were freeze-dried (Lab Conco, USA) for 2 days, extracted and analysed using a modified method of Crozier et al. (1997). Powdered tissues (0.25 g) were extracted with 20 mL of 60% (v/v) methanol with 20 mM Na-DEDTC, filtered with Whatman no-1 and 2.0 mL of the extract was separated. Other portion of the extract was added with 5 mL of 6 M HCl solution (AR grade). Each extract was separately refluxed at 90 ºC for 2 h. A 50-µL hydrolysed extract containing aglycone and glucosides was diluted with 50 µL H_2O that had been previously adjusted to pH 2.5 with trifluroacetic acid (TFA). Each extract was then filtered through microfilter [0.45 µm PTFE membrane (Whatman)] prior to injection into the HPLC.

Apparatus for HPLC

A HPLC system (Beckman, USA) attached with ultraviolet (UV) detector was used in this experiment. The column used was a Novapak C 18 column (3.9 mm x 150 mm, 4.0 µL) (Waters, USA).

HPLC conditions

Flavonoids were analysed using modified HPLC method developed by Crozier et al. (1997). The elution solvents used were solvent A (water adjusted to pH 2.5 with TFA) and solvent B (100% acetonitrile) and run under gradient [A:B (80–20%) to (60–40%)] for 20 min and operated at room temperature. Detection was performed at 370 nm and the absorption spectra of each compound was recorded between 350 nm and 450 nm.

Statistical analysis

Data were subjected to analysis of variance (ANOVA)/Duncan using SAS.

Results and discussion

Effect of elicitors on callus growth

Both fresh and dry weights of *C. hystrix* callus decreased gradually after treatment with 5 mM salicylic acid (SA) and achieved a steady state at 15 and 20 mM SA. Most part of the callus turned brown immediately when placed on the medium supplied with SA. This phenomenon may possibly be due to the abundant stress and which finally caused cell death *(Figure 1).*

Treatment with different concentrations of yeast extract (YE) decreased the callus growth *(Figure 2)*. Koch et al. (1998) reported that an elicitor derived from a fungus, *Phythium aphanideratum,* induced cell death. The callus growth remained relatively constant after treatment with 0.05% (w/v) alginate and slightly increased to 1.30 ± 0.04 g dwt./culture at 0.1% (w/v) alginate *(Figure 2)*. Further cultivation of callus on the medium supplied with 0.3% and 0.5% (w/v) alginate suppressed the callus growth. Kebmann and Barz (1987) and Ayabe et al. (1986) reported that elicitors did not necessarily reduced cell viability. For example, callus of *Glycyrrhiza echinata* was not damaged or lysed by the addition of sodium alginate.

Effect of salicylic acid on the production of naringin and rutin

The effect of salicylic acid (SA) on the production of naringin and rutin is as shown in *Figure 3*. Maximum quantities of naringin (6.37 ± 0.13 mg/g dwt.) and rutin (1.40 ± 0.28 mg/g dwt.) ($p \leq 0.05$), which were respectively 2.8 and 1.8 times higher than control were obtained after treatment with 5.0 mM SA. Hernandez and Vargas (1997) reported that addition of various concentrations of acetylsalicylic acid to the tumor line of *Catharanthus roseus* callus which required corn starch as a carbon source produced a remarkable increased of alkaloids by 505% (cells per culture medium), 1587% total phenolics (liquid medium), 612% total furanocuomarins (liquid medium) and 1476% total

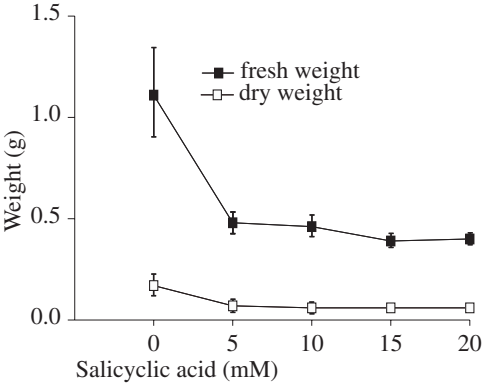

*Figure 1. Effects of salicylic acid on **Citrus hystrix** callus growth after 6 weeks of incubation. Bar indicates the standard error of mean (n = 5)*

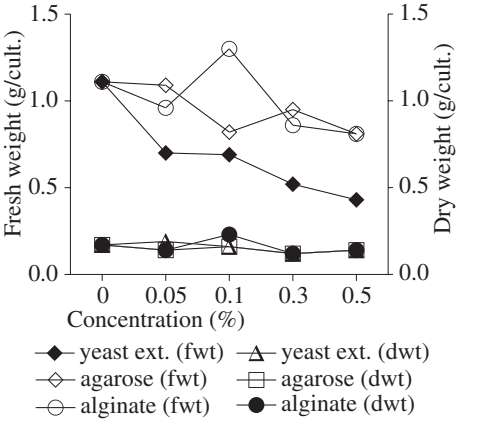

*Figure 2. Effects of yeast extract (YE), alginate and agarose on **Citrus hystrix** callus growth after 6 weeks of culture (n = 5)*

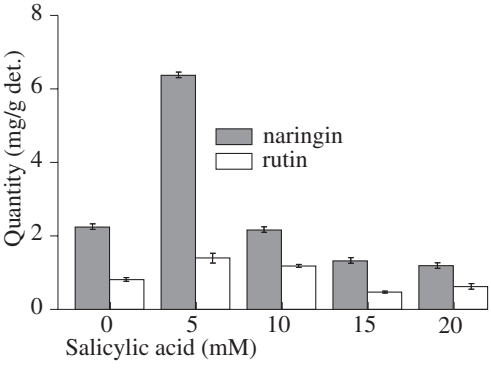

*Figure 3. Production of naringin and rutin from **Citrus hystrix** callus after treatment with salicylic acid. Mean values for the same concentration of salicylic acid with the same letter are not significantly different using DMRT $p \leq 0.05$. Bar indicates the standard error of mean (n = 3)*

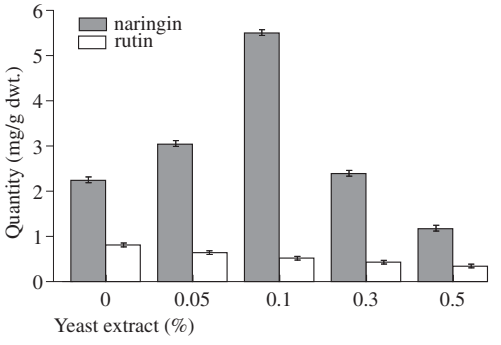

Figure 4. Production of naringin and rutin from ***Citrus hystrix*** *callus after treatment with yeast extract. Mean values for the same yeast percentage with the same letter are not significantly different using DMRT $p \leq 0.05$. Bar indicates the standard error of mean (n = 3)*

anthocyanins (liquid medium). On the other hand, treatment with SA could not produce other similar compounds as in the intact plant. Hernandez and Vargas (1997) also reported that SA would act as a chelate that inhibited ethylene synthesis in cultured plant cells by blocking the action of ethylene forming enzymes. Puig et al. (1995) showed that the levels of naringin and rutin diminished after treatment with ethylene gas, whilst, the level of nookatone (sesquiterpene nootkatone) was increased.

Effects of yeast extract on the production of naringin and rutin

Yeast extract (YE) is an effective elicitor in inducing the production of flavonoids (Funk et al. 1987) and brewers yeast possess a powerful elicitor of glyceollin accumulation in soybean tissues. The effects of various concentrations of YE on naringin and rutin productions are as shown in *Figure 4.* A significant increased ($p \leq 0.05$) of naringin content was observed after treatment with YE. The production was maximum (5.50 ± 0.16 mg/g dwt) ($p \leq 0.05$) after treatment with 0.1%YE and which was 2.5 times higher than control. According to Hahn and Albersheim (1978), YE contained 1-3-ß-D-glucan molecules with 6-linked glucosyl residues that elicit the production of secondary metabolites. These molecules will increase the phenyl ammonium lyase activity and finally resulted in an increase of flavonoid compounds. Treatment with YE also increased two enzymes i.e. chalcone synthase and isoflavone reductase (Wysocki et al. 1997). In a similar finding, Osman and Fett (1983) reported that treatment of soybean leaf with sodium iodoacetate and yeast extract led to the accumulation of isoflavonoids.

Treatment of *C. hystrix* callus with YE inhibited the production of rutin after 6 weeks of culture. The amount of rutin decreased to 0.34 ± 0.01 mg/g dwt. or 2.4% lower than controls. The results obtained is similar to the previous work reported by Park et al. (1995) where upon treatment of *Pureria lobata* callus with YE, the constitutive isoflavonoids conjugates with (7-)-glucoside-6"-O-melonyl esters showed a rapid declined in their content within 4 hours, followed by re-accumulation of the conjugates.

Effect of alginate on the production of naringin and rutin

Alginate is a matrix that is widely used as an immobiliser. This compound can also promote the accumulation of secondary metabolites that are usually produced in low quantities by calli (Ramakrishna et al. 1993).

The quantity of naringin decreased after the *C. hystrix* callus has been treated with 0.05 and 0.1% (w/v) alginate. Further supplementation on callus cultures with 0.3% (w/v) of alginate resulted in a high increase (11.39 ± 0.89 mg/g dwt.) ($p \leq 0.05$) of naringin after 6 weeks of incubation *(Figure 5)*. Aoyagi et al. (1998) reported that enzymes such as 5'-phosphodiesterase (5'-P-dase), catalase and chitinase were promoted remarkably when 1.0 g/L (w/v) of alginate was supplied to *Catharanthus roseus* cell culture.

Treatment of callus with various concentrations of alginate did not show any significant effect on the quantity of rutin produced, but remained slightly lower than

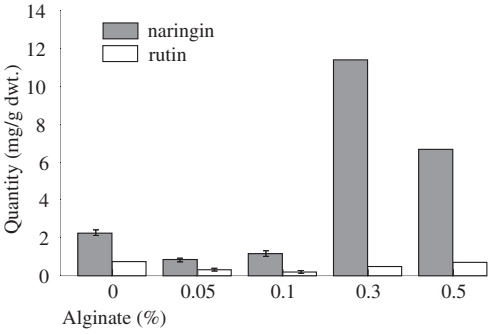

*Figure 5. Production of naringin and rutin from **Citrus hystrix** callus after treatment with alginate. Mean values for the same alginate percentage with the same letter are not significantly different using DMRT $p \leq 0.05$ Bar indicates the standard error of mean (n = 3)*

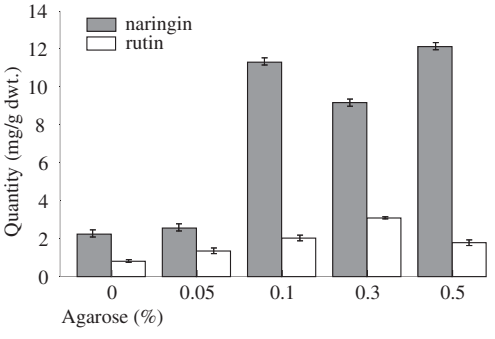

*Figure 6. Production of naringin and rutin from **Citrus hystrix** callus after treatment with agarose. Mean values for the same agarose percentage with the same letter are not significantly different using DMRT $p \leq 0.05$. Bar indicates the standard error of mean (n = 3)*

the controls at 0.05 and 0.10% (w/v) of supplied alginate. Treatment of callus with 0.30% (w/v) alginate slightly increased the production of rutin and reached a maximum ($p \leq 0.05$) at 0.50% (w/v) of supplied alginate. Ayabe et al. (1986) reported that echinatin (isoflavonoid) production is stimulated by the addition of alginate (either calcium or sodium salt) and not necessarily by cell immobilisation. This phenomenon may possibly follow the previous experiment reported by Keen and Dawson (1992) that the PAL activity may be induced by the addition of biotic and abiotic elicitors, mechanical damage and environmental factors such as light, temperature and pH.

Effect of agarose on the production of naringin and rutin

Agarose was the most effective elicitor for the production of naringin and rutin compared to salicylic acid, yeast extract and alginate. Maximum naringin and rutin ($p \leq 0.05$) levels obtained were 12.13 ± 0.07 mg/g dwt. and 3.09 ± 0.05 mg/g dwt., respectively, after treatment with 0.5 and 0.3% (w/v) of agarose *(Figure 6)*. In contrast, Aoyagi et al. (1998) reported that alginate decreased the production of 5"-phosphodiesterase which finally inhibited the production of secondary metabolites.

Conclusion

Treatment of *Citrus hystrix* callus with various concentrations of elicitors decreased the callus weight. However, only treatment with 0.1% alginate increased the callus weight. Naringin and rutin were found in all treated callus. Treatment of *C. hystrix* callus with agarose caused the highest production of naringin. The data obtained are useful for increasing the quantity of naringin and rutin, in order to achieve the super-producer callus.

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