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Glucosinolates content of *in vitro* grown *Nasturtium officinale* (watercress)

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Watercress *(Nasturtium officinale)*, a green vegetable belongs to Brassicaceae, is a rich source of phenyl ethyl glucosinolate (PEGSL) and benzyl glucosinolate (BGSL) i.e. the precursors of phenyl ethyl isothiocyanate (PEITC) and benzyl isothiocyanate (BITC), which are widely reported to restrain the growth of the cancer cells. The content of secondary metabolites and other compounds in plants are affected by different growth conditions such as pH, temperature, light intensity and nutrient supply. Thus, the aim of the current study is to evaluate the concentration of PEGSL and BGSL from *in vitro* grown watercress under non-elicited and elicited as well wild plant of watercress. The samples were collected from watercress wild growing in Kundasang area, Ranau, Sabah and subjected to sterilization to establish sterile *in vitro* culture. All plant cultures were kept inside growth chamber at 25°C under 16 hours photoperiod for thirty days before sub-cultured into fresh medium containing elicitors. Elicitors and natural additives tested in this study are chitosan, casein hydrolysate and coconut water at different concentration: chitosan (10, 20, 40, 60, 100 mg/L), casein hydrolysate (0.5, 1.0, 1.5, 2.0 g/L) and coconut water (5, 10, 15, 20, 25 % v/v). The results revealed that non-treated plant culture accumulated high content of PEGLS and benzyl glucosinolate (BGSL) which are 2.31 μmol/g FW and 1.08 μmol/g FW, respectively. PEGSL and BGSL increased over five-fold and three-fold, respectively, in non-treated plant culture compared to wild matured plant. Meanwhile the maximum concentration of the PEGSL and BGSL in treated plant were 1.60 μmol/g FW and 0.82 μmol/g FW, respectively. Interestingly, all *in vitro* plant culture (non-treated and treated) in this study shows higher concentration of PEGSL and BGSL compared to wild plant of watercress. Thus, tissue culture could be a valuable alternative for higher production of glucosinolates in watercress with short period of plant development. Besides, the plant is not exposed to the common environmental pollutants such as heavy metal and agrochemicals.

Keywords: *Nasturtium officinale*, phenyl ethyl glucosinolate, benzyl glucosinolate, *in vitro* grown

I. INTRODUCTION

Watercress is a rich source of phenyl ethyl glucosinolate (PEGSL) or commonly known as gluconasturtiin which produced phenyl ethyl isothiocyanate (PEITC) upon hydrolysis. Previous studies revealed that PEITC restrain the growth of cancer cell (Powolny *et al.*, 2011),

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possess a strong chemopreventive effect on colorectal cancers in mice model (Khor *et al.*, 2008) and could be use as antitumor compound for oral cancer therapy (Chen *et al.,* 2012). However, despite the promising benefits of PEITC, the production of glucosinolates from wild growing watercress could be hampered by ecological conditions and nutrient supply. Coincidently, advancement in research allows us to exploit cell, tissue, organs or entire organism through *in vitro* method to enhance the production of secondary metabolites or specific compounds (Rao & Ravishankar 2002). Besides, treatment using elicitors such as salicylic and methyl jasmonate in turnip (*B. rapa ssp. rapifera*) increased aromatic gluconasturtiin and indole GLs in secondary roots and exudates (Smetanska *et al.,* 2007). Hence, the aim of this study is to evaluate the concentration of PEGSL and benzyl glucosinolate (BGSL) from *in vitro* grown watercress under non-treated and treated plant culture as well wild plant of *N. officinale*. PEGSL and BGSL have health-promoting effect, and it is therefore desirable to enhance the concentration of these compounds.

II. MATERIALS AND METHODS

A. Plant Material and Explant Preparation

The wild watercress samples were harvested from a spring in Kundasang area, Ranau Sabah. The young healthy shoots were cut about 1-1.5 cm and used as an explant. The explant was cleaned under running tap water for 5 minutes before sterilized for 15 minutes in 15 % of chlorox solution containing Tween 20. Finally it was rinsed three times with sterile distilled water before soaked into sterile distilled water containing plant preservative mixture (PPM).

B. Culture Medium and Incubation Conditions

Explants were grown inside glass jar containing hormone-free Murashige and Skoog (Murashige & Skoog 1962) with 30 g/l of sucrose and pH was adjusted to 5.7-5.8. The medium was solidified with 4 g/L^{-1} of agar and sterilized at 121°C for 20 minutes. All cultures were kept inside growth chamber at 25°C under 16 hours photoperiod for 30 days before sub-cultured into fresh medium treated with elicitor and plant natural additives. All plants culture were grown for 30 days in MS medium before harvested prior to analysis. Elicitor and natural additives tested in this study are chitosan, casein hydrolysate and coconut water with different concentration: chitosan (10, 20, 40, 60, 100 mg/L), casein hydrolysate (0.5, 1.0, 1.5, 2.0 g/L) and coconut water (5, 10, 15, 20, 25 % v/v).

C. Chemical Analysis

The method for extraction of glucosinolates was adopted from previous study with slight modification (Rosetto *et al.*, 2013). About 1.00 g of the fresh samples was homogenized in a porcelain mortar containing 5 ml of 70 % of

methanol. The extract was transferred into conical flask and placed into water bath for 30 minutes at 70°C. The crude extracts were centrifuged at 8000×g for 20 minutes. The supernatants were collected prior to HPLC analysis with PEGSL and BGSL as external standards. All samples were prepared in triplicates.

D. HPLC Condition

The HPLC conditions were according to previous study with slight modifications (Aires *et al.,* 2013). PEGSL and BGSL in watercress extracts were analyzed by reversed-phase ion-pair chromatography at 30°C with flow rate of 1ml/min for 10 minutes. Isocratic elution was performed with mobile phase consisting of 0.1 % trifluoroacetic acid (TFA) aqueous solution (A) and 0.1 % TFA acetonitrile (B). The injection volume was 10 µl with UV detection at 229 nm.

III. RESULTS AND DISCUSSIONS

A. Plant Development of in vitro Culture of Watercress

The stage of plant growth under sterile conditions in MS media was observed at ten days interval from ten days after grown until sixty days to determine the growth pattern of plant until maturity. The plant development such as height, wet weight, and dry weight were summarized in Table 1. Table 1 shows the development of *in vitro* grown plant, particularly height, fresh weight and dry weight from 20 days after grown until 60 days. The maximum weight of the plant is 60 days grown plant which is 1.40 ± 0.05 g FW while the minimum weight recorded is 20 days grown plant which is only 0.69 ± 0.02 g FW. The weight of plant increased as day increased and it is rapidly increase with 0.33 g from 20 days to 30 days.

Table 1. Plant development of *in vitro* culture of watercress

Days	Plant development		
	Height (cm)	Fresh weight (g)	Dry Weight (g)
20	9.77 ± 0.25	0.69 ± 0.02	0.047 ± 0.001
30	11.3 ± 0.36	1.02 ± 0.10	0.070 ± 0.001
40	12.8 ± 0.67	1.13 ± 0.07	0.077 ± 0.001
50	13.1 ± 0.56	1.21 ± 0.04	0.083 ± 0.001
60	13.4 ± 0.41	1.40 ± 0.05	0.095 ± 0.001

The plant continues to grow even after 30 days but most of the leaves turn yellowish. Thus, the optimum concentration of the glucosinolates from *in vitro* grown watercress were determined by harvesting at each stage of plant development from 20 days until 60 days as shown in Figure 1. In contrast, the wild plant of watercress was harvested at matured condition. Biomass accumulation and synthesize of metabolites occur in two-step process where at the beginning the cultured cell and organ support the growth, multiplication and biomass accumulation followed by synthesize of the metabolites from biomass (Murthy *et al.*, 2014). Hence, increased in plant biomass allows improvement of secondary metabolites.

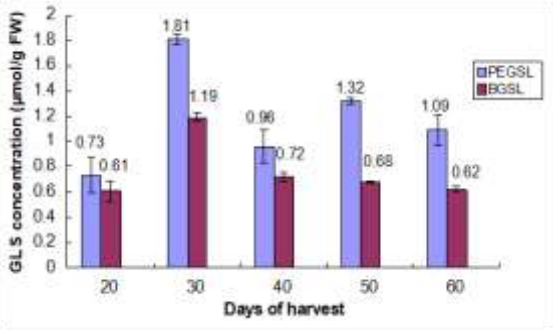

Figure 1. Glucosinolates content of *in vitro N. officinale at* different stages of harvest

Thirty days grown plant *(in vitro)* had the highest PEGSL and BGSL content at about 1.81 ± 0.04 µmol g^{-1}FW and 1.19 ± 0.03 µmol g^{-1} FW, respectively, as shown in Figure 1. The results showed that PEGSL and BGSL increased almost three-fold and two-fold, respectively, in 30 days plant culture as compared to 20 days plant culture. This finding are lower compared to previous study by Aires *et al* (2013). They reported that baby-leaf watercress grown under organic practices consisted of 3308.7 ± 117.1 µmol 100^{-1} dw of PEGSL.

B. Effect of Elicitors and Natural additives on Glucosinolates Content

Elicitor such as chitosan and natural additives (coconut water and casein hydrolysate) were tested for possibility to enhance the accumulation of glucosinolates and the growth of watercress grown in MS medium. Different elicitor and natural additives treatment varied the glucosinolates content in plant culture. The effect of each treatment on glucosinolates content in plant culture of *N. officinale* were summarized in Table 2 with non-treated plant culture as a control of this study.

Table 2. Glucosinolates content in *in vitro* watercress treated with chitosan, casein hydrolysate and coconut water

Treatments	Concentration	Glucosinolates content (µmol/g FW) PEGSL	BGSL
Control	-	1.81 ± 0.04	1.19 ± 0.03
Chitosan	10 mg/L	0.88 ± 0.001	0.73 ± 0.01
	20 mg/L	0.93 ± 0.01	0.59 ± 0.001
	40 mg/L	0.77 ± 0.001	0.60 ± 0.01
	60 mg/L	0.77 ± 0.001	0.57 ± 0.001
	100 mg/L	0.86 ± 0.01	0.54 ± 0.01
Casein hydrolysate	0.5 g/L	1.60 ± 0.08	0.82 ± 0.02
	1.0 g/L	1.45 ± 0.19	0.80 ± 0.03
	1.5 g/L	1.53 ± 0.03	0.81 ± 0.001
	2.0 g/L	1.18 ± 0.02	0.77 ± 0.01
Coconut water	5 % v/v	1.43 ± 0.001	0.66 ± 0.001
	10 % v/v	1.32 ± 0.24	0.59 ± 0.02
	15 % v/v	1.49 ± 0.07	0.64 ± 0.01
	20 % v/v	1.59 ± 0.01	0.60 ± 0.001
	25 % v/v	1.58 ± 0.001	0.61 ± 0.001

Initially, elicitor was used to imitate the effect of stresses to activate the biological system in plant to increase the production of secondary metabolites and natural additives could be used to promote the growth of the culture yet the results are different from the expectation.

Table 2 shows the maximum concentration of the PEGSL in treated plant is 1.60 ± 0.08 µmol/g FW which found in plant grown with casein hydrolysate at concentration of 0.5 g/L. Meanwhile, the maximum concentration of BGSL in treated plant is 0.82 µmol/g FW which found in plant grown with casein hydrolysate at concentration of 0.5g/L. Casein hydrolysate serve as a source of calcium, phosphate, several microelements, vitamins and a mixture of 18 amino acids which is

important for plant growth. However, fresh weight of plant culture were lower in casein hydrolysate treatment as compared to the non-treated plant. The finding is different from previously reported which found that the growth of the tissue, fresh weight and growth rate of date palm (*Phoenix dactylifera* L.) were increased when the concentration of casein hydrolysate increased (Ageel & Elmer 2011).

Previous studies found that chitosan does not improve the production of BGSL hydrolysis product compared to elicitation using methyl jasmonate (Al-Gendy & Lockwood, 2005). The highest PEGSL content in treated plant is 1.59 ± 0.01 µmol/g FW at 20 % and BGSL content is 0.66 ± 0.001 µmol/g at 5 % of coconut water. Both identified glucosinolates respond differently on coconut water treatment. PEGSL tend to increase at higher concentration of coconut water while BGSL tend to increase at low concentration of coconut water. Plant growth hormone which used in tissue culture are naturally present in coconut (Agampodi & Jayawardena, 2006) which affect the growth of the *in vitro* plant.

Plant culture supplemented with elicitor and natural additives in this study has low glucosinolates content as compared to non-treated plant. Nevertheless, all *in vitro* grown plant in this study showed higher concentration of PEGSL and BGSL compared to the matured wild plant. Finding in this study revealed that PEGSL and BGSL increased over five-fold and three-fold, respectively, in non-treated plant culture compared to wild plant as shown in Figure 2.

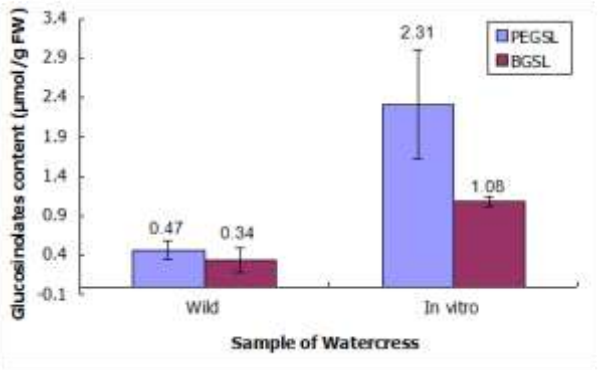

Figure 2. Glucosinolate content in wild plant and in vitro (non-treated) plant of watercress

The maximum concentration of PEGSL and BGSL in wild plant is 0.47 ± 0.11 µmol/g FW and 0.34 ± 0.15 µmol/g FW, respectively. *In vitro* plant culture obtained nitrogen and sulphur from MS medium for biomass enhancement but nutrient supply is limited to wild plants as wild plant obtained nutrients from non-fertilized soil and subjected to environmental changes. In addition, *in vitro* watercress was grown under long photoperiod (16 hours) resulted in high concentration of glucosinolates as compared to matured wild plant. These findings are in agreement with previously study where watercress grown under 16 hours photoperiod showed high concentration of PEGLS compared to the short photoperiod (8 hours) grown plant (Engelen-Eigles *et al.,* 2006). Matallana *et al.* (2006) found different observations in *in vitro* plant of *Tropaeolum majus* L. where these compounds were lower in *in vitro* grown than the soil grown plant. The glucosinolates content, specifically BGSL, found in their study is varied from 10 and 55 µmol/g DW for *in vitro* grown plant and up to 80 µmol/g DW in soil grown

plant. The reason might be different in plant species and other factors related to soil condition etc.

Apart from that, elicitation and supplementation in this study does not enhance the biomass and glucosinolates production. This is may be due to the high concentration of the elicitor/ natural additives added into growth media which in turn inhibit the production of secondary metabolites. Besides, there is no universal predictable effect of certain elicitor/ natural additives on tissue culture system with different plant species.

IV. SUMMARY

Tissue culture method could be a valuable alternative for higher production of glucosinolates in *N. officinale* with short period of plant development. Besides, the plant is less exposed to common environmental pollutants such as heavy metals and agrochemicals.

V. ACKNOWLEDGMENT

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