Synthesis of Sulforaphane during the Formation of Plantlets from Broccoli (*Brassica oleracea L var italica*) *In Vitro*

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***Abstract*— Synthesis of sulforaphane in shoots of broccoli (*Brassica oleracea L var italica*) had been conducted *in vitro*. Sulforaphane is found in abundance in broccoli shoots. The experiment was designed in three stages. Stage one was aimed to find the best concentration of naphthalene acetic acid (NAA) and benzylaminopurine acetic acid (BAP) in inducing the production of sulforaphane in broccoli cultures. In the second stage we studied the shoot propagation and analyzed the effect of combination of methionine precursor and the broccoli seed extract in increasing the sulforaphane yield. Stage three was aimed to find the best combination of methionine concentration and broccoli seed extract in producing sulforaphane in the broccoli plantlet. The results show that during the induction stage 1 ppm of NAA and 5 ppm BAP increased the sulforaphane production upto 47.76 ng/g, while the combination of 100 mg methionine and 1 g broccoli seed extract yielded 182.09 ng/g in the shoot propagation stage and 2,901 ng/g in the plantlet formation stage.**

***Index Terms*— broccoli, methionine, sulforaphane, NAA, BAP, shoot induction, shoot propagation, plantlet formation.**

I. Introduction

BROCCOLI is well known for its high content of anti-oxidants, e.g. sulforaphane, beta-carotene, indole, quercetin, and glutathione. It also contains fat, protein, carbohydrates, fiber, water, iron, calcium, minerals, and various vitamins (A, C, E, riboflavin, nicotinamide) [1]. The substance of interest, sulforaphane, plays an important role in human health. 1-5 µmol/L of sulforaphane is capable of reducing aldehyde dehydrogenase (ALDH) and the population of cancerous cells by 65-80% for human cancer stem cells (CSCs) ($P < 0.01$); and reducing the size and number of mammosphere about 8-125 times and 45-75% ($P < 0.01$), respectively. Sulforaphane inhibits cancer cells and sets the trajectory of Wnt/β-catenin [2].

There are two types of antioxidant: direct and indirect type antioxidants. The direct type is a substance which helps in the physiological, biochemical and cellular processes, such as glutathione, tocopherols, ascorbic acid, and carotenoids. The indirect antioxidants are substances that are not able to assist in radical reactions or redox reactions within the cell, but it can work with various types of mechanism to detoxify and induce protection to animals and their cells against carcinogens and mutagenesis. This type of antioxidant includes glutathione transferase, NAD(P)H reductase, epoxide hydrolase, and heme oxygenase that are involved in inducers phase II [3]. Sulforaphane belongs to the latter.

Sulforaphane was produced from glucosinolate hydrolysis [4, 5]. Glucosinolate is a glucoraphanin that produces sulforaphane with the help from myrosinase [6]. Broccoli seed is rich of myrosinase and glucoraphanin [7]. The total level of glucosinolate type glucoraphanin in seeds of 5 varieties (i.e. GreenKing, Packman, PaGing, Rod Fai, and Top Green #67) is in the range of 11.4 to 48 µmol/g of seed and higher than the other types of glucosinolates [8]. The glucoraphanin in some inbred genotypes is produced in many ways and is determined by the genotype. In USVL102 the glucoraphanin is produced by open pollination and reaches 91 µmol/g seed, in USVL049 it is obtained from dihaploid breeding that reaches 80.5 µmol/g seed. While in the Pinnacle type the glucoraphanin is derived from F1 that yields 107.5 µmol/g broccoli seed [9]. Glucoraphanin content in seeds is different from the flower as observed by Sarikamis *et al*. [10].

The initial precursor in the synthesis of sulforaphane from *Cruciferaceae* is methionine. The final precursor according to the synthetic line of sulforaphane substance is glucoraphanin [10]. Broccoli seed contains glucoraphanin 20 to 50 mg/g seed [11], sulforaphane 11.53 mg/g dry weight seed [12]. Gluco-raphanin in the broccoli seed is a precursor that produces sulforaphane as a phytochemical compound [13]. This allows us to produce seed extract to substitute glucoraphanin to stimulate the synthesis of sulforaphane. Therefore, to increase the production of sulforaphane it is possible to use methionine and broccoli seed extract as precursor in the growth media.

In the future, the demand for these compounds by the pharmaceutical industry is expected to significantly increase.

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Conventional production of seed will not be able to meet the demand. Thus, a non-conventional seed propagation method is one of the solutions. This propagation technique is also called micro propagation which in principle is to grow cells or plant parts on an artificial medium aseptically to gain shoots or new plants. The advantage of this technique is the independency on climate, as well as free of pests and diseases [14]. Therefore, studies on vegetative breeding using micro propagation system is beneficial in maintaining the nature of the parent and to provide good quality seeds in large quantities with relatively in a short time.

Plant growth is influenced by hormones as the growth regulators that could be synthesized from plants [15, 16]. For instance, auxin and cytokinin in growth medium play important roles in organogenesis of tobacco tissue culture [17]. When the auxin concentration is lower than the cytokinin, explants will induce shoots, otherwise it will form roots. In broccoli hypocotyls explants can grow to form callus on 1 ppm of at 2.0 mg/L and naphthalene acetic acid (NAA) or combination of 0.1 ppm NAA and 0.5 ppm benzylaminopurine acetic acid (BAP), while the best shoot formation was at 5 ppm BAP [18]. In another study using explant hypocotyls of *Royal Green* in induction stage we obtained yields in induced shoots from broccoli hypocotyls with a combination of 1 ppm NAA and 4 ppm BAP [19]. However, the hypocotyls growth response on NAA and BAP combinations could be different from our previous study [18]. Moreover, the sulforaphane synthesis has never been conducted yet.

In this study we used Murashige and Skoog (MS) base media supplemented with NAA and BAP. Combinations of NAA and BAP concentrations were varied in all three stages of shoot formation: induction, shoot propagation, and plantlet formation. Variations in the concentration of methionine and broccoli seed extract were then added to MS base medium to obtain sulforaphane content in the shoots. We aimed to find the optimal combination of NAA and BAP concentrations in increasing sulforaphane in broccoli cultures *in vitro* and to find the optimal combination of methionine and broccoli seed extract in increasing sulforaphane in shoots from the in tissue culture and also broccoli plantlets *in vitro*.

II. Research Methods

A. Tissue Culture

The broccoli shoots were obtained and propagated by tissue culture technique. There were three stages in the culture procedure.

Stage I: Induction

The goal of this phase was to obtain the best concentration combination of NAA and BAP in escalating of sulforaphane in the broccoli culture in in vitro. The medium used was the basic MS medium. The explants used were the hypocotyls of broccoli sprout. The experimental design adopted the factorial design. There were two factors in this research: factor A (NAA with concentrations of 0 and 1 ppm) and factor B (BAP with concentrations of 0, 2.5, and 5 ppm).

Stage II: Shoot Propagation

The aim of this phase was to acquire the best concentration combination of methionine precursor and the broccoli seed extract in the increasing of sulforaphane in the shoots of broccoli on in vitro. In this stage we adopted the factorial design. There were two factors as the precursor, factor A (methionine 0, 50, 100, and 150 mg) and factor B (broccoli seed extract 0, 1, 2, and 3 g). Each treatment was made quadruple. The explants used were adventive shoots from the induction stage I which were the combination of 1 ppm NNA and 5 ppm BAP. The medium used was the MS medium and the broccoli seed extract was used as the glucoraphanin source.

Stage III: Plantlet Formation

The purpose of this stage was to find the best methionine precursor concentration and broccoli seed extract in producing sulforaphane in the broccoli plantlets. At this stage, 50% of MS medium was added with 1 ppm NAA and the best three treatments of methionine precursor and broccoli seed extract from stage II to form roots and plantlets or seeds. The explants used were the broccoli shoots from stage II. We employed a complete random design for the experimental design. The treatment used was 100 mg methionine, a combination of 100 mg methionine and 1 g broccoli seed extract, and 1 g broccoli seed extract. Each treatment comprised five repeats. The data were analyzed with analysis of Variety and followed by LSD test 5%. The variables observed were the content of sulforaphane of adventives shoots formed in stage I and stage II, and sulforaphane content of plantlets.

B. Sulforaphane Analysis

Tools and Materials

The tools used in the experiments were a laminar air flow cabinet, an autoclave, a freezer, an oven, a culture chamber, a magnetic stirrer, a rotary evaporator, a pH-meter, an analytical balance, a detector, an ultrasonic cleaner, a hot plate, a centrifuge, mortar & pestle and other glass wares, a Liquid Chromatography MSMS (Accela LC 1250, Thermo Scientific). The detector used was a triple quadrupole MSMS (TSQ Quantum Access Max, Thermo Scientific). The column used was a Hypersil Gold column (50 mm × 2.1 µm).

The materials used were the components of MS medium, NAA, BAP, agar, sucrose, and broccoli shoot hypocotyls, calluses, buds and plantlets. The chemicals used for the extraction were 0.1% formic acid in water, 0.1% formic acid in acetonitrile gradient grade for LC type (code I486330 920), standard DL sulforaphane (Sigma Aldrick code s4441 5 mg), aqua bidest (code 201802-IV), and methylene chloride.

Extraction and Isolation of Sulforaphane

- Sample preparation

Sulforaphane extraction began with the weighing of broccoli shoots. Then, the broccoli shoots were put in the mortar and

added with 1 -2 mL methyl chloride and ground. The crushed broccoli shoot was transferred to the flash tube and was added with 25-50 mL methyl chloride. The sonification was then performed for 30 minutes to extract the sulforaphane from the rushed broccoli shoots. The sulforaphane extract was filtered using Whatman filter papers and transferred to a tube and placed on the hot plate with 70 to 80 °C to produce dry residue. The dry residue was added with 5 mL NaSO4 and reheated on the hot plate at 70 to 80 °C until the residue was completely dry. The dry residue was added with 10 mL acetonitrile, and then filtered with Whatman filter papers and was centrifuged for 15 minutes at 4000 rpm. Before the analysis was conducted, another filtration was held on a 0.2-µm cellulose acetate PTFE filter membrane. Finally, the solution containing the residue was transferred to a micro tube and was fed to the LC-MS tandem to determine the shoot sulforaphane content.

- *Quantitative Analysis of Sulforaphane*

The broccoli shoots were extracted and added with 250 µL acetate buffer pH 4.5 and 500 µL acetonitrile to make an extract stock. 10 µL extract was taken and fed to the LC-MS tandem to determine the shoot sulforaphane content. A quantitative analysis was carried out by identifying the standard sulforaphane compound 177.29 g/mol. A "*low flow*" procedure was performed with a speed 5 µL/minute to acquire a sulforaphane spectrum. The spectrum showed 178.29 g/mol. The sample was then fragmented to obtain ion products with 72 g/mol and 114 g/mol. The sulforaphane concentration was estimated by plotting the result on the standard calibration curve that was obtained from the data of various concentrations of standard solution.

III. Results and Discussion

A. Induction Stage

The analysis showed combination of NAA and BAP concentrations that influenced sulforaphane content in shoots *in vitro* with the highest yield (47.76 ng/g) was from the combination of 1 ppm NAA and 5 ppm BAP (see Table I). An LSD 5% test was performed to determine which treatments had differences.

TABLE I
EFFECT OF NAA AND BAP COMBINATION ON SULFORAPHANE CONTENT IN ADVENTIVES SHOOTS

Treatments		BAP (ppm)		
		0.0	2.5	5.0
NAA (ppm)	0.0	9.83 c	5.99 ab	16.35 d
	1.0	7.55 bc	3.65 a	47.76 e
LSD 5%			3.23	

Note: sulforaphane content in ng/g wet weight of shoots.

These results were largely determined by the balance of growth regulators introduced to the media. In this term, the combination of 1 ppm NAA and 5 ppm BAP was the right combination to stimulate sulforaphane content. Mantell and Smith (1983) reported the use of 10^{-5} M NAA and 5×10^{-6} M BAP; 10^{-6} M NAA and 10^{-4} M BAP; and 10^{-6} M NAA and 10^{-8} M BAP and found that *Solanum aviculare* cell cultures produced the highest saponin on a combination of 10^{-6} M NAA and 10^{-8} M BAP with more than 8 mg/L cell aggregates was yielded. Whereas other combinations produced only about 1 mg/L cell aggregates. This indicates that the presence of NAA and BAP in the culture determines the sulforaphane content.

The LSD test shows that the combination of 1 ppm NAA and 5 ppm BAP is significantly different from all other combinations in influencing the sulforaphane content in the shoots *in vitro*. The combination of 0 ppm BAP and 0 ppm NAA shows a significant difference to the combination of 0 ppm NAA and 2.5 ppm BAP and the combination of 0 ppm NAA and 5 ppm BAP in controlling the sulforaphane content. Further, 1 ppm NAA that was combined with 0 ppm BAP also shows significant different from the combination of 1 ppm NAA and 2.5 ppm BAP as well as from the combination of 1 ppm NAA and 5 ppm BAP.

The effect of 0 ppm BAP combined with 0 ppm NAA is not significantly different from the combination of 0 ppm BAP and 1 ppm NAA in dictating the content of sulforaphane. Then, the combination of 2.5 ppm BAP and 0 ppm NAA is also not significantly different from treatment with a combination of 2.5 ppm BAP and 1 ppm NAA. But the content of sulforaphane in the combination of 5 ppm BAP and 0 ppm NAA is significantly different from that of 5 ppm BAP and 1 ppm NAA.

B. Shoot Propagation Stage

The result of the experiment is listed in Table II. From the variety combination of methionine and broccoli seed extract, the highest sulforaphane content (182.09 ng) was produced in the combination of 100 mg methionine and 1 g of broccoli seeds extract. This combination was significantly different from all other treatments.

TABLE II
EFFECT OF METHIONINE AND BROCCOLI SEED EXTRACT COMBINATION ON THE CONTENT OF SULFORAPHANE (IN NG/G FRESH WEIGHT OF SHOOTS)

Treatments		Broccoli seed extract (g)			
		0	1	2	3
Methionine (mg)	0	26.05 ab	32.59 bc	23.39 ab	9.58 a
	50	12.68 a	45.22 cd	44.68 cd	54.92 def
	100	162.89 i	182.09 j	47.48 cde	83.07 g
	150	36.20 bc	63.14 ef	69.37 fg	139,32 h
LSD 5%			17.45		

Note: sulforaphane content in ng/g fresh weight of shoots.

The results of LSD 5% indicates that 0 mg methionine in combination with 0 g seed extract were not significantly different from 0 mg methionine combined with 1 g and 2 g of broccoli seed extract but significantly different from treatment with a combination of 0 g methionine with 3 g broccoli seed extract. Sulforaphane content in the treatment of 50 mg methionine in combination with 0 g seed extract was significantly different from the treatment with a combination of 50 mg methionine with 1, 2, and 3 g of broccoli seed extract. But sulforaphane content of 50 mg methionine in combination with 1 g of seed extract was not significantly different from the treatment with a combination of 50 mg methionine with 2 g and 3 g of broccoli seed extract. The sulforaphane content in the treatment of 100 mg methionine combined with 0 g seed extract was significantly different from the treatment in a combination of 100 mg methionine with 1, 2, and 3 g of broccoli seed extract. The combination of 150 mg methionine with 0 g seed extract was also significantly different from the combination of 150 mg methionine with 1, 2, and 3 g of broccoli seed extract. But the content of sulforaphane in adventive shoots in combination treatment with 150 mg methionine with 1 g seed extract was not different from that of 150 mg methionine in combination with 2 g of broccoli seed extract.

The results of LSD 5% test for sulforaphane produced in shoots with treatment without seed extract (0 g seed extract) in combination with 0, 50, 100, and 150 mg methionine were significantly different. From the four combinations the only treatment with 100 mg methionine showed a significant different when compared with results from other combinations. The other three combinations did not show significant different in controlling the sulforaphane production. This was probably due to the administration of plant growth regulators (the 1 ppm NAA and 5 ppm BAP combination from previous experiment) in the growth media that induced the sulforaphane similar to the treatment with 50 mg methionine. The similarity to the treatment of 150 mg of methionine might be due to too high concentration thus suppressed the sulforaphane synthesis. Furthermore, sulforaphane content in the treatment of 1 g seed extract combined with 0, 50, 100, and 150 mg methionine showed significant difference. Then after having tested in LSD 5%, the content of sulforaphane in adventive shoots showed that the combination of 1 g seed extract and 100 mg methionine was significantly different from the combination of 1 g seed extract and 0, 50, and 150 mg methionine. While the content of sulforaphane in shoots for combination of 2 g seed extract and 150 mg methionine was different from the combination of 2 g of extract in the treatment of seeds and 0, 50, and 100 mg methionine. Finally, the content of sulforaphane in adventive shoots from a combination of 3 g seed extract and 150 mg methionine was significantly different from the combination of 2 g seed extract and 0, 50, and 100 mg of methionine (Table II).

It can be concluded that the combination of 100 mg methionine with 1 g seed extract was the best combination with the highest sulforaphane content in adventive shoots (182.09 ng/g of shoot). While for a single provision was in 100 mg methionine (162.89 ng/g of shoot). For the seed extract, it was still smaller than the administration of methionine or methionine combined with broccoli seed extract put in shoot propagation media. However, the treatment of 1 g seed extract to the shoot propagation media induced higher content of sulforaphane compared to the provision of 0, 2, and 3 g of seed extract which produced 32.59 ng/g of plant material (shoots) (see Table II). But the content of sulforaphane from shoots was different from the high concentration of methionine even when combined with the seed extract which produced a high content of sulforaphane. Therefore, the more depressed its growth, the higher the sulforaphane content. These results were still lower than those in field as the results achieved by [12], who found that the content of sulforaphane in the broccoli seeds was 1153 mg per 100 g dry weight and was 10-fold higher than that in mature plants (44 – 171 mg per 100 g dry weight).

C. Plantlet Formation Stage

Quantitative analysis was undertaken to find sulforaphane contained in plantlets. The results showed that the sulforaphane compound varied between 625.54 ng/g and 2,945.16 ng/g of plantlet. The highest average content of sulforaphane was found in combination of 100 mg methionine and 1 g of broccoli seed extract in MS media which was 2901.7461 ng/g plantlets. While the lowest as in the treatment of E1 with the average content of sulforaphane 658.7907 ng/g plantlets (see Table III).

TABLE III
THE AVERAGE SULFORAPHANE CONTAINED IN PLANTLETS

Treatment	Average Sulforaphane Content (ng/g)
E1	658.7907 a
M100	2070.8423b
M100-E1	2901.7461c
LSD 5%	83.84

The analysis of varians showed that the three treatments were significantly different in their effects on sulforaphane content. The LSD 5% test showed that the combination of 100 mg methionine and 1 g of broccoli seed extract was significantly different from the combination of 100 mg methionine and with the treatment of 1 g broccoli seed extract MS media. Provision of a combination of methionine and seed extract was better than the treatment given by a single rate of 100 mg methionine and 1 g extract of media broccoli seeds (see Table III).

IV. CONCLUSION

Our study revealed that the highest sulforaphane content in shoot induction stage was detected in combination 1 ppm

NAA and 5 ppm BAP with the yield 47.76 ng g^{-1} fresh weight of shoot. Whereas in the shoot multiplication stage it was shown that combinations of methionine and broccoli seed extract did influence the sulforaphane content. For inducing the production of sulforaphane we found that the combination of 100 mg methionine and 1 g broccoli seed extract was the optimal one. In stage of plantlet formation the best combination was 100 mg methionine and 1 g broccoli seed extract.

The next study would be focusing on the production of sulforaphane from broccoli calluses using the similar method and 2,4-dichlorophenoxyacetic acid (2,4-D) treatment.

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