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Response of elicitors on glucosinolate biosynthesis in hairy root cultures of *Brassica rapa* subsp. pekinensis

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Chinese cabbage, a popular Cruciferous vegetable are well-known for their health benefits owing to the presence of the biologically active compounds glucosinolates. The aim of this was to explore the elicitors (i.e., ethephon, methyljasmonate, salicylic acid and yeast) effect on GSL biosynthesis of Chinese cabbage hairy root cultures. Five glucosinolates, i.e., glucoerucin, glucobrassicin, 4-methoxyglucobrassicin, gluconasturtiin, and neoglucobrassicin, were detected in the hairy root of studied crop species. The levels of glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin were much higher irrespective of elicitors application. The accumulation of glucobrassicin in the hairy root cultures was 62.8 and 41.9 times higher than those of glucoerucin and gluconasturtiin, respectively. Further, the level of 4-methoxyglucobrassicin was 52.9 and 35.3 folds than those of glucoerucin and gluconasturtiin, respectively. Considering the total and individual levels of glucosinolate especially glucobrassicin and 4-methoxyglucobrassicin varied greatly in response to concentration of yeast extract. Besides yeast extract, other elicitors did not response well for the accumulation of glucosinolate. Yeast extract using at the rate of 5.0 g/L produced the highest level of total glucosinolate giving 13.7, 2.9 and 2.4 times higher than that of the elicitor of salicylic acid, methyljasmonate and ethephon, respectively. The same concentration of yeast extract (5.0 g/L) when used, also produced the highest level of glucobrassicin and 4-methoxyglucobrassicin. The highest level of glucoerucin was found at the treatment of 30 μM ethephon than any other treatments of any elicitors. For getting higher level of glucosinolate from the Chinese cabbage especially its hairy root through different elicitors especially yeast extract at the rate of 5.0 g/Lcould be a valuable alternate approach.

Keywords: Chinese cabbage, elicitors, glucosinolate,hairy root induction

INTRODUCTION

It has been recognized since a long time that plant species of Brassicaceae are the important components of health benefited diets as these contained vitamins of C and A, fiber, folic acid, , different minerals, and several bioactive phytochemicals, especially glucosinolates (Baenas et al., 2012; De Nicola et al., 2013). Intake of cruciferous vegetables containing glucosinolates act a vital role in anti-oxidation and preventing of various cancers, acting as potential chemo preventive agents (Conaway et al., 2002).

Glucosinolates, secondary metabolites containing sulfur and nitrogen, have more than 120 structures. A number of glucosinolates have anti-carcinogenic activities, especially the aliphatic glucoraphanin, the indolic glucobrassicin, and the aromatic gluconasturtiin. Recently, studies have

concentrated on improving health-promoting compounds such as glucosinolates (Kastell et al. 2013). The glucosinolate content in the plant system might be varied depends on several factors like attack through s insect (Birch et al. 1990; Lammerink et al., 1984; Koritsas et al., 1989), by any mechanical damage (Bodnaryk 1994), and infection from fungal (Doughty et al., 1991) and so on. Plant sources secondary metabolities now a day are frequently used in the pharmaceutical industry as a potential drug, in the nutraceuticals and food additives. Secondary metabolites from original plant parts are difficult to extracts and chemical synthesis and also it is not possible to produce in a large scale, For these kinds of complexity, tissue culture techniques could be an alternative way to overcome the said mentioned problem and day by day this tissue culture technique is gaining popularity for large-scale production of desired compound. The transformed root cultures systems have been gained to produce large scale secondary metabolites in most of the medical and other valued crop species and showed a popular technique as their rapid and uniform growth and sustainable and higher capacity to synthesize levels of secondary metabolites compared to synthesis from normal roots (Toivonen 1993; Flores and Medina-Boliver 1995; Doran 1997; Bhagyalakshmi and Ravishankar 1998; Fu et al 1999). Elicitation is a sustainable strategy to enhance secondary metabolites for commercial application. Elicitors may be the compounds of microbial origin or non-biological origin, which could triggered the increased level of production of pigments, flavones, phytoalexins and other defense related compounds upon contact with higher plant cell (Eilert et al., 1984; Robbins et al. 1985; Eilert et al. 1986; Flores, and Curtis 1992; Sim et al., 1994; Bhagyalakshmi and Bopanna 1998; Singh 1999). In this study, we evaluated variations of glucosinolate in the hairy roots of Chinese cabbage using different elicitors detecting by HPLC and ESI-MS.

MATERIALS AND METHODS

Seed sterilization and germination

At the begging the surface –sterilization of *Brassica rapa* subsp. pekinensis seeds were done using 70% (v/v) ethanol for 1 min and then sodium hypochlorite solution at 4% (v/v) was used for 10 min, and after that seeds were rinsed three times in sterilized water. Six sterilized seeds were kept on 25 ml of agar-solidified culture medium in petri dishes of (100 x 15 mm. The basal medium used in this system is Murashige and Skoog (Murashige and Skoog 1962), salts which was solidified with 0.8% (w/v) agar. The medium pH was maintained to 5.8 before adding agar, and then sterilized by autoclaving at 121°C for 20 min. The seeds were allowed to keep in a growth chamber maintaining the temperature at 25°C for germination under standard cool white fluorescent tubes with a flux rate of 35 μmol s^{-1} m^{-2} and a 16-h photoperiod.

Growth of Agrobacterium rhizogenes

Seven *A. rhizogenes*strains R1000were cultured from glycerol stocks. They were allowed to stay overnight at 28°C by shaking (180 rpm) in liquid Luria-Bertani medium, to a mid-log phase of OD_{600} = 0.5. The *A. rhizogenes* cells were cultured by centrifugation for 10 min at 224 ×*g* and re suspended in liquid MS medium having30 g/l sucrose. *A. rhizogenes* cell density was adjusted to give an A_{600} of 1.0for inoculation.

Hairy root culture establishment and application of elicitor treatment

Leaves of *Brassica rapa* subsp. Pekinensis were used for establishing hairy roots which was already grown with special care *in vitro* culture condition. Leaves were cut at the ends into sections of 7 × 7 mm. The pieces of leaf were put into a culture of *A. rhizogenes* strain of R1000 in the liquid inoculation medium for 10 min, and then blotted dry on sterile filter paper, and there after incubated in the dark condition maintaining a temperature of 25°C on agar-solidified MS medium. The tissue explants were transferred 2 days after co-cultivation to the hormone-free medium having a complete mixture of MS salts and vitamins, 30 g/l sucrose, 500 mg/l cefotaxime, and 8 g/l agar. A huge number of hairy roots were observed from the wound sites of the explants within 2 weeks. Immediately after emerging hairy roots from the systems, they were collected from the explant and then again sub-cultured in the dark condition at 25°C on agar-solidified MS medium. Rapidly growing hairy root cultures were obtained after repeated transfers to fresh medium. After collecting the roots, they were transferred at the rate of 0.5 g dry weight [DW]/l to 30 ml of MS liquid medium, having 30 g/l sucrose, in 100 ml flasks. Root were cultured maintaining the temperature of 25°C on a gyratory shaker (100 rev/min) in a growth chamber following standard cool white fluorescent tubes with a flux rate of 35 μmol $s^{-1}m^{-2}$ and a 16-h photoperiod.

Four different elicitors with different concentration i.e., ethephon (0, 10, 30, 50, 100, and 200 μM), methyl jasmonate (0, 10, 50, 100, 300, and 500 μM), salicylic acid (0, 0.01, 0.05, 0.1, 0.5, and 1 mM) and yeast extract (0, 0.1, 0.5, 1, 3, and 5 g/L) were used. Immediately after preparation of individual concentration elicitor was applied to the culture medium for 72 h. The hairy root cultures were kept in a shaking incubator at 100 rpm maintaining temperature of 25℃. Each elicitor treatment with each concentration was used three flasks and the experiment was repeated three times. Hairy roots grown with each elicitor treatment were harvested after three days of cultivation and then frozen in liquid nitrogen and stored at -80℃ until further experiment

Glucosinolate extraction and HPLC analysis

Extraction for glucosinolates was done following the procedures reported previously with slight modification (Kim et al. 2013). An amount of 100 mg freeze-dried sample of Chinese cabbage was extracted following the protocol i.e. 1.5 mL of 70% (v/v) boiling aqueous methanol at 70°C for 5 min in a water bath and then centrifuged at 12,000 rpm at a temperature of 4°C for a period of 10 min. Collecting the supernatant into a 15-mL conical tube and the resultant pellets were re-extracted two times using the same procedure. The collected supernatants (the crude glucosinolate extracts) were then carefully loaded onto a mini-column having DEAE-Sephadex A-25 and then rinsed with 3 ml of distilled water. The eluate was then desulfated using 75 μL of purified arylsulfatase and incubated at room temperature overnight. Thereafter, desulfated glucosinolate samples were again eluted using 0.5 mL (×3) of ultrapure water keeping in a 2.0-mL safe-lock micro centrifuge tube and passed through 0.22-μm PTFE syringe filters into a brown vial. HPLC (Agilent Technologies 1200 series) system, equipped with an Inertsil® ODS-3 column (150 × 3.0 mm i.d., particle size 3 μm; GL Sciences, Tokyo, Japan) and an Inertsil® ODS-2 guard column (10 × 2.0 mm i.d., particle size 5 μm), was used to separate glucosinolate contents. HPLC conditions were as follows: detection wavelength, 227 nm; oven temperature, 40°C; flow rate, 0.2 mL/min^{-1}; injection volume, 20 μL. The gradient program used in the system was as follows: Solvent A, HPLC grade water; Solvent B, HPLC grade acetonitrile; 0–18 min, 7%–24% B; 18–32 min, 24% B; 32.01 min, rapid drop to 7% B; and 32.01–40 min, 7% B (total 40 min). Each glucosinolate was identified and calculated following the information like HPLC retention times, HPLC areas, and response factors compared with the standard, sinigrin used in the systems (0.5 mg/5ml) (ISO 9167-1) [ISO, 1992].

RESULTS

Four elicitorsi.e. ethephon, methyljasmonate, salicylic acid and yeast extract with different concentrations were used to investigate their response on variation of glucosinolate contentin hairy root cultures of Chinese cabbage. Five different glucosinolates, i.e., glucoerucin, glucobrassicin,4-methoxyglucobrassicin, gluconasturtiin, and neoglucobrassicin were detected in the hairy root cultures of Chinese cabbage (Table 1). Total as well as individual amount of glucosinolate especially glucobrassicin and 4-methoxyglucobrassicin varied highly in response to yeast extract. Besides yeast extract, other elicitors did not response well for the accumulation of glucosinolate. The total glucosinolate content was the highest when treated with yeast extract at 5.0 g/L achieving 13.7, 2.9 and 2.4 times higher than that of the using highest level of elicitor of salicylic acid, methyl jasmonate and ethephon, respectively. Of the five glucosinolates, glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin contents were considerably much higher. The accumulation of glucobrassicin in the hairy root cultures was 62.8 and 41.9 times higher than those of glucoerucin and gluconasturtiin, respectively. Further, the level of 4-methoxyglucobrassicin was 52.9 and 35.3 folds than those of glucoerucin and gluconasturtiin, respectively. The trend of accumulation of glucosinolates was not same for different concentration of elicitors.In case of yeast extract, most of the cases the highest concentration responded well for higher accumulation of glucosinolates. The content of glucobrassicin was the highest when5.0 g/L yeast extract was applied. Yeast extract at 5.0 g/L treatment produced glucobrassicin 83.8, 35.9, 17.1 times higher compared to the lowest amount of glucobrassicin from salicylic acid, methyl jasmonate and ethephon, respectively. Similarly, the treatment yeast extract at 5.0 g/L gave the highest level of 4-methoxyglucobrassicin showing 23.5, 2.4 and 1.8 times higher than that of glucobrassicin from salicylic acid, methyljasmonate and ethephon, respectively.

Table 1. Effect of elicitors on glucosinolate accumulation (μmol/g dry wt.) in hairy root cultures of Chinese cabbage.

Elicitors	Concentration	Glucoerucin	Glucobrassicin	4-Methoxyglucobrassicin	Gluconasturtiin	Neoglucobrassicin	total
Ethephon	Control	0.12 ± 0.00	0.54 ± 0.01	5.90 ± 0.02	0.18 ± 0.01	7.78 ± 0.01	14.52 ± 0.05
	10 μM	0.15 ± 0.01	0.45 ± 0.01	5.02 ± 0.07	0.14 ± 0.00	3.83 ± 0.02	9.59 ± 0.12
	30 μM	0.28 ± 0.01	0.54 ± 0.01	5.64 ± 0.10	0.12 ± 0.01	4.68 ± 0.04	11.26 ± 0.17
	50 μM	0.19 ± 0.01	0.50 ± 0.01	5.04 ± 0.05	0.11 ± 0.00	4.15 ± 0.02	9.98 ± 0.08
	100 μM	0.13 ± 0.00	0.44 ± 0.01	3.92 ± 0.00	0.10 ± 0.00	4.54 ± 0.00	9.12 ± 0.02
	200 μM	0.09 ± 0.00	0.49 ± 0.00	3.46 ± 0.03	0.11 ± 0.00	5.01 ± 0.02	9.15 ± 0.06
Methyl jasmonate	10 μM	0.15 ± 0.01	0.29 ± 0.01	3.81 ± 0.01	0.09 ± 0.01	4.96 ± 0.12	9.31 ± 0.16
	50 μM	0.10 ± 0.01	0.24 ± 0.01	2.85 ± 0.02	0.08 ± 0.01	3.77 ± 0.05	7.05 ± 0.09
	100 μM	0.10 ± 0.01	0.26 ± 0.01	2.70 ± 0.00	0.08 ± 0.01	3.84 ± 0.03	6.98 ± 0.05
	300 μM	0.13 ± 0.01	0.31 ± 0.01	3.03 ± 0.01	0.10 ± 0.00	5.03 ± 0.04	8.61 ± 0.07
	500 μM	0.09 ± 0.00	0.21 ± 0.01	2.84 ± 0.04	0.09 ± 0.00	4.08 ± 0.03	7.31 ± 0.08
Salicylic acid	0.01 mM	0.12 ± 0.01	0.34 ± 0.01	3.09 ± 0.00	0.08 ± 0.00	4.40 ± 0.04	8.02 ± 0.06
	0.05 mM	0.17 ± 0.01	0.46 ± 0.01	3.96 ± 0.04	0.10 ± 0.00	5.62 ± 0.03	10.31 ± 0.09
	0.1 mM	0.15 ± 0.01	0.26 ± 0.01	5.01 ± 0.08	0.10 ± 0.00	5.63 ± 0.03	11.16 ± 0.13
	0.5 mM	0.19 ± 0.01	0.22 ± 0.01	2.98 ± 0.06	0.07 ± 0.00	3.96 ± 0.03	7.42 ± 0.10
	1.0 mM	0.00 ± 0.00	0.09 ± 0.00	0.27 ± 0.02	0.00 ± 0.00	1.21 ± 0.01	1.58 ± 0.03
Yeast extract	0.1 g/L	0.12 ± 0.01	0.75 ± 0.02	6.23 ± 0.07	0.14 ± 0.00	7.78 ± 0.03	15.03 ± 0.12
	0.5 g/L	0.10 ± 0.01	0.63 ± 0.01	5.60 ± 0.01	0.15 ± 0.00	5.03 ± 0.02	11.51 ± 0.05
	1.0 g/L	0.14 ± 0.02	1.19 ± 0.02	5.91 ± 0.07	0.15 ± 0.00	5.87 ± 0.02	13.26 ± 0.14
	3.0 g/L	0.15 ± 0.01	2.73 ± 0.03	4.09 ± 0.08	0.10 ± 0.00	6.23 ± 0.02	13.30 ± 0.14
	5.0 g/L	0.11 ± 0.01	7.54 ± 0.04	6.35 ± 0.07	0.14 ± 0.00	7.46 ± 0.02	21.60 ± 0.14

The values are mean with standard deviation.

The range of glucoerucin content was 0.09 to 0.28 µmol/g dry wt among the elicitors treatments. No glucoerucin was detected at 1.0 mM concentration of salicylic acid. The highest level of glucoerucin was found at the treatment of 30 µM ethephonthan any other treatments of any elicitors.The level of gluconasturtiin content ranged 0.07 to 0.18 µmol/g dry wt among the concentrations of different elicitors, where no gluconasturtiin was detected at 1.0 mM concentration of salicylic acid. Here the highest level of gluconasturtiin was found at the control treatment, it means no elicitors responded positively for the accumulation of gluconasturtiin. Among the elicitors treatment, the amount of neoglucobrassicin ranges 1.21 to 7.78 µmol/g dry wt where the highest level was detected from control and from the treatment of 0.1 g/L yeast extract. The variation among the treatment was much higher compared to any other glucosinolate. The highest level was 6.4 and 2.1 times higher than that of the lowest content of neoglucobrassicin from salicylic acid and methyl jasmonate, respectively.

Stimulating secondary metabolites through elicitation is considered one of best strategies that currently is practicing for commercial production of any secondary metabolites. Secondary metabolites accumulation from either parts of mother or transformed plants is greatly dependent on their sources of origin; however, it might be influenced by the treatments especially using phyto hormones and elicitors, and also depend on environmental factors. Elicitors of either biotic or abiotic origin, when contact with the cells of higher plants trigger to increase production of pigments, flavones, phytoalexins, and other defense related compounds (Flores, and Curtis 1992; Sim et al., 1994; Bhagyalakshmi and Bopanna 1998; Singh 1999). From a previous study reported by Uddin et al., 2010 that among the elicitors used their experiments, cellulose stimulated higher sorgoleone production achieving 6.2 times more sorgoleone compared to untreated control. In this study, among the elicitors, yeast extract performed the best for glucosinolates accumulation in the hairy root of Chinese cabbage. However, there was no definite concentration those lead either the highest or the lowest amount of accumulation. Previously, it was reported that IAA at lower concentration boasted up for the highest amount of glucosinolates in the hairy root cultures of broccoli[23].From a previous study Bong et al., 2015 reported that auxins and wounding had a positive response for the accumulation of biologically active compounds glucosinolates. They also mentioned that five different glucosinolates, were detected in the hairy root cultures of Chinese cabbage, where the concentrations of neoglucobrassicin and 4-methoxyglucobrassicin were considerably higher than those of other glucosinolates in response to both Auxin and wounding. Here in this study we also observed the same accumulation pattern of glucosinolate in response to concentration of elicitor.The content of secondary metabolites in any part of a plant, either a mother plant or transformed plant, largely depends on their source of origin, but it can also be affected by treatment with growth regulators, hormones, elicitors, wounding, and even by environmental factors. It was mentioned that chlorogenic acid responded well for the highest accumulation after the treatment like wounding in different species like Romaine, Butter leaf, and iceberg lettuce cultivars (Toma´s-Barbera´n et al., 1997; Vargas and Saltveit 2002), whereas no significant change was occurred by exposure of non-wounded leaves with concentrations of methyl jasmonates (Vargas and Saltveit 2002). Wounding had the positive role for the accumulation of phenolic compounds (Hyodo et al., 1978; Dixon and Paiva, 1995; Ke and Saltveit 1989). A positive effect of different biotic and abiotic elicitors was observed on the production of betalain in the hairy root cultures of *Beta vulgari* (Savitha et al., 2006), *Saccharomyces cereviseae*, a yeast elicitor increased the production of berberine by 4-folds in *Thalictrum rugosum*. Elicitation enhanced 3-fold anthocyanin in cultured cells of *Daucus carota* (Rajendran et al., 1994). Elicitation activities were studied in *Tagetes patula* cultures (Buitelaar et al.,1992; Buitelaar et al. 1993) where it was found as high as 4-fold increase over the control (Mukundan and Hjortso 1990). Secondary metabolites those are involved in plant defense functions have significant elicitation for external physical, chemical and biological stimuli.

CONCLUSION

Now a day's invitro hairy root culture express a new dimension for the production of secondary metabolites. This technique might d be a valuable alternative approach for the production of health benefitting secondary metabolites, especially the compound of glucosinolate from Chinese cabbage. Elicitors especially yeast extract at 5.0 g/L concentration influenced for having higher

level of glucosinolates in the hairy roots of Chinese cabbage, whereas other elicitors used in this study did not perform well for the accumulation of glucosinolate. We are giving more emphasis in our laboratory for further improving accumulation of secondary metabolites and especially glucosinolate compounds from hairy root cultures of Chinese cabbage using other treatments.

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CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

SUP designed the experiments and also wrote the manuscript. JKK, and SJB performed hairy root culture, HPLC analysis, and data analysis. All authors read and approved the final version.

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