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RESEARCH ARTICLE

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Elicitors Induced Sulforaphane Production in *Lepidium draba*

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Abstract

Lepidium draba, a weed of the Brassicaceae family contains high level of glucoraphanin that can be produced sulforaphane (SFN) through myrosinase hydrolysis activity. This study was conducted to optimize conditions for SFN production in this plant; several factors including age of seedlings and calluses as well as application of different kind of elicitors such as methyl jasmonate and salicylic acid (pH=7), and also Cu^{2+} and Zn^{2+} (pH=5 and 7) were examined. The results showed that the highest amount of SFN was achieved in 7-day-old seedlings and 14-day-old calluses, but more significantly in the seedlings. In general, elicitation of the 7-day-old seedlings was associated with a clear improvement in SFN content, but more drastically with metals-treated under acidic condition. In the metals-treated seedlings, it seems that the promotion of SFN content is due to the more adsorption of the ions under the acidic condition and subsequently induced the glucoraphanin biosynthesis pathway.

Keywords: Glucoraphanin; Isothiocyanate; *Lepidium draba*; Sulforaphane

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INTRODUCTION

Whitetop (*Lepidium draba* L.), a noxious weed, belongs to the Brassicaceae family. It is a perennial plant, native to South Europe which now wide spread throughout most of Europe. This wild-growing weed extensively found along roadsides, meadows and in fields [1]. It has been shown that this plant like the other Brassicaceae genus contains glucosinolates, a unique group of secondary metabolites containing sulfur and nitrogen. So far, more than 100 kinds of these metabolites have been identified [2; 3]. Glucosinolates were found to have a defensive role and classified in two classes: phytoanticipins and phytoalexines [4]. These metabolites that accumulate in the cell vacuoles, any injuries or pathogens attack leads to release them and expose to myrosinase (β-thioglucoside glucohydrolase, EC. 3.2.3.1) [5]. Hydrolysis of glucosinolates by myrosinase produce glucose and an unstable intermediate molecule (aglycon) which can be converted to thiocyanates, isothiocynates and nitriles spontaneously, depending on environmental conditions [5]. The pharmaceutical properties of nitriles and thiocyanates have not yet clear understood, and little is known about the pharmacological properties of the glucosinolates. Isothiyocynates exhibit various pharmacological activities and are of interest to the researcher [6–8]. SFN (4-(methylsulfinyl)butyl isothiocyanate) is one of the most important isothiocyanate which can be produced through glucoraphanin hydrolysis via myrosinase activity [2]. The most therapeutic effect of this isothiocyanate is antioxidant properties [7], anti-bacterial effects on *Helicobacter pylori* [6], apoptosis induction in cancer cells [9], anti-metastatic [10] and anti-angiogenesis properties [11].

However, large quantities of glucoraphanin can be found in broccoli [12] and withetop [13]. Despite the other members of the Brassicaceae family containing several types of glucosinolates, *L. draba* contains two major types of glucosinolates: glucoraphanin and glucosinalbin [13]. Hence, this weed could serve as a source of glucoraphanin extraction [1]. Moreover, extraction of glucoraphanin, as the precursor of SFN has been optimized in this weed [13].

While plenty of literatures have been focused on SFN production from other members of the Brassicaceae such as *Brassica oleracea* and *Raphanus sativus* seedlings as well as *in vitro* cell lines [2; 14], few studies have been only focused on the biological activities and analysis of volatile constituent of *L. draba* [1; 15]. Up to now, there have been no reports on the SFN production in *L. draba* cell culture and also stimulation of glucosinolates biosynthesis in this plant.

However, there are several aspects in order to improve production of desirable plant secondary metabolites including use of different stimulator, optimize culture condition and select suitable seedling or cell line [16–18]. Therefore, the present study was conducted to optimize conditions for increasing SFN content in *L. draba*. To acquire the optimum conditions, effects of several factors including age of seedlings and calluses as well as influences of various concentrations of biotic and abiotic elicitors at different elicitation time were analyzed.

MATERIALS AND METHODS

Materials, reagents and standard

Seeds of matured *L. draba* were collected from around Kerman province, Iran, during May to June 2012. SFN standard was purchased from Sigma and all other chemicals were of analytical reagent grade and obtained from Merck.

Seed culture and plant growth

The seeds were surface-sterilized by subsequent washing with detergent, ethanol (75%, 30 s), sodium hypochlorite (3% for 10 min), with rinsing with sterile distilled water intervals. In order to find optimal seedlings age that able to produce high level of SFN; thirty seeds were placed on the surface of solidified basal MS media [19] containing 0.8% agar in Petri dishes, with about 5 mm spacing between them. The plates were incubated at controlled temperature of 28±2 ºC, with 16 hours photoperiod and relative humidity of 60-65% for 3, 7,11 and 15 days.

Seedlings treatment

Different concentrations (0 (as a control), 4, 8 and 16 µM) of $CuSO_4$ and $ZnSO_4$ -as an abiotic elicitors- were dissolved in distilled water. All concentrations were prepared with both pH 7±0.2 and 5±0.2. Around 60 seedlings were separated from their medium and washed thoroughly using distilled water and subjected to different concentrations of the elicitors (50 mL) in 250 mL Erlenmeyer flasks. The flasks were shaken at 100 rpm on an orbital shaker at room temperature for 8 and 16 hours. The different elicitor concentrations and time of treatment were selected according to previous studies in our lab. Additionally, the SFN production level was also analyzed, in 7-day-old seedlings which elicited with various concentrations (0 (as a control), 1, 5, 10, 20, 40 µM) of methyl jasmonate (MJ) and salicylic acid (SA) as biotic elicitors at neutral pH (7±0.2) for 24 and 48 hours. To remove surface elicitors, the treated seedlings were rinsed several times using sterile distilled water and immediately frozen in liquid nitrogen, and stored at -80 ºC until use.

Callus culture

Calluses were induced from cotyledon explants of *in vitro* grown 7-day-old *L. draba* seedlings. The seedlings were grown as mentioned above. The explants were proliferated in solidified basal media of MS

supplemented with different hormone (4 mg/L of BAP, 1 mg/L of NAA and 0.2 mg/L of 2, 4-D). Culture vessels were transferred to darkness at controlled temperature of 26±2 °C, allowing growth of the calluses. The 7-day-old calluses were subcultured in a fresh medium containing similar hormone concentration as above. The calluses were harvested at 7-day intervals (at 7, 14 and 21 days) and were washed thoroughly using distilled water. Then the SFN content of the calluses were measured as described at bellows.

Determination and quantification of SFN

SFN extraction and quantification was carried out according to the method described by Liang et al. [20] with little modifications. Briefly, 0.5 g of fresh tissue (frozen sample) was grinded into powder using a mortar and pest. The resultant powder was mixed with 1 mL acidic water (pH=5) and incubated at 42±2 °C for 2 hours. Afterward, 5 mL acetonitrile was added to the mixture and ultrasonicated for 3 min then the homogenous solution was centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant was filtered using 0.2 µm syringe filter prior to injection into the column (C_{18}; 250×4.6 nm) of High Performance Liquid Chromatography (HPLC) (Agilent 1100 series, USA) in order to separate SFN.

Condition for HPLC was as follows: solvent system; acetonitrile/H_2O (65/35 v/v); flow rate, 1 mL/min. Identification of SFN in the seedlings was achieved by comparison of retention times with authentic standard that was detected at 254 nm.

Statistical analysis

Experiments were conducted over three independent stages with completely random designs. The results were represented as mean values ± Standard Deviation (SD). Duncan´s multiple range tests were used to compare mean value of the data at P≤0.05 using SAS 9.1.3 (service pack 4, version= 6.1.7601) software. Significance of the difference mean values was determined by one-way analysis of ANOVA variance.

RESULTS

SFN content in different ages of seedlings and calluses

The retention time for the standard SFN was about 7 minutes after injection it into the column (Figure 1) and a similar peak was also observed for the control and treated samples (data not shown). The highest amount of SFN was seen in 3- and 7-day-old seedlings and declined drastically thereafter; no chromatogram peak was seen at higher age more than 7-day-old seedlings (Figure 2A). Due to higher growth of the 7-day-old seedlings compared to 3-day-old ones, further experiments were only performed on them.

SFN content elevated by the age of calluses and reached to the highest amount at 14-day-old calluses, and then it significantly decreased at 21-day-old calluses (Figure 2B).

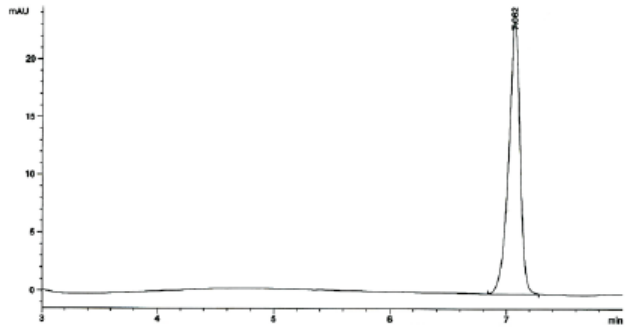

Figure 1: Chromatogram of the standard SFN. As indicated the retention time for the standard SFN is about 7 minutes after injection

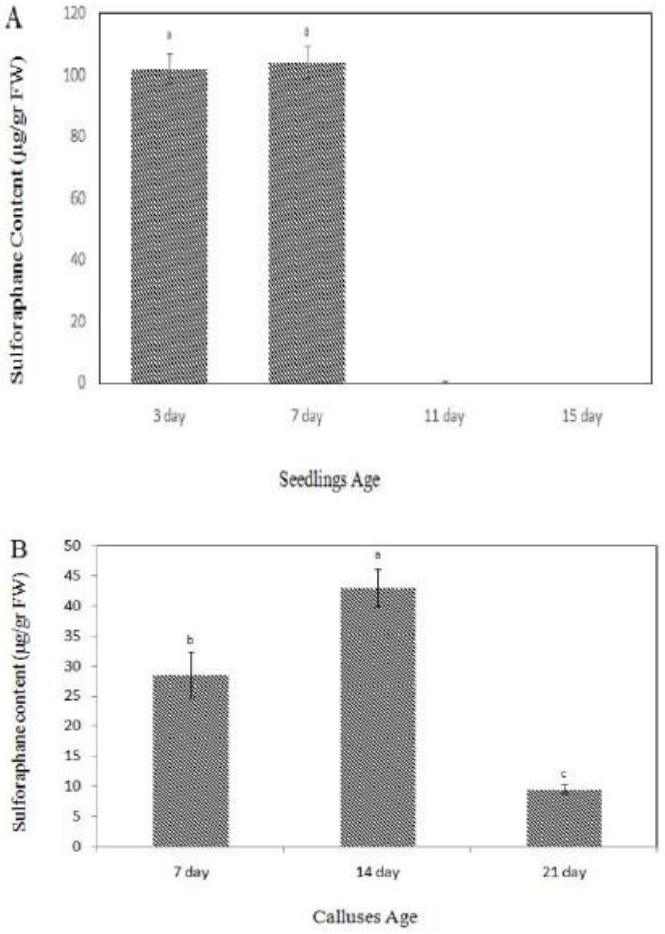

Figure 2: Comparison of SFN content in different age of seedlings (A) and calluses (B). Different letters indicate significant differences at p<0.05 according to Duncan's Multiple Range Tests. Bars represent one standard deviation of the mean (n = 3 replicates)

The effects of metal elicitors on SFN content

SFN content had no significant changes in treated seedlings with copper under neutral pH condition for the both elicitation times (Figure 3A). While under the acidic condition (pH=5), SFN exhibited around two-fold changes in content at the highest elicitor concentration (16 µM) after 8 hours treatment compared to that of

the control. In addition, after 16 hours elicitation, a dramatic increase in the SFN content was observed at elicitor concentrations more than 4 µM (Figure 3A). The maximum amount of SFN reached about 235.5 µg/g fresh weight, in presence of 8 µM Cu^{2+}, which was about 2-fold that of the control (117.6 µg/g FW).

The SFN content in Zn-treated seedlings under the neutral pH, was similar to that of the control for the both elicitation times (except at 16 µM concentration after 8 hours treatment). But under the acidic condition (pH=5), SFN content was significantly elevated at 8 hours treatment, with the elicitor concentrations more than 4 µM. Nevertheless, by the increase elicitation time up to 16 hours a remarkable increase in SFN content was observed at all concentrations compared to the control (Fig. 3B). It is quite obvious that the SFN content drastically elevated with Zn^{2+}concentration up to 8 µM, hitting a peak (348.2 µg/g FW) that was about 3.5-times that of the control (117.6 µg/g FW) (Figure 3B).

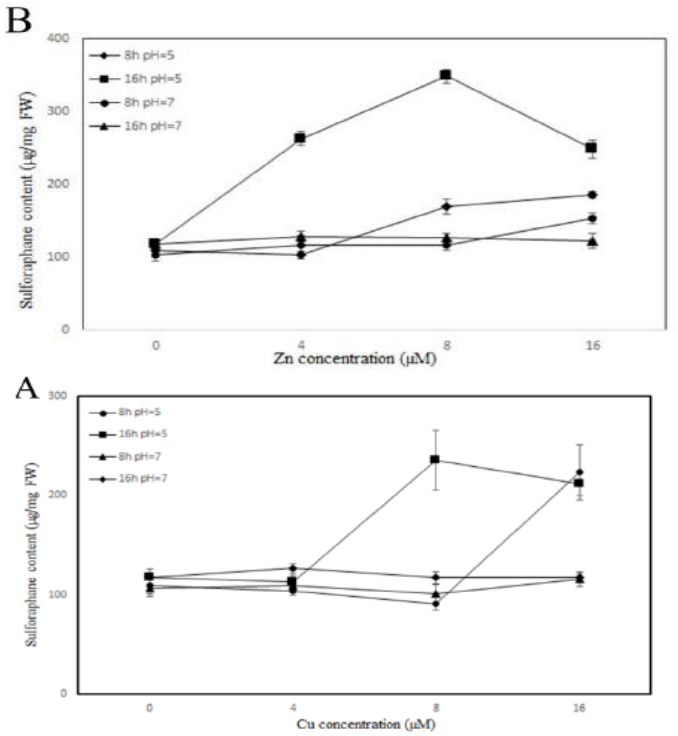

Figure 3: The effects of different concentrations of Cu (A) and Zn (B) elicitors at different pH and time intervals on SFN content in 7-day old seedlings. Different letters indicate significant differences at $p<0.05$ according to Duncan's Multiple Range Tests. Bars represent one standard deviation of the mean (n = 3 replicates).

The effects of MJ and SA on SFN content

The effects of MJ and SA on SFN content was dependent on the elicitor doses and also time of elicitation (Table 1). As revealed by the data, SFN content was similar to that of the control after 24 hours treatment with MJ (except at 40 µM treatment that its content significantly increased compared to the control). But by the increase elicitation time up to 48 hours, a drastic increase in production of this effective ingredient was observed at elicitor concentrations more than 5 µM (Table 1).

As shown in the Table 1, at all SA concentrations (except for the highest ones), the SFN content was promoted and the highest amount was achieved at 10 µM. While after 48 hours treatment, the SFN content drastically roses at concentrations more than 10 µM SA; its content in 40 µM-SA-treated seedlings (103.98 µg/kg FW) were approximately 1.5 fold that of the control (66.93 µg/kg FW).

Table 1. The effects of various concentrations of MJ and SA on SFN content in 7-day-old seedlings.

Concentration (µM)	MJ		SA	
	24h	48h	24h	48h
0	72.5 ± 6^{b}	66.9 ± 11^{c}	72.5 ± 6^{c}	66.9 ± 11^{b}
1	82.9 ± 9^{b}	65.8 ± 9^{c}	89.6 ± 8^{b}	76.3 ± 14^{b}
5	72.8 ± 14^{b}	65.2 ± 2^{c}	82.9 ± 14^{b}	60.2 ± 17^{b}
10	70.8 ± 7^{b}	76.1 ± 7^{b}	123.4 ± 14^{a}	69.7 ± 17^{b}
20	73.5 ± 2^{b}	88.4 ± 3^{a}	92.4 ± 14^{a}	98.4 ± 16^{a}
40	103.3 ± 8^{a}	85.0 ± 1^{a}	71.8 ± 2^{c}	103.9 ± 14^{a}

Table 1: Data show mean ± SD, n = 3 replicates. In each group, different letters indicate significant differences at $p<0.05$ according to Duncan's Multiple Range Tests.

DISCUSSION

SFN is an isothiocyanates which is produced through enzymatic hydrolysis of glucoraphanin [21]. It is of interest to researcher due to its anti-cancer effects [8[. In the present study, SFN production level was investigated in *L. draba* seedlings as well as in the cell cultures. Our results showed that, the production of SFN correlated with seedling age, as its content reached a maximum of 104 µg/g FW in *L. draba* seedlings after 7 days and thereafter drastically decreased. This is consistent with a previous report on broccoli [22]. They showed the highest amount of SFN was obtained at three days broccoli seedlings. It has also reported that, the SFN content had a decrease trend during the growth of broccoli seedlings until mature plant [23]. These results were surprisingly consistent with the finding in calluses excrement. As shown in Fig. 2B the calluses SFN content promoted by age up to 14-day and thereafter decreased.

Comparison between the highest amount of SFN in the seedlings and calluses revealed that it is more produced in the seedlings, consistent with those reported by Redovnikovi et al. (2008). They showed that total glucosinolates content in horseradish seedlings was more than teratoma and tumor [24].

Since, more SFN content was obtained by the seedlings; production of this compound was investigated in 7-day-old seedlings which treated with different kind of elicitors. Elicitors are stable molecules which induce

plants defense response [25]. However, it has been shown that this weed can accumulate several heavy metals like Zn, Cu, Cd and Ni [26]. Among the mentioned metals, Zinc and copper are essential microelements for the growth and development of plants [27]. Furthermore, it has been established that excessive accumulation of heavy metals (such as Zn and Cu) in the plant tissues lead to trigger of reactive oxygen species (ROS). ROS were found to alter some vital processes involved in photosynthesis, chlorophyll synthesis and cell membrane integrity [28]. Therefore, we analyzed the effects of various concentrations of zinc and copper ions as abiotic elicitors on SFN production level in 7-day-old seedlings at different time intervals and pH condition. The SFN content reached to maximum at 16 hours elicitation with the both metals under the acidic condition (pH=5). These observations can be attributed to the more uptake of these metals by the plant under the acidic condition compared to the neutral pH [29]. Under acidic condition (pH=5) and elicitation time of 16 hours the optimal concentration for the both elicitors was 8 µM among the tested concentration (Fig. 3) and higher concentration caused to decrease in SFN content. These finding can be attributed to the toxic effects of these ions on the seedlings when exposed to the high concentrations for long time, as revealed by the increase activity of the key ROS scavenging enzyme (data not shown).

However, it has been demonstrated that ROS (especially H_2O_2) can induce plant defense system directly or through mediated by signaling molecules (such as JA and SA) which lead to increase secondary metabolites [30]. Hence, it may be speculated, elevated of SFN content resulted in induction of ROS and/or signaling molecules such as JA and/or SA under treatment seedling with the metals.

Moreover, it has been shown that JA and SA have critical role in activation of plant defense responses and induction of secondary metabolites in several plant species [31; 32]. In this study, MJ and SA as biotic elicitors also lead to elevated level of SFN production (Table 1). This observation is consistent with those reported previously [27; 33–36]. They showed application of MJ or SA as a elicitor increased the amount of glucosinolates in some *Brassica* species [27; 33].

In addition, there have been some indications that showed the similar effects of heavy metals and jasmonate on induction of the genes expression involve in defense responses [37]. It has been reported that increasing glucosinolates level after treatment with MJ and SA is related to their effects on expression of the genes involve in their biosynthesis [38]. Hence, based on the defensive role of glucosinolates and their derivatives in the plant [32], it may be suggested that the promotion of SFN content in elicited seedlings are attributed to influence of the key genes involve in glucoraphanin biosynthesis (precursor of SFN**).**

Interestingly, among different elicitors, the SFN production level extremely promoted by Zn and reached in high level after 16 hours of treatment in presence of 8 µM under the acidic condition (pH=5). This finding may be related to presence of many myrosinase isoforms in the plant, and zinc acts as a cofactor for them. It has been confirmed that in some plant species (such as broccoli) several isoforms of the enzyme use zinc as cofactor [39].

In our knowledge, there have been no reports regarding the effects of any factors on SFN production in this wild-growing plant containing high level of glucoraphanin which make its extraction and purification easier among two glucosinolates [13].

Overall, the experimental results showed that Zn was an effective elicitor for the induction of SFN in *L. draba* seedling. Furthermore, the timing of seedlings and calluses harvesting could be critical factors in large-scale productivity. In addition, the data also revealed that the kind of elicitors, doses of stimulator and time of elicitation are the critical items toward establishing large-scale of this active ingredient. In conclusion, although finding optimal conditions for SFN production is the main step to obtain large-scale productivity.

Further studies are required to optimize all conditions (including, other biotic and abiotic elicitors and elicitation time) for inducing SFN production in this medicinal plant with high level of glucoraphanin.

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