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Sulforaphane Production and Antioxidant Enzyme Activity in *Lepidium draba* Seedlings in Presence of Yeast Extract and Silver Nitrate

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Abstract

Sulforaphane is produced through a hydrolytic reaction catalyzed by myrosinase from glucoraphanin. It has recently attracted researcher's interest due to its anticancer activities. The present study was conducted to evaluate the effects of various concentrations of yeast extract (YE) and silver ion (Ag^+) on sulforaphane production level in *Lepidium draba* seedlings. Our results revealed that the sulforaphane content is increased in seedlings that treated with the both elicitors, but more significantly by Ag^+. All elicitor influences are depend on the elicitor dose and time of elicitation and maximized with YE at 1 mg/mL for 8 hours and with Ag^+ at 50 µM for 4 hours treatment. It seems that the promotion of sulforaphane content results in induction of glucoraphanin biosynthesis pathway which in turn is mediated by H_2O_2, as revealed by a significant increase in the activity of H_2O_2 scavenger enzymes.

1. Introduction

Glucosinolates are a unique group of secondary metabolites which exclusively can be found in dicotyledonous plants species, particularly the Brassicaceae family. These metabolites containing sulfur and nitrogen (Brader *et al*., 2006; Fahey *et al*., 2001), and have important role in plants resistance against insects and pathogens (Halkier., 2006). These metabolites accumulate in vacuoles of myrosine cell and after cell disruption they contact with a β-thioglucoside glucohydrolyase enzyme (myrosinase, EC. 3.2.3.1) and then glucose and an unstable intermediate (aglycone) are produced. Aglycone will be rearranged into some other compounds such as isothiocyanates, thiocyanates and nitrils depending on pH, temperature and presence of different ions (Rask *et al*., 2000) Among all the mentioned compounds, isothiocyanates have shown various pharmaceutical effects including anticarcinogenic and anticancer activity (Grubb *et al*., 2006; Mithen *et al*., 2000). Wide ranges of glucosinolates (more than 100) are found that can be converted into a specific type of isothiocyanate. Sulforaphane (SFN) is a natural isothiocyanate which is produced through hydrolytic breakdown of glucosinolate glucoraphanin. This glucosinolatecan be found in Broccoli (*Brassica oleraceaL.var*) and whitetop (Lepidium draba L.) in large amounts (Zhang 2004). However, plenty of studies have been focused on induction of glucosinolates biosynthesis and their derivatives in some plant species that contain several types of these metabolites (Liang *et al*., 2006; Fritz *et al*., 2010; Zhao *et al*., 2005). Elevation of glucosinolates and derivatives levels have been extensively studied particularly in some *Brasica* species after treatment with different elicitors (Mohammadi *et al*., 2014; Pérez-Balibrea *et al*., 2011; Aminizadeh *et al*., 2014; Schreiner *et al*., 2011; Wiesner *et al*., 2013; Smetanska *et al*., 2007; Sun *et al*., 2012).Two groups of elicitors including biotic and abiotic are molecules that induce signal triggering to produce and accumulate certain secondary metabolites in the intact plants or tissue cultures (Wu., 2004). Whitetop, a member of the *Brassicaceae* family is a noxious weed widely grown in Eastern Europe and Asia especially in Middle East (Radonić *et al*., 2011; Zargari, 1995; Zhang.,

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2004). In contrast to other genus of this family, this weed contains only two major types of glucosinolate; glucoraphanin and glucosinalbin (Powell *et al*., 2005). However, few studies have so far been focused on the effect of different elicitors on induction of glucosinolates in this plant. Therefore, in this study, SFN production level is investigated in *L. draba* seedlings in the presence of the two well-known stimulators; YE and Ag^+. YE, which has been obtained from *Saccharomyces cerevisiae*, has been extensively used as a biotic elicitor to stimulate a specific secondary metabolite in plants since 1976 (Angeles Sánchez-Sampedro *et al*., 2005; Nasiri-Bezenjani *et al*., 2014; Szabo *et al*., 1999; Yan *et al*., 2006). Another elicitor Ag^+, as a strong inhibitor of ethylene biosynthesis (Bais *et al*., 2000), have been utilized to induce the production and accumulation of various active ingredients in some plant species (Pitta–Alvarez *et al*., 2000; Rahimi Ashtiani *et al*., 2010; Zhang *et al*., 2004). The main aim of this research is to evaluate the effects of different concentrations of YE and Ag^+ on SFN content and activity of superoxide dismutase (SOD), Peroxidase (POD) and Catalase (CAT) in *L. draba* seedlings.

2. Material and Methods

2.1. Materials

Seeds of *L. draba* were collected from around Kerman province (Iran) at the end of May and beginning of June 2012. SFN standard and YE were purchased from Sigma and all other chemicals were of analytical reagent grade and purchased from Merck.

2.1.1. Seed Culture and Plant Growth

Surface of the seeds were sterilized by subsequent washing with detergent, sodium hypochlorite (2% for 10 min), ethanol (75%, 30 s) and rinsing (at least three times) with sterile distilled water. Thirty seeds with about 8 mm spacing, were placed on the surface of solidified basal media of MS (Murashige., 1962) containing 0.8 agar in Petri dishes. The plates were placed in an incubator at controlled temperature of 28±2 ˚C, relative humidity of 60-65% and periodic light response 8:16 (dark: light). In these situation nearly 95% of the seeds were germinated and their roots developed.

2.1.2. Elicitor preparation and seedling treatment

Different concentrations of YE [0 (as a control), 0.5, 1, 2 and 5 mg/mL] and $AgNO_3$ [0 (as a control), 25, 50, 100 and 200 µM] were dissolved in sterile distilled water separately (without dilution). The 7-day-old seedlings (50 seedlings) were selected from media 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 25 ˚C. The YE-treated seedlings were harvested at three time intervals; 4, 8 and 16 hours and with the Ag^+-treated, sampling was done at two time intervals; 4 and 8 hours. Then the treated seedlings were washed to eliminate remained elicitors at the surface of plants, and were frozen using liquid nitrogen and kept at -80 ˚C until used for biochemical analysis.

2.1.3. SFN Content Measurement

Extraction and quantification of SFN content were performed according to the method described by Liang *et al*., 2006, with little modifications. In brief, 500 mg of fresh tissue was grinded into powder using a mortar. One milliliter of acidic water (pH=5) was added to the powder and incubated at 42±2 ˚C for 2 hours. Following, 5 mL acetonitrile was added to the mixture and ultrasonicated for 3 min, the homogenous solution centrifuged for 10 min at 10000 rpm and 4 ˚C. The supernatant was filtered with 0.2 µm syringe filter prior to subject in C_{18} column (250×4.6 nm) of High Performance Liquid Chromatography (HPLC) (Agilent 1100 series, USA). HPLC conditions were as follows: solvent system; acetonitrile/H_2O (65/35 v/v), flow rate; 1 mL/min. Identification of SFN in the seedlings was done by comparison of retention times with authentic standard that was detected at 254 nm.

2.1.4. Protein Extraction and Antioxidant Enzymes Assay

Protein extraction was performed by homogenizing 500 mg of fresh tissue in a solution containing 5 mL of 50 mM potassium phosphate, 1% Polyvinylpyrrolidone, 1 mM EDTA and 1 mM PMSF at 4 ˚C. The homogenous solution was centrifuged at 10000 rpm for 15 min at 4 ˚C and the supernatant, as a crud enzyme solution was kept at -20 ˚C until used. Protein contents were determined according to the method of Bradford using bovine serum albumin (BSA) as a standard (Bradford., 1976). The activity of SOD (EC. 1.15.1.1) was determined by monitoring of inhibition in photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Giannopolitis and Ries, 1977. One unit of SOD activity was defined as an amount of enzyme required to cause 50% inhibition in the rate of NBT reduction measured at 560 nm by spectrophotometer (Varian cary50, Australia). SOD activity was expressed as enzyme unit per milligram of protein (U/mg protein).

POD (EC.1.11.1.7) activity was determined as described by Plewa *et al*., 1991 protocol. The reaction mixture (3

mL) contained 50 mM potassium phosphate buffer (pH=7), 4% guaiacol, and 1% H_2O_2 and appropriate enzyme extract. POD activity was expressed as changes in absorbance at 470 nm against mg of protein. CAT (EC 1.11.1.6) activity was assayed in a 3 mL reaction mixture containing 2.87 mL of 50 mM sodium phosphate buffer (pH 7.0), 30 µLH_2O_2 (15%) and 100 µL of enzyme extract. Decrease in absorbance was recorded after decomposition of H_2O_2 at 240 nm. One unit of CAT is defined as an amount of the enzyme that decomposes 1 µM H_2O_2 in one minutes and the activity is expressed in unit/mg protein (Dhindsa., 1981).

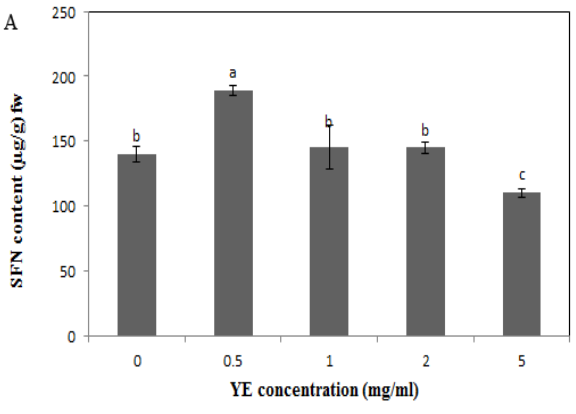
2.1. Statistical Analysis

Experiments were conducted with completely random designs in triplicate for each treatment. Duncan's multiple range tests were used to compare mean of the treatments at p≤0.05 using SAS9.1.3 (service pack 4, version= 6.1.7601) software. Significance of the difference between mean values was determined by one-way analysis of ANOVA variance. The results are expressed as mean values ± standard deviation (SD) from triplicate experiments.

3. Result

3.1. Effects of Yeast Extract on SFN Content

As shown in figure 1, the effect of YE on SFN content is depend on the elicitor dose as well as the time of elicitation. The highest amount of SFN in the YE-treated seedlings after 4 hours treatment was observed at the lowest concentration of the elicitor (0.5 mg/mL) and thereafter decreased (Fig. 1A). However, increasing of treatment time up to 8 hours the SFN content significantly elevated at all concentrations (except at 5 mg/mL treatment which its content was the same as the control) (Fig. 1B). The maximum amount of SFN reached about 227.8 µg/g fresh weight, in presence of YE (1 mg/mL), which was 1.33-fold that of the control.



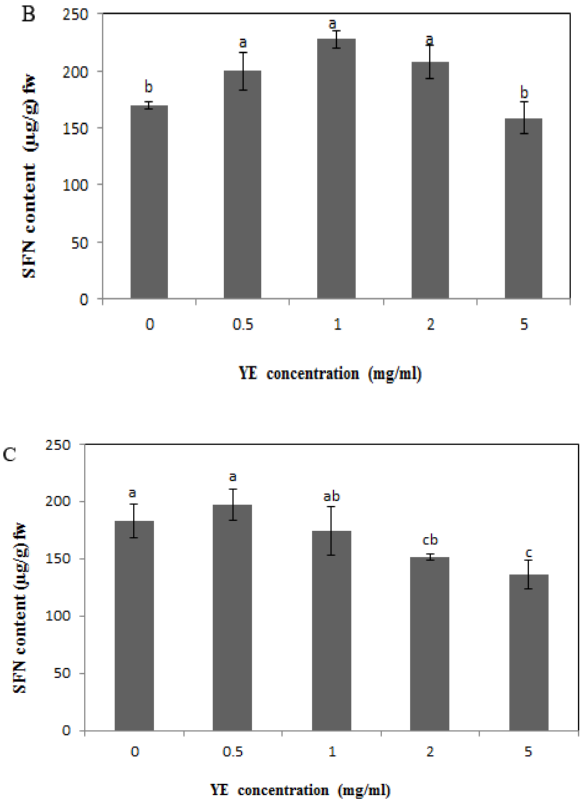


Fig. 1 Comparison of SFN content in *L. draba* seedlings treated with different concentrations of YE for 4 (A), 8 (B) and 16 (C) hours. Signs with different letters indicate significant differences at p≤0.05 according to Duncan's Multiple Range Test. Data are mean ± SD, n=3.

Nevertheless, the SFN content tends to decrease when the seedlings exposed to YE for 16 hours.

The results show that YE at 0.5 mg/mL have no significant effect on the SFN content but at higher concentrations adversely affect its production.

3.2. Effects of Ag^+ on SFN Content

The effects of different concentrations of Ag^+ on SFN content at different time intervals are shown in figures 2 A and B. The amount of this compound was induced in the presence of Ag^+ depends on the elicitor concentration and the time of treatment. As shown in the figure 2A, the SFN content drastically promoted when the concentration of Ag^+ increases up to 25 µM, so that its content in 50 µM-Ag^+-treated seedlings was nearly 1.66-fold more than that of the control (112.5 µg/g FW). However, increase in elicitation time to 8 hours, the SFN content did not change compared to the control up to 50 µM Ag^+, but it significantly increased at 100 µM Nevertheless at the highest concentration of Ag^+ (200 µM), SFN content drastically decreased in comparison with the control (Fig. 2B).

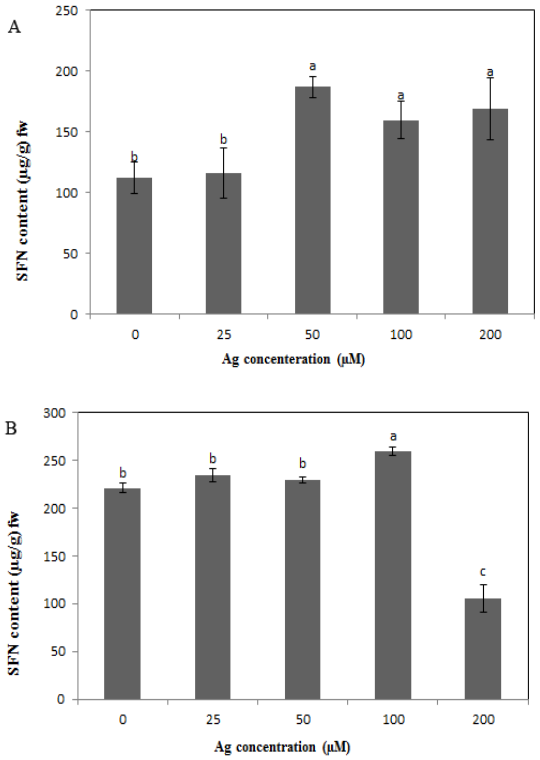


Fig. 2 comparison of SFN content in *L. draba* seedlings treated with different concentrations of Ag^+ after 4 (A) and 8 (B) hours. Signs with different letters indicate significant differences at $p \leq 0.05$ according to Duncan's Multiple Range Test. Data are mean ± SD, n=3.

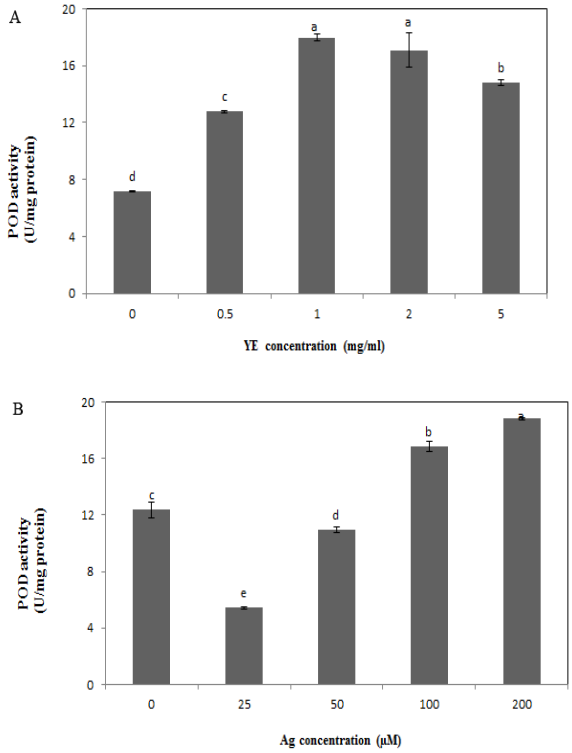


Fig. 3 SOD activity in *L. draba* seedlings which were treated with various concentrations of YE for 8 hours (A) and Ag^+ for 4 hours (B). Signs with different letters indicate significant differences at $p \leq 0.05$ according to Duncan's Multiple Range Test. Data are mean ± SD, n=3.

3.3. Effects of the elicitors on antioxidant enzyme activity

Since considerable amounts of SFN was induced after 8 and 4 hours treatment with YE and Ag^+ respectively, the activity of the key enzymes involved in enzymatic antioxidant system were assayed only during the aforementioned times.

3.3.1. Effects of the elicitors on SOD activity

Activity of SOD was significantly decreased in the YE-treated seedlings compared to the control (Fig. 3A). Similar pattern for SOD activity was also seen in Ag^+-treated seedling (Fig. 3B).

3.4. Effects of the elicitors on CAT activity

As shown in figures 4A and B, the CAT activity is increased specially at lower concentrations of the both elicitors (except for 0.5 mg/mL YE which its effect was the same as the control) and its activity tends to decrease as the level of the control at higher concentrations of the both elicitors.

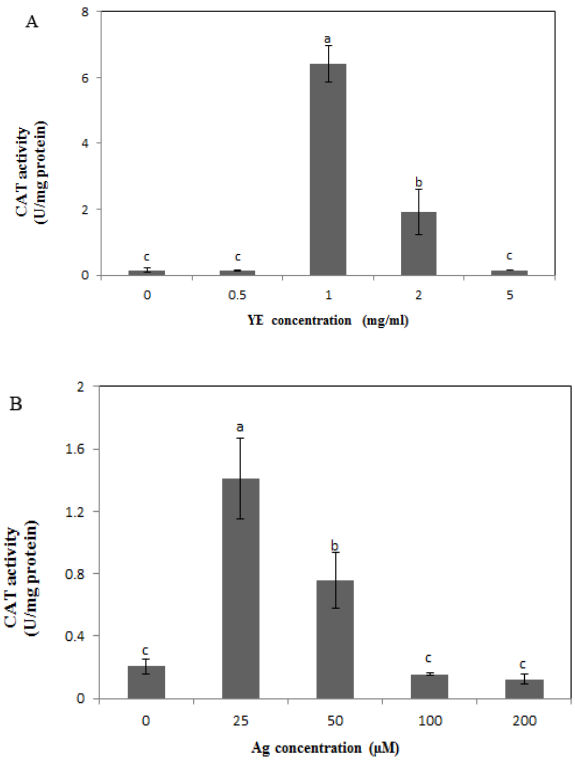


Fig. 4 CAT activity in *L. draba* seedlings after treatment with YE for 8 hours (A) and with Ag^+ for 4 hours (B). Signs with

different letters indicate significant differences at p≤0.05 according to Duncan's Multiple Range Test. Data are mean ± SD, n=3.

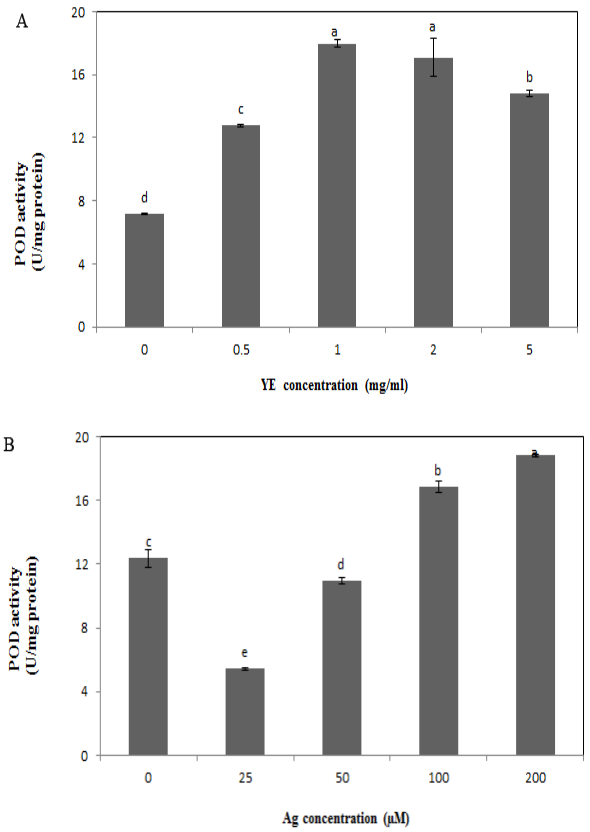


Fig. 5 POD activity in *L. draba* seedlings which were treated with different concentration of YE for 8 hours (A) and Ag^+ for 4 hours (B). Signs with different letters indicate significant differences at p≤0.05 according to Duncan's Multiple Range Test. Data are mean ± SD, n=3.

3.5. Effects of the Elicitors on POD Activity

The POD activity significantly increased in YE-treated seedlings compared to the control. The activity of POD gradually increased when the elicitor concentration increased in media until 1 mg/mL and thereafter decreased (Fig. 5A). POD activity significantly decreased at the lowest concentration of Ag^+ (25 µM) and then elevated. The significant increase in POD activity was seen at elicitor concentrations more than 50 µM (Fig. 5B).

4. Discussion

According to the results, YE have positive effect on the SFN content especially at lower concentrations. The optimum concentration was obtained at 1 mg/mL and the best time of elicitation was 8 hours. Up to now, the stimulatory effects of this elicitor have been reported on various secondary metabolites including rosmarinic acid and silymarin (Angeles Sánchez-Sampedro *et al.*, 2005; Hasanloo *et al.*, 2009; Nasiri-Bezenjani *et al.*, 2014; Yan *et al.*, 2006). YE consist of several compounds including peptides, polysaccharides, vitamins and metal ions and also some unknown components (Mizukami *et al.*, 1992). Thus, it has not been clearly understood which component of YE has stimulatory effect on the biosynthesis of secondary metabolites. Some studies suggested that the stimulatory effect of this elicitor may be attributed to the certain ions including Zn and Co or other unknown compounds (Pitta–Alvarez *et al.*, 2000). As recently reported, Zn ions have stimulatory effects on SFN production in *L. draba* seedlings (Mohammadi *et al.*, 2014). However, previous studies suggested that in YE-elicited, jasmonic acid is a molecule that mediates the elicitor stimulatory signal to cells (Abraham *et al.*, 2011; Hasanloo *et al.*, 2009). A promotion in the glucosinalates level has been shown after elicitation of some Brassica species with methyl jasmonate and salicylic acid (Loivamäki *et al.*, 2004; Wiesner *et al.*, 2013). It has been demonstrated that the stimulatory effects of the elicitors are correlated with change in the expression level of several genes involve in glucosinolates biosynthesis pathway (Glombitza *et al.*, 2004; Wiesner *et al.*, 2013). Therefore, it may be suggested that the elevated level of SFN mediated through signaling molecules such as jasmonates, as an inducer of the expression of some genes, which in turn results in glucoraphanine biosynthesis induction. On the other hand, Ag^+ as an abiotic elicitor has also positive effects on SFN production. Based on the results, the maximum amount of SFN is obtained at 50 µM and the optimum time of treatment is 4 hours. Moreover, the inhibitory effects of this element have been shown on ethylene (a phytohormone) biosynthesis pathway (Pan *et al.*, 2000; Zhao *et al.*, 2005). It has been suggested that higher concentrations of this hormone leads to inhibition of the biosynthesis of secondary metabolites, however at lower concentration it induces biosynthesis of the metabolites (Pan *et al.*, 2000). Thereby, the positive effects of Ag^+ on SFN production level may be attributed to its inhibitory effects on biosynthesis or action of ethylene (Zhang 2003). As indicated in figures 1 and 2, the effects of these elicitors on SFN production depend on the does and time of elicitation. The adverse effect of the elicitors on production level of SFN was observed especially at the highest concentration of the elicitor which it content tends to reduce at all elicitation times. These results (induction or inhibition effects of the elicitor) are consistent with those findings that reported earlier (Hasanloo *et al.*, 2009; Rahimi Ashtiani *et al.*, 2010; Yan *et al.*, 2006).

Induced oxidative stress has been reported in some plant species when exposed to the biotic or abiotic elicitors (Aminizadeh *et al*., 2014; Hasanloo *et al*., 2009; Nasiri-Bezenjani *et al*., 2014). In plants, detoxification of ROS mainly occurs through two antioxidant systems; enzymatic (glutathione peroxidase, ascorbate peroxidase, SOD, CAT, POD and etc.) and non-enzymatic (carotenoid, flavonoid, ascorbate, tocopherol and glucosinolatesetc) (Choi *et al*., 2004; Tang *et al*., 2006). According to results it can be revealed that activity of SOD as a key player in catalyzing of O^{-2} to H_2O_2 (Dong *et al*., 2002), significantly decreases in the treated seedlings with both elicitors compared to the control (Fig.3 A, B). It seems that the reduction of SOD activity is correlated with the presence of other antioxidant compounds such as ascorbic acid. As previously reported by Noctor and Foyer (1998) the activity of SOD is decreased in the presence of exogenous ascorbic acid as elicitor. They concluded that their results possibly are related to the antioxidant properties of this vitamin due to direct neutralization of superoxide ions by ascorbate (Noctor and Foyer 1998). In our research, in contrast to the SOD activity, the activity of H_2O_2 scavenger enzymes significantly enhanced in the seedlings treated with the both elicitors. According to our results, CAT is more active at lower concentrations of elicitors, while POD is strongly active at higher ones. Thus it can be deduced from obtained results that POD is more active than CAT in the seedlings which are treated with the both elicitors. The higher activity of POD may be attributed to the high level content of POD in *L. draba*, as has been reported in horseradish (a member of the Brassicaceae family) (Soudek *et al*., 2005). Moreover, the promotion of CAT activity in 1 and 2 mg/mL YE concentration after 8 hours treatment may be attributed to more adsorption of YE, as deduced by the highest SFN content in these treatments (Fig. 1B). Furthermore, due to the promotion of the H_2O_2 scavenger enzymes activity, it may be concluded that treatment of seedlings leads to trigger of reactive oxygen species (ROS) induction. It has been known that H_2O_2, as an important signal of oxidative stress and activation of network signaling (Mejía-Teniente *et al*., 2013), induces expression of many genes involved in defense and secondary metabolites biosynthesis pathway (Xu *et al*., 2007). Additionally, it can be deduced from the results that the promotion of SFN content resulted in stimulatory effects of the elicitors on glucosinolates biosynthesis pathway which mediated by the ROS. Since activation of myrosinase has also been reported in presence of ascorbic acid in *Wasabia japonica* (Ohtsuru., 1979), it may be suggested that the increasing of SFN production level in treated *L. draba* seedlings resulted in the induction of glucoraphanin biosynthesis (as a precursor of SFN) as well as elevation of myrosinase activity. Furthermore, the toxicity effects of the elicitors (YE and Ag^+) over long elicitation time may be attributed to the high level of ROS production as indirectly revealed by the increase in POD activity (Zhang *et al*., 2013). Summing up, it may be suggested that the stimulatory effects of these elicitors respectively mediated through induction of jasmonate or salicylic acid and inhibition of ethylene biosynthesis when YE and Ag^+ are used as elicitors. However, reduction of superoxide dismutase activity may be attributed to the production of ascorbic acid as well as activation of myrosinase after treatment with the elicitors. Finally, it may be concluded that the induction of glucoraphanin is the first step toward large-scale production of SFN. Up to now, no study has been reported on the effects of YE and Ag^+ on the production levels of SFN and even any glucosinolates or breakdown products. In this regards, our results are shown that YE and Ag+ are effective stimulators to induce SFN production in *L.draba* seedlings.

Acknowledgments

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