

## Increased Growth Rate, Lignin, and Shikonin Levels in *Onosma dichroantha* Boiss. as Affected by Silicon Treatment

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### ABSTRACT

*Onosma dichroantha* Boiss. is a local medicinal herb in Iran, belonging to the Boraginaceae family that is used mainly for wound healing due to the presence of shikonin in its root cortex. Optimization of *Onosma* spp. *in vitro* cultures and shikonin production is encouraged as an alternative to harvesting the plant from its natural habitats. The present study evaluates the growth rate, several biochemical properties, and shikonin content of *O. dichroantha* plants treated with various concentrations of silicon (in the form of potassium silicate) in a hydroponic medium. Silicon application up to 0.5 mM increased the fresh mass, chlorophyll a/b, carotenoids, soluble proteins in shoots, and the lignin content in roots; however, phenolic compound contents were not significantly affected. In addition, silicon nutrition increased catalase and soluble ascorbate peroxidase activities, whereas polyphenol oxidase activity was not affected in roots and shoots. Interestingly, the shikonin content of *O. dichroantha* roots treated with increased concentrations of Si was 2-fold higher than that in the control plants, while the activity of phenylalanine ammonia lyase, a key enzyme in shikonin biosynthesis, was not affected. This suggests that the observed increase in shikonin in response to the silicon treatment could be due to increased stability or more accumulation sites of shikonin in roots. These data may be used for improvement of shikonin production in cell cultures of *O. dichroantha* under experimental or industrial conditions.

**Keywords:** Boraginaceae, Cell culture, Phenylalanine ammonia lyase, Medicinal plants, Potassium silicate

### INTRODUCTION

Medicinal plants and their products are widely used for prevention and treatment of complex diseases, such as cancer and diabetes. The *Onosma* genus (including *Onosma dichroantha* Boiss) is one of the most important genera of the Boraginaceae family with about 150 species that are distributed in eastern and central Asia and throughout the Mediterranean region (Pavol *et al.*, 2008). The main secondary metabolites of these genera include alkaloids, naphthoquinones, polyphenols, phytosterols, terpenoids and fatty acids (Kumar *et al.*, 2013). Root extract of *Onosma dichroantha* were traditionally used as a home remedy in burn-wound

healing in Iran. These wound healing properties of the *Onosma* and other Boraginaceae family members are attributed to anti-bacterial, anti-viral, anti-oxidant, and anti-inflammatory activities of phenolics, flavonoids, and in particular, naphthoquinones such as shikonin and alkannine (Chen *et al.*, 2002). Shikonin and its derivatives are red pigments that accumulate in the roots of many Boraginaceous plants. Furthermore, Shikonin and its analogs inhibit cancer cell glycolysis by selectively targeting tumor pyruvate kinase-M2 (Chen *et al.*, 2011). Excessive harvest of *O. dichroantha* natural populations for medicinal use has recently led to extreme reduction of its frequency in Northern Iran. Thus, cultivation of *O. dichroantha* is

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important to prevent the destruction of this species in its natural environment.

Silicon is the second most abundant element in the soil and is currently classified as non-essential for plants; however, its availability for some vascular plants is critical for normal growth and development. Indeed, all rooted plants in the soil have some silicon in their tissues (Ma *et al.* 2011). Plants absorb silicon through their roots as silicic acid [ $\text{Si}(\text{OH})_4$ ] (Richmond and Sussman, 2003). Layers of accumulated silicon, typically in the form of solid silica ( $\text{SiO}_2$ ) are deposited on the epidermal cell wall, forming a physiological barrier. This deposition of silicon can confer numerous beneficial effects on plants. For example, it can reduce the negative effects of water stress by decreasing transpiration; it can also increase plant resistance to pathogens and herbivory (Alberto Moldes *et al.*, 2013; Ma *et al.*, 2007). Furthermore, silicon can reduce the toxicity of heavy metals *via* decreasing their uptake (Iwasaki *et al.* 2002). Delavar *et al.* (2016) reported that silicon relieves the adverse effects of aluminum toxicity in *Borago officinalis*. An increase in the root growth accompanied by a decrease of shoot-root ratio has been reported in *Brassica napus* and alfalfa plants following silicon application (Hashemi *et al.*, 2010; Guo *et al.*, 2006). Some studies have found that silicon application increases Phenylalanin ammonia lyase (PAL) (Carver *et al.*, 1998; Hajiboland *et al.*, 2017; Cai *et al.*, 2008).

Since shikonin and its derivatives accumulate mostly in the periderm of the plant roots, we aimed to test the hypothesis that silicon application to *O. dichroantha* may affect shikonin content *via* its effects on the root growth and cell wall composition. To the best of our knowledge, this is the first report on the effects of silicon on the accumulation of the secondary metabolites in *O. dichroantha* plants.

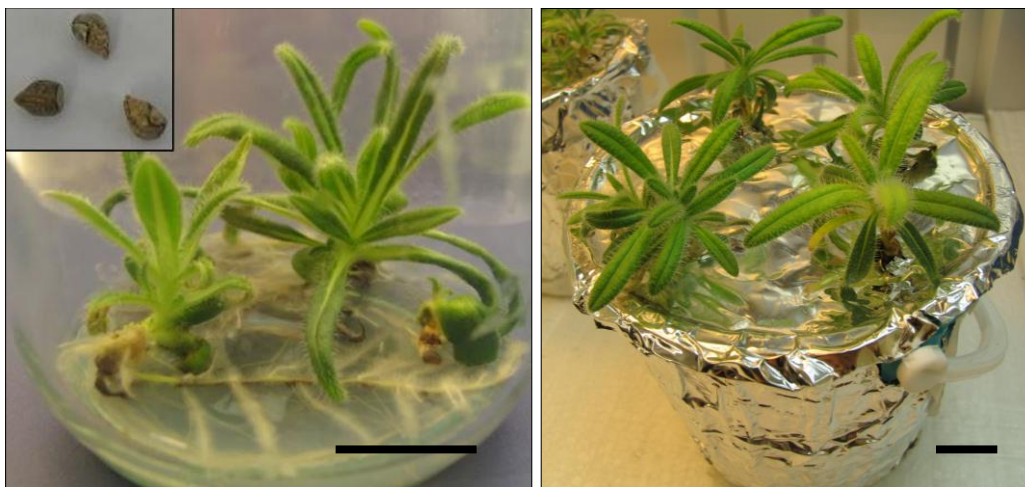
## MATERIALS AND METHODS

### Seed Sterilization and Germination

Seeds of the *Onosma dichroantha* Boiss., herbarium Code: Voucher SP. No. 36194, were collected from the village of Aref located in city of Mashhad in geographical location  $36^\circ 8' 17'' \text{N}$  and  $59^\circ 31' 44'' \text{E}$  and 1,263 meters above the sea level. Seeds were surface-sterilized by 70% ethanol and their outer shells were removed to facilitate germination, as previously described (Bagherieh-Najjar and Nezamdoost, 2016). Afterward, the embryos were sterilized again with 70% ethanol for 30 seconds and 5% sodium hypochlorite for 2 min, followed by vigorous washing with sterile distilled water under a laminar flow hood and were cultured in glass jars containing Murashige and Skoog (MS, Murashige and Skoog, 1962) growth medium. The jars were placed in a growth chamber for 15 days with a 16 h light photoperiod of  $60 \mu\text{M m}^{-2}\text{s}^{-1}$ ; and 70% relative humidity at  $23 \pm 2^\circ \text{C}$ .

### Silicon Treatment and Biochemical Measurements

In order to establish a hydroponic culture, four-week-old healthy seedlings grown on solid MS medium (Figure 1a) were transplanted into 2 L black plastic containers (Figure 1b) containing liquid MS nutrient solution, supplemented with 0, 0.25, 0.5 or 0.75 mM silicon in the form of potassium silicate (Sigma, Germany) and placed in a growth chamber, as explained above. The pH of the nutrient solution was adjusted daily at  $6.5 \pm 0.2$  and the solution was refreshed weekly. Day and night temperatures of the culture room were regulated at 25 and 18 °C, respectively, and relative humidity was about 65–74 %. The irradiance was kept at  $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  provided from a mixture of cool/warm white fluorescent lamps using a 16 h day/8 h night photoperiod. Plants were harvested after 30



days, weighted and stored in liquid nitrogen for further biochemical analysis.

For chlorophyll and carotenoids measurements, the leaves were extracted in cold 80% acetone in the dark condition and the absorbance was measured at 470, 645, 646.8, 663 and 663.2 nm by UV-spectrophotometer (Shimadzu, Japan) as described by Arnon (1949). The chlorophyll a, chlorophyll b, and carotenoids (Kar and Mishra, 1976) were calculated accordingly.

Extracts for catalase (CAT), soluble guaiacol peroxidase (GPX) polyphenol oxidase (PPO) and soluble proteins assays were prepared as described by Kar and Mishra (1976) and Liu and Huang (2000). Briefly, fresh leaf samples (0.05 g) were homogenized with 2 ml phosphate buffer (100 mM, pH 6.8) and centrifuged for 15 min at 3000 g. The clear supernatant was subsequently used as an enzyme source. The activities of peroxidase, catalase, and polyphenol oxidase were determined, as described earlier (Mehraban *et al.*, 2008; Hashemi *et al.*, 2010).

The activity of ascorbate peroxidase (APX) was measured as described by Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm ( $\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ ). The reaction mixture in a

total volume of 2 mL contained 250 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 1.2 mM hydrogen peroxide, and aliquots of enzyme extract. The activity of phenylalanine ammonia lyase (PAL) was determined, as described by Whetten and Sederoff (1992) using an extinction coefficient of  $20000 \text{ mM}^{-1} \text{ cm}^{-1}$  at 290 nm.

Soluble proteins content was determined as described by Bradford (1976). Phenolic compounds were extracted with ethanol and determined at 720 nm, using the folin-ciocalteu reagent, as basically described by Fukuda *et al.* (2003).

Lignin extraction was carried out by ethanolic HCl (absolute ethanol: 1 mol L<sup>-1</sup> HCl; 1:1; v/v) after three times pre-extraction of the plant materials with 50% methanol at 60 °C to remove any phenolic compounds, as described by Zimmer (1999). The lignin content was determined colorometrically at 488 nm, using phloroglucinol.

Shikonin and its derivatives were assayed spectrophotometrically (Shimadzu UV-1800, Japan) as described by Gupta *et al.* (2014) and confirmed by HPLC, basically as described by Sagratini *et al.* (2008) using a Hitachi (D-7000, Series 0127, Japan) HPLC-DAD instrument, equipped with a



binary solvent pump (L 7100) and a DAD (diode array detector).

### Statistical Analysis

All experiments were carried out in a completely randomized design with three replications. The statistical analyses were carried out using SAS statistical software (SAS Institute 2004). All data were subjected to ANOVA and comparison of the means was performed, using the Least Significant Difference (LSD) test at  $p < 0.05$ .

## RESULTS

### Effects of Silicon on Plant Growth, Soluble Proteins, and Pigment Content

Increasing concentrations of silicon up to 0.5 mM led to gradual increase in the fresh weight of both shoots and roots of *O. dichroantha* plants (Figure 2). Plants grown in the presence of 0.75 mM Si exhibited approximately 50 % higher total fresh mass, as compared to the control plants.

The soluble proteins remained unchanged

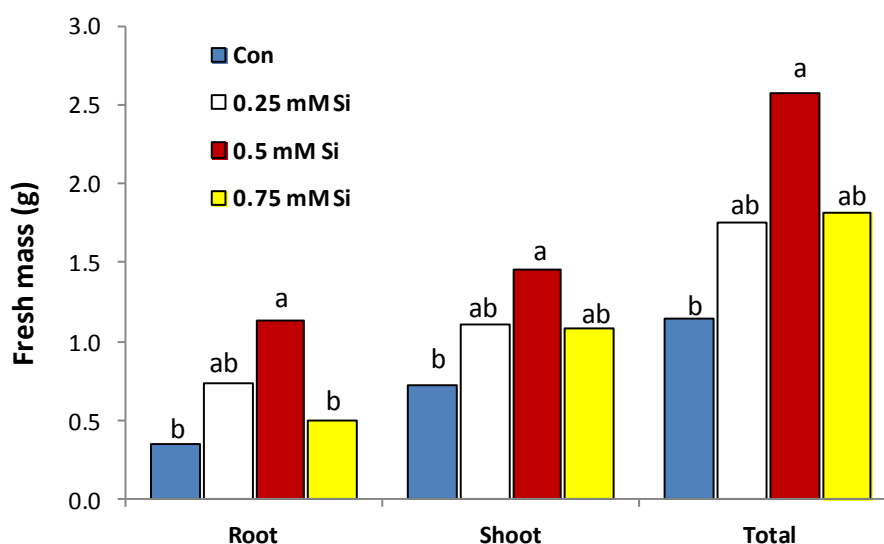
in root of *O. dichroantha* plants following Si application. In shoots, however, soluble proteins increased gradually, as the Si concentration increased in the culture medium (Table 1).

These data also showed that up to 0.5 mM Si treatment, a significant increase was observed in chlorophyll a/b, carotenoids, and xanthophyll contents (Table 1).

### Silicon Effects on Enzymes Activity

In *O. dichroantha*, the catalase activity detected in the roots with or without Si treatment was about two times more than that detected in the shoots. Upon Si nutrition, the catalase activity in shoots was increased until 0.5 mM of Si concentration and remained unchanged by excess Si (Figure 3). In the roots, Si supply lower than 0.25 mM did not have a significant effect, while higher levels of Si caused about 60 % increase in catalase activity.

Soluble guaiacol peroxidase activity levels were about 7-folds higher in the roots as compared to shoots. The activity of this enzyme showed an increase of about two-fold in the roots, while in the shoots the



**Figure 2.** Effect of supplementary silicon on roots and shoots fresh mass of *O. dichroantha* plants grown hydroponically for 30 days. Different small letters represent statistically significant differences at  $P < 0.05$ . Con: control.

**Table 1-** Effect of Si treatments on pigments, total phenols, lignin, and soluble protein contents of *O. dichroantha* plants grown in hydroponic culture. <sup>a</sup>

		Si concentration (mM)			
		Control	0.25	0.5	0.75
Chlorophyll a		0.033b	0.055b	0.065a	0.029b
Chlorophyll b		0.015b	0.022b	0.042a	0.012b
Carotenoids		1.17c	1.57ab	1.73a	1.24bc
Total Phenols	Shoot	8.17a	9.06a	10.42a	10.68a
	Root	16.35a	16.68a	19.72a	18.14a
Lignin	Shoot	69.05a	70.41a	80.90a	78.42a
	Root	59.33b	85.48ab	63.52ab	110.03a
Soluble protein	Shoot	5.41b	4.71b	8.79ab	10.40a
	Root	6.48a	8.34a	6.86a	9.32a

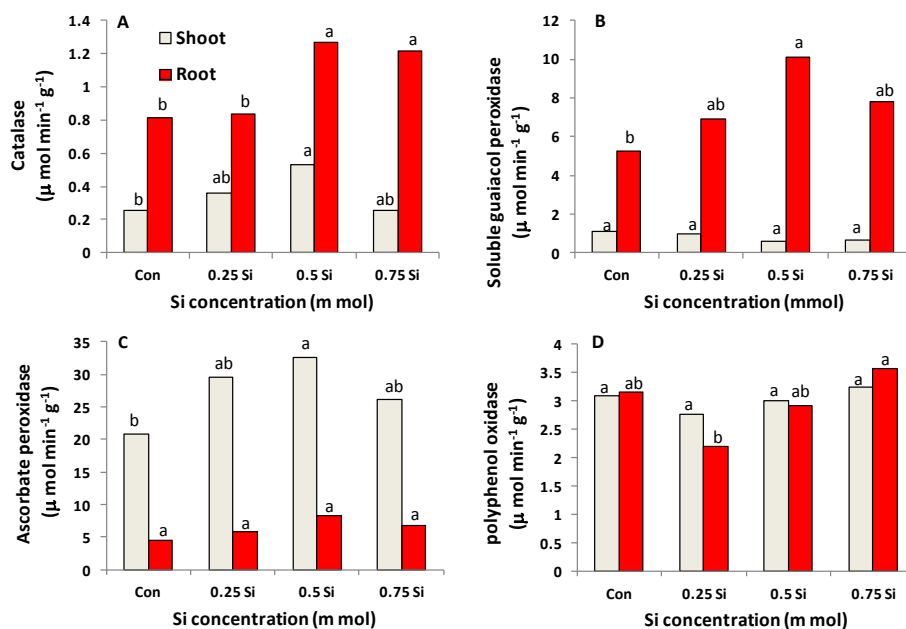
<sup>a</sup> Means followed by the same letter within each row are not significantly different according to a Least Significant Difference (LSD) test ( $P < 0.05$ ).

activity of the enzyme was not affected by increasing silicon concentrations in the medium. (Figure 3).

Following Si application, the polyphenol

oxidase activity in roots or shoots did not change significantly (Figure 3).

The ascorbate peroxidase activity was higher in shoots than that in roots. Si



**Figure 3.** Effects of silicon treatments on activity of catalase (A), soluble guaiacol peroxidase (B), ascorbate peroxidase (C), and polyphenol oxidase (D) in roots and shoots of *O. dichroantha*. Statistical analysis on shoots and roots were performed separately and different small letters represent statistically significant differences at  $P < 0.05$ .



nutrition did not affect the activity of this enzyme in the roots; however, its activity was increased in shoots until 0.5 mM Si in the culture medium.

### Silicon Effects on PAL Activity and Phenols, Lignin, and Shikonin Contents

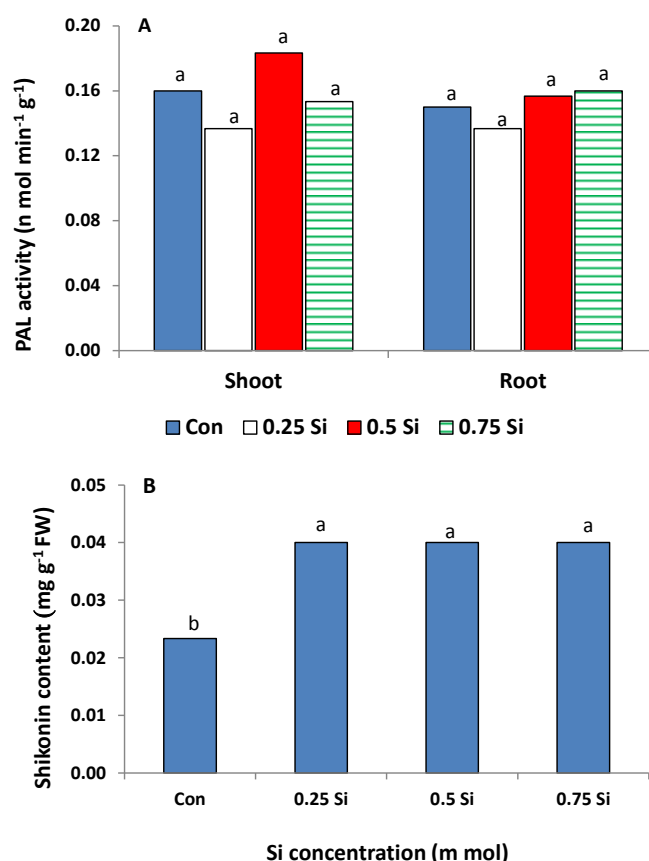
The amount of phenolics in roots was about two times more than that in shoots. Silicon nutrition did not have any significant effect on phenolic compounds of the roots or shoots. Silicon application significantly increased the lignin content of root tissues; however, it did not significantly affect the lignin content of shoots (Table 1).

Silicon nutrition did not show any effects on PAL activity either in shoots or roots. The shikonin content of *O. dichroantha*

roots treated by Si was about 60 % more than that of control plants (Figure 4).

## DISCUSSION

Our results indicated that silicon supply in the root medium enhanced the shoots, roots, and total fresh mass of *O. dichroantha* plants (Figure 2). This increase was more strikingly observed in plants treated with 0.5 mM silicon. Silicon application has been reported to increase the biomass, yield, and growth quality in a broad range of plants, including mono- and di-cotyledons. For instance, in rice, oat, barley, wheat, and annual brome, application of silicon increased the dry weight by 2–20% (Lewin and Reimann, 1969; Gali and Smith, 1992, Tahir et al., 2012). Shi et al. (2005) showed



**Figure 4.** Effects of silicon treatments on phenylalanine ammonia lyase (PAL) activity in shoots and roots (A) and shikonin content in roots (B) of *O. dichroantha*. Different small letters represent statistically significant differences at  $P < 0.05$ .

that shoot and root dry weight of cucumber plants were increased by silicon treatments. The real mechanism controlling the positive effects of silicon on plant growth is not fully understood yet (Frew *et al.*, 2018). Hossain *et al.* (2002) reported that Si promoted cell elongation and cell wall extensibility in rice, suggesting that deposition of silicon to the cell wall components might increase hardening of tissues and promote plants performance in regard to leaf positioning. In addition, Markovic *et al.* (2017) reported the beneficial effect of silicon application in cytokinin biosynthesis leading to delayed leaf senescence in *Arabidopsis* and sorghum. Further experiments are needed to clarify the mechanisms by which silicon application directly or indirectly may affect cell wall extension, leaf architecture, and longevity in plants, including *O. dichroantha*.

In our experiments, the photosynthetic pigments (chlorophyll a/b and carotenoids) increased by the applied silicon, especially at 0.5 mM (Table1). The studies conducted by Wang and Galletta (1998) on strawberry, Al-aghabary *et al.* (2005) on tomato, and Neocleous (2015) on melon confirmed the positive impact of silicon on the concentration of photosynthetic pigments. It is possible that leaf angle modification (Hossain *et al.*, 2002) caused by silicon treatment plays a role in increasing plant photosynthetic activity, which in turn may enhance plant growth rate.

In this study, catalase (CAT), and ascorbate peroxidase (APX) activities of plant shoots were increased in the presence of silicon, while the soluble guaiacol peroxidase (GPX) activity increased in the plant roots (Figure 3). Catalase and peroxidase detoxify H<sub>2</sub>O<sub>2</sub> and most of H<sub>2</sub>O<sub>2</sub> production in plants growing under normal conditions is produced in plant shoots. Enhanced activity of CAT and APX upon silicon treatment observed in our experiment might suggest a role for silicon in H<sub>2</sub>O<sub>2</sub> scavenging in *O. dichroantha*. In support of these data, several studies reported that exogenous silicon application can improve the ability of reactive oxygen scavenging by

regulation of antioxidant enzymes activities including CAT and APX (Hashemi *et al.*, 2010; Torabi *et al.*, 2015; Kim *et al.*, 2017; Tripathi *et al.*, 2017). However, further in-depth transcriptomic analyses are required to unravel the mechanisms responsible for the Si-mediated plant antioxidant systems.

In our experiments, silicon supply had no significant effects on polyphenol oxidase (PPO) and PAL activities. PAL is the first and the key enzyme in the phenylpropanoid pathway and is therefore involved in the biosynthesis of the polyphenol compounds, such as flavonoids, phenylpropanoids, lignin, and shikonin in plants (Gaisser and Heide, 1996). In agreement with our data, Cai *et al.* (2008) reported that application of silicon in the absence of pathogen stress did not change either PPO or PAL activities in rice. Interestingly, Hajiboland *et al.* (2017) reported that Si application decreased PPO and PAL activities in the leaves of tobacco plants grown with or without mechanical stress. Similarly, PAL activity was greater in -Si leaves of oat plants compared to +Si leaves (Carver *et al.* 1998). More experiments are needed to resolve the current discrepancies regarding the effects of different concentrations of silicon application on PAL activity.

In these experiments, the amount of phenolic compounds was not significantly affected by increasing concentrations of silicon. The production of soluble phenolic compounds is regulated by various enzymes such as POD, PPO and PAL (Cai *et al.*, 2008). Therefore, it is reasonable that they change in a more or less similar manner. On the other hand, the lignin content of the plant roots increased at concentration of 0.75 mM Si. Also, the shikonin content of roots of *O. dichroantha* increased following Si application, while the activity of PPO and PAL showed no significant alterations. These data suggest that other biochemical pathways might be involved in production of plant lignin and shikonin in *O. dichroantha* or, under our experimental conditions, the supply of the intermediate compounds of the phenylpropanoid pathway were enough to



increase the level of shikonin and lignin. Shikonin is the main secondary metabolite present in *O. dichroantha* roots, which only accumulates in the cork layers of the roots (Tabata et al. 1974). Both shikonin and lignin are produced via the phenylpropanoid pathway and it is likely that the factors capable of inducing the synthesis of lignin might be associated with shikonin accumulation as well. Together, detailed gene expression analyses are needed to interpret the effects of silicon on the PAL activity, and shikonin accumulation in *O. dichroantha*.

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### افزایش شدت رشد، مقدار لیگنین و شیکونین در گیاه اونوسما (*Onosma dichroantha* Boiss.) تحت تأثیر سیلیکون

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#### چکیده

اونوسما (*Onosma dichroantha* Boiss.) یک گیاه دارویی محلی متعلق به خانواده گاوزبانیان است که به علت داشتن ماده شیکونین در پوست ریشه از دیر باز در ایران برای درمان زخم مورد استفاده قرار گرفته است. بهینه‌سازی کشت درون شیشه‌ای اونوسما و تولید شیکونین به عنوان روشی جایگزین برای برداشت بی‌رویه گیاه از محیط طبیعی دارای اهمیت بسزایی است. مطالعه حاضر، شدت رشد، برخی خواص بیوشیمیایی و محتوی شیکونین گیاه اونوسما (*O. dichroantha*) تحت تأثیر غلظت‌های مختلف سیلیکون به فرم سیلیکات پتاسیم را در محیط کشت هیدروپونیک بررسی نموده است. کاربرد سیلیکون تا غلظت ۵/۰ میلی‌مولار، وزن تر، کلروفیل a/b کاروتنوئیدها و پروتئین محلول بخش هوایی و شیکونین ریشه گیاه را افزایش داد، لیکن محتوی ترکیبات فنلی تغییر معنی‌داری نداشتند. به‌علاوه تغذیه Si فعالیت آنزیم‌های کاتالاز و آسکوربات پراکسیداز محلول را افزایش داد، در

حالی که فعالیت آنزیم پلی فنل اکسیداز در ساقه و ریشه تغییری نداشت. جالب توجه اینکه، محتوی شیکونین ریشه گیاه *O. dichroantha* تحت تأثیر سیلیکون حدود دو برابر بیشتر از مقدار آن در گیاه کنترل بود، در حالی که فعالیت آنزیم فنیل آلانین آمونیا لیاژ (یک آنزیم کلیدی در مسیر بیوسنتز شیکونین) تغییری نیافته بود. این مشاهدات پیشنهاد می کند افزایش مقدار شیکونین تحت تأثیر تیمار سیلیکون به علت افزایش پایداری شیکونین و یا افزایش محل های تجمع آن در ریشه حاصل شده است. تصور می شود داده های حاصل برای بهبود تولید شیکونین در کشت سلولی گیاه اونوسما در مقیاس آزمایشگاهی و یا صنعتی کاربرد داشته باشد.