PERIÓDICO TCHÊ QUÍMICA
ARTIGO ORIGINAL

O EFEITO DA CANTARIDINA E DO ENDOTHALL NA EXPRESSÃO GENÉTICA DAS ENZIMAS REGULADORAS CHAVE EM *CICHORIUM INTYBUS* L.

THE EFFECT OF CANTHARIDIN AND ENDOTHALL ON GENE EXPRESSION OF THE KEY REGULATING ENZYMES IN *CICHORIUM INTYBUS* L.

تاثیر کانتاریدین و اندوتال روی بیان ژن آنزیم‌های کلیدی تنظیم کننده در *CICHORIUM INTYBUS* L.

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RESUMO

Introdução: os mecanismos subjacentes que regulam a biossíntese e a própria existência de um sistema celular nas plantas ainda precisam ser esclarecidos a um ponto satisfatório. **Objetivo:** este experimento tentou revelar a influência da cantaridina e do endothall na expressão gênica de enzimas críticas, glutationa S-transferase, proteína fosfatase e aspartato fosfatase em *Cichorium intybus* L., uma importante planta medicinal. **Métodos:** duas concentrações de cantaridina e endothall, 2,5 e 10 μgml^{-1}, foram aplicadas em mudas de *C. intybus* e as alterações nos genes avaliadas na parte aérea e nas raízes. **Resultados e Discussão:** Mudas tratadas com cantaridina e endothall tiveram o menor nível de expressão de glutationa S-transferase em 2,5 μgml^{-1} (0,92 ± 0,11 e 1,07 ± 0,61, respectivamente) enquanto em 10 μgml^{-1}, os níveis de expressão foram ligeiramente maiores. A expressão da glutationa S-transferase nas raízes seguiu uma tendência semelhante. O efeito da cantaridina nas proteínas fosfatases na parte aérea e nas raízes de *C. intybus* seguiu um padrão semelhante, onde o nível de expressão das proteínas fosfatases na parte aérea e nas raízes de Mudas tratadas com 2,5 μgml^{-1} (0,51 ± 0,03 e 1,34 ± 0,12, respectivamente) foi reduzido. Com cantaridina 10 μgml^{-1}, a expressão das proteínas fosfatases aumentou acentuadamente na parte aérea e nas raízes (3,55 ± 0,21 e 3,96 ± 032, respectivamente). A expressão das proteínas fosfatases na parte aérea recebeu 2,5 μgml^{-1} do endothall consideravelmente aumentado (43,21 ± 3,1), enquanto sua expressão foi próxima de zero nas raízes. A expressão das aspartato fosfatases nos brotos com cantaridina 2,5 μgml^{-1} foi quase nula. Já em 10 μgml^{-1}, sua expressão foi radicalmente aumentada (3,76 ± 0,23). As raízes tratadas com cantaridina indicaram altos níveis de expressão, principalmente em 10 μgml^{-1} (2,07 ± 0,11). A raiz tratada com 2,5 μgml^{-1} de cantaridina não expressou aspartato fosfatases. Em mudas expostas ao endothall, o nível de expressão relativa de aspartato fosfatases em brotos com menos de 2,5 μgml^{-1} foi de 17,49 ± 1,21, enquanto em raízes com 2,5 μgml^{-1} foi de 0,45 ± 0,09. **Conclusões:** Pode ser expresso de forma confiável que a cantaridina e o endothal interrompem o crescimento da planta de uma maneira dependente da dosagem, regulando para baixo as enzimas críticas *C. intybus*.

Palavras-chave: *glutationa S-transferase, proteínas fosfatases e aspartato fosfatases, regulação negativa, expressão gênica, Cichorium intybus*

ABSTRACT

Background: the underlying mechanisms regulating biosynthesis and the very existence of a cellular system in plants have yet to be clarified to a satisfying point. **Aim:** this experiment tried to reveal the influence of cantharidin and endothall on gene expression of critical enzymes, glutathione S-transferase, protein phosphatase and, aspartate phosphatase in *Cichorium intybus* L., an important medicinal plant. **Methods:** two concentrations of cantharidin and endothall, 2.5 and 10 μgml^{-1}, were applied on *C. intybus* seedlings, and the changes in genes evaluated in shoots and roots. **Results and Discussion:** Cantharidin and endothall-treated seedlings had the lowest expression level of glutathione S-transferase in 2.5 μgml^{-1} (0.92±0.11 and 1.07±0.61, respectively) while in 10 μgml^{-1}, the expression levels were slightly higher. The glutathione S-transferase expression in roots followed a similar trend. The Cantharidin effect on protein phosphatases in shoots and roots of *C. intybus* followed a similar pattern where the expression level of protein phosphatases in shoots and roots of treated seedlings with 2.5 μgml^{-1} (0.51±0.03 and 1.34±0.12, respectively) reduced. At 10 μgml^{-1} cantharidin, protein phosphatases' expression

markedly increased in both shoots and roots (3.55±0.21 and 3.96±032, respectively). The expression of protein phosphatases in shoots received 2.5 µgml^{-1} endothall considerably enhanced (43.21±3.1), whereas its expression was close to zero in roots. The expression of aspartate phosphatases in shoots with cantharidin 2.5 µgml^{-1} was almost zero. Whereas in 10 µgml$^{-1,}$ its expression was radically increased (3.76±0.23). Roots treated with cantharidin indicated high levels of expression, particularly in 10 µgml^{-1} (2.07±0.11). Root treated with 2.5 µgml^{-1} cantharidin similarly did not express aspartate phosphatases. Seedlings exposed to endothall, the relative expression level of aspartate phosphatases in shoots under 2.5 µgml^{-1} was 17.49±1.21 while in roots with 2.5 µgml^{-1} was down to 0.45±0.09. **Conclusion:** It can be reliably expressed that cantharidin and endothall halt plant growth in a dosage-dependent manner by downregulating critical enzymes *C. intybus*.

Keywords: *glutathione S-transferase, protein phosphatases and aspartate phosphatases, downregulation, gene expression, Cichorium intybus*

چکیده

پیشینه: مکانیسم‌های بنیانی تنظیم بیوسنتز و وجود یک سیستم سلولی در گیاهان هنوز تا حد رضایت بخشی مشخص نشده است. **هدف:** آزمایشی برای آشکار کردن تأثیر کانتاریدین و اندوتال روی بیان ژن آنزیم‌های حیاتی گلوتاتیون S- ترانسفراز، پروتئین فسفاتاز و آسپارتات فسفاتاز در *Cichorium intybus* L.، یک گیاه دارویی مهم انجام گرفت. **روش‌ها:** دو غلظت کانتاریدین و اندوتال 2/5 و 10 µgml^{-1} بر روی نهال *C. intybus* اعمال شده و تغییرات ژن‌ها در ساقه‌ها و ریشه‌ها ارزیابی شد. **نتایج و بحث:** نهال‌های تیمار شده با کانتاریدین و اندوتال کمترین میزان بیان گلوتاتیون S- ترانسفراز را در 2/5 µgml^{-1} داشتند (0/11 ± 0/92 و 0/61 ± 1/07، به ترتیب) در حالی‌که در 10 µgml^{-1} سطح بیان کمی بالاتر بود. بیان گلوتاتیون S- ترانسفراز در ریشه نیز روند مشابهی را دنبال نمود. اثر کانتاریدین بر پروتئین فسفاتاز در ساقه‌ها و ریشه‌های *C. intybus* از الگوی مشابهی پیروی کرد که در آن سطح بیان پروتئین فسفاتاز در ساقه‌ها و ریشه گیاهچه‌های تیمار شده با 2/5 µgml^{-1} (0/03 ± 0/51 و 0/12 ± 1/34، به ترتیب) کاهش یافت. در 10 µgml^{-1} کانتاریدین، بیان پروتئین فسفاتاز به طور قابل توجهی در هر دو ساقه و ریشه افزایش یافت (0/21 ± 3/55 و 0/32 ± 3/96، به ترتیب). بیان پروتئین فسفاتاز در ساقه‌های تحت 2/5 µgml^{-1} اندوتال به میزان قابل توجهی افزایش یافته (3/1 ± 43/21) در حالی که بیان آن در ریشه نزدیک به صفر بود. بیان آسپارتات فسفاتاز در ساقه‌های موجود در 2/5 µgml^{-1} کانتاریدین تقریباً صفر بود در حالی‌که در 10 µgml^{-1} بیان آن به طور بنیادی افزایش یافت (0/23 ± 3/76). ریشه های تحت تیمار با کانتاریدین بیان سطح بالایی به ویژه در 10 µgml^{-1} را نشان دادند (0/11± 2/07). ریشه تحت تیمار با 2/5 کانتاریدین به طور مشابهی اثری از بیان آسپارتات فسفاتاز را نشان نداد. دانهال‌های تحت اندوتال، میزان بیان نسبی آسپارتات فسفاتاز در ساقه‌های تحت تیمار 2/5 µgml^{-1} افزایش یافت، 1/21 ± 17/49، در حالی‌که در ریشه‌ها با 2/5 µgml^{-1} این مقدار به 0/9 ± 0/45 کاهش یافت. **نتیجه‌گیری:** با اطمینان می‌توان بیان کرد که کانتاریدین و اندوتال رشد گیاه را به روش وابسته به دوز و با کاهش بیان آنزیم‌های حیاتی *C. intybus* متوقف می‌کنند.

کلمات کلیدی: *گلوتاتیون S-ترانسفراز، پروتئین فسفاتازها، آسپارتات فسفاتازها، تنظیم مقررات، بیان ژن، Cichorium intybus*

1. INTRODUCTION:

Endothall and cantharidin (Figure 1) are specific inhibitors of a protein phosphatase that are involved as markers for their function in defense against pathogens, response to cold treatment, and regulation of (Bajsa *et al.*, 2015; Dayan, 2019). The endothall's functional mechanism in the plant prohibits protein phosphatase (PP1) and (PP2A). Detailed investigation on the endothall controlling system on maize roots indicated abnormal cell division incidence, which consequently halted the root microtubule spindle structures leading to the cell cycle in the prometaphase (Bajsa *et al.*, 2012; Dayan, 2019). Further, Tresch *et al.* (2011) reported that endothall also blocks two checkpoints in the cell cycle. Another potent chemical compound with numerous biological activity from anti-cancer properties to pesticides is cantharidin, which has several analogs, including cantharidic acid and endothelium (Yan *et al.*, 2018).

These substances have an anti-tumor role and are used in pharmacological treatments as well as pesticides. Having such a large spectrum of cantharidin effects results from the biochemical blocking of the target protein cantharidin CBP (CA-binding protein), known as protein phosphatase 2A (PP2A). A regulatory enzyme that many controls cellular stages in eukaryotes (Durian *et al.*, 2016; Máthé *et al.*, 2019). Cantharidin is also a potent inhibitor of serine/threonine protein phosphatase, which is highly toxic and significantly affects Arabidopsis transcription. Serine/threonine-protein phosphatases are essential in conducting cellular signals (Dayan, 2019). In the plant, protein phosphatases are involved in various cellular stages, including cell cycle regulation, hormone signaling and transmission, flowering, photoperiod, cell

differentiation, cell proliferation, and plant defense (Prickett and Brautigan, 2006). Protein phosphatases are highly conserved and have homologous amino acid sequences among different species. Cantharidin binds to protein phosphatase binding sites. In the Arabidopsis genome, 19 phosphatase proteins belonging to 5 different classes (PP6, PP5, PP4, PP2A, PP1) have been identified that represent the target sites of cantharidin (Prickett and Brautigan, 2006; Shi *et al.*, 2006). Cantharidin is a toxin due to its effect on Arabidopsis protein phosphatases; And when plants are sprayed with 200 (Mm) cantharidin, the transcription of 1509 genes is very clearly affected within 2 hours, and all 19 arabidopsis phosphatase proteins are inhibited by cantharidin, indicating that the signal conduction pathways are affected (Li and Casida, 1992; Bajsa *et al.*, 2015).

Endothelium was first discovered by Bruchhansen vom in 1929 and is a derivative of cantharidin; It is a natural product of a type of beetle called blister beetled and is reported to have herbicidal activity, which Niagra Chemical identified in 1948. The compound was registered as a plant growth regulator in 1950 by chemical Sharples. Endothall was first patented by pennsalt chemical in 1960 for controlling aquatic weeds (Hiltibran, 1963; Hiltibran, 1967; Hiltibran, 1972). The endothall's functional properties are white or colorless crystals that are stable to light and temperatures up to about 90 °C. Above this temperature, it slowly converts to anhydride. When endothall heats up to decompose, it emits intense smoke and steam. The technical endothall name is Aquathol, Hydrothal-47,191, also, endothelial amine salt is called Hydrothal, a dicarboxylic acid family's member. Endothelial amine and potassium salts are used as an aqueous herbicide to control a wide range of plants, including plankton, algae, and pondweed in irrigated lands and rice fields. It is also used to control annual weeds and broadleaf weeds in sugar beets and spinach. Rapid degradation of endothall depends on leaching and often disappears in soil within 7-21 days. It is 4-5 days in clay soils and nine days in soils with high organic content (Hiltibran, 1972; Langel and Warner, 1986; Erdodi *et al.*, 1995; Skogerboe and Getsinger, 2001; Poovey *et al.*, 2002).

An important subclass of protein phosphatase is aspartate-based phosphatases (ASPT) that mediate metabolism at the cellular level. ASPT can be widely found in either prokaryotes or eukaryotes (Gantt *et al.*, 1992; Perego and Hoch, 1996; Liepman and Olsen, 2004; Dayan, 2019). It is responsible for catalyzing aspartate and ketoglutarate to generate oxaloacetate and glutamate. Due to its substrates' criticality, ASPT has been suggested to be involved in diverse metabolic processes. Some of these are: ASPT is considered a primary source of cellular aspartate, necessary for protein production. ASPT also plays a precursor role in producing the aspartate family of amino acids that covers the agronomically essential amino acids, particularly aspartate (Bryan, 1980; Uhrig *et al.*, 2013; Wadsworth, 1997). The biosynthesis of critical amino acids, asparagine, and ureides as leading nitrogen trans-port carriers also controlled by aspartate (Schubert, 1986).

Cichorium intybus L. (Chicory) is one of the most important medicinal plants of the Asteraceae family. As an annual herbaceous species, it has half to one meter long roots. The exterior color of the root is brown, and the inside is white. Chicory is a plant with blue flowers that grows mostly in spring and grows more in humid weather. These flowers open only in the sun and can not be seen in the sun's absence (Gemeinholzer *et al.*, 2005; Gianquinto, 1997). All parts of chicory, including roots and leaves, have medicinal values, and fresh leaves contain a lot of vitamin C, and its consumption improves gingivitis, which is caused by a deficiency of this vitamin (Pushparaj *et al.*, 2007; Street *et al.*, 2013; Al-Snafi, 2016). Chicory grows in relatively moist roadsides, barren areas, and low slopes from Europe to Sweden, western and central Asia and North Africa, and semi-wild in North America (Frese *et al.*, 1991; Das *et al.*, 2016). This plant is distributed in different regions of Iran, including the low slopes of Alborz, around Tehran, Lahijan, and Rasht, mountainous areas of Khorasan. Chemical compounds in chicory leaves, in addition to minerals, securic yen glucoside is also found. In chicory root, in addition to securic glucoside, two bitter substances called lactosine, interbin, and arsenic (in the range of 0.01 mg per 100 g of the root) are found (Heibatollah *et al.*, 2008; Nouri *et al.*, 2011; Amiri *et al.*, 2014).

These critical enzymes (glutathione S-transferase, aspartate phosphatases, and protein phosphatases) have a ubiquitous nature regulating the vital cellular mechanisms. Thus, this study aimed to clarify the impact of endothall and cantharidin on the expression of the critical genes associated with the crucial enzymes in the virtue of understanding how these herbicides are trampling the growth and development in *C. Intybus*.

2. MATERIALS AND METHODS:

2.1. Plant material and application of treatments

Chicory seeds (*Cichorium intybus* L.) were procured from a private company. Seeds were disinfected with sodium hypochlorite for 5 minutes with washed with distilled water. Then treated by different levels of cantharidin and endothall (Sigma-Aldrich, Germany 99%; 10, 50 and 100 μgml^{-1}) and were kept under 25 ^{o}C for a week to complete the germination. Only those treated with 10 μgml^{-1} of cantharidin and endothall were germinated, placed in pots (4 seedlings per pot) filled with coco peat and perlite, and daily irrigated with 200 ml water for two weeks and kept under 16/8 day/night and 25°C.

Having the proper level of the two herbicides (10 μgmL^{-1}), first to calibrate the amount of solution required for each pot, using water a pot thoroughly sprayed which need 400 mL. The solution was prepared for each concentration in which 2.5 μgmL^{-1}(cantharidin or endothall), 1 mg solved in 400 ML water. In the case of the other treatment (10 $\mu g/mL^{-1}$), it was 4 mg (Table 1). The seedlings were sprayed to apply the treatments (Figure 2). To avoid the effect of the difference in the environment, pots were maintained under the controlled conditions of a greenhouse; day/night 16/8 and 25°C. Two months after applying treatments, samples of roots and leaves from treated seedlings of *C. intybus* were collected separately and used for the analysis. The samples were kept at -80 ^{o}C until RNA extraction.

2.2. RNA extraction

According to the instructions, RNA extraction was performed by a Sinaclone RNX-plus kit from frozen leaf and root samples of *C. intybus*. The quality of RNA extracted by gel electrophoresis was confirmed by 1.5%. The formation of two RNA bands indicated the quality of purified RNA. A spectrophotometer (UV-1280 Shimadzu, Japan) was used to evaluate the extraction quantitatively. In the next step, the extracted RNA was treated with DNase1 enzyme based on the method proposed by Fermentase Company.

2.3. cDNA library

The cDNA was prepared using the RevertAid M-MuLVReverse Transcriptase kit from Sinaclon. 0.1 to 5 µg of total RNA solution was mixed with 1 µl of Oligo-dT and 6 µl of deionized water. Then it was cold off by putting on ice for 5 minutes at + 65 °C and immediately on ice for 5 minutes. 2 µl of reaction buffer solution, 1 µl of deionized water, 2 µl of dNTP mixture, and 1 µl of RT enzyme were added to the reaction mixture. The tubes were placed at +45 °C for 60 minutes and then placed at + 70 °C to stop the reaction. Samples were stored at -20 °C until use.

2.4. Designing primers

Oligo Primer Analysis Software v. 7. was utilized for designing of the primers. The gene understudy has three coding regions (exons) and two non-coding regions (introns). For the quantitative RT-PCR reaction, pairs of specific primers were designed so that the return primer was in the range of the untranslated region 3´3´ (which is unique to each gene), and the forward primer was in the penultimate exon so that the product size PCR and RT-PCR are different and detectable (Table 2).

2.5. Study of the gene expression pattern

Gene expression pattern by quantitative RT-PCR using SuperScript One-Step RT-PCR (Light Cycler, Bio-Rad, USA) with Platinum Taq, Invitrogen, and Real-time PCR in three experimental replications glutathione S-transferase, protein phosphatase, and aspartate phosphatase were studied using BIO-RAD kit and quantitative RT-PCR which they considered as internal controls. Gene expression in Real-time PCR was calculated by 2-ΔΔCt (Livak et al., 2001). In this method, all data were normalized with s18 ribosomal acid ribonucleic acid gene as the internal control (normal). The amount of gene expression changes in different stresses was measured compared to the control (Kermani *et al.*, 2020).

2.6. Statistical Analysis

Minimum three independent replicates were used for each test. Using SPSS version 26 (SPSS Institute, Cary, NC, USA), ANOVA was conducted for the data. The Duncan test performed a comparison of the means at 1% and 5% probability. ChemDraw Ultra V12.0 (PerkinElmer) was used to design the chemical structure of cantharidin and endothall.

3. RESULTS AND DISCUSSION:

3.1. Effect of cantharidin and endothall on GST in shoot and root

Investigating the expression of glutathione S-transferase **(**GST) gene in a tissue-specific manner in *C. intybus* revealed its significant reduction in both shoots and roots exposed to cantharidin and endothall (Figure 3a and 3b). Cantharidin-treated seedlings experienced the

lowest expression level of GST in 2.5 µgml^{-1} (0.92±0.11) while plants treated with ten µgml^{-1} cantharidin comparably showed a higher expression quantity of GST gene in the shoot (2.94±0.61; Figure 3a). GST expression in shoot received 2.5 and 10 µgml^{-1} cantharidin similarly declined (1.07±0.61 and 1.63± 0.23, respectively; Figure 3a). In endothall treated *C. intybus* seedlings (Figure 3b), the shoot of seedlings exposed to 2.5 and 10 µgml-1 endothall had a relative expression level of 1.03±0.08 and 1± 0.03, respectively; Figure 3b that were significantly lower compared to the control. The GST expression level in C. intybus roots applied with 2.5 and 10 µgml-1 of endothall was notably downregulated compared to control (0.71±0.06 and 1±0.08; Figure 3b). Generally, the two herbicides, cantharidin, and endothall generated a similar pattern affecting GST in both roots and shoots of *C. intybus*.

The GST family, as antioxidant factors, play an essential role in tolerating various stresses in plants. Bajsa *et al.* (2012) studied the effect of cantharidin on the Arabidopsis proteome reported that detoxifying enzymes, especially GST, were down-regulated by cantharidin. GST functions are closely associated with response to a wide variety of environmental stresses from abiotic to xenobiotic-types, to name a few, herbicides, low temperature, hypoxic stress, drought, physical damage, diseases, phytohormones (ethylene, auxin, trinitrotoluene, hydrogen peroxide (H2O2) (Chen et al., 1996; Seppanen *et al*., 2000; Brentner *et al*., 2008), its significant decrease in relative expression levels under cantharidin and endothall stress indicate the control mechanisms of these herbicides.

GST induction suggests that cantharidin induces pseudo-defense responses by inhibiting protein phosphatases through the primary role of GST in the detoxification processes of xenobiotic and endobiotic compounds regulating GST enzymes in addition to other metabolic pathways (Roxas et al., 1997). A prime example of the importance of GST in the plant is when the transgenic tobacco plants overexpressing GST exposed to salt and drought stress, which exhibited a higher level of resistance. In comparison to wild-type plants, these plants had a lower level of lipid peroxidation and showed a higher metabolic activity under salinity and drought stress (Roxas *et al*., 2000).

3.2. Effect of Cantharidin and endothall on PPO in shoot and root

The influence of Cantharidin on PPO in shoot and root of *C. intybus* followed a similar pattern (Figure 4a and 4b) where the expression level of PPO in shoots and roots of treated seedlings with 2.5 µgml^{-1} (0.51±0.03 and 1.34±0.12, respectively) reduced in a statistically significant manner (5%) compared with control. On the contrary, at the highest level of Cantharidin (10 µgml^{-1}), the expression level of PPO markedly increased in both shoots and roots (3.55±0.21 and 3.96±032, respectively; Figure 4a). The expression of PPO in shoot received 2.5 µgml^{-1} endothall strikingly enhanced (43.21±3.1, Figure 4a) whereas the expression of PPO in shoots treated with ten µg/ml^{-1} declined to 0.19±0.02 (Figure 4b). Moreover, the roots of endothall treated *C*. *intybus* seedlings (Figure 4b) in general indicated an expression level of close to zero in which 2.5 and 10 µgml^{-1} treatments showed 0.43±0.04 and 0.1±0.00 relative expression level with no significant difference at 5% level against control 0.55±0.03. Overall, cantharidin and endothall revealed an almost different pattern in PPO expression and in a dosage-dependent way.

Cantharidin and endothall are protein phosphatase inhibitors, especially PP2A and PP4 maintain the balance between dephosphorylated and phosphorylated forms of specific proteins. They play an essential role in transduction pathways regulating gene expression, cell proliferation and differentiation, and apoptosis. It was observed that both compounds inhibited the expression of protein phosphatase gene in the roots and shoots of chicory plants similar to Bajsa *et al.* (2015) that observed cantharidin significantly reduced transcription of light and auxin transduction genes by inhibiting protein phosphatases in Arabidopsis plants. Additionally, Bajsa *et al*. (2012) reported that endothelium and cantharidin altered the serine/threonine protein phosphatases gene expression in *Arabidopsis thaliana* Lemna paucicostata, which in turn affected the regulation of many biochemical processes in these plants. Li and Casida (1992) pointed to the inhibitory role of cantharidin and endothelium in PP2A phosphatase protein, consistent with the result of this study.

To give a perspective on the importance of PPOs in plants recently has been determined that a significant ratio of the sequenced *Arabidopsis* genome involved in encoding protein kinases and PPOs, which catalyzing reversible phosphorylation. An optimal regulation in plant cells requires a balance between kinases and phosphatases (Luan 2003). Thus, the influence of cantharidin and endothall on reducing PPO

expression was severe, particularly endothall. Application of catandirin on Arabidopsis plants revealed a notable reduction in PPOs while dramatically increased GST expression due to the role of GST in controlling the damage and maintaining the situation for the function of PPOs. However, in this study, the opposite where PPO did not downregulate while GST was reduced by increasing dosage (Bajsa *et al.*, 2015). The mechanism that has been proposed for the inhibitory effect of endothall at the cellular level involves distortion of the cell division orientation program and microtubule spindle formation, which initiates the prevention of the cell cycle in prometaphase. Although a lot has been revealed about the function of PPOs and the influence of herbicides on them, many territories of their cellular function still yet to be known (Tresch *et al*., 2011).

3.3. Effect of cantharidin and endothall on ASPT in shoot and root

The expression of ASPT in shoots under treatments, control, and 2.5 μgml^{-1} cantharidin was almost zero (Figure 5a), while in 10 μgml^{-1} treatment, its expression was radically increased (3.76±0.23). Differently, roots treated with cantharidin indicated high relative expression of ASPT, particularly in 10 μgml^{-1} (2.07±0.11), which was lower than control (4.11±0.23) and made a statistically significant difference. Like shoots treated with 2.5 µgml-1 cantharidin, roots similarly did not express ASPT (Figure 5a). In *C. intybus* seedlings exposed to endothall, expression of ASPT followed a different behavior in which the relative expression level of ASPT in shoots under 2.5 μgml^{-1} was 17.49±1.21 and made a significant difference with control (12.22±2.5) while in roots its expression level under 2.5 μgml^{-1} was down to 0.45±0.09 (Figure 5b). Also, the expression of the root of ASPT in control (6.11±0.54) was considerably lower than control in shoots. Finally, similar to shoots under ten μgml^{-1} endothall, the expression level of ASPT was downregulated markedly (Figure 5b).

ASPT is one of the main pathways for synthesizing and removing amino groups from a molecule, thereby facilitating internal conversion of carbohydrate and protein metabolism when plants are exposed to stress conditions to address the excessive energy demand (Gelfand and Steinberg, 1977; Liepman and Olsen, 2004). This enzyme exists at the cellular level in various isozyme forms. They are also differentially located in a minimum of four different subcellular compartments, namely the cytosol, plastids, mitochondria, and peroxisomes (Wadsworth, 1997). Aspartate-based protein phosphatases are one of the main classes of PPOs (Fuchs et al., 2013). Given the fundamental role of phosphatases, particularly ASPT, manipulating these enzymes can be a reliable approach for precise metabolic engineering to generate crop cultivars with improved stress tolerance.

Additionally, many phosphatases do not exist in animals, which may be suitable targets for herbicides (Givan, 1980; Nakamura and Tolbert, 1983; Reumann, 2000). Despite their primary role in plant metabolism and the fact that herbicides can be developed targeting these enzymes. So far, there is no published study exists addressing the effect of herbicides on ASPT in plants.

In all the eukaryote organisms, only 11 highly conserved metallo-dependent protein phosphatase exist which 9 of which are amino acids, that 4 of them are aspartate residues responsible for the coordination of metallic ions essential for catalytic processes. Unlike other protein families that have been the subject of many studies, ASPT has only been known for 10 years in animals and plants (Matsuda *et al*., 1997; Archambault *et al*., 1998; Moorhead *et al*., 2009).

Differences in responses to catalase and glutathione peroxidase enzymes in C. intybus and cantharidin and endothall have also been observed in a study conducted by this group (Kermani *et al*., 2020). By taking a comparative approach between the result of the previous study and this study, catalase and glutathione peroxidase enzymes, despite their critical role in plants under stress, were less responsive than PPO, GST ASPT in this study. The possible explanation could be that catalase and glutathione peroxidase enzymes are secondary defense mechanisms while PPO, GST, and ASPT regulate basically the whole plant organization. Further, the responses of PPO, GST, and ASPT to endothall were less remarkable in general compare to cantharidin.

4. CONCLUSIONS:

The influence of endothall and cantharidin herbicides on the gene expression changes of vital enzymes, including GST, PPO, and ASPT in *C. intybus* investigated in this study. These enzymes involved in the majority of biochemical processes in plant and herbicides used significantly reduced their expression. However, reduction in expression level often showed dose-dependency and tissue-specificity. Response to endothall and cantharidin concentrations in shoots and roots were different. Also, downregulation of the enzymes in the low level (2.5 μgml^{-1}) used in this

study in some cases did not follow the highest concentration (10 μgml^{-1}). Moreover, endothall is an analog of cantharidin but act differently in many instances. It can be speculated that the significant meddling of cantharidin and endothall in growth negatively affecting the critical enzymes in *C. intybus.* The effect of these herbicides at the physiological level on *C. intybus* is under study by this group and strongly recommends studying other essential enzymes along with a broader range of concentrations from these herbicides.

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Table 1. *Concentrations of herbicides applied on C. intybus seedlings.*

Treatments	Discription	Concentration (µg/mL^{-1})
1.	Control	Distilled water only
2.	Cantharidin	2.5
		10
3.	Endothall	2.5
		10

Table 2. *Sequences of primers used to evaluate endothall and cantharidin's influence on essential genes (protein phosphatases; PPO, glutathione S-transferase; GST, aspartate phosphatases; ASPT, in C. intybus).*

Gene	Forward 5' → 3'	Reverse 5' → 3'
PPO	TCCGGTTCATATATGGACT	TCC CTC ACC TTG ACA CGAAC
GST	TCCTCC AGATGAACC CGGTA	TTTCTTCAT ATCCTCCT
ASPT	GAACAAGTACTA AAC CGTGTG	AATTTGCCTTCTTCATCCAC

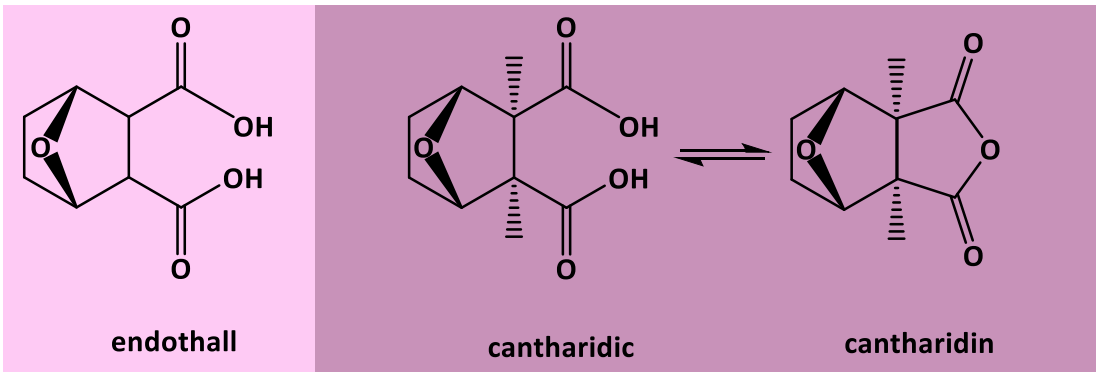

Figure 1. *Structure of endothall, cantharidin, and its dicarboxylic acid analog.*

Figure 2. *C. intybus seedlings under treatments of various concentrations of endothall and cantharidin (mentioned in Table 1).*

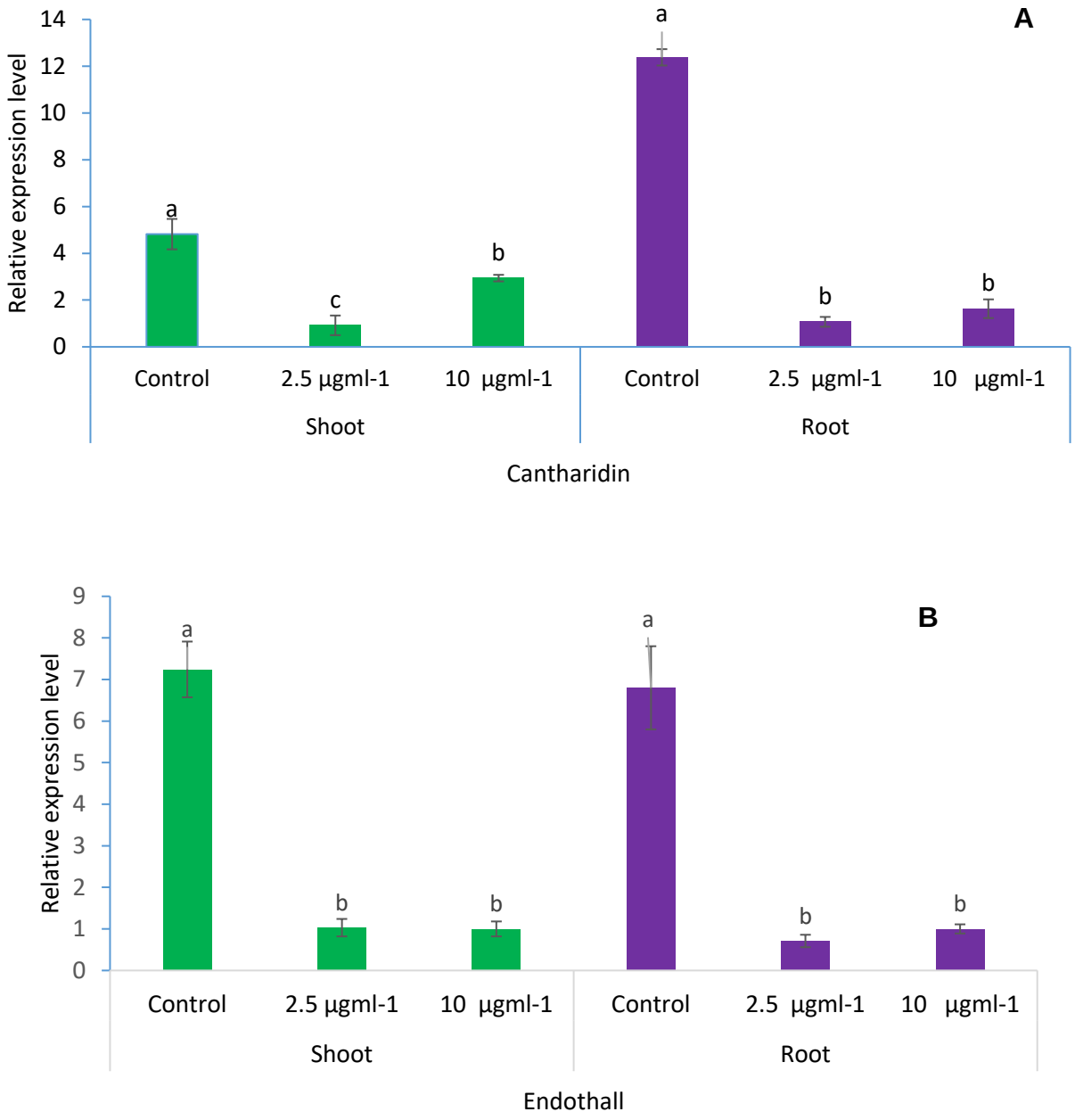

Figure 3. *The expression level of GST in Chicorium intybus shoots and roots treated with cantharidin (a) and endothall (b).*

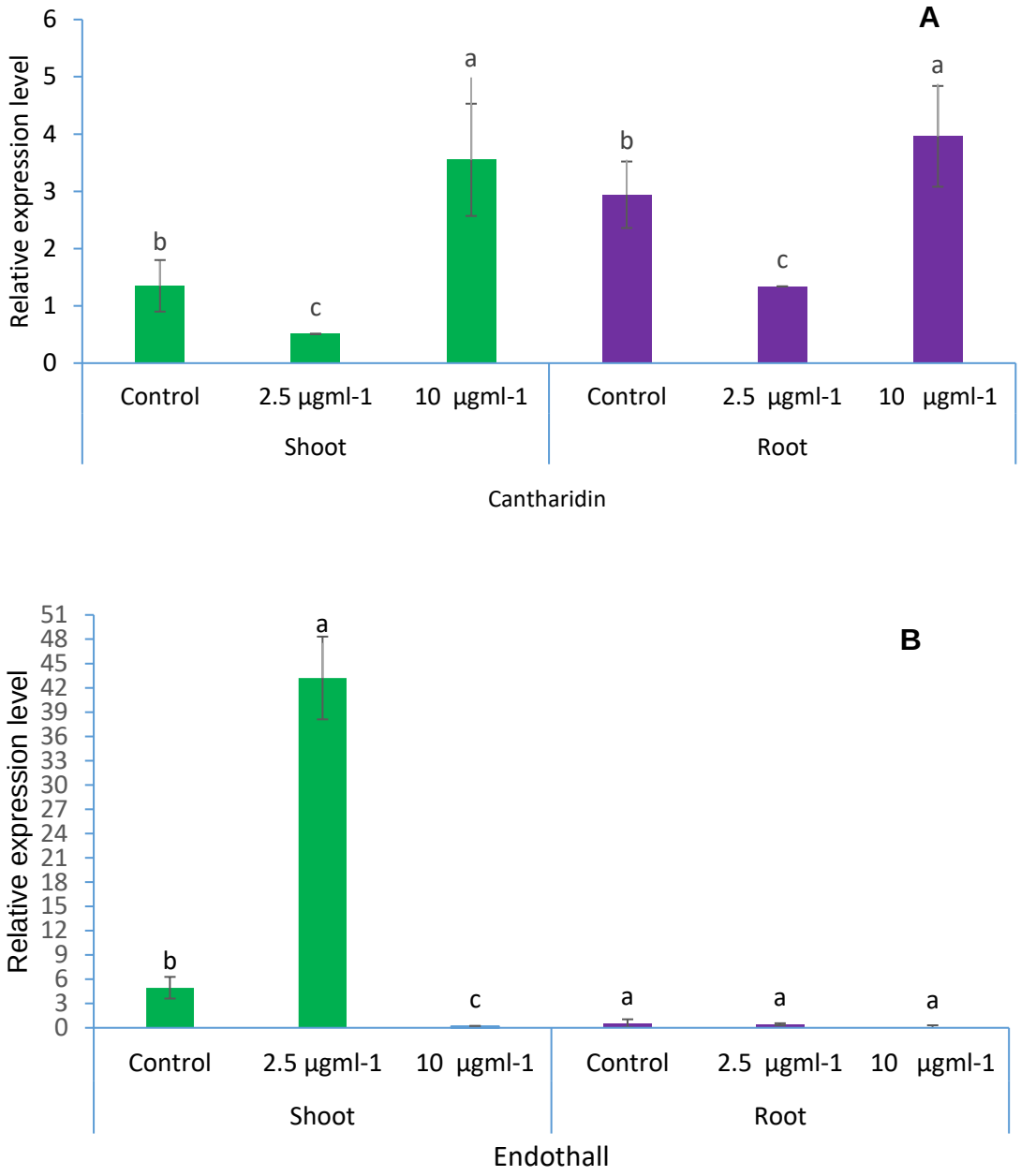

Figure 4. *The expression level of PPO in Chicorium intybus shoots and roots treated with cantharidin (a) and endothall (b).*

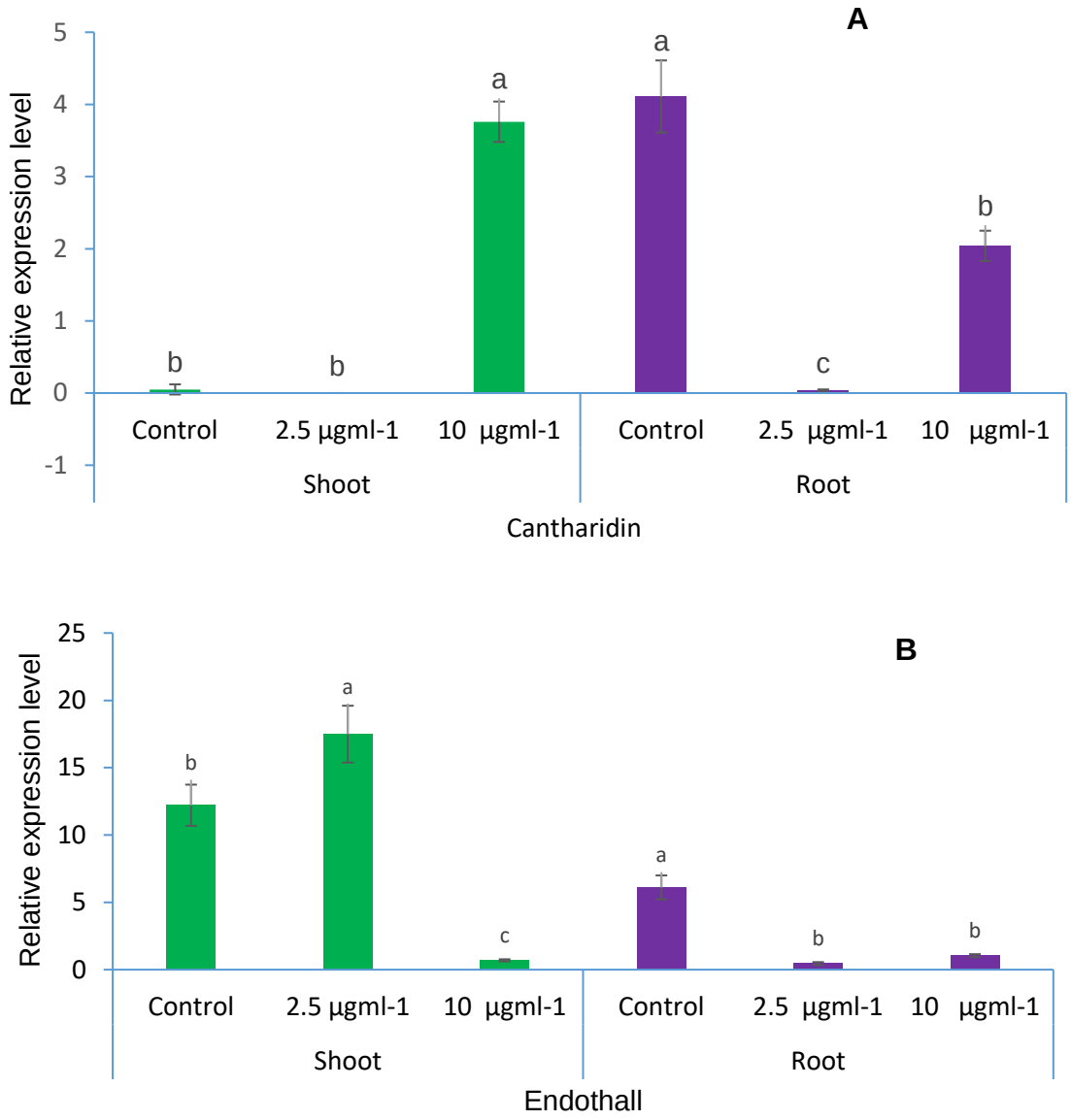

Figure 5. *The expression level of ASPT in Chicorium intybus shoots and roots treated with cantharidin (a) and endothall (b).*