DOI: 10.22620/agrisci.2022.35.008

EFFECT OF ADDITIONALLY BCAA SUPPLY ON IMAZAMOX-TREATED SUNFLOWER AND ANTIOXIDATIVE DEFENSE SYSTEM

Dobrinka Balabanova

Agricultural University – Plovdiv, Bulgaria
Email: d_balabanova@au-plovdiv.bg

Abstract

Sunflower is an economically important oilseed crop worldwide and weeds have a negative effect on its yields. The herbicide imazamox, combined with imazamox-resistant (IMI-R) sunflower hybrids, is a technology known as the Clearfield production system and successfully controls weeds. The mechanism of action of the herbicide imazamox is related to blocking the AHAS enzyme, which catalyzes the first step in the biosynthetic pathway of the branched-chain amino acids (BCAA). In the present study, we investigated the effect of the supplementary addition of BCAA to imazamox-treated IMI-R sunflower plants and antioxidative metabolism. Our results showed that the additional application of BCAA has a beneficial effect on both the total protein content and the activity of the antioxidant defense system of plants. This amelioration is probably a secondary effect of the enhanced protein metabolism and the supposed direct involvement of the three amino acids in the overall protein turnover.

Keywords: sunflower, imazamox, branched-chain amino acids (BCAA), antioxidarive enzymes

INTRODUCTION

Sunflower is an economically important oilseed crop worldwide. The presence of weed species in farmlands causes considerable yield losses to all crops, including sunflower. This inevitably leads to the use of herbicides to improve both the yield and the quality of the sunflower production. The technologies using herbicide-resistant sunflower hybrids allow post-emergence control of a wide range of broad leaf and parasitic weeds. Such a technology is the Clearfield® production system, which includes the use of an imidazolinone herbicide (imazamox) in combination with imidazolinone resistant (IMI-R) sunflower hybrids, since 2003 (Penning et al., 2008). The mode of action of imazamox is blocking the enzyme acetohydroxyacid synthase (AHAS), also known as acetolactate synthase (ALS). AHAS is the first common enzyme in the synthesis of the branched-chain amino acids (BCAA) valine, leucine and isoleucine in plants (Duggleby and Pang, 2000). Therefore, the application of AHAS-inhibiting herbicides leads to a strongly suppressed protein turnover in plants and subsequent plant death of susceptible species. AHAS-inhibiting herbicides are widely used in agriculture given their high weed control efficacy, high crop-weed selectivity, low use rates, low levels of mammalian toxicity and their favorable environmental profile (Shaner, 2003). Despite the relatively high resistance of IMI-R sunflower hybrids to the herbicide imazamox, temporary yellowing of meristem tissues and inhibition of plant growth may occur after imazamox application (Pfenning et al. 2008, Sala et al. 2012). This inhibitory effect might be even more pronounced in combination with stressful abiotic conditions (Pfenning et al. 2008). In our previous study we observed reduced chlorophyll content in the young leaves of the imazamox-treated IMI-R sunflower (Balabanova and Vassilev 2015, Balabanova *et al*. 2016). We also found that this growth retardation was overcome by the plants within

14 days (Balabanova et al., 2020).

Conventional sunflower cultivars are sensitive to imazamox, while the IMI-R hybrids are able to resist application rates that are lethal for the susceptible sunflower varieties. The resistance of Clearfield® hybrids to imidazolinone herbicides is based on a naturally occurring mutation in the *AHAS1* gene. In addition to this genetic resistance, some authors have reported that non-target site mechanisms of resistance are involved in the imidazolinine resistance of the *Imisun* Clearfield® sunflower hybrids (Sala et al., 2012). In our previous research, we also confirmed that imazamox triggers the xenobiotic detoxification metabolism in sunflower (Balabanova et al., 2018). In many cases, the enhanced activation of the antioxidative defense system in the herbicide-treated plants has also been reported to play a non-target mechanism of resistance (Yang et al., 2021). On other hand, many herbicides are causing toxicological effects on plants by triggering ROS production (Erinle et al., 2016). Not much is known concerning the effect of imidazolinones on the antioxidative defense system in plants. In 2005, Zabalza and colleagues reported that the oxidative stress is not related to the mode of action of the herbicides that inhibit AHAS enzyme. On other hand, in a study by Manabe and co-workers (2007), it is reported that the treatment of *A. thaliana* plants with imazethapyr results in the activation of genes related to the stress response, including antioxidative enzymes. Again for imazethapyr applied to *Arabidopsis*, Qian et al. (2011) report increased superoxide dismutase (SOD) activity and lipid peroxidation. The root application of imazamox has also been reported to induce the activation of antioxidative enzymes such as SOD and guaiacol peroxidase (GPOD) in nodules of the beam and common vetch (Garcia-Garijo et al., 2014). Recently in a study conducted with IMI-R sunflower plants, Arda et al., (2020) demonstrated that imazamox leads to over expression of SOD and CAT genes. These findings indicate that the herbicides of the imidazolinine group have an effect on the antioxidative defense machinery of the plants, although this effect might be studied in more details.

Plants have developed various defense mechanisms to avoid oxidative damage and to reduce the reactive oxygen species (ROS). The oxidative stress resulting from ROS production damages the cell membranes and metabolites in plants, disturbs physiological and biochemical metabolism and leads to inhibited growth and development. During this process, the product of membrane lipid peroxidation malondialdehyde (MDA) might be used to indicate the level of lipid peroxidation. The enzyme antioxidation system includes superoxide dismutase (SOD), catalase (CAT) and peroxidases (PODs). SOD is well known as the first step of defense against ROS damage (Foyer et al., 1994). PODs are playing a role in utilizing electron donors to convert H_2O_2 to H_2O, and the increased PODs activities can prevent plant cells from injuries caused by herbicides (Song et al., 2007).

The growth retardation caused by the herbicide imazamox on IMI-R sunflower is completely recoverable within 14 days after the application, but this has raised a question of whether this recovery could be facilitated by an additional supply of BCAA to the plants. In addition, there are still some unknown details about the behavior of the antioxidant system of imazamox-treated plants. Therefore, we set up the following aim of the present model study: to monitor the effect of an additional supply of branched-chain amino acids (BCAA) to imazamox-treated IMI-R sunflower plants and antioxidative metabolism.

MATERIALS AND METHODS

Plants and treatments: In this study, sunflower (*Helianthus annuus* L.) Clearfield® hybrid Meldimi carrying haplotype 5 of the *AHAS1* gene (*Imisun* trait) were used. The seeds were washed with distilled water and

germinated in Petri dishes for three days at 22°C. The seedlings were transplanted to pots (4 plants per pot) filled with a half-strength modified Hoagland nutrient solution and placed in a growth chamber (14-h photoperiod, a photosynthetic photon flux density at the leaf level of 250 μmol m^{-2} s^{-1}, day/night temperatures of 24/22 ± 1°C, and a relative humidity of 60%). An experimental design, including four treatments, was set up with plants at stage of 4 - 6 true leaves: **1) non-treated** plants; **2) BCAA** – the branched-chain amino acids (valine, leucine and isoleucine) were supplied by the growth solution in concentration 0,5 mM of each; **3) imazamox -** application of imazamox was done by spraying it on the leaves in a approximate rate of 132 μg per plant (approximately 1 ml of solution, with concentration 3,3 ml/L of Pulsar 40®) and **4) imazamox + BCAA**. Three pots (four plants per pot) were used for each variant. The analyses were performed on the second, third, and fourth true leaf pairs. Sampling was done at 1, 7, and 14 d after treatment (DAT). Samples were immediately frozen in liquid nitrogen and stored at -80°C until the analysis.

Protein content: The protein extraction was performed according to Schröder and Götzberger (1997) and is described in detail in our previous publication (Balabanova et al., 2020). The protein content was determined spectrophotometrically using the *Biorad* (Hercules, CA, USA) protein assay and a bovine albumin as a standard (Bradford 1976).

Enzyme activity measurement:

Superoxide dismutase (SOD, EC 1.15.1.1) activity was based on the inhibition of cytochrome ***c*** by xanthine oxidase, described by McCord and Fridovich, 1969.

Guaiacol peroxidase (GPOD, EC 1.11.1.7) capacity was determined with guaiacol substrate under an established methodology, described by Bergmeyer (1974).

Syringaldazine peroxidase (SPOD, .EC 1.11.1.7) was measured with syringaldazine as a substrate, described by Imberty et al. (1985).

Lipid peroxidation: the MDA content of the plant leaves was measured spectrophotometrically, according to Dhindsa et al. (1981).

Statistical analysis: Statistical analysis was performed using one-way *ANOVA*. Based on these *ANOVA* results, a post-hoc Duncan test for a mean comparison was performed at a 95% confidence level to test for significant differences between treatments.

RESULTS

To determine whether imazamox and BCAA supplementation interfere with the protein metabolism the protein content was estimated (Fig. 1). On the first day after the treatments, the protein content of sunflower plants did not show significant changes. Seven days later, the protein content is strongly reduced with 34% in the plants treated with imazamox. In the plants treated with the combination of imazamox and BCAA, the protein content is significantly higher with 20%, compared to the plants that have received single imazamox application. On the 14th day the protein content shows the same tendency and a considerable reduction is obvious on the plants treated with imazamox. The sunflower treated simultaneously with imazamox and BCAA had protein content comparable with one of the non-treated control plants.

The performance of the antioxidant system in the sunflower plants was studied by measuring the activities of some antioxidative enzymes such as superoxide dismutase (SOD), guaiacol peroxidase (GPOD) and syringaldazine peroxidase (SPOD). The SOD is catalyzing the dismutation of the superoxide radical (O^{-}_{2}) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). GPOD and SPOD are enzymes with similar functions in cells against the oxidative damage caused by H_2O_2, but are using different substrates – guaiacol and syringaldazine, respectively.

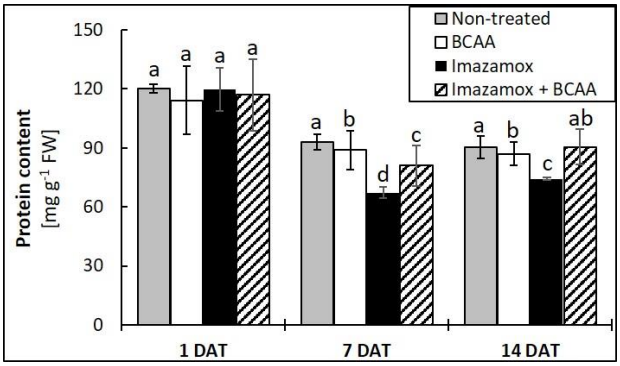

Fig. 1. Total protein content in IMI-R sunflower plants treated with imazamox and/or branched-chain amino acids (BCAA), 1, 7 and 14 days after treatment (DAT). Means ± SDs, n = 3 biological replicates; different letters (a, b, c, d) indicate significant differences ($P < 0.05$).

The response of the antioxidative defense system of the sunflower plants is shown on Fig. 2. Imazamox application results in strongly increased SOD activity with 30% at 7 DAT, while on the 1 and 14 DAT the SOD was not significantly influenced by the herbicide. The combination of imazamox and BCAA has significantly reduced these effects caused by the herbicide imazamox, where 7 DAT - the SOD activity of the plants treated with imazamox + BCAA was near to one of the non-treated control plants. The single treatment with BCAA had no remarkable effect on the activity of SOD in sunflower plants. The imazamox treatment had a highly pronounced activating effect on GPOD enzyme, where the activity was significantly increased at 1 and 7 DAT with 74 and 144%, respectively. This effect is lowered down to the level of the non-treated control until the 14 DAT. The extra treatment with BCAA did not reduce the impact of the herbicide and the activity of treatments with imazamox and imazamox + BCAA are on similar levels and have significantly increased compared to non-treated controls. The SPOD activity was also significantly increased by imazamox treatment through all the periods up to 14 DAT. Unlike the GPOD activity in SPOD it was obvious that the addition of BCAA alleviated the effect of imazamox and the activities of SPOD of the plants that have received treatment of imazamox + BCAA were near the ones of the control plants.

Lipid peroxidation is a metabolic process that causes oxidative deterioration of lipids by reactive oxygen species and the detection of malonaldehyde (MDA) has traditionally been used as its primary indicator (Bonnes -Taourel et al., 1992). Therefore, we measured the level of MDA to evaluate the damage caused by imazamox on cell lipids and membranes. The amount of MDA detected in the samples was significantly increased due to imazamox application in all tested periods. The highest MDA levels were on 7 and 14 DAT with 93 and 114%, respectively, compared to non-treated plants. The addition of BCAA lowered this indicator, and the MDA levels on 7 and 14 DAT was 19 and 40% lower than those of single imazamox-treated sunflower.

DISCUSSION

The herbicides that inhibit the synthesis of amino acids are widely used in agronomy because of their high efficiency. All those herbicides limit plants' efficacy in producing proteins and essential metabolites, leading to massive metabolic disorder. A decrease of total protein content has been repeatedly reported as a result of AHAS-inhibiting herbicides application, even in the resistant species (Scarponi et al., 2001; Zulet et al., 2013), which corresponds also to our results. Previously, we have found that the plant biostimulants and in particular protein hydrolisates (containing short peptides and free amino acids) has an ameliorative effect on plants injured by imazamox (Balabanova et al., 2016; Balabanova, 2021; Neshev et al., 2022). This improving effect from adding the amino acids indicates that the additionally supplied amino acids are involved in the disrupted protein metabolism and thus relieve the overall protein turnover in plants. These led us to assume that the specific addition of the branched-chain

amino acids whoose synthesis imazamox is directly blocking, could have even more pronounced positive effect on plant metabolism. The improving effect of additional BCAA supply on protein content and antioxidative defense metabolism on imazamox injured plants was confirmed by the obtained results.

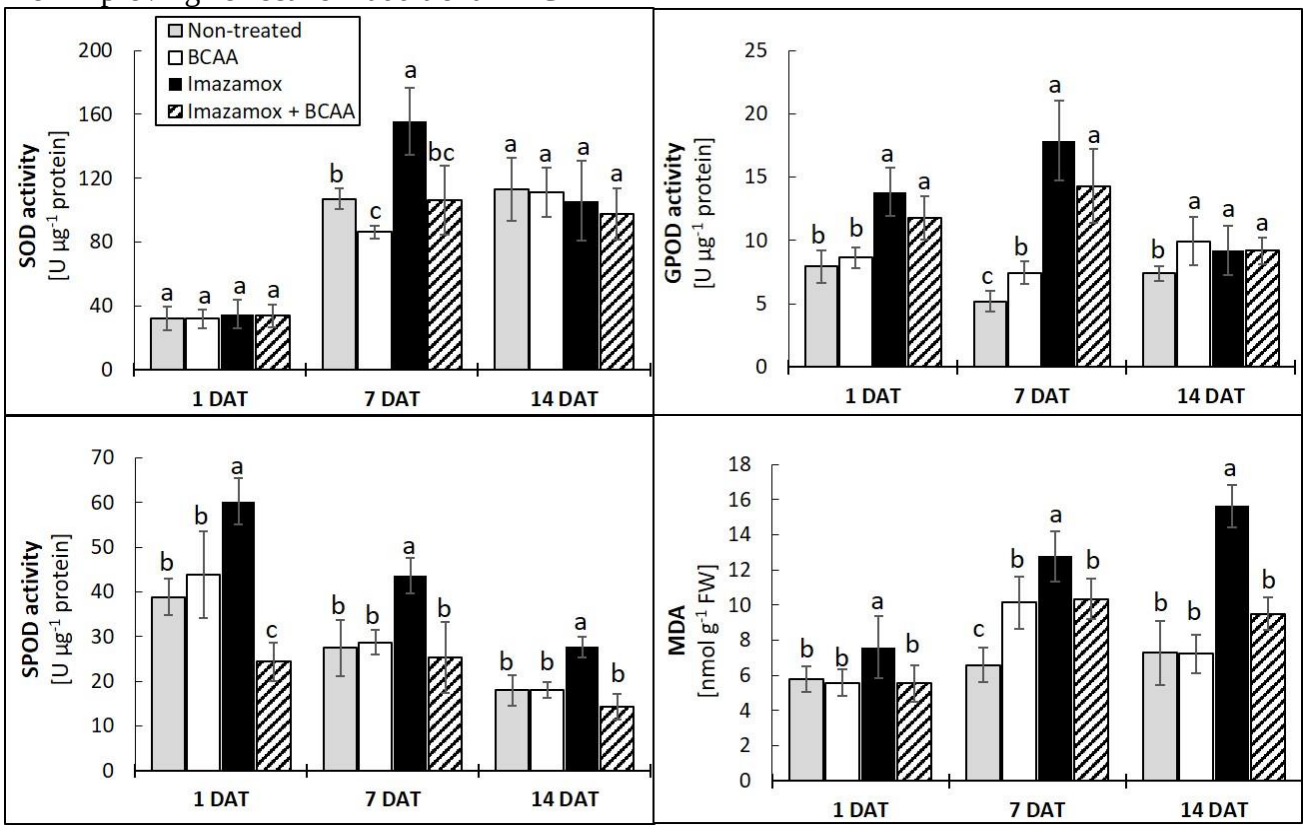

Fig. 2. Activity of antioxidative enzymes superoxide dismutase (SOD), guaiacol peroxidase (GPOD), syryngaldazine peroxidase (SPOD) and lipid peroxidation (MDA) of IMI-R sunflower plants treated with imazamox and/or branched-chain amino acids (BCAA), 1, 7 and 14 days after treatment (DAT). Means ± SDs, n = 3 biological replicates; different letters (a, b, c, d) indicate significant differences ($P < 0.05$).

The herbicide imazamox alleviates the antioxidative defense mechanism in plants. Even though the ROS level is not directly connected to imazamox mode of action, it becomes evident that the oxidative damage has also increased. In 2014 Gaecia-Garijo also detected an increased SOD activity as a result of imazamox application on beam and common vetch. Arda et al., (2020) demonstrated that increasing the imazamox dose leads to over expression of SOD and CAT genes in IMI-R sunflower plants. Recently, a paper by Li and colleagues (2022) reported significant lipid peroxidation and antioxidant damage in *Lemna minor* plants supplied with imazamox. According to the mentioned reports as well as based on our results, we may assume that the AHAS inhibitors are influencing the antioxidative metabolism in plants. This suppressive effect was most pronounced about a week after the treatment and within two weeks the cells recover their functioning. The addition of BCAA significantly decreased this negative effect by maintaining membrane integrity and has a partially ameliorative effect on antioxidative enzymes. This mitigating effect might be due to an improvement of the protein turnover, having a secondary effect on the overall metabolism in plant cells, including the antioxidative defense system.

CONCLUSION

The protein metabolism is an essential process in plants that is directly disrupted by the herbicide imazamox, resulting in a significant decrease of the protein content. The additional supply of BCAA to the plants injured by the herbicide had a beneficial effect on the protein content, and consequently, on the protein turnover. The treatment with imazamox leads to activating the antioxidative enzymes SOD, GPOD and SPOD in the studied sunflower plants, where the addition of BCAA has also had an ameliorative effect. This enhancing effect induced by the additional BCAA supply on imazamox-demaged plants might be due to the initially ameliorated protein turnover, resulting in a mitigation of the overall cell metabolism, as a secondary effect.

REFERENCES

Arda, H., Kaya, A., & Alyuruk, G. (2020). Physiological and Genetic Effects of Imazamox Treatment on Imidazolinone-Sensitive and Resistant Sunflower Hybrids. *Water Air Soil Pollut*, 231, 118. https://doi.org/10.1007/s11270-020-4439-8

Balabanova, D., & Vassilev, A. (2015). Response of sunflower Clearfield hybrids to both recommendable and higher doses of imazamox herbicide. *Agricultural Sciences*, 8 (18), 41-46.

Balabanova, D., Remans, T., Vassilev, A., Cuypers, A., & Vangronsveld, J. (2018). Possible involvement of glutathione S-transferases in imazamoxdetoxification in an imidazolinone-resistant sunflower hybrid. *Journal of plant physiology*, 221, 62-65. https://doi.org/10.1016/j.jplph.2017.12.008

Balabanova, D., Paunov, M., Goltsev, V., Cuypers, A., Vangronsveld, J., & Vassilev, A. (2016). Photosynthetic Performance of the Imidazolinone Resistant Sunflower Exposed to Single and Combined Treatment by the Herbicide Imazamox and an Amino Acid Extract. *Front Plant Sci*. https://doi.org/10.3389/fpls.2016.01559

Balabanova, D. (2021). The ameliorative effect of protein hydrolysate on the imazamox-damaged young wheat plants, *Agricultural Sciences*, 13(31), 69-76. https://doi.org/10.22620/agrisci.2021.31.010

Balabanova, D., Remans, T., Cuypers, A., Vangronsveld, J., & Vassilev, A. (2020). Imazamox detoxification and recovery of plants after application of imazamox to an imidazolinone resistant sunflower hybrid. *Biologia plantarum*, 64, 335-342. https://doi.org/10.32615/bp.2019.150

Bergmeyer, H.U., Gawehn, K., & Grassl, M. (1974). Enzymes as biochemical reagents. In: Bergmeyer HU (ed) *Methods in enzymatic analysis*. Academic Press, New York.

Bonnes-Taourel, D., Guérin, M.C., & Torreilles, J. (1992). Is malonaldehyde a valuable indicator of lipid peroxidation? *Biochem Pharmacol.* Sep 1, 44(5), 985-8. https://doi.org/10.1016/0006-2952(92)90132-3

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. - *Anal Biochem*, 72, 248-254.

Dhindsa, R.S., Plumb-Dhindsa, P., & Thorpe, T.A. (1981). Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase, J. *Exp. Bot*. 32, 93–101.

Duggleby, R.G., & Pang, S.S. (2000). Acetohydroxyacid synthase. J. Biochem. *Mol. Biol*. 33, 1–36.

Erinle, K.O., Jiang, Z., Li, M.Y., Su, G.X., Ma, B.B., Ma, Y.H., & Zhang, Y. (2016). Oxidative stress response induced in an atrazine phytoremediating plant: Physiological responses of *Pennisetum glaucum* to high atrazine concentrations. Int. J. *Phytorem*. 18, 1187–1194.

Foyer, C.H.; Lelandais, M.; & Kunert, K.H. (1994). Photooxidative stress in plants. *Physiol. Plant*., 92, 696–717. https://doi.org/10.1111/j.1399-3054.1994.tb03042.x

García-Garijo, A., Tejera, N.A., Lluch, C., & Palma, F. (2014). Metabolic responses in root nodules of *Phaseolus vulgaris* and *Vicia sativa* exposed to the imazamox herbicide. *Pestic Biochem Physiol*. 111, 19-23. https://doi.org/10.1016/j.pestbp.2014.04.005

Imberty, A., Goldberg, R., & Catesson, A. M. (1985). Isolation and characterization of Populus isoperoxidases involved in the last step of lignification. *Planta* 164, 221-226

Li, R., Luo, C., Qiu, J., Li, Y., Zhang, H., & Tan H. (2022). Metabolomic and transcriptomic investigation of the mechanism involved in enantioselective toxicity of imazamox in Lemna minor. *Journal of Hazardous Materials*, 425, 127-818. https://doi.org/10.1016/j.jhazmat.2021.127818 , PMID: 34875416.

Manabe, Y., Tinker, N., Clville, A., & Miki, B. (2007). CSR1, the sole target of imidazolinone herbicide in *Arabidopsis thaliana*. *Plant Cell Physiol*. 9, 1340–1358. http://dx.doi.org/10.1093/pcp/pcm105

McCord, J., & Fridovich, I. (1969). Superoxide dismutase: an enzymatic function for erythrocuperein (hemocuprein). *The Journal of Biological Chemistry*; 244, 6049–55.

Neshev, N.; Balabanova, D.; Yanev, M.; & Mitkov, A. (2022). Is the Plant Biostimulant Application Ameliorative for Herbicide-Damaged Sunflower Hybrids? Ind. *Crops Prod*., 182, 114-926. https://doi.org/10.1016/j.indcrop.2022.114926

Pfenning, M., Palfay, G., & Guillet, T. (2008). The CLEARFIELD® technology –a new broadspectrum post-emergence weed control system for European sunflower growers. J. *Plant Dis. Prot.* 1861–4051 (Special Issue XXI, 000 - 000, ISSN).

Qian, H., Lu, T., Peng, X., Han, X., Fu, Z., & Liu, W. (2011). Enantioselective phytotoxicity of the herbicide imazethapyr on the response of the antioxidant system and starch metabolism in *Arabidopsis thaliana*. *PLoS One*., 6, 6(5), e19451. https://doi.org/10.1371/journal.pone.001945 ,. PMID: 21573119; PMCID: PMC3089624.

Sala, C.A., Bulos, M., Altieri, E., & Weston, B. (2012). Response to imazapyr and dominance relationships of two imidazolinone-tolerant alleles at the *Ahasl1* locus of sunflower. *Theor. appl. Genet.* 124, 385-396.

Scarponi, L., Vischetti, C. & del Buono, D. (2001). Imazamox in maize, uptake, persistence and interference on protein and carbohydrate formation. *Ital. J. Food Sci.*, 13(2), 213-220.

Schröder, P., & Götzberger, C. (1997). Partial purification and characterization of glutathione S-transferase from leaves of *Juniperus communis*, *Larix decidua* and *Taxus baccata*. Appl. Bot. 71, 31-37.

Shaner, D.L. (2003). Imidazolinone herbicides. In: Plummer, D., Ragsdalr, N. (ed.): *Encyclopedia of Agrochemicals*. 769-784. John Wiley and Sons, Hoboke.

Sharner, D.L. & Singh, B.K. (1997). Acetohydroxyacid synthase inhibitors.

In: Roe RM, Burton JD, Kuhr RJ (eds) *Herbicide activity: Toxicology, Biochemistry and Molecular Biology*. IOS press, Amsterdam, 69–110.

Song, N.H.; Yin, X.L.; Chen, G.F.; & Yang, H. (2007). Biological responses of wheat (*Triticum aestivum*) plants to the herbicide chlorotoluron in soils. *Chemosphere*, 68, 1779–1787, https://doi.org/10.1016/j.chemosphere.2007.03.023

Yang, J.; Yu, H.; Cui, H.; Chen, J.; & Li, X. (2021). High antioxidant ability confer resistance to atrazine in *Commelina communis* L. *Plants*, 10, 2685. https://doi.org/10.3390/plants10122685

Zabalza, A., Gaston, S., Sandalio, L.M., Del Río, L. A., & Royuela, M. (2007). Oxidative stress is not related to the mode of action of herbicides that inhibit acetolactate synthase. *Environ Exp Bot* , 59,150–159

Zulet, A., Gil-Monreal, M., Villamor, J.G., Zabalza, A., van der Hoorn, R.A., Royuela, M. (2013). Proteolytic pathways induced by herbicides that inhibit amino acid biosynthesis. *PLoS One.* 6, 8(9), e73847. https://doi.org/10.1371/journal.pone.0073847 PMID: 24040092; PMCID: PMC3765261.