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PHYSIOLOGICAL RESPONSES OF PEA PLANTS TO TREATMENT WITH SYNTHETIC AUXINS AND AUXIN-TYPE HERBICIDE

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Abstract

Todorova D., Sergiev I., Shopova E., Brankova L., Jankauskienė J., Jurkonienė S., Gavelienė V., Mockevičiūtė R., 2021: Physiological responses of pea plants to treatment with synthetic auxins and auxin-type herbicide. – Botanica, 27(2): 125–133.

The effect of exogenously applied 2,4-D (2,4-dichlorophenoxyacetic acid) on growth and antioxidant defence of pea plants, preliminary treated with two synthetic auxin compounds 1-[2-chloroethoxycarbonyl-methyl]-4-naphthalenesulfonic acid calcium salt (TA-12) and 1-[2-dimethylaminoethoxycarbonylmethyl]naphthalene chlormethylate (TA-14) was examined. All chemicals were applied by foliar spraying. Applied alone, TA-12 and TA-14 had no significant effects, but they modulated the 2,4-D induced changes on most investigated biochemical parameters. The shoot fresh weight reduction caused by 2,4-D was partially overcome by the use of TAs. The use of TAs partially overcame the shoot fresh weight reduction induced by 2,4-D. Apart from this, no significant changes were observed in the other biometric parameters. Treatment with 2,4-D did not enhance lipid peroxidation, and hydrogen peroxide content was slightly increased. These data indicate that treatment with 2,4-D did not cause severe oxidative stress, which is also confirmed by the results of the antioxidant defence system. The application of 2,4-D provoked mild accumulation of thiol-containing compounds, free proline and phenolic compounds and increased the antioxidant enzyme activities (GST, SOD, CAT, POD and GR) to a moderate degree. Pretreatment with TAs noticeably decreased the non-enzymatic antioxidants (free proline, total phenolics and total low-molecular thiols) compared to plants treated with 2,4-D only. Except for GR, TAs pretreatment returned the enzyme activities to levels close to the controls. Based on the results obtained, we suggest that the application of both synthetic auxins could modulate 2,4-D herbicide effects.

Keywords: antioxidants, growth, *Pisum sativum* (L.), stress markers, 2,4-D.

INTRODUCTION

The chemicals possessing herbicidal activity are more than 1500 compounds and are the most used farming products applied against weeds in agriculture worldwide (Pesticide Properties Database, 2021). The herbicides which affect all plant species are classified as total, and those which affect certain plant species are classified as selective. Regarding their mechanism of action, herbicides can be merged into four main groups: blockers of photosynthesis, inhibitors of amino acid biosynthesis, inhibitors of lipid and fatty acid biosynthesis, and auxin type herbicides.

The highly effective selective herbicide of the auxin type 2,4-dichlorophenoxyacetic acid (hereafter, 2,4-D) is applied against dicotyledonous weeds in monocotyledonous crops (Cobb, 1992; Peterson et al., 2016). Formulations containing 2,4-D are manufactured in the form of more than 20 different preparations alone and in combination with other herbicides (Peterson et al., 2016). It is mainly foliar ap-

plied or directly into the soil as a solution or granules (Walters, 2000). Field doses of 2,4-D correspond to auxin concentrations 1000 times above the average plant concentrations. This affects the plants, resulting in hormonal imbalance and oversaturation of the systems for degradation and control of hormone levels. As a result, uncontrollable growth of susceptible plants is observed, resulting in resource depletion and death (Cobb, 1992; Walters, 2000). Although 2,4-D is not used against weeds in legumes (Fabaceae), as they are sensitive, data are demonstrating its action on pea plants (Romero-Puertas et al., 2004; McCarthy-Suárez et al., 2011; Pazmiño et al., 2011).

The herbicidal antidotes are chemicals that can lessen to some degree or wholly overcome the phytotoxic effects of herbicides on cereals or monocotyledonous crops without reflecting on herbicide action on weeds (Jablonkai, 2013). Although the safeners (protectors), which inactivate herbicides used in dicotyledonous crops, are underexplored (Jablonkai, 2013), it was shown that some plant growth regulators can modulate physiological responses of herbicide treated dicotyledonous plants (Zheleva et al., 1994; Kim & Jin, 2006; Pinol & Simon, 2009; Cui et al., 2010; Singh et al., 2017). In our recent study (Sergiev et al., 2020), we established that two synthetic auxin compounds, namely (1-[2-chloroethoxycarbonylmethyl]-4-naphthalenesulfonic acid calcium salt) and TA-14 (1-[2-dimethylaminoethoxycarbonylmethyl] naphthalene chlormethylate), which are structural analogues of naphthyl acetic acid (synthetic auxin), also modulated herbicide induced plant responses of young pea seedlings treated with Glyphosate (inhibitor of aromatic amino acids synthesis) and Glean-75 (inhibitor of branched amino acids synthesis). This gives a perspective for further investigations on their potential protective effect against other herbicides, like 2,4-D.

The herbicide action builds up of reactive oxygen species (hereafter, ROS) (Sergiev et al., 2006; Romero-Puertas et al., 2004; McCarthy-Suárez et al., 2011; Pazmiño et al., 2011; Varshney et al., 2015; McCarthy-Suárez, 2017; Langaro et al., 2017), which is accompanied with significant changes in the plant antioxidant system consisting of different enzymatic and non-enzymatic ingredients. Therefore, this study aimed to investigate the physiological effects of synthetic auxins TA-12 and TA-14 on pea plant responses provoked by auxin type herbicide 2,4-D. In addition, in this study, we aimed to answer which principal components of the enzymatic and non-enzymatic defence systems are altered.

MATERIALS AND METHODS

Plant material and treatments

Pea plants (*Pisum sativum* L.) of the cultivar 'Ran-1' were grown hydroponically on a half-strength Hoagland-Arnon nutrient solution in phytotrons with the following characteristics: 24/22°C day and night temperatures, 12/12 h photoperiod, 150 μmol m^{-2} s^{-1} photon flux density.

The water solutions of 1 mM TA-12 (1-[2-chloroethoxycarbonyl -methyl]-4-naphthalenesulfonic acid calcium salt) and TA-14 (1-[2-dimethylaminoethoxycarbonylmethyl]naphthalene chlormethylate) containing 0.01% Tween-80 used as a surfactant were applied on 13-day-old seedlings by leaf spraying. After 24 hours, plantlets were sprayed with 0.05 mM water solution of 2,4-D (containing 0.01% Tween-80). Biometrical and biochemical analyses were made two weeks after 2,4-D treatment.

Biometrical analyses

The length of roots and shoots was measured using a linear ruler (precision 1 mm). Fresh biomass (FW) of shoots and roots was weighed on an electronic balance, precision 0.001 g (Precision Standard TS4000, Ohaus®, USA).

Biochemical analyses

The kinetic records of enzyme activities were done on Shimadzu UV-1601 UV-Visible spectrophotometer (Shimadzu, Japan). The absorbance records were made on Multiskan Spectrum UV/VIS spectrophotometer (Thermo Fisher Scientific, Finland). All biochemical analyses were done using fresh plant material collected from 3rd leaf pair.

Approximately 200 mg plant biomass was ground in ice-cold potassium phosphate buffer (100 mM, pH 7.0) supplied with 1mM ethylenediaminetetraacetic acid. Polyvinylpyrrolidone (1% w/v) was added to each sample. Homogenates were centrifuged for

30 min (15 000 × *g* at 4 °C) on Sigma 2-16K centrifuge. Supernatants were used for the determination of the enzyme activities of superoxide dismutase (SOD), guaiacol peroxidase (POX), catalase (CAT), glutathione reductase (GR), and glutathione S-transferase (GST). In brief, SOD activity was measured according to Beauchamp & Fridovich (1971) method based on the inhibition of photochemical reduction of nitrobluetrazolium assayed at 560 nm. One enzyme unit (EU) of SOD was defined as the enzyme quantity sufficient to provoke 50% inhibition of the nitrobluetrazolium reduction. POX activity was measured according to the method of Dias & Costa (1983) using guaiacol (ε = 26.6 mM^{-1} cm^{-1}) as an external electron donor. The increase in absorbance at 470 nm was recorded for 60 s. CAT activity was monitored for 60 s by the hydrogen peroxide degradation at 240 nm (36.8 mM^{-1} cm^{-1}) according to Aebi (1984) method. GR activity was measured by the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) by glutathione and the increase in absorbance at 412 nm (ε = 6.2 mM^{-1} cm^{-1}) after Smith et al. (1988). The GST activity was determined by using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate and recorded at 340 nm (ε = 9.6 $mM^{-1}cm^{-1}$) according to Gronwald et al. (1987) method.

Approximately 300 mg plant biomass was ground in 0.1% ice-cold trichloroacetic acid. The homogenates were centrifuged for 30 min (15 000 × *g* at 4°C) on Sigma 2-16K centrifuge. Supernatants were used to determine stress biomarkers (malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and free proline), and water-soluble antioxidants (free thiol-containing compounds and soluble phenols). Content of MDA was determined according to Kramer et al. (1991) method as a product of membrane phospholipid peroxidation reaction using thiobarbituric acid-reagent (ε = 155 mM^{-1} cm^{-1}). Hydrogen peroxide content was measured after Alexieva et al. (2001) and calculated by a standard curve. The derivatisation assay with acid ninhydrin according to Bates et al. (1973) method and reading the absorbance at 520 nm was used to determine the free proline amount. The concentration of thiol-containing compounds was determined spectrophotometrically at 412 nm using Ellman's reagent (Ellman, 1959). The content of total soluble phenolics was determined by incubation of the supernatant with Folin-Ciocalteu reagent, and the reaction mixture was measured at 725 nm (Swain & Goldstein, 1964). The data were calculated by using gallic acid (GA) as a standard.

Statistical analyses

Three independent experiments with three internal replicates were done. The data presented in the Figures were statistically analysed by Duncan's multiple range test ($p < 0.05$) and are mean values ± Standard error (SE).

RESULTS

The 2,4-D treatment reduced by 29% only fresh weight of pea shoots, while no significant alterations were found in the rest biometrical parameters measured (Fig. 1).

The pretreatment with TAs compounds mitigated the reduction of shoot biomass accumulation. The synthetic auxins provoked a slight increase in shoot length (by 8%) and gathered fresh weight (by 13% and 18%, respectively) of roots after single treatments. In addition, both TAs compounds applied alone did not cause any significant changes in the level of biochemical parameters analysed (Fig. 2 and Fig. 3) compared to control ones. However, 2,4-D application reduced MDA quantity by 27% below the control (Fig. 2A). MDA content remained lower than the control in 2,4-D + TAs-treated plants also.

A significant increase (by 106%) of free proline content after 2,4-D treatment was established (Fig. 2B). Pretreatment with TAs noticeably decreased the proline content compared to plants treated with 2,4-D only. The 2,4-D application did not alter significantly total phenolic content (Fig. 2C). The TA compounds applied before herbicide decreased the content of phenolics as compared to 2,4-D treated variant. The content of free thiol-containing compounds was also increased by 25% in plants treated with 2,4-D (Fig. 2D). The application of TA compounds decreased its content in 2,4-D-treated plants.

An increase of H_2O_2 content (by 14%), accompanied by a rise in the activity of SOD (by 19%), was found in the seedlings treated with 2,4-D (Fig. 3A and 3B). Conversely, the spraying with TAs before 2,4-D treatment caused a decrease in H_2O_2 quantity and SOD activity to the respective control levels.

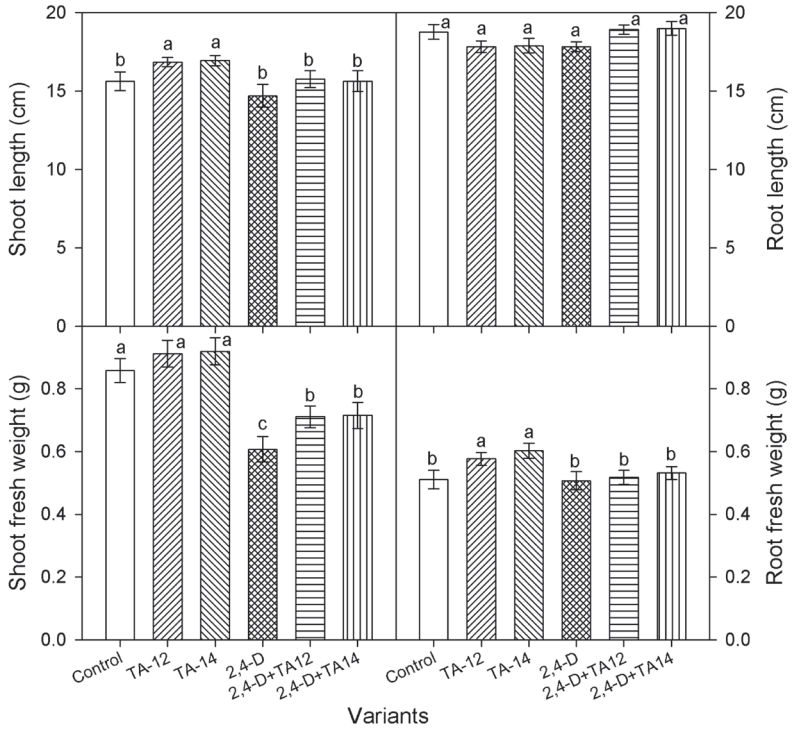

Fig. 1. Fresh weight and length of shoots and roots of garden pea plants sprayed with 1 mM 1-[2-chloroethoxycarbonyl -methyl]-4-naphthalenesulfonic acid calcium salt (TA-12) or 1-[2-dimethylaminoethoxicarbonylmethyl]naphthalene chlormethylate (TA-14) and treated with herbicide 2,4-D. Data are mean values ± SE. Different letters designate statistically significant differences at $p < 0.05$

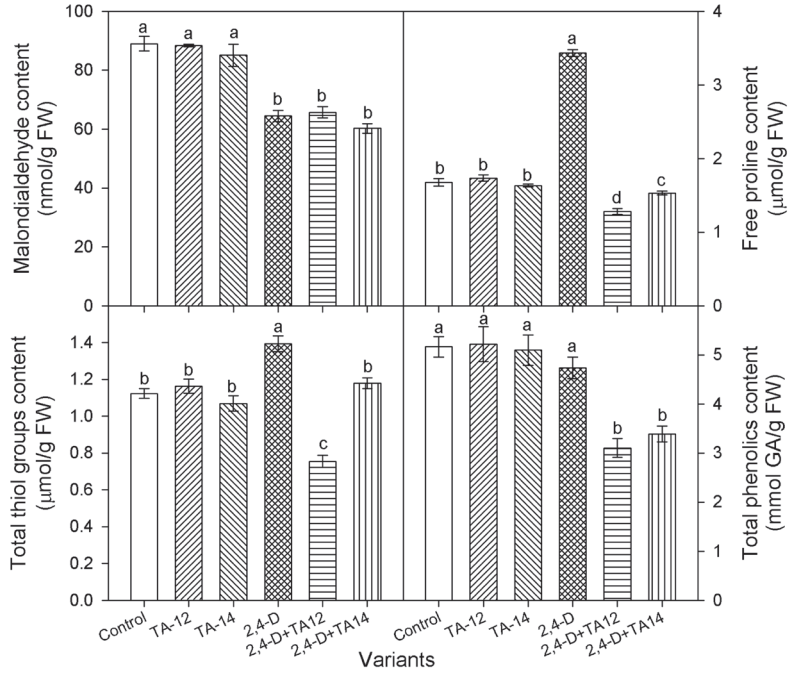

Fig. 2. Malondialdehyde, free proline, total phenolics, and total thiol-containing compounds content in leaves of garden pea plants sprayed with 1 mM 1-[2-chloroethoxycarbonyl -methyl]-4-naphthalenesulfonic acid calcium salt (TA-12) or 1-[2-dimethylaminoethoxicarbonylmethyl]naphthalene chlormethylate (TA-14) and treated with herbicide 2,4-D. Data are mean values ± SE. Different letters designate statistically significant differences at $p < 0.05$; FW – fresh weight

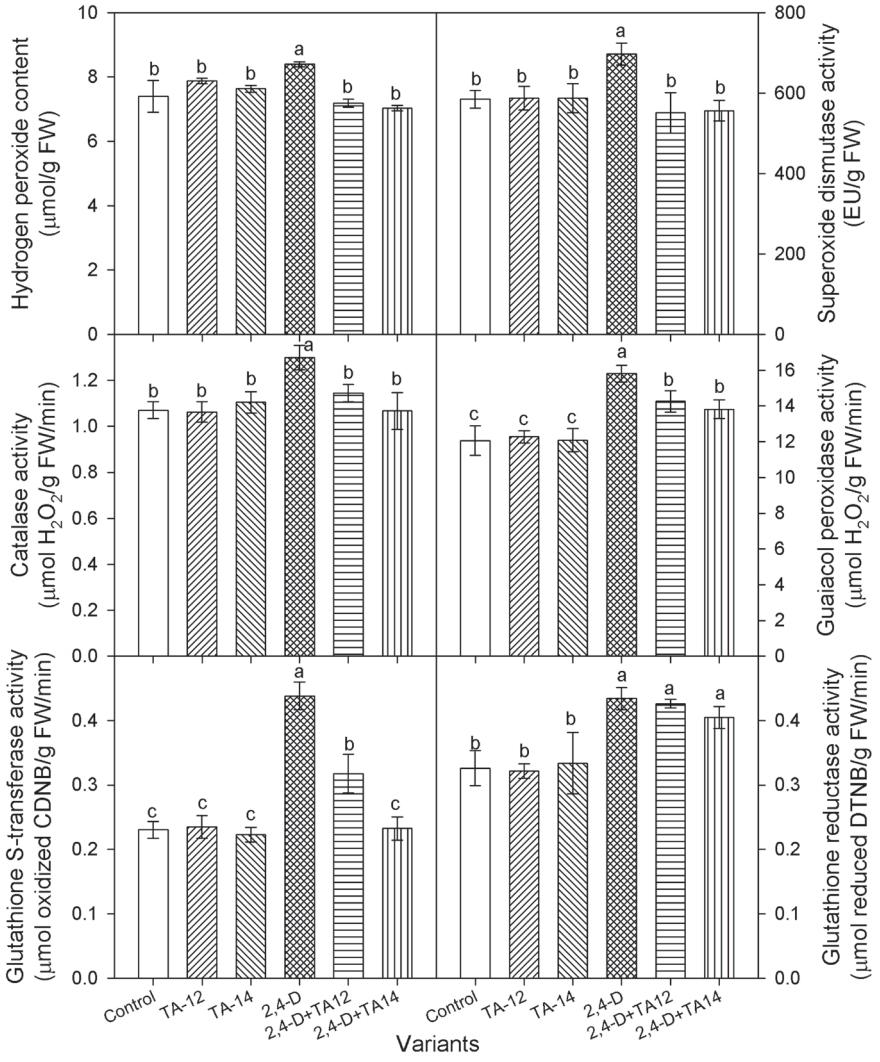

Fig. 3. Hydrogen peroxide content, and activity of superoxide dismutase, catalase, guaiacol peroxidise, glutathione S-transferase, and glutathione reductase in leaves of garden pea plants sprayed with 1 mM 1-[2-chloroethoxycarbonyl -methyl]-4-naphthalenesulfonic acid calcium salt (TA-12) or 1-[2-dimethylaminoethoxicarbonylmethyl]naphthalene chlormethylate (TA-14) and treated with herbicide 2,4-D. Data are mean values ± SE. Different letters designate statistically significant differences at $p < 0.05$; FW – fresh weight

The catalase activity was also increased by 21% after 2,4-D treatment (Fig. 3C). However, its activity was decreased to the control level in TAs + 2,4-D treated plants. Peroxidase activity (Fig. 3D) was raised by 31% after 2,4-D application, while pretreatment with TAs decreased the POX activity.

The 2,4-D treatment significantly increased GST activity by 90% (Fig. 3E). The application of synthetic auxins before herbicide led to a considerable decrease in enzymatic activity. The glutathione reductase activity (Fig. 3F) was increased by 33% and did not alter in plants pretreated with TAs.

DISCUSSION

The extensive use of herbicides in modern agricultural practice often impacts weeds and non-target crops. Despite their different mode of action, a common feature of all herbicides is bringing on oxidative stress in plants due to increased generation of ROS (Varshney et al., 2015; McCarthy-Suárez, 2017; Langaro et al., 2017). ROS can cause harm to key primary metabolites such as proteins, lipids, carbohydrates, and DNA that disrupts plant growth and even lead to plant death.

Although it is known that the molecular mechanism of action of 2,4-D is habitualdisruption of the hormonal equilibrium, which disturbs normal plant growth (Walters, 2000; Romero-Puertas et al., 2004; Pazmiño et al., 2011), in our study, pea growth was not affected significantly by 2,4-D application, except fresh shoot weight. It could be due to the lower concentration we used than those applied in other investigations (Romero-Puertas et al., 2004; Pazmiño et al., 2011). Furthermore, the herbicide did not cause accumulation of MDA, and hydrogen peroxide content was increased to a minor degree. So, probably oxidative stress did not occur in 2,4-D treated plants or was not so severe. Two possible explanations could arise: 1) the used concentration was subherbicide and did not provoke oxidative events, or 2) specific components of antioxidant defence were strongly activated to detoxify 2,4-D injuries. Plants develop a complex of antioxidant ROS-scavenging systems to cope with oxidative stress, including enzymatic and non-enzymatic antioxidants (Gomes et al., 2014; Varshney et al., 2015; de Freitas-Silva et al., 2017). Concerning the amounts of non-enzymatic antioxidants, we established that while total phenolics content did not alter significantly, the quality of the rest non-enzymatic antioxidants – thiol-containing compounds (glutathione in particular) free proline was increased noticeably by 2,4-D treatment. Regarding the activity of enzymatic antioxidants, we found that they were increased to a different extent (ranging from 19%to 33%) by herbicide application, especially that of GST, which was raised almost twice.

Obviously, most of the components of the antioxidant machinery were substantially activated by the herbicide application, but predominantly those involved in 2,4-D detoxification and metabolism. It has been proven that 2,4-D detoxification is realised by direct conjugation with glucose and amino acids (Peterson et al., 2016) and conjugation to glutathione by GSTs (Edwards & Dixon, 2005). Phenoxyacetic herbicides like 2,4-D form amino acid conjugates through bonding to sugars and amino acids (mainly aspartate and glutamate). The conjugation with amino acids is more prevalent in sensitive plants like dicotyledonous species (Peterson et al., 2016). Based on the significantly increased content of free proline in our study, the possible role of proline as a direct 2,4-D detoxifier could not be excluded. In addition, Verma et al. (2010) have also reported a substantially increased amount of proline in 2,4-D treated pea plants.

Increased content of thiol-containing compounds (glutathione, which represents more than 95% of non-protein thiols), along with the amplified activity of GST in 2,4-D treated pea plants appear to be particularly important in maintaining the leaf metabolic homeostasis. Glutathione is involved in the oxidative stress responses by 1) direct scavenging of ROS and/or by 2) detoxifying herbicides and other xenobiotics (reviewed in Katerova & Miteva, 2010). As a critical component of the glutathione-ascorbate shuttle (Halliwell-Asada cycle), it regulates the redox state within the plant cells where during the ROS deactivation, it is oxidised. Then the enzyme GR converts back the oxidised glutathione to reduced form (reviewed in Katerova & Miteva, 2010). Glutathione can detoxify xenobiotics due to their conjugation by the enzyme family GSTs (Edwards & Dixon, 2005). Several distinct classes of plant GSTs are recognised. Among them, tau (T) and phi (F) classes are the most prominent groups that are involved in the detoxification of herbicides (Labrou et al., 2015). In addition to T and F glutahione S-transferases, lambda (L) class plant GSTs are auxin-inducible (Marss, 1996; van der Kop et al., 1996; Shi et al., 2014). Increased GST activity in leaves of pea plants exposed to 2,4-D correlated with its involvement in the conjugation of 2,4-D with glutathione, leading to decreased herbicide levels in plant cells. In accordance, some studies suggest that GST can be strongly induced during the treatment with 2,4-D herbicide (Romero-Puertas et al., 2004; Pazmiño et al., 2011). In our study, pea plants responded to 2,4-D by increasing the synthesis of proline and glutathione and raising GST activity.

As it was mentioned, the application of some PGRs could modulate endogenous plant defence systems to better counteract ROS and oxidative stress due to herbicide treatment. Therefore, our study investigated the effects of synthetic auxins as modulators of action of 2,4-D in pea plants. Preliminary application of TA compounds to herbicide treated plants overcame the decrease of fresh biomass of pea shoots. In the plants treated with 2,4-D, MDA level did not change extensively by preliminary application of both TA compounds. It remained low along with

the content of hydrogen peroxide and SOD activity. However, the content of non-enzymatic antioxidants (free proline, total phenolics and total low-molecular thiols) was substantially decreased in conjunction with a reduction in catalase, POX, and GST activity in TAs+2,4-D treated plants as compared to 2,4-D treated only. Only GR activity was not altered in plants preliminary treated with TA compound, following our earlier study with Glyphosate and Glean-75 (Sergiev et al., 2020). This could mean that both auxin analogues can trigger pea adaptation reactions to 2,4-D application by modulating its physiological and metabolic processes, which finally led to better shoot growth. Our results represent a reasonable basis for further investigation on the possibility for modulation of herbicide action by applying synthetic auxins.

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FIZIOLOGINĖ ŽIRNIŲ REAKCIJA Į SINTETINIŲ AUKSINŲ IR AUKSINŲ TIPO HERBICIDO POVEIKĮ

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Santrauka

Ištirtas egzogeninis 2,4-dichlorofenoksiacto rūgšties (2,4-D) herbicido poveikis žirnių, apdorotų dviem sintetiniais auksinų analogais (TA-12 ir TA-14), augimui ir antioksidaciniam aktyvumui. Cheminėmis medžiagomis buvo apipurkšti žirnių lapai. Panaudoti atskirai, TA-12 ir TA-14 junginiai neturėjo reikšmingo poveikio augalams, tačiau panaudoti kartu su 2,4-D, jie moduliavo 2,4-D sukeltas biochemines reakcijas. Panaudojus TA junginius, pavyko iš dalies įveikti 2,4-D sukeliamą ūglių žaliosios masės mažėjimą. Taip pat nebuvo nustatyta kitų augalų biometrinių parametrų pokyčių. Paveikus žirnius 2,4-D, nedaug padidėjo vandenilio peroksido kiekis, bet lipidų peroksidacija nesustiprėjo. Gauti duomenys rodo, kad 2,4-D stipraus oksidacinio streso nesukėlė. Tą patvirtino ir antioksidantinio aktyvumo tyrimo rezultatai. Augalus apdorojus 2,4-D, truputį padidėjo tiolio turinčių junginių, laisvojo prolino ir fenolinių junginių kaupimasis ir vidutiniškai padidėjo antioksidacinių fermentų (GST, SOD, CAT, POD ir GR) aktyvumas. Apdorojus augalus TA junginiais, pastebimai sumažėjo nefermentinių antioksidantų (laisvojo prolino, bendrojo fenolių ir bendrojo mažos molekulinės masės tiolių) kiekiai, palyginti su augalais, kurie buvo paveikti tik 2,4-D. Antioksidacinių fermentų aktyvumas išliko artimas fermentų aktyvumui, nustatytam kontrolės sąlygomis (išskyrus GR). Remdamiesi gautais rezultatais darome prielaidą, kad naudojant abu sintetinius auksinus galima moduliuoti 2,4-D herbicido poveikį.