

Effect of Thidiazuron Used as an Elicitor in the Production of Capsaicin on Total Protein and Phenolic Amounts, Antioxidant Enzyme Activities of Pepper Plants

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Abstract— The effect of thidiazuron (TDZ) used as an elicitor on the total phenolic, total protein, superoxide dismutase (SOD) and catalase (CAT) enzyme activities, and production of capsaicin in the cell suspension culture of Maraş-1 pepper seeds was determined. TDZ was applied in four levels (0, 0.1 mM, 0.2 mM, 0.4 mM) and at three times (days 8, 10, and 12) to the cell suspensions. The content of the capsaicin in suspended cells was identified by high-performance liquid chromatography after extraction with ethyl acetate. It was identified that TDZ had a variable effect on the capsaicin accumulation. The highest capsaicin content was determined by 84.859 µg/g f.w. at 0.4 mM TDZ 10d. The highest increase by 181.48% at 0.2 mM TDZ 10d, the highest decrease by 49.83% at 0.4 mM TDZ 10d was determined in total phenolic amount. The total protein amount decreased at 0.4 mM TDZ 8d and 12d. The highest SOD (457.983 enzyme units/g f.w.) and CAT (0.143 enzyme units/g f.w.) activities at 0.4 mM TDZ 8d, the lowest SOD (73.526 enzyme units/g f.w.) and CAT (0.030 enzyme units/g f.w.) activities at 0.2 mM TDZ 12d were measured.

Keywords—*Capsicum annuum*, catalase, superoxide dismutase, TDZ.

I. INTRODUCTION

Capsicum annuum L. is an economically important crop plant belonging to the family *Solanaceae*. Two main consumption types of pepper, spice and vegetable, that are prevalent throughout the world [1]. *Capsicum* species are well-known because of many medicinal properties. Chilies contain numerous chemicals including steam-volatile oil, fatty oils, carotenoids, vitamins, protein, fiber, and mineral elements [2, 3]. Additionally, *Capsicum* contains capsaicinoid alkaloids specific to the genus *Capsicum*, which show many pharmacological properties [4]. As medicine, it is used as a counter irritant or pain

relief in lumbago, neuralgia, rheumatic disorders, non-allergic rhinitis, diabetic neuropathy, osteoarthritis, and mouth sores [5, 6, 7]. Apart from this, chilies also possess in vitro antitumor-promoting activities and synergistic antimutagenic properties [8, 9]. Thus, capsaicin is considered to be a potential chemical for pharmaceutical industries.

The secondary metabolites are known to play a major role in the adaptation of plants to their environment and also represent an important source of pharmaceuticals [10]. The developing field of plant biotechnology may provide a better alternative for the large scale production of secondary metabolites. The use of plant cell and tissue cultures for the large-scale production of secondary metabolites has so far achieved only limited success due to the low and unreliable yields of the products [11]. Various strategies have been developed to improve the production of secondary metabolites. This means that the maximization of the production and accumulation of secondary metabolites [12]. Elicitation is the induction of secondary metabolite production by either biotic or abiotic treatments [13].

Capsaicin is produced by separation and purification of the free cells [10]. *Capsicum* plants to be used for the synthesis of capsaicin normally take 4–5 months to grow. Therefore, *Capsicum* free and immobilized cell cultures can be an important way to obtain capsaicin [10, 14].

Thidiazuron (TDZ), a substituted phenylurea (N-phenyl-1,2,3-thiadiazol-5-yl urea), is a potent plant growth regulator that exhibits auxin- and cytokinin-like activity in various culture systems [1, 15]. A number of physiological and biochemical events in cells are induced or enhanced by TDZ [15]. It induces diverse morphogenic responses, ranging from tissue proliferation to induction of shoot buds and somatic embryos [16, 17]. Also, TDZ is

known for stimulating strong shoot proliferating activity in several plant species [18, 19, 20].

We investigated capsaicin production and some physiological parameters of *Capsicum annuum* L. affected by TDZ elicitor.

II. MATERIALS AND METHODS

2.1. Plant material

Seeds of *Capsicum annuum* L. (Maraş-1) were obtained from the Eastern Mediterranean Transitional Zone Agricultural Research Station.

2.2. Culture conditions

Seeds of *C. annuum* L. were sterilized with 70% ethanol for 3 min and sodium hypochlorite for 20 min, followed by washing with sterile distilled water. *C. annuum* seedlings were germinated in Murashige and Skoog [21]'s medium without hormones. Hypocotyl explants of the seedlings were placed into MS medium with 0.1 mg/L kinetin, 1 mg/L 2,4 D, 3% sucrose and 0.7% agar to produce callus tissue at 25 °C. They were subcultured two times and placed into liquid medium (MS without agar) to produce cell suspensions in 100 mL Erlenmeyer flasks. The cultures were incubated on a shaker at 110 rpm and 25 °C [22].

2.3. TDZ applications and Capsaicin Analysis

Experiments were carried out in 100 mL Erlenmeyer flasks containing 40 mL of fresh medium. Each flask was inoculated with 1 g of fresh weight cells. After 14 days of growth, sterile TDZ (0, 0.1 mM, 0.2 mM, 0.4 mM) was

added. The cultures were maintained on an orbital shaker at 25°C for 8, 10, and 12 days.

Cell samples were collected after incubation and analyzed for capsaicin. It was estimated by high-performance liquid chromatography assay based on the method of Sudha and Ravishankar [23]. Capsaicin from the culture media was extracted thrice with 20 ml of ethyl acetate, each time in a separating funnel. The ethyl acetate layers were pooled and evaporated. The residue was dissolved in 1.0 mL of ethyl acetate for analysis. The extractions were filtered through a 0.45 -µm Millipore filter prior to injection on the HPLC. The capsaicin extracted from the callus and media was estimated by HPLC. The quantification of capsaicin was done on a C18 nucleosil column with detection at 280 nm. The isocratic mobile phase was methanol: water (1% acetic acid) (39: 60 v/v) and a flow rate of 1 ml/min was used [14].

2.4. Physiological assays

The total phenolic content of the callus was determined using the Folin-Ciocalteu assay [24]. The total protein amount was determined according to method of Bradford [25]. The superoxide dismutase (SOD) activity assay was based on the method of Beauchamp and Fridovich [26] and the catalase (CAT) activity was estimated according to the method of Bergmeyer [27].

2.5. Statistical analysis

Tukey's test was employed for statistical analysis using the SPSS 16.0 software package at the $P \leq 0.05$ level.

III. RESULTS AND DISCUSSION

The cell suspension samples capsaicin content of *Capsicum annuum* L. are shown in Fig. 1.

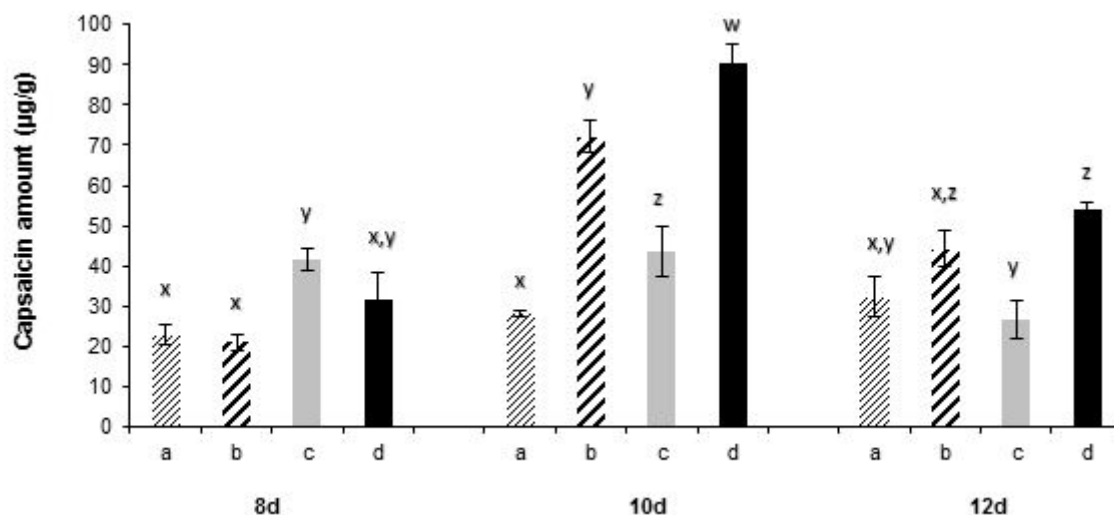


Fig. 1: Effect of TDZ on the amount of capsaicin in *Capsicum annuum* L. at different concentrations and times (a: Control, b: 0.1 mM TDZ, c: 0.2 mM TDZ, d: 0.4 mM TDZ). Vertical bars represent SE (n=3). Same letters are not significantly different at $P \leq 0.05$ (Tukey test) for each days.

The change in the capsaicin amount of *C. annuum* L. at 8d was found between 21.095–41.649 µg/g f.w. The capsaicin amount showed an increase with 0.2 mM and 0.4 mM TDZ at 8d to the control ($P \leq 0.05$). The capsaicin content change was determined between 28.261–90.385 µg/g f.w at 10d. The highest capsaicin accumulation at 10d was 0.4 mM TDZ. The difference between the control and 0.1 mM, 0.2 mM and 0.4 mM TDZ was statistically significant ($P \leq 0.05$). The capsaicin content was found between 26.652–54.005 µg/g fw at 12d. The highest value was found at 0.4 mM TDZ 12d ($P \leq 0.05$). Considering the all concentrations and days, the highest amount was 90.385 µg/g f.w. at 0.4 mM TDZ 10d, while the lowest amount was 21.095 µg/g f.w. at 0.2 mM TDZ 12d. There is no parallelism between the increase of capsaicin and TDZ (Fig. 1).

There are no studies regarding TDZ elicitor effect on capsaicin accumulation in the literature. Phenylpropanoid and valine biosynthesis metabolic pathways are the two methods of capsaicin biosynthesis. The first part of the aromatic biosynthesis pathway occurs through phenylpropanoid metabolism in all plants. This situation suggests that an elicitor application from outside advances the metabolic pathway in this direction. Transformation from vanillin and vanillylamine to capsaicin was found only in cell cultures of pepper and fruits [28]. According to this, it can be expressed that the TDZ compound effect on increasing the effect of the capsaicin amount is caused by the destruction of capsaicin or inhibitory activity with other molecules. The findings evidence that TDZ elicitor treatments enhances the effect on the accumulation of the capsaicin.

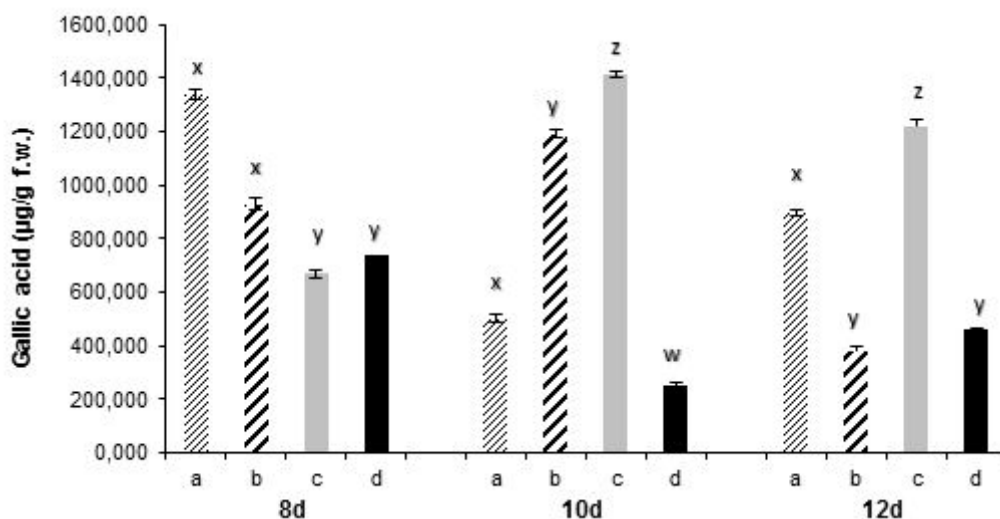


Fig. 2: Effect of TDZ on the amount of total phenolic in the cells of *Capsicum annuum* L. at different concentrations and times (a: Control, b: 0.1 mM TDZ, c: 0.2 mM TDZ, d: 0.4 mM TDZ). Vertical bars represent SE (n=3). Same letters are not significantly different at $P \leq 0.05$ (Tukey test) for each days.

Capsaicin production increased with 0.2 mM and 0.4 mM TDZ at day 8 to the control, and the maximum increase was determined at 0.4 mM TDZ 10d and 12d. In contrast, the phenolic content was the opposite. The highest phenolic increase was determined at 0.2 mM TDZ 10d (181.48%) ($P \leq 0.05$) (Fig. 2). The highest capsaicin increase was determined at 0.4 mM TDZ 10d (247.13%) ($P \leq 0.05$) (Fig. 1), but the phenolic amounts decreased by 49.83% to the control at this concentration (Fig. 2).

Two pathways are involved in the biosynthesis of capsaicinoids: fatty acid metabolism and the phenylpropanoid pathway [29]. The phenolic structure comes from the phenylpropanoid pathway, in which phenylalanine is the precursor [30]. Estrada et al. [31] suggested that competition exists between the biosynthesis of capsaicinoids and other phenylpropanoid metabolites. Our results can be related with the idea.

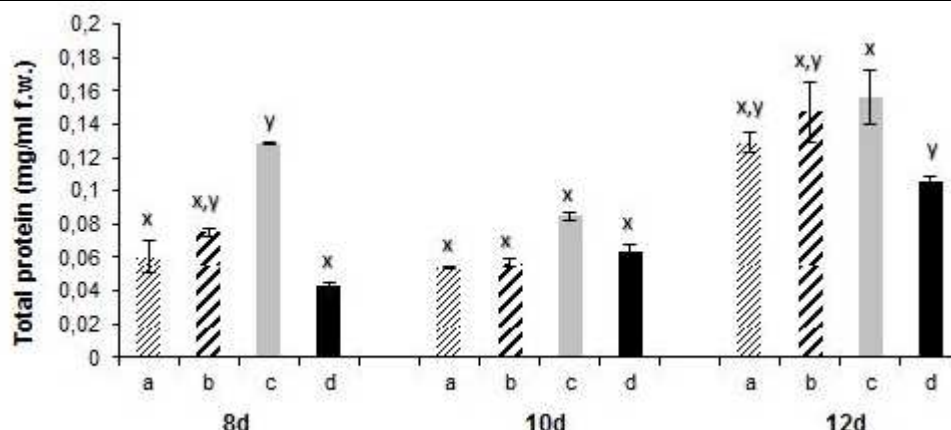


Fig. 3: Effect of TDZ on the amount of total protein in the cells of *Capsicum annuum* L. at different concentrations and times (a: Control, b: 0.1 mM TDZ, c: 0.2 mM TDZ, d: 0.4 mM TDZ). Vertical bars represent SE (n=3). Same letters are not significantly different at $P \leq 0.05$ (Tukey test) for each days.

In the present study, only 0.2 mM TDZ 8d treatment affects the change of total protein significantly ($P \leq 0.05$). In addition, 0.2 mM TDZ treatments were more effective than other treatments in increasing the total protein amount. The total protein amounts decreased to the control at 0.4 mM TDZ 8d and 12d (Fig. 3). It is our opinion that TDZ stimulates protein synthesis. Klyachko et al. [32] and Ananiev et al. [33] observed that TDZ, 4-PU-30 and N6-benzylaminopurine accelerate polysome formation and stimulate RNA polymerase

activity in pumpkin cotyledons, which supports this assumption. We have already mentioned that TDZ exhibits cytokinin-like activity in various culture systems. Cytokinins increase the total protein amount in maize and other plants too [34, 35, 36]. Moreover, cytokinins inhibit protein degradation by preventing protease and nuclease. Zavaleta-Mancera et al. [37] showed that cytokinins delay the oxidative process and using cytokinin increased the total protein in rice leaves.

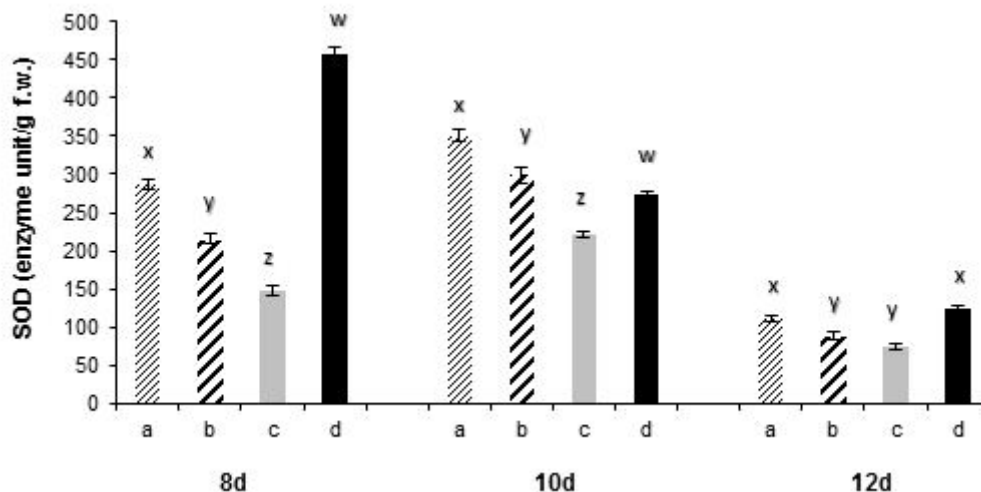


Fig. 4: Effect of TDZ on the activity of SOD enzyme in the cells of *Capsicum annuum* L. at different concentrations and times (a: Control, b: 0.1 mM TDZ, c: 0.2 mM TDZ, d: 0.4 mM TDZ). Vertical bars represent SE (n=3). Same letters are not significantly different at $P \leq 0.05$ (Tukey test) for each days.

The SOD activities of the pepper plants were decreased at 0.1 mM and 0.2 mM TDZ of all treatment days and increased at 0.4 mM TDZ 8d and 12d compared to the controls. The SOD responses were significant in all treatment groups except 0.4 mM TDZ 12d. The highest rate of SOD activity was measured at 0.4 mM TDZ 8d (59.58% increase compared to the control) and the lowest

rate was measured at 0.2 mM TDZ 8d (49.29% decrease compared to the control) (Fig. 4).

In the *Pelargonium* plants, after days 4 and 5 of the treatment, the control leaves showed significantly reduced the APX and SOD activities when compared with the initial stage [38]. In addition, the strongest SOD activity increase was detected at the highest TDZ concentration in

carnations [39]. The results of both studies are in agreement with our results.

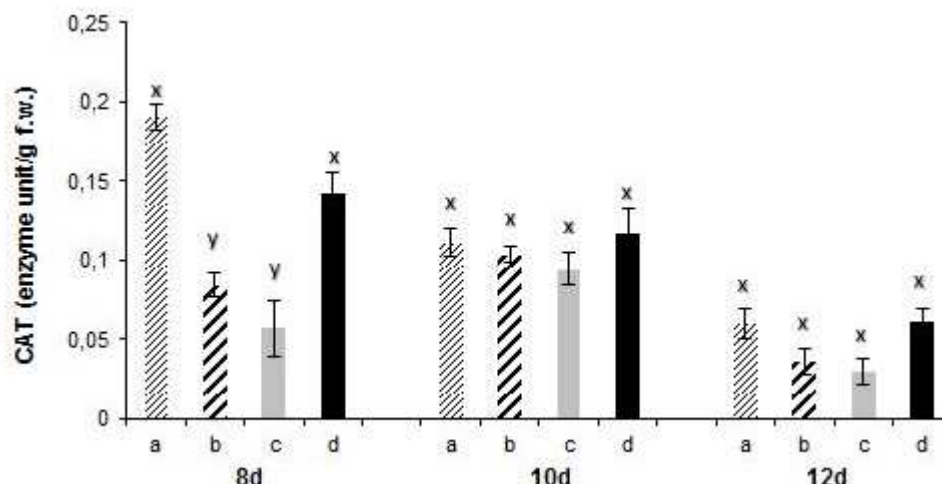


Fig. 5: Effect of TDZ on the activity of CAT enzyme in the cells of *Capsicum annum* L. at different concentrations and times (a: Control, b: 0.1 mM TDZ, c: 0.2 mM TDZ, d: 0.4 mM TDZ). Vertical bars represent SE (n=3). Same letters are not significantly different at $P \leq 0.05$ (Tukey test) for each days.

Another scavenger of H_2O_2 , the CAT activity, decreased at 0.1 mM TDZ and 0.2 mM TDZ of all treatment days, and increased at 0.4 mM TDZ 10d and 12d compared to the controls. Although significant decreases (69.99% at 0.2 mM TDZ and 55.58% at 0.1 mM TDZ) were measured on day 8, other groups exhibited slight and insignificant changes (Fig. 5).

Tang and Newton [40] determined TDZ influences on the antioxidant enzyme activity during the morphogenesis of eastern white pine. They showed that during the culture, the peroxidase (POD) activity decreased at the beginning and increased at later stages of the shoot bud formation and the CAT activity linearly decreased throughout the complete culture duration. All of these findings show that the TDZ-induced direct adventitious shooting in eastern white pine resulted in decreased activities of POD and CAT.

The abovementioned antioxidant enzymes play an important role in the detoxification of reactive oxygen species (ROS) produced under stressful conditions [41]. The role of ROS in plant growth and development is sustained by the interplay of ROS and plant growth regulators [42].

The concentration of plant growth regulators used is critical. When used with the appropriate concentrations of cytokinins, auxins and TDZ, it reduces the amount of SOD and CAT enzymes [43, 44]. In our results, the increase in the amount of protein and decrease in the amount of SOD and CAT enzymes at 0.1 mM and 0.2 mM TDZ supports this view. Our results were the opposite with 0.4 mM TDZ. This shows the importance of the concentration to be applied.

In our study, capsaicin production increased, and SOD and CAT amounts decreased at 0.1 mM and 0.2 mM TDZ. SOD converts superoxide radicals into hydrogen peroxide and molecular oxygen (O_2), while the CAT and POD convert hydrogen peroxide into water, and in the case of CAT, to oxygen and water [45]. Capsaicinoids are products of the phenylpropanoid and fatty acid synthesis pathways [46]. The POD activity had a negative association with the capsaicinoid amounts [47, 48]. This adverse relationship might be associated to the fact that POD activity is closely related to the oxidization of the capsaicinoid metabolism [49].

IV. CONCLUSION

Capsaicin production is important for food and pharmaceutical industries. When capsaicin is produced by the traditional method, it takes a lot of time. To attain continuous and quick industrial production, the addition of stimulants to the growing medium will contribute to production of secondary metabolites. TDZ, when used in the appropriate concentration, in terms of both increasing the amount of capsaicin and not creating stress in plants, is found to be an important compound. On this topic, there are very few detailed studies. Furthermore, other enzymes in the biosynthetic pathway, and different concentrations of TDZ or TDZ in combination with other plant growth regulators, can be studied.

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