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Peroxidase activity as a biochemical marker of insecticide use in vegetables

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Abstract: The insecticides use is important for crop improvement and protection, but in excessive amounts, they would induce a dysfunction of metabolic enzymatic systems in plant tissues, leading to undesirable qualitative changes. In this context, we are interested in peroxidase (POD), an important enzyme in plant physiology but whose activity seems to be conditioned by the presence of insecticides in the soil. This work aims to study the impact of locally used insecticides (chlorpyrifos and dimethoate) on the activity of POD in parsley, onion, celery and garlic grown in soils treated or not. POD extraction was performed using Tris-HCl buffer (pH 7.3); its activity was measured using the substrate o-dianisidine in the presence of H_2O_2. Our result showed that POD activity for insecticide treated parsley, celery and onions increased by 30 % 127 % and 341 % respectively, however did not change significantly for garlic. Thus, the action of these chemicals is not trivial because they may alter non-target pathways, especially when doses are not adjusted accordingly. We found that insecticide stress would increase POD activity in all vegetables except garlic, which showed tolerance to insecticides. Our findings suggest that organic farming conditions could minimize peroxidase activity in parsley, celery and onion. We add that overproduction of POD negatively affects the quality and reduces the shelf life of vegetables, thus would be a very interesting biomarker of insecticide stress.

Key words: peroxidase activity; enzymatic browning; insecticides; oxidative stress; crop protection

Aktivnost peroksidaze kot biokemični označevalec uporabe insekticidov v zelenjavi

Izvleček: Uporaba insekticidov je pomembna za izboljšanje in zaščito pridelkov, vendar bi v prevelikih količinah povzročili motnje v delovanju presnovnih encimskih sistemov v rastlinskih tkivih, kar bi povzročilo neželene kakovostne spremembe. V zvezi s tem nas zanima peroksidaza (POD), ki je pomemben encim v fiziologiji rastlin, vendar se zdi, da njeno delovanje pogojuje prisotnost insekticidov v tleh. Namen tega dela je preučiti vpliv lokalno uporabljenih insekticidov (klorpirifos in dimetoat) na aktivnost POD v peteršilju, čebuli, zeleni in česnu, ki rastejo v tleh, obdelanih ali ne. Ekstrakcija POD je bila izvedena z uporabo pufra Tris-HCl (pH 7.3); aktivnost encima POD je bila izmerjena z uporabo substrata o-dianizidina v prisotnosti H_2O_2. Naši rezultati so pokazali, da se je aktivnost POD pri peteršilju, zeleni in čebuli, tretiranih z insekticidi, povečala za 30 %, 127 % oziroma 341 %, pri česnu pa se ni bistveno spremenila. Tako delovanje kemikalij ni nepomembno, saj lahko spremenijo neciljne poti, zlasti če odmerki niso ustrezno prilagojeni. Ugotovili smo, da stres zaradi insekticidov poveča aktivnost POD pri vseh vrtninah, razen pri česnu, ki je pokazal toleranco na insekticide. Naše ugotovitve kažejo, da bi lahko pogoji ekološkega kmetovanja zmanjšali aktivnost peroksidaze pri peteršilju, zeleni in čebuli. Dodajamo, da prekomerna produkcija POD negativno vpliva na kakovost in zmanjšuje rok trajanja zelenjave, zato bi bila zelo zanimiv biomarker insekticidnega stresa.

Ključne besede: aktivnost peroksidaz; encimsko porjavenje; insekticidi; oksidativni stres; zaščita pridelkov

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1 INTRODUCTION

Crops are exposed to a variety of diseases and pests that are responsible for important losses of yields and limited agricultural productivity worldwide. Food and Agriculture Organization (FAO) estimates that annually up to 40 percent of global crop production is lost to pests (FAO, 2021). Plant pathogens can be fungal, bacterial, viral, insects or nematodes and can damage plant parts above or below the ground and alter their quality (Pandit et al., 2022). In order to control these pathogens and protect crops, the intensive agricultural systems rely heavily on the use of chemical pesticides. Nevertheless, the excessive application of pesticides has become a major cause of widespread ecological imbalances. Indeed, these chemicals resulted in serious problems of insecticide resistance, pest resurgence and pesticide residues accumulation in soil, water and plant tissues (Gull et al., 2019). Besides, pesticides may induce physiological variations in plants such as plant growth (Parween et al., 2015), germination (Fatma et al., 2018). Thus, processes of seed germination, cell division and elongation are changed (Gaspar et al., 1991). They also induce metabolic and enzymatic dysfunctions and toxicities on cell membranes (Moriwaki et al., 2017).

For instance, several studies have shown a variation in peroxidase levels after treatment with insecticides (García-Hernández et al., 2005). The peroxidase enzyme (EC1.11.1.7) is an important antioxidant that plays a pivotal role in plant growth and development (Breda et al., 1993). Peroxidases belong to a family of glycoproteins containing iron atoms as a prosthetic group and different quantities of carbohydrate residues (Van Huystee, 1987). Peroxidases are located mainly in the cell wall and in the vacuoles of plant cells; their location varies according to the age, species and developmental stage of the plant (Gaspar et al., 1982). Elevation in POD (peroxidase) activity has been linked to resistance to stress and self-defence mechanisms. Under stress conditions, the rate of respiration increases with upregulation in peroxidase enzyme activity (Aspinall & Paleg, 1981). High levels of POD in plants are involved in multiple deteriorating changes affecting flavor, texture, color and nutrition in processed fruits and vegetables (Bett-Garber et al., 2005). Therefore, knowledge about how they react is an important consideration in food technology.

The use of insecticides is not trivial on the quality of plants and on human health, especially when their dosage and treatment periods are not respected. Moreover, a major problem in Algeria is the unreasonable and random use of insecticides by farmers. In spite of the use of prohibited products such as DDT (dichloro-diphenyl-trichloro-ethane), the overdosing of insecticides and the non-respect of the life span of insecticides are alarming problems, which must be addressed seriously.

Chlorpyrifos and Dimethoate are the most used insecticides in Algeria, they are applied at 0.3-0.7 kg ha^{-1} and 1.5 liters of product/ha respectively on many crops: fruits and vegetables (beans, broccoli, cabbage, cauliflower, peppers, potatoes, spinach, tomatoes) (Worthing & Walker, 1983). The half-life of chlorpyrifos ranges from 60 to 120 days and its persistence appears to be highly dependent on pH, climatic conditions and other soil factors, ranging from two weeks to more than a year. Dimethoate is rapidly absorbed and broken down in the plant by hydrolysis and oxidation (Menzie, 1969). Its half-life in plants varies from 2 to 5 days (Melnikov et al., 1977) and it disappears after an average of 30 days, depending on the plant species and the climatic conditions

The aim of this study is to investigate the effects of insecticides on peroxidase activity in selected vegetables namely parsley, celery, garlic and onion bulbs. Parsley (*Petroselinum crispum* (Mill.) Fuss (*Petroselinum sativum*) a biennial herb is an important dietary source of vitamins and essential metals. Supplementation with parsley at sufficient levels can promote the levels of vitamins and essential metals in the human body (Zhai et al., 2015). Celery (*Apium graveolens* L.) (also called krafes in northern Africa) belongs to the Apiaceae family. It grows annually or perennially throughout Europe and in tropical and subtropical regions of Africa and Asia. Celery is considered the most widely used plant in traditional food and medicine because it contains compounds such as limonene, selinene, furocoumarin glycosides, flavonoids, and vitamins A and C (Kooti et al., 2014; Al-Asmari et al., 2017; Li et al., 2019). Garlic (*Allium sativum* L.) is one of the oldest of all cultivated plants that has been used as a spice or food for over 400 years (Choi et al. 2007). Onion (*Allium cepa* L.) is botanically included in the Amaryllidacea family and a variety of species are found across a wide range of latitudes and altitudes in Europe, Asia, N. America and Africa (Griffiths et al. 2002). Onion is widely used in all parts of the world as a flavoring vegetable in various types of food. These vegetables represent the most important commercial crops and indispensable vegetables in Algeria and other countries thereby provide an important backdrop for evaluating the effects of insecticides in Algeria.

2 MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

O-dianisidine and bovin serum albumin (BSA) were obtained from Sigma Aldrich. H_2O_2 (30 % [v/v]) was provided by Prolabo. All chemicals were of the best commercially available quality, and all solutions were prepared using deionized water.

2.2 SAMPLES

Two groups of tissue samples from fresh parsley, celery, garlic, and onion were involved in this study. The first group was provided by a local farmer using chlorpyrifos and dimethoate as insecticides. The second group was provided by a local organic farmer who does not use insecticides. Only uninjured plants were selected.

2.3 PREPARATION OF CRUDE EXTRACT

Peroxidase enzyme extraction was carried out according to Diao et al. (2019). Five grams of each plant were mixed with an electric blender. The resulting mixture was homogenized with 30 ml of Tris-HCl buffer (50 mM, pH 7.3) containing 0.5 $MCaCl_2$ and 5 mM DTT, at 4 °C for 1 hour. After filtration, the extracts were centrifuged (14.000 g, 4 °C, 45 min). The supernatants containing the peroxidase were stored at -20 °C until use.

2.4 TOTAL PROTEIN CONCENTRATION

Protein content of each extract was determined according to the spectrophotometric method of Lowry (1951). The reaction medium contains 3 ml of solution C and 20 µl of the extract; let it stand for 10 minutes in dark, at room temperature, then add 0.3 ml of Folin-Ciocalteu reagent diluted to half. After 15 minutes, absorbance is measured at 750 nm. Concentrations are expressed in grams per 100 grams of fresh matter (g 100 g^{-1}) using the regression equation obtained with BSA.

2.5 ENZYME ASSAY

Peroxidase activity was assayed according to the method of Bradely et al. (1982) modified by Bedouhene et al. (2020). The change in absorbance at 460 nm due to the oxidation of o-dianisidine in the presence of hydrogen peroxide (H_2O_2) and enzyme extract at 25 °C was monitored using Jenway 6405 UV/VIS Spectrophotometer. A standard assay solution contained 15 mM o-dianisidine, 10 mM H_2O_2 in sodium phosphate buffer pH 6.5 was prepared. Twenty-five microliters of the crude extract (contained peroxidase enzyme) were added to the standards solution in total volume of 1 ml. The change of color is due to the oxidation of o-dianisidine in the presence of hydrogen peroxide (H_2O_2). Kinetics of POD activity is followed by monitoring the change in absorbance at 470 nm per min (Abs/min). One enzyme unit (U) is defined as the amount of enzyme producing a 0.001 absorbance change per min under the assay conditions used. The readings were taken for every 1 min for 10 minutes and enzyme extract at 25 °C was monitored using Jenway 6405 UV/VIS Spectrophotometer.

2.6 DATA ANALYSIS

The results were expressed as mean values with their standard deviations. The Two-way ANOVA analysis test was used to estimate the significance of the obtained data for each experiment. The Tukey-Kramer multiple-comparison test was used for analysis of the two sample groups (treated versus untreated) results. Wherever differences are reported as significant, a 95 % confidence

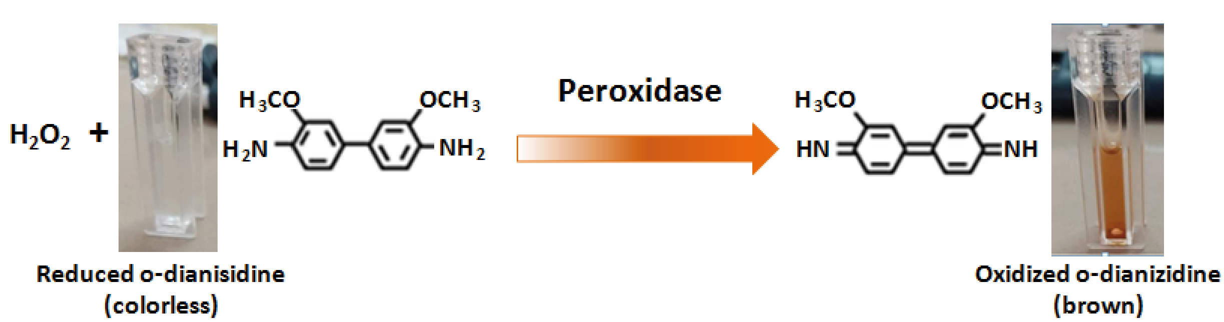

Figure 1: Oxidation of the molecular chromophore (o-dianisidine) by H_2O_2 and peroxidase, and the resultant color change from colorless to brown

level was used. The data analysis was performed using GraphPad Prism software version 5.01 (2010).

3 RESULTS

3.1 PEROXIDASEACTIVITY

Activity was measured in extracts of treated and untreated vegetables with insecticides by spectrophotometry using o-dianisidine as chromogenic agent and hydrogen peroxide (H_2O_2) as substrate (Fig. 1). POD is an enzyme related to plant defence and plays an essential role in resistance to membrane damage, mainly through the enzymatic degradation of H_2O_2. Peroxidase activity was strongly elevated in treated vegetables versus untreated samples (Fig. 2). The four plants showed different levels of POD activity. The level of POD activity was low in garlic treated with insecticides. This finding is supported by the proteins contents results (Table 1).

3.2 COMPRAISON OF PEROXIDASE ACTIVITIES

Peroxidase activities from parsley, celery, garlic and onion bulbsare summarized in Figure 3. Plant samples not subjected to insecticides show POD activities ranging from 201 to 2922, where parsley shows the highest activity, followed by celery and garlic, onion shows the lowest concentration. Higher POD activities ranging from 777 to 3769 $Umin^{-1}g^{-1}$ were observed in samples from insecticide-treated plants. Significantly the highest activity was found in insecticide-treated plant tissues from parsley with 3768.74 ± 141.59 $Umin^{-1}g^{-1}$ and celery with 2680.81

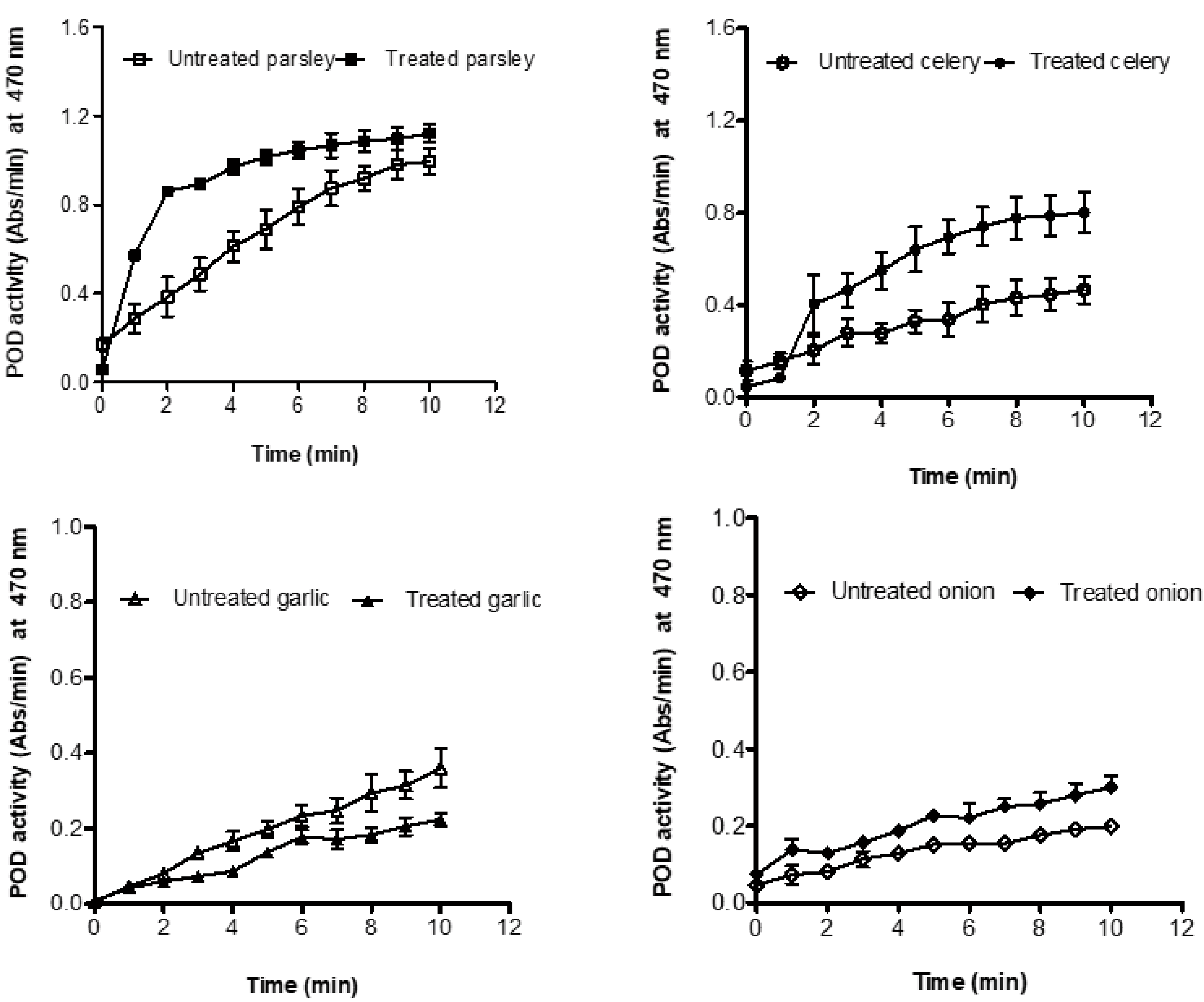

Figure 2: Kinetics of peroxidase activity is followed by monitoring the change in absorbance at 470 nm per min (Abs/min)of crude vegetables extracts (parsley, celery, garlic and onion) treated with insecticides compared to crude vegetables extracts without insecticides (control samples). Data represent mean values ± standard deviation of three determinations

Table 1: Proteins contents in crude vegetables extracts untreated and treated with insecticides

Vegetables	Organs	Total protein (g 100 g^{-1}) in untreated plant	Total protein (g 100 g^{-1}) in treated plant
Parsley	Leaves	1.02 ± 0.011	1.23 ± 0.001 *
Celery	Leaves	0.26 ± 0.001	0.91± 0.014*
Garlic	Bulbs	1.03 ± 0.009	0.73 ± 0.007
Onion	Bulbs	0.15 ± 0.001	0.32 ± 0.003*

Data represent mean values ± standard deviation of three determinations. *Means are significantly different ($p< 0.05$)

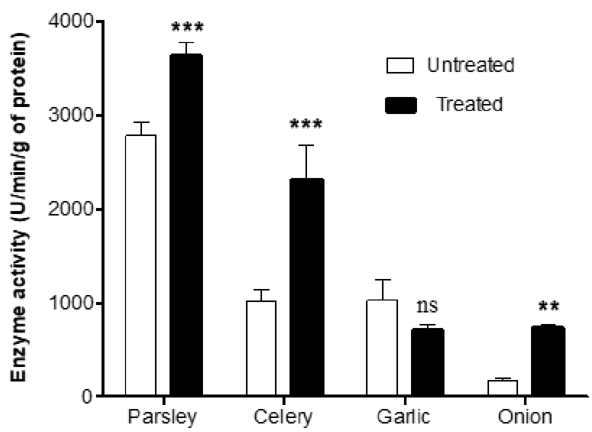

Figure 3: Peroxidase activity of crude vegetable extracts treated or untreated with insecticides. Error bars indicates the standard deviation of determinations. Differences were considered significant at $p< 0.05$. (ns > 0.05, ** $p < 0.01$, *** $p < 0.001$)

± 373.66 Umin^{-1}g^{-1} ($p< 0.001$). Insecticide-treated onions showed lower activity, with a measurement of 776.99 ± 33.62 Umin^{-1}g^{-1} ($p< 0.01$). Samples derived from garlic did not show a significant increase in POD activity in insecticide-treated 772.84 ± 67.25 Umin^{-1}g^{-1} ($p>0.05$) compared to the untreated samples that had POD activity of 1253.09± 232.84 Umin^{-1}g^{-1}.

4 DISCUSSION

Use of insecticides leads to a dysfunction of metabolic enzyme systems in plant tissues, and negatively modifies certain physiological functions. In order to show the difference in tolerance behaviour and toxicity level among different vegetables selected against insecticide stress, the activity of the antioxidant enzyme peroxidase was evaluated.

In this work, we compared POD activity in insecticide-treated and untreated parsley, onion, garlic, and celery. The assessment of the oxidation of o-dianisidine in the presence of H_2O_2 revealed that the four plants had significant differences ($p<0.05$). Our findings are comparable to those of two groups, Hemeda & Klein (1990) and Ponce et al. (2004). They reported differences in POD activity indifferent crude vegetable extracts. García-Hernández (2005) showed high activity of POD in peppers treated with insecticides. On the other hand, the application of insecticides on garlic did not show an increase in POD activity compared to the other plants studied; this could be explained by the fact that the analyzed part is the bulb and not the leaf part. Garlic is described as a biopesticide possessing other defense mechanisms apart from peroxidase, such as poly sulfides. Several studies have shown that garlic possess some insecticidal, fungicidal, acaricidal, nematocidal and bactericidal properties (Lalla et al., 2013; Nwachukwu & Asawalam, 2014). Garlic has received much interest in recent years with respect to environmental concerns about the use of chemically synthesized plant protection products and has been proposed as a green pesticide; a new and environmentally sustainable alternative for application in control programs against various pest species. Indeed, this plant is equipped by evolution to defend itself against pathogens and pests (Mamduh *et al., 2017;* Wang et al., 2019*)*.

Phytotoxicity by excessive use of insecticide has been evaluated in some physiological traits in other cultivars and plants (Mousavizadeh & Sedaghathoor, 2011; Diao et al. 2011; 2019). García-Hernández (2005) reported that the highest insecticides rates caused alterations in the expression of peroxidase. The potential variation in peroxidase activity can be reflected in the growth and yield of plants, playing an important role in some stages of the metabolism, such as the auxin catabolism, and lignin formation (Fang & Kao, 2000). Peroxidase is involved in detoxification of xenobiotic a defense system of plants (Çördük, 2016; Lubos et al., 2011), its increase in plants is thought to be a response to stress, especially when the levels of H_2O_2 which is its substrate is high. The expression of each peroxidase isoform, is linked to the physiological status and the stress of developing conditions in a plant (Lobarzawsky et al., 1991). Hajjar et al. (2018) were able to identify many isoforms of POD using electrophoresis and spectrophotometric approaches. Additionally, they found that each isoform is activated depending on the chemical structure and properties of the insecticide.

Chlorpyrifos and dimethoate are organophosphorus insecticides with a large spectrum activity. Their mechanism of action is to inhibit cholinesterase, which is the cause of potential toxicity in humans (Gupta, 2016; Dhiraj et al., 2020; Nazam et al., 2020). The excessive use of insecticides can underlie health problems in humans; ranging from minor problems(e.g., eye irritation, skin irritation, skin sensitization) (Damalas & Eleftherohorinos, 2011) to neurotoxicity or cancer (Foster & Brust, 1995; Yadav et al., 2019).Exposure to organophosphate insecticides leads to depression of plant growth and nitrogen metabolism (Parween et al., 2011). The highest exposure of the Algerian consumer to pesticide residues through consumption of raw fruit and vegetables was found to be (42 %) for chlorpyrifos (Mebdoua et al., 2017).

Fatma et al. (2018) showed a significant decrease in seed germination of *Allium cepa* in the presence of these insecticides, and the effects were enhanced with increasing their doses. Thus, seed germination, a primary physiological process of plant growth, is strongly influenced by environmental stress. Stunting of plant growth at higher concentrations of applied pesticides indicates a reduction in cell division, cell elongation, and conversion of indole-3 acetic acid to various photo-oxidative products, as these compounds function as potent auxin antagonists (Tevini & Teramura, 1989). Plants possess a complex antioxidant system including enzymes such as catalase (CAT; EC.1.11.1.6), peroxidase (POD; EC. 1.11.1.7), and superoxide dismutase (SOD; EC. 1.15.1.1) to mitigate and repair ROS damage (Pandey & Rizvi, 2010). There are several evidences of insecticide degradation by high activity of oxidoreductase enzymes which reflects the level of toxicity and also the ability to combat stress (Dong et al., 2007; Yildiztekin et al., 2015; Singh et al., 2015).

Several studies have showed that spraying of crops with organophosphorus insecticides was associated with a remarkable stimulation in peroxidase activity (Garcia-Hernandez et al., 2005). Hajjar et al. (2018) found that that the highest level of increase in peroxidase activity was recorded at 20 days after spraying tomato plants with organophosphorus insecticides compared with untreated plants. Furthermore, the effects in interaction and response of peroxidase activity relied significantly on two factors; the insecticide and the dose. The effect of insecticides depended on their formulations and physicochemical properties (vapor pressure and solubility), climatic conditions (temperature, humidity, and sunlight), plant characteristics (genus and species), location of their applications and importantly the number and doses applied (Heshmati et al., 2020). García-Hernández et al. (2005) showed that insecticides applied at low doses did not cause significant differences in peroxidase activity compared to the control without insecticides, but a higher dose significantly increased peroxidase activity. Similar trends have also been reported in studies related to physiological injury by insecticides in hot pepper (Atale et al., 1995; García- Hernández et al., 2000). Furthermore, the results obtained here are consistent with the hypothesis reported by García-Hernández et al. (2005), who reported that insecticide-induced stress influences antioxidant enzymatic activity. The impact of regulated expression of peroxidase in plants has a direct effect on their shelf life. Indeed we noticed that the shelf life of parsley and celery that have not been treated with insecticides is relatively longer than that of treated vegetables. Furthermore, the external morphology of insecticide treated vegetables is altered to appear less shiny.

Some farmers apply insecticides in concentrations that are higher than the recommended amount to control resistant pests, occasionally reporting better control, but the yields are reduced and may have undesirable consequences. In general, the manufacturer's recommended application protocol does not have a negative effect on the plants, and some reports showed that there are certain insecticides that act as growth stimulants when applied at low doses (Ahemad & Khan, 2012; Singh et al., 2015; Yang et al., 2020). Other studies have shown that the excessive use of fertilizers, inappropriate irrigation, and exploitation of metal resources can lead to salt stress to a large extent (Shrivastava & Kumar, 2015; Gull et al., 2019). Under these circumstances, plants are likely to face biotic and abiotic stresses more frequently and simultaneously.

The action of commercial chemicals is not trivial because they modify non-target physiological pathways, especially when the doses are not adapted. Work from this study suggests that insecticide stress influences antioxidant enzyme activity and supports that organic farming conditions minimize peroxidase activity and enzyme browning in parsley, celery and onion. We conclude that POD is a very interesting biomarker of insecticide stress, and that overproduction of POD negatively affects their quality and shelf life.

5 CONCLUSION

Our study showed a significant increase in peroxidase activity on samples from conventional agriculture. These results represent an alarming report on the excessive and unreasonable use of insecticides by farmers, which is why it is important to inform farmers about the danger of these practices. Indeed, the use of chemicals to control pests can be useful on the one hand, but on the other hand can present many risks for human health. In this perspective, the evaluation of peroxidase enzymatic activity could be a reliable tool for the evaluation of the physiological stress resulting from the application of insecticides and will help to prevent the loss of antioxidant potential as well as the quality of vegetables, including the commonly used aromatic plants such as parsley and celery. Thus, we recommend through this study to reduce doses by combining biopesticides and by producing long-term resistant varieties, we also underline the importance of peroxidase which seems to be an interesting marker of insecticide-induced stress. Finally, additional and further studies are required to determine the doses of pesticides that do not significantly influence peroxidase activity.

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