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AFFINITY OF CLASS I AND CLASS III PEROXIDASES FOR H_2O_2 IN PEPPER LEAVES OF DIFFERENT MATURITY

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ABSTRACT. Paper deals with activities of Class III peroxidases (POX, EC 1.11.1.7) and Class I peroxidases (ascorbate peroxidase, APX, EC 1.11.1.11) as well as the K_m of these enzymes for hydrogen peroxide (H_2O_2) in the pepper leaves of different maturity. The obtained results suggest that the youngest pepper leaves compared to more mature ones have different strategies for H_2O_2 removal. There was an increase in APX activity with leaf maturity, while POX activity had the opposite trend, and its lowest activity was observed in the oldest leaves. The accumulation of reducing substrates i.e., ascorbate and total phenols was in positive correlation with corresponding enzymes following leaf maturity. The comparison of POX and APX affinity for H_2O_2 in pepper leaves of different maturity and their relationship between these enzymes' activities were showed.

Keywords: *Capsicum annuum* L., ascorbate peroxidase, class III peroxidase, leaf maturity, hydrogen peroxide.

INTRODUCTION

Ascorbate peroxidase (APX) is a central component of the ascorbate-glutathione cycle and has an essential role in maintaining the level of intracellular reactive oxygen species-ROS (MITTLER, 2002). It catalyses the degradation of hydrogen peroxide (H_2O_2) using ascorbate as a reducing agent (SHARMA *et al*., 2012). Ascorbate peroxidase belongs to the Class I peroxidase family and represents one of the most widespread antioxidant enzymes in the plant cell and its isoforms have been found in various cellular compartments. There are at least five isoforms detected in the cytoplasm, thylakoid membranes, chloroplast stroma, and peroxisome membranes (ASADA, 1999; LOGAN *et al*., 2006). It shows a higher affinity to H_2O_2 than catalases and Class III peroxidases (GILL and TUTEJA, 2010). APX gene expression is in relation to the protection of plant cells from adverse environmental conditions. The studies made on pepper leaves showed that stress conditions such as low temperatures, heavy metals, and

gamma rays led to increased APX activity (KIM *et al*., 2005; AIRAKI *et al*., 2012; LATEF, 2013). PROCHÁZKOVÁ *et al*. (2001) have shown that young maize leaves have higher APX activity and other antioxidant enzymes, but a lower concentration of H_2O_2 and malonyldialdehyde (MDA). On the other hand, in the final stage of senescence, the APX activity decreased and the content of H_2O_2 increased (PROCHÁZKOVÁ *et al*., 2001).

Class III peroxidases (POX) are hem containing oxidoreductases that catalyse the oxidation of different substrates (mainly phenolic) in the presence of H_2O_2 (VELJOVIĆ-JOVANOVIĆ *et al*., 2018). They are involved in many physiological processes primarily due to a large number of isoforms and different regulation of their expression. Its action is present from plant development to plant death (PASSARDI *et al*., 2005). Peroxidases are widely accepted as stress-related enzymes involved in ROS elimination in stress conditions (SHARMA *et al*., 2012; VELJOVIĆ-JOVANOVIĆ *et al*., 2018). In vascular plants, POX has multiple roles, participating in the metabolism of the cell wall, cell growth, detoxification of hydrogen peroxide, auxin catabolism, oxidation of toxic compounds, protection against pathogens, mechanical injuries and oxidative stress, symbiosis, and senescence (PASSARDI *et al*., 2005; COSIO and DUNAND, 2009). POX are detected in a vacuole, cell wall, and cytosol (SHARMA *et al*., 2012).

Low-molecular-weight-compounds, such as ascorbic acid and phenols, are also important antioxidants in plant cells acting in a cooperative network with antioxidant enzymes (TAKAHAMA and ONIKI, 1997). The ascorbate concentration in plant subcellular compartments is in the range from 20 to 300 mM and depends on plant organ and species (BADEJO and ESAKA, 2010). AIRAKI *et al*. (2012) have found that the content of ascorbate in pepper leaves was 77 mg g^{-1}FW. As an APX substrate, ascorbate plays a key role in H_2O_2 scavenging in chloroplast, cytosol, and peroxisomes. In addition, ascorbate reduced phenolic radicals generated during H_2O_2 scavenging in Class III peroxidase/phenolics/AA system in apoplast and vacuoles (TAKAHAMA and ONIKI, 1997). For some enzymes, ascorbate is a cofactor (SMIRNOFF and WHEELER, 2000), and has a significant role in redox signaling, biosynthesis of oxalate and tartrate, and in growth regulation (VELJOVIĆ-JOVANOVIĆ *et al*., 2017). The content of total ascorbate in plant leaves is higher than glutathione and more than 10% of total soluble sugars are in the form of ascorbate, which indicates its importance for plants. As a result of its high antioxidant capacity, the relatively low energetic cost for its biosynthesis and the existence of an efficient system for its redox regeneration, ascorbate represents important plant defence strategy to oxidative stress (GEST *et al*., 2012; VELJOVIĆ-JOVANOVIĆ *et al*., 2017). Besides the role in enzymatic H_2O_2 scavenging, ascorbate can also directly remove several different ROS including singlet oxygen, superoxide, and hydroxyl radicals (PADH, 1990).

Pepper (*Capsicum annuum* L.) from the Solanaceae family is a very important crop and its fruits are second in the world consumption of vegetables (TOPUZ and OZDEMIR, 2007). It represents a very important source of many essential nutrients for humans, especially ascorbic acid, ß-carotene, and calcium. Also, some varieties of peppers contain significant amounts of capsaicinoid, which originate from a phenyl group with strong physiological and pharmacological properties (TOPUZ and OZDEMIR, 2007). Capsaicinoids play a significant role as neuropharmacological substances and their role in medicine has been the subject of many studies. Some of the research indicate that capsaicinoid has an antibacterial and antiplatelet effect (TSUCHIAY, 2001). In addition, some studies indicated its use as a pain reliever in chronic diseases such as post-herpetic neuralgia, diabetic neuropathy, osteoarthritis (TSUCHIYA, 2001, BACKONJA, 2010).

Ascorbate is also an important compound of pepper fruit especially because of its antioxidant capacity (MCCALL and FREI, 1999). The ascorbate content in the fruit of different types of pepper ranges from 0.575 and 0.649 mg g^{-1}FW (TOPUZ and OZDEMIR, 2007).

The importance of the antioxidant system was shown in the literature, especially the APX and POX and the content of their substrate, ascorbate, and phenols during the influence

of some environmental stress on pepper leaves. AIRAKI *et al*. (2012) explored the influence of lower temperature on the antioxidant system of pepper leaves. They show an increase of APX activity and the content of ascorbate in the pepper leaves exposed to low temperature compared to control plants. In addition, HUSEYNOVA *et al*. (2017) have shown an increase of POX activity and higher phenol content in pepper leaves during the pathogenic infection. They considered that the increase of POX activity was associated with an increase in phenol content.

This study aimed to determine the APX and POX affinity to H_2O_2 in pepper leaves of different maturity. In addition, the aim was to evaluate POX and APX activity in the pepper leaves of different maturity and correlate their activity with the amount of ascorbate and phenols.

MATERIALS AND METHODS

Plant material

Sixty-day-old pepper seedlings (*Capsicum annuum* L.), purchased at the local market, were used for the experiment. Leaf sample were separated according to their maturity and marked as maturity level I, II, and III where level I represented the oldest, completely formed leaves. Level II contained the leaves from the middle part of the stem, which were still in forming and younger than those at the level I, while the leaves of level III were the youngest. The age of the leaves was determined apically with the leaves on the top stem being the youngest. The leaves at the bottom of stem are the oldest, while those in between represent level II. Each sample (level) consisted of ten leaves and sampling was performed in triplicates. Pepper is characterized by sympodial or "determinate" development in which the shoot apical meristem terminates by a flower and further development continues from the upper most axillary meristems (ELITZUR *et al*., 2009). In this sense the younger leaves are placed on the apical part of the branch, and the more mature leaves, which are larger, remain on the lower part of the stem.

Plant extract preparation

Plant leaves were powdered in liquid nitrogen and homogenized in a mortar with extraction buffer in the ratio 1:4 w/v. For APX extraction, 100 mM sodium phosphate buffer pH 7.8 containing: 1 mM PMSF (phenylmethylsulfonyl fluoride), 8% glycerol, 1 mM EDTA (ethylenediaminetetraacetic acid) and 5 mM ascorbic acid was used. Therefore, 100 mM sodium phosphate buffer pH 6.4 containing: 1 mM PMSF and 0.2% tween was used to extract POX. The homogenates were centrifuged at 10 000 rpm for 10 minutes at 4°C and collected supernatants were used for determination of protein concentration, APX and POX activity, as well as POX isoforms separation by native electrophoresis.

Determination of protein concentration

The protein concentration was determined according to the Lowry method (LOWRY *et al*., 1951) using bovine serum albumin (BSA) as a standard compound. The standard curve for the determination of protein concentration was $y = 1.476x - 0.0056$.

Determination of ascorbate peroxidase activity and K_m for H_2O_2

Ascorbate peroxidase activity was determined by the method described by MIYAKE and ASADA (1992). The reaction mixture (3 mL) contained: 50 mM sodium phosphate buffer pH 7.0, 0.5 mM ascorbate (ε=2.8 $mM^{-1}cm^{-1}$), 0.0165 mM hydrogen peroxide and 50 µL of protein

extract. The decrease in the absorbance at 290 nm for one minute was monitored. The APX activity was expressed in $\mu mol/mg_{prot}$ min. Therefore, 0.5 mM ascorbate and hydrogen peroxide in the concentration range 0.0165 to 0.165 mM were used as substrates to determine the Michaelis-Menten constant (K_m) for hydrogen peroxide of APX.

Determination of peroxidase activity

The reaction mixture (3 mL) contained 100 mM sodium phosphate buffer pH 6.4, 5 mM pyrogallol, 0.33 mM hydrogen peroxide and 50 µL of protein extract for POX activity. An increase in the absorbance at 439 nm for one minute was monitored. The POX activity was expressed in $\mu mol/mg_{prot}$ min. Therefore, 5 mM pyrogallol, and hydrogen peroxide in the concentration range 0.33 to 6.67 mM were used as substrates to determine the Michaelis-Menten constant (K_m) for hydrogen peroxide of POX. POX activity was determined based on the extinction coefficient for pyrogallol ($\varepsilon = 12\ mM^{-1}\ cm^{-1}$).

Linearization of Michaelis-Menten constant, K_m

K_m for APX and POX were determined after the linearization by Lineweaver-Burk plot based on the dependence 1/EA(POX) i.e., 1/EA (APX) in the function $1/c(H_2O_2)$, where the line was obtained, and K_m was determined as a section on the abscissa.

Native electrophoresis

Separation of POX isoforms was performed by native electrophoresis polyacrylamide gel (PAGE) using 5% stacking and 10% running gel. Before application on the gel, the samples were mixed with sample buffer (50 mM Tris pH 6.8, 10% glycerol 0.01% bromphenol blue) and 5.40 µg protein was applied to gels. Specific peroxidase staining was performed by immersing the gel in the dyeing solution containing 5 mg naphthol, 5 mL methanol, 45 mL 100 mM sodium phosphate buffer pH 6.4, and 50 µL of 30% hydrogen peroxide (added before staining). POX isoforms were visualised as purple bands on a colourless background.

The Volumes of POX isoforms and their Rf values were determined densitometric in the program TotalLab. The Volume of POX isoforms detected on the native gel correspond to their activities and are expressed in relative units.

Determination of total ascorbate amount

The ascorbate acid content was determined by titrimetric method using the standard analytical method (AOAC 1990). The leaves were homogenized with 5% HCl, filtered through quantitative filter paper, and titrated with 2,6-dihydrophenol-indophenol (DCIP). The endpoint of the titration was defined as a pink colour that persists through at least 15 s of shaking. Commercial L-ascorbic acid was used as a standard and calculated values were expressed as $mg\ g^{-1}$ FW.

Determination of total phenol concentration

In order to determine the total phenol concentration, extraction was performed in methanol with the addition of quartz sand for better homogenisation. The homogenates were incubated for 30 minutes at 37 °C and then centrifuged for 10 minutes at 10 000 rpm. The concentration of the total phenol was determined using Folin-Ciocalteu reagent (diluted with distilled water in a ratio of 1:3) according to the method given by SINGLETON and ROSSI (1965). Quantification was performed by using the line equation of the calibration curve for standard (Gallic acid) and expressed as the equivalent of Gallic acid (GAE) per gram of fresh weight. The standard curve for the determination of phenol concentration was $y = 0.913x - 0.027$.

Statistical analysis

Data from the experiments were submitted to analysis of variance (ANOVA) and Pearson correlations by using the JASP graphical program and GraphPad Prisma 6. The statistical significance was determined at $p < 0.05$.

RESULTS AND DISCUSSION

Ascorbate peroxidase activity and ascorbate concentration

The obtained results showed that there was a significant difference in APX activity in pepper leaves of different maturity. The highest APX activity was determined at maturity level I and decreased by 31% and 22% at levels II and III respectively (Fig. 1).

During different stress conditions, an increase in APX activity in the pepper leaves was recorded (KIM *et al*., 2005; GILL and TUTEJA, 2010; AIRAKI *et al*., 2012), and some authors showed that leaf maturity affects APX activity. In pea leaves APX activity decrease in older leaves (DONAHUE *et al*., 1997; JIMENEZ *et al*., 1997). On the other side, APX activity measured in the leaves of drought-tolerant species *Ramonda serbica* was increased with aging (VELJOVIĆ-JOVANOVIĆ *et al*., 2006). PROCHÁZKOVÁ *et al*. (2001) have shown that in the maize leaves the concentration of hydrogen peroxide increased in the older leaves, compared to the younger ones. The increase in H_2O_2 content may be accompanied by an increase in the activity of antioxidant enzymes, so based on this it could be concluded that maturity in pepper intensifies the metabolic pathway that removes hydrogen peroxide in an ascorbate dependent way. This conclusion is in line with the results obtained for ascorbate content (Fig. 1B).

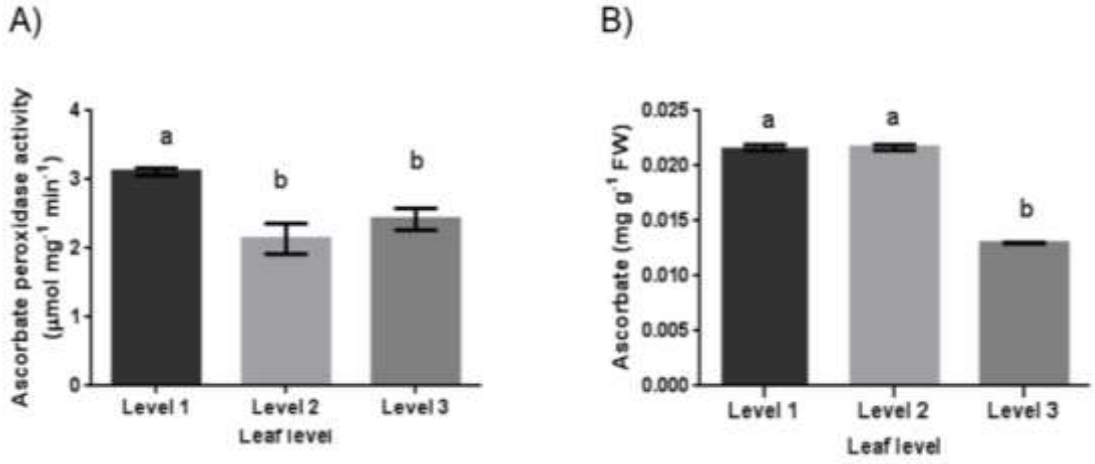

Figure 1. The APX activity at different leaf maturity levels for the highest (0.165 mmol L^{-1}) concentrations of H_2O_2 at the ascorbate concentration of 0.5 mmol L^{-1} (A); The content of ascorbate in all level leaves (B).
The results are expressed as mean ± standard error. Values are the mean of 3 replications. The same letter indicates that there is not statistical significance between values according to Tukey's test ($p \leq 0.05$, Tukey's test).

The amount of ascorbate decreases from the oldest (maturity level I) to the youngest leaves (maturity level III), wherein the concentration of ascorbate decreased by 40% in the youngest leaves. Ascorbate plays an essential role in the several pathways of antioxidant

systems and protects plant cells from oxidative damages caused by ROS (CONKLIN, 2001). Many authors emphasized that the ascorbate is a metabolite with an essential role in the regulation of the cellular redox state which is, on the other hand, crucial for plant development (NOCTOR and FOYER, 1998; ASADA, 1999). Numerous studies have shown that the various stress conditions increase ascorbate synthesis in the pepper leaves and that ascorbate amount was followed by increased APX activity (MAHDAVIAN *et al*., 2008; AIRAKI *et al*., 2012). CONKLIN *et al*. (1996) have shown that ascorbate has a key role in the protection of Arabidopsis foliar tissue. Our results showed that there were positive correlations between the APX activity and the ascorbate content in pepper leaves, but not statistically significant (Table 3). The possible reason for higher APX activity and higher ascorbate content in older leaves is that older leaves usually have higher ROS content as was shown for sunflower leaves (DE LA HABA *et al*., 2014). The results obtained indicate that APX and ascorbate, as a part of the ascorbate-glutathione cycle, play an important role in removing ROS from more mature pepper leaves.

Peroxidase activity and the concentration of total phenols

Our results showed a significant difference in POX activity in relation to the pepper leaf maturity level (Fig. 2A). Contrary to APX activity, POX activity was the lowest in mature leaves (maturity level I). Higher POX activity was detected by 43% at maturity level II and by 29% at level III compared to level I (Fig. 2A).

POX involvement in plant response against numerous kinds of environmental stress is well known (JANSEN *et al*., 2001; JOUILI *et al*., 2011; WANG *et al*., 2015). Using endogenous phenols substrates, the POX from the vacuole efficiently removes H_2O_2 (FERRERES *et al*., 2011). POX in the apoplast oxidize different phenolics compounds to forming lignin which is deposited in the secondary cell walls during normal growth (SATO *et al*., 1993; LÓPEZ-SERRANO *et al*., 2004; PASSARDI *et al*., 2006). In addition to being POX substrates, phenolics compounds can also remove ROS in direct reactions. Thanks to their hydroxyl group phenolic compounds are the good electron and proton donors and they are capable to react with ROS and RNS whereby more stable radicals are formed (PEREIRA *et al*., 2009). More stable radicals are formed due to delocalization of π-electrons, intramolecular hydrogen bonding, or condensation with other radicals (PROCHÁZKOVÁ *et al*., 2011).

The total phenols concentration in pepper leaves of different maturity is presented in Figure 2B. Higher phenol concentration, by 33% at maturity level II, and by 15% at level III were detected in comparison with level I (Fig. 3B). ESRA *et al*. (2010) have shown that an increase in phenolics content can be related to the antioxidant defence of pepper plants under low temperatures stress. KU *et al*. (2009) have shown that pepper leaves have antimutagenic, antimicrobial, and antioxidant activities. Authors showed that the content of total phenolic and flavonoid compounds in pepper leaves ranged from 2.31 mg/g to 5.6 mg/g and 2.51 mg/g to 6.89 mg/g respectively and suggested that the antioxidant capacity of pepper leaves is largely a result of flavonoid content (KU *et al*., 2009).

Our results showed that the lowest POX activity in the most mature leaves was accompanied by the lowest s concentration (Fig. 3) and a positive correlation between phenols concentration and POX activity ($p=0.049$ and $r=0.669$) was detected (Table 3). Such results could indicate that phenols play an important role in the ROS removal and that their concentration in individual pepper leaves depends on leaf maturity. Some authors have shown that phenols content in plant leaves increases with maturity (OZYIGIT *et al*. 2007; YANG *et al*., 2011). YANG *et al*. (2011) has shown that phenols content in plant *Morinda citrifolia* L. were highest in leaves of medium maturity, which is in line with our results.

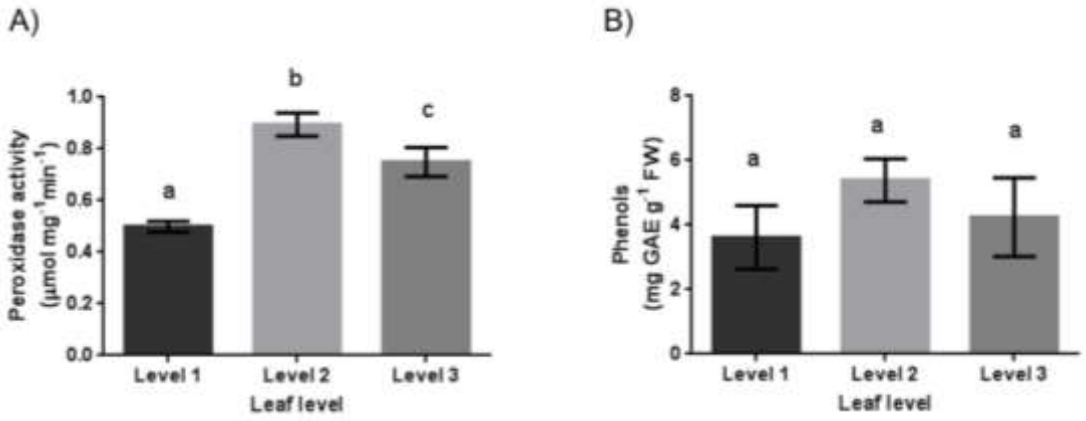

Figure 2. The POX activity at different leaf levels for the highest (6.67 mmol L^{-1}) concentration of H_2O2 $_{at}$ the pyrogallol concentration of 5 mmol L^{-1}(A); The content of phenol in the first, second, and third-level pepper leaf (B).
Values are the mean of 3 replications from three-level leaves. The results are expressed as mean ± standard error. Values followed by the same letter are not significantly different according to Tuke's test ($p \leq 0.05$, Tukey's test).

In order to determine the POX isoforms, pepper protein extracts were separated by native PAGE. Five POX isoforms were detected (marked with POX1-POX5) at all leaf levels, except in the third level where POX1 was not detected (Fig. 3).

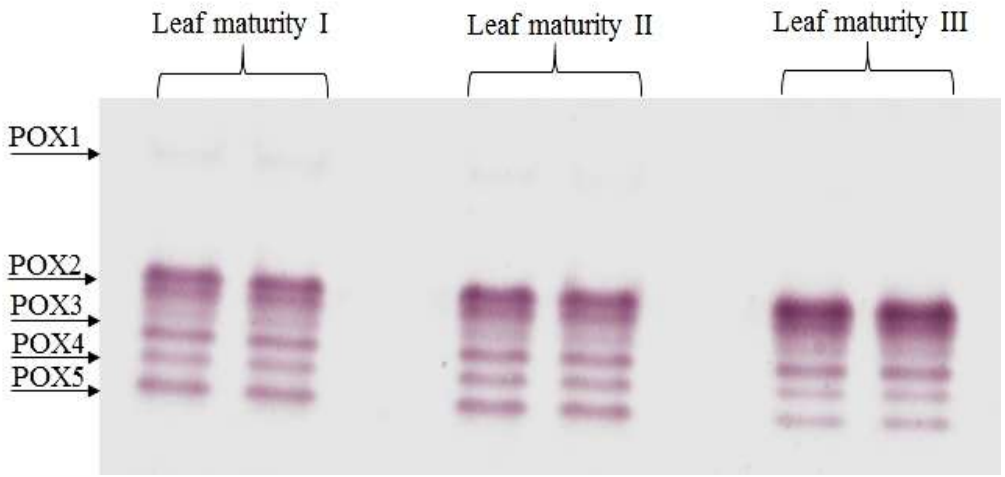

Figure 3. Native gel with separated peroxidase isoforms labeled POX1-POX5 in pepper leaves of different maturity.
The arrows indicate different peroxidase isoforms.

The relative activity of individual POX isoforms is shown in Table 1. The POX2 isoform had the highest activity compared to other isoforms, wherein its activity decreases with the maturity of the leaves (Table 1). Isoform POX3 also had the lowest activity in mature leaves. Unlike them, POX5 had higher activity in mature leaves. Such results indicate activation of different POX isoforms depending on leaf maturity.

Table 1. The activity of POX isoforms at different levels of pepper leaves expressed as Volume, relative to densitometric measurement in the TotalLab program and their Rf values.

Activity (Volume)				
Isoforms of POX	**Maturity level I**	**Maturity level II**	**Maturity level III**	**Rf**
POX1	4125.67	1740.33	–	0.199
POX2	60543.00	69870.15	89433.55	0.506
POX3	11666.62	21827.54	24855.41	0.721
POX4	14445.02	16910.13	13910.47	0.787
POX5	22990.04	22459.84	11648.94	0.865

We have shown a positive correlation of APX and POX with amounts of their reducing substrates, ascorbate, and phenols, respectively. At the same time, the APX and POX affinity for oxidising substrate, hydrogen peroxide, can be partially connected with leaf maturity. APX and POX compete for the same oxidizing substrate, in fact, H_2O_2 as their electron acceptor, when they are present in the same sub-cell compartments (DE GARA, 2003). Both APX and POX have low K_m for H_2O_2, in the micromolar range (NAKANO and ASADA, 1981), even though the affinity of APX for H_2O_2 is much higher than that of POX (DE PINTO and DE GARA, 2004). The results for K_m values of POX and APX for H_2O_2 are shown in Table 2. The results show that APX affinity for hydrogen peroxide was higher in older leaves (maturity level I and level II) and that it may be correlated with APX functions as a scavenger of hydrogen peroxide. The value of K_m for POX in pepper leaves were higher than for APX (Table 2). The K_m values of APX in pepper leaves is lower than POX K_m for about 53%. In comparison to the POX and CAT, APX has a higher affinity for hydrogen peroxide and because of that, they may have a crucial role in the management of ROS during stress (GILL and TUTEJA, 2010). It is interesting that K_m values of APX and POX decreased with leaf maturity, indicating higher affinity of both enzymes for H_2O_2 in older leaves (Table 2).

The K_m values of APX for H_2O_2 in saffron was 0.05 mM under optimal pH 8 (GHAMSARI and KEYHANI, 2003). Some authors stated that mature plant leaves accumulated less H_2O_2 than young leaves because growing tissues are more sensitive to environmental changes, so lower H_2O_2 accumulation is correlated with stress tolerance (LIU *et al*., 2014; MOUSTAKA *et al*., 2015). Also, APX affinity for H_2O_2 in young leaves is usually higher than that of POX which is one of the reasons for lower H_2O_2 accumulation in them (BORRACCINI *et al*., 1994). This hypothesis is following our findings since the K_m of APX is lower than K_m of POX in all level leaves (Table 2). Interestingly, by comparing antioxidant strategies for hydrogen peroxide removal in the pepper leaves of different maturity, it can be said that the affinity of POX for its oxidising substrate is inversely proportional to the amount of reducing substrates, phenols. On the other hand, APX affinity is proportional to the amount of ascorbate.

Table 2. The K_m value (μM) of APX and POX for the first, second and third level pepper leaf.

Level leaf	APX	POX
Level 1	0.212 ± 0.095 a	0.337 ± 0.088 a
Level 2	0.175 ± 0.115 a	0.487 ± 0.089 a
Level 3	0.257 ± 0.069 a	0.563 ± 0.156 a

Note: The results are expressed as mean ± standard error. Values are the mean of 3 replications. The same letter indicates that is no statistical significance between values according to Tukey's test ($p \leq 0.05$, Tukey's test).

Table 3 shows the correlation between enzymatic and non-enzymatic antioxidants and their affinity to hydrogen peroxide. It was detected a positive correlation between POX activity and phenols content (p=0.048). The positive correlation between POX activity and phenol content indicates that increased POX activity in pepper leaves may be associated with increased phenol content. In addition, there is a positive correlation between APX and ascorbate content, but not significant. On the other hand, a negative correlation was found between APX and POX activity (p=0.002). This can indicate their antagonistic activity.

Table 3. Pearson Correlations for all measured parameters.

		APX		POX		K_m APX	K_m POX	AA	Phenols
APX	Pearson's r	—							
	p-value	—							
POX	Pearson's r	-0.868	**	—					
	p-value	0.002		—					
K_m APX	Pearson's r	0.239		-0.065		—			
	p-value	0.568		0.879		—			
K_m POX	Pearson's r	-0.462		0.601		-0.132	—		
	p-value	0.210		0.087		0.755	—		
AA	Pearson's r	0.222		-0.147		-0.347	-0.522	—	
	p-value	0.565		0.706		0.399	0.150	—	
Phenols	Pearson's r	-0.613		0.670	*	-0.358	0.245	0.141	—
	p-value	0.079		0.048		0.384	0.526	0.718	—

*p < .05, **p < .01, ***p < .001

TAKAHAMA (2004) proposed the concept of delocalized H_2O_2 scavenging (scavenging in a compartment different from its source). This pathway implies the existence of synergistic acting of ascorbate, phenols, and POX in H_2O_2 scavenging in vacuoles: phenolic compounds in vacuoles act as electron donors for Class III POX and formed phenoxyl radicals which oxidize ascorbate to its dehydroascorbate, and then it is reduced and back in the cytoplasm (TAKAHAMA, 1993; TAKAHAMA, 2004).

It was shown that leaf maturing change ascorbate metabolism because it is associated with cell wall stiffening and that negative correlation between APX and POX during developmental regulation appears in older leaves (DE PINTO and DE GARA, 2004). These findings are in line with our results since the highest APX and the lowest POX activities have been observed in mature, completely formed leaves. In addition, the opposite trend and negative correlation between these two enzymes confirmed their antagonistic activity (r=-0.868 and p=0.002).

CONCLUSION

The results obtained in this study suggest that pepper leaves of different maturity include different strategies in H_2O_2 removal. Ascorbate peroxidase, as a representative enzyme from Class I peroxidases family, showed higher activity in older leaves were also a higher amount of its reducing substrate i.e., ascorbate was observed. Class III peroxidase has higher activity and a positive correlation with reducing substrate i.e., phenols in younger leaves. APX had a higher affinity for hydrogen peroxide than POX in pepper leaves, while both enzymes show a higher affinity for hydrogen peroxide in older pepper leaves.

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