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Role of peroxidases in capsaicinoids degradation in habanero pepper (*Capsicum chinense* Jacq.) plants grown under water deficit conditions

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Abstract

Habanero pepper (*Capsicum chinense* Jacq.) is one of the most pungent cultivars of the genus *Capsicum.* We studied the effects of water deficit on capsaicinoids (CAPs, acid amides derived from phenylalanine and valine or leucine) accumulation. It comprises their synthesis and degradation through the determination of not only capsaicinoid content, but also of related enzymes such as capsaicinoid synthetase (CS) and peroxidases (PODs) in pods from water-stressed habanero pepper plants. Water stress was induced by withholding irrigation for 7 (R7) or 9 (R9) days after anthesis occurred, while control plants were watered daily. Irrigation withholding for 9 days induced effects on the rate of capsaicinoid accumulation in the placental tissue (131 mg g^{-1} DW), through a lowered rate of degradation (low PODs activities: 31 µKat mg^{-1} prot). The CS activity was not detectable at 60 DPA (days of post-anthesis). A correlation between PODs activities and CAPs contents was totally dependent on the maturation stage of the fruit. At 60 DPA, the PODs activities were highest for control and plants at R7 treatment, whereas CAPs contents remained high. PODs activities could not fully explain the formation of the dimer 5,5'-dicapsaicin when these enzymes' activities was immuno-inhibited. PODs may not be the sole pathway in the degradation process of CAPs in habanero pepper plants under water stress.

Keywords: Capsaicinoids; capsaicinoid synthetase; habanero pepper; peroxidases; water deficit.
Abbreviations: CAPs_capsaicinoids; CS_capsaicinoid synthetase; DPA_days of post-anthesis; POD(s)_peroxidase(s).

Introduction

Water is fundamental for living organisms and essential for agricultural production. Water stress is one of the most common abiotic stresses worldwide, which induces major constraints on plant productivity (Turner and Begg, 1981; Pedrol et al., 2000). Habanero pepper (*Capsicum chinense* Jacq.) is one of the most pungent known cultivars due to its high CAPs (capsaicinoids)content, exclusively accumulated in the placental tissue of fruits (Bennett and Kirby, 1968; Leete and Louden, 1968). CAPs are acid amides of vanillylamine and C_9 - C_{11} branched fatty acids, of which capsaicin is the principal pungent compound. The vanillylamine moiety of CAPs is biosynthetically derived from L-phenylalanine, whereas the branched fatty acid moiety arises from valine or leucine (Iwai et al., 1979). The aromatic and aliphatic moieties are condensed by the enzyme CS (Curry et al., 1999; Sung et al., 2005; Aza-González et al., 2011). CAPs biosynthesis occurs specifically in the epidermal cells of the interlocular septum of placental tissue in *Capsicum* fruits (Fujiwake et al., 1980; Suzuki et al., 1980; Zamsky et al., 1987; Estrada et al., 2000; Stewart et al., 2007). CAPs accumulation is greatly influenced by environmental factors, such as soil type, osmotic pressure, nutrients and water availability (Estrada et al., 1998; Díaz et al., 2004; Sung et al., 2005). An increase in capsaicin accumulation in *C. chinense* plants grown under water deficit conditions has been reported, which resulted paradoxical since CS activity decreased in plants (Ruiz-Lau et al., 2011). Hence, it is still under study how synthesis and degradation pathways contribute to their overall concentration in pods from plants under water stress (Estrada et al., 2000). Contrasting to the attention placed in the elucidation of the intracellular localisation and biosynthetic pathways of CAPs, there are only a few reports regarding their catabolism (Estrada et al., 1998; Martínez-Juárez et al., 2004; Díaz et al., 2004; Sung et al., 2005). PODs have been involved not only in plant defence against biotrophic and necrotrophic pathogens (Passardi et al., 2004; Almagro et al., 2009; Mohamed et al., 2011), but can also act as biomarkers of biotic and abiotic stresses (Jouili et al., 2011). These enzymes can readily oxidise vanillin (Zapata et al., 1992; Díaz et al., 2004), vanillylamine and CAPs (Martínez-Juárez et al., 2004), so they may play a significant role in CAPs catabolism. They have been purified from *C.annuum* fruits and are capable of oxidizing capsaicin into 5,5'-dicapsaicin and 4'-O-5-dicapsaicin in the presence of H_2O_2 (Bernal et al., 1993a,b; 1995; Bernal and Ros-Barceló, 1996). In whole fruits, there was an increase in PODs activities at the late stages of fruit maturation when CAPs content decreased (Contreras-Padilla and Yahia, 1998).

Furthermore, an increase in CAPs content along fruit development was not only related to changes in total PODs

activities, but specifically to changes in basic PODs (Estrada et al., 2000).
The objective of this study was to determine the role of PODs on the CAPs degradation in habanero pepper fruits under water deficit.

Results and discussion

Water deficit has a direct effect on the total number of flowers but not on the fructification index

Habanero pepper plants can tolerate up to 9 days without irrigation. They never reached the permanent wilting point, since they recovered their turgidity within five hours after re-watering (Fig. 1). There were, on average, 291 ± 24 flowers per plant in the control, in contrast to 158 ± 19 flowers in R7 plants and 101 ± 8.5 flowers in R9 plants, which represented 54% and 35% of the control values, respectively. Fructification index was 27%, 25%, and 12% for tagged flowers in the control, R7, and R9 treatments, respectively (Table 1). Water deficit significantly reduced the number of flowers on treated plants. This effect is rather common during water deficit since plants remobilise their nutrients to maintain meristematic activity (Jaimez et al., 2000; Sung et al., 2005).

There were no significant differences in the number of fruits formed in relation to the total number of flowers for the control and R7 treatment. Nevertheless, in the R9 treatment this number decreased to half of that observed in the control (Table 1).

Water deficit modifies CAPs content in fruits

The pungency level of peppers is mainly determined by two factors: plant genetics and environmental interactions. Temperature, light and fertilization have been reported to affect the pungency levels of fruits (Medina-Lara et al., 2008; Monforte-Gonzalez et al., 2010). It has been previously reported that capsaicin and dihydrocapsaicin concentrations increased in fruits from plants subjected to water stress compared to control plants, and this effect was correlated with fruit age. Contrastingly, CS activity lowered in response to water stress, and this effect depended on both, stress severity and fruit age (Ruiz-Lau et al., 2011).

CAPs (capsaicin plus dihydrocapsaicin) accumulation began about 20 DPA (Fig. 2). Notably, in plants under the R9 treatment, CAPs content was doubled in *C. annuum* at 30 DPA (Sung et al., 2005). In contrast, capsaicinoid accumulation in plants under R7 treatment followed a similar trend to that in control plants, with the exception of the level reached at 50 DPA, where maximal accumulation could be observed. Thereafter, there was a decrease in both water stress treatments and the control according to Contreras-Padilla and Yahia (1998) and Zhang et al. (2008).

PODs are present in the placental tissue in habanero peppers

PODs activities were observed in transversally-cut slices of fruits from water stressed and control plants (Fig. 3). There was significant staining in the pericarp of 25 DPA-fruits, without distinctions among control or water stressed (R7 or R9) plants, contrasting to the low response found in the placental tissues. However, from 45 DPA on, blue stained areas concentrated in the placenta, located in the centre of fruits (Fig. 3). These results point to the fact that habanero pepper pods can harbour several different PODs activities, which can be located either in the pericarp or placental tissue depending on their maturation stage. The presence and characterization of several PODs has been described for *C. annuum* (Bernal et al., 1994a, b; 1993b). From our data, it is evident that PODs are located in the same tissue where CAPs are being stored.

CAPs content is related to both CS and PODs activities

Contradictory reports have been published on the role of PODs in relation to the total CAPs content in pepper fruits (Bernal et al., 1993a, b, c; Bernal et al., 1994b; Bernal et al., 1995; Bernal and Ros-Barceló, 1996; Pomar et al., 1997; Estrada et al., 2000; Díaz et al., 2004; Zhang et al., 2008). Although PODs activities may be related to the catabolism of these compounds under normal growing conditions, and they increase under stress conditions, it has not been unequivocally demonstrated that PODs activities and the catabolism of these compounds are related under stress conditions in *Capsicum*. Results regarding CAPs content (A), CS (B) and PODs activities (C) at three physiological stages (25, 45 and 60 DPA) of habanero pepper fruits subjected to different watering regimes (C, R7 or R9) are shown in Fig. 4. Under these watering regimes, in terms of total CAPs concentrations, there was a tendency to increase their content in all cases as the plants became more stressed (Fig. 4A). This behaviour was more evident in 25 and 45 DPA-fruits, as in *Capsicum chinense* (Ruiz-Lau et al., 2011) and Patron pepper (*C. annuum*) fruits (Estrada et al., 1999). It is important to note that their results suggest that environmental conditions, such as water deficits, have a strong effect upon the competition between the biosynthesis of CAPs and other phenylpropanoid metabolites (Estrada et al., 1999). The CS in control plants showed a low activity along the fruit's ontogeny, whereas in the R7 treatment it increased from 0.13 to 2.47 μkat mg^{-1} protein at 60 DPA. In contrast, in R9 treatment, this activity was increased (0.79 to 1.17 μkat mg^{-1} protein) until 45 DPA, and then, lowered to a non detectable level by 60 DPA (Fig. 4B). It is interesting to point out that although both the control and stressed plants reached similar CAPs contents after 60 DPA, no clear-cut relationship between these and CS activity could be drawn. In the control plants, though this enzyme activity remained low, capsaicinoid levels were in the same range to those found in the stressed plants. When PODs activities were determined in control plants at 60 DPA (Fig. 4C), a peak was found. The behaviour of this group of enzymes in plants under water stress mimicked that of CS, by which plants under the R7 treatment, there was a continuous increment, while in the case of the R9 plants it diminished over the same period of time. PODs activity in placentas from 45 DPA-fruits was negatively correlated to CAPs content ($y = 144.76 – 0.62x$; $r^2 = 0.99$), whereas in fruits from water stressed plants, these activities were lower at the same developmental stage (Fig. 4C). In contrast, CAPs contents and CS activity were higher in plants under both water stress regimes compared to the control (Figs. 4A and B). Thus, both a higher rate of synthesis and a lowered rate of degradation could contribute to CAPs accumulation at 45 DPA. No such correlation could be observed in plants at 25 or 60 DPA ($r^2=0.319$ and $r^2=0.271$, respectively). A negative correlation between PODs activities and CAPs contents has been reported for *C.annuum* (Di et al., 2000; Sung et al., 2005). It is important to point out that for *C. chinense*, this correlation is totally dependent on the maturation stage of the fruit.

Data shown in Figs. 4A, B, and C point to the surprising fact that at 60 DPA,

Table 1. Fructification and flowering indexes of habanero pepper plants subjected to daily watering (C), and 7 (R7) or 9 (R9) days of water deficit. Mean comparisons were performed using Tukey's multiple range test.

Water deficit Traits	C	R7	R9
Number of flowers by plant	291.0 ± 24.1*	158.0 ± 19.3	101.5 ± 8.5
Flowering (%)	100*	54.29	34.7
Number of fruits by plant	80.0 ± 7.4*	40.0 ± 5.2	12.5 ± 3.5
Fructification (%)	27.4*	25.3	12.3

n=20. The asterisk indicated differences *P≤ 0.05.

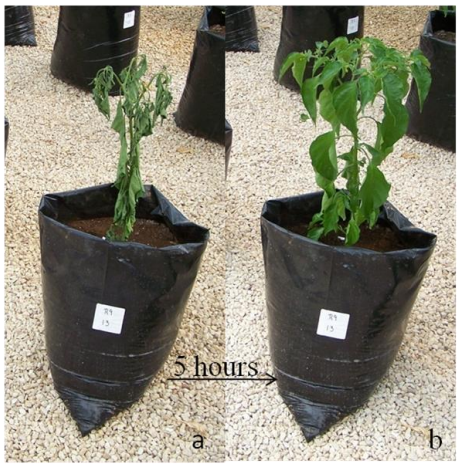

Fig 1. Habanero pepper plants can tolerate up to 9 days without irrigation. (A) Plant subjected to a 9-day water stress treatment; (B) The same plant 5 hours after irrigation with 1 L of water at the end of the water stress period.

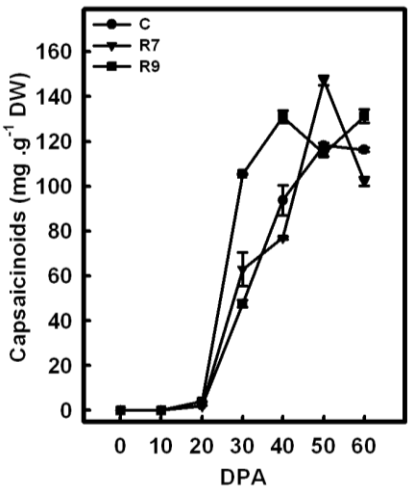

Fig 2. Total CAPs content in placental tissue along the development of habanero pepper pods. Plants were subjected to different water regimes (C, daily watering; R7 treatment, watering every 7 days; R9 treatment, watering every 9 days). Water stress treatments were initiated when anthesis occurred. Determinations were performed every 10 days up to 60 DPA. Data are the mean of three replicates ± SE.

though PODs activities were highest for control and plants under R7 treatment, CAPs contents remained high, which led us to think that degradation of CAPs does not depend solely on PODs activities, so other different catabolic pathways must be involved.

Immunoinhibition of PODs does not impede the formation of the dimer 5,5'-dicapsaicin

In order to further demonstrate that PODs were not the main players in the degradation of CAPs in habanero peppers under water stress, these enzymes activities were inhibited with a specific antibody raised against radish PODs. It has been demonstrated that there is significant cross-reactivity between pepper´s and horseradish´s PODs (Bernal et al., 1994a). Using this approach, PODs activities were evaluated whether as capsaicin consumption or formation of the dimer 5,5'-dicapsaicin as a result of capsaicin oxidation. The dimer was identified and characterised as reported by Martinez-Juárez et al. (2004). Since control 60-DPA plants presented the highest PODs activities (Fig. 4C), they were used for a dose-response experiment using different antibodies' concentrations to inhibit PODs activities. Inhibition was dependent on the antibodies' dose (Fig. 5A). However, no complete inhibition could ever be reached even when employing 5 mg/mL antisera (approximately 25% of the activity remained; Fig. 5A). When PODs activities were monitored *via* consumed capsaicin, inhibition was much weaker at the highest antibodies' concentration (Fig. 5A). These results suggest that these enzymes do not contribute to a major proportion to capsaicin degradation in these fruits. Then, there might be other catabolic pathways other than PODs oxidation of capsaicin that are functional in habanero pepper pods. It has been suggested that polyphenol oxidases, or even lipases, might be involved and play a role in mechanisms different from simple oxidation (Díaz et al., 2004). When PODs activities were assayed in extracts from water stressed plants in the presence of 2 mg/mL of the specific antibodies, there was an inhibition that varied between 35 to 55%, compared to the levels found in the absence of the antisera (Fig. 5B). Surprisingly, at 60 DPA, when PODs activities were highest in control plants, no concomitant level of the dimer was observed. In contrast, in the stressed plants, which showed only a half and a fourth of the control PODs activities; the dimer's contents were higher than that of the control. It is very important to note that our data has considered both, CAPs production and degradation in the most pungent variety of peppers, the habanero pepper in contrast to previous reports, which focused mainly on PODs role in the production of CAPs in stressed *C. annuum* plants (Sung et al., 2005).

Materials and Methods

Biological material

Flowers from habanero pepper plants cultivated in the greenhouse were labelled to record the exact day of anthesis. Twenty plants were used for each replicate and each experiment was repeated thrice. Fruits from plants under the different water deficit treatments were collected 25, 45 and 60 days post-anthesis (DPA).

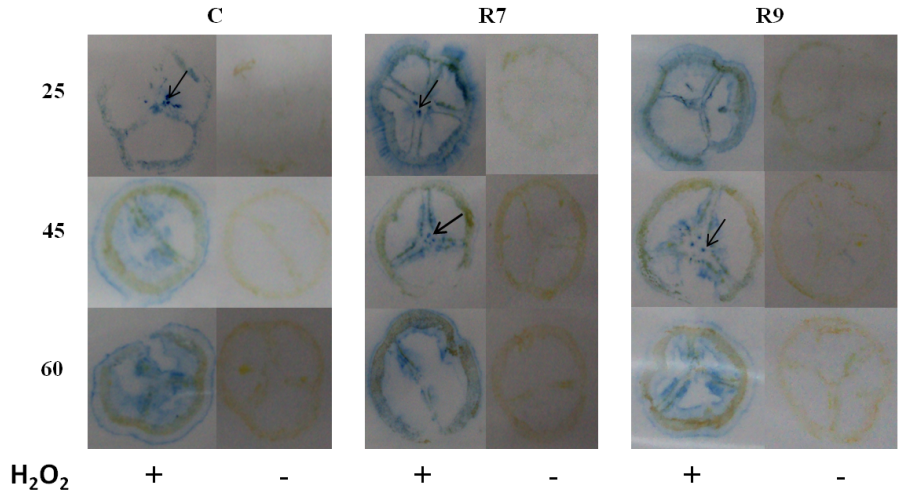

Fig. 3. Histochemical localization of PODs in transversally-cut slices of fruits from plants under different irrigation regimes (C: daily, R7: every 7 days and R9: every 9 days). Arrows indicate PODs localisation in both the placental and outermost epidermal cell layers. Photos present one representative result from three different tissue blots. Assay with 0.1 mM H_2O_2 (+) and without H_2O_2 (-).

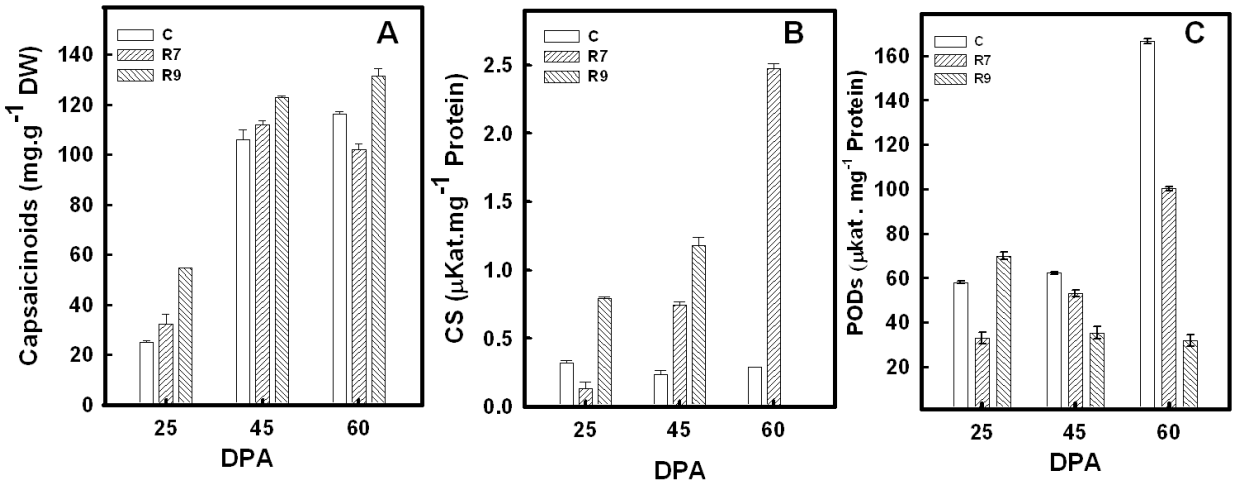

Fig. 4. CAPs content (A), CS (B) and PODs activities (C) in placental tissue of habanero pepper fruits. The plants were subjected to different water regimens and measurements were performed in fruits collected 25, 45 and 60 DPA. Data are the mean of three replicates ± SE.

Placental tissue was excised from the fruits, and 1 g samples were stored at -80ºC prior to analyses. The environmental conditions in the greenhouse were: relative humidity, 89%; mean temperature, 32°C and light intensity, 700 µmoles m^{-1} seg^{-1}.

Water stress treatments

Four-week-old habanero pepper plants were individually transferred from pots to black bags containing 6 kg of a mixture of soil and peat moss (2:1, v/v). Transplanted plants were maintained in a greenhouse under controlled irrigation (1 L water/day). Water stress treatments were initiated when anthesis occurred (0 DPA; approximately 25 days after transplanting). For each water deficit treatment, 20 plants were used. Water stress was induced by withholding irrigation for 7 (R7) or 9 (R9) days. For re-watering experiments, 1 L water was used in each case. Fructification index was estimated by counting the number of flowers and fruits after 150 days following transplantation.

Extraction and quantitation of CAPs

A modified protocol of Collins et al. (1995) was used. Briefly, lyophilised samples (0.1 g) of placental tissue were added to 10 mL acetonitrile and placed in an 80ºC water bath for 4 h. Supernatants were collected, filtered through a Millex-HV PVDF filter (0.45 µm) and analysed by HPLC via a diode array detector and a Phenomenex ODS2 column (5 µm), 250 mm X 4.6 mm i.d, (Cheshire, England). The mobile phase was a gradient of (A) 1 mM trifluoroacetic acid and (B) acetonitrile (Martínez-Juárez et al., 2004).

Histochemical localisation of PODs

Histochemical localisation of PODs in fruits was performed by tissue printing samples onto nitrocellulose membranes (Bernal et al., 1994b). Controls stained in the absence of H_2O_2 were also included. Membranes were rinsed with distilled water, air-dried and photographed.

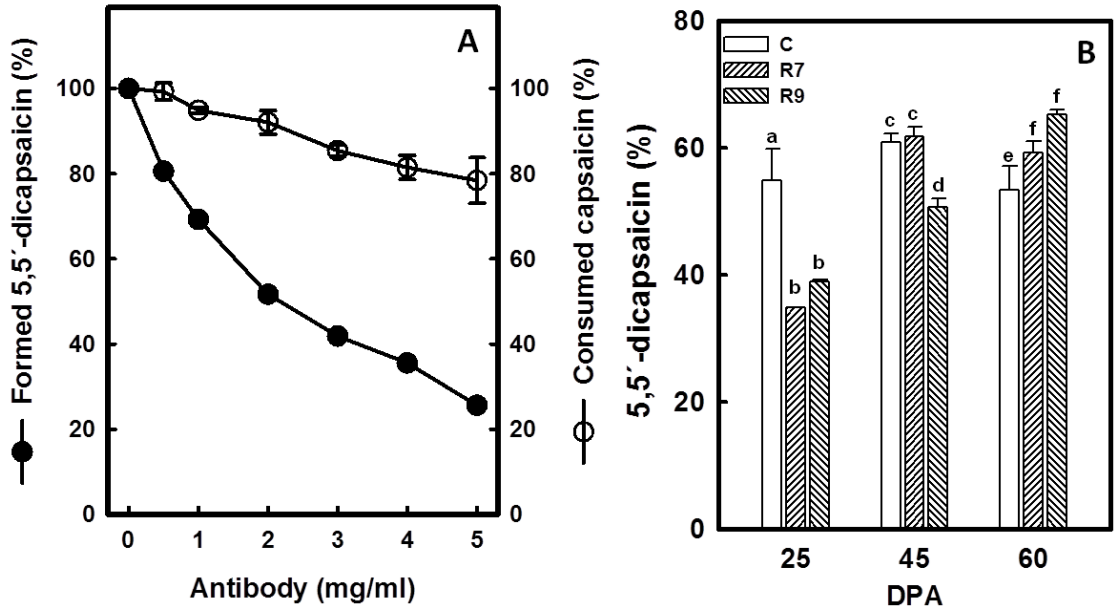

Fig 5. Immunoinhibition of PODs activities in placental tissue from habanero pepper pods. (A) Immunotitration of PODs activities. Different concentrations of anti-horseradish PODs antibodies were used to inhibit capsaicin oxidation by PODs. Activity was monitored by measuring consumed capsaicin or formation of the 5,5'-dicapsaicin dimer, using extracts from placentas of control 60-DPA plants. Data are expressed as percentages of control (100% = 75.16 +/- 3.32% or 0.19 mM of consumed capsaicin, or 4635.3 +/- 157.46 mAU*S peak area of the dimer); (B) Immuno-inhibition of PODs in placental extracts from control and water stressed plants at 25, 45 and 60 DPA. PODs activity was expressed as formation of the dimer, in the absence or presence of the antibodies (2 mg/mL). Data are presented as percentage of the treatments with respect to the control. Values are the mean of three replicates and vertical bars indicate SE. Different letters represent significant differences ($P \leq 0.05$).

Extraction and quantitation of total soluble protein

Total soluble protein was extracted from 1 g of freshly ground placental tissue as previously reported (Zamudio-Moreno, 2007). Supernatants were recovered after centrifugation at 8,000 rpm for 20 min and concentrated in an Amicon Ultra-0.5 tube. Total protein content was determined by Bradford's (1976) method, using bovine serum albumin (BSA) as a standard. Total protein extracts were subsequently used for enzymatic assays.

CS assay

Capsaicin quantitation was performed as stated above (Govindaswam and Ravishankar, 2003). The CS activity was expressed as μkat of formed capsaicin mg^{-1} protein. Formed capsaicin was calculated using the absorption coefficient of capsaicin at 230 nm (Martínez-Juárez et al., 2004)

PODs assay

A modification of the method reported by Martínez-Juárez et al. (2004) was used, as follows: The reaction mixture contained 250 μM capsaicin, 150 μM H_2O_2, 10 μg protein, and 50 mM Tris-HCl, pH 7.0 in a final volume of 1 mL. The reaction mixture was incubated for 2 h at 30ºC and stopped by the addition of 1 mL ethyl acetate. Products' extraction was repeated twice using 1 mL ethyl acetate. Extracts were pooled and evaporated under a stream of oxygen-free N_2. The residue was resuspended in 500 μL ethanol and filtered through a Millex-HV PVDF filter (0.45 μm). Reactions' products were analyzed by HPLC. PODs activity was expressed as μkat of consumed capsaicin mg^{-1} protein. Consumed capsaicin was calculated using the same extinction coefficient used above.

Immunoinhibition of capsaicin oxidation

Specific anti-horseradish PODs antibodies were obtained from Sigma (P7899, St Louis, Mo.) with a specificity of 1:100,000/1:200,000. In order to standardized the inhibition assay of PODs, various antibodies' concentrations (0.5, 1, 2, 3,4 and 5 mg/mL) were added to a reaction mixture that contained 250 μM capsaicin, 150 μM H_2O_2, 60 μg protein extract from control plants (60 DPA), 100 mM citrate, pH 5.0 in a final volume of 1 mL. Reactions were incubated for 100 minutes at 50ºC, and then stopped by the addition of 1 mL ethyl acetate, which served to extract the reactions' products. Extraction was performed twice. Extracts were pooled and evaporated with a stream of oxygen-free N_2. Residue was resuspended in 500 μL ethanol HPLC-grade and filtered through Millex-HV PVDF filter (0.45 μm). Reactions' products were analyzed by HPLC. To evaluate PODs contribution to capsaicin degradation in pods from plants under water stress, activity was determined in the absence or presence of 2 mg ml^{-1} of the anti-PODs serum and the total protein extracts from control or plants under each water stress treatment, respectively, following the above mentioned conditions. PODs activity was expressed in terms of formed 5,5'-dicapsaicin (the product of capsaicin oxidation) or consumed capsaicin.

Data analysis

All data were subjected to analysis of one-way variance (ANOVA) (Systat Software Inc; 1735 Technology Drive Suite 430, San Jose, CA) and mean comparisons were made

using Tukey's multiple range test at 5% level of probability. Data are the mean of three replicates.

Conclusions

Water deficit affects flowering in habanero pepper plants and modifies CAPs content in fruits. Analyses of CAPs content and CS and PODs activities, both present in placental tissue, in fruits from plants under different water stress treatments led us to conclude that PODs are not the sole pathway in the degradation process of these compounds.

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