Capsaicinoids Are Absent in Habanero Pepper Vegetative Organs (*Capsicum chinense* Jacq.)

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Abstract. Capsaicinoids, the chemical compounds that confer the pungency trait to peppers, are accumulated at different levels in all species of the genus *Capsicum*. There is much evidence suggesting that the synthesis of capsaicinoids occurs in the placenta interocular septum of pepper fruits; however, the exact localization of the capsaicinoids biosynthesis accumulation pathways is still under debate. Thus, the aim of the present work was to evaluate whether pepper plants synthesize or accumulate capsaicinoids in vegetative organs as an indirect way to elucidate the systemic regulation of the capsaicinoid biosynthesis. For that purpose, we studied habanero pepper grown in the Yucatan Peninsula, which is among the hottest pepper worldwide. Our results, obtained by chromatographic and enzymatic measurements, provide solid evidence that habanero pepper plants do not accumulate capsaicinoids in the vegetative organs analyzed, even under water stress conditions. Thus, it is probable that the accumulation of capsaicinoids is restricted to reproductive organs.

Phenolic compounds are present in all plant tissues and are among the most abundant secondary metabolites in fruits. Levels of these compounds vary widely during growth and maturation, and they contribute to the sensory quality of the fruits such as color, astringency, bitterness, and flavor (Estrada et al., 2002).

Capsaicinoids are a group of phenolic compounds commonly present in the genus *Capsicum*, of which capsaicin is the most abundant (Bennett and Kirby, 1968). Many studies have reported on the accumulation of capsaicinoids in *Capsicum* fruits with regard to the fruit age, size, and stage of development and under water or nutrient stresses. The capsaicinoids consistently begin to accumulate in the early stages of fruit development and they reach a maximum rate as the

fruit approaches the end of the growth phase. This accumulation continues to increase after the fruit reaches a maximum value (Hall et al., 1987). The level of capsaicinoids varies according to the different pepper cultivars examined (Contreras-Padilla and Yahia, 1998; Estrada et al., 2000; Salgado-Garciglia and Ochoa-Alejo, 1990).

Although capsaicinoids had been found in vegetative organs (Estrada et al., 2002; Ishikawa et al., 1998), it is generally accepted that capsaicinoids are produced solely in pepper fruits, although the localization of the biosynthesis and accumulation pathways within the fruits has been debated (Stewart et al., 2007). Within the pepper fruit, capsaicinoids chiefly accumulate along the epidermal cells of the interocular septum, which defines the fruit locules and is derived from the tissue connecting the placenta to the pericarp (Judd et al., 1999).

The content of capsaicinoids correlates with pungency levels and is determined by two factors: the genetics of the plant and how the plant interacts with the environment (Iwai et al., 1979; Zewdie and Bosland, 2000). Jurenitsch et al. (1979) found considerable differences in total capsaicinoids within cultivars grown in greenhouses, laboratory field studies, and regular plantations, highlighting

the effect of environmental conditions on capsaicinoid content.

In other work, we found that the content of capsaicin and dihydrocapsaicin in mature fruits increased when the plants were stressed by keeping them 7 and 9 d without watering.

Some reports in the literature describe the detection of capsaicinoids in vegetative organs (stem and leaves) of *C. annuum* cv. Padron (Estrada et al., 2002; Ishikawa et al., 1998). The ratio of individual capsaicinoids was different from that in fruits; dihydrocapsaicin was more abundant. Capsaicinoids were not detected in the stems and leaves of floral bud-derived plants, suggesting that they did originate from the fruit (Estrada et al., 2002).

In the state of Yucatán, where the best habanero peppers are produced, the farmers that work with habanero seedlings and plants report that their hands feel hot. From this, one may conclude that leaves have capsaicinoids.

However, although there are reports of capsaicinoids in vegetative organs and that the water stress increases capsaicinoid content, in this work, we analyze the content of capsaicin on vegetative organs from *Capsicum chinense* Jacq. by using highly sensitive methods (high-performance liquid chromatography, thin-layer chromatography, and capsaicin synthase activity) under water stress conditions.

Materials and Methods

Plant material

Plants of habanero pepper, cv. Orange, 120 d after transplantation (DAT), were cultured in plastic bags containing 6 kg of a mixture of red soil (Luvisol)–peatmoss (2:1 v/v) and placed in a greenhouse. From these plants, we preferentially collected leaves and stems located at the apex and close to the fruits. These plants were also subjected to water stress with watering every seventh and ninth day, whereas plants with daily watering were observed as controls. The water potentials were as follows: daily watering (−0.4 MPa), watering every seventh day (−2.09 MPa), and watering every ninth day (−3.13 MPa). The collected leaves and stems were placed in aluminum foil and dried in an oven at 60 °C for 4 d. Afterward, the dried leaves were crushed with a mortar and pestle and the dried stems were crushed with a Willey mill (Model 3383-L10; Thomas Scientific, Swedesboro, NJ).

Labeling of fruits

Flowers were counted daily and marked to register the date of the anthesis, and fruits were harvested 25 and 45 d later. The harvested fruits were measured for morphological parameters such as the size and fresh weight; the placentas were later extracted and stored in glass flasks covered with a porous cloth and frozen until lyophilization.

Determination of capsaicinoids in leaves and stems

Capsaicinoids were extracted using the method proposed by Collins et al. (1995) and modified by Estrada et al. (2002). For

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the quantification of capsaicinoids, we used an Agilent Technologies 1200 Series high-performance liquid chromatography (HPLC) (Agilent Technologies, Inc., Santa Clara, CA) with a quaternary pump, diode-array detector, and an Agilent Eclipse XDB-C₁₈ reversed-phase chromatographic column (150 mm × 4.6 mm; particle size 5 µm). The operating conditions were as follows: flow rate of 1 mL·min⁻¹; running time 10 min; ultraviolet-Vis detection 280 to 338 nm; and an isocratic mobile phase with 30% solvent A (10% methanol) and 70% solvent B (100% methanol). The identity of capsaicinoids (capsaicin and dihydrocapsaicin) was determined by comparing the retention times of the peaks of the samples with those of the standards. Detection limit of the HPLC was 5 mg·L⁻¹. Ten plants were used to determine capsaicinoid content.

Purification of capsaicinoids by chromatographic column

To extract the capsaicinoids, the method proposed by Collins et al. (1995) was modified as follows. The extraction was performed with 10 g of dried leaves in 50 mL of acetonitrile (HPLC-grade) in a water bath at 80 °C for 4 h, and then the sample without the sediment was collected and centrifuged at 4420 × *g* for 20 min. The supernatant was reduced to 6 to 8 mL in a rotary evaporator and filtered through polyvinylidene difluoride (PVDF) filters (Millex-HV, 0.45 µm; Millipore Corporation, Billerica, MA) in a 10-mL syringe. Subsequently, the sample was divided into two parts (L1 and L2), adding to one of them (L1) a certain amount of capsaicin and dihydrocapsaicin standards to establish what fraction would be present.

Purification was carried out in a chromatographic column with an external diameter of 1.4 cm, 45 cm in height, and filled with 16.8 g of Sephadex LH-20 (GE Healthcare Life Sciences; UK Limited Little Chalfont, Buckinghamshire, UK). Sample L1 was eluted with 100% methanol (analytical grade) and nine fractions (L1a to L1i) of 10 mL were collected. The purity of each fraction was checked using thin-layer chromatography (TLC) (silica gel 60 F₂₅₄ plates, 0.2 mm thick, 5 × 5 cm) with a solvent system of cyclohexane–chloroform–acetic acid (7:2:1). Spots were visualized first under ultraviolet light (254 and 365 nm, respectively) and then after spraying a solution prepared with 4% (w/v) of phosphomolybdic acid in 5% sulfuric acid containing traces of ceric sulphate redox indicator.

A sample (L2) was purified without any of the standard additions, in the same manner as described previously, but only five fractions (L2a to L2e) were collected throughout this process.

Fractions of L1 and L2 were analyzed by HPLC as described previously under fluorescence and diode-array detectors. Two methods were used: one as published by Collins et al. (1995) with all the conditions described previously but using in this work a fluorescence detector, and the second method used was that described by Estrada

et al. (2002). To follow this method, we used an Agilent Technologies 1200 Series HPLC with a quaternary pump, diode-array detector, and a reversed-phase Agilent Eclipse XDB-C₁₈ column (150 mm × 4.6 mm; particle size 5 µm). Capsaicinoids were detected under the following operating conditions: column temperature 25 °C, flow of 1 mL·min⁻¹; run time of 12 min, wavelength between 280 and 360 nm, and an isocratic mobile phase with 50% of Solvent A (100% acetonitrile, HPLC-grade) and 50% of Solvent B (10% acetonitrile, HPLC-grade). The injection volume was 50 µL.

Determination of the enzyme activity of capsaicin synthase in leaves

Extraction and quantification of total proteins. The enzyme activity was determined by the method of Govindaswami and Ravishanker (2003). The extract was centrifuged at 23,708 × *g* and the collected supernatant was used as a total protein extract. The quantification of the total protein was performed using the method of Bradford (1976).

Enzymatic activity of capsaicin synthase. The presence of capsaicin synthase was determined with the method reported by Govindaswami and Ravishanker (2003). The reaction mixture used was: phosphate buffer 0.5 mM, MgCl₂ 1 µM, ATP 1 µM, vanillylamine hydrochloride 5 µM, and 8-methyl-nonenoic acid 5 µM in a final volume of 500 µL. A volume of 200 µL from the reaction mixture and 277 µg of total protein in the case of leaves and 62 µg of total protein in the case of placenta were taken in a final volume of 500 µL. The reaction was carried out in a water bath at 37 °C for 2 h and was stopped with cold 98% ethanol. Leaves of plants with 120 DAT and placentas of fruits of 25 and 45 d after flowering were used (N. Ruiz-Lau, unpublished data).

The extraction of capsaicinoids was carried out with 2 mL of ethyl acetate and the sample was then dried. The sample was redissolved in 500 µL of absolute ethanol (HPLC-grade) and filtered through PVDF filters (Millex-HV, 0.45 µm) in a 3-mL syringe for quantification. Quantification of the product formed (capsaicin) was conducted in an Agilent Technologies 1200 Series HPLC with a quaternary pump, diode-array detector, and a reversed-phase Agilent Eclipse XDB-C₁₈ column (150 mm × 4.6 mm, particle size 5 µm). Operating conditions previously established were: flow of 1 mL·min⁻¹ with a running time of 10 min; wavelengths between 280 and 338 nm and an isocratic mobile phase, with 30% of Solvent A (10% methanol, HPLC-grade) and 70% of Solvent B (100% methanol, HPLC-grade). The injection volume for all samples was 20 µL. Four determinations per treatment were run.

Statistical analysis

The data were analyzed with a one-way analysis of variance (Sigma Stat version 3.1). The means of treatments were compared with Tukey's multiple range test (*P* = 0.05).

Results and Discussion

Determination of capsaicinoids in leaves and stems of habanero pepper

The leaves and stems of plants subjected to water stress (with watering every seventh and ninth day versus plants with daily watering as controls) were used for analysis. The plants were subject to severe water stress according to the water potential of the soil (Hsiao, 1973).

The extraction of capsaicinoids from the fruits of the genus *Capsicum* was first made through a protocol established in the literature by Collins et al. (1995), which allows a simple, efficient, and low-cost qualitative and quantitative analysis within a short time, leading to an optimal extraction of capsaicinoids, whereas Estrada et al. (2002) reported an extraction technique mainly for vegetative organs such as stems, leaves, and fruits (Contreras-Padilla and Yahia, 1998; Estrada et al., 1997, 1999, 2000). In this work, the identification and quantification of the capsaicinoids was conducted by HPLC under the conditions mentioned previously.

HPLC analysis did not reveal any peak near the retention times of the standards (capsaicin *R_T* = 4.2 ± 0.2 min and dihydrocapsaicin *R_T* = 5.8 ± 0.2 min). Similar analyses were run through both HPLC methods with stem extract samples obtaining similar results.

Therefore, using this method of extraction and quantification, it was not possible to detect the presence of capsaicinoids in leaves and stems from habanero pepper plants.

There are many authors that had found capsaicin in other plant tissues, e.g., in cellular suspensions (Martínez-Juarez et al., 2004; Sudha and Ravishanker, 2002, 2003), but in our laboratory, under our conditions, we never had found capsaicinoids in non-inductive conditions in cell suspensions of habanero pepper (data not shown).

Until today, everybody reports that the vegetative organs do not have capsaicinoids, but there is only a report that corroborated this information (Stewart et al., 2007); therefore, today, this is a question that needs to be answered. As we mentioned, there are few reports about the content of capsaicinoids in vegetative organs (Estrada et al., 2002; Ishikawa et al., 1998).

Purification by a chromatographic method. The use of chromatographic techniques allowed us to separate a mixture of pigments (chlorophylls) that were present in the extract, which may have interfered with the determination of capsaicinoids by HPLC. In this process, the components of the mixture are absorbed by the stationary phase at different ratios so that the process of adsorption-desorption makes some components elute more rapidly than others.

A sample of the leaf extract (L1) with known amounts of capsaicin and dihydrocapsaicin standards was prepared and purified through a chromatographic column to establish what fractions these two compounds would be eluted corroborating the standards'

flux ratio (R_f s). In this way, one could determine with greater certainty the R_f s and the presence of capsaicinoids in stem and leaf extracts, which otherwise would be difficult because of the complexity of the sample.

First, the sample containing the standards (L1) was purified and nine fractions (L1a to L1i) were collected. These fractions were then analyzed by TLC and, as expected, spots resulting from capsaicin and dihydrocapsaicin were observed in fractions L1b and L1c. Afterward, a sample without standards (L2) was separated by HPLC and five fractions (L2a to L2e) were collected. When these fractions were analyzed by TLC, no spots pertaining to capsaicin and dihydrocapsaicin were visualized. These results show that, under these conditions, capsaicin and dihydrocapsaicin are not present in leaves of habanero pepper plants, in accordance with literature reports that capsaicinoid concentration found in vegetative tissues is very low (Ishikawa et al., 1998).

To support these results, fractions obtained from the extract L1 were analyzed by two HPLC methods, each with different detectors (fluorescence and diode-array detectors). As expected, fractions L1b and L1c, containing the standards, showed the presence of both capsaicinoids with an $R_T = 4.2$ for capsaicin and $R_T = 6.2$ for dihydrocapsaicin analyzed under a fluorescence detector and $R_T = 3.8$ and 5.0, respectively, analyzed under a diode-array detector.

Fractions L2b and L2c obtained in the second purification were injected to the HPLC following Collins' method. In the chromatogram, fraction L2b appeared as a slight peak with an $R_T = 4.4$, very close to the retention time of capsaicin. No peak was observed at $R_T = 6.2$, corresponding to dihydrocapsaicin.

In the chromatogram obtained for L2c, two peaks were observed, but only one of them was near the retention time of one of the standards. The first peak, despite appearing in the area marked for capsaicin, had an $R_T = 4.8$, well above the established retention time for this standard, and the second one appeared at an R_T near 5.6 compared with that of dihydrocapsaicin ($R_T = 6.2$).

By analyzing these same fractions by HPLC by the Estrada's method (HPLC with a diode-array detector), in the chromatogram of L2b, a peak was observed at $R_T = 4.3$, close to that of the standard of capsaicin ($R_T = 3.8$). No peak was detected near the retention time of dihydrocapsaicin ($R_T = 5.0$). In chromatograms run for fraction L2c, no peak was observed near the retention times of any of the standards.

When the content of capsaicinoids was analyzed in a variety of *Capsicum annuum* L. cv. Jalapeño, Ishikawa et al. (1998) found small amounts of total capsaicinoids in the stem and flower stalk, but these were not detected in leaves. The content of capsaicinoids in vegetative organs represents only 0.7% of that found in the placenta.

Estrada et al. (2002) analyzed the content of capsaicinoids in vegetative organs of *Capsicum annuum* L. cv. Padron in different

segments of the plant and determined that the presence of capsaicinoids found in vegetative organs depends on the presence of fruits in the plant. They divided the plant into two groups: in the first set, flower buds were removed to avoid the formation of the fruit, whereas the second group was left intact to bear ripening fruit. They observed a clear gradient of capsaicinoid accumulation with the leaves and stems located in the apical areas of the plant having the highest concentrations compared with those located in the middle and base of the plant. They also noted that the concentration of capsaicinoids was always higher in leaves than in stems. These authors found significant differences in analyzing the relative ratios of the two major capsaicinoids, capsaicin and dihydrocapsaicin. The percentage of dihydrocapsaicin was the highest in most samples, whereas in the fruit, capsaicin dominates. The total concentration of capsaicinoids reported in the apical segment of leaves was 40 $\text{mg}\cdot\text{g}^{-1}$ dry weight (Estrada et al., 2000). Therefore, they concluded that in the initial states of the fruit, there is a pool of phenolic substances such as lignin and chlorogenic acid, which have protective functions. As it develops, the fruit synthesizes capsaicinoids that accumulate until full maturation. When the fruit matures, this tendency is reversed and acts as a source of capsaicinoids that are allocated toward the vegetative organs. Thus, in leaves of *Capsicum annuum* cv. Padron, capsaicin can be found only when the plant produces fruits. It is therefore necessary that the plant be in a state of fruiting to accumulate these compounds in the vegetative organs.

To confirm the location of capsaicinoids in the interocular septum, in a recent study by Stewart et al. (2007), capsaicinoids from different organs (leaves, roots, pericarp, interocular septum of the placenta, and seeds) of the habanero pepper plant were analyzed by HPLC and they do not report the method used nor the detection limit. As a result, they only observed these compounds

in the interocular septum ($52\text{ g}\cdot\text{kg}^{-1}$), and the compounds were absent in other tissues of the plant. Our results coincide with these results, but we used both TLC and HPLC methods. They also reported traces of capsaicinoids in the pericarp, possibly as a result of an imperfect separation between the interocular septum and the pericarp.

Until this study, little was known about the presence of capsaicinoids in vegetative organs of *Capsicum annuum* and even less in *Capsicum chinense* plants. Similar results are expected because they belong to the same genus, but apparently the plants have very different physiologies because capsaicinoids are not detectable in vegetative organs as noted by our results.

In conclusion, we report that it was not possible to detect capsaicinoids through the traditional chromatographic methods through analyses of habanero pepper leaves and stems in either stressed or control plants.

Enzymatic activity of capsaicin synthase in leaves. In this study, to determine the activity of capsaicin synthase, we used leaves of habanero pepper plants exposed to water stress conditions (watering every seventh and ninth day), and for the quantification of total protein, we calculated the volume necessary (μL) to obtain 222 μg of protein. Activity of the enzyme was detected in the placenta as a positive control.

Under these conditions, it was not possible to detect the specific activity of capsaicin synthase in leaves of habanero pepper plants as shown in Figure 1. However, it has been reported that activity of this enzyme in non-specialized tissues, such as cells in suspension, was detected and it increases in presence of putrescine (0.1 mM) (Govindaswami and Ravishankar, 2003). In the literature, there is no report on the activity of this enzyme in leaves of *Capsicum* plants. In a study, capsaicin synthase was specifically immunolocalized in the placenta of the fruit (Narasimha et al., 2006). Therefore, we suggest that in the event of any presence of capsaicinoids in

Fig. 1. Specific activity of capsaicin synthase. Leaves of plants with 120 d after transplantation subjected to different regimens of watering and placentas of fruit with 25 (Tukey $P \leq 0.001$; $n = 3$) and 45 d after flowering (Tukey $P \leq 0.021$; $n = 3$) as positive controls were used. Symbols were (1) for the control (daily watering, $\psi_{\text{w soil}} = -0.4\text{ MPa}$); (7) and (9) for watering every seventh ($\psi_{\text{w soil}} = -2.09\text{ MPa}$) day and every ninth day ($\psi_{\text{w soil}} = -3.13\text{ MPa}$), respectively; whereas (ND) means not detected. The activity was carried out with 277 mg of total protein (leaves) and 62 mg of total protein (fruits). Bars = $\pm$ SE. Different letters (a, b) mean different media.

leaves or stems of these plants, it would be likely as a result of translocation of the compounds from the fruit and not as a result of synthesis in situ.

Under water stress conditions, the specific activity of capsaicin synthase in leaves of habanero pepper plants was not detected either as shown in Figure 1, indicating that in leaves, there is no activity of this enzyme.

In conclusion, we demonstrate that despite the pungency of this pepper, and under stress conditions, no capsaicinoids were detected in its vegetative organs.

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