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Induction of capsaicinoid synthesis in *Capsicum chinense* cell cultures by salicylic acid or methyl jasmonate

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Abstract

Suspension cultures of Habanero pepper (*Capsicum chinense* Jacq.) were exposed to salicylic acid or methyl jasmonate to change secondary metabolism. Both treatments led to the accumulation of capsaicinoids and their late biosynthetic intermediate, vanillin. Both elicitors had a positive effect on the activities of phenylalanine ammonia lyase and coumarate *O*-methyltransferase, but none of them represented the main limiting step for capsaicinoid accumulation since vanillin contents were two orders of magnitude higher than those of capsaicinoids.

Additional key words: Habanero pepper, secondary metabolism, vanillin.

Introduction

The typical burning sensation caused by chili peppers is due to the occurrence of capsaicinoids, a group of acid aromatic amides derived from phenylalanine and leucine or valine (Fig. 1). Capsaicinoids are exclusively synthesized in pepper fruits, specifically in the placenta and the interlocular septum, where they accumulate in vesicles (Stewart *et al*. 2007). As is the case for many secondary metabolites, capsaicinoids accumulation and the activity of their biosynthetic enzymes are sensitive to environmental conditions (Johnson and Decoteau 1996, Harvell and Bosland 1997, Sung *et al*. 2005).

Cell cultures of *C. annuum* and *C. frutescens* are capable to produce capsaicinoids, albeit in lower amounts than fruits (Lindsey 1986). In cell cultures of *C. annuum* (cv. Tampiqueño), this lowered content has been related to the low activity of some enzymes involved in the formation of the phenolic moiety of capsaicin, such as phenylalanine ammonia lyase (PAL) and coumaric acid *O*-methyltransferase (COMT) (Ochoa-Alejo and Gómez-Peralta 1993). Attempts to promote capsaicinoids synthesis in *in vitro* cultures include the use of biosynthetic precursors and intermediates (Sudhakar *et al*. 1996), modification of the media composition (Lindsey 1986), cell immobilization (Sudhakar *et al*. 1990), and the application of stress conditions and elicitors of secondary metabolism (Sudhakar *et al*. 1990, 1991, Sudha and Ravishankar 2003).

We have developed a Habanero pepper (*C. chinense* Jacq.) cell line, which is considered the hottest of all peppers, and followed capsaicinoid biosynthesis after application of methyl jasmonate and salicylic acid, two well known inducers of plant secondary metabolism (Repka 2001, Xu *et al*. 2008).

Material and methods

Cell suspension cultures were obtained from *Capsicum chinense* Jacq. friable calli generated from hypocotyls of the local landrace Naranja (Santana-Buzzy *et al*. 2005). The suspension has been kept in Murashige and Skoog

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Abbreviations: COMT - coumarate *O*-methyltransferase; DMSO - dimethyl sulfoxide; MeJa - methyl jasmonate; MS - Murashige and Skoog culture medium; PAL - phenylalanine ammonia lyase; TLC - thin layer chromatography; SA - salicylic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid.

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(1962; MS) medium similarly as Sanatombi and Sharma (2008) supplemented with 25 g dm^{-3} myo-inositol, 10 g dm^{-3} thiamine, 0.1 g dm^{-3} cysteine, 30 g dm^{-3} sucrose and 1.0 g dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D). Cultures were maintained at 25 °C in an orbital shaker and under continuous irradiance of 50 μmol m^{-2} s^{-1}, provided by a combination of fluorescent (39 W) and incandescent (60 W) lamps (*Philips de México*, México). They were subcultured every two weeks.

Cells (2 g) were transferred to Erlenmeyer flasks containing 50 cm^3 of media and maintained as described above for 14 d prior to received 500 μM of either methyl jasmonate (MeJa) or salicylic acid (SA) (both from *Sigma-Aldrich Chemical Co*., St. Louis MO, USA). Controls were treated with the corresponding solvents (DMSO for MeJa and water for SA). Cell cultures were exposed to the inducers for 0, 12, 24, 36, 48 and 72 h, harvested by vacuum filtration, immediately frozen with liquid nitrogen and stored at -80 °C until analysis. Each treatment was applied in triplicate.

Capsaicinoids and biosynthetic intermediaries were acetone extracted from freeze-dried cells, and quantified by *in situ* TLC densitometry, using a *Shimadzu CS-930* dual wavelength chromatoscanner, equipped with a *DR 2* data collector (*Shimadzu*, Kyoto, Japan), as described by Monforte-González *et al*. (2007). Phenylalanine ammonia lyase (PAL) and coumarate *O*-methyltransferase (COMT) were assayed in crude desalted protein extracts prepared from frozen cells. Extraction buffer (50 mM Tris HCl, pH 8.8) with 15 mM β-mercaptoethanol was added. Extracts were desalted in *PD-10* columns (*Amersham Pharmacia*, Buckinghamshire, UK) and then eluted with the extraction buffer. Enzyme assays were performed as described by Ochoa-Alejo and Gómez-Peralta (1993).

Results

C. chinense cultures were exposed to 5, 50, and 500 μM of either MeJa or SA for 24 h, and their effect on capsaicinoids and vanillin contents were analyzed (Fig. 2). In cultures exposed to SA no effect on vanillin accumulation was detected (Fig. 2*A*), while in those exposed to MeJa, near to five-fold increase was noticed at 500 μM concentration (Fig. 2*A*). In contrast to vanillin contents, SA treatment provoked an increase in capsaicinoid accumulation (Fig. 2*B*). Such response increased with the dose, reaching its maximum in cultures exposed to 500 μM SA (Fig. 2*B*). Interestingly, content of capsaicinoids did not increase in cultures exposure to MeJa (Fig. 2*B*). No other intermediaries could be detected (data not shown). Based on these results, further experiments were performed using a concentration of 500 μM for both inducers and time courses of exposure of the cell cultures were performed.

In cultures exposed to SA, vanillin accumulation was not affected in comparison to the control during the first 36 h (Fig. 3*A*). However, from this time onwards, it increased steadily, reaching maximum after 72 h of exposure (Fig. 3*A*). Content of capsaicinoids increased within the first 24 h, as previously determined (Fig. 2*B*), reached maximum after 36 h, and it slightly decreased for the rest of the experiment (Fig. 3*B*). In control cultures, no capsaicinoid accumulation could be detected (Fig. 3*B*). In cell cultures to which MeJa was added, vanillin accumulation occurred continuously during the first 36 h, but decreased afterwards (Fig. 3*A*). No capsaicinoids

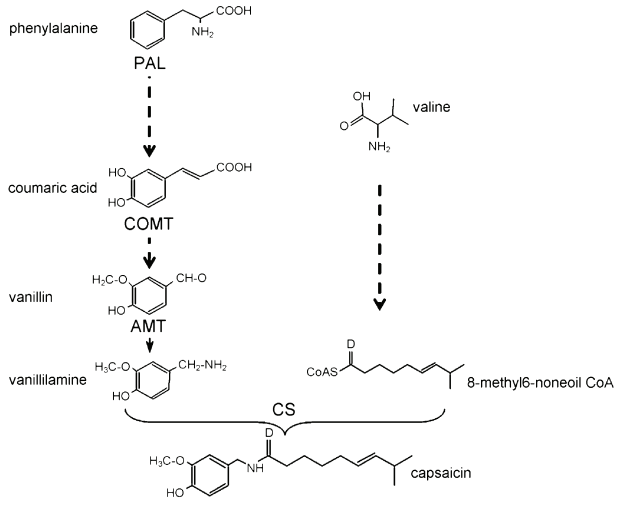

Fig. 1. A condensed view of the biosynthetic route of capsaicin. AMT - aminotransferase, COMT - coumarate *O*-methyl-transferase, CS - capsaicinoid synthase, PAL - phenylalanine-ammonia lyase.

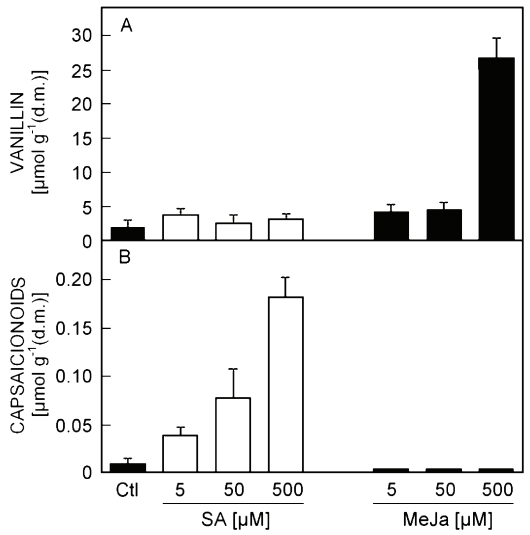

Fig. 2. Effect of different doses of salicylic acid (SA) and methyl jasmonate (MeJa) on vanillin (*A*) and capsaicinoids (*B*) accumulation in *C. chinense* cell cultures after 24 h. Average of triplicates with standard deviation.

were detected in these cultures during the first 24 h, but a transient peak of accumulation was observed at 48 h of exposure (Fig. 3*B*). Maximum amount of capsaicinoids was similar using both inducers (Fig. 3*B*). Although both MeJa and SA promoted capsaicinoid accumulation in *C. chinense* cell cultures, a longer exposure was required when MeJa was employed (Fig. 3*B*). These effects were the opposite of those recorded for vanillin accumulation (Fig. 3*A*).

To get a deeper understanding of capsaicinoid metabolism in this cell line, PAL and COMT activities were analyzed. PAL activity remained unchanged not only in cultures exposed to both inducers, but also in control cells during the first 24 h (Fig. 3*C*). With longer exposure, PAL activity decreased in the control, while it maintained relatively stable in the SA or MeJa treated cultures (Fig. 3*C*). On the other hand, in control cultures, COMT activity slightly increased during the first 24 h, and decreased afterwards (Fig. 3*D*). In response to both inducers, this activity remained at the same level throughout the experiments, and only at the end of the exposure (72 h) a significant increase was recorded in the cultures treated with SA (Fig. 3*D*).

Discussion

The ability of MeJa and SA to stimulate secondary metabolism has been well documented (Radman *et al*. 2003). Furthermore, both of them modified capsaicin accumulation in *C. frutescens* cell cultures (Sudha and Ravishankar 2003). This work was aimed to establish a methodology for the induction of capsaicinoid biosynthesis in *C. chinenese* cell cultures, which could be employed as a model in further studies on the regulation of capsaicin synthesis. Hence, a suspension culture was obtained from friable calli generated from hypocotyls of a local landrace. Capsaicin content in these cell cultures was around 200 times lower than those in placentas from fruits of the same landrace [0.012 *vs* 0.24 μmol g^{-1}(dm); Monforte-González *et al*. 2007]. However, when compared to cell cultures from *C. annuum* (Salgado-Garciglia and Ochoa-Alejo 1990) and *C. frutescens* (Sudha and Ravishankar 2003), the *C. chinense* cell line produced similar levels of capsaicinoids.

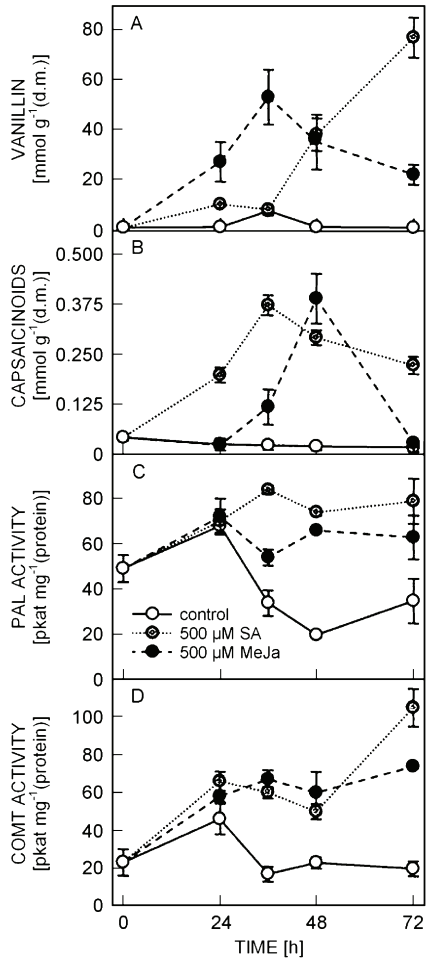

Fig. 3. Accumulation of vanillin (*A*) and capsaicinoids (*B*), and activities of PAL (*C*) and COMT (*D*) in *C. chinense* cell cultures exposed to 500 μM salicylic acid or methyl jasmonate. Average of triplicates with standard deviation.

The application of 500 μM MeJa or SA promoted the accumulation of capsaicinoids and their late precursor vanillin, although differential patterns of response suggest differences in the signal transduction pathways activated by each inducer (Maleck and Dietrich 1999, Repka 2001). SA exposure resulted in a rapid accumulation of capsaicinoids, whereas its effect on vanillin required a longer exposure. This behaviour was opposite to this observed when cultures were exposed to MeJa, where early response for vainillin accumulation was recorded and the delayed ones for capsaicinoids.

Interestingly, MeJa promoted the biotransformation of caffeic acid into vainillin in root cultures of *C. frutescens* (Suresh and Ravinshankar 2005). SA effects on capsaicinoid contents have been reported to occur in *C. frutescens* cultures although after a longer exposure (Sudha and Ravishankar 2003). No effect could be determined when MeJa was employed in these cultures. During the first 36 h, SA treatment did not affect vanillin accumulation, although it promoted capsaicinoid accumulation. This may have been caused by a rapid transformation of vanillin into capsaicinoids, impeding the accumulation of the precursor. Enhancement of vanillin accumulation after a longer exposure may be explained in the terms of a demand for this intermediary to keep capsaicinoid biosynthesis in the SA treated cultures. Nevertheless, vanillin contents in our cell line were two orders of magnitude higher than those of capsaicin [*i.e.* 80 *vs* 0.5 μmol g^{-1} (d.m)], suggesting that this intermediary did not represent limitation.

PAL and COMT are involved in the formation of the phenolic moiety of capsacinoids (Fig. 1), and represent limiting steps in the formation of capsaicin in cell cultures (Ochoa-Alejo and Gómez-Peralta 1993). Given its position in the biosynthetic route, PAL plays a key role in the channeling of carbon skeletons from primary to secondary metabolism (Fig. 1; Curry *et al*. 1999). Furthermore, *Capsicum* cell lines which overproduce phenylalanine display an increased PAL activity and capsaicine accumulation (Salgado-Garciglia and Ochoa-Alejo 1990, Hall and Yeoman 1991), suggesting that an incresed PAL activity may be required for promoting capsaicin biosynthesis. However, SA or MeJa effects were only related to the maintenance of the initial levels and so our experiments do not support a major role for PAL in the induced capsaicinoid accumulation.

COMT, which forms ferulic acid (Fig. 1), has also been identified as a limiting step for capsaicinoid synthesis in cell cultures (Ochoa-Alejo and Gómez-Peralta 1993). This may be related with the multiple end products in which it may be used (Dixon and Paiva 1995). COMT activity increased in response to both MeJa and SA. Ferulic acid is the immediate precursor of vanillin (Fig. 1), and so increased vanillin content may be a result of increased COMT activity.

Differences in the accumulation between vanillin and capsaicinoids suggest that the limiting steps for capsaicinoid formation are not directly related to vanillin formation. Thus, three possible regulatory points may be proposed: the formation of vanillinamine from vanillin, catalyzed by an aminotransferase (Curry *et al*. 1999), vanillinamine condensation with 8-methyl-6-nonenoic acid, catalyzed by the capsaicinoid synthase (Tetstuya *et al*. 1981), or the synthesis of this fatty acid (Aluru *et al*. 2003). Interestingly, whereas in *C. frutescens* cultures, it has been shown that the latter process has a crucial role (Prasad *et al*. 2006), in *C. annuum* cultures, the formation of the phenolic portion of capsaicin has been identified as the main limiting step (Ochoa-Alejo and Gómez-Peralta 1993).

In conclusion, our results indicate that, as a difference to *C. annuum* where the formation of the phenolic moiety has been found limiting for capsaicine biosynthesis, in *C. chinense* cultures the low contents of capsaicin are not related with this branch of the biosynthetic route.

References

Aluru, M., Mazourek, M., Landry, L., Curry, J., Jahn M., O'Connell, M.: Differential expression of fatty acid synthase genes, *Acl*, *Fat* and *Kas*, in *Capsicum* fruit. - J. exp. Bot. **54**: 1655-1664, 2003.

Curry, J., Aluru, M., Mendoza, M., Nevarez, J., Melendrez M., O'Connell, M.: Transcripts for possible capsaicinoid biosynthetic genes are differentially accumulated in pungent and non-pungent *Capsicum spp*. - Plant Sci. **148**: 47-57, 1999.

Dixon, R., Paiva, N.: Stress-induced phenylpropanoid metabolism. - Plant Cell **7**: 1085-1097, 1995.

Hall, R., Yeoman, M.: The influence of intracellular pools of phenylalanine derivatives upon the synthesis of capsaicin by immobilized cell cultures of the chilli pepper, C*apsicum frutescens*. - Planta **185**: 72-80, 1991.

Harvell, K.P., Bosland, P.W.: The environment produces a significant effect on pugency of chili. - HortScience **32**: 1292-1297, 1997.

Johnson, C.D., Decoteau, D.R.: Nitrogen and potassium fertility affects Jalapeño pepper plant growth, pod yield and pungency. - HortScience **31**: 1119-1123, 1996.

Lindsey, K.: Incorporation of [^{14}C]phenylalanine and [^{14}C]cinnamic acid into capsaicin in cultured cells of *Capsicum frutescens*. - Phytochemistry **25**: 2793-2801, 1986.

Maleck, K., Dietrich, R.: Defense on multiple fronts: how do plants cope with diverse enemies? Trends Plant Sci. **4**: 1360-1385, 1999.

Monforte-González, M., Medina-Lara, F., Gutierrez-Carbajal, G., Vázquez-Flota, F.: Capsaicinoid quantitation by *in situ* densitometry of thin layer chromatography plates. - J. Liq. Chromatog. Rel. Technol. **30**: 1697-1704, 2007.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. **15**: 473-497, 1962.

Ochoa-Alejo, N., Gómez-Peralta, J.: Activity of enzymes involved in capsaicin biosynthesis in callus tissue and fruits of chili pepper (*Capsicum annum* L.). - J. Plant Physiol. **141**: 147-152, 1993.

Prasad, B.C.N., Gururaj, H.B., Kumar, V., Giridhar, P., Parimalan, R., Sharma, A., Ravishankar, G.A.: Influence of 8-methyl-nonenoic acid on capsaicin biosynthesis and *in-vivo* and *in-vitro* cell cultures of *Capsicum* spp. - J. agr. Food Chem. **54**: 1854-1859, 2006.

Radman, R., Saez, T., Bucke, C., Keshavarz, T.: Elicitation of plants and microbial cell systems. - Biotech. appl. Biochem. **37**: 91-102. 2003.

Repka, V.: Elicitor-stimulated induction of defense mechanisms and defense gene activation in grapevine cell suspension cultures. - Biol. Plant. **44**: 555-565, 2001.

Salgado-Garcigilia, R., Ochoa-Alejo, N.: Increased capsaicin content in PFP-resistant cells of chili pepper (*Capsicum annuum* L.). - Plant Cell Rep. **8**: 617-620, 1990.

Sanatombi, K., Sharma G.J.: *In vitro* plant regeneration in six cultivars of *Capsicum* spp. using different explants. - Biol. Plant. **52**: 141-145, 2008.

Santana-Buzzy, N., Canto-Flick, A., Barahona-Pérez, F., Moltalvo-Peniche, M.C., Zapata-Castillo, P.Y., Solís-Ruiz, A., Zaldívar-Collí, A., Gutiérrez-Alonso, O., Miranda-Ham, M.L.: Regeneration of Habanero Pepper (*Capsicum chinense* Jacq.) *via* organogenesis. - HortScience **40**: 1829-1831, 2005.

Stewart, C., Jr., Mazourek, M., Stellari, G.M., O'Connell, M., Jahn, M.: Genetic control of pungency in *C. chinense via* the *Pun1* locus. - J. exp. Bot. **58**: 979-991, 2007.

Sudha, G., Ravishankar, A.: Influence of methyl jasmonate and salicylic acid in the enhancement of capsaiscin production in cell suspension cultures of *Capsicum frutescens* Mill. - Curr. Sci. **85**: 1212-1217, 2003.

Sudhakar, J., Ravishankar, A., Venkataraman, L.: *In vitro*

capsaicin production by immobilized cells and placental tissues of *Capsicum annuum* L. grown in liquid medium. - Plant Sci. **70**: 223-229, 1990.

Sudhakar, J., Ravishankar, A., Venkataraman, L.: Elicitation of capsaicin production in freely suspended cells and immobilized cell cultures of *Capsicum frutescens* Mill. - Food Biotechnol. **5**: 197-205, 1991.

Sudhakar, T., Ravishankar, G., Venkataraman, L.: Biotransformation of ferulic acid and vanillylamine to capsaicin and vanillin in immobilized cell cultures of *Capsicum frutescens*. - Plant Cell Tissue Organ Cult. **44**: 117-121, 1996.

Sung, Y., Chang, Y.Y., Ting, N.L.: Capsaicin biosynthesis in water-stressed hot pepper fruits. - Bot. Bull. Acad. sin. **46**: 35-42, 2005.

Suresh, B., Ravinshankar, G.A.: Methyl jasmonate modulated biotransformation of phenylpropanoids to vanillin related metabolites using *Capsicum frutescens* root cultures. - Plant Physiol. Biochem. **43**: 125-131, 2005.

Tetsuya, S., Teruo, K., Kazuo, I.: Biosynthesis of acyl moieties of capsaicin and its analogues from valine and leucine in *Capsicum* fruit. - Plant Cell Physiol. **22**: 23-32, 1981.

Xu, C.M., Ou, Y., Zhao, B., Whang, X.D., Yuan, X.F., Wang Y.C.: Syringin production by *Saussurea medusa* cell cultures in a novel bioreactor. - Biol. Plant. **52**: 377-380, 2008.