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Capsaicinoid and capsinoid accumulation during fruit development of three chili pepper genotypes (*Capsicum* spp.) carrying *Pun1* and *pAMT* alleles related to pungency

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ABSTRACT

Quantification, using an accurate analytical approach, of capsinoids and capsaicinoids was performed on three chili pepper (*Capsicum* spp.) genotypes: 'Chiltepín', 'Tampiqueño 74', and 'Bhut Jolokia' at various stages of fruit development. The accumulation of capsinoids, in all chili peppers started between 10 to 20 days post-anthesis (dpa), increased and reached the highest capsinoid amount at 40 dpa, and then decreased until 60 dpa. Conversely, capsaicinoids could already be determined at 10 dpa in 'Bhut Jolokia' and their accumulation pattern was different from that of the capsinoids in this genotype. The capsiate/dihydrocapsiate ratio presented a higher variation between genotypes and developmental stages than the capsaicin/dihydrocapsaicin ratio. Capsinoid ratios (4 - 24 %) and *Pun1*/*pAMT* genotyping were determined. These results provide information on the progress of the accumulation of capsinoids in the aforementioned pungent and super-pungent cultivars and could support future breeding studies towards the understanding of the factors affecting their accumulation.

Keywords: *Capsicum* spp; capsaicinoids; capsinoids; fruit development; accumulation pattern; HPLC-ESI-MS(QTOF).

INTRODUCTION

Hot peppers (*Capsicum* spp.) are world widely cultivated and used as food, spice or ingredient [1]. The pungency of chili peppers is one of the main attributes of quality in *Capsicum*, which depends on the accumulation of capsaicinoids (mainly, capsaicin and dihydrocapsaicin). Analogous non-pungent compounds, called capsinoids (mainly, capsiate and dihydrocapsiate) are also present in chili peppers [2,3]. Capsaicinoids and capsinoids exhibit similar health-promoting properties such as being antioxidant, anti-inflammatory, analgesic [4-5], anticancer [5,6], and anti-obesity [5]. In that way, chili pepper fruits are considered an excellent source of health-related metabolites. Both compounds have similar fundamental structures, except for their central linkage: an amide bond is found in capsaicinoids whereas an ester bond is present in capsinoids [7,8]. This difference in their structure seems to be responsible for the lower pungency of capsinoids (approximately 1,000 times lower) compared to that of capsaicinoids [9], making them less irritant compounds and, therefore, more interesting for the development of drugs, food supplements, and functional foods for those markets where non- or low-pungent pepper varieties are preferred.

Capsaicinoids and capsinoids are biosynthesized from common precursors, phenylalanine and valine, and share at least the early part of the biosynthesis pathway [8,9]. Until now, some genes and QTLs (quantitative trait loci) seem to be involved in the genetic control of both capsaicinoids and capsinoids biosynthesis, but only *Pun1* and a *putative aminotransferase* (*pAMT*) genes have proven a key role in this regard. Whereas *Pun1* gene has a qualitative effect on the presence/absence of capsaicinoids and capsinoids, *pAMT* gene seems to have a quantitative effect on the accumulation of capsinoids over capsaicinoids [9–11].

Many studies in different *Capsicum* species [e.g. 'Habanero' of *C. chinense* [12,13], 'Malagueta' of *C. frutescens* [14], and several *C. annuum* cultivars [12,15,16] indicate that the accumulation of capsaicinoids during *Capsicum* fruit development is dynamical, varies according to cultivar and fruit stage, and depends on the growth conditions. On the whole, the biosynthesis of capsaicinoids occurs in the epidermal cells of the placenta and pericarp; they appear between 10 and 20 days post-anthesis (dpa) [17,18], reaching a maximum around 40 dpa and decreasing afterwards by increasing the peroxidases activity [15]. However, the evolution pattern of capsinoid accumulation during fruit development and the relationship between capsinoid and capsaicinoid contents in different stages of fruit development have not been reported systematically. Thus, an accurate characterization of these metabolites could be associated with differential transcript expression studies to understand better the genes involved in capsinoid and capsaicinoid biosynthesis. The emergence of metabolomics approaches based on analytical techniques [i.e. high-performance liquid chromatography (HPLC)/mass spectrometry (MS)] for food product applications has created new opportunities for the determination of metabolites involved in food quality traits in a consistent, reproducible, accurate and sensitive way [19]. The availability of a powerful analytic tool, such as HPLC coupled with electrospray ionization (ESI) to a quadrupole time-of-flight mass spectrometer (QTOF), could enable a precise determination (with a limit of detection of 0.006 μg/ml) of capsinoids and capsaicinoids during chili pepper fruit development [20,21]. In fact, an accurate determination is particularly important during the first stages of fruit development, when these compounds begin to be synthesized and are present in trace amounts. The high selectivity and sensitivity of the HPLC-ESI-MS(QTOF) technique can reduce the possibility of assigning false negatives (i.e. assign 'not determined' to a trace amount at the first fruit developmental stage) allowing a more accurate phenotyping, an essential

step in metabolic tracing studies. Thus, the aim of this work is to determine the capsinoid and capsaicinoid contents, using HPLC-ESI-MS(QTOF) for their quantification in fruits at four developmental stages, belonging to two pungent *C. annuum* genotypes, 'Chiltepín' and 'Tampiqueño 74', and a super-hot interspecific hybrid 'Bhut Jolokia'. These chili peppers are highly appreciated in Mexican and Indian gastronomy due to their distinctive flavor and pungency [22]. 'Chiltepín' (*C. annuum* L. var. *glabriusculum*), locally known as 'Piquín' or 'Bird pepper' is a wild type chili pepper native to Mexico. 'Tampiqueño 74' (*C. annuum* var. *annuum*), a Serrano type chili pepper, is also widely used in Mexican gastronomy for making 'pico de gallo', a salsa-type relish, and for spicy soups, stews, and pickles [1]; and 'Bhut Jolokia' (*C. chinense* Jacq. with introgression from *C. frutescens* L.) is considered one of the most pungent cultivars in the world [23] and traditionally grown in the North Eastern states of India[24]. The patterns of capsinoid and capsaicinoid accumulation during the fruit development of these cultivars were evaluated in order to determine whether they were similar. In addition, ratios between capsaicinoids and capsinoids at the four developmental stages and the alleles of *Pun1* and *pAMT* genes in the three genotypes were determined in order to assess the effect of the development stage and the allele type on the values of the ratios.

MATERIAL AND METHODS

Plant material

'Chiltepín' (from Querétaro state in Mexico) and 'Tampiqueño 74' seeds were supplied by the National Genomics Laboratory for Biodiversity (LANGEBIO) of Irapuato (Mexico). 'Bhut Jolokia' seeds were supplied from the Chile Pepper Institute (Las Cruces, NM, USA). Ten plants per genotype were grown in a climatized glasshouse (at 22-32 ºC),

located in Zaragoza (Spain), in black plastic pots (one plant per pot) of 17 cm in diameter, containing Projar Professional substrate (Projar S.A., Valencia, Spain) enriched with 2 g of a slow-release fertilizer (Osmocote 16N-4P-9K, Scotts, Tarragona, Spain) and watering by a drip irrigation system. The monitoring of the fruit development was performed by labeling and dating the flowers at anthesis, and the chili pepper fruits were harvested at 10, 20, 40, and 60 days post-anthesis (dpa) (Figure 1). The fruits of the three genotypes reached their final size and displayed the red color at 60 dpa, only 'Chiltepín' presented most fruits of green color at this time. Peppers were harvested daily until getting, at least, 60 fruits of 'Chiltepín' and 30 fruits of 'Tampiqueño 74' and 'Bhut Jolokia', for each developmental stage. The fruits of 'Chiltepín' were much smaller than those of 'Tampiqueño 74' and 'Bhut Jolokia'. Harvested chili pepper fruits were immediately frozen in liquid nitrogen and stored at −80 °C until use.

Chemicals and reagents

Capsaicinoids and capsinoids were extracted with ethyl acetate (> 99.8% LC-MS grade, Scharlau Chemie S.A., Barcelona, Spain). All HPLC samples were prepared in a mixture (60:40 v/v) of methanol (> 99.9% LC-MS grade, Scharlau Chemie S.A., Barcelona, Spain) and analytical grade type I water (Milli-Q Synthesis, Millipore, Bedford, MA, USA) for HPLC analysis. Capsaicin (> 95%) and dihydrocapsaicin standards (90%) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Capsiate, dihydrocapsiate, and (±)-3,4-dimethoxybezyl 4-methyloctanoate (DMBO) were synthesized in the Department of Organic Chemistry at the University of Cádiz, Spain [25,26].

Capsaicinoid and capsinoid determination

All harvested chili pepper fruits at each developmental stage and for each genotype were pooled and dried in a freeze-drier (Virtis Genesis 25 EL, SP Scientific, Stone Ridge, NY, USA) for 4-5 days, and ground in a conventional blender. Three hundred milligrams of these chili pepper powders were extracted with 3 ml of pure ethyl acetate and a fixed concentration of 5 µmol/l of DMBO was added as an internal standard in each sample. Then, the chili pepper powder suspension was stirred constantly at 30 ºC for one hour operating at 60 rpm (orbital shaker from Velp Scientifica, Usmate Velate, Lombardia, Italy). Two milliliters of the supernatant were collected in microtubes and centrifuged at 620 *g* for 5 min. This supernatant was completely evaporated in a vacuum centrifuge (miVac Duo Concentrator, Barnstead GeneVac, Ipswich, UK). The resulting pellet was resuspended with a 0.5 ml mixture of methanol and ultrapure water (60:40 v/v). Finally, the mixture was filtered through two disposable nylon filters, first with a 0.45 µm filter and then with a 0.20 µm (Teknokroma Analítica S.A., Barcelona, Spain). All the extractions were performed three times [21].

Agilent 1100 series HPLC system (Agilent Technologies, Waldbron, Germany) was used for capsaicin (C), dihydrocapsaicin (DHC), capsiate (CTE), and dihydrocapsiate (DHCTE) characterization, as reported by Fayos *et al*. [21]. The HPLC system was coupled to a quadrupole time-of-flight (QTOF) mass spectrometer equipped with an electrospray ionization source (ESI) interface (MicrOTOF-Q, Bruker Daltonics, Bremen, Germany) operating in positive ion mode. Chromatographic separation of the analytes and internal standard DMBO was achieved using an analytical HPLC C_{18} column (Symmetry® C_{18}, 2.1 x 150 mm; 3.5 µm spherical particle size, Waters, Milford, MA, USA) protected by a guard column (Symmetry® C_{18} 2.1 mm i.d. x 10 mm length, 3.5 µm spherical particle size, Waters) and a stepwise gradient of acidified methanol and Milli-Q water containing 0.1% (v/v) acetic acid. The column was equilibrated at a flow rate of 0.2 µl/min with a

mobile phase consisting of acidified water (0.1% acetic acid, solvent A) and acidified methanol (0.1% acetic acid, solvent B) (60:40 v/v) at 25 ºC. The elution program started according to the following gradient: 0-7 min, 60% B; 7-15 min, 60-70% B; 15-52 min, 70-100% B; and was maintained so for 80 min to equilibrate and return to the initial concentrations. The molecular ions $[M + H]^+$ for capsaicin (C) (*m*/*z* 306) and dihydrocapsaicin (DHC) (*m*/*z* 308), and $[M + Na]^+$ for capsiate (CTE) (*m*/*z* 329), dihydrocapsiate (DHCTE) and internal standard DMBO (*m*/*z* 331) were monitored. Spectra were acquired in the *m/z* 50-800 range. The mass axis was calibrated by using Na-formate adducts [10 mmol/l NaOH, 2.5% (v/v) formic acid and 50% (v/v) 2-propanol] that were introduced through a divert valve at the beginning of each direct injection. Bruker Daltonik software packages micrOTOF Control v.2.3 and HyStar v.3.2 were used to control the system and Data Analysis v.4.0 was used to process the data.

Capsaicinoids and capsinoids were quantified with the calibration curves obtained from the standard solutions. The resulting calibration curves were obtained by plotting the analyte peak area ratio, standardized with the peak area ratio of the internal standard DMBO, versus the concentration of the analyte. Due to the wide variability between capsaicinoid concentrations, two calibration curves at different ranges of concentrations were necessary to maintain the linearity of the method: $y = 2.438x + 6.0427$ ($R^2 = 0.9995$) and $y = 2.058x + 5.1892$ ($R^2 = 0.9995$) for low (< 100 µmol/l) C and DHC contents, respectively; $y = 0.843x + 63.846$ ($R^2 = 0.9998$) and $y = 0.660x + 58.144$ ($R^2 = 0.9998$) for high (> 100 µmol/l) C and DHC contents. The calibration curves used for capsinoids were: $y = 0.172x - 0.118$ ($R^2 = 0.997$) for CTE and $y = 0.248x - 0.144$ ($R^2 = 0.999$) for DHCTE.

DNA extraction and PCR assays for identification of pAMT and Pun1 alleles

The total genomic DNA was isolated from young leaves, as described by Garcés-Claver *et al.* [27]. The DNA concentration was quantified by NanoDrop-1000 (Thermo Fisher Scientific, Wilmington, DE, USA) and diluted to 10 ng/µl to be used for amplification. DNA from the pungent genotype *C. chinense* 'Habanero' (*Pun1*/*Pun1; pAMT/pAMT*) and the non-pungent *C. annuum* 'Yolo Wonder' ($pun1^1/pun1^1$) was used as controls. Specific markers were used to distinguish *pamt* [9, 28–30] and *pun1* [31] alleles. Primer sequences of $pamt^{1\text{-}7}$ and $pun1^{1\text{-}3}$ alleles are shown in Table S1 and PCR analyses were carried out according to the procedure described by the authors. The PCR products for $pamt^4$, $pamt^7$, and $pun1^3$ and $pun1^1$ alleles were visualized on 1% agarose gels (Sigma Aldrich). Agarose gels were in 1x TAE buffer, stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA) and visualized using a UV transilluminator G-Box (Syngene, Cambridge, UK). A 1 Kb Plus DNA ladder (Invitrogen) was used as size reference. The amplification products corresponding to $pamt^1$, $pamt^2$, $pamt^3$, $pamt^5$, $pamt^6$, and $pun1^2$ alleles were sequenced (STAB VIDA, Caparica, Portugal) and analyzed by BioEdit ver.5.0.6 [32] and MEGA6.06 software [33]. The *pamt* reference sequences of 'Himo' (GenBank # LC0321051), 'Belice Sweet' (GenBank # LC032107), 'Aji Dulce Strain2' (GenBank # LC321091), 'Habanero' (GenBank # LC032106), and 'NMCA 30036' (GenBank # EF104910) were downloaded from the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/) and they were used for the alignments.

Statistical analysis

A one-way variance analysis (ANOVA) followed by Tukey's test were performed to determine any significant differences (at $P < 0.05$) between the capsaicinoid and capsinoid contents depending on the developmental stage and genotype. The results were expressed as the mean ± standard deviation (SD) for triplicate analysis. All analyses were

performed using the statistics software package SPSS (IBM SPSS statistics v. 21.0.0.0) for Windows. The capsinoid ratio was calculated as described by Tanaka *et al*.: capsinoid content/ (capsinoid content + capsaicinoid content) [9].

RESULTS AND DISCUSSION

Capsaicinoid and capsinoid accumulation patterns during chili pepper fruits development in 'Chiltepín', 'Tampiqueño 74', and 'Bhut Jolokia'

The array and quantity of capsaicin (C), dihydrocapsaicin (DHC), capsiate (CTE), and dihydrocapsiate (DHCTE) throughout the different developmental stages of the fruit in a given genotype are defined in this study as the capsaicinoid and capsinoid accumulation pattern. Overall, the concentrations of the two major capsaicinoids (C, DHC) and capsinoids (CTE, DHCTE) varied significantly with genotype and developmental stages of fruit (Table 1). During the fruit development, in all genotypes, capsaicinoids were detected in higher amounts than capsinoids, being C the most abundant compound, followed by DHC, CTE, and DHCTE.

Regarding the capsinoid accumulation pattern during chili pepper fruit development, a similar trend was observed in the three chili pepper genotypes (Figure 2). Neither CTE nor DHCTE were detected at 10 dpa in any genotype, but both capsinoids were detected at 20 dpa in all of them. Therefore, these results suggest that capsinoid accumulation begins between 10 and 20 dpa, as occurs with that of capsaicinoid in some cultivars [17,18]. After 20 dpa stage, the capsinoid contents increased and reached their highest concentrations at 40 dpa in 'Chiltepín' (276.39 µg/g DW of CTE and 112.90 µg/g DW of DHCTE), 'Tampiqueño 74' (69.31 µg/g DW of CTE and 28.83 µg/g DW of DHCTE), and 'Bhut Jolokia' (122.62 µg/g DW of CTE and 11.25 µg/g DW of DHCTE). Finally,

the capsinoid contents decreased gradually from 40 dpa to 60 dpa. This reduction of capsinoid contents, at the end of the fruit development, could be associated with a decrease in the expression of capsaicinoid biosynthetic structural genes [34] or, alternatively, to the activities of different chili pepper peroxidases, as it was described for capsaicinoids catabolism [12,15]. In this sense, Lema *et al*. suggested that the same chili pepper peroxidases that oxidize the vanillyl moiety of capsaicinoids are able to oxidize the vanillyl moiety of capsinoids [35].

A similar trend for capsinoid accumulation during fruit development was observed in two non-pungent pepper cultivars and in four cultivars with different levels of pungency: i) In the first one, the non-pungent cultivar 'CH19 Sweet' (*C. annuum*), two capsaicinoid-like substances, CLS-A and CLS-B (further re-named as CTE and DHCTE, respectively), were detected in fruits from 10 dpa to 40 dpa, reaching the highest (598 µg/g DW) content at 30 dpa [36]; ii) afterwards, in the non-pungent *C. annuum* '509-45-1', CTE and DHCTE were found in pepper fruits classified in several developmental stages according to weight (from A to G) and/or coloration (stage of maturity), reaching the maximum content (1013 µg/g fresh weight) in the immature green stage [37]; (iii) and finally, in the cultivars 'SNU11-001' (slight pungent), 'Yuwol-cho' (mild pungent), 'Tanaknotsume' (mild pungent), and 'Habanero' (pungent), the highest capsinoid content were measured in the intermediate developmental stages.

Total capsinoid content varied from 50.94 to 389.28 µg/g DW regardless of the genotype and fruit development stage (Table 1). CTE and DHCTE were quantified for the first time in 'Chiltepín' and 'Tampiqueño 74'. For 'Bhut Jolokia', the values observed in this study were lower than those previously reported by our group (440.90 µg/g DW for CTE and 27.25 µg/g DW for DHCTE) in fully ripe fruits [21], and than those (308 µg/g DW) reported by Tanaka *et al*. in chili pepper fruits at 30 dpa [9]. These variations on capsinoid contents

268 may be due to differences in growing conditions and environmental factors, as described
269 for capsaicinoid contents [2].
270 In contrast to capsinoids, the capsaicinoid accumulation pattern presented certain
271 differences between 'Bhut Jolokia' and the other two genotypes, 'Chiltepín' and
272 'Tampiqueño 74' (Figure 2). For 'Chiltepín' and 'Tampiqueño 74', capsaicinoids were
273 not detected until 20 dpa, increasing and reaching their highest concentrations at 40 dpa
274 (685.29 µg/g DW of C and 564.00 µg/g DW of DHC for 'Chiltepín' and 183.80 µg/g DW
275 of C and 177.43 µg/g DW of DHC for 'Tampiqueño 74'). Finally, capsaicinoid contents
276 decreased from 40 dpa to 60 dpa, probably due to peroxidase activity [12,15]. Similar
277 capsaicinoid accumulation patterns in 'Chiltepín' and 'Tampiqueño 74', reaching
278 maximum capsaicinoids contents at 40 dpa (53.7 µg/g DW and 17060 µg/g DW,
279 respectively) and then decreasing gradually, have been previously reported [12,38].
280 Likewise, González-Zamora *et al*. reported higher capsaicinoid contents in green mature
281 stage (70040 - 104690 µg/g DW) than in red mature stage (29550 - 86880 µg/g DW) in
282 'Chiltepín' from Sonora [39]. The wide variability observed in the capsaicinoid content
283 could be due to the growing conditions and the genetic variability among cultivars.
284 Moreover, 'Tampiqueño 74', capsaicinoid extracts were obtained in this work from whole
285 fruit instead of placental tissue as described by Arce-Rodríguez and Ochoa-Alejo [38]. On
286 the other hand, it is interesting to highlight that the capsaicinoid patterns in both
287 genotypes were similar to those aforementioned for capsinoids.
288 For 'Bhut Jolokia', unlike 'Chiltepín' and 'Tampiqueño 74', C and DHC were detected
289 at 10 dpa (1.31 µg/g DW and 1.28 µg/g DW, respectively) and reached their maximum
290 concentrations at 60 dpa (1517.61 µg/g DW for C and 1032.12 µg/g DW for DHC). A
291 similar evolution pattern of capsaicinoid accumulation, with a maximum of C content in
292 the last maturity stage (at 63 days after fruit set) has been described in 'Habanero', a very

293 pungent *C. chinense* cultivar [13]. Furthermore, the capsaicinoid accumulation pattern in
294 'Bhut Jolokia' was different from that of capsinoids, since, whereas capsaicinoid contents
295 increased at 60 dpa, capsinoids decreased in this developmental stage. The divergent
296 trends observed herein for the accumulation of capsinoids and capsaicinoids in 'Bhut
297 Jolokia', a super-hot cultivar, could be attributed to the presence of different factors acting
298 as regulators of the accumulation of capsinoids and capsaicinoids in the biosynthesis
299 pathway of this cultivar. This is consistent with previous findings suggesting that the
300 mechanisms involved in capsaicinoid accumulation are different for pungent and super-
301 hot pepper cultivars [18,40]. In this way, extremely high concentrations of capsaicinoids, in
302 extremely pungent peppers, such as 'Bhut Jolokia,' 'Trinidad Scorpion,' 'Trinidad
303 Moruga Scorpion,' and 'Trinidad Moruga Scorpion Yellow' have been associated with
304 the presence of capsaicinoids-containing vesicles, which are located on the pericarp [40,41].
305 Also, the mechanisms responsible for high capsaicinoid contents in extremely pungent
306 cultivars, such as 'Trinidad Moruga Scorpion Yellow', were investigated, revealing that
307 the up-regulated expression of multiple capsaicinoid biosynthetic genes in the pericarp
308 tissue of this type of cultivars plays an important role in capsaicinoid accumulation during
309 the capsaicinoid biosynthesis [18]. Alternatively, the increase in capsaicinoids in the fruit
310 of 'Bhut Jolokia' throughout its development may be due not only to their continued
311 synthesis, but perhaps could be due to a mechanism that inhibits or delays its catabolism.
312 Different patterns of peroxidase activity for capsaicinoid catabolism depend on cultivars.
313
314 ***Relationship between capsaicin, dihydrocapsaicin, capsiate, and dihydrocapsiate***
315 The C/DHC ratio varied slightly between the three genotypes and among the fruit
316 developmental stage, ranging from 1.02 to 1.74 (Table 2). Whilst in 'Chiltepín' and
317 'Tampiqueño 74' the highest C/DHC ratio (1.74 and 1.43, respectively) was observed at

20 dpa, in 'Bhut Jolokia' it was at 40 dpa (1.69). These results are in agreement with previous works, in which the variation of C/DHC ratio has been observed for both developmental stages and genotypes, for example, values of C/DHC ratio between 2.1 and 2.4 (with a maximum of 2.44 at 47 dpa) were found in 'Malagueta' chili pepper fruit during development [14], and the C/DHC ratios from 0.33 to 4.92 were reported for fruits in the mature stage of 139 *Capsicum* accessions [24].

The CTE/DHCTE ratios for 'Chiltepín' (from 2.45 to 5.33), 'Tampiqueño74' (from 2.40 to 6.16) and 'Bhut Jolokia' (from 10.89 to 23.56) were significantly different ($P < 0.05$) along the developmental stages (Table 2). For 'Chiltepín' and 'Tampiqueño 74', the highest and the lowest significant ratios were at 60 dpa and 40 dpa, respectively; contrarily, 'Bhut Jolokia' showed the maximum ratio (23.5) at 20 dpa. Significant differences were also highlighted among genotypes, being the CTE/DHCTE ratios of 'Bhut Jolokia' considerably higher than those observed in the other two genotypes. So far, the variation of CTE/DHCTE ratio during fruit development has not been investigated in detail. Nevertheless, values of ratio at 60 dpa (equal to mature stage) for 'Chiltepín' and 'Tampiqueño 74' are agreed with the findings of Singh *et al*. who analyzed capsiate and dihydrocapsiate contents from extracts of chili peppers in mature stage, revealing values of CTE/DHCTE ratio between 2.5 and 9.24 [2]. According to our results, we can conclude that the variation in the CTE/DHCTE ratio was higher than that observed for C/DHC ratio, during the fruit development as well as among genotypes with different hotness.

The capsinoid ratio varied slightly during fruit development, showing the highest value at 40 dpa for the three genotypes and the lowest value at 60 dpa for 'Chiltepín' and 'Tampiqueño 74' (Table 2). This is the first report in which the capsinoid ratio has been calculated at various fruit developmental stages. Capsinoid ratios were approximately 4

343 times lower in 'Bhut Jolokia' (0.04-0.06) than those calculated for the other two
344 genotypes (0.15-0.24). This is due to the fact that the capsaicinoid total content was
345 significantly higher than that of capsinoids in the super-hot genotype during fruit
346 development. These results could suggest that factors acting as regulators of the
347 capsaicinoids accumulation in the super-hot genotype 'Bhut Jolokia' may be do not
348 operate exactly in the same way in capsinoid accumulation.
349 According to previous studies, the capsinoid ratio is affected by several mutations in the
350 *pAMT* allele, which lead to the accumulation of capsinoids instead of capsaicinoids,
351 resulting in a capsinoid ratio < 10% in *pAMT* genotypes and > 90% in the *pamt* mutants
352 [9]. Thus, the mutations of the *pAMT* alleles cause a significant reduction of pungency in
353 the chili pepper fruits. Moreover, the loss of functionality of the *Pun1* gene results in the
354 non-pungency of chili pepper fruits [42], and does not produce capsaicinoids or
355 capsinoids[11]. Both genes have been turned into potential candidates to use in breeding
356 programs for the development of new non-pungent or low-pungent pepper cultivars. For
357 that purpose, several specific markers (*pamt*$^{1\text{-}7}$ and *pun1*$^{1\text{-}3}$) have been developed in
358 *Capsicum* spp. [9,28–31]. In the present study, the three pungent genotypes were analyzed
359 using the specific molecular markers developed, in order to assess the allelic state of the
360 *pAMT* and *Pun1* genes. PCR markers for identificating the *pamt*^{1} allele, whose mutation,
361 a T nucleotide insertion, was identified in the non-pungent *C. annuum* cultivar 'CH19
362 Sweet' [28], showed the absence of the insertion in the *pAMT* allele in 'Tampiqueño 74'.
363 No fragments were amplified with these markers in 'Bhut Jolokia' and 'Chiltepín',
364 suggesting a high specificity of the markers for the sequence of the 'CH19 Sweet'
365 cultivar. For the *pamt*^{2} allele, which has a single nucleotide substitution, T instead of C
366 [29], the alignment of the sequences of amplified DNA fragments from the three genotypes
367 against the reference sequence of the non-pungent 'Himo' (GenBank # LC0321051),

showed the presence of the C nucleotide in 'Chiltepín', 'Tampiqueño 74', and 'Bhut Jolokia', confirming the wild type *p-AMT* allele in the three genotypes (Figure 3A). This same result was obtained in the *pamt*^{3} and *pamt*^{5} alignments. These mutation alleles, containing a short nucleotide insertion of 6 bp and 8 bp, respectively, were detected in the mildly pungent *C. chinense* 'Belize Sweet' (GenBank # LC032107) and 'Ají Dulce' (GenBank # LC0321091) cultivars, respectively [30]. These insertions were not found in the sequences of 'Chiltepín', 'Tampiqueño 74', and 'Bhut Jolokia' (Figure 3B, C). For the *pamt*^{6} allele, the specific primers developed by Koeda *et al*. amplified a 107 bp fragment in the three genotypes similar to that amplified in the reference *C. chinense* 'Habanero' (GenBank # LC032106), which was used by the authors, revealing the absence of the 7 bp insertion detected in the *C. chinense* 'No.80', owner of the *pamt*^{6} mutation (Figure 3D) [43]. In the case of the *pamt*^{4} and *pamt*^{7} alleles, which were caused by the 2.3 and 2.8 kb insertions, respectively [9,30], the PCR primers amplified 1500 bp (for *pamt*^{4}) and 900 bp (for *pamt*^{7}) in the three genotypes of the present study, as well as in the control 'Habanero' (*pAMT*/*pAMT*). Both amplified fragments corresponded to type *p-AMT* allele for each *pamt* genotyping, respectively (Figure 3E). Taking into account all the results of *pAMT* genotyping, 'Chiltepín', 'Tampiqueño 74', and 'Bhut Jolokia' did not contain the *pamt* mutations analyzed.

A set of molecular markers (*pun1*^{2}, *pun1*^{3}, and *pun1*^{1} markers), reported by Wyatt *et al*., whose combination allows to distinguish between the three *pun1*$^{1\text{-}3}$ mutants and *Pun1* allele [31], were used for *Pun1* genotyping. Firstly, alignment of the sequences amplified with the *pun1*^{2} marker using 'Chiltepín', 'Tampiqueño 74', and 'Bhut Jolokia' DNA against the reference *pun1*^{2} sequence of the cultivar 'NMCA 30036' (GenBank # EF104910) displayed the absence of a 4 bp deletion in the three genotypes, thus defining the presence of *Pun1* or *pun1*^{3} alleles (Figure 4A). Secondly, using the *pun1*^{3} marker, a

fragment of 586 bp was amplified in 'Chiltepín', 'Tampiqueño 74', 'Bhut Jolokia', as well as in the control 'Habanero' (*Pun1*/*Pun1*), indicating the presence of either the *Pun1*, *pun1*^{1}, or *pun1*^{2} allele (Figure 4B). Finally, using the *pun1*^{1} marker, a fragment of 1063 bp was amplified from 'Chiltepín', 'Tampiqueño 74', and 'Bhut Jolokia' indicating the presence of the *Pun1*, *pun1*^{2}, *or pun1*^{3} allele. A fragment of 746 bp for the *pun1*^{1} allele was amplified in the non-pungent control 'Yolo Wonder' (*pun1*^{1}/*pun1*^{1}) (Figure 4B). The combination of *Pun1* genotyping results, using the *pun1*^{3}, *pun1*^{2}, and *pun1*^{1} markers, revealed that the three pungent genotypes contain the wild-type *Pun1* allele.

Based on the results of *pAMT* and *Pun1* genotyping and the capsinoid ratios, the value of the capsinoid ratio (0.04-0.06) for fruits of 'Bhut Jolokia' (*pAMT*; *Pun1*) was in agreement with the capsinoid ratio values < 10% in *pAMT* genotypes previously established [9]. However, such ratios for 'Chiltepín' (0.16) and 'Tampiqueño 74' (0.15), both *pAMT* and *Pun1* genotypes, were slightly higher than expected. On the other hand, the presence of both capsaicinoids and capsinoids in the three (*Pun1* and *pAMT*) genotypes was consistent with previously reported findings, which confirms that capsinoids could be synthesized regardless of the *pAMT* function [11].

To date, no other studies have quantified capsinoids using an accurate HPLC-ESI-MS(QTOF) methodology or evaluated the capsinoid ratio in pungent genotypes at different fruit developmental stages. In the current study, three chili peppers, 'Chiltepín', 'Tampiqueño 74' and 'Bhut Jolokia', showed similar accumulation patterns of individual capsinoids during four fruit developmental stages: capsinoids were not detected at 10 dpa, their contents started to increase from 20 dpa to a maximum at 40 dpa, and then their levels decreased at later stages. These results depict the same accumulation trend than that of capsaicinoids found in this study for 'Chiltepín' and 'Tampiqueño 74', and highlight that capsinoid accumulation in 'Bhut Jolokia' displayed a different pattern to

418 that of capsaicinoids. The divergent accumulation trends of capsinoids and capsaicinoids,
419 as well as the different CTE/DHCTE ratio, in 'Bhut Jolokia', could be attributed to the
420 presence of several factors regulating the accumulation of capsinoids and capsaicinoids
421 in the biosynthesis pathway of this genotype. On the other hand, the three pungent
422 genotypes contained *pAMT* and *Pun1* alleles and produced both capsaicinoids and
423 capsinoids, being the capsinoid ratios between 0.04 and 0.24. Then the capsinoid contents
424 detected in the three chili peppers (48.34-389.28 μg/g DW) can be considered more than
425 trace amounts, suggesting that other genes or dysfunctional alleles are involved in
426 capsinoid accumulation, besides the mutation *pAMT* alleles analyzed. Additional studies,
427 involving chili pepper genotypes with different levels of pungency, are necessary to
428 elucidate the mechanisms that control the accumulation of capsinoids and capsaicinoids.
429

430 **Abbreviations Used**

431 C, capsaicin; CTE, capsiate; DHC, dihydrocapsaicin; DHCTE, dihydrocapsiate; DMBO,
432 (±)-3,4-dimethoxybezyl 4-methyloctanoate; DNA, deoxyribonucleic acid; dpa, days
433 post-anthesis; DW, dry weight; ESI, electrospray ionization source; HPLC, high
434 performance liquid chromatography; MS, mass spectrometry; *m*/z, mass-to-charge; PCR,
435 polymerase chain reaction; QTL, quantitative trait loci; QTOF, quadrupole time-of-flight.
436

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442

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Conflict of interest

The authors declare that there is no competing financial interest.

Supporting Information

Table S1. Primers used to determine *pamt*$^{3-7}$ alleles and *pun*$^{1-3}$ alleles

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608 **Figure captions**

609

610 **Figure 1.** Chili pepper fruits assayed for their capsinoid and capsaicinoid accumulation
611 patterns during development and maturation. Fruits of 'Bhut Jolokia' at 10, 20, 40, and
612 60 days post-anthesis (dpa) (A-D); fruits of 'Chiltepín' at 10, 20, 40, and 60 dpa (E-H);
613 fruits of 'Tampiqueño 74' at 10, 20, 40, and 60 dpa (I-L).

614

615 **Figure 2.** Accumulation patterns of major capsaicinoids, capsaicin (C) and
616 dihydrocapsaicin (DHC), and capsinoids, capsiate (CTE) and dihydrocapsiate (DHCTE),
617 during chili pepper fruit development in 'Chiltepín' (A), 'Tampiqueño 74' (B), and 'Bhut
618 Jolokia' (C).

619

620 **Figure 3**. *pAMT* Allele-specific PCR markers described by Lang *et al*. [28], Tanaka *et al*.
621 [29,30], and Tanaka *et al*. [9]. (**A**) The *pamt*^{2} marker was visualized by alignment of sequences
622 amplified from 'Chiltepín', 'Tampiqueño 74', and 'Bhut Jolokia' against that of the
623 reference sequence of the non-pungent 'Himo' (GenBank # LC0321051). The *pamt*^{2}
624 allele contains a single nucleotide substitution, T instead of C. (B) The *pamt*^{3} marker was
625 visualized by the alignment of sequences amplified from 'Chiltepín', 'Tampiqueño 74',
626 and 'Bhut Jolokia' against that of the reference sequence of the mildly-pungent 'Belize
627 Sweet' (GenBank # LC032107). The *pamt*^{3} allele contains a short nucleotide insertion of
628 6 bp. (**C**) The *pamt*^{5} marker was visualized by the alignment of sequences amplified from
629 'Chiltepín', 'Tampiqueño 74', and 'Bhut Jolokia' against that of the reference sequence
630 of the mildly-pungent 'Aji Dulce' (GenBank # LC0321091). The *pam*^{5} allele contains a
631 short nucleotide insertion of 8 bp. (**D**) The *pamt*^{6} marker was visualized by the alignment
632 of sequences amplified from 'Chiltepín', 'Tampiqueño 74', and 'Bhut Jolokia' against

633 that of the reference sequence of the pungent 'Habanero' (GenBank # LC032106). The
634 *pamt*^{6} allele yields a fragment of 114 bp. A fragment of 107 bp indicated the presence of
635 the wild type *pAMT* allele. (**E**) The *pamt*^{4} and *pamt*^{7} specific markers were visualized on
636 a 1% agarose gel. The *pamt*^{4} marker amplified a band of 1500 bp. A larger band of 3.8
637 kb, with the presence of a 2.3 kb insertion, indicates the presence of the *pamt*^{4} allele. The
638 *pamt*^{7} marker amplified a band of 900 bp. A larger band of 3.7 kb, with the presence of a
639 2.8 kb insertion, indicates the presence of the *pamt*^{7} allele. M: 1 kb ladder; 1: 'Chiltepín'
640 (pungent); 2: 'Tampiqueño 74' (pungent); 3: 'Bhut Jolokia' (pungent); 4: 'Habanero'
641 (pungent).
642
643 **Figure 4**. *Pun1* allele specific PCR marker described by Wyatt *et al*. [31]. (**A**) The *pun1*^{2}
644 marker was visualized by the alignment of sequences amplified from 'Chiltepín',
645 'Tampiqueño 74', and 'Bhut Jolokia' against that of the reference sequence of the non-
646 pungent 'NMCA 30036' (GenBank # EF104910). The *pun1*^{2} allele contains a small
647 deletion of 4 bp and the absence of this deletion indicates the presence of *Pun1* or *pun1*^{3}
648 alleles. (**B**) The *pun1*^{3} and *pun1*^{1} specific markers were visualized on a 1% agarose gel.
649 For the *pun1*^{3} marker, a smaller band of 586 bp indicated the presence of either the *Pun1*,
650 *pun1*^{1}, or *pun1*^{2} allele. The *pun1*^{1} marker yielded a larger band of 1063 bp indicates the
651 presence of the *Pun1*, *pun1*^{1}, or *pun1*^{3} allele and a smaller band of 746 bp for the *pun1*^{1}
652 allele. M: 1 kb ladder; 1: 'Chiltepín' (pungent); 2: 'Tampiqueño 74' (pungent); 3: 'Bhut
653 Jolokia' (pungent); 4: 'Habanero' (pungent); 5: 'Yolo Wonder' (non-pungent).

654 **Table 1**. Mean values (µg/g DW) for capsaicin, dihydrocapsaicin, total capsaicinoids, capsiate, dihydrocapsiate, and total capsinoid contents of
655 chili pepper fruits of 'Chiltepín', 'Tampiqueño 74', and 'Bhut Jolokia' at 10, 20, 40, and 60 days post-anthesis (dpa). The values are expressed as
656 the mean ± SD (n = 3, biological replicates). The means in the same column and within the same genotype followed by different letters (a-d) were
657 found to be significantly different by Tukey's test at ($P \leq 0.05$).
658

Cultivar	Developmental stages (dpa)	Capsaicin	Dihydrocapsaicin	Total capsaicinoids	Capsiate	Dihydrocapsiate	Total capsinoids
Chiltepín	10	ND*	ND	ND	ND	ND	ND
	20	137.35 ± 0.34c	78.87 ± 0.34c	216.22 ± 0.68c	45.72 ± 0.71c	14.10 ± 1.28b	59.82 ± 1.31c
	40	685.29 ± 3.94a	564.00 ± 9.59a	1249.28 ± 12.54a	276.39 ± 3.53a	112.90 ± 1.53a	389.28 ± 4.74a
	60	291.81 ± 2.72b	242.86 ± 11.83b	534.66 ± 13.76b	87.07 ± 4.06b	16.57 ± 2.78b	103.64 ± 6.24b
Tampiqueño 74	10	ND	ND	ND	ND	ND	ND
	20	132.10 ± 0.99b	92.19 ± 2.82c	224.30 ± 2.45c	39.25 ± 2.29b	11.69 ± 0.23b	50.94 ± 2.38b
	40	183.80 ± 0.01a	177.43 ± 2.95a	361.23 ± 2.94a	69.31 ± 0.65a	28.83 ± 0.23a	98.14 ± 0.88a
	60	134.07 ± 7.07b	129.64 ± 4.63b	263.71 ± 11.51b	41.58 ± 1.65b	6.76 ± 0.47c	48.34 ± 2.12b
Bhut Jolokia	10	1.31 ± 0.01c	1.28 ± 0.01d	2.59 ± 0.01c	ND	ND	ND
	20	1243.09 ± 25.44b	846.85 ± 27.70b	2089.94 ± 50.63b	92.59 ± 2.70c	3.94 ± 0.28c	96.53 ± 2.86c
	40	1275.02 ± 32.67b	755.81 ± 33.96c	2030.83 ± 65.96b	122.62 ± 3.71a	11.25 ± 0.17a	133.88 ± 3.86a
	60	1517.61 ± 28.42a	1032.12 ± 36.44a	2549.73 ± 59.58a	108.84 ± 1.01b	7.59 ± 0.40b	116.43 ± 1.37b

659 *ND = not detected

660 **Table 2**. Mean values for capsaicin/dihydrocapsaicin (C/DHC) ratio, capsiate
661 /dihydrocapsiate (CTE/DHCTE) ratio, and the capsinoid ratio of pepper fruits of
662 'Chiltepín', 'Tampiqueño 74', and 'Bhut Jolokia' at 10, 20, 40, and 60 days post-anthesis
663 (dpa). The values are expressed as the mean ± SD (n = 3, biological replicates). Means in
664 the same column and within the same genotype followed by different letters were
665 significantly different by Tukey's test at $P \leq 0.05$. The capsinoid ratio was calculated as
666 described in Tanaka *et al*.: capsinoid content/ (capsinoid content + capsaicinoid content)
667 [9].
668

	Developmental stages (dpa)	C/DHC ratio	CTE/DHCTE ratio	Capsinoid ratio
Chiltepín	10	-	-	-
	20	1.74 ± 0.01a	3.26 ± 0.31b	0.22 ± 0.01b
	40	1.22 ± 0.02b	2.45 ± 0.02b	0.24 ± 0.01a
	60	1.20 ± 0.05b	5.33 ± 0.70a	0.16 ± 0.01c
Tampiqueño 74	10	-	-	-
	20	1.43 ± 0.05a	3.36 ± 0.18b	0.19 ± 0.01b
	40	1.04 ± 0.02b	2.40 ± 0.01c	0.21 ± 0.01a
	60	1.03 ± 0.02b	6.16 ± 0.18a	0.15 ± 0.01c
Bhut Jolokia	10	1.02 ± 0.01c	-	-
	20	1.47 ± 0.03b	23.56 ± 1.40a	0.04 ± 0.01b
	40	1.69 ± 0.04a	10.89 ± 0.21c	0.06 ± 0.01a
	60	1.47 ± 0.04b	14.37 ± 0.63b	0.04 ± 0.01b

669

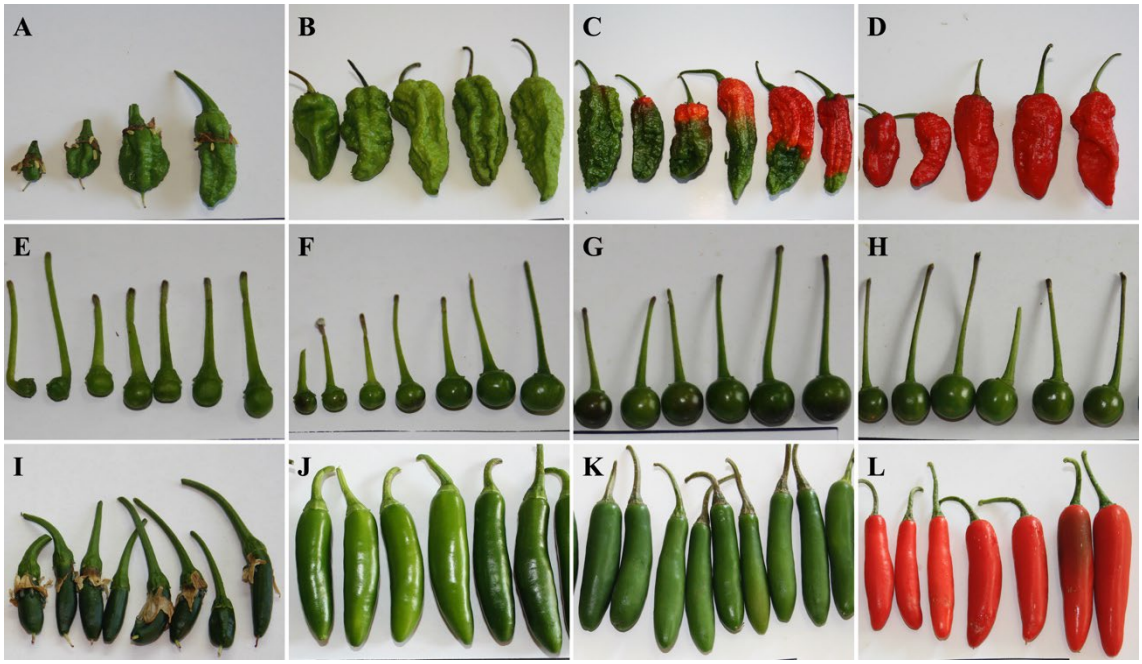
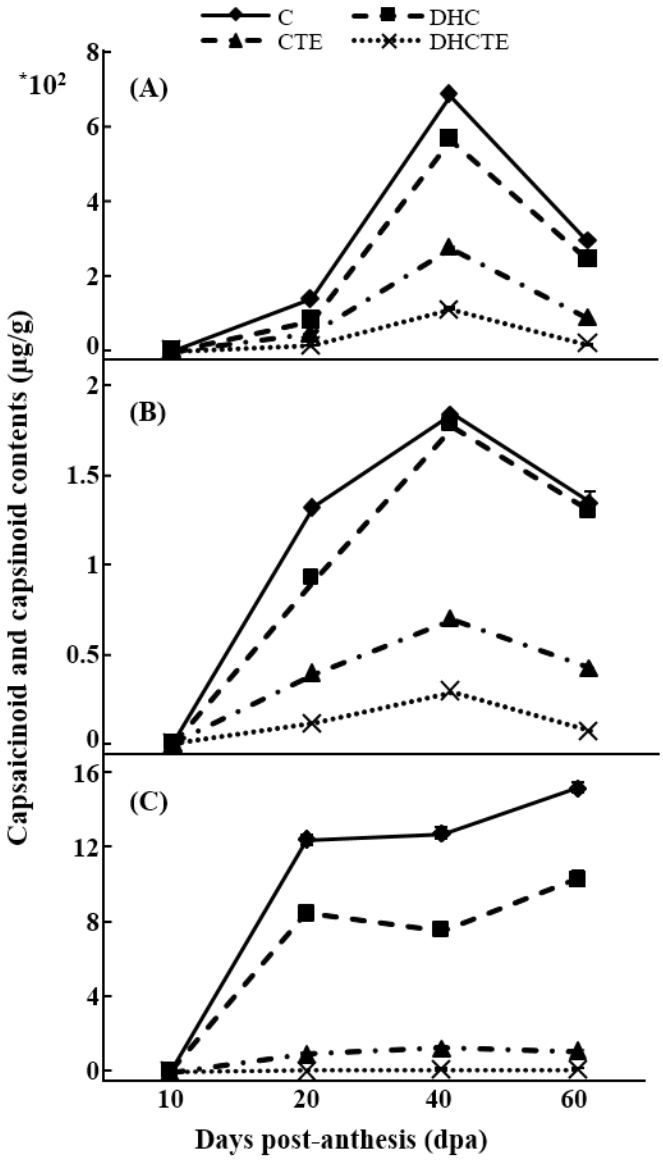


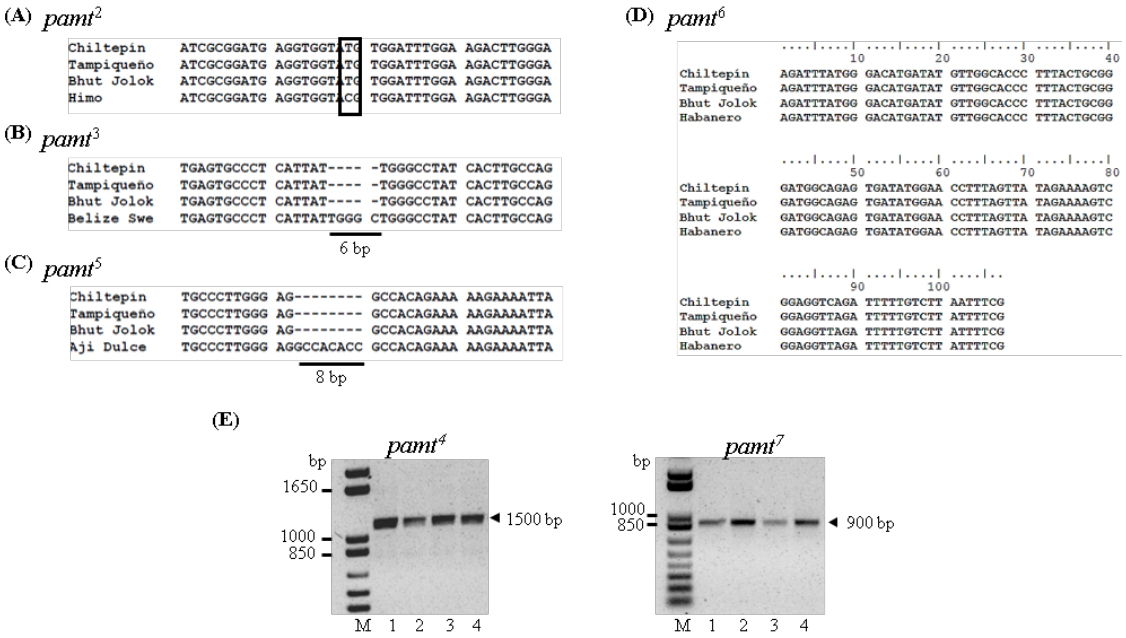
Figure 1.



673

674 **Figure 2.**

675

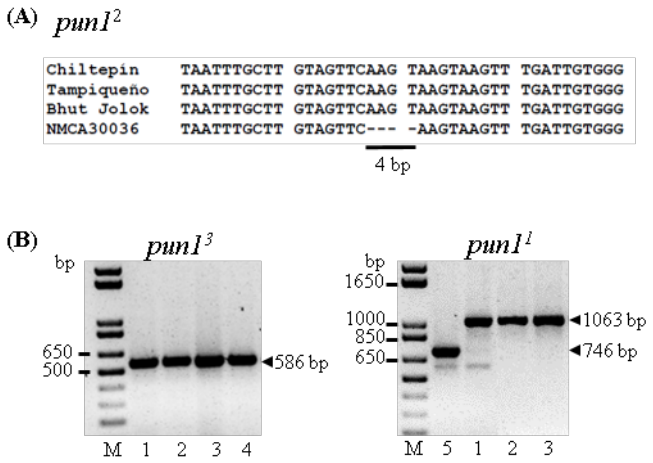


676

677

678 **Figure 3**.

679



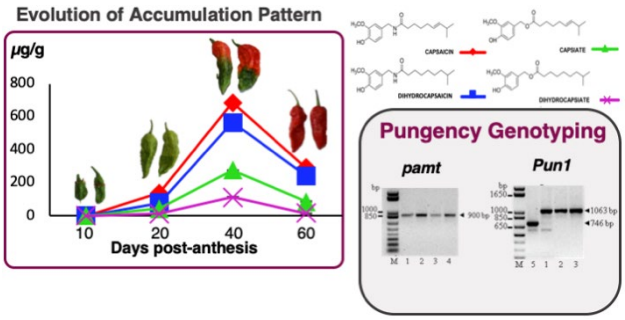
680

681

682 **Figure 4**.

683

684 **Table of Contents**



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