Acta Agrobotanica

DOI: 10.5586/aa.1792

Publication history
Received: 2019-02-21
Accepted: 2019-09-03
Published: 2019-12-16

Handling editor
Barbara Łotocka, Faculty of Agriculture and Biology, Warsaw University of Life Sciences – SGGW, Poland

Authors' contributions
SBS, FAZP: performed experiments and measurements; TJOF, JFRC: designed experiments, analyzed data, and revised the manuscript; JA: set up and calibration of the MS equipment, helped with data analysis and software use; MGL, LGVT: helped to perform experiments and revised the manuscript; RRS: planned the experiments, analyzed data, and wrote the manuscript

Funding
PAPIIT-DGAPA-UNAM IN216815. Partially PAPIIT-DGAPA-UNAM IN219518. PAIP-FQ-UNAM 5000-9122. CONACyT-INFRA-2016-252001. CONACyT-CB2018-A1-S-8650. SBS was recipient of a DGAPA-UNAM scholarship No. 236916:IN216815.

Competing interests
No competing interests have been declared.

Citation
Brito-Sánchez SC, Zaragoza-Pérez FA, Olivera-Flores TJ, Rivero-Cruz JF, Amacosta J, Gutiérrez-Luna FM, et al. Biolistics transformation of callus and cell suspension cultures of *Capsicum annuum* L. 'Serrano' is useful for in vitro studies of the relative contents of secondary metabolites. Acta Agrobot. 2019;72(4):1792. https://doi.org/10.5586/aa.1792

ORIGINAL RESEARCH PAPER

Biolistics transformation of callus and cell suspension cultures of *Capsicum annuum* L. 'Serrano' is useful for in vitro studies of the relative contents of secondary metabolites

Sara Cristina Brito-Sánchez[1], Fredy Alan Zaragoza-Pérez[1], Teresa de Jesús Olivera-Flores[1], José Fausto Rivero-Cruz[2], Jessica Amacosta[3], Francisca Morayna Gutiérrez-Luna[1], Lilián Gabriela Valencia-Turcotte[1], Rogelio Rodríguez-Sotres[1]*

[1] Departamento de Bioquímica, Facultad de Química, Universidad Nacional Autónoma de México, Mexico
[2] Departamento de Farmacia, Facultad de Química, Universidad Nacional Autónoma de México, Mexico
[3] Perkin-Elmer de México, S. A. Macedonio Alcalá 54, Col. Guadalupe Inn Mexico City, Mexico

* Corresponding author. Email: sotres@unam.mx

Abstract
Capsicum annuum is a crop species of economic importance able to produce capsaicinoids, capsinoids, and pigments with nutritional and medicinal value. Methods to propagate and transform this species have been reported, but most are phenotype dependent, rely on *Agrobacterium* for transformation, and their success has been limited. This relates to only one commercial transgenic variety currently on trial. In the present work, we report the conditions to produce callus and cell suspension cultures of *C. annuum* 'Serrano' using commercial seeds. The culture could be induced to produce capsaicin and dihydrocapsaicin in detectable quantities and was amenable to transformation using biolistics. The expression of the *Arabidopsis thaliana* soluble inorganic pyrophosphatase 4 fused to a fluorescent protein was demonstrated using confocal microscopy. Evidence of the integrity of the fusion was obtained by immunoblot. The transformation induced a change in the ratio of capsaicin to dihydrocapsaicin measured using high resolution direct sample analysis-mass spectrometry (DSA-MS). The method is thus useful for the study of capsaicinoid production under controlled conditions for special purposes and metabolic studies.

Keywords
plant secondary metabolites; direct sample analysis-mass spectrometry; soluble inorganic pyrophosphatase; transgenic plant cells

Introduction

Capsicum is an important solanacean plant genus comprising valuable crop species known widely as chilli peppers [1]. Its importance resides in the fruit's high content of pigments, vitamins, and valuable secondary metabolites, including flavonoids and the distinctive capsaicinoids and capsinoids [2,3]. *Capsicum annuum* L. is currently the chilli pepper of high economic impact, and the genetic manipulation of this plant species has become an important biotechnological goal [1,4,5]. However, major obstacles have limited the obtention of genetically modified chilli peppers with improved agricultural traits [4,6]. It has been possible to obtain tissue cultures of *Capsicum* plants and the genetic transformation of this plant is attainable, although transformation yields are usually

low [4]. Tissue cultures from *Capsicum* species can produce secondary metabolites, but to levels well below those observed for the fruit placenta tissue [7].

The somatic embryogenesis has been reported but the number of plants recovered is low [5] and the results seem to depend on the genotype and its previous history [8,9]. In fact, the presence of genetic features limiting the rate of somatic embryogenesis in *C. annuum* was demonstrated by the overexpression of the BABY BOOM transcription factor from *Arabidopsis thaliana* L. [10]. This transcription factor belongs to the CLAVATA family of plant transcriptional regulators, and it acts upstream of major regulators of plant embryo identity and totipotency in *A. thaliana* [11]. Its transgenic overexpression in *C. annuum* tissue culture resulted in a higher number of somatic embryos [10].

Plant regeneration seems to occur more consistently by direct organogenesis [5,12]. In this case, consistent regeneration from shoot segments has been achieved using spermidine and avoiding the formation of callus [13]. *Agrobacterium*-mediated transformation of explants has made possible the obtention of transgenic plants from many genotypes [14]. The selection protocol has become a limiting factor [15] because strong selection markers (usually transgenes) are required to eliminate those cells not carrying the desired trait [16] and kill the remaining bacteria. In addition, *Capsicum* species have a tendency for the loss of transposons, making *Agrobacterium*-mediated transformants less stable beyond T2 or T3 generations [6]. All these factors reflect on the low number of chilli pepper transgenics currently on field trial [17].

There is a current concern in informed societies regarding the consumption of transgenic crops [18]. Among other reasons, this has grounds on the risk of an accidental horizontal transfer of transgenes to wildlife [19], which may inflict disruptive disturbance to natural ecosystems. The use of *Agrobacterium*-mediated transformation implies the insertion of bacterial sequences and antibiotic resistance as a requirement to eliminate the bacteria from the culture. Biolistics is an alternative transformation tool, barely been tested on *Capsicum* species. There are, however, two reports on the biolistics transformation of *C. annuum* 'Serrano' [20] but no stable transgenic plants were obtained, no attempt was made to produce transformed calli, and access to the details is limited as the reports were not published in peer-reviewed journals.

Consistent methods of transgenesis in *C. annuum* could help to address a number of metabolic and physiological questions that remain elusive, including those related to the synthesis of capsaicinoids and their regulation [7]. Some of these questions could be addressed directly in transformed tissue cultures of this plant species, particularly if these transformed cultures are able to produce secondary metabolites. Such a system would bypass the time-consuming, and currently inefficient regeneration of transformed plants, as an additional advantage.

Secondary metabolism is very active in *Capsicum* fruits because the mesocarp accumulates an ample quantity of useful carotenoids, flavonoid glycosides, ascorbate, and tocopherols [16]. In addition, the placenta accumulates a significant amount of capsaicinoids (in pungent peppers) or capsinoids (in sweet peppers) [21]. The accumulation of these secondary metabolites is regulated by environmental conditions. Drought upregulates the activity of enzymes controlling the synthesis of capsaicinoids [22]. Nevertheless, despite this increase in the production of secondary metabolites, the plant is affected by the stress because the leaf pools of ATP are known to drop significantly under this condition [23], implying an energy-deficit condition in the plant.

In a recent review, a possible relationship between secondary and pyrophosphate (PPi) metabolisms was outlined, but the proposal was based on indirect evidence [24]. Pyrophosphate is a byproduct of the synthesis of DNA, RNA, proteins, and complex carbohydrates but can be produced in substantial amounts during the synthesis of terpenes [24]. *Capsicum annuum* can be a good model to study the connection between these compounds and the synthesis of secondary metabolites, especially if the cells can be manipulated genetically to study the effects of different genes, because the genome of this species is currently available [25].

The present report deals with transformed tissue cultures of *C. annuum* 'Serrano' as callus in solid medium, and as cell suspensions in liquid medium expressing a fusion of GFP and a soluble inorganic pyrophosphatase from *A. thaliana*. This transgenic transformation has the potential to alter the homeostasis of phosphate metabolism and perhaps alter the regulation of capsaicin production. However, testing such a possibility

requires a reproducible system to maintain transformed cells of *C. annuum*; it has to be one in which the cells can be induced to accumulate secondary metabolites. The purpose of the present work was to put these tools together using plant tissue culture techniques. In addition, the transformation was found to have an effect on the relative amounts of capsaicin and dihydrocapsaicin. This aspect was analyzed in more detail.

Material and methods

Chemicals and reagents

Adenine sulfate, myo-inositol, nicotinic acid, pyridoxine HCl, thiamine HCl, 2,4-dichlorophenoxyacetic (2,4-D), gibberellic acid (GA_3), capsaicin, TRIS base, $MgCl_2$, tetrasodium pyrophosphate, NaCl, HCl, malachite green, Triton X100 electrophoresis reagents, polyacrylate-polyol powder, and PEG 35000 were all purchased from Sigma-Aldrich. Polyvinylpyrrolidone was from Alfa Aesar. Sucrose, anhydrous sodium sulfate, iodine, absolute ethanol, dichloromethane, ethyl acetate, hexane, and other organic solvents were from J. T. Baker (Fisher Scientific). Anti-GFP primary antibodies were obtained from Santa Cruz Biotechnology Inc. Disodium EDTA, Silica gel 60 F254 plates and immunoblot reagents were from Merck-Millipore. NaCLO was acquired as 6% solution of commercial bleach. Murashige-Skoog salts, Gamborg's B5 vitamins and Gelzan were from Phytotech Labs. COMPLETE cocktail of protease inhibitors was acquired as tablets from Roche. Cocktail 20 (FQ-UNAM; patent in progress) is commercially available from the Tissue Culture Unit, Faculty of Chemistry, UNAM.

Plant material

Seeds of *C. annuum* 'Serrano' were surface sterilized by washing in 70% ethanol for 1 min and then soaked for 15 min in 2% NaClO plus 0.6% Triton-X100 while shaking, and then washed four times with deionized sterile water. After 24 h of imbibition in deionized sterile water, the seeds were planted in solid medium. This medium was Murashige–Skoog [26], adjusted to pH 5.7, supplemented with 3% (w/v) Gelzan and sterilized in an autoclave. Finally, it was supplemented with filter sterilized gibberellic acid (5.7 μM GA_3) and 3% sucrose before it solidified. The seeds were allowed to germinate and grow for 15 to 18 days in a growth room under a 16/8-h light (~180 μmol m^{-2} s^{-1} photon flux density) cycle, at 25°C (±2%) under sterile conditions.

Obtention of *C. annuum* callus and suspensions cultures, and their sensitivity to ammonium glufosinate

The hypocotyl, cotyledon, the fully differentiated primary leaf and shoot apex explants were tested for the induction of calli (15 to 40 explants of each one). Callus induction was tested in media MS-B5 and MS-C20. Both media contained Murashige–Skoog salts [26], adjusted to pH 5.7, and, in the case of solid media, 3% Gelzan, and then supplemented with filter-sterilized 3% sucrose, 5.4 μM adenine sulfate, 9.05 μM 2,4-dichlorophenoxyacetic (2,4-D), and 1 g L^{-1} polyvinylpyrrolidone. In addition, in MS-B5, the Murashige–Skoog vitamins [26] were replaced with Gamborg's B5 vitamins (0.55 mM myo-inositol, 8.1 μM nicotinic acid, 4.9 μM pyridoxine, and 29.7 μM thiamine HCl) [27], whereas in MS-C20, the Murashige–Skoog medium was supplemented with the Cocktail 20 (FQ-UNAM; patent in progress) of amino acids and vitamins, 0.02% casein hydrolysate, and supplemented with 23.5 μM L-proline.

Suspension cell cultures were started in 50-mL flasks with 30 mL liquid medium MS-B5 by inoculation of 1.5 g of hypocotyl-derived callus on each flask. Cultures were maintained in an orbital shaker with a 16/8-h light-day cycle, 25°C and 100 rpm for 15 days. After this time, the cultures were passed through a tissue disperser to fragment large aggregates, and then subcultivated as before, mixing 15 mL of culture and 15 mL of fresh medium.

Resistance to ammonium glufosinate (BASTA herbicide) was determined by addition of 0, 2, 4, or 8 mg L^{-1} of the herbicide (equivalent to 0, 10.1, 20.2, and 40.4 mM of ammonium glufosinate) to the tissue culture medium and monitoring the fresh weight of the culture. Five samples of 1 g of callus were used per treatment for the cultures in solid medium, and the increase in fresh weight was monitored after 1, 3, 5, 8, 10, 12, 15, 17, 19, 22, and 26 days. For liquid cultures, aliquots of 1 mL were withdrawn from the flasks after 1, 3, 5, 8, 10, 12, 15, 17, 19, and 22 days and, after centrifugation at 1,000 *g*, the accumulated mass was estimated by gravimetric analysis as the fresh weight of the pelleted material. The pellet then was dried to constant weight in a vacuum stove at 60°C and weighed again to record its dry weight. The appearance of the cultures was also monitored but not in a quantitative manner.

Biolistics transformation of *C. annuum* 'Serrano' callus

Bombardment of calli samples was done with a Bio-Rad PDS-1000 gun, 1,100 psi rupture discs, at 3-cm separation between the rupture and macrocarrier discs, and 12 cm separation between the macrocarrier disc and the target callus. The samples of the callus to be treated were subcultivated in MS-B5 medium for 8 days, transferred to a Petri dish with fresh solid MS-B5 medium in small fragments dispersed in circles of 1-cm diameter (approx.). Bombardment schemes tested were: (***i***) M5 microprojectiles (tungsten; 4 µm average diameter) shot once, (***ii***) M5 microprojectiles, shot twice, (***iii***) M10 microprojectiles (tungsten; 7 µm average diameter) shot once, and (***iv***) M10 microprojectiles, shot twice. Each transformation was done with six replicates (microprojectiles treated with DNA dissolved in deionized water) and three negative controls (empty microprojectiles treated with deionized water).

The DNA used for transformation was the plasmid pEARLY-gate 103::35S::AtPPa4-YFP, obtained as described elsewhere [28]. After bombardment, the transformed *C. annuum* 'Serrano' calli were allowed to recover for 48 h and selected using ammonium glufosinate (2 mg L^{-1}) for 3 weeks. This concentration was chosen based on the results shown in Fig. 2 and Fig. 3 (see "Results" section). Control calli were not selected with herbicide but were allowed to grow for the same time. After recovery and/or selection, calli were then subcultivated as cell suspensions for 2 weeks at 25°C under the following conditions: LC, MS-B5 medium, and 16/8-h photoperiod; DC, MS-B5 medium, and in the dark; LS, MS-B5 medium containing 40 mM NaCl [34], and 16/8-h photoperiod; DS, MS-B5 medium containing 40 mM NaCl, and in the dark. After incubation, the cells and the culture medium were separated by filtration through Whatman 1 filter paper. Both the callus and cell suspensions were used for the extraction of secondary metabolites. Samples of the callus were also used for the extraction as soluble protein as described next. Fresh samples from the callus in solid medium were also taken for their observation by means of a confocal microscope to detect at 527 nm the fluorescence of the yellow fluorescent protein (YFP), using an exciting light beam of 515 nm, as reported elsewhere [28].

Extraction of soluble protein, SDS-PAGE, immunoblot, and pyrophosphatase activity from *C. annuum* 'Serrano' callus, wild type (WT) or transformed

Medium-free callus samples were weighed and frozen in liquid nitrogen and powdered with a mortar and pestle. The powder was suspended in five volumes (w/v) of extraction buffer (0.1 M TRIS-HCl, pH 7.5, with 1 mM EDTA, 3 mM $MgCl_2$) supplemented with one tablet of COMPLETE protease inhibitor cocktail for each 50 mL (according to the manufacture's instructions). The suspension was centrifuged at 20,000 *g* at 4°C, and the supernatant was dialyzed against 100 volumes of extraction buffer and concentrated with polyacrylate-polyol powder and PEG 35000 scales applied to the outside of the dialysis membrane. The resulting solution was recovered from the bag and used for the determination of protein, pyrophosphatase activity, and SDS-PAGE. Pyrophosphatase activity was determined at pH 7.5, in 0.05 M TRIS-HCl, with 0.1 mM EDTA, with or without 3 mM $MgCl_2$ and 80 µM tetra-sodium pyrophosphate with malachite green reagent, as described by Navarro de la Sancha et al. [29]. Mg^{2+}-dependent pyrophosphatase

activity is the difference between the activity with Mg^{2+} (3 mM) minus the activity in the absence of this divalent cation. Protein was quantified using a modified Coomasie blue binding assay as described by Zor and Seelinger [30]. SDS-PAGE was performed using the Laemli technique [31] in 12% gels, and immunoblot was performed using antibodies against GFP, as described by Gutiérrez-Luna et al. [28].

Extraction and analysis of capsaicin from *C. annuum* 'Serrano' fruit and callus

Medium-free cell suspensions were obtained by filtration through Whatman 1 filter paper, weighed and allowed to dry at room temperature. For the fresh green fruit, the placenta was excised with a scalpel into thin sections, weighed, and allowed to dry at room temperature. Dry samples were mixed with 10 volumes of methanol (w/v) for 1 h and the solids were filtered through Whatman 1 filter paper in a Buchner funnel. The filtrate was centrifuged at 10,000 *g* (for debris removal), concentrated at reduced pressure and 40°C in a rotatory evaporator, then dissolved in a small amount of dichloromethane and transferred to small preweighed vials, where the solvent was allowed to evaporate. The dry residue was weighed and stored at −20°C in a nitrogen atmosphere.

The filtered medium recovered from cell suspension was extracted with 1:1 ethyl acetate in a separation funnel and extracted three more times. The recovered fractions were mixed and treated with anhydrous sodium sulfate. Then, these fractions were combined, evaporated to dryness, redissolved, weighed, and stored as before.

The presence of capsaicinoids accumulated in the calli under the different treatments (LC, DC, LS, or DS; see subsection "Biolistics transformation of *C. annuum* 'Serrano' callus" above) was compared using thin layer chromatography (TLC) with pure capsaicin as standard. Silica gel 60 F254 plates were developed with ethyl acetate/hexane (6/4), and the spots were developed using sublimated iodine. The spots were compared according with their relative mobility (Rf).

Processing of data from direct sample analysis-mass spectrometry

The identification of capsaicin and dihydrocapsaicin was achieved in a Perkin–Elmer AxION II DSA TOF MS mass spectrometer for direct sample analysis (DSA-MS). This equipment produces high resolution mass spectra of complex mixtures, rendering a rather complex profile corresponding to the overlap of molecular fingerprints of all compounds present in the sample. Then the separation of signals made using the Perkin–Elmer software. This software calculates the expected values of the quotient of ion mass/ion charge (the m/z ratio) for all the predicted molecular ions for the compounds of interest suspected to be present in the sample. These sets of molecular ions consider the existence of different isotopologues, in accordance with the natural abundance of isotopes for the elements forming the molecule. Then, it seeks for that set of signals and reports a confidence score and an experimental error value. All analyses were performed with at least three independent samples.

For the DSA-MS data from the AxION II DSA, the difference between the expected ion mass and the observed mass, as well as the expected sigma value of the natural isotope abundances, were used as criteria to ensure the correct identification of capsaicin and dihydrocapsaicin. Natural *C. annuum* plants are known to vary significantly in their spiciness and the tissue cultures did exhibit this trait (not shown). For this reason, no attempt was made to make this analysis strictly quantitative for the present work. Moreover, the manipulations required to obtain the tissue cultures and/or their transformation introduced additional sources of variability. However, we found the ratio of capsaicin to dihydrocapsaicin to be a highly reproducible feature. Commercial capsaicin (99%+ from Sigma-Aldrich) was used as the reference, and this standard was found to contain mostly capsaicin but contained about 10–20% of dihydrocapsaicin. The natural ratio of capsaicin to dihydrocapsaicin in the fresh fruit of *C. annuum* 'Serrano' was determined using an extract from the placenta tissue of the fresh fruit, collected from mature *C. annuum* plants.

Statistical analysis

To isolate statistically significant changes in accumulated mass of the cultures a two-way analysis of variance (ANOVA) was used to compare the effects of different treatments, with time and treatment as the main factors. When statistical significance at the $p < 0.05$ level was found, the individual differences were isolated using a post hoc Tukey's honest significant difference analysis. For simple mean comparisons between treatments only, we used Student's *t* test. These analyses were performed using R statistical software [32].

Results

Capsicum annuum still poses difficulties for genetic manipulation and plant tissue culture. Here, we took as a basis a previously reported protocol [8,33] in order to implement a reproducible method for the induction of callus from *C. annuum* 'Serrano', and then developed a protocol for stable genetic manipulation using biolistics. In this technique, stable integration of genetic constructs is possible with or without the genetic elements needed for *Agrobacterium*-mediated transformation of plant tissues.

Hypocotyls from *C. annuum* 'Serrano' gave a yellow-white and friable callus

In the search for a reproducible and efficient method to induce and propagate callus from *C. annuum* 'Serrano', the potential of hypocotyl, cotyledon, mature primary leaf and shoot apex explants for callus induction was tested in solid cultures using two different media, MS-B5 and MS-C20. Callus could be induced in both media after 2 weeks, but MS-B5 gave a greater efficiency of induction (judged from the larger mass of calli, see Fig. S1, panels A to D, with a lower fraction of oxidated explants), and the calli so obtained showed low signs of oxidation after 2 weeks (Fig. 1A–D). Out of 40 explants in medium MS-B5, all 40 formed calli and about 10% showed signs of oxidation after 2 weeks. At longer times, however, the signs of oxidation increased in all explants when left with the mother tissue (Fig. S1). Furthermore, all four types of explants produced abundant undifferentiated growing tissue, mainly at the site of wounding (Fig. 1A–D and Fig. S1A–E). However, upon subcultivation, only the hypocotyl-derived calli were consistently friable, maintained a yellow to yellow-brown healthy appearance, and showed reduced evidence of oxidative stress responses (Fig. 1E).

The hypocotyl-derived calli could be subcultivated several times without apparent deterioration (not shown). Nevertheless, for the transformation experiments, only samples from the first subcultivation round were used. The subcultured calli showed a nearly linear growth rate in solid medium MS-B5 for at least 3 weeks (Fig. 2, blue Box–Wilson plots).

In addition, the calli could effectively be transferred to suspensions, but a halt in growth was observed at fifth day after transfer and there was a loss of suspended material up to the twelfth day. Thereafter, the culture did grow again, nearly duplicating the initial culture density at the end of the 3-week period (Fig. 3, blue Box–Wilson plots).

Wild type callus and cell suspension culture of chili pepper were highly susceptible to glufosinate

The susceptibility of plant tissue cultures to common selection agents varies between species and sometimes between genotypes, so that in order to design effective selection protocols it is necessary to determine the sensitivity of each culture to the selection compound, in our case, ammonium glufosinate (BASTA herbicide).

Capsicum annuum 'Serrano' calli growing in solid medium were treated with 2, 4, 8, or 10 mg L^{-1} of glufosinate for 26 days. The results are presented in Fig. 2 and show a nearly complete halt to growth, even at the lowest dose tested, 2 mg L^{-1} (red). The ANOVA test followed by post hoc comparisons (Tukey's HSD) found statistical

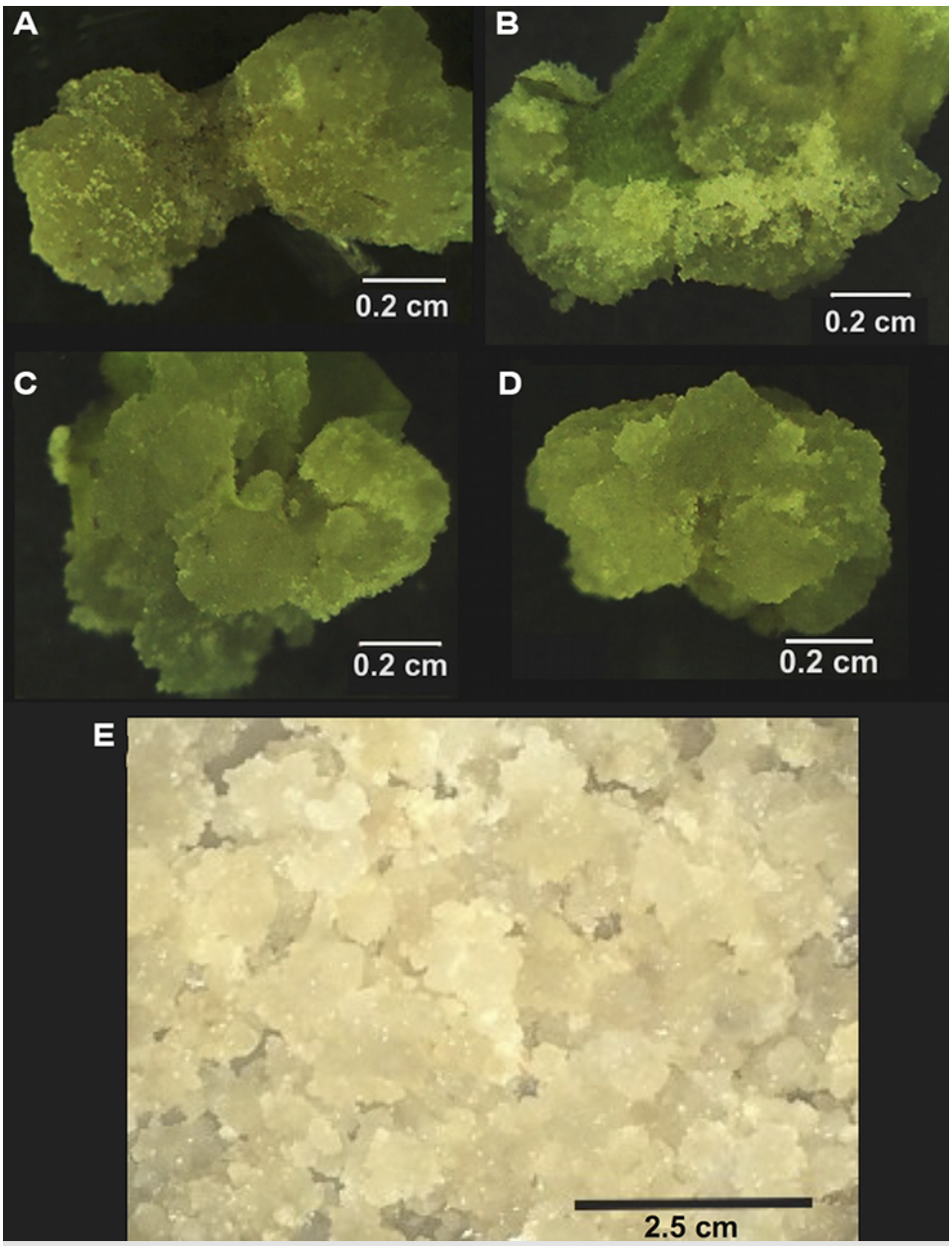
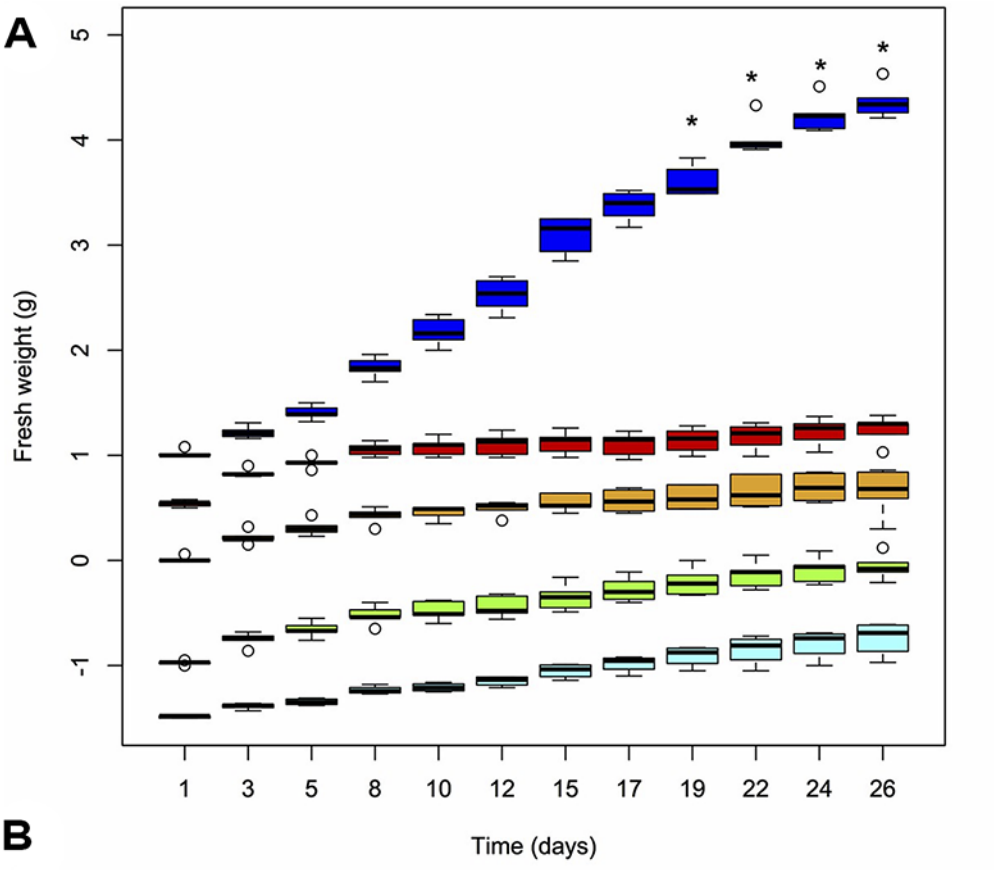


Fig. 1 Stereoscopic micrographs of callus from *C. annuum* 'Serrano'. Induction after 2 weeks in explants excised from (**A**) hypocotyl, (**B**) cotyledonary leaf, (**C**) primary leaf, and (**D**) apex shown at ×10 magnification. (**E**) Subcultivated hypocotyl-derived callus, 3 weeks after transfer shown at ×1.0 magnification.

significance ($p < 0.05$) in the differences between the accumulated mass of the control cultures without herbicide after 19 days growth or more, as compared to the cultures treated with glufosinate at any of the doses tested. The cultured cells were highly susceptible to this herbicide and differences between cultures treated with any pair of doses of glufosinate were not statistically significant; i.e., even the lowest dose tested (2 mg L^{-1}, i.e., 10.1 mM) was enough to completely arrest growth.

A similar test was done with cell suspensions grown in the absence or presence of 2, 4, or 8 mg L^{-1} glufosinate for 22 days, starting the culture at ~50 mg fresh weight (FW) of plant tissue per mL. This time, the samples were only 1 mL aliquots from the liquid suspension. The results are shown in Fig. 3, but meaningful comparisons can only be made after twelfth day, because after this time the subcultivated cells were adapted to the cell suspension conditions. Again, as observed for the solid medium, the control showed a mass increase after 22 days, but even the lowest herbicide dose tested (2 mg L^{-1}; equivalent to 10.1 mM) did prevent any mass increase in the treated samples. Then, except for the control, the final mass was similar to that loaded at zero



Factor	Df	Sum Sq	Mean Sq	F value	Prob. (>F)	Significance
Glufosinate	4	74.36	18.59	54.003	below 2×10^{-16}	*** (0.001)
Time	11	8.12	0.738	2.143	0.0184	* (0.05)
Interaction	44	7.19	0.163	0.475	0.998	none
Residuals	*231*	*79.52*	*0.344*			

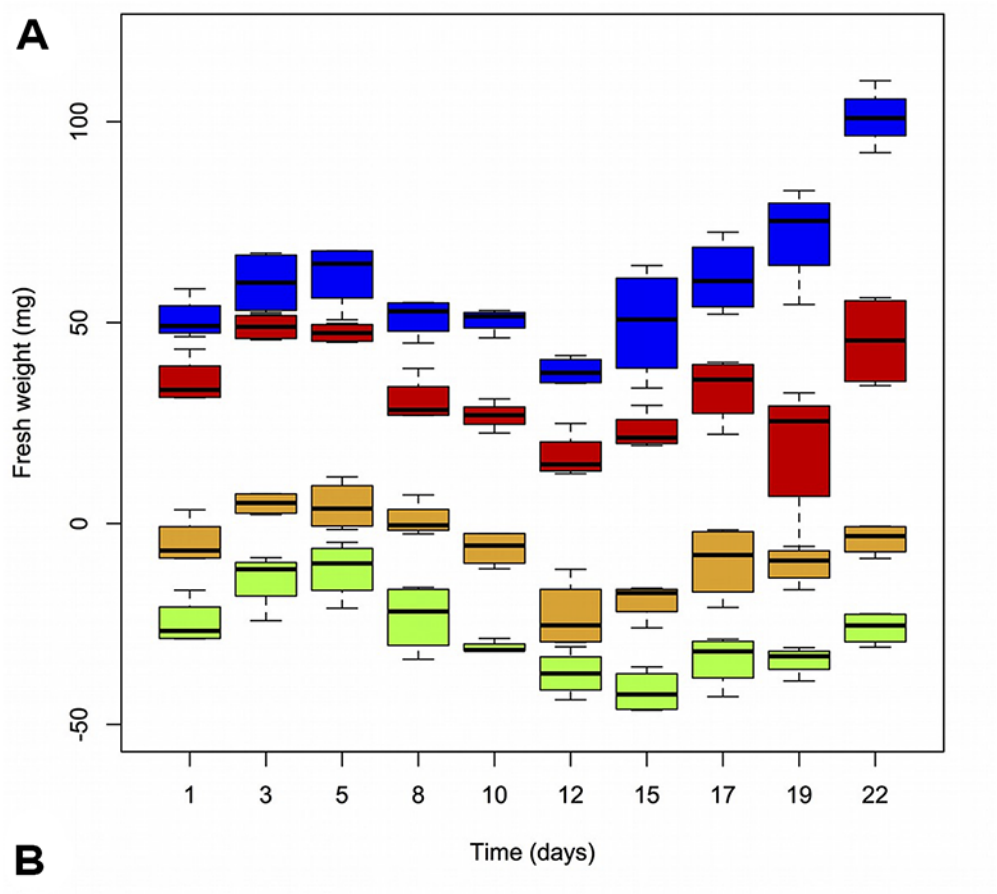
Fig. 2 Box–Wilson plots of the mass (**A**) of 1-g samples of *C. annuum* 'Serrano' calli against cultivation time in solid medium MS-B5 and in the absence (blue bars) or presence of glufosinate at 2 (red; offset[1] −0.5), 4 (orange; offset[1] −1.0), 8 (green; offset[1] −2.0), and 10 (cyan; offset[1] −2.5) mg L^{-1}. [1] For clarity, the plots for data in the presence of glufosinate were offset down, as indicated (* significant differences at $p < 0.05$ from time 0 and from glufosinate-treated cultures). The results from a two-way ANOVA are shown below (**B**) (*** significant differences at $p < 0.001$ apply to glufosinate factor globally). The plot (**A**) and ANOVA (**B**) were performed using R statistical software [32].

time for all treatments. Statistical comparison using ANOVA (Fig. 3B) did support the statistical significance for the mass differences between the absence of glufosinate or its presence (regardless of the dose). The dry weight of the tissue, relative to its fresh weight, did not vary significantly from the start of the culture to the last day sampled (22 days) and was 5.03 ±1.22% of the fresh weight (mean ± standard deviation).

Data in Fig. 2 and Fig. 3 reveal a high sensitivity of the cells from the *C. annuum* callus to the herbicide glufosinate, and therefore this compound appears to be a good selection agent for transformed callus of this plant species, at least for the 'Serrano' cultivar.

Biolistic genetic transformation was reproducible and showed acceptable efficiency

As already mentioned, when it comes to *Capsicum* spp., *Agrobacterium*-mediated protocols have been the preferred choice [4] and there is only scarce information regarding the effectiveness of biolistics transformation of the species from this genus [20]. Therefore, as a transformation alternative protocol, biolistics deserves consideration and here this method was tested using a construction similar to the one reported by Gutiérrez-Luna et al. [28] in their localization studies of the soluble inorganic



Factor	Df	Sum Sq	Mean Sq	F value	Prob. (>F)	Significance
Glufosinate	3	7458	2486.1	16.962	3×10^{-09}	*** (0.001)
Time	9	2202	244.7	1.669	0.104	none
Interaction	27	5595	207.2	1.414	0.106	none
Residuals	*120*	*17588*	*146.6*			

Fig. 3 Box–Wilson plots of the mass (**A**) of 1-mL samples of *C. annuum* 'Serrano' cell suspensions measured against cultivation time in solid medium MS-B5 and in the absence (blue bars) or presence of glufosinate at 2 (red; offset[1] −15), 4 (yellow; offset[1] −55), or 8 (green; offset[1] −75) mg L^{-1}. [1] For clarity, the plots for data in the presence of glufosinate were offset down, as indicated. The results from a two-way ANOVA are shown below (**B**) (*** significant differences at $p < 0.001$ apply to glufosinate factor globally). The plot and ANOVA were performed using R statistical software [32]. The average dry weigh was 5.03 ±1.22% of the fresh weight, and remained stable along the culture.

pyrophosphatases in *A. thaliana*. This construction has the pEARLY-gate 103 backbone carrying the glufosinate-resistant gene and the fusion of the CDS encoding the isoform 4 of the inorganic soluble pyrophosphatase (At3g53620, known as AtPPa4 protein) to the yellow fluorescent protein (YFP). This fused chimeric ORF is under the control of CAM35S promoter.

Several bombardment schemes were tested including different sizes of microprojectiles delivered in a single or a double shot to the tissue. Because this construction can drive the transgenic expression of the YFP in fusion with the AtPPa4 pyrophosphatase, the protein expression can be observed using fluorescence confocal microscopy. Fig. 4 shows the fluorescent signal corresponding to the YPF expressed in the callus of *C. annuum* 'Serrano' transformed using biolistics, and after a recovery period. There was a fluorescent signal in all panes where bombardment performed with microprojectiles carrying plasmidic DNA (T1+, T2+, T3+, T4+ in Fig. 4), as expected for the YFP expression in the callus after plasmidic DNA integration (pEARLY-gate 103::35S::AtPPa4-YFP). Out of six trials, we found fluorescent cells for treatment T2+ in all six, in only five for T4+, and only in four and three for treatments T1+ and T3+, respectively. The signal was found inside the cells but also in the intercellular space as reported for this protein in transgenic *A. thaliana* plants [28]. By contrast, cultures bombarded with empty microprojectiles gave no signal above the background (T1−, T2−, T3−, T4− in Fig. 4).

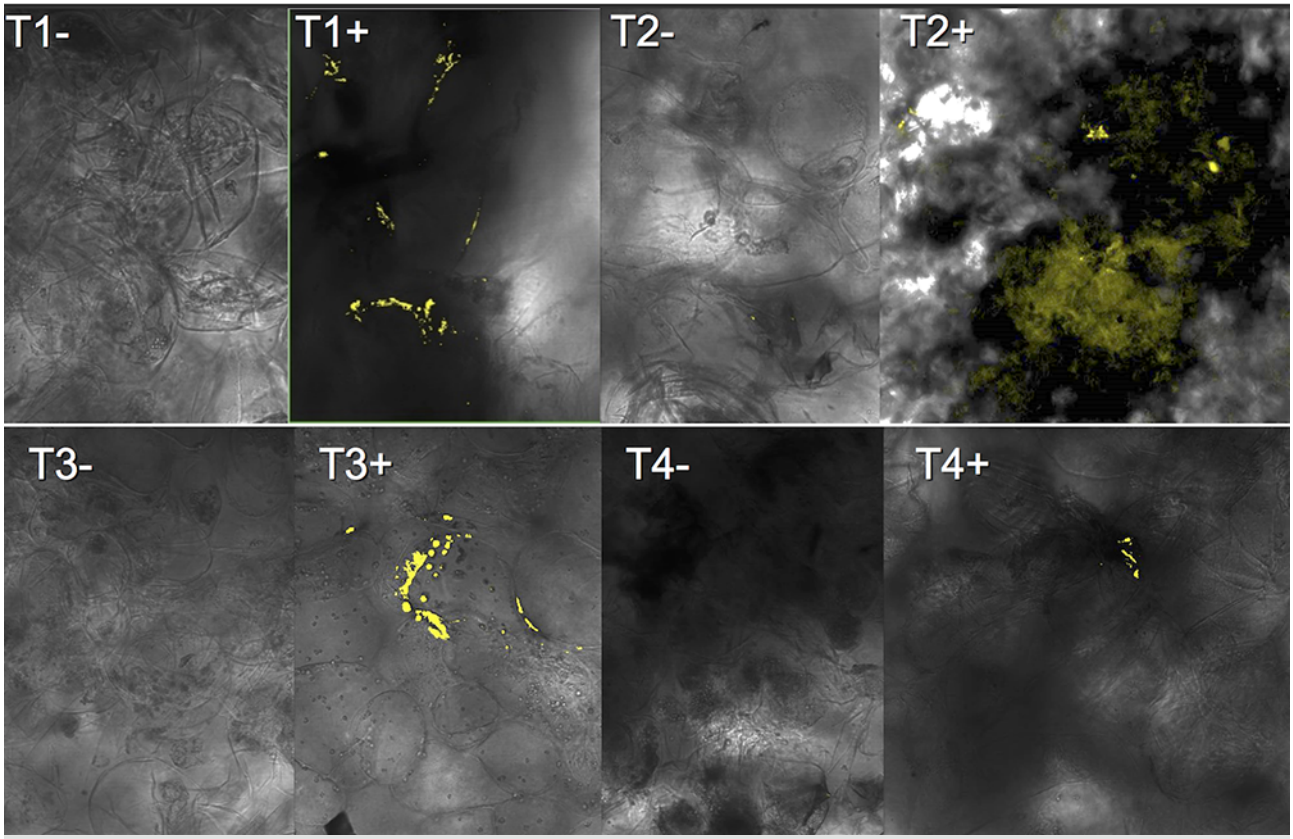


Fig. 4 Confocal microscopy images of in vitro cultured callus of *C. annuum* 'Serrano' showing the overlap of phase contrast and YFP fluorescent signal. Samples from cells growing in MS-B5 solid medium shoot once (T1+, T1−, T3+, T3−) or twice (T2+, T2−, T4+, T4−), with M5 (T1+, T1−, T2+, T2−), or M10 (T3+, T3−, T4+, T4−) microprojectiles, without (T1−, T2−, T3−, T4−) or loaded with plasmid pEARLY-gate 103::35S::AtPP4-YFP (T1+, T2+, T3+, T4+) and allowed to recover for 5 days, before observation of fresh cell clusters.

However, the signal covered a wider area and the intensity was more evenly distributed when DNA was loaded on M5 microprojectiles and the tissue received two shots (T2+ in Fig. 4). This scheme was therefore used for all transformations thereafter. In addition, to further test if the expression of the protein was stable, calli were bombarded using the T2+ or the T2− schemes. After the recovery period, the cells bombarded with DNA were selected using MS-B5 solid medium with 2 mg L^{-1} ammonium glufosinate for 3 weeks, whilst the wild-type cells were grown in MS-B5 medium for 3 weeks. The total protein was then extracted from the samples for analysis. The crude extract before and after dialysis was found to fluoresce well above the background fluorescence of natural proteins in the sample from wild-type cells (Fig. S2A). When analyzed by immunoblot using antibodies against the fluorescent protein, the presence of a single protein band was detected only in the transformed calli. The molecular weight of the band was slightly over 50 kDa, corresponding to the expected size of the fusion protein (Lanes 6 and 7 in Fig. S2B). The band was absent in the wild-type cells even if the membrane was overexposed (not shown). The expression was weak as reported for the transgenic *A. thaliana* plant carrying the same construct [28]. We also compared the total Mg^{2+}-dependent pyrophosphatase activity, determined as described here in "Material and methods", but there was only a small (significant) increase in specific activity (Tab. S1), along with a reduction in total protein. An increase in an unidentified protein band at ~20 kDa (Lanes T1, T2, and T3 in Fig. S3) was observed but not pursued further.

Cell suspensions grown under dark and salt stress did accumulate capsaicinoids

Here, salt stress [34] and darkness [35] conditions were tested independently and together as possible inducers of the synthesis and accumulation of capsaicinoids in *C. annuum* 'Serrano'. Cell suspensions of this species were therefore incubated in MS-B5 basal medium under a 16/8-h day cycle (L) or in the dark (D), without (C) or with 40

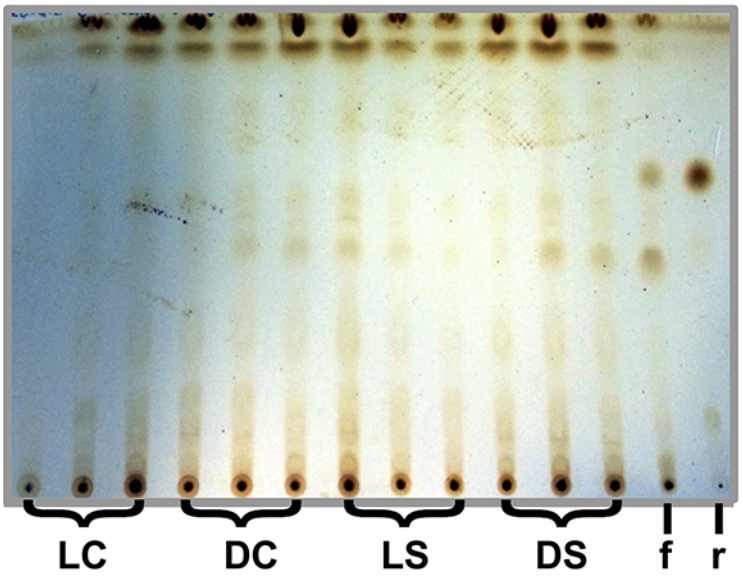


Fig. 5 Thin layer chromatography of secondary metabolites extracted from *C. annuum* 'Serrano' samples. Cell suspensions of WT cells were grown in liquid medium under a 16/8-h light cycle (LC) or in the dark (DC), or in medium with 40 mM NaCl under a 16/8-h light cycle (LS) or with NaCl 40 mM and in the dark (DS). The extract from the natural green fruit (f) and a standard of purified capsaicin (r) is also shown. Arrows show the positions expected for capsaicin and dihydrocapsaicin.

mM NaCl (S) to determine if capsaicinoids were produced. The resulting extract was initially analyzed by TLC and the spots near the expected Rf of capsaicin and dihydrocapsaicin were compared against a standard of pure capsaicin and a sample of extract from the natural placenta tissue of the *C. annuum* 'Serrano' green fruit. In Fig. 5, Lane r shows the spot of the capsaicin standard and a light signal below at the position where dihydrocapsaicin is expected. Fig. 5 (Lane f) shows the same two spots, now with similar intensity in the green fruit extract, consistent with the presence of capsaicin and a possibly dihydrocapsaicin. In the extracts from cell suspensions grown in MS-B5 medium and 16/8-h light cycle, only a weak signal was observed at both positions (LC in Fig. 5) but the signals apparently increase in intensity when the cell suspensions were grown in darkness without NaCl (DC in Fig. 5), in the light but with NaCl added (LS in Fig. 5), or both with NaCl and in the dark (DS in Fig. 5). In addition to these spots, some minor components were observed in the extracts from the fruit and the cell suspensions (Fig. 5), and this was expected, since the natural extract does include a mixture of capsaicin and dihydrocapsaicin, with a minor contribution of other components possibly including nordihydrocapsaicin, homodihydrocapsaicin, homocapsaicin, and other compounds. TLC does not offer the resolution needed to identify the spots with enough confidence, and capsaicin or dihydrocapsaicin are stained with different intensity by iodine. The extracts were therefore analyzed using the AxION 2 mass spectrometer (DSA-MS). The equipment was calibrated using the recommended protocols of Perkin–Elmer and then the samples were applied in metal grids for ionization. Fig. 6 shows the ion peaks identified by the software and having the expected *m*/*z* ratio for capsaicin (306.2064) and dihydrocapsaicin (308.222) which were found in the capsaicin standard (Fig. 6A). The results shown in Fig. 6 confirm the presence of both capsaicin and dihydrocapsaicin in the samples from fresh *C. annuum* 'Serrano' fruits (Fig. 6B) as well in the extract from the suspension cell cultures treated with NaCl in continuous darkness (Fig. 6C); this is in agreement with the TLC data. For other treatments, the TLC spots are faint but DSA-MS was able to detect the compound signature with good confidence (see next section). In general, the mass error was between 5 and 300 mg L^{-1} (see legend to Fig. 6 for averages by sample type), depending mostly on the abundance of the compound in the sample and the sample complexity. The deviation from the expected distribution of ions, according to the natural isotopic abundance (Fig. 6A–D) was from 5% to 20%. This was the case for capsaicin (Fig. 6D,F) and for dihydrocapsaicin (Fig. 6E,G), as

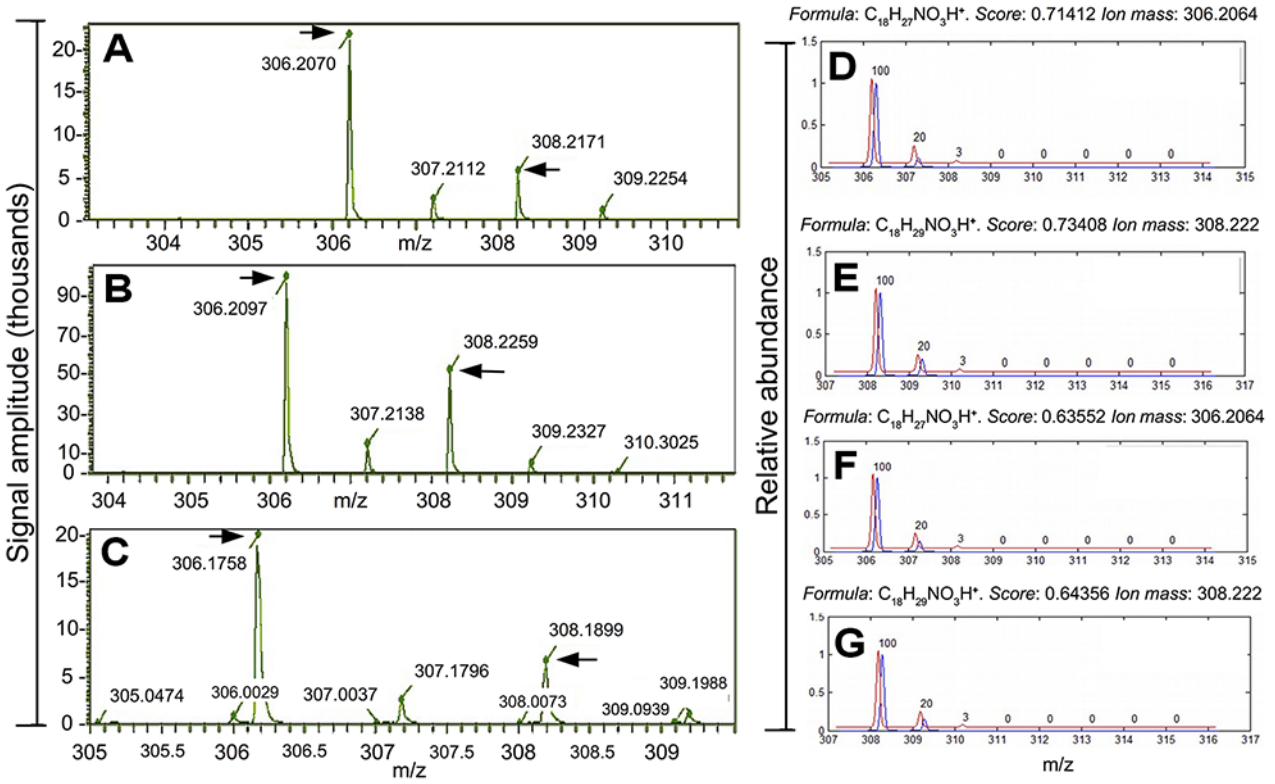


Fig. 6 Mass spectrometry peaks of capsaicin standard (**A**), *C. annuum* 'Serrano' extracts from green fruit (**B**), and from cell suspensions grown in MS-B5 liquid medium with 40 mM NaCl and in the dark (**C**). The signals corresponding to species of natural isotopic abundance were isolated using the Perkin–Elmer software and compared to the expected profile for capsaicin (**D,F**), or dihydrocapsaicin (**E,G**), for both the sample in (**A**), panes (**D,E**); and in (**C**), panes (**F,G**). The average (±*SD*) mass error for capsaicin and dehydrocapsaicin, in ppm (= mg L^{-1}), was 26.1 (±13.1) for the standard and extracts from fruit placenta, 203 (±70) for extracts from suspension cultures of WT cells, and 219 (±114) for extracts from suspension cultures of transformed cells, and the deviation from the expected isotopic mass distribution was from 5% to 20%. The arrows (**A–C**) mark the peaks of capsaicin (right arrow) and the dihydrocapsaicin (left arrow). For an explanation of *m/z* ratios and the meaning of mass error, see "Material and methods".

shown for the standard (Fig. 6D,E), and for the culture treated with 40 mM NaCl and kept in the dark (Fig. 6F,G), the isotopic distribution data for other samples followed the same trend (not shown). Although the analysis was not intended to be quantitative, samples with very low amounts of capsaicinoids (such as the extract shown in Fig. 5, LC) showed the largest error. The presence of capsaicin and dihydrocapsaicin in the cell suspension cultures of *C. annuum* 'Serrano' was confirmed by the MS data. Again, no attempt was made to accurately quantify the compounds, but when a sample corresponding to the same fresh weight of tissue was applied to the MS equipment, a considerably larger peak area and peak height was found for the fruit (Fig. 6B) than for the wild-type cell suspension extract (Fig. 6C). This is in agreement with the TLC data in Fig. 5 and the previously reported data of capsaicinoid accumulation by tissue cultures of *Capsicum* spp. [34,35]. Furthermore, the AxION 2 DSA-MS can be used to quantify the relative content of compounds within the sample. This feature was then used to analyze the relative content of capsaicin to dihydrocapsaicin in extracts from cell suspension cultures.

Expression of a transgenic *Arabidopsis thaliana* soluble inorganic pyrophosphatase was accompanied by an alteration in capsaicin to dihydrocapsaicin ratio

Using the previously described protocols, the gene encoding the amino acid sequence of the *A. thaliana* soluble pyrophosphatase, isoform 4, was expressed in transformed callus of *C. annuum* 'Serrano'. The transformed cells were cultured under conditions to induce capsaicinoids accumulation, as described in the previous section. Then,

using the AxION 2 mass spectrometer we analyzed the relative levels of capsaicin and dihydrocapsaicin in these cell cultures, and compared the ratio of capsaicin to dihydrocapsaicin found in cell cultures of wild-type cells growing in the same conditions. Plant transgenesis with biolistics is subjected to variability and this is somehow evident in the data from Fig. 5, where three extracts from independent cultures were included. This transformation was therefore independently repeated three times and the independent cultures were analyzed for capsaicin to dihydrocapsaicin ratios.

The first observation was an increase in the apparent health of the cell suspensions (Fig. S4A–D, as compared to Fig. S4E–H), the cultures appeared less pigmented, usually a sign of oxidative stress. In addition, a slight increase in the intensity of the capsaicin spots in comparison with the cell suspension cultures of wild-type cells under the same growth conditions (not shown). However, because of the sources of variability in the transformation discussed above, the obtention of stable lines is currently in progress to take this analysis to a quantitative level. This, however, will require lengthy selection protocols, more controls and a detailed genetic characterization of the plant materials, so it will be the subject of a future work.

Fig. 6 shows the presence of both dihydrocapsaicin and capsaicin in both the wild-type callus and the natural fruit. There was a higher amount of capsaicin relative to dihydrocapsaicin, as expected. Therefore, the manipulations performed to induce and maintain the in vitro cell suspension cultures did not seem to alter the mechanisms controlling the ratio of capsaicin to dihydrocapsaicin accumulation in this undifferentiated proliferative plant tissues.

The ratio of capsaicin to dihydrocapsicin was then determined for the extracts from untransformed and transformed cell suspension cultured in medium MS-B5 under standard conditions, with a day/night cycle, or in the dark, without or with NaCl 40 mM. The cultures obtained from callus bombarded with empty microprojectiles did show the same capsaicin to dihydrocapsicin profile (not shown), as those from non-bombarded calli, or the fruit extract. The ratio seems also to be insensitive to the bombardment stress. However, when the extracts from samples of the cell suspensions transformed with the pEARLY-gate 103::35S::AtPP4-YFP vector were analyzed using the AxION-2 DSA-MS, there was an increase in the accumulation of dihydrocapsaicin relative to that of capsaicin (tr vs. wt in Fig. 7), which was observed for all the transformed cell suspensions cultivated in the DC, LS, and DS conditions, and although the peak areas were small, the altered ratio was also observed for the LC treatment. The ANOVA and Tukey HSD post hoc analyses (Fig. 7, Greek letters) found this difference to be statistically significant at the $p < 0.05$ level. Because the change in the ratio was consistently found in all samples from transformed cell suspension cultures (tr in Fig. 7), and in none of the extracts from the natural green fruit or the wild-type cell suspensions (wt in Fig. 7), the change can be attributed to the transformation with good confidence, although there are insufficient data to speculate about the mechanism involved.

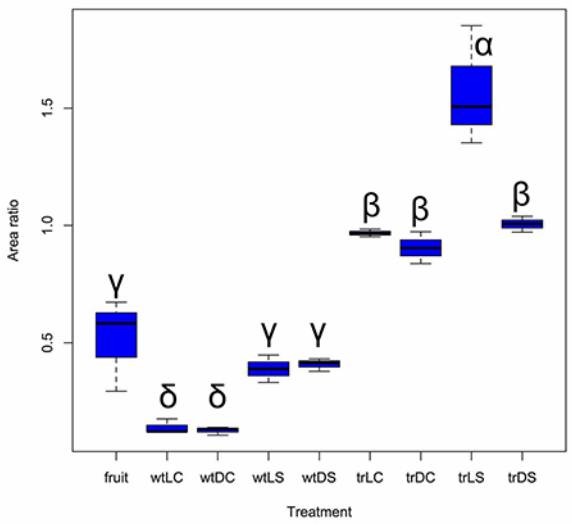


Fig. 7 Ratio of the content of dihydrocapsaicin to capsaicin in fruit and cell suspension cultures of *C. annuum* 'Serrano' quantitated as the ratio of the respective peak areas in DSA-MS (area ratio). Labels: fruit – *C. annuum* 'Serrano' extract from the green fruit; wt – extract from suspension cells cultured after bombardment with microprojectiles treated with deionized water; tr – extract from suspension cells cultured after bombardment with plasmid loaded microprojectiles; LC – DC, LS, DS, as in Fig. 5. Different Greek letters over the boxes indicate statistically significant ($p < 0.05$) differences (ANOVA and Tukey's HDS; R statistical software [32].

Discussion

Capsicum annuum is a crop of economic importance worldwide and methods to accelerate the selection of varieties and lines with improved agronomic and nutritional traits that are a relevant biotechnological goal. Many protocols to obtain transformed plants of crop species from the genus *Capsicum* suffer from low efficiency and strong dependency on specific genotypes [4], but also lack of reproducibility and suffer from loss of the transgene [6]. Thus, there is still work to be done regarding the exploration of protocols, especially those

providing tools for cell genetic transformation, which can be used for research, and eventually for technological applications.

Mainly the meristematic zones from different tissues have been shown to form calli, and our results agree with an earlier paper [8] which reported a good response of hypocotyls from *C. annuum* 'Serrano' to the induction of adventitious shoots. However, in our protocol, the formation of adventitious shoots was avoided by the use of an appropriate combination of phytohormones and regulators (3% sucrose, 5.4 μM adenine, and 9.05 μM 2,4-D), and the induction of calli was observed from several tissues of *C. annuum* 'Serrano'. Furthermore, the hypocotyl was the most effective starting material, since there was a 100% response in the induction and these calli remained friable and free of oxidation after several weeks.

Our goal was to develop an efficient and reproducible protocol for the obtention of calli from *C. annuum*, as a tool to aid the research of the genetics and biochemistry in this species, because reports of transgenic plants from species of the genus *Capsicum* are indeed limited to a few cases [16,17,20,33]. Here we used commercial seeds of the 'Serrano' variety and observed a uniform response. Similar experiments are in progress with other *Capsicum* species and the protocol seems to be less dependent of the phenotype (not shown). In addition, the use of biolistics for the transformation of *C. annuum* tissue cultures has been explored infrequently [20]. This possibly relates to the smaller number of transgene copies integrated, a reduced tissue manipulation and cell stress with *Agrobacterium*-mediated transformation, which translates into an increased survival and a higher yield of transformed cells. However, selection agents must eliminate untransformed plant cells and remaining bacterial cells, and it also requires a DNA construct with additional genetic elements to support the bacterial transformation.

Min et al. [33] reported a protocol for *Agrobacterium*-mediated transformation of the *C. annuum* inbreed lines P915 and P409, taking advantage of the ability of those cultivars to generate shoots in primary tissue culture. This group also stressed the need for careful selection methods because the explants produce shoots from both the growing callus and the hypocotyl explant itself, which reduced the effective recovery of transformants and their later establishment. In the case of *C. annuum*, the transformation protocols still suffer from low efficiency, and both *Agrobacterium*-mediated and biolistics protocols for plant cell transformation require effective selection methods to eliminate the untransformed cells.

Ammonium glufosinate (BASTA herbicide) has effectively been used for the selection of transformed plant cells because most wild-type crops are susceptible to this herbicide. However, the susceptibility varies according to the species and sometimes the genotype. From our results, *C. annuum* 'Serrano' was found to be very sensitive to ammonium glufosinate (Fig. 2 and Fig. 3). Resistance to this herbicide was found to be an effective marker for the selection of transformed cells (Fig. S2).

A successful transformation and selection does not guarantee culture suitability for metabolic and biochemical studies since the selection agent causes some stress to the culture. It is necessary to investigate if the transformed cells are still responsive to other kinds of stress, and if these cells do respond in a fashion similar to wild-type cultured cells. When the cultures were dispersed in liquid culture we found a lag in growth, which can be ascribed to an adaptive period to the new conditions. These presumably impose some stress to the callus, but both wild-type and transformed cultures could resume growth under the liquid culture.

There are reports of secondary metabolite production of *Capsicum* spp. cells growing under tissue culture conditions. The two factors reported to induce the accumulation of capsaicinoids in cultures of tissue cultures of *C. annuum* are osmotic (salt) stress [34] and darkness [35]. Here, both WT and transformed lines showed accumulation of capsaicinoids in response to continuous darkness and to salt stress (Fig. 5 and Fig. 6), in agreement with the previous reports [34,35].

Plant cell metabolism is characterized by a complex network of interrelated pathways, controlled by an exquisite signaling network acting on gene expression and modulating regulatory proteins and enzyme activity. This rather intricate arrangement of metabolic and signal transduction pathways gives these sessile organisms high plasticity in their responses to environmental fluctuations [24]. Due to this complexity, a shift in environmental conditions or the altered expression of an enzyme may correlate with an

unsuspected change in the metabolic activity of a pathway with little or no apparent connection. These unexpected changes may help uncover novel connections in the metabolic networks, and in turn, may help to reveal sophisticated adaptive mechanisms of the plant cells to stressful conditions.

In *C. annuum*, the production of secondary metabolites such as terpene pigments, capsinoids and capsaicinoid, has a special interest. Thus, an additional contribution of our work was the successful application of DSA-MS for a rapid analysis of the ratio of capsaicin to dihydrocapsaicin in tissue cultures of *C. annuum*.

Plant transgenesis with biolistics is subjected to a number of stochastic factors such as: (***i***) the number of copies integrated, (***ii***) the position in the genome of each integrated copy, and (***iii***) the intrinsic genetic variability of the plant cells. The variability due to these three factors is somehow evident in the data from Fig. 5, where three extracts from independent cultures were included. However, when this transformation was independently repeated three times and the these cultures were analyzed using DSA-MS, reproducible data were obtained. Despite the complexity of the sample, the equipment was able to resolve the relevant peaks with good confidence, giving similar data for the standard (Fig. 6A,D,E) and for the complex tissue extracts (Fig. 6B,C,F,G).

In the present report, the goal was setting up the tools for future studies and we demonstrated the successful transformation associated to the resistance to ammonium glufosinate, the expression of a fluorescent protein, and the detection of a band by immunoblot. The difficulties in detecting the increase in enzyme activity is not surprising and has been discussed elsewhere, when the same construct was expressed in *A. thaliana* [28]. The same limitations apply here. Further development of this technique could establish a rapid and efficient protocol for the analysis of the relative contents of alkamide metabolites in plants, and we are currently testing different internal standards to make the technique fully quantitative. Unfortunately, the high water content of the cell suspension cultures demanded a lengthy extraction protocol, but we will endeavor to reduce times whilst maintaining good yields. Finally, as part of future work we are currently testing if our protocol can be applied to the in vitro synthesis of ^{15}N- and ^{13}C-labeled capsaicinoids, at low cost. We intend to test these compounds as DSA-MS calibration standards with applicability to agriculture and the food industry.

Conclusions

The present work established a successful method to obtain callus and cell suspensions of *C. annuum* 'Serrano', viable for months. In contrast to previous reports, we achieved stable transformation using biolistics, and the transformed cell suspensions were able to grow and accumulated capsaicin and dihydrocapsaicin in response to salt stress, as did the wild-type cell suspensions. Furthermore, the expression of recombinant fusion protein was accompanied by a change in the ratio of capsaicin to dihydrocapsaicin, and from the result, a relationship between pyrophosphate and capsaicin metabolism may be hypothesized.

Acknowledgments

We thank Perkin–Elmer de México for the facilities to use de Perkin Elmer AxION II DSA TOF MS instrument. We thank the technical assistance of Eng. Cesar J. López Lara from ESIQIE Instituto Politécnico Nacional, Dr Beatriz King-Díaz from Faculty of Chemistry, and Dr Liliana C. Córdoba Albores from Universidad Autónoma del Estado de Morelos.

Supplementary material

The following supplementary material for this article is available at http://pbsociety.org.pl/journals/index.php/aa/rt/suppFiles/aa.1792/0:

Fig. S1 Explants and callus induction.

Fig. S2 Total fluorescence under UV light illumination and immunoblot with anti-GFP antibodies of protein extracts from *C. annuum* L. calli.

Fig. S3 Silver-stained SDS-PAGE of soluble protein extracts for *C. annuum* L. calli.

Fig. S4 Cell suspensions of *C. annuum* L.

Tab. S1 Protein concentration and Mg^{2+}-dependent pyrophosphatase activity in crude extracts from solid tissue cultures of *C. annuum* L.

References

1. Paran I, van der Knaap E. Genetic and molecular regulation of fruit and plant domestication traits in tomato and pepper. J Exp Bot. 2007;58(14):3841–3852. https://doi.org/10.1093/jxb/erm257
2. Wahyuni Y, Ballester A, Sudarmonowati E, Bino RJ, Bovy AG. Metabolite biodiversity in pepper (*Capsicum*) fruits of thirty-two diverse accessions: variation in health-related compounds and implications for breeding. Phytochemistry. 2011;72(11–12):1358–1370. https://doi.org/10.1016/j.phytochem.2011.03.016
3. Wahyuni Y, Ballester A, Sudarmonowati E, Bino RJ, Bovy AG. Secondary metabolites of *Capsicum* species and their importance in the human diet. J Nat Prod. 2013;76(4):783–793. https://doi.org/10.1021/np300898z
4. Kothari SL, Joshi A, Kachhwaha S, Ochoa-Alejo N. Chilli peppers – a review on tissue culture and transgenesis. Biotechnol Adv. 2010;28(1):35–48. https://doi.org/10.1016/j.biotechadv.2009.08.005
5. Gammoudi N, Pedro TS, Ferchichi A, Gisbert C. Improvement of regeneration in pepper: a recalcitrant species. In Vitro Cell Dev Biol Plant. 2018;54:145–153. https://doi.org/10.1007/s11627-017-9838-1
6. Barchenger DW, Lamour KH, Bosland PW. Challenges and strategies for breeding resistance in *Capsicum annuum* to the multifarious pathogen, *Phytophthora capsici*. Front Plant Sci. 2018;9:628. https://doi.org/10.3389/fpls.2018.00628
7. Kehie M, Kumaria S, Tandon P, Ramchiary N. Biotechnological advances on in vitro capsaicinoids biosynthesis in *Capsicum*: a review. Phytochem Rev. 2014;14(2):189–201. https://doi.org/10.1007/s11101-014-9344-6
8. Ochoa-Alejo N, Ireta-Moreno L. Cultivar differences in shoot-forming capacity of hypocotyl tissues of chilli pepper (*Capsicum annuum* L.) cultured in vitro. Sci Hortic (Amsterdam). 1990;42(1–2):21–28. https://doi.org/10.1016/0304-4238(90)90144-4
9. Cheng Y, Ma R, Jiao Y, Qiao N, Li T. Impact of genotype, plant growth regulators and activated charcoal on embryogenesis induction in microspore culture of pepper (*Capsicum annuum* L.). S Afr J Bot. 2013;88:306–309. https://doi.org/10.1016/j.sajb.2013.08.012
10. Heidmann I, de Lange B, Lambalk J, Angenent GC, Boutilier K. Efficient sweet pepper transformation mediated by the BABY BOOM transcription factor. Plant Cell Rep. 2011;30(6):1107–1115. https://doi.org/10.1007/s00299-011-1018-x
11. Horstman A, Li M, Heidmann I, Weemen M, Chen B, Muino JM, et al. The BABY BOOM transcription factor activates the LEC1-ABI3-FUS3-LEC2 network to induce somatic embryogenesis. Plant Physiol. 2017;175:848–857. https://doi.org/10.1104/pp.17.00232
12. Gunay AL, Rao PS. In vitro plant regeneration from hypocotyl and cotyledon explants of red pepper (*Capsicum*). Plant Sci Lett. 1978;11(3–4):365–372. https://doi.org/10.1016/0304-4211(78)90024-X
13. Haque SM, Ghosh B. An improved micropropagation protocol for the recalcitrant plant *Capsicum* – a study with ten cultivars of *Capsicum* spp. (*C. annuum*, *C. chinense*, and *C. frutescens*) collected from diverse geographical regions of India and Mexico. J Hortic Sci Biotechnol. 2018;93(1):91–99. https://doi.org/10.1080/14620316.2017.1345331
14. Balázs E, Bukovinszki Á, Csányi M, Csilléry G, Divéki Z, Nagy I, et al. Evaluation of a wide range of pepper genotypes for regeneration and transformation with an *Agrobacterium tumefaciens* shooter strain. S Afr J Bot. 2008;74(4):720–725. https://doi.org/10.1016/j.sajb.2008.05.005
15. Liu J, Yu Y, Lei J, Chen G, Cao B. Study on *Agrobacterium*-mediated transformation of pepper with *Barnase* and *Cre* gene. Agric Sci China. 2009;8(8):947–955. https://doi.org/10.1016/S1671-2927(08)60299-0
16. Ko MK, Soh H, Kim K, Kim YS, Im K. Stable production of transgenic pepper

plants mediated by *Agrobacterium tumefaciens*. HortScience. 2007;42(6):1425–1430. https://doi.org/10.21273/HORTSCI.42.6.1425

17. Kreuze JF, Valkonen JP. Utilization of engineered resistance to viruses in crops of the developing world, with emphasis on sub-Saharan Africa. Curr Opin Virol. 2017;26:90–97. https://doi.org/10.1016/j.coviro.2017.07.022
18. Bawa AS, Anilakumar KR. Genetically modified foods: safety, risks and public concerns-a review. J Food Sci Technol. 2013;50(6):1035–1046. https://doi.org/10.1007/s13197-012-0899-1
19. Timmons AM, Charters YM, Crawford JW, Burn D, Scott SE, Dubbels SJ, et al. Risks from transgenic crops. Nature. 1996;380(6574):487. https://doi.org/10.1038/380487a0
20. Ochoa-Alejo N, Ramirez-Malagon R. In vitro chili pepper biotechnology. In Vitro Cell Dev Biol Plant. 2001;37:701–729. https://doi.org/10.1007/s11627-001-0121-z
21. Nugroho LH. Red pepper (*Capsicum* spp.) fruit: a model for the study of secondary metabolite product distribution and its management. AIP Conf Proc. 2016;1744:020034. https://doi.org/10.1063/1.4953508
22. Phimchan P, Chanthai S, Bosland PW, Techawongstien S. Enzymatic changes in phenylalanine ammonia-lyase, cinnamic-4-hydroxylase, capsaicin synthase, and peroxidase activities in *Capsicum* under drought stress. J Agric Food Chem. 2014;62(29):7057–7062. https://doi.org/10.1021/jf4051717
23. Turner LB, Wellburn AR. Changes in adenylate nucleotide levels in the leaves of *Capsicum annuum* during water stress. J Plant Physiol. 1985;120(2):111–122. https://doi.org/10.1016/S0176-1617(85)80015-8
24. Gutiérrez-Luna FM, Hernández-Domínguez EE, Valencia-Turcotte LG, Rodríguez-Sotres R. Review: pyrophosphate and pyrophosphatases in plants, their involvement in stress responses and their possible relationship to secondary metabolism. Plant Sci. 2018;267:11–19. https://doi.org/10.1016/j.plantsci.2017.10.016
25. Kim S, Park M, Yeom S, Kim Y, Lee JM, Lee H. et al. Genome sequence of the hot pepper provides insights into the evolution of pungency in *Capsicum* species. Nat Genet. 2014;46(3):270–278. https://doi.org/10.1038/ng.2877
26. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant. 1962;15(3):473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x
27. Gamborg OL, Miller RA, Ojima K. Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res. 1968;50(1):151–158. https://doi.org/10.1016/0014-4827(68)90403-5
28. Gutiérrez-Luna FM, Navarro de la Sancha E, Valencia-Turcotte LG, Vázquez-Santana S, Rodríguez-Sotres R. Evidence for a non-overlapping subcellular localization of the family I isoforms of soluble inorganic pyrophosphatase in *Arabidopsis thaliana*. Plant Sci. 2016;253:229–242. https://doi.org/10.1016/j.plantsci.2016.10.005
29. Navarro de la Sancha E, Coello-Coutiño MP, Valencia-Turcotte LG, Hernández-Domínguez EE, Trejo-Yepes G, Rodríguez-Sotres R. Characterization of two soluble inorganic pyrophosphatases from *Arabidopsis thaliana*. Plant Sci. 2007;172(4):796–807. https://doi.org/10.1016/j.plantsci.2006.12.011
30. Zor T, Selinger Z. Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. Anal Biochem. 1996;236(2):302–308. https://doi.org/10.1006/abio.1996.0171
31. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227(5259):680–685. https://doi.org/10.1038/227680a0
32. R Core Team. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2014.
33. Min J, Shin SH, Jeon EM, Park JM, Hyun JY, Harn CH. Pepper, chili (*Capsicum annuum*). In: Wang K, editor. *Agrobacterium* protocols. New York, NY: Springer; 2015. p. 311–320. (Methods in Molecular Biology; vol 1223). https://doi.org/10.1007/978-1-4939-1695-5_25
34. Kehie M, Kumaria S, Tandon P. Osmotic stress induced-capsaicin production in suspension cultures of *Capsicum chinense* Jacq. cv. Naga King Chili. Acta Physiol Plant. 2012;34(5):2039–2044. https://doi.org/10.1007/s11738-012-0991-1
35. Weathers PJ, Fadzillah NM, Cheetham RD. Light inhibits the formation of capsaicin from *Capsicum* callus. Planta Med. 1992;58(3):278–279. https://doi.org/10.1055/s-2006-961455

Transformacja biolistyczna kalusa i kultur komórkowych zawiesinowych u *Capsicum annuum* L. 'Serrano' przydatnych do badań in vitro względnej zawartości metabolitów wtórnych

Streszczenie

Capsicum annuum L. jest gatunkiem uprawnym o dużym znaczeniu gospodarczym, który jest zdolny do wytwarzania kapsaicynoidów, kapsynoidów i barwników wartościowych dla żywienia i dla medycyny. Opisywano wcześniej już metody rozmnażania i transformacji tego gatunku, ale większość jest zależna od fenotypu, opiera się na transformacji przy pomocy *Agrobacterium*, a ich sukces jest ograniczony. Skutkiem tego jest fakt, że tylko jedna komercyjna odmiana transgeniczna jest obecnie na etapie prób. W niniejszej pracy opisujemy warunki wytwarzania kalusa i zawiesinowych kultur komórkowych u *C. annuum* L. 'Serrano' z wykorzystaniem nasion uzyskanych z handlu. Kulturę można było indukować do wytwarzania kapsaicyny i dihydrokapsaicyny w ilościach wykrywalnych, kultura ta była także podatna na transformację metodą biolistyczną. Wykorzystując mikroskopię konfokalną wykazano ekspresję konstruktu zawierającego pochodzącą z *Arabidopsis thaliana* rozpuszczalną pirofosfatazę 4 w fuzji z białkiem fluorescencyjnym, a integralność tego konstruktu udowodniono przy pomocy immunoblot. Transformacja spowodowała zmianę w stosunku kapsaicyny do dihydrokapsaicyny, mierzonego wysokorozdzielczą metodą DSA-MS (*direct sample analysis mass spectrometry*). Metoda ta jest zatem użyteczna do badań wytwarzania kapsaicynoidów w warunkach kontrolowanych i do badań metabolizmu.