Influence of calcium channel modulators in capsaicin production by cell suspension cultures of *Capsicum frutescens* Mill.

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The production of capsaicin by cell suspension cultures of *Capsicum frutescens* mediated through the calcium channel was studied. Administration of the calcium ionophore A23187 resulted in a 1.43-fold enhancement of the total capsaicin production in the cell suspension cultures. Treatments wherein the calcium channel modulators verapamil and chlorpromazine were administered resulted in lower growth and capsaicin production, suggesting that calcium is involved in the signal transduction of capsaicin pathway in the suspension culture. The activity of Ca^{2+}ATPase, which is a calmodulin-related enzyme, was also studied under the influence of the calcium channel modulators. Maximum activity of Ca^{2+}ATPase was observed preceding the capsaicin elicitation. The activity of capsaicin synthase correlated with the increased capsaicin levels under the influence of calcium ionophore.

PRODUCTION of secondary metabolites using plant cell culture is extensively investigated[1,2]. Overproduction of the desired compounds by adopting cell culture technology has been demonstrated in case of *Lithospermum erythrorhizon* for shikonin[3], *Papaver somniferum* for sanguinarine[4], and *Coptis japonica* for berberine[5]. Several factors, viz. nutrients, hormones, precursors, cultural environment and elicitors influence productivity of secondary metabolites in cell cultures[6–9]. In recent years, elicitor-mediated enhancement of desired compounds is well studied[10]. However, the mechanism of elicitation has not been conclusively understood. One of the hypotheses of elicitation involves promotion of calcium channels which influence calmodulin-dependent protein kinase having its effect on gene expression[11].

Capsicum sp. produces the pungent principle – capsaicin which finds use as food additive and also in medicine as a counter-irritant. It is also reported to have antioxidant properties[12,13]. Cell cultures and immobilized cell cultures of *Capsicum* have shown the potential for continuous production of capsaicin in column reactor process and also ability to biotransform phenylpropanoids to capsaicin[14–16]. Capsaicin synthase, a terminal enzyme of the capsaicin biosynthetic pathway, catalyses the condensation reaction between vanillylamine and nonanoic acid to form capsaicin[17].

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It was of interest to understand the correlation of capsaicin synthesis and activity of capsaicin synthase in cell cultures of *Capsicum frutescens* in relation to calcium channel signal transduction. This study addresses these aspects to find out their relationship leading to enhanced production of capsaicin cell cultures.

High capsaicin-yielding seeds of *C. frutescens* Mill. (Arch 226) were obtained from Ankur Seeds, Nagpur. The seeds were surface sterilized with mercuric chloride (0.15%) for 5 min and transferred to sterile petri dishes with moist filter paper support for germination in dark. After a week of radical emergence, seedlings were injured and placed on MS medium[18] containing 2 mg l^{-1} 2,4-D and 0.5 mg l^{-1} kinetin for callus initiation. Calluses were developed from all the regions of the seedling and such calluses were maintained on the same medium for experiments. The cultures were incubated at 25 ± 2°C under cool light (4.4117 Jm^{-2} s^{-1} 18 h day^{-1}). The callus tissue weighing approximately 1 g was transferred to 40 ml of liquid MS medium of the same hormonal combination in 150 ml Erlenmeyer flasks kept on a rotary shaker (New Brunswick Scientific Co. Inc., New Jersey) at 90 rpm in light. The fine uniform cell suspension free from clumps was maintained by subculturing at two-week intervals and growth was measured in terms of fresh weight.

The influence of calcium on capsaicin production in the cell suspension cultures of *C. frutescens* was studied. The cells were first placed in medium without calcium, and after ten days they were transferred to MS medium containing 1/2, 1/4, 1/8, 1/16 and 2X calcium compared to the standard MS medium.

The calcium ionophore A23187, channel blocker verapamil and calmodulin antagonist chlorpromazine were administered to study the involvement of calcium channel during the elicitation of capsaicin in the cell suspension cultures of *C. frutescens*. The calcium ionophore A23187 (Sigma Chemical Co., USA) at a concentration of 0.5 µM was found to be effective in pilot experiments, in enhancing the metabolite production and was used for further studies. The solutions of calcium ionophore A23187 and calcium–calmodulin antagonist chlorpromazine (50 µM) were dissolved in ethanol and were filter-sterilized before addition to the autoclaved medium.

The calcium channel blocker verapamil hydrochloride (100 µM)[19] was dissolved in water and filter-sterilized before addition to the autoclaved medium.

One gram of cells separated from the culture medium by filtration using nylon mesh (50 micron size) was ground well with neutralized glass powder (100 mg) using a mortar and pestle, and extracted thrice with 25 ml of ethyl acetate. The extract was centrifuged at 2000 rpm for 15 min and the supernatant was evaporated. The residue was then dissolved in known aliquots of ethyl acetate and used for capsaicin analysis[14].

Cell cultures were filtered through nylon mesh as mentioned above, and centrifuged. The supernatant was ext-

racted thrice with 20 ml of ethyl acetate each time in a separating funnel. The ethyl acetate layers were pooled and evaporated. The residue was dissolved in known volume of ethyl acetate for capsaicin analysis[14].

The capsaicin extracted from the callus and medium was estimated by high performance liquid chromatography (HPLC; Shimadzu LC 10 A) following the method of Hoffman *et al.*[20]. The quantification of capsaicin was done on a C18 Bondapak column with detection at 280 nm. The isocratic mobile phase was acetonitrile: water (1% acetic acid) (40:60 v/v) and a flow rate of 1 ml/min was used.

Capsaicin synthase assay from the callus of *C. frutescens* was standardized following the method of Iwai *et al.*[17]. The callus was extracted with 0.1 M potassium phosphate buffer pH 6.8, and 5 mM 2-mercaptoethanol. The extract was centrifuged at 10,000 rpm for 40 min and the supernatant was used as the crude enzyme extract. The assay mixture contained 40 µmol potassium phosphate buffer (pH 6.8), magnesium chloride (1 µmol), ATP (1 µmol), vanillylamine hydrochloride (5 µmol), nonanoic acid (5 µmol) and the enzyme extract (300 µl) in a final volume of 0.5 ml. The assay was carried out at 37°C for 2 h, and the reaction was terminated by adding 0.3 ml of 98% ethanol. Capsaicin was extracted with ethyl acetate (10 ml) and estimated by HPLC. One unit of enzyme activity was defined as 1 µM of capsaicin formed per mg protein in one hour. The protein was determined by the method of Lowry *et al.*[21].

The Ca^{2+}ATPase activity was assayed following the method of Vambutas and Racker[22]. The enzyme was extracted in 0.5 mM Tris buffer, pH 8.0 and activated using 200 µg of trypsin for 5 min. The activation by trypsin was stopped by the addition of trypsin inhibitor. The assay mixture contained 50 µmol of Tris buffer pH 8.0, 5 µmol of ATP, 5 µmol of $CaCl_2$ in a final volume of 1.0 ml. The reaction was stopped by the addition of 0.1 ml of 20% trichloroacetic acid and the amount of inorganic phosphate (Pi) released was determined[23]. One unit of activity was defined as equivalent to 10 µM of Pi formed per mg protein in one hour.

The fresh weight, capsaicin content, capsaicin synthase and Ca^{2+}ATPase activity were estimated in five samples and the average was calculated. The standard error was calculated following the method of Freund and Perles[24].

The cultures which were treated with standard and 2X calcium level as MS medium were found to have higher biomass accumulation (Figure 1 *a*). The capsaicin content in the cells after ten days on the medium without calcium was found to be low (8.36 ± 0.4 µg/g FW). The total capsaicin level in the cultures treated with 2X Ca was higher (30.7 ± 1.5 µg/culture) than the control (26.3 ± 1.3 µg/culture) on the 12th day of culture (Figure 1 *d*). The other lower levels of calcium gave lesser production of capsai-

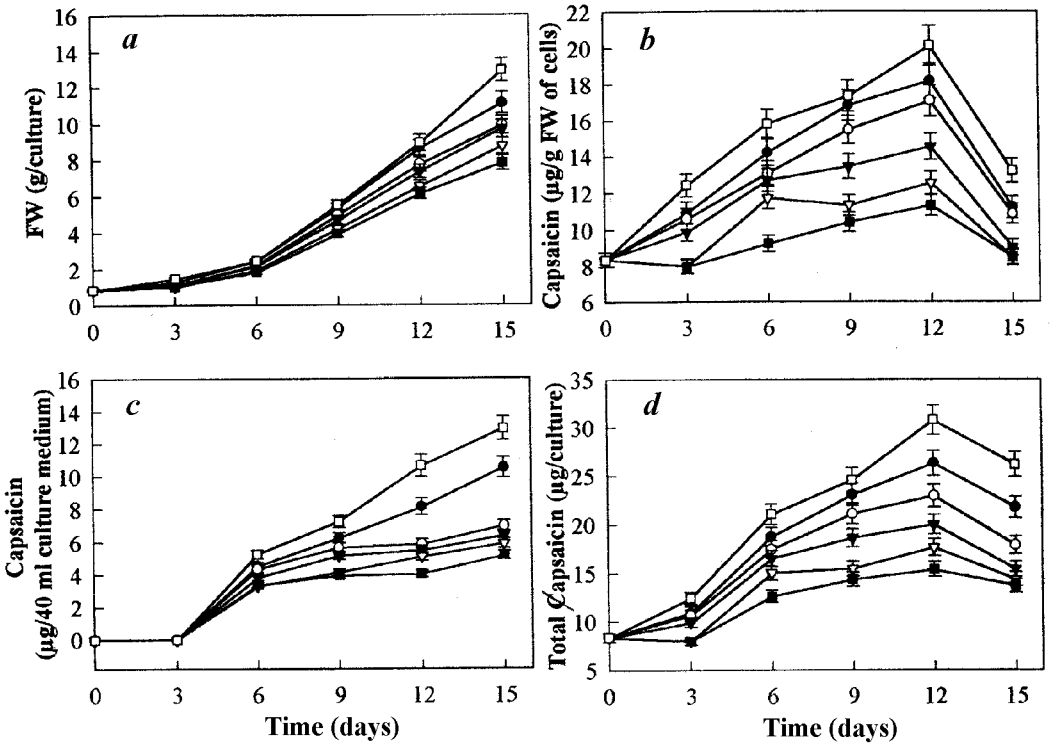

Figure 1 *a–d*. Effect of various levels of calcium on growth and capsaicin content in cell suspension cultures of *C. frutescens*. —Σ—, C; — —, 1/2 Ca; — —, 1/4 Ca; — —, 1/8 Ca; — —, 1/16 Ca; — —, 2X Ca.

cin, as evident from the capsaicin content in the cells and the medium (Figure 1 *b* and *c*).

Cells which were in the medium containing double the level of calcium compared to the standard MS medium, showed higher growth and capsaicin content compared to the control values (Figure 1 *a* and *d*). Possibly higher levels of calcium (2X compared to the regular MS medium level) were required for the restoration of growth and capsaicin production (Figure 1 *a–d*) subsequent to their transfer from the medium devoid of calcium.

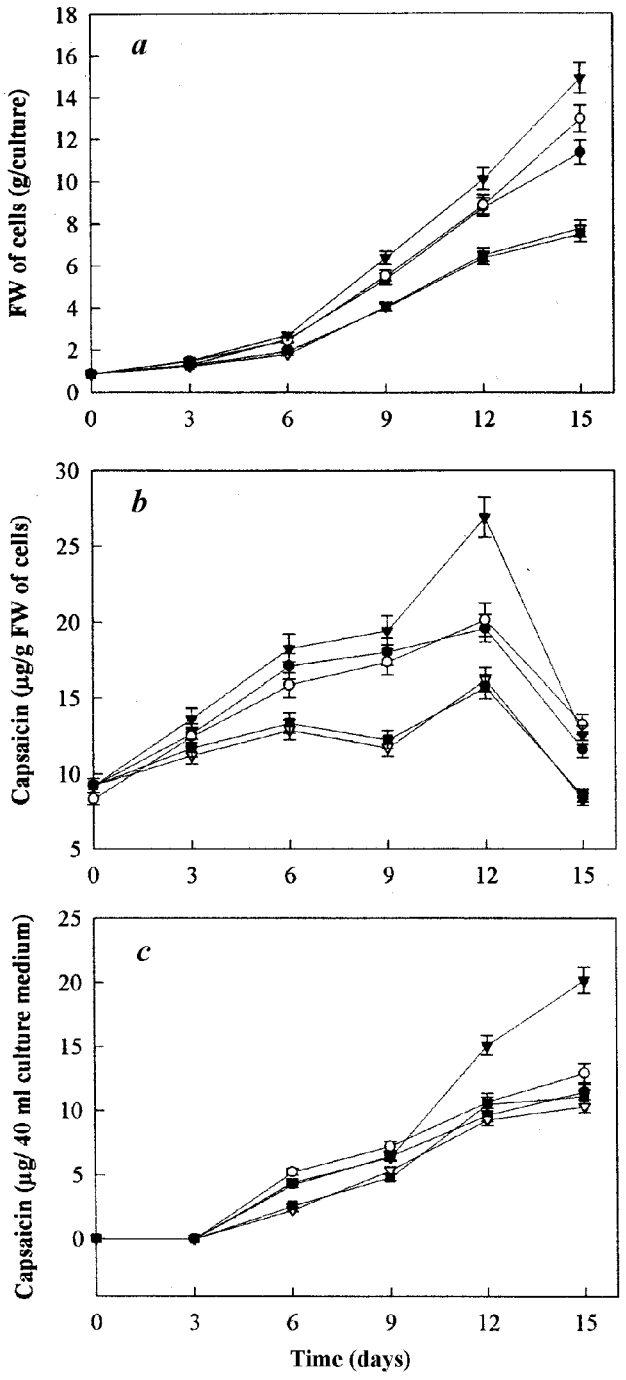

Figure 2 *a–c*. Influence of calcium, ionophore and channel blockers on growth and capsaicin content in cell suspension cultures of *C. frutescens*. —Σ—, C; — —, 2X Ca; — —, I (0.5 µM); — —, V (100 µM); — —, CP (50 µM).

The cultures treated with ionophore showed enhancement in biomass accumulation (14.92 ± 0.74 g/culture) on the 15th day of culture (Figure 2 *a*). However, treatment with verapamil (V) and chlorpromazine (CP; Figure 2 *a*) resulted in growth inhibitions (V, 7.82 ± 0.39; CP, 7.56 ± 0.37 g/culture). The ionophore treatment resulted in a 1.43-fold increase in the total capsaicin content over the control cultures (Figure 3 *a*). The capsaicin content in the cells was maximum in the ionophore-treated cultures (26.89 ± 1.34 µg/g FW) compared to the control (19.59 ± 0.9 µg/g FW), which was a 1.37-fold increase (Figure 2 *b*). The ionophore-treated cultures had a 1.76-fold increase in the capsaicin content in the medium (Figure 2 *c*).

When the calcium channel blockers were administered, the levels of total capsaicin were the lowest (V, 25.45 ± 1.2; CP, 26.18 ± 1.3 µg/culture) on the 12th day of culture. The ionophore-treated cultures had higher capsaicin synthase activity (6.75 ± 0.33 units) compared to the control (5.2 ± 0.26 units) on the 12th day of culture, coinciding with higher capsaicin content (Figure 3 *a*). Treatments wherein the channel blockers were added showed lower levels of capsaicin synthase activity (V, 4.65 ± 0.23; CP, 4.56 ± 0.22 units; Figure 3 *b*), which correlated with the lower levels of capsaicin.

The ionophore treatment resulted in the highest levels of Ca^{2+}ATPase activity (42.24 ± 2.1 units) on the 6th day of culture. Treatments with the calcium channel blockers resulted in lower Ca^{2+}ATPase activity (Figure 3 *c*). The maximum activity of Ca^{2+}ATPase was on the 6th day of culture, which preceded the maximal capsaicin synthase activity and the maximum capsaicin production on the 12th day.

Calcium plays a key role in plant growth and development because changes in the Ca^{2+}, acting through the Ca^{2+}-modulating proteins and their targets, regulate a number of cellular processes. The Ca^{2+} homeostasis is maintained by an ensemble of Ca^{2+} transport proteins. The Ca^{2+}-ATPases mediate the Ca^{2+} efflux from the cytosol[11]. The activity of Ca^{2+}-ATPase can be modulated by the Ca^{2+} ionophore A23187. Hence, the involvement of calcium was studied through the activity of Ca^{2+}-ATPase (ref. 11).

Calcium is known to exhibit specific functions in the regulation of growth, development and metabolism in plants[25]. Calcium levels influenced growth of the suspension cultures of *C. frutescens* and also capsaicin production (Figure 1 *a–d*). The growth-inhibitory effect of high calcium concentration has been reported to be due to an inhibition by calcium of the biochemical wall-loosening process[26].

Calcium regulates metabolic processes in plants or animals, either directly[27] or through calcium–calmodulin

interaction[28]. The involvement of calcium in the process is suggested by the action of calcium chelators and calcium ionophores[25,29]. The involvement of calmodulin is indicated by the effect of calmodulin antagonists[30]. Calmodulin, Ca^{2+}-activated phosphatases and kinases probably integrate the elevated calcium signal into the resultant diverse array of signalling pathways. The exquisite specificity in calcium-based responses is illustrated by the rapid and selective transcriptional activation of specific calmodulin isoforms in soybean cell cultures, and the effects of expression of a hyperactive mutant calmodulin in transgenic tobacco[31,32]. There are reports on the enhancement in the production of alkaloids[33], sesquiterpenes[34], isoflavonoids[35] and flavanol[36] under the influence of calcium.

This study confirms the involvement of calcium in the capsaicin production by cell suspension cultures of *C. frutescens*. The ionophore also results in enhanced capsaicin content in the medium (Figure 2 *c*), suggesting changes in the plasma membrane permeability mediated through the ionophore resulting in the leaching out of the capsaicin. The involvement of calcium is further confirmed by the lowering of capsaicin production by treatments with the calcium channel blockers (Figure 2 *c*). The calcium influenced capsaicin synthesis is accompanied by the concomitant increase in capsaicin synthase and calcium–calmodulin-related ATPase (Figure 3 *b* and *c*). This shows that capsaicin elicitation is through calcium–calmodulin activated pathway. It would be of further interest to know the extent of elicitation possible by the use of effective elicitors coupled to calcium channelling enhancers and precursors, which may further boost capsaicin production. Further work in this direction is in progress.

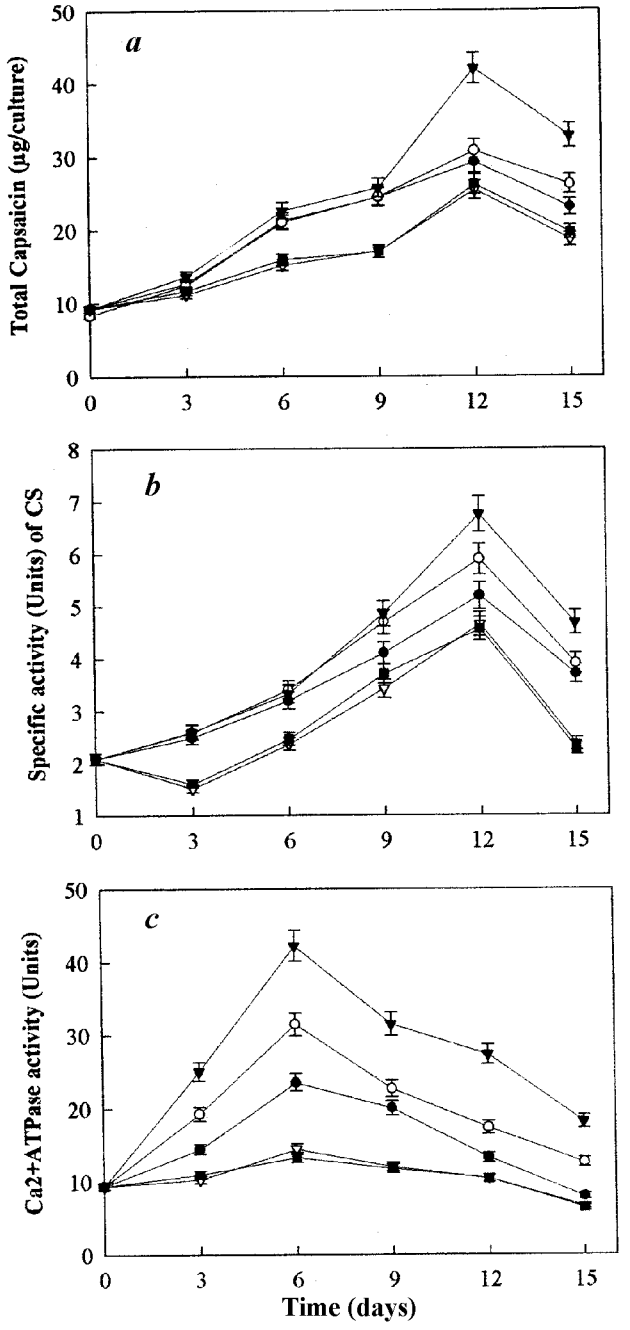

Figure 3 *a–c*. Capsaicin content, activity of capsaicin synthase and Ca^{2+}ATPase in cell suspension cultures of *C. frutescens* under the influence of calcium, ionophore and channel blockers. —Σ—, C; — —, 2X Ca; — —, I (0.5 µM); — —, V (100 µM); — —, CP (50 µM).

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Conservation and utilization of *Arnebia benthamii* (Wall. ex G. Don) Johnston – a high value Himalayan medicinal plant

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The investigation on conservation and utilization of *Arnebia benthamii* (Wall. ex G. Don) Johnston was carried out to identify optimum stage of the collection of propagules, improve upon the rooting of root cuttings and identification of optimum conditions for seedling survival. Individuals at reproductive maturity were found suitable for collection of propagules because of the occurrence of 3–5 buds at the terminal growing end of the root. These buds can be effectively utilized for vegetative propagation. Chilling for 40 days significantly ($P < 0.05$) improved rooting of root cuttings. Seedling survival and growth performance were significantly ($P < 0.05$) higher at a high-altitude village Lata, thereby facilitating the establishment of herbal gardens in the vicinity of natural population. This activity will not only reduce pressure on the natural population, but also has the potential to generate rural economy. Further, the possibilities of revegetating the degraded natural habitats and creating nursery centres at low-altitude areas are discussed. This study will help in developing conservation strategy for optimum utilization of *A. benthamii*.

CONSERVATION of medicinal plants is receiving increased attention all over the globe in view of their erosion, and resurgence of interest in herbal medicines for health care[1,2]. World trade figures suggest that India ranks next to China in export of medicinal raw material (32,600 tonnes, US$ 46 million)[3]. In India, large quantities of medicinal plants are extracted from the wild to meet the increasing demand of raw material for domestic consumption and export. As a result, natural habitats are depleting at a fast pace. Besides, unscientific methods of extraction and adulteration have further accentuated this complex problem[4,5].

Arnebia benthamii (Wall. ex G. Don) Johnston [Syn *Macrotomia benthamii* (Wall.) DC.] (family Boraginaceae), a high-value Himalayan medicinal plant, ranks second in the list of medicinal plants prioritized for western Himalaya and also figures among the 59 medicinal plants prioritized for conservation[4] due to high extinction threat.

It is an erect, herbaceous perennial, 30–90 cm in height, occurring in the alpine and subalpine Himalaya at altitude of 3000–3900 m asl. The species is a major ingredient of the commercial drug available under the name Gaozaban, which has antibacterial, antifungal, anti-inflammatory and wound-healing properties. The roots yield a red pigment, Shikonin (a dye), which has several medicinal properties and is marketed under the trade name Ratanjot[6]. The species also possess stimulant, tonic, diuretic and expectorant properties. The flowering shoots are used in preparation of sherbet (syrup) and jam useful in various diseases of tongue, throat, fever and cardiac disorders. Secondary metabolites, Arnebin 1 and Arnebin 3 obtained from the other species of this genus are reported to possess anticancerous property[7]. *Arnebia euchroma* exhibits potent anti-HIV activity[8].

As a result of over exploitation of its rhizomes for medicinal purposes, *Arnebia benthamii* has been listed in the Indian Red Data Book[9]. Apart from the reports on

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