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SHORT COMMUNICATION

Osmotic stress induced-capsaicin production in suspension cultures of *Capsicum chinense* Jacq.cv. Naga King Chili

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Abstract The influence of osmotic stress on capsaicin production was investigated in cell suspension cultures of *Capsicum chinense* Jacq.cv. Naga King Chili, a chili species native to Northeastern India. The sterilized seeds were germinated in Murashige and Skoog medium. Two-week-old hypocotyls were excised from in vitro germinated seedlings and implanted in MS medium containing 2, 4-dichlorophenoxyacetic acid (2 mg/l), and Kinetin (0.5 mg/l) for callus induction. Capsaicin production in the suspension cultures was significantly affected using sucrose, mannitol, and NaCl in the medium. Stoichiometric analysis with different combinations of sucrose and non-sugar osmotic agent (NaCl) showed that osmotic stress was an important factor for enhancing capsaicin production in cell suspension cultures of *C. chinense*. The capsaicin content of 1,644.1 μg g^{-1} f.wt was recorded on day 15 in cultures grown in MS medium containing 87.64 mM sucrose in combination with 40 mM NaCl. However, osmotic stress treatment at 160 mM NaCl with sucrose resulted in lowering capsaicin accumulation and separation of cell wall from their cytoplasm, under microscopic observation.

Keywords Capsaicin · *Capsicum* · Cell culture · Naga King Chili · Osmotic stress

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Introduction

Capsicum chinense Jacq.cv. Naga King Chili is an important spice crop of India belonging to the family Solanaceae (Kehie et al. 2011). *C. chinense* is a very pungent chili, measuring 1,001,304 Scoville Heat Units (SHU). It is locally called as Naga King Chili (*Bhoot jolokia* or *Naga jolokia* in Assamese), and is native to Northeastern India more particularly to Nagaland (Bhagowati and Changkija 2001). It has been acknowledged as the hottest chili in the world (Guinness Book of World Records 2006). The Naga King chili has received the attention of world scientific community due to its extremely high pungency and unique aroma (Meghvansia et al. 2010). It has also been used conventionally by different ethnic communities of the Northeastern India in treating various human ailments. In Nagaland, *Capsicum* spp. including Naga chili are used to tone up body muscles after heavy workouts whereas hot infusions are used for toothache and muscle pain (Bhagowati and Changkija 2001).

The pungent principle of chili fruit are capsaicinoids. In nature, capsaicin and dihydrocapsaicin account for 90 % of the total capsaicinoid content in chili fruits (Suzuki et al. 1981). Capsaicin is mainly used as a spice, as food additive, and in pharmacological applications. As a medicine, capsaicin is known to kill some types of cancer cells (Min et al. 2004); it also provides relief in arthritis and respiratory ailments (Mazzone and Geraghty 1999). It is a counterirritant and an analgesic agent (Fusco and Giacovazzo 1997). The biosynthetic capacity of in vitro cultured cells and tissues to produce capsaicinoids has been investigated by different workers using immobilized cell cultures, nutrient limitation, precursors, and elicitors (Ravishankar et al. 1988; Salgado-Garciglia and Ochoa-Alejo 1990; Ochoa-Alejo and Salgado-Garciglia

1992) Most of these reports have been attempted on *C. annuum* and *C. frutescens.* There is only one report existing on induction of capsaicinoid in *C. chinense* by salicylic acid or methyl jasmonate (Gutierrez-Carbajal et al. 2010). Osmotic stress is an important factor affecting plant growth, development, morphogenesis and the formation of secondary metabolites (Liu and Cheng 2008). Enhanced production of secondary metabolites from in vitro tissue cultures under optimal osmotic stress has been reported (Shohael et al. 2006). However, there is no report available on capsaicin production in cell cultures under osmotic stress.

In the present investigation, we report the osmotic effect of sucrose, mannitol, and NaCl on capsaicin production in cell suspension cultures of *C. chinense*.

Materials and methods

Plant material and callus induction

Capsicum chinense seeds were obtained from a local field at Rüziephema village, Nagaland, India. The seeds were thoroughly washed in running tap water, then treated with 2 % Labolene (*v/v*) for 10 min and finally rinsed five times with distilled water. These were then surface sterilized with 0.1 % $HgCl_2$ for 5 min followed by several washes with sterile distilled water. The sterilized seeds were cultured in MS medium (Murashige and Skoog 1962) containing 3 % (*w/v*) sucrose and 0.8 % (*w/v*) agar, the pH of the medium was adjusted to 5.8 before autoclaving. Two-week-old hypocotyls were excised from in vitro germinated seedlings and implanted in MS medium containing various concentrations of 2,4-dichlorophenoxyacetic acid (2, 4-D; 0.5–4 mg/l), and Kinetin (KN; 0.5–2 mg/l) for callus induction (Table 1). The callus cultures induced were maintained on the medium containing 2, 4-D (2 mg/l) and KN (0.5 mg/l), and subcultured every 30 days (Fig. 1a). Cultures were maintained in a culture room at temperature of 25 ± 2 °C, 14/10-h photoperiod with an irradiance of 62.2 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes.

Cell suspension

Capsicum chinense cell suspension cultures were established from callus tissue cultures described above. About 2 g fresh weight callus was inoculated into 25 ml of MS liquid medium fortified with 2, 4-D (2 mg/l), and KN (0.5 mg/l) (Fig. 1b). Cultures were incubated on rotary shaker (125 rpm) under the same culture room conditions of temperature and light as described above.

Table 1 Effect of 2, 4-dichlorophenoxyacetic acid and kinetin on callogenesis from cotyledon segments of *Capsicum chinense*

PGR Conc. (mg/l)	Callogenesis	% Callus induction	Morphology of callus
Control	–	–	–
2, 4-D			
0.5	+	50	White, friable and fragile
1	+	50	White, friable and fragile
2	++	60	White, friable and fragile
4	++	80	White, friable and fragile
Kn			
0.5	+	50	Light brown, friable
1	+	50	Light brown, friable
2	++	60	Light brown, friable
2, 4-D + Kn			
0.5 + 0.5	+	60	White, friable
0.5 + 1	+	60	White, friable
0.5 + 2	+	70	White, friable
1 + 0.5	++	70	White, friable
1 + 1	++	70	White, friable
1 + 2	++	80	White, friable, profuse callusing
2 + 0.5	+++	100	White, friable, profuse callusing
2 + 1	++	90	White, friable
2 + 2	++	90	White, friable
4 + 0.5	++	90	White, friable
4 + 1	+	90	White, friable
4 + 2	+	90	White, friable

– No response, + low, ++ medium, +++ high, *PGR* plant growth regulators

Osmotic stress treatment

All experiments were carried out in 100 ml Erlenmeyer flasks containing 25 ml MS liquid medium/flask inoculated with 2 g callus (f.wt) of 15-day-old cell suspension cultures.

1. Effect of sucrose and mannitol on capsaicin production: Calli were harvested and subjected to various concentrations of sucrose (43.82, 87.64, 175.28, 350.56 mM) and mannitol (82.34, 164.68, 329.36, 658.72 mM) in MS medium containing 2, 4-D (2 mg/l) and KN (0.5 mg/l).
2. Effect of NaCl on capsaicin production: To investigate the effect of NaCl (non sugar) as osmotic agent, *C. chinense* suspension cultures were transferred to MS medium containing 2, 4-D (2 mg/l) and KN (0.5 mg/l), and also with various concentrations of NaCl (10, 20, 40, 80, 160 mM). *C. chinense* cultured cells under

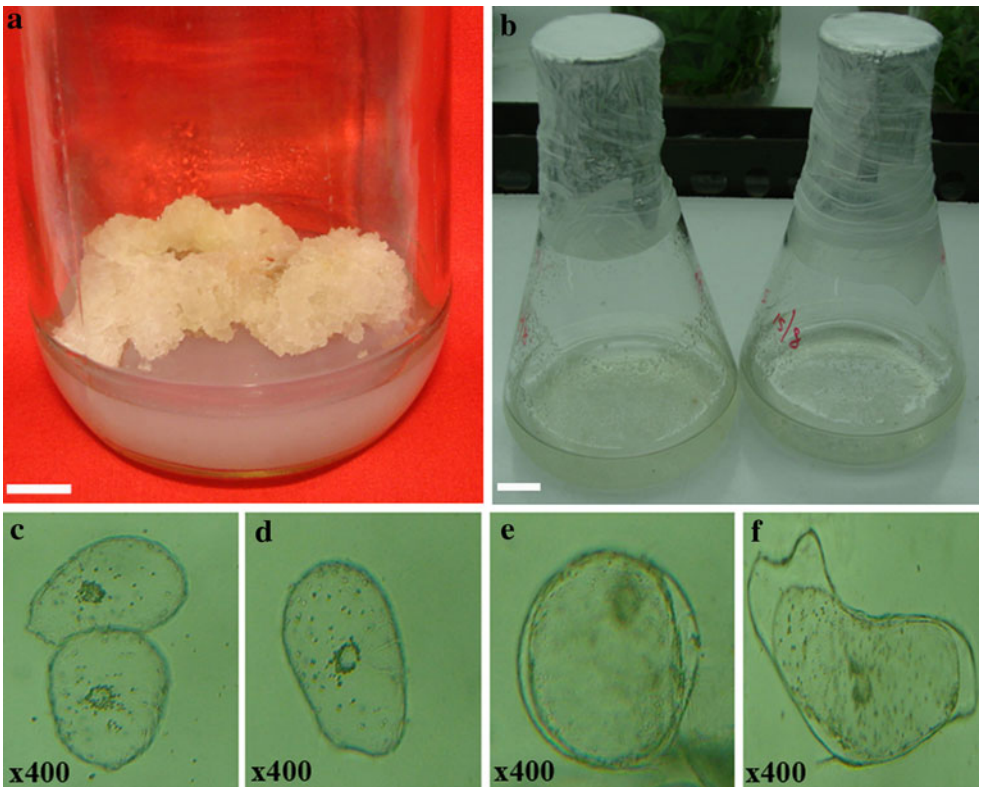

Fig. 1 **a** Callus tissue of *C. chinense* growing on MS agar medium containing 2,4-D (2 mg/l), and KN (0.5 mg/l) (*Bar* 1 cm). **b** Cell suspension cultures of *C. chinense* growing in MS medium fortified with 2,4-D (2 mg/l), and KN (0.5 mg/l) (*Bar* 1 cm). Light micrograph (400×) of *C. chinense* cells under different osmotic stress. **c** Control (MS + 3 % sucrose). **d** MS + 3 % sucrose +40 mM NaCl. **e** MS + 3 % sucrose +80 mM NaCl. **f** MS + 3 % sucrose +160 mM NaCl

various *osmotic* stress were observed for plasmolysis by Leitz FLUOVERT FU microscope (Fig. 1c–f) under 400× magnifications.

Cells were collected from the suspensions by filtration, at 5 days interval for 25 days and used for determination of capsaicin content. Triplicate flasks were used in all experiments, and all values were the means of triplicate flasks with standard error.

Extraction, separation and quantification of capsaicin

Capsaicin was extracted from either cells or liquid medium following the method described by Nunez-Palenius and Ochoa-Alejo (2005). Thin layer chromatography (TLC) was carried out for the separation of capsaicin from other impurities as described by Perucka and Oleszek (2000). In order to locate the capsaicin on the TLC plate, the plate containing the standard was exposed to iodine vapor. The presence of capsaicin was indicated by the formation of brown-colored bands on the plate (Fig. 2a, b). The *Rf* value ($Rf = 0.56$) of capsaicin was determined from the standard plate. The bands corresponding to capsaicin were scraped off, the plates not exposed and the compounds were washed out of silica gel with methanol. The TLC purified capsaicin was measured at 280 nm by the method of Suzuki et al. (1957), using Perkin Elmer Lambda 35 UV/Vis Spectrometer. Capsaicin and dihydrocapsaicin identities were further confirmed by High performance liquid chromatography using Waters M515 series equipped with a μBondapak C_{18} column (10 μm particle size, 300 × 3.9 mm), comparing their retention times with that of the standard as described by Nunez-Palenius and Ochoa-Alejo (2005) (Fig. 2c, d).

Results and discussion

In the present investigation, osmotic role of sucrose, mannitol and NaCl on capsaicin production in *C. chinense* cell suspension cultures was studied. Addition of sucrose and mannitol in the medium significantly enhanced the accumulation of capsaicin in suspension cultures of *C. chinense*. With 87.6 mM sucrose in the medium, a maximum of 165 μg g^{-1} f.wt capsaicin content was obtained on day 25 (Fig. 3a). The level of sucrose has been shown to

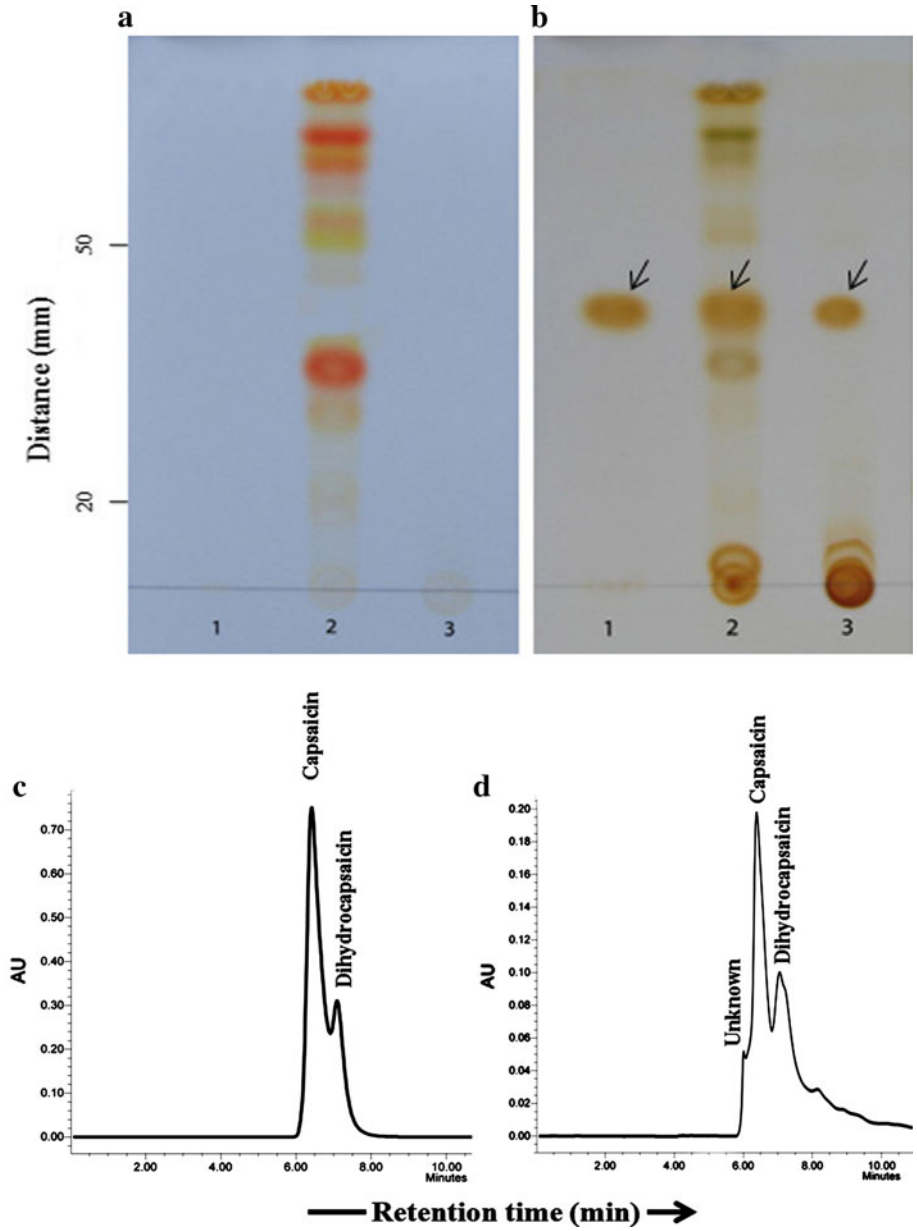

Fig. 2 Chromatography separation of capsaicin. **a** Chromatogram visualized prior to iodine vapor exposure, capsaicin spot not visible. **b** Chromatographic spots (indicated with *arrow mark*) visualized after being exposed to iodine vapor. Standard spot (1), crude extract from ripen fruit (2), crude extract from callus tissue (3). Mobile phase: Benzene:Ethyl acetate:methanol (75:20:5). HPLC chromatogram in methanol of standard (**c**) and callus sample (**d**) showing capsaicin and dihydrocapsaicin at 280 nm

affect the production of secondary metabolites in cell cultures (Do and Cormier 1990; Rao and Ravishankar 2002). A dual role of sucrose as carbon source and osmotic agents was observed in *Solanum melongena* (Mukherjee et al. 1991). In the present study, a comparatively higher accumulation of capsaicin was also obtained with mannitol in MS medium. A maximum of 181.66 μg g^{-1} f.wt capsaicin content was recorded on day 15 when the cells were treated with 164.68 mM mannitol in MS medium (Fig. 3b). Addition of mannitol to the cell cultures influences the production of secondary metabolites (Borowitzka 1981; Tholakalabavi et al. 1994). The effect of NaCl on the production of secondary metabolites has been reported by various workers (Kim et al. 2001; Liu and Cheng 2008). In our study, NaCl was found to favor the accumulation of capsaicin. Capsaicin production was significantly enhanced (1,644.1 μg g^{-1} f.wt) on day 15 in MS medium supplemented with 40 mM NaCl (Fig. 3c). Addition of NaCl (80 mM, 160 mM) into MS liquid medium containing 87.6 mM sucrose resulted in lowering capsaicin accumulation and separation of cell wall from their cytoplasm, under microscopic observation (Fig. 1c–f).

From the present study, it may be concluded that both the extent of osmotic stress and the type of solute used to induce osmotic stress seems to influence capsaicin levels in cell suspension cultures. NaCl may be used as an osmotic

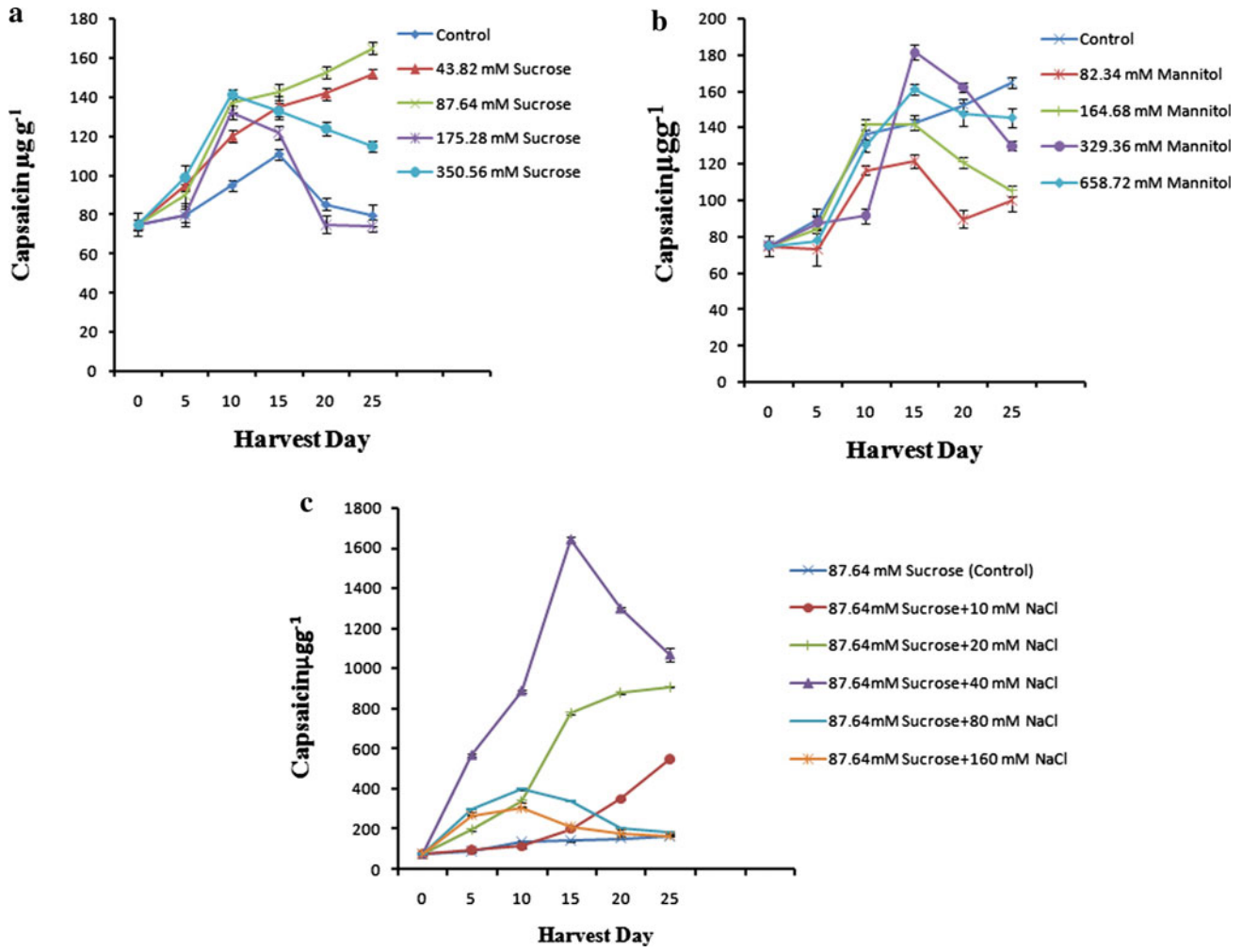

Fig. 3 Accumulation of capsaicin under various treatments of sucrose, mannitol and NaCl. **a** Effect of sucrose on capsaicin production, **b** effect of mannitol on capsaicin production, **c** effect of the combination of sucrose and NaCl on capsaicin production. Values are means of triplicate with standard error

agent in combination with sucrose to enhance the production of capsaicin. Further investigation using other group of solutes that are known to increase secondary metabolites by inducing osmotic stress and the activity of regulatory enzymes of capsaicin biosynthesis might be useful in further elucidating the mechanism of osmotically induced capsaicin production.

Author contribution MK carried out the experiment, analyzed the data and drafted the manuscript. SK and PT supervised the work. SK edited the manuscript. MK, SK & PT were involved in designing the experiment. All authors read and approved the final version of this manuscript

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