

A study on the effect of different elicitors on capsaicin accumulation in cell suspension cultures of *Capsicum assamicum* (Bhut Jolokia)

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Elicitation of cell suspension cultures of *Capsicum assamicum* (Bhut Jolokia) for enhancement of capsaicin content was tried using different elicitors such as cellulase, vanillin, salicylic acid and sinapic acid in different concentrations for 24, 48 and 72 hours. Cell suspension culture was established in B5 media supplemented with 3.5 mM 2,4-D (2,4-diphenoxyacetic acid) and 1.1 mM

Kin and elicitors were introduced at the end of exponential phase. All the elicitors, except methyl jasmonate, led to significant increase in production of capsaicin. Sinapic acid, when added in 0.022 μ M concentration and incubated for 24 hours, led to highest capsaicin accumulation of 0.5% (5068 μ g/g) which was highest among all the treatments.

Key Words: Elicitation; Capsaicin; *Capsicum assamicum*; Cell suspension culture

INTRODUCTION

Capsaicin is a secondary metabolite produced in chilli pepper that imparts them characteristic pungent taste and burning sensation. Chemically, it is a crystalline, fat soluble alkaloid with a molecular weight of 305.41 g/mol [1]. Capsicum oleoresins are widely used as additives in food industry to impart taste and colour [2,3]. Capsaicin is also known for its medicinal and pharmacological properties that have been established by extensive research carried out in past years. It has been found to have analgesic [4], anti-cancer [5], anti-obesity [6] antioxidant [7], antimicrobial [8,9], cardiovascular [10] and gastrointestinal properties [11]. Its extensive application in food and pharmaceutical industries as well as in research studies has increased its demand in the market. Industrial production of capsaicin relies on its extraction from Capsicum fruit.

Capsicum assamicum (Bhut Jolokia), a chilli species endemic to North East India, is one of the hottest chillies in the World with a Scoville Heat Unit (SHU) of more than 1 million [12]. Capsaicin, the compound responsible for its hotness, has numerous applications in food and pharmaceutical industries. It is generally extracted from the chilli fruit. However, most Indian chilli species produce less than 1% capsaicin, *C. assamicum*, which contain 3%-5% capsaicin, is a suitable candidate for commercial production, but the chilli fruit is available only from May to October [13]. Cell suspension culture can be a suitable alternative for large scale production of capsaicin, round the year and independent of climatic and geographic conditions. No report regarding the use of suspension culture for capsaicin production from *C. assamicum* could be traced. With this aim, callus had been induced and cell suspension culture has been established [14]. However, the amount of capsaicin accumulated was found to be very low.

Elicitation is the process of enhancement of a particular compound of interest in cell cultures by addition of a foreign material [15]. Previously conducted studies have shown that elicitors increase secondary metabolite accumulation in plant cell cultures [16]. The present study investigates the use of different elicitors, viz. cellulase, salicylic acid, sinapic acid and vanillin on accumulation of capsaicin in suspension cultures of *C. assamicum*.

Cellulase is an enzyme produced by a wide range of microbes including bacteria, fungi and protozoa that plays an important role in the hydrolysis of lignocellulosic materials [17]. Being of microbial origin, it mimics microbial infection and may trigger defence response resulting in capsaicin production. Sinapic acid is a phytochemical which belongs to a class of phenolic acids known as hydroxycinnamic acid [18]. It is a chemical analogue of caffeic acid

which is a precursor in capsaicin biosynthetic pathway. Hence, it is worth investigating the effect of sinapic acid on capsaicin production. Vanillin is also a precursor in phenylpropanoid pathway of capsaicin biosynthesis. Hence, its addition in cell cultures might enhance capsaicin production. Salicylic Acid (SA) is a derivative of chorismate which is a signal molecule in plants and act as an inducer of Systemic Acquired Response (SAR) against pathogenic attack [19]. In the event of pathogen attack, level of salicylic acid has been found to increase [20,21]. Since capsaicin is synthesized as a defence mechanism, introduction of salicylic acid might induce SAR which would lead to the production of capsaicin. Methyl Jasmonate (MeJA) is a commonly used elicitor. It is known to have both inhibitory and stimulatory effect, physiologically as well as morphologically. It also induces defence metabolism in cultured cells. Enhancement in alkaloid production after treatment with MeJA has also been shown in a number of plant cultures. In the present study, the effect of all the above mentioned elicitors, in different concentration and for different incubation time, on capsaicin accumulation has been investigated.

MATERIALS AND METHODS

Establishment of cell suspension culture

Previously standardized protocol has been used for establishment of suspension cultures. Fruits of *C. assamicum* was grown inside net house (50% shade) located in Technical Complex of Defence Research Laboratory, DRDO, Tezpur in the month of October 2017 and half-ripe fruits were collected in March 2018. Placental explants were inoculated in Gamborg's B5 media supplemented with 3.5 mM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.1 mM Kinetin (Kin) for callus induction [14]. Cell suspension culture was established in Erlenmayer flasks each containing 500 ml of liquid B5 media supplemented with 3.5 mM 2,4-D and 1.1 mM Kin. Each flask was inoculated with 5 gm of friable callus. The cultures were kept on an orbital shaker set at 120 rpm and incubated at a temperature of 25 \pm 2°C under light: dark cycle of 16:8 hours. The light intensity was maintained at 3000 lux provided by cool fluorescent white light.

Elicitation of cell cultures

For enhanced accumulation of capsaicin, various elicitors, namely, cellulase, vanillin, sinapic acid and salicylic acid were used. All the elicitors were analytical grade with >99% purity obtained from Sigma-Aldrich. 1 M solution of cellulase was prepared by dissolving it in deionized water and was subsequently diluted to 10 mM, 20 mM, 30 mM and 40 mM concentrations. Vanillin was dissolved in methanol to prepare a 10 M solution which was

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diluted to prepare test solutions of 2500 mM, 4000 mM and 5200 mM concentrations. Sinapic acid solution was prepared in 1M conc. by dissolving it in ethanol and the test sample was prepared by diluting it to 0.02, 0.04 and 0.06 mM. Stock solution of 1M salicylic acid was prepared by dissolving it in acetone which was then diluted to 0.145 mM, 0.180 mM and 0.217 mM. The elicitor solutions were filter sterilized using sterile syringe filter of 0.2 µm pore size under laminar air flow. At the end of the exponential phase, elicitors were added to the cultures in different concentration and incubated for 24, 48 and 72 hours in the same culture condition as before. Unelicited cultures were used as control. All the experiments were performed in triplicates.

Capsaicin extraction and quantification

For extraction of capsaicin, callus was filtered from the suspension culture using Whatman No. 1 filter paper and washed with distilled water. The callus was collected in a pre-weighed petridish and dried in circulatory hot air oven at 50°C. Dry weight of the callus was recorded and was crushed into fine powder. Capsaicin was extracted in 80% methanol using cold maceration. Since capsaicin is stored extracellularly, it often gets leached into the media as well. Hence, capsaicin was extracted from media using the method used by Johnson et al., [22]. Media was washed with ethyl acetate in a separating funnel in 2:1 ratio. The upper layer of ethyl acetate was collected. The process was repeated thrice with the remaining media. The extracts were pooled and evaporated to obtain dry extract residue and stored at -20°C until further processing. Capsaicin quantification was done through HPLC based on the protocol standardized by Gonzales-Zamora et al., [23]. HPLC was performed through (LC Prep 150 System, Waters Corporation) using a C18 column (4.6 × 250 mm). Mobile phase for HPLC constituted of an isocratic mixture of water: acetonitrile (50: 50) and 20 µl injection volume was eluted with a flow rate of 1 ml/min. Detection was done using UV detector at 280 nm. Capsaicinoid related peak was obtained within 20 minutes. Capsaicin content of the extracts was quantified by plotting the peak area of chromatogram on the standard curve prepared on the basis of HPLC chromatogram of standard capsaicin.

Statistical analysis

Data were analyzed using two way ANOVA and sample means were compared via Tukey’s HSD test at p<0.05 using GraphPad Prism 8.3.0 software package.

RESULTS AND DISCUSSION

All the four elicitors used were shown to have different effect on capsaicin accumulation when used in different concentrations for different time period. The effect of different elicitors on capsaicin accumulation is described below: Cellulase

Application of cellulase in cell suspension cultures of *C. assamicum* resulted in 14 times increment in capsaicin accumulation compared to untreated callus. Both cellulase concentration and incubation time affected capsaicin accumulation (Table 1). When cellulase was applied for 24 hours in the concentration 40 mM, highest capsaicin accumulation of 0.058% (5.84 µg/g) was observed followed by cellulase application in the same concentration for 48 hours and 72 hours respectively. It was found that the increase in cellulase concentration resulted in increase in capsaicin production. These findings corroborated with that of Islek et al., [24], who found that capsaicin accumulation in cell suspension cultures of *Capsicum annum* L., increased with increase in cellulase concentration. It was observed that at all concentrations increasing incubation time resulted in enhancement in capsaicin accumulation except at 40 mM where increasing incubation time resulted in decrease in capsaicin. These results are in contrast to the findings of Islek et al., [24], who reported that at all cellulase concentrations, capsaicin accumulation increased when incubation time was increased from 24 hours to 48 hours but decreased at 72 hours incubation time. The results were found to be statistically significant with p<0.05.

TABLE 1
Effect of cellulase in different concentration for different incubation period on capsaicin accumulation in cell suspension culture**

Time (hours.)	Cellulase Conc. (mM)*				
	Control	10	20	30	40
24	0.39 ± 0.05	0.58 ± 0.11	1.25 ± 0.04	2.15 ± 0.07	5.84 ± 0.27

48	0.42 ± 0.03	0.75 ± 0.4	2.68 ± 0.04	3.05 ± 0.18	5.18 ± 0.17
72	0.46 ± 0.008	0.90 ± 0.05	2.80 ± 0.07	3.68 ± 0.07	5.49 ± 0.02

Note: *±: Standard Deviation of triplicate samples, **Capsaicin Concentration in µg/g.

Vanillin

Elicitation using vanillin resulted in high capsaicin accumulation in cell suspension cultures. Being a precursor in capsaicin biosynthesis, addition of vanillin enhances the capsaicin production in the cell cultures. Production of capsaicin increases significantly with increase in vanillin concentration (Table 2). Treatment of callus with vanillin gave yield percentage of 0.3% (2146 µg/g) (capsaicin percentage in 1 gm of dry callus) which is very high compared to the yield percentage of untreated callus. However, there is no linear relation between capsaicin produced and vanillin concentration. In the studies conducted by Pandhair and Goyal [25] it was found that the addition of vanillin in the cell cultures of *Capsicum annum* L., in 4000 mM concentration enhanced capsaicin yield by 2.4 times which is a lot less than what is found in the present study. The results were found to be statistically significant with p<0.05.

TABLE 2
Effect of vanillin in different concentration for different incubation period on capsaicin accumulation in cell suspension culture**

Time (hours.)	Vanillin Conc. (mM)*			
	Control	2500	4000	5200
24	0.37 ± 0.036	1020.98 ± 0.81	385.3 ± 0.6	988.31 ± 0.62
48	0.45 ± 0.03	1265.14 ± 1.53	1902.24 ± 0.79	1773.68 ± 0.77
72	0.48 ± 0.02	2146.73 ± 1.14	1128.91 ± 0.49	641.24 ± 0.71

Note: *±: Standard Deviation of triplicate samples, **Capsaicin Concentration in µg/g.

Salicylic acid

Elicitation through salicylic acid was found to have positive effect on the capsaicin production in cell suspension culture of *C. assamicum*. Capsaicin production was found to be affected by both the concentration as well as the incubation period (Table 3). Highest capsaicin content of 0.016% (1.615 µg/g) was found in cultures to which salicylic acid was added in 0.217 µM concentration and incubated for 48 hours. Another study [26] showed that the highest capsaicin was produced (50 µg/g f.w of callus) in the cultures of *Capsicum frutescens* treated with salicylic acid. In the present study, after treatment with salicylic acid, highest capsaicin content of 1.6 µg/g d.w of callus was observed.

TABLE 3
Effect of salicylic acid in different concentration for different incubation period on capsaicin accumulation in cell suspension culture**

Time (hours.)	Salicylic acid (mM)*			
	Control	0.145	0.18	0.217
24	0.423 ± 0.02	1.128 ± 0.008	0.53 ± 0.004	0.61 ± 0.004
48	0.38 ± 0.02	0.81 ± 0.02	1.603 ± 0.01	1.615 ± 0.01
72	0.34 ± 0.03	0.77 ± 0.002	0.73 ± 0.004	1.15 ± 0.03

Note: *±: Standard Deviation of triplicate samples, **Capsaicin Concentration in µg/g.

Sinapic acid

Of all the elicitors used, elicitation using sinapic acid showed the best result. Sinapic acid, in all the concentrations and for all the incubation period, enhanced the total capsaicin accumulation in the cultures of *C. assamicum* (Table 4). Highest capsaicin accumulation of 0.5% (5068.23 µg/g) was found in cultures to which sinapic acid was added in 0.022 µM concentration and incubated for 24 hours. In the studies conducted by Pandhair and Goyal [25,26], it was found that on addition of sinapic acid at 44 µM concentrations to the cultures of *Capsicum annum* L., there was 8.9 fold increases in capsaicin accumulation. The results were found to be statistically significant with p<0.05.

TABLE 4
Effect of sinapic acid in different concentration for different incubation period on capsaicin accumulation in cell suspension culture**

Time (hours.)	Sinapic acid (mM)*			
	Control	0.022	0.044	0.066
24	0.38 ± 0.02	5068.33 ± 6.23	1762.33 ± 2.05	1408.33 ± 8.49
48	0.44 ± 0.04	3042.33 ± 2.05	1244.33 ± 4.1	1563 ± 2.44
72	0.41 ± 0.03	2644.66 ± 4.1	1612 ± 8.83	1729 ± 3.29

Note: *±: Standard Deviation of triplicate samples, **Capsaicin Concentration in µg/g.

CONCLUSION

Capsaicin is an important industrial and pharmaceutical product, which is generally extracted from different members of the *Capsicum* genus. Though artificial production through chemical and enzymatic synthesis is in use, there are certain limitations. Chemical synthesis employs a number of reagents and catalysts and releases certain by-products that are known to be toxic, which is a major drawback. Enzymatic synthesis is free from toxic reagents and by-products; however, the total yield of capsaicin through this method is very low. The only viable method for commercial production is extraction from plants. As the criterion for commercial production clearly dictates that the source plant must contain more than 1% capsaicin and none of the Indian chillies, except Bhut Jolokia, contain more than 1% capsaicin. But the major issue is the limited cultivation of this chilli in few states of North East India and its seasonal nature of production. Thus, plant tissue culture technique can ensure round the year production of capsaicin.

Based on above results, it can be concluded that sinapic acid and vanillin showed promising effect on capsaicin accumulation. Both elicitors have enhanced yield of capsaicin in cell cultures by factor of hundreds. Vanillin, being an intermediate in capsaicin biosynthetic pathway, acts as a potent elicitor. The results showed that sinapic acid has been, by far, the best elicitor which led to highest capsaicin yield of 0.5% compared to all other elicitors used in this study. In earlier studies also, both these elicitors have shown promising response and led to significant increase in capsaicin accumulation.

Although significant enhancement in capsaicin accumulation has been observed, there are a number of other methods that can be studied to further enhance its production. Effect of various intermediates and precursors of capsaicin biosynthetic pathway on its production through suspension culture needs to be investigated. Besides, biotransformation and use of transgenics, for enhancing capsaicin production can also be explored.

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