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IN VITRO CALLUS INDUCTION FROM *CAPSICUM ASSAMICUM (BHUT JOLOKIA)* AND ITS CAPSAICIN QUANTIFICATION

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ABSTRACT

Capsicum assamicum (Bhut jolokia) is one of the hottest chillies of the world and contains very high amount of capsaicin. Attempt was made to induce in vitro callus culture of this hot chilli species with an aim to produce capsaicin. Callus was induced using Gamborg's B5 and MS media with various concentration and combination of phytohormones. B5 media with 4.6 mM 2,4 D + 3.4 mM kin ***and*** *3.5 mM 2,4 D + 1.1 mM Kin induced callus with high frequency. All calli were creamy white to light yellow in colour and highly friable. Cell suspension culture was established in the same media with a growth period of 15 days. Capsaicin extraction was performed in different solvents out of which maximum yield of 39 mg/g of capsaicin in chilli was obtained in 80% Methanol. Capsaicin content in callus was 0.38 mg/g of callus.*

KEY WORDS

Capsicum assamicum, callus culture, cell suspension culture and capsaicin.

INTRODUCTION

Capsicum assamicum (locally known as Bhut jolokia or Naga chilli) belonging to the Solanaceae family, is endemic to North East India (Purkayastha et al., 2012). It has been used for dietary and medicinal purposes by different ethnic tribes of the region (Meghvanshi et al., 2010). It has been ranked as world's hottest chilli in 2007 with Scoville Heat Unit score of more than 1 million. However, it has descended in the hotness rank since then, presently holds seventh position in the list of top ten hottest chillies in the world (Guinness Book of World's Record, 2013). The hotness of this chilli is due to the presence of high capsaicin content of 3-5%, unlike other Indian chillies, which contain less than 1% capsaicin (Borgohain and Devi, 2007). Capsaicin belongs to the alkaloid family of capsaicinoids and is mostly present in the placental partition of the chilli (Andrews, 1984). It possesses a number of medicinal properties *viz.* analgesic, anti–mutagenic, anti–obesity, anti–oxidant, anti-microbial and good for cardiovascular health (Braun and Walker, 1992; Oh et al., 2008; Peng and Li, 2010; Rakshit et. al., 2007; Roos et al., 1999; Snitker et al., 2009).

Capsaicin is in high demand in pharmaceutical industry for its medicinal value. The main source of capsaicin production is to extract it from fruit. Capsaicin oleoresins extracted from most of the chilli species in India is less than 1% whereas the standard requirement for commercial production states that it should be more than 1%. Bhut Jolokia with 3 – 5 % capsaicin content has highest amount among all the Indian chillies but the chilli fruit is available partly in a year, generally from May to October (Borgohain and Devi, 2007).

The aim of this study was to develop a technology for *in vitro* production of capsaicin through plant tissue culture which is advantageous over conventional means of extraction in several ways: (a) extraction of capsaicin from cell cultures can be simpler; (b) large volumes of capsaicin can be

extracted; (c) round the year production can be ensured; and (d) no geographical and climatic dependency is required. In the present work, callus was induced from *Capsicum assamicum* placenta in order to establish cell suspension culture and subsequently capsaicin was extracted from it.

MATERIALS AND METHODS

Cultivation of fruits of *Bhut Jolokia*: *Bhut Jolokia* was planted inside greenhouse of DRL, Tezpur in the month of April, 2016 following standard package of practices and ripe fruits were collected in the month of June 2016 to September 2016 (Fig 1).

Establishment of callus culture: Fruits were initially washed under running tap water and then treated with 1% Bavistin for 15 minutes and washed with distilled water until Bavistin was completely washed off. They were surface sterilized with 0.1% Mercuric chloride ($HgCl_2$) for 5 minutes followed by washing with sterile distilled water and then with 70% ethanol for 30 seconds. The pericarp was excised off and the seeds were removed. The placenta was cut into 1 mm thick explants which were inoculated in Gamborg's B5 and MS basal media. All the cultures were kept in the culture room maintaining a temperature of 25±2°C and a 16:8 light:dark cycle. The light intensity was maintained at 3000 lux provided by cool fluorescent white light.

For callus induction, the established explants were transferred in Gamborg's B5 and MS media supplemented with different concentrations and combinations of 2,4 –D and kinetin after 7 days of culture. For each treatment, explants were inoculated in ten replicates. Callus induced at the edges and at centre was transferred in the same media and was repeatedly subcultured until friable callus was obtained. The callus was transferred in fresh medium after every 30 days for its proliferation.

Parameters for growth analysis of callus: For qualitative growth analysis, callus colour and callus texture were observed, while for quantitative growth analysis, following parameters were studied:

(a) Callus induction frequency (C_{ip}): It was calculated using the formula:
(Total no. Of explants forming callus / Total no. of explants) x 100

(b) Callus dry matter percentage: The callus was dried at 50°C for 24 hours and then callus dry matter percentage was calculated using the formula:(Callus dry weight / Callus fresh weight) x 100

The fresh and dry weight of the callus was taken 30 days after inoculation.

Establishment of cell suspension culture:

For establishment of suspension culture, treatment media in which good friable callus with maximum yield was selected. 10 gm of friable callus was weighed and inoculated in 100 ml of Gamborg's B5 media + 4.6 mM 2,4 – D + 3.4 mM Kin and Gamborg's B5 media + 3.5 mM 2,4 – D + 1.1 mM Kin. The cultures were kept on an orbital shaker set at 120 rpm in the culture room with conditions similar to that maintained for callus induction. After 15 days of culture, the suspension was filtered out to separate out single cells and which were resuspended in the same media. Growth was measured by estimating the packed cell volume and absorbance at 234 nm in a UV – Vis spectrophotometer.

Parameters for growth analysis of cell suspension culture: For growth studies of suspension culture, following parameters were studied:

(a) Dry cell weight: Appropriate volume of suspension culture was centrifuged at 13,000 rpm for 15 minutes. The supernatant was discarded; pellets were washed and dried overnight at 60°C on pre – weighed Whatman filter paper.

(b) Cell turbidity: 2 ml aliquot was taken from suspension culture and absorbance was measured at 324 nm.

The above growth analysis was done on every alternate day since inoculation. The obtained data was recorded and was used to plot growth curve of cell culture.

Extraction of capsaicin from fruits and callus: For extraction of capsaicin, *Bhut Jolokia* fruits were collected from the greenhouse of DRL, Tezpur. The fruits were dried in a circulatory hot air oven and crushed into fine powder. Extraction was performed in three solvent systems, *viz.* ethanol, methanol and 80% methanol, both by cold maceration and soxhlet extraction.

For extraction by cold maceration, dried sample was extracted in all the three solvents in 1:10 ratio for 2 hours under agitation after which the solvent was decanted. The process was repeated until the solvent is colourless. All extracts were pooled together and stored at 4^0C until further analysis. Soxhlet extraction was performed using soxhlet apparatus. 7.5 gm of dried sample was taken in 150 ml solvent. The extraction was performed at 60^0C (methanol), 70^0C (ethanol) and 65^0C (80% methanol) until the solvent becomes colourless. The extracts were stored at 4°C until further analysis. Capsaicin extraction from callus was performed in 80% methanol using cold extraction following the same protocol used for extraction from fruits.

Quantification of capsaicin:

For capsaicin quantification through HPLC all the extracts were filtered through 0.45 µm syringe filter. HPLC protocol was based on the studies by Gonzales-Zamora et al (2015). Isocratic mixture of water : acetonitrile (50 : 50) was used as mobile phase and injection volume of 20 µl was eluted. Detection was performed using UV detector at 280 nm. Capsaicinoid related peak was obtained within 20 minutes. The capsaicin content of the extracts was measured by plotting on the standard curve prepared on the basis of HPLC data of standard capsaicin.

RESULTS AND DISCUSSION

Establishment of Callus culture:

Callus induction was observed in 05 and 08 treatments out of 15 and 19 different combinations in B5 and MS media respectively (Table 1). Early callus induction (4 days) with C_{ip} of 80% was recorded in B5 medium containing 4.6 mM 2,4 – D and 3.4 mM Kin followed by B5 containing 3.5 mM 2,4 – D and 1.1 mM kin media (7 days with 90% C_{ip}) (Table 1). It can be seen from the data that frequency of callus induction in B5 media is higher than that in MS media. Moreover, early callus induction was observed in B5 media than in MS media supplemented with same volume of hormones. The results corroborated with the findings of Rakshit *et. al.* (2007) who reported high frequency of callus induction in *Capsicum annum* L. when inoculated in B5 media. No report of callus induction in *Capsicum assamicum* using B5 media could be traced. Mohammad *et. al.,* 2003 reported that 2,4 – D and Kin are best for calls induction which corroborated with our observation. Mawahib E.M. EL Nour (2013) observed that, although there is no considerable difference between callus induction frequency in MS and B5 media, callus initiation occurs faster in B5 media.

No callus was induced in B5 media supplemented with 2,4 – D in concentrations 9mM and higher (Table 1). However, higher concentration of 2,4 – D with significantly lower concentrations of Kin resulted in callus induction in B5 media containing 9 mM 2, 4 – D and 1.1 mM Kin. In MS media, no callus was induced in media supplemented with 9 mM 2, 4 – D and 2.3 mM Kin and 9 mM 2, 4 – D and 3.4 mM Kin. Increasing the concentration of 2, 4 – D to 13.6 mM in MS media in combination with Kin (in 2.3 mM and 3.4 mM conc.) induced callus but the callus induction frequency was significantly low. This may imply that higher concentration of auxin has inhibitory effect on callus induction. Lowering the 2, 4 – D concentration to 3.5 mM resulted in callus induction in B5 media when combined with very low Kin concentration of 1.1mM and 2.3 mM. However, increasing the Kin concentration to 3.4 mM resulted in no callus induction. Further lowering of 2, 4 – D concentration did not induce callus in either of the media. From these observations, it can be concluded that higher concentrations of 2, 4 – D and Kinetin have inhibitory effects on callus induction. Callus is induced when intermediate concentration of 2, 4 –D is combined with significantly lower concentration of Kin. Similar results on the effect of 2, 4 – D and Kinetin were reported by Khatar et. al.(2013).

The callus induced were creamy white to slightly yellow in colour and friable in nature. Callus friability increased with subsequent subcultures (Fig. 2 & 3). The treatment media with highest callus induction frequency *viz.* B5 + 4.6 mM 2,4 – D and 3.4 mM Kin was selected as the best treatment for callus induction and for subsequent growth kinetics.

Growth of the callus in these two treatments was analyzed in terms of fresh and dry weight (gm) and dry matter content (%). Increment of fresh and dry weight as well as dry matter content of the callus was nearly doubled after 30 days of inoculation which indicated considerable amount of growth of the callus (Table 2).

Figure 1: Pot cultivation of *Bhut Jolokia* in DRL greenhouse

A **B** **C** **D**

Figure 2. Development of callus from placenta of *Capsicum assamicum*.
(A) Placental explants (B) Callus Induction (centre and edges of explants) (C) Callus proliferation
(D) Friable and creamy callus after 3 sub-cultures

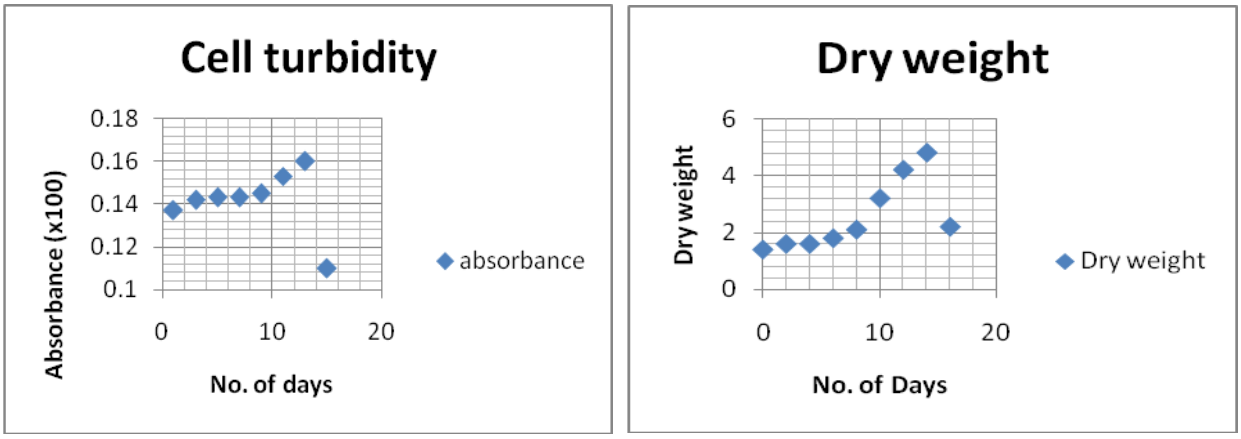
Figure 3: Effect of sub-cultures on callus friability.

Establishment of cell suspension culture:

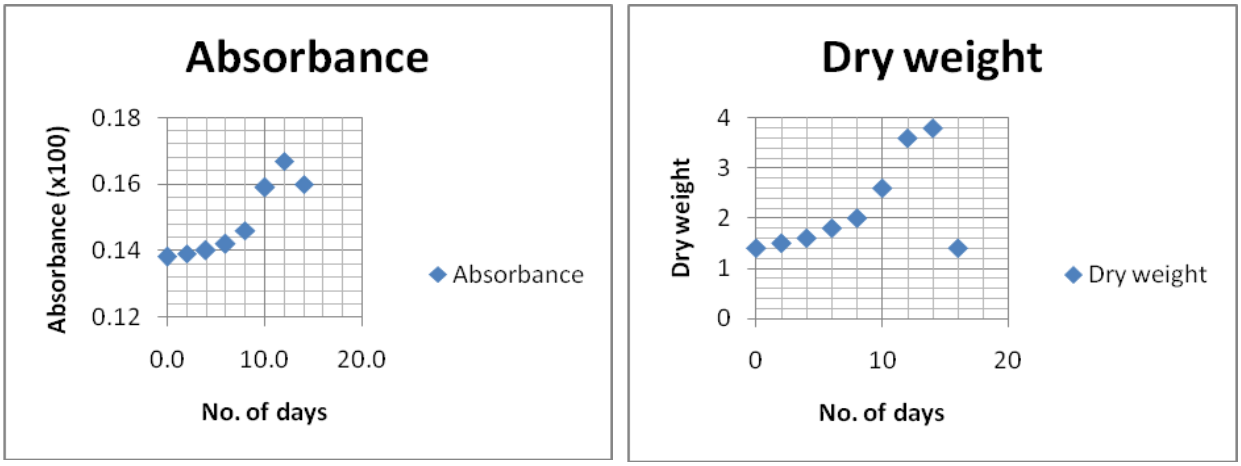
Cell suspension culture was established in broth B5 medium containing two combinations of hormones *viz.* 4.6 mM 2,4 – D + 3.4 mM Kin and 3.5 mM 2,4 – D + 1.1 mM Kin. The growth curve shows that the cells in suspension form a sigmoid growth curve where cells enter exponential phase after 8 days and reached stationary phase after 14 days from the day of inoculation (Fig. 4A & 4B). Analysing the growth curve, the specific growth rate was found to be 0.34 both in B5 + 4.6 mM 2,4 – D + 3.4 mM Kin and in B5 + 3.5 mM 2,4 – D + 1.1 mM Kin respectively.

Quantification of capsaicin:

The capsaicin content of the extracts was measured from the standard curve prepared on the basis of HPLC data of standard capsaicin (Fig. 5 and 6). Among the three solvent systems used for extraction of capsaicin highest content was obtained from cold extraction in 80% methanol with an yield of 39 mg capsaicin / gm of chilli powder which was found to be higher than that obtained in other methods (Table 3). Thus cold extraction in 80% methanol was used for capsaicin extraction from callus. A small amount of 0.38mg capsaicin was obtained from 1 gm of dry callus. Although the amount produced in callus is very low, there is prospect of increasing the amount of capsaicin in cell suspension culture.



A



B

Figure 5: Growth curve of cell suspension culture in (A) B5 + 4.6 mM 2,4–D + 3.4 mM kinetin (B) B5 + 3.5 mM 2,4–D + 1.1 mM kinetin

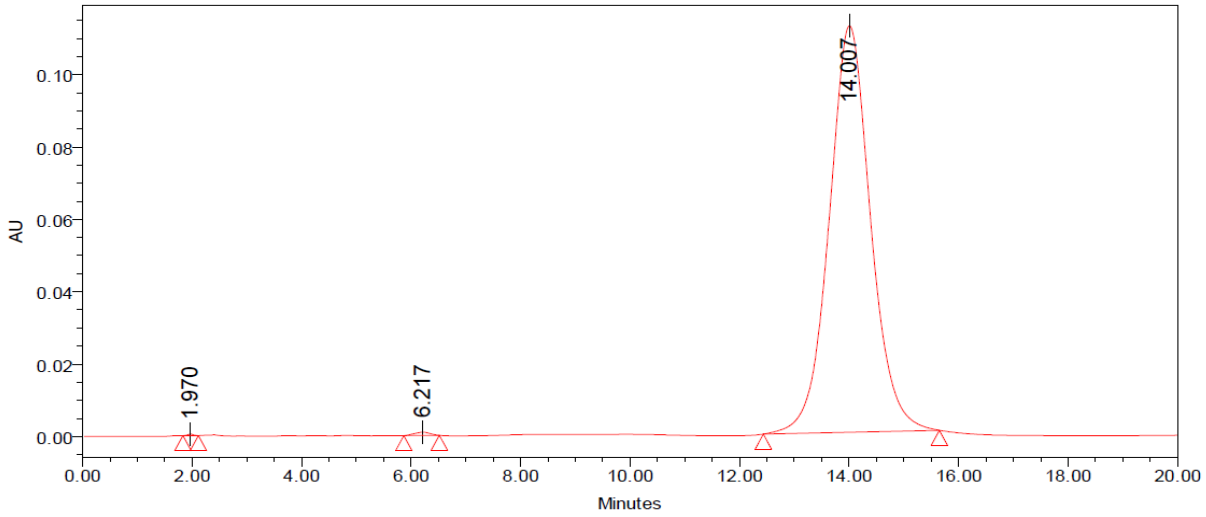


Figure 7:- HPLC chromatogram of Capsaicin standard

Table 1: Hormonal concentration for callus induction and their effect in different concentration in B5 and MS media

Sl. No.	2,4-D (mM)	Kin (mM)	Observation			
			B5		MS	
			Days for initiation	C_{ip}	Days for initiation	C_{ip}
1.	4.6	1.1	No callus	-	8	55
2.	4.6	2.3	5	50	10	70
3.	4.6	3.4	4	80	8	50
4.	9.0	1.1	4	60	8	62
5.	9.0	2.3	No callus	-	No callus	-
6.	9.0	3.4	No callus	-	No callus	-
7.	13.6	1.1	No callus	-	No callus	-
8.	13.6	2.3	No callus	-	8	16
9.	13.6	3.4	No callus	-	8	25
10.	3.5	1.1	7	90	No callus	-
11.	3.5	2.3	7	10	No callus	-
12.	3.5	3.4	No callus	-	No callus	-
13.	2.3	1.1	No callus	-	No callus	-
14.	2.3	2.3	No callus	-	No callus	-
15.	2.3	3.4	No callus	-	10	54
16.	13.6	0.2	Not tested	-	No callus	-
17.	13.6	0.4	Not tested	-	No callus	-
18.	13.6	0.6	Not tested	-	No callus	-
19.	13.6	0.9	Not tested	-	7	25

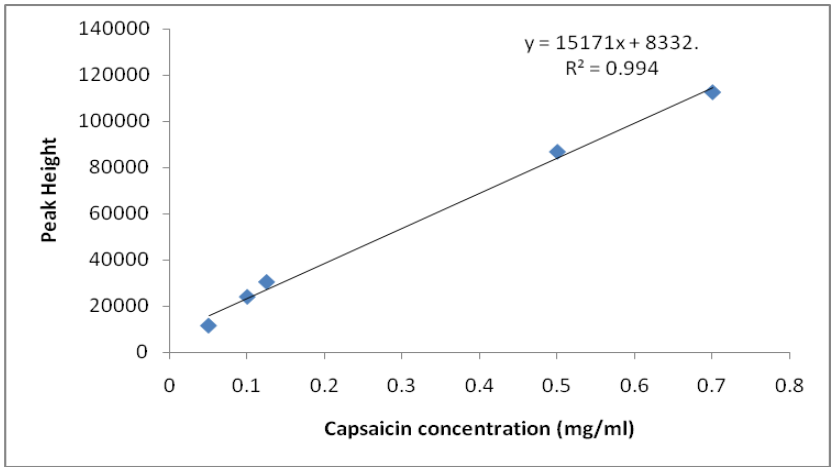


Figure 8: - Capsaicin standard curve

Table 2: Callus fresh weight, dry weight and dry matter content in B5 media with different 2,4–D and kinetin concentration 30 days after callus initiation.

Sl. No.	Media combination	Initial inoculum (gm)	Fresh weight (gm)	Dry weight (gm)	Dry matter content (%)
1.	B5 + 4.6 mM 2,4–D + 3.4 mM kinetin	10	21.4 ± 1.2	1.03 ± 0.05	3.66 ± 0.2
2.	B5 + 3.5 mM 2,4–D + 1.1 mM kinetin	10	23.4 ± 4.27	0.8 ± 0.15	3.5 ± 0.06

Table 3: - Capsaicin yield from Bhut jolokia using different extraction methods

Method	**Cold extraction**			**Soxhlet Extraction**		
Solvant used	Methanol	Ethanol	80% methanol	Methanol	Ethanol	80% methanol
Yield	15 mg/ml	3 mg/ml	39 mg/ml	4 mg/ml	7 mg/ml	3.4 mg/ml

CONCLUSION

Bhut Jolokia is in great demand, both in the national and international market, due to its high capsaicin content. Since cultivation of this chilli is restricted to only one season in a year, cell suspension culture can play a vital role in terms of round the year production of capsaicin from this chilli. Though it was observed in this study that the capsaicin content of the *Bhut Jolokia* cells decreases significantly during the suspension culture, there are methods for its enhancement which needs to be investigated and further studies to increase its content is in progress.

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