

Research Article



Chemotypic Diversity in Different Bhut Jolokia Fruits: *In vitro* Conservation and Mass Propagation of Superior Ecotype

Subrata Kundu, Anupam Das, Sk Moquammel Haque, Biswajit Ghosh*

Plant Biotechnology Laboratory, Dept of Botany, Ramakrishna Mission Vivekananda Centenary College, Rahara, Kolkata, India.

*Corresponding author's E-mail: ghosh_b2000@yahoo.co.in

Accepted on: 10-08-2015; Finalized on: 30-09-2015.

ABSTRACT

The Bhut jolokia (*Capsicum chinense*) fruit is extensively used in pharmaceutical industries for extraction of capsaicin. The content of capsaicin and major bioactive compounds differ greatly due to geographical variation. The aim of this study was to identify potential chemotype of Bhut jolokia and optimized *in vitro* micropropagation protocol of the superior ecotype for commercial purposes. Five Bhut jolokia plants were collected from different geographic position in North-East India and their chemotypic variations were analyzed. Among these ecotype total phenolic content combined with ascorbic acid in the fruit was found to be significantly higher in BJ-1 compared to other ecotypes. The free radical scavenging activity as well as reducing power of the BJ-1 ecotype also corroborate the higher phenolic content. The superior ecotype BJ-1 was selected for *in vitro* conservation and micropropagation. The shoot-tips explants was inoculated on Murashige and Skoog (MS) basal medium with different concentrations and combinations of cytokinins and auxins. Optimum multiplication was achieved on medium containing 6-benzyl-aminopurine (2.5 mg L^{-1}) and indole-3-acetic acid (0.25 mg L^{-1}). *In vitro* rooting of multiplied individual shoots was achieved on half strength MS medium supplemented with indole-3-butyric acid (IBA) (2 mg L^{-1}) and α -naphthalene acetic acid (NAA) (1 mg L^{-1}), with a maximum of 18.9 ± 0.28 roots. These micropropagated plants were successfully established in soil after hardening with 95.38% survival rate.

Keywords: Bhut jolokia, capsaicin, antioxidant potential, HPTLC, micropropagation.

INTRODUCTION

Chili (*Capsicum* sp.), a member of the Solanaceae family is a diploid, self-pollinating crop which includes more than 3,000 diverse species worldwide.¹ The chili has a wide range of uses in pharmaceuticals industries, natural coloring agents and cosmetics as well as an ornamental plant. It also provides many essential vitamins, minerals and nutrients that have enormous importance for human health.^{2,3} It has been reported that ingestion of chili fruits prevent various ailments including cardiovascular disease, diabetes and neurological disorders.⁴⁻⁶

The pungency in chili fruits is due to the presence of series of alkyl vanillylamides including capsaicin (trans-8-methyl-*N*-vanillyl-6-nonenamide).^{7,9} Among the different varieties of *Capsicum chinense*, 'Bhut jolokia' is an interspecific hybrid cultivated in the Northeastern states of India. It has been reported that Bhut jolokia is the second world's hottest chili.¹⁰ This important chili is widely cultivated in Northeastern states of India including Assam, Nagaland and Manipur. Besides pungency, the fruits of this chili also possess a pleasant and edible aroma.¹¹ The fruit is conventionally used by the indigenous people of the Northeast Indian treating various human ailments including tonsillitis, ulcer and dysentery.^{12,13} Due to higher capsaicin content and medicinal properties, the Bhut jolokia are also extensively used in pharmaceutical industries.¹¹ The capsaicin content and amount of bioactive compounds in pepper fruits is controlled by environment as well as by genotype–environment interaction.¹⁴⁻¹⁶ It has been

reported that capsaicin content in Bhut jolokia fruits may also vary greatly during the conventional propagation method.¹⁷ Never the less propagation by seed is inadequate for large-scale industrial production due to its short viability and low germination rate. Under such circumstances, *in vitro* culture will provide innovative way for the asexual multiplication of this important chili to maintain its major bioactive compounds as well as capsaicin content. Therefore, the objective of present study was to analyze the diversity in major bioactive compounds including capsaicin in different ecotype of Bhut jolokia and established optimized micropropagation protocol for superior ecotype.

MATERIALS AND METHODS

Plant material

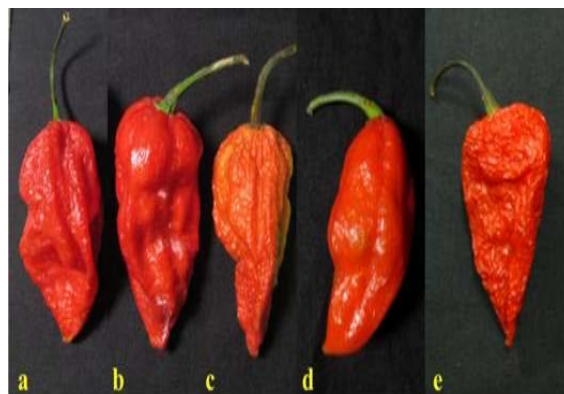


Figure 1: Bhut jolokia fruits collected from different geographical locations. a. BJ-1; b. BJ-2; c. BJ-3; d. BJ-4 and e. BJ-5.

The Bhut jolokia plants were collected from five different regions of North-East India (BJ-1: Dimapur, Nagaland; BJ-2: Silchar, Assam; BJ-3: Namrup, Assam; BJ-4: Changangei, Manipur and BJ-5: Itanagar, Arunachal Pradesh). The mature fruits of the collected plants were used for biochemical analysis (Fig. 1).

Determination of total phenolic and flavonoid content

Total phenolic content was measured following Folin-Ciocalteu method.¹⁸ The total flavonoid content was estimated following the aluminum chloride colorimetric assay.¹⁹ Total phenolic and flavonoid content was expressed as mg g⁻¹ fresh weight (fw).

Estimation of ascorbic acid and total protein

The ascorbic acid was estimated using Folin phenol reagent.²⁰ The amount of ascorbic acid was expressed as mg g⁻¹ fw. The total protein content was determined following the method reported by Bradford.²¹

Estimation of total carbohydrate and reducing sugar

Total carbohydrate content was determined in the aqueous solution with anthrone sulfuric acid reagent.²² The amount of reducing sugar was estimated by dinitrosalicylic acid method.²³ The reducing sugar and total carbohydrate content was expressed as mg g⁻¹ fw.

Determination of antioxidant capacity

Free radical scavenging by the use of the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical

The antioxidant activity of the ethanolic extracts was determined according to the DPPH method.²⁴ Briefly, 50 µL of different concentrations of ethanolic extract was mixed with 950 µL of 6.34 × 10⁻⁵ M DPPH radical solution in ethanol. The mixture was allowed to stand for 30 min in the dark. The absorbance was recorded at 517 nm. Free radical scavenging activity was calculated using the following formula: % inhibition = [(A_B-A_E)/A_B] × 100

Where, A_B and A_E are the absorbance at 30 min of the blank and the sample, respectively.

The antioxidant activity was calculated as IC₅₀ (µg mL⁻¹), the extract dose required to cause a 50% decrease of the absorbance at 517 nm. A lower IC₅₀ value corresponds to a higher antioxidant activity. The antiradical activity was expressed as 1/IC₅₀.

Free radical scavenging by the use of the ABTS radical

The free radical scavenging activity was studied using the ABTS radical cation decolorization assay.²⁵ ABTS radical cation was produced by reacting 7.0 mM ABTS solution with 2.45 mM potassium persulfate and the mixture was kept in dark at room temperature for 16 h. For the analysis, the solution was diluted in double distilled water to an absorbance of 0.7 (±0.02) at 734 nm. Fifty microliter of the ethanolic extract of different concentrations was added to 950 µL of ABTS solution and the absorbance was recorded after incubation of 30 min at 30 °C. The

different amount of ascorbic acid was used as standard. The percentage of inhibition of ABTS+• was calculated using above formula (eq 1). The antiradical activity was expressed as 1/IC₅₀.

Ferric reducing antioxidant power (FRAP) assay

Antioxidant activity was also determined by ferric reducing power using a spectrophotometer at 700 nm.²⁶ Briefly, 1 mL of extract was mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Then 2.5 mL of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 20 min. Then 1 mL supernatant was added to the mixture of 0.5 mL of 0.1% FeCl₃ and 2.5 mL of double distilled water. The absorbance was measured at 700 nm after incubation for 10 min. Different concentrations of ascorbic acid was used as control. Increase in absorbance was interpreted as increased ferric reducing activity. The IC₅₀ value (µg mL⁻¹) is the concentration giving an absorbance of 0.5. The antiradical activity was expressed as 1/IC₅₀.

Extraction of capsaicin and separation by High Performance Thin Layer Chromatography (HPTLC)

The extraction and quantification of capsaicin was performed according to previously published protocol.²⁷ For HPTLC five microliters of the ethanolic extract (500 mgmL⁻¹) was applied (band length – 5.0 mm; distance between bands – 14.5 mm; distance from left edge - 20.0 mm; distance from lower edge - 10.0 mm) on a pre-coated TLC aluminum sheets of silica gel G60 F₂₅₄ of 200 µm thickness plate, 20x10cm (Merck, Mumbai) using Linomat 5 automated TLC applicator (Camag, Muttenz, Switzerland) equipped with a 100-µL syringe (Hamilton, Nevada, USA). The standard capsaicin at concentration of 0.1 mg mL⁻¹ was spotted as a reference on the TLC plate. Prior application, the plate was pre-washed with methanol AR and dried at 60°C. TLC plates were developed using the mobile phase Chloroform: Methanol: Acetic acid: Hexane (2.85: 0.15: 0.15: 1, v/v/v) in a Camag HPTLC twin-trough chamber (20x10cm). The chamber was saturated with filter paper for 15 minutes and plate equilibrium was carried out for 10 minutes. Plate was developed upto 85.0 mm and dried under stream of air. Separated bands were quantified by HPTLC densitometric scanning using Camag TLC Scanner 4 in the absorption mode at 282 nm operated by WinCATS software (version 1.4.8). Quantitative analysis of the extracts was done by comparative densitometric analysis via height and area with the standards.

In vitro propagation

In vitro shoot proliferation and elongation

For *in vitro* propagation purpose MS basal medium was used.²⁸ The pH of the medium was adjusted to 5.6 before the addition of agar. The medium was autoclaved at 1.04 kg cm⁻¹ pressure and 121°C for 18 min. The shoot tips (8-10 mm) were used as explant. The explants were



implanted in culture tubes (25 mm × 150 mm) containing MS basal medium supplemented with 0.8% agar, 3.0% sucrose and different concentrations of 6-Benzyl-aminopurine [BAP], kinetin [Kn], α -naphthalene acetic acid [NAA] and Indole-3-acetic acid [IAA] alone or in combination with three different concentration (Table 3). For shoot elongation, cytokines were withdrawn and only 0.5 mg L⁻¹ IAA were supplemented. The cultures were incubated inside the growth chamber maintained at 24±2 °C under a 16 hrs photoperiod with a photosynthetic photon flux density of approximately 50 $\mu\text{mol m}^{-2}\text{s}^{-1}$ emitted from cool fluorescent tubes (Philips India Ltd.). The cultures were sub-cultured in their respective fresh media at every 4 weeks intervals.

In vitro root induction

For *in vitro* root induction, we follow the method previously optimized by the present laboratory.^{29,30} Elongated adventitious shoots were cut into about 2.5 cm pieces and implanted on half strength MS media supplemented with different concentrations of IBA or NAA. MS medium devoid of growth regulator served as control. The percentage of rooting, mean root length and total number of roots were calculated after 4 weeks.

Hardening of tissue cultured derived plantlets

Rooted plantlets (about 6-8 cm) were transferred to small earthen pots containing 'Soilrite' (Keltech Energies Ltd., Bangalore, India) and protected with transparent polythene bags to maintain 90-99% relative humidity and were kept in 25 ± 2°C temperature and 16-h photoperiod for 20 to 25 days. Thereafter, the acclimatized plants were relocated on earthen tubs containing a mixture of soil and vermin compost (3:1 ratio) and maintained inside the poly-green house (30 ± 2°C temperature and relative humidity of 60-65%) for another 30 days. Finally, the plantlets were transferred to the field under full sunlight.

Statistical analyses

A randomized block design was used to set up all the experiments. The data presented are means ± standard deviation of 3 biological replicates and are examined by analysis of variance (ANOVA) to detect significant differences between means and those differing significantly ($p < 0.05$) are compared using Duncan multiple range test (DMRT) at the same (5%) probability level using SPSS software (17.0.0, 2008, SPSS Inc.).

RESULTS AND DISCUSSION

Comparative analysis of bioactive compounds

The variation of major bioactive compounds of Bhut jolokia fruits due to different geographical discrepancy were analyzed. It was found that total phenolic content of BJ-1 ecotype was significantly higher ($17.878 \pm 0.445 \text{ mg g}^{-1} \text{ fw}$) compared to the other ecotypes (Fig. 2A). The total phenolic content in different ecotype were found to be in following order: BJ-1>BJ-2>BJ-3 >BJ-5>BJ-4. The flavonoid content also differ significantly among different ecotypes

(Fig. 2B). The highest total flavonoid content was found to be $0.598 \pm 0.055 \text{ mg g}^{-1}$ in ecotype BJ-2 followed by $0.536 \pm 0.043 \text{ mg g}^{-1}$ in BJ-1. The ascorbic acid content was found to be highest ($3.367 \pm 0.318 \text{ mg g}^{-1} \text{ fw}$) in BJ-1 ecotype (Fig. 2C). The ascorbic acid content in different ecotype were found to be in following order: BJ-1>BJ-2>BJ-3 >BJ-4>BJ-5. Although the total protein content was comparable in three samples (BJ-2, BJ-3 and BJ-4) but reducing sugar were found to be significantly higher in BJ-4 compared to others (Fig. 2E). The total carbohydrate content in different ecotype were found to be in following order: BJ-2>BJ-1>BJ-4>BJ-5>BJ-3 (Fig. 2F)

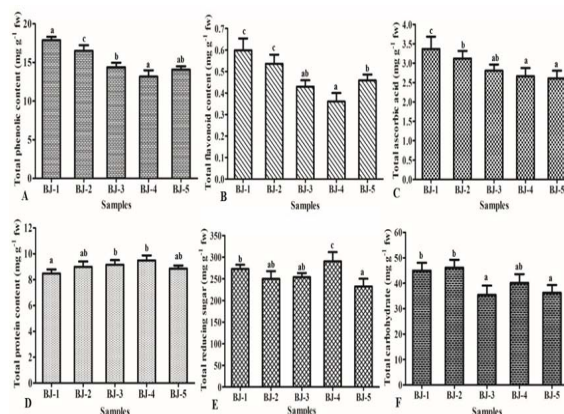


Figure 2: Comparative analysis of bioactive compounds in different ecotypes. A. total phenolic content B. total flavonoid content and C. total ascorbic content. D. total protein content; E. reducing sugar and F. total carbohydrate content. Bars are mean ± SD; bars followed by same alphabets are not significantly different at $p \leq 0.05$ according to ANOVA and DMRT.

Comparative analysis of antioxidant potential of different ecotypes

The ethanolic extract of the Bhut jolokia fruits collected from different geographical positions showed scavenging activity over DPPH free radicals. The antiradical activity of five different ecotype was found to be in following order: BJ-1>BJ-2>BJ-3 >BJ-5>BJ-4. The antiradical activity of BJ-1 over DPPH radical was found to be 2.225 ± 0.0678 (Fig. 3). The ethanolic extract also showed ABTS radical scavenging activity. The decreasing order of scavenging activity was also found to be in similar pattern as DPPH. The reducing power of ethanolic extract was also significantly higher in BJ-1 compared to other samples (Fig. 3). It has been reported that there is a positive correlation between the total phenolic content and antioxidant activity.^{31,32} A highly positive correlation between the DPPH ($R^2 = 0.958$) and ABTS ($R^2 = 0.980$) values and total phenolic content clearly indicated that phenolic compounds could be one of the major components responsible for antioxidant activity. The results of the present study revealed that the higher phenolic content in the BJ-1 ecotype correlated with higher antioxidant potential.

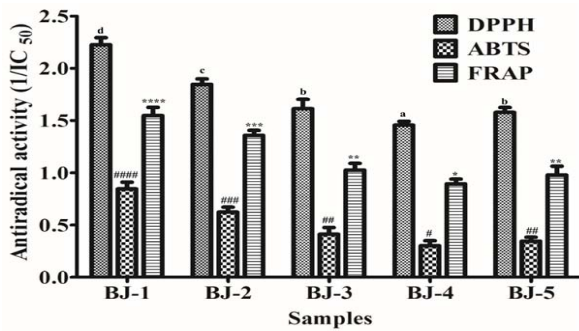


Figure 3: Comparative analysis of antiradical activity in different ecotypes. Bars are mean \pm SD; bars followed by same symbols are not significantly different at $p \leq 0.05$ according to ANOVA and DMRT

Variation in capsaicin content in different ecotype

The capsaicin of five Bhut jolokia fruits was extracted and subjected to HPTLC densitometric analysis (Fig. 4). The linear regression analysis data for the calibration plots using capsaicin standard showed good linear relationship with regression coefficient (R^2) of 0.985 in the concentration range of 1-5 $\mu\text{g}/\text{spot}$. The capsaicin content of the analyzed samples were calculated using capsaicin linear regression equation and it was expressed as microgram of capsaicin per gram tissue (Table 1). The highest capsaicin content was found in BJ-1 with $5.148 \pm 0.078 \text{ mg g}^{-1}$ while sample BJ-5 exhibited lowest content ($3.448 \pm 0.047 \text{ mg g}^{-1}$). The amount of capsaicin in the different ecotype varied in the 3.4–5.1 mg g^{-1} range.

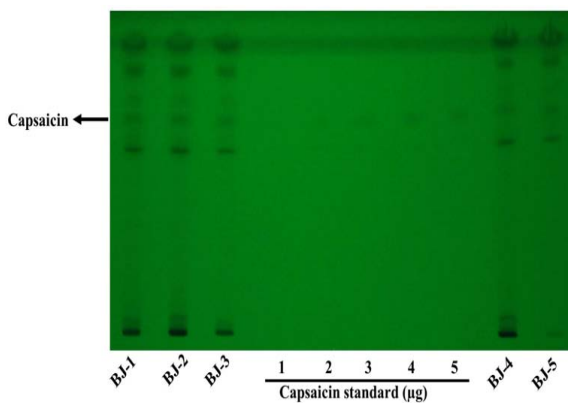


Figure 4: HPTLC fingerprints of capsaicin standard and different ecotypes.

Table 1: Comparative analysis of capsaicin content of different ecotypes.

Sample	Amount of capsaicin (mg g^{-1}) Mean \pm SD
BJ-1	5.148 ± 0.078
BJ-2	4.648 ± 0.054
BJ-3	4.048 ± 0.088
BJ-4	3.448 ± 0.047
BJ-5	3.848 ± 0.038

Effects of plant growth regulators for optimal proliferation

The biochemical as well as HPLTC analysis indicates that BJ-1 ecotype was found to be superior ecotype compared to other in terms of antioxidant potential including phenolic and ascorbic acid content. Therefore, ecotype BJ-1 was selected as explant for *in vitro* conservation and micropropagation. Maximum of 5.5 ± 0.41 shoots were induced in MS medium containing 2.5 mg L^{-1} BAP after 30 days of culture (Fig. 5). Out of three cytokinin tested BAP was found more efficient compared to others for shoot proliferation (Table 2). The effectiveness of BAP alone on multiple shoot bud differentiation has been well established.³³⁻³⁵ It has been observed that low concentration of IAA (0.25 mg L^{-1}) combined with optimum concentration of BAP (2.5 mg L^{-1}) had advantageous effect on multiplication rate as well as elongation of multiplied shoots of Bhut jolokia. Maximum 8.4 ± 0.37 shoots from each explant were induced in MS basal medium supplemented with 2.5 mg L^{-1} BAP with 0.25 mg L^{-1} IAA after 30 days of culture (Table 2).

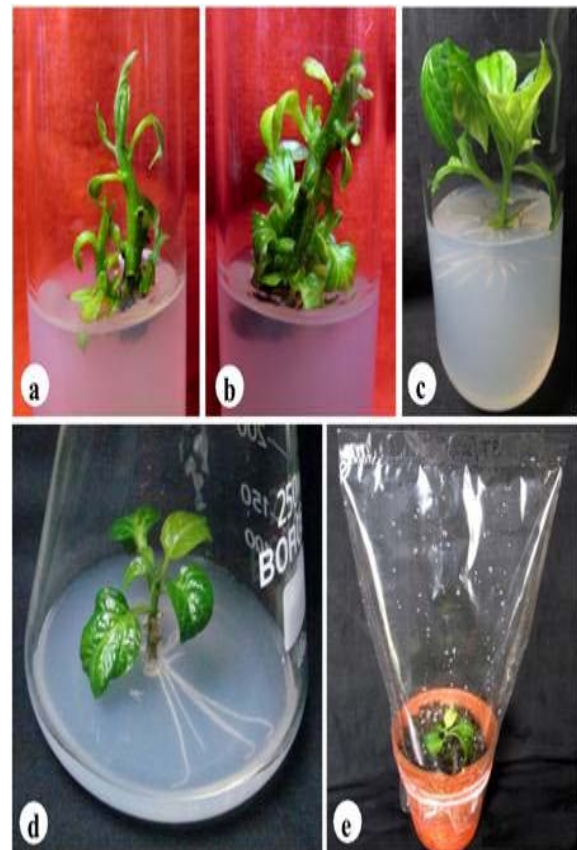


Figure 5: Micropropagation of Bhut jolokia (a) Multiple shoots induced in MS medium supplemented with 2.5 mg L^{-1} Kn after 30 d of implantation. (b) Multiple shoots induced in MS medium supplemented with 2.5 mg L^{-1} BAP+ 0.25 mg L^{-1} IAA after 30 d of implantation. (c) *In vitro* rooting in MS medium supplemented with 1.5 mg L^{-1} IBA after 21 d of implantation. (d) *In vitro* derived complete plantlet ready for hardening. (e) Hardening of regenerated plants.

***In vitro* rooting of regenerated shoots and acclimatization of plantlets**

The microshoot tips (about 2–3 cm) with two to three leaves were harvested from the medium and used for rooting. The rooting response differed depending upon the concentration of NAA and IBA as well as the strength of the basal medium (Table 3).

Rooting percentage was significantly lower in full-strength as well as one-quarter strength medium compared to the half strength MS medium. Therefore ½ MS media with different concentrations of NAA and IBA were used.

Table 2: Influence of plant growth regulators and their interaction on morphogenic response from nodal explants of *Bhut jolokia* on MS growth medium.

SL No.	MS media with growth regulators (mg l ⁻¹)				Percentage of response	No. of multiplied shoots / nodal explant
	BAP	Kn	IAA	NAA		
1	1.0	0.0	0.0	0.0	70.15	3.6 ± 0.42 ^a
2	2.5	0.0	0.0	0.0	83.42	5.5 ± 0.41 ^c
3	4.0	0.0	0.0	0.0	73.56	4.3 ± 0.37 ^b
4	0.0	1.0	0.0	0.0	53.34	3.2 ± 0.56 ^a
5	0.0	2.5	0.0	0.0	66.28	4.7 ± 0.48 ^b
6	0.0	4.0	0.0	0.0	60.16	3.8 ± 0.57 ^a
7	2.5	0.0	0.10	0.0	86.44	6.1 ± 0.39 ^c
8	2.5	0.0	0.25	0.0	93.64	8.4 ± 0.37 ^e
9	2.5	0.0	0.40	0.0	90.27	6.3 ± 0.12 ^c
10	2.5	0.0	0.0	0.10	83.25	6.0 ± 0.23 ^c
11	2.5	0.0	0.0	0.25	90.89	7.3 ± 0.45 ^d
12	2.5	0.0	0.0	0.40	86.47	5.8 ± 0.15 ^c

Values are mean ± SE of three independent experiments each with 12 replicates. Treatment means followed by same letter within column are not significantly different from each other (P < 0.05) according to ANOVA and DMRT.

Table 3: The effect of various concentrations of auxins treatments on root induction.

Nutrient medium (3% Sucrose)	Concentrations of auxins (mg l ⁻¹)		Percentage of root induction	No. of roots induced / micro shoot
	NAA	IBA		
MS	0.0	0.0	51.0	3.2±0.14 ^a
½ MS	0.0	0.0	65.0	4.6±0.26 ^b
½ MS	0.5	0.0	74.0	6.3±0.21 ^c
½ MS	1.0	0.0	77.2	9.4±0.26 ^d
½ MS	2.0	0.0	82.2	12.1±0.32 ^d
½ MS	3.0	0.0	80.5	11.0±0.24 ^f
½ MS	0.0	0.5	88.2	10.7±0.16 ^e
½ MS	0.0	1.0	90.8	11.1±0.28 ^f
½ MS	0.0	2.0	92.8	15.4±0.22 ⁱ
½ MS	0.0	3.0	83.5	13.6±0.20 ^d
½ MS	1.0	2.0	97.9	18.9±0.28 ^b
½ MS	2.0	1.0	93.8	16.4±0.22 ^j

Values are mean ± SE of three independent experiments each with 12 replicates. Treatment means followed by same letter within column are not significantly different from each other (P < 0.05) according to ANOVA and DMRT.



CONCLUSION

The superior ecotype of Bhut jolokia identified based on antioxidant potential as well as capsaicin content could be commercially cultivated and utilized in traditional medicines and pharmaceutical industries.

The optimized *in vitro* culture system of chili would also be beneficial for genetic manipulation, germplasm preservation including *in vitro* production of capsaicin.

Acknowledgement: SK is thankful to Council of Scientific & Industrial Research (CSIR), India for providing Research Associate (RA) fellowship. AD thanks for research fellowships to the Department of Science and Technology, West Bengal (616(Sanc.)/ST/P/S&T/1G-6/2011). SMH acknowledge to "Ministry of Minority Affairs" (MOMA) and 'University Grant Commission' (UGC) for providing "Maulana Azad National Fellowship" (MANF).

All authors are thankful to Swami Kamalasthananda, Principal, Ramakrishna Mission Vivekananda Centenary College, Rahara, Kolkata (India) for the facilities provided during the present study and also acknowledge DST-FIST program for infrastructural facilities.

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Source of Support: Nil, **Conflict of Interest:** None.

