Production of Virus Free Naga King Chilli (*Capsicum chinense* Jacq.) by *in Vitro* Meristem Tip Culture

Ngasepam Tombisana Meetei[1], Amit Kumar Singh[2] and Nirmal Mandal[3]

[1,2,3]Department of Agril. Biotechnology, Faculty of Agriculture, BCKV, Mohanpur, Nadia, West Bengal
E-mail: [1]ntmeetei@gmail.com, [2]geneamit@gmail.com, [3]nirman_bckv05@yahoo.com

Abstract—*The Naga king chilli (Capsicum chinense Jacq) is one of the hottest chillies in the world with a Scoville heat unit (SHU) rating of 1,001,304 and is native to the north eastern region of India. The Naga king chilli is susceptible to different viruses such as Cucumber mosaic virus (CMV), Potato virus Y (PVY) and Chilli leaf curl virus. Virus infection results in reduction in yield as well as quality and the only really effective measure for their control is through prevention. Virus (CMV) free Naga king chilli was obtained through shoot apical meristem culture. The detection of virus was done by polymerase chain reaction (PCR) using coat protein gene specific primers. Shoot tips from infected plants were used as explants and plants were regenerated through apical meristems having 2 to 3 primordial leaves (sizes varying from 0.5 to 1 mm). The meristems were first allowed to proliferate shoots using Murashige and Skoog (MS) medium amended with various concentrations of Thidiazuron (TDZ) or Benzyl Amino Purine (BAP) (5, 10 and 20 µM). MS medium supplemented with 10 µM TDZ gave the maximum number of shoot buds i.e., 4.25 ± 0.96 shoot buds per explant. Elongated shoots were rooted on MS supplemented with 5 µM NAA. The plantlets were then transferred to artificial soil (peat, perlite and vermiculite mixture in 1:1:1 ratio) and kept in greenhouse for hardening. Regenerated plants were tested for the virus using PCR based methods which displayed negative results for CMV.*

Keywords*: Naga king chilli, PCR, meristem culture, virus free plants.*

1. INTRODUCTION

Chilli (*Capsicum* spp.) belongs to the family Solanaceae (Nightshade family) and they are used variously as a pungent flavor in food, natural plant colour, pharmaceutical ingredient and as sprays for riot control and self-defence. There are about twenty two wild and five cultivated species of *Capsicum* namely *C. annuum, C. baccatum, C. frutescens, C. chinense* and *C. pubescens* [4]. In India, *C. annuum* is the most widely cultivated, whereas, the cultivation of *C. frutescens, C. chinense,* and *C. baccatum* is not common and are usually restricted to homestead gardening in different regions [12].

The northeast region of India is recognized as hot-spot for chilli diversity [10]. Among the many landraces of chilli that are cultivated in the northeast, the Naga king chilli is best known worldwide. The Naga king chilli (*Capsicum chinense* Jaqc.) is considered one of the world's hottest chilli having Scoville heat units (SHU's) rating of 1,001,304 [5]. A number of variants of this chilli are known in the north-eastern region of India [9] with different local names such as Naga chilli in Nagaland, Bhut Jolokia in Assam, and U-Morok in Manipur [13, 16]. This chilli is grown mainly in the state of Nagaland, Assam and Manipur and to some extent in Mizoram, Arunachal Pradesh and Meghalaya. The Nagaland Government obtained the Geographical Indication (GI) of Goods tag for Naga king chilli in the year 2008 under the Geographical Indication of goods (Registration and Protection) Act 1999.

Bhut Jolokia is vulnerable to several biotic stresses caused by virus, fungus and bacteria [14]. In Assam, the incidence of viral infection in Bhut jalokia is highest as compared to fungal infection and bacterial infection [15]. The Naga king chilli is susceptible to different viruses such as *Cucumber mosaic virus* (CMV), *Potato virus Y* (PVY) [1, 15], *Chilli leaf curl virus* (ChLCV) [1, 3, 15], as a result of which there is reduction in yield as well as quality.

The efficient use of pathogen free plant materials can overcome the menace of plant diseases and sustain crop production. For viruses, prevention is the only really effective measure for control. The possibilities of primary infection occur during seedling stage in open field condition and in the transplanted crop in main field. However, production of virus free quality seedling of chilli is the best option to avoid further spread of the deadly viruses in the farmers' field. Virus free planting material can be produced by meristem-tip culture. Apical meristem is a dome of actively dividing cells located at the apex of shoots and roots. Plantlets derived from apical meristem usually retain the genetic characteristics of mother plants, therefore virus elimination from selected plants-do not differ phenotypically from mother plants. Reports are available for the production of *C. annuum* free from Tobacco mosaic virus and or with Tomato spotted wilt virus or with

both viruses by the use of meristem tip culture [7]. Leaf curl virus free chilli has also been produced by meristem tip culture [11].

The main objective of this study is the production of virus free Naga king chilli planting material using meristem tip culture technique.

2. MATERIALS AND METHODS

2.1. Collection of infected samples

Plants suspected of viral infection based on visible symptoms such as mild mosaic, necrotic lesions, reduced leaf lamina, leaf deformation, stunted growth, etc were identified and leaves were collected from the farmers' field in Ri-Bhoi district, Meghalaya. Mechanical inoculation was done using the leaf sap from naturally infected plants into *in vitro* grown healthy Naga king chilli plantlets (3 -5 leaf stage). Young leaves were homogenized in 0.02 M sodium phosphate buffer, pH.7.0 with mortar and pestle. The slurry thus obtained was squeezed through double layered muslin cloth and used for inoculation into young King Chilli plants (3-5 leaf stage) to test the presence of the virus.

2.2. Virus detection

The leaves of Naga king chilli, identified by visible symptoms such as deformed leaves, necrotic lesions, reduced leaf lamina, mild mosaic, etc, were used for CMV detection by the use of primers specific for CMV viral coat protein gene (Table 1).

Table 1: Primers specific for coat protein gene of CMV [2]

CMVf (Forward primer)	5'-GTAGACATCTGTGACGCG-3'
CMVr (Reverse Primer)	5'-GCGCGAAACAAGCTTCTTATC-3'

2.3. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using Promega's RNeasy plant mini kit following the manufacturer's protocol. For reverse transcription, a 25 µl reaction was set up as follows:

Component	Stock	Volume
Total RNA		10 µl
Buffer	5 X	5 µl
CMVr	10 µM	3.13 µl
dNTP mix	40 mM	1.5 µl
RNAsin (NEB)	40 U/µl	0.5 µl
M-MLV-RT (NEB)	200 U/µl	0.5 µl
RNase free water		4.37 µl

The mixture was incubated at 37^{o}C for 75 min and then at 70^{o}C for 5 min (for enzyme inactivation). It was then immediately incubated on ice for 5–10 min.

The cDNA that is produced was then used for running PCR using the primers specific for coat protein gene of CMV. A 50 µl reaction was set up as follows:

Component	Stock	Volume
cDNA		5ul
Forward primer	10 µM	3.6 µl
Reverse primer	10 µM	3.13 µl
dNTP mix	10 mM	3 µl
Taq buffer	10 X	5 µl
Taq polymerase	5 U/µl	0.3 µl
RNase free water		29.97 µl

For initial denaturation, 94^{o}C for 5 min followed by 33 cycles of 94^{o}C for 1 min, 46^{o}C for 1 min and 72^{o}C for 1 min with final extension of 72^{o}C for 5 min was used. The PCR product obtained was then run on 1% agarose gel at 80 V along with 100 bp ladder (NEB) for size determination and visualized under Gel doc system.

2.4. *In vitro* Regeneration

The *in vitro* grown Naga king chilli plants which gave positive results for CMV were used as the source of explants. Shoot tips (2 – 3 mm long) were excised from infected plants of Naga king chilli and were then washed with 2% liquid detergent solution for 5 minutes under running tap water and immersed in 0.1 % Mercuric chloride for 3 - 5 minutes followed by three washings with sterile distilled water. Meristems tips with a size ranging from 0.5 – 1.0 mm were excised aseptically using stereomicroscope and were kept in laminar flow clean bench. The excision was performed in a sterile glass petridish, lined with sterile moist filter paper to avoid desiccation of the small explant. MS-medium supplemented with Thidiazuron (TDZ) or Benzylaminopurine (BAP) was prepared (Table 2). The meristem tips were planted with the cut-end slightly embedded in the medium. All the cultures were incubated at 25 ± 0.5°C and exposed to 16 hr photoperiod illuminated by fluorescent light with an intensity of about 1500 - 2500 lux. Relative humidity of 60 ± 5% was maintained in the culture room. Ten (10) replications for each treatment were used. The cultures were periodically subcultured on fresh medium after 4 weeks. Rooting was done on MS medium supplemented with 5 µM NAA. Regenerated plantlets were then transferred to pots containing sterilized soil and vermiculite (3:1) inside a green-house. The plants were routinely checked for presence of virus. Confirmation of virus free plantlets was carried out using PCR with specific primers.

3. RESULTS AND DISCUSSION

3.1. Virus indexing

On mechanical inoculation from naturally infected chilli into *in vitro* grown young Naga king chilli plants, visible symptoms were produced on the leaves in 4 to 5 weeks. However detection by using molecular methods coat protein

gene specific primer is considered more reliable method of detection.

3.2. Molecular detection of virus

When PCR was conducted using primers specific for coat protein gene of CMV, a product of the expected size of ~ 540 bp was observed (Fig 1), which indicates the presence of the virus [2]. Of the 10 plants that were tested only three displayed positive bands.

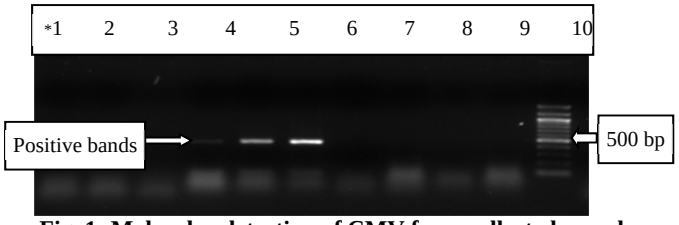

Fig. 1: Molecular detection of CMV from collected samples
***Sample 1 to 10, L = 100 bp ladder**

3.3. *In vitro* regeneration using Meristem tips

The number of shoot buds per explants given by different combinations of plant growth regulators is given in Table 2. Among the different media combinations used, MS medium fortified with 10 µM TDZ alone produced the maximum number of shoots. The regenerated shoots were rooted by using MS medium containing 5 µM NAA. TDZ is generally used at very low concentrations, however, the use of high amount of TDZ (18.16 µM) for growing shoot tip of Naga king chilli is reported [8]. BAP alone was also able to proliferate meristem tips with 10 µM giving the maximum number of shoots (3 buds per explant).

Table 2: Effect of Plant Growth Regulators (PGR) on shoot formation from shoot tip explants

Treatment	TDZ (µM)	BAP (µM)	No. of shoot buds per explant
C	0	0	0.25 ± 0.00
T_1	5	0	2.50 ± 0.71
T_2	10	0	4.25 ± 0.96
T_3	20	0	1.75 ± 0.50
T_4	0	5	1.67 ± 0.58
T_5	0	10	3.00 ± 0.82
T_6	0	20	2.50 ± 0.58

Once the plantlets were rooted they were transferred in artificial soil (Peat, perlite and vermiculite in the ratio 1:1:1) and kept in green-house for acclimatization.

Fig. 2: Regeneration of Naga king chilli using meristem tips

3.4. Verification of virus free plants:

In vitro regenerated plants were used for checking the presence of the virus. Leaf samples from 10 regenerated plants were collected and RT-PCR was conducted using the same method mentioned earlier. The control gave an amplicon size of approximately 540 bp as expected whereas all the 10 samples did not give any positive bands (Fig 3). From the above observation, it can be concluded that the regenerated plants are free from the virus. We can therefore, infer that meristem tip culture is a reliable and efficient method for the production of virus free plants. It is reported that the optimum size of meristem tips for the production of virus free chilli plants is 0.5 mm with 80 % success [11].

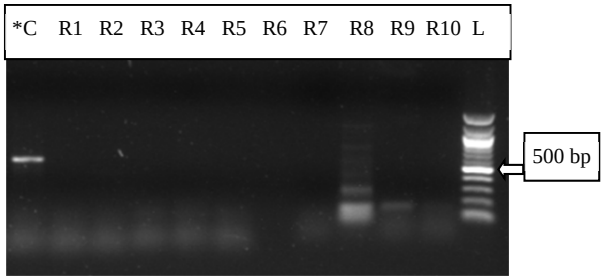

Fig 3: Detection of viruses in regenerated plants by PCR based methods
***C= Control; R1 to R10 = Regenerated plants, L = 100 bp Ladder**

REFERENCES

[1] Baruah Borsha Rani, Kashyap Anurag, Nath P.D. "Incidence, detection and integrated management of viral disease complex in *Bhut Jolokia*, a Chilli cultivar in Assam", *Annals of Plant Protection Sciences*, 24, 1, 2016, pp. 136 - 141.

[2] Biswas Koushik, Vipin Hallan, Aijaz Asghar Zaidi and Pramod Kumar Pandey. "Molecular evidence of *Cucumber mosaic virus* subgroup II infecting *Capsicum annuum* L. in the Western region of India", *Current Discovery*, 2, 2, 2013, pp. 97 - 105.

[3] Borgohain, R. and Devi, J. "The hottest chilli: A new horizon in agri-entrepreneurship", *Science Tech Entrepreneur*, http://www.assamagribusiness.nic.in, 2007.

[4] Bosland, PW. "Chiles: history, cultivation, and uses. *In:* Species, herbs, and edible fungi (Charalambous G, ed.)", *Elsevier Publishers*, New York, USA. 1994, pp. 347 - 366.

[5] Bosland, WP. and Baral, BJ. "Bhut jolokia: The World's Hottest Known Chile Pepper is a Putative Naturally Occurring Interspecific Hybrid", *Hortscience*, 42, 2007, pp. 222 – 222.

[6] Geographical Indications Journal No.25, Government of India, August- 11, 2008, pp. 33 - 46.

[7] Katoh N, Yui M, Sato S, Shirai T, Yuasa H, Hagimori M. "Production of virus free plants from virus-infected sweet pepper by *in vitro* grafting", Sci Hortic, 100, 2004, pp.1 – 6.

[8] Kehie Mechuselie, Kumaria Suman and Tandon Pramod "*In vitro* plantlet regeneration from nodal segments and shoot tips of Capsicum chinense Jacq. cv. Naga King Chili", 3 Biotech, 2, 2012, pp. 31 – 35.

[9] Kumar, S., Kumar, R., Kumar, S., Singh, M., Rai, AB., Rai, M. "Incidences of leaf curl disease on *Capsicum* germplasm under field conditions", *Indian Journal of Agricultural Science*, 81, 2011, pp. 187 – 189.

[10] Mathur, R., Dangi, RS., Dass, SC., Malhotra, RC. "The hottest chilli variety in India. *Current Science"*, 79, 2000, pp. 287 – 288.

[11] Meena Rishi kesh, Gour Kavi, Patni Vidya. "Production of Leaf Curl Virus - Free Chilli by Meristem Tip Culture", *Int. J. Pharm. Sci. Rev. Res.*, 25, 2, 12, 2014, pp. 67 - 71.

[12] Reddy, M.K., Srivastava, A., Kumar, S., Kumar, R., Chawda, N., Ebert, A.W. and Vishwakarma, M.. "Chilli (*Capsicum annuum* L.) breeding in India: An overview", *SABRAO Journal of Breeding and Genetics*, 46, 2, 2014, pp. 160 - 173.

[13] Sanatombi, K., Sen-Mandi, S. and Sharma, G.J. "DNA profiling of *Capsicum* landraces of Manipur" *Scientia Horticulturae*, 124, 2010, pp. 405 – 408.

[14] Talukdar Juri, Saikia, A. K. and Nath, P. D. "Identification of diseases occurring on 'Bhut Jolokia. Detection of Fungal, Bacterial, Viral diseases of 'Bhut jolokia' (*Capsicum chinense*) of Assam", *Lambert Academic Publishing.* 2012.

[15] Talukdar Juri, Saikia, A. K., and Borah, P. "Survey and detection of the diseases of Bhut Jolokia (*Capsicum chinense* Jacq.) in Assam", *Journal Crop and Weed*, 11(Special Issue), 2015, pp.186 - 192.

[16] Verma, PK., Rawat, KK., Das, N., Pradhan, B. "A botanical enigma of India's hottest chilli Bhoot Jolokia (*Capsicum chinense* Jacq)", *New York Science Journal*, 6, 2013, pp. 49 –51.