International Journal of Advanced Biochemistry Research

ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating: 5.29
IJABR 2025; 9(3): 458-462
www.biochemjournal.com
Received: 02-01-2025
Accepted: 06-02-2025

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Effect of SAR chemicals and thermotherapeutic treatment in the elimination of viruses from Bhut Jolokia (*Capsicum chinense* Jacq.)

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DOI: https://www.doi.org/10.33545/26174693.2025.v9.i3f.3982

Abstract
Bhut Jolokia (*Capsicum chinense* Jacq.) is one of the important chilli landraces native to North Eastern Region of India which was formerly recognized as the world's hottest pepper in 2007 by the Guinness World Record. Bhut jolokia contains 3-5 percent more capsaicin in comparison to other chilli species found in India. Bhut Jolokia has been reported to be attacked by various pathogens more specifically by many viruses among which the *Cucumber mosaic virus* (CMV), *Chilli leaf curl virus* (ChiLCV) and *Potato Virus Y* (PVY) are the important one hindering its cultivation. In the present study thermotherapeutic treatment of Bhut Jolokia seeds and application of SAR chemicals in the Bhut Jolokia seedlings was evaluated in the elimination of viruses from Bhut Jolokia. Hot water treatment of CMV-infected Bhut Jolokia seeds at 50 °C for 120 minutes proved to be beneficial resulting in minimum the disease incidence. Foliar application of Salicylic acid at 300 ppm concentration and Benzothiadiazole at 400 ppm concentration was very much effective in reducing the severity and incidence of the viral diseases.

Keywords: Bhut Jolokia, *CMV*, *PVY*, thermotherapeutic treatment, Benzothiadiazole, Salicylic acid

Introduction
Bhut Jolokia (*Capsicum chinense* Jacq.), commonly known as '*Naga King Chilli*' is one of the important chilli landraces native to North Eastern Region of India, which is an inter specific hybrid between *Capsicum chinense* and *Capsicum frutescens* (Bosland and Baral, 2007) [5]. Bhut Jolokia is currently recognized as the seventh hottest chilli in the world with a Scoville heat units (SHU) rating of 1,001,304. This chilli is widely cultivated in the state of Nagaland, Manipur and Assam and up to some extent in Mizoram, Arunachal Pradesh and Meghalaya (Meetei *et al*., 2016) [9] besides the Sylhet region of Bangladesh. Bhut jolokia is mainly cultivated for its pungent fruits to impart taste and pungency to food. It holds great utilization potential due to its high capsaicin content and medicinal properties. Traditionally the fruits are used in various homeopathic preparations for curing different human ailments like, body pain, stomach ailments, body temperature regulation and remedy to summer heat (Bhagowati and Changkija, 2009) [4], besides having various ethno-pharmaceutical applications Having such a great potential in commercial prospects together with the suitable agro-climatic conditions of the region providing vast scope for production of the crop, there still exists a few bottlenecks for its commercial cultivation, as numbers of abiotic and biotic stresses have restricted its yield potential. A number of viruses infecting the crop have been reported viz. *Chilli leaf Curl Virus* (ChiLCV) (Talukdar *et al*., 2015; Baruah *et al*., 2016) [13, 3], *Chilli Veinal Mottle Virus* (ChiVMV) (Adluri *et al*., 2016; Routhu *et al*., 2022) [1, 12], *Cucumber Mosaic Virus* (CMV), *Potato Virus Y* (PVY) (Talukdar *et al*., 2015; Baruah *et al*., 2016) [13, 3], *Tomato Spotted Wilt Virus* (TSWV) (Kalita *et al*., 2014) [7], *Groundnut Bud Necrosis Virus* (GBNV) (Baruah *et al*., 2016) [3]. At times, the crop has been reported to be infected by a single virus or collectively by more than one viruses and thereby forming a viral complex (Kalita *et al*., 2014) [7] and causing severe loss to the crop. Among different viruses infecting Bhut Jolokia, *Cucumber mosaic virus* (CMV), *Chilli leaf curl virus* (ChiLCV) and *Potato Virus Y* (PVY) is a serious cause of concern for Bhut Jolokia cultivation in the region. CMV has been reported to be seed transmitted in various host

species, including pepper (Ali and Kobayashi, 2009) [2], which make the viruses almost ubiquitous contributing to its higher rate of incidence. Realizing the importance and severity of the viral diseases, the present investigation was undertaken to evaluate the efficacy of thermotherapeutic treatment and SAR chemicals in the elimination of viruses from Bhut Jolokia.

Materials and Methods

Thermotherapautic treatment of CMV infected Bhut jolokia seeds for elimination of the virus

Seed Source and detection of CMV

Suspected CMV infected Bhut jolokia fruits were collected from various farmers of Manipur state of Northeast India. Seeds were taken out from the fruit, kept separately location wise, rinsed thoroughly with running tap-water and were allowed to dry in shade. The seeds were subjected to RT-PCR for confirmation of CMV infection in them.

Seed treatment using hot water

The CMV positive Bhut jolokia seeds were subjected to four temperature regimes viz.,45 °C, 50 °C, 55 °C, 60 °C for four different duration viz. 15 minutes, 30 minutes, 60 minutes, and 120 minutes. For each treatment, 30 CMV infected seeds were taken and each lot of 30 shade dried seeds were properly wrapped in muslin cloth before treatment. Water baths (serological water bath) were pre-warmed to the required temperatures (45 °C, 50 °C, 55 °C, 60 °C) and the seeds were subjected to the respective temperature to the respective duration of treatment (15 minutes, 30 minutes, 60 minutes, and 120 minutes). The untreated diseased Bhut Jolokia seeds were used as control. The thermo-treated seeds were raised in nursery trays and were kept under insect proof conditions. Regular observations were made and information regarding seed germination, type of symptoms developed (visual observations), days taken for initial symptom development and disease incidence (at 60 days after growing) were recorded.

In-vitro assay of Benzothiadiazole (BTH) and Salicylic Acid (SA)

The studies on effect of Benzothiadiazole (BTH) and Salicylic Acid (SA) treatment on incidecnce of different viral diseases on Bhut Jolokia was carried out in the agro shade net house of Department of Plant Pathology, Assam Agricultural University, Jorhat. Three different concentrations *viz*., 200, 300 and 400 ppm of both the chemicals were tested. Twenty days old seedlings were used for treatment with the chemicals. The experiment was carried out in Completely Randomised Design (CRD) comprising of seven treatments (Table 1) with three replications using 3 plants per replication. Each concentration of the chemicals were applied three times to the 20 days old plants at an interval of three days. A set of 3 plants were kept separately without applying any chemical treatment and used as control. The plants were observed regularly and different types of symptoms appeared were recorded. PCR were performed by collecting symptomatic plant samples.

Table 1: Treatment evaluated for the effect of Benzothiadiazole and Salicylic acid at different concentrations

Treatment	Treatment description
T_1	Spraying of Bhut jolokia seedlings with Benzothiadiazole at 200 ppm concentration
T_2	Spraying of Bhut jolokia seedlings with Benzothiadiazole at 300 ppm concentration
T_3	Spraying of Bhut jolokia seedlings with Benzothiadiazole at 400 ppm concentration
T_4	Spraying of Bhut jolokia seedlings with Salicylic acid at 200 ppm concentration
T_5	Spraying of Bhut jolokia seedlings with Salicylic acid at 300 ppm concentration
T_6	Spraying of Bhut jolokia seedlings with Salicylic acid at 400 ppm concentration
T_7	Control

Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA) to detect CMV in Bhut Jolokia

DAS-ELISA kit (Bio Reba, AG, Switzerland) was used as per manufacturer's protocol for detection of CMV from symptomatic as well as asymptomatic Bhut Jolokia plant samples. The reaction was recorded using an ELISA reader (Bio-Rad Instruments Inc.) at 405nm. Plant samples were considered infected with CMV if ELISA reading was more than five times the average reading of negative control. Data was analysed and infection percentage was recorded.

Detection of viruses in Bhut Jolokia

Detection of CMV and PVY

Bhut Jolokia plant samples, both symptomatic and asymptomatic were collected from the experimental field for RT-PCR detection of CMV and PVY virus using specific primers (Table2). Gel electrophoresis was performed for the amplified PCR product to examine the expected band size.

Detection of ChiLMV

Samples were collected from both symptomatic and asymptomatic Bhut Jolokia plants from the experimental plot to detect the presence of ChiLCV through PCR analysis using specific primers (Table2). Gel electrophoresis was performed for the amplified PCR product to examine the expected band size.

Table 2: Specific primers for detection of CMV, PVY and ChiLCV

Specifications	Primer name	Sequence	Product size (bp)	GC content (%)	Temp (°C)	Reference sequence ID
CMV-CP	IK-4F	AGTGCTGGTCGTAACCGTC	633	58	60	NC_001440
	IK-6R	GACTGGGAGCACTCCAGATG		60	60	
PVY-CP	IK-1F	TGGAACCTCGCCAAATGTCA	386	50	60	NC_001616
	IK-2R	TGGTGTGCCTCTCTGTGTTC		55	60	
	CPR	TTTTTTTTTTTTTTTAACGCCAACTATTG		-	59	
ChiLCV-CP	IK-3F	AACTTCGACAGCCCTTATGC	524	50	58	NC_004628
	IK-4R	TGTTCCTTCGAAGCGTACTG		50	58	

Agarose gel electrophorosis

The PCR products were resolved on 1.2% agarose gel in TBE containing 0.5 µg/ml of ethidium bromide. The gel was visualized on an UV-transilluminator Gel-doc to observe the amplified bands.

Results and Discussion

Thermotherapautic treatment of CMV infected Bhut Jolokia seeds for elimination of the virus

Symptomatology

Different types of symptoms were observed in the Bhut Jolokia plants raised from hot water treated and the untreated seeds. The plants raised from hot water treated Bhut Jolokia seeds produced milder symptoms as compared to the plants produced from untreated seeds. The major symptoms developed in the hot water treated plants were chlorotic mosaic (Plate1A); very mild to clear mosaic (Plate 1B) and small sized leaves (Plate1C). Some of the plants exhibited reduction in growth. Similar types of symptoms like mosaic, leaf size reduction and stunted growth were also observed in the untreated control plants but the severity was more as compared to the plants raised from hot water treated seeds. It was observed that Bhut Jolokia seeds treated with hot water at temperature of 55 °C for 60, 120 minutes duration and at 60 °C for 30 minutes duration produced very mild mosaic symptoms and was the best in reducing the symptom development in Bhut Jolokia plants as compared to other temperatures and duration used under the thermotherapautic treatments. Similar observations were made by Abbas *et al*. (2016) [16] where mild symptoms such as rolling up of lower and upper leaves were observed in thermo-treated potato tubers.

Effect of thermotherapautic treatments on seed germination and incidence of CMV disease

The effect of hot water treatment in seed germination and incidence of CMV disease is depicted in Table3, which revealed that hot water treatment influenced the percentage and duration of seed germination and also CMV disease incidence. Lowest incidence of the CMV disease (12.75%) was observed in hot water treatment of seed at 55 °C for 120 minutes, with 56 percent germination of seeds in 21-25 days. Hot water treatment at 50 °C for 120 minutes resulted in 64.5 percent germination in 23-25 days, 26.08 percent disease incidence. Among the hot water treatments highest disease incidence (99.17%) was observed when seeds were treated at 45 °C for 15 minutes, followed by 45 °C for 30 minutes (98.83%) which are statistically at par with the disease incidence in untreated control plants with 99.58 percent disease incidence. From the results, it was clear that though high temperatures with prolonged periods of treatment time were more effective for elimination of virus, but germination rate were highly reduced. The results were in conformity with earlier works carried out by Ling (2010) [8] where hot water soaking of seeds at 55 °C for 2hrs deactivated virus infectivity in PepMV infected tomato seeds but greatly reduced the seed germination rate. Similar observation was also recorded by Palyan *et al*. (2014) [11] in ToMV infected tomato seeds and CMV infected melon, squash and bean seeds. In conformity to the results of the present investigations.

RT-PCR detection of CMV

Samples were collected from both symptomatic and asymptomatic Bhut Jolokia plants and subjected to RT-PCR using specific primers to detect the presence of CMV. Out of the 16 samples collected, 14 samples were tested positive. The gel photographs showing the bands of 633 bp of the amplified PCR product were presented in Plate2.

In-vitro assay of Benzothiadiazole (BTH) and Salicylic Acid (SA) treatments

Symptomatology and incidence of virus

The observations recorded on effect of Benzothiadiazole and salicylic acid are presented in Table 4. At 30 Days Post Treatment (DPT), mild and very mild mosaic symptoms were observed in plants treated with BTH @ 200 ppm and 300 ppm respectively where an average of 11.11 percent disease incidence was recorded in both the treatments. No visible symptom was observed in plants treated with 400 ppm of BTH. At 45 days post treatment, mild mosaic and upward curling of leaves were observed in plants treated with BTH @ 200 ppm with an average disease incidence of 44.44 percent; whereas, mild mosaic symptoms with 33.33 percent disease incidence was recorded in BTH treated Bhut Jolokia plants at the concentration of 300 and 400 ppm. At 60 days post treatment, symptoms like yellow mosaic with curling of leaves were observed in BTH treated Bhut Jolokia plants @ 200 ppm with 66.67 percent disease incidence. On the other hand, mosaic and slight reduction in leaf size were observed in plants treated with BTH @ 300 ppm and 400 ppm with 55.56 and 44.44 percent disease incidence respectively.

A B C

Plate 1: Symptoms of viral diseases in Bhut Jolokia

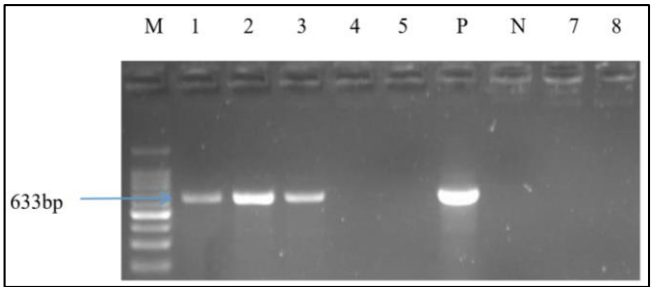

Plate 2: Agarose gel electrophoresis showing amplified RT-PCR product of CMV isolates ((Lane M. 100 bp DNA ladder; Lane P. Positive control; Lane N. Negative control; Lane 1-3. Symptomatic plant sample; Lane 4-8 asymptomatic plant sample)

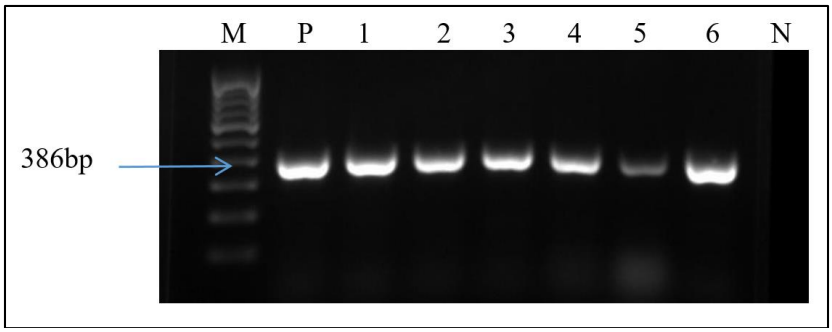

Plate 3: Agarose gel electrophoresis showing amplified RT-PCR product of PVY isolates ((Lane M. 100 bp DNA ladder; Lane P. Positive control; Lane N. Negative control; Lane 1-6. Symptomatic plant sample)

Table 3: Effect of hot water treatment of CMV infected Bhut Jolokia seeds on the CMV disease incidence

Temperature (°C)	Duration of treatment (min)	Germination of seed (%)	Number of days taken for first appearance of symptom	Disease incidence (60 DAG)	Reduction of disease incidence over control (%)
45	15	97.25	15-16	99.17	0.41
	30	88.00	17-21	98.83	0.75
	60	76.00	15-18	89.58	10.04
	120	72.00	20-21	78.00	21.67
50	15	96.50	16-18	98.92	0.66
	30	91.50	17-19	95.17	4.43
	60	88.50	18-20	65.75	33.97
	120	64.50	23-25	26.08	73.81
55	15	88.00	15-18	88.08	11.55
	30	84.00	17-20	61.42	38.32
	60	68.00	21-23	43.92	55.89
	120	56.00	21-25	12.75	87.20
60	15	36.00	21-25	58.87	40.88
	30	20.00	23-26	35.83	64.02
	60	0	-	0	0
	120	0	-	0	0
Control	-	98.00	15-16	99.58	
SEd				1.012	
CD(P=0.05)				2.065	

In SA treated plants, mild mosaic symptoms were observed in plants treated with 200 ppm with 11.11 percent disease incidence at 30 days post treatment but no visible symptoms were observed in plants treated with 300 and 400 ppm concentration. Prominent mosaic symptoms were observed in plants treated with 200 ppm SA with 33.33 and 44.44 percent disease incidence at 45 and 60 days post treatment respectively. Plants treated with 300 and 400 ppm SA, produced mild mosaic symptoms with 22.22 percent disease incidence at 45 days post treatment. At 60 days post treatment, plants treated with 300 ppm SA produced mosaic along with curling of leaves where 44.44 percent disease incidence were recorded. On the other hand, chlorotic mosaic symptoms were observed in plants treated with 400 ppm SA with 44.44 percent disease incidence. In the control, where no chemical treatment was applied except spraying of water, yellow mosaic with rugose (wrinkled) leaves and stunted plant growth were observed. White (1979) [14] reported increased resistance of *Tobacco* cv. *Xanthi*-nc against TMV where acetylsalicylic acid (Aspirin), benzoic acid and salicylic acid were applied exogenously at different concentrations on *Tobacco* cv. *Xanthi*-nc plant inoculated with TMV. The present results were also in agreement with the findings of Yi *et al*. (2012) [15], where it was reported that BTH application on CMV infected pepper plants reduced the disease symptoms. SA, being an endogenous signalling chemical for Systemic Acquired Resistance and BTH, a synthetic chemical having similar mechanism like SA enhanced the activation of pathogenesis-related proteins leading to the synthesis of phenolic compounds thereby activating the defense response in the plants against the pathogen (virus) (Metraux and Raskin, 1993) [10]. Conrath *et al*. (1995) [6] reported that exogenous application of synthetic chemicals could enhance resistance against TMV in tobacco by inducing PR gene expression. These might be the cause of the reduced incidence of CMV with lower severity of symptoms as observed in the present investigation where BTH and SA was sprayed on twenty days old bhut jolokia seedlings raised from CMV infected Bhut jolokia seeds.

Table 4: Effect of Benzothiadiazole and Salicylic acid treatment on the incidence of CMV and PVY virus diseases in Bhut Jolokia

Treatments	Disease incidence at 30 DPT (%)	Reduction over control (%)	Disease incidence at 45 DPT (%)	Reduction over control (%)	Disease incidence at 60 DPT (%)	Reduction over control (%)
BTH (200 ppm)	11.11	66.67	44.44	20.01	66.67	33.33
BTH (300 ppm)	11.11	66.67	33.33	40.01	55.56	44.44
BTH (400 ppm)	0	100	33.33	40.01	44.44	55.56
SA (200 ppm)	11.11	66.67	33.33	40.01	66.67	33.33
SA (300 ppm)	0	100	22.22	60	44.44	55.56
SA (400 ppm)	0	100	22.22	60	44.44	55.56
Control	33.33	-	55.56	-	100	-

*****DPT: Days Post Treatment

Detection of virus

20 samples were collected both from symptomatic and asymptomatic plants to detect the presence of viruses. Out of the 20 samples tested, 6 samples showed bands of 633 bp on gel electrophoresis indicating the presence of CMV and 7 samples showed bands of 386 bp of amplified PCR product on gel electrophoresis indicating the presence of PVY. However, ChiLCV could not be detected.

Conclusion

The results obtained in the present study reveal that treatment of CMV infected Bhut Jolokia with 50 °C at 120 min, 55 °C at 60 min or 120 min effectively reduced the disease incidence withot much inhibition of the germination rate. Although higher temperatures with prolonged treatment time were effective for elimination of virus, but germination rate were highly reduced. Thus it is not advisable. Again, plants treated with BTH and SA showed reduction in disease incidence (%) and severity of symptoms as compared to the untreated plants. It was observed that BTH @ 400 ppm and SA @ 300 ppm effectively reduced the percent disease incidence and the symptom severity of the viral diseases. As such, thermotherapy and application of SAR chemicals like, BTH and SA can be advocated for effective management of the viral diseases of Bhut Jolokia.

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