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RESEARCH ARTICLE

In vitro chemotherapy for inducing tolerance towards Tomato leaf curl New Delhi virus (ToLCNDV) in bitter gourd (*Momordica charantia* L.)

Adarsh M V[1], Smitha Bhasi[1*], Soni K B[1], Swapna Alex[1], Ayisha R[2] & Manjushri Dinkar Dongare[1]

[1]Department of Molecular Biology and Biotechnology, College of Agriculture, Vellayani 695 522, Thiruvananthapuram, Kerala, India
[2]Department of Plant Pathology, College of Agriculture, Vellayani 695 522, Thiruvananthapuram, Kerala, India

*Correspondence email - smitha.bhasi@kau.in

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Abstract

ToLCNDV, a whitefly transmitted begomovirus, is reported to be associated with Bitter gourd yellow mosaic disease (BGYMD), a deadly disease in bitter gourd that can lead to 100 % loss of marketable fruits. The aim of the study was to evaluate the effect of *in vitro* treatment with antiviral compounds for developing tolerance towards ToLCNDV in bitter gourd. Germinated seeds of bitter gourd were grown for 14 days in Murashige and Skoog (MS) medium supplemented with 2 ppm Benzyladenine (BA) and antiviral compounds viz. ribavirin (Treatment 1), Virus-Ex (Treatment 2) and extracts of *Bougainvillea spectabilis* (Treatment 3). Plants grown in MS medium supplemented with 2 ppm BA were used as control. The treated plants were hardened in coir pith compost and whitefly-mediated artificial inoculation of the virus was carried out 2 weeks after hardening. Absence of virus in treatment 1 and treatment 2 was confirmed in PCR using universal Deng primer. Biometric observations of treated plants (35-day-old) viz. plant height, number of leaves and leaf area were found higher in all the treatments compared to control, out of which treatment using ribavirin was found to be highly significant for all the parameters.

Keywords: bitter gourd; chemotherapy; *in vitro* culture; peroxidase; ribavirin; ToLCNDV; whitefly transmission

Introduction

Bitter gourd (*Momordica charantia* L.), often referred to as insulin plant, is reported to originate in tropical Asia and belongs to the family Cucurbitaceae. India is one of the leading producers of bitter gourd in the world, with a production of 1433.2 metric tons during 2021–22. It is mainly grown in the states of Tamil Nadu, Kerala, Karnataka, Andhra Pradesh, West Bengal and Odisha. BGYMD is the most severe and widespread disease in bitter gourd that leads to the reduction of fruit quality and complete loss of marketable fruits (1). Yellowing, distorted leaf lamina, puckering and reduced plant growth are some of the symptoms of BGYMD infection in bitter gourd.

A single virus or two or more related viruses, typically from three different families, viz., Bromoviridae, Potyviridae and Geminiviridae can cause BGYMD. Begomoviruses reported to infect bitter gourd include Bitter gourd yellow mosaic virus (BGYMV), Indian cassava mosaic virus (ICMV), Pepper leaf curl Bangladesh virus (PLCBV), Squash vein yellowing virus (SVYV), Cucurbit leaf crumple virus (CLCV) and ToLCNDV (2–4). Whiteflies belonging to the *Bemisia tabaci* complex are the primary vectors of begomovirus transmission (5).

ToLCNDV, a begomovirus, is reported to be associated with viral infections of *M. charantia* (4). Its presence has also been reported in BGMYD of Kerala and Tamil Nadu (6, 7).

Among several approaches, *in vitro* culture techniques, which comprise meristem culture, micrografting, chemotherapy, thermotherapy and their combinations represent the most effective means of producing virus-free plants (8, 9). Chemotherapy is a successful *in vitro* method employing antiviral agents such as ribavirin, 2-thiouracil, 6-azauracil, etc., for the production of virus-free plants. In commercially significant crops, including potato, peanut, apple and *Prunus* spp., *in vitro* treatment using chemicals like ribavirin, 5-azacytidine (AZA) and 3-deazauridine (DZD) has been found successful (10). However, there are no reports on *in vitro* chemotherapy in bitter gourd for producing virus-free plants.

Materials and Methods

Germinated seeds of the popular bitter gourd variety ‘Preethi’, released from Kerala Agricultural University (KAU), were used as experimental material. Prior to inducing germination in the dark, the seeds were subjected to hot water treatment for 15 min at 55 °C, followed by washing in a 0.01 % Bavistin solution for 15 min. A completely randomized design (CRD) was employed for the experimental study, using 4 treatments with 3 replications. The study used 20 ppm concentration of various antiviral compounds for experimentation to understand the tolerance level of treated plants towards ToLCNDV infection.

T1: MS + 2 ppm BA+ 20 ppm Ribavirin

T2: MS + 2 ppm BA+ 20 ppm Virus-Ex

T3: MS + 2 ppm BA+ 20 ppm Bougainvillea extract

T4: MS + 2 ppm BA (Control)

Ribavirin and Virus-Ex at 20 ppm were filter sterilized and added to sterile MS medium supplemented with 2 ppm BA. For preparing 20 ppm *B. spectabilis* leaf extract, young leaves were dried at 50 °C for 5 days, powdered and 20 mg of the powder was added to 100 mL of sterile water and heated at 70 °C for 1 hr. The solution was filtered through Whatman filter paper (No.1), filter-sterilized and added to sterile MS medium. Plants grown in MS medium supplemented with 2 ppm BA were used as a control. Five-day-old seedlings germinated in dark were washed using 0.01 % bavistin for 15 min and treated with 0.07 % mercuric chloride for 3 min, followed by a wash using sterile water. The seedlings were inoculated under aseptic conditions into medium having various antiviral chemicals and incubated at 24 °C under a photoperiod of 16 × 8 hr for two weeks.

After two weeks, the *in vitro* grown plantlets were removed from the culture bottles, given a thorough wash under running water and placed in a potting tray containing coir pith compost. The plants were maintained inside an insect-proof cage and the growth parameters, viz. height, number of leaves and leaf area, were recorded at periodic intervals. Analysis of variance (ANOVA) was performed on the experimental data in accordance with standard procedures to determine significant differences between treatments using the KAU software GRAPES. Whiteflies were collected from infected bitter gourd plants and allowed to feed on 25-day-old plants that had been hardened inside the insect-proof cage. After 48 hr of feeding, the whiteflies were collected and killed. Further, the plants were maintained inside the insect-proof cage for assessing symptoms of disease development. DNA was isolated from the hardened plants using C-TAB method both before and 2 days after whitefly transmission. Further, the isolated genomic DNA from the leaf samples of various treatments (T1-T3) and control were amplified using the CP gene-specific universal Deng primer (Forward primer: 5'TAATATTACCKGWKGVCCSC3' Reverse primer: 5'TGGACYTTRCAWGGBCCTTCACA3'). The reaction was carried out in a 96-well Thermocycler. A standard PCR mix was prepared for a 10 µL reaction, consisting of 5 µL 2X PCR master mix (G-Biosciences), 1 µL each of the forward and reverse primers, 100 ng of DNA template and the remaining volume of nuclease-free water. The amplified products, along with a Quantum PCR Marker (G-Biosciences), were separated on a 1.5 % agarose gel. To differentiate the target products from non-target products and primer dimers, appropriate control reactions were also conducted.

The gel was viewed under a gel documentation system (BIO-RAD, USA). Peroxidase assay was carried out before and 3 days after whitefly transmission following the protocol of Lobenstein and Linsey using pyrogallol as the substrate (11).

Results and Discussion

The seeds germinated in the dark showed a germination percentage of 91.2 % and took 3 days for germination (Fig. 1). The plants under various treatments after 20 days are depicted in Fig. 2 and a comparison of their growth pattern is shown in Fig. 3. Twenty-day-old *in vitro* grown plantlets transferred to a potting tray containing coir pith compost are shown in Fig. 4. The 35-day-old hardened plants, maintained in an insect-proof cage are depicted as Fig. 5. Biometric observations of 20-day-old and 35-day-old *in vitro* treated plants showed a significant increase (4-fold) in plant height over control in treatment using ribavirin. Statistical analysis of biometric observations of 20-day-old *in vitro* treated plants showed that the plant height and number of leaves were significantly higher in all the treatments compared to control (Table 1). Maximum plant height was observed with the 20 ppm ribavirin treatment, followed by bougainvillea extract and Virus-Ex treatments. The maximum number of leaves was observed with the 20 ppm Virus-Ex treatment, followed by the ribavirin treatment. The graphical representation of the result is given in Fig. 6.

Biometric observations on the 35th day, specifically plant height, number of leaves and leaf area, were found to be higher in all treatments compared to the control group. Among these, the treatment using ribavirin was found to be highly significant (Table 2). Maximum plant height was noticed in the treatment using 20 ppm ribavirin, followed by Virus-Ex and bougainvillea extract. Conversely, the maximum number of leaves was observed in treatments using Virus-Ex, followed by ribavirin and bougainvillea extract. Maximum leaf area was noticed in the treatment using 20 ppm ribavirin, followed by bougainvillea extract and Virus-Ex. The graphical representation of the result is given in Fig. 7 and Fig. 8.

Chlorotic spots, indicative of viral infection, were observed in control plants 10 days after whitefly transmission. Conversely, no symptoms were noted in plants treated with various antiviral compounds, suggesting their efficacy in preventing disease development. Fig. 9 illustrates the symptoms observed in hardened plantlets 10 days post whitefly transmission. PCR was employed to confirm the presence or absence of the virus both before and after whitefly transmission. The presence of the virus was not detected in treated as well as control plants before white fly transmission, indicating that all plant samples were virus-free.

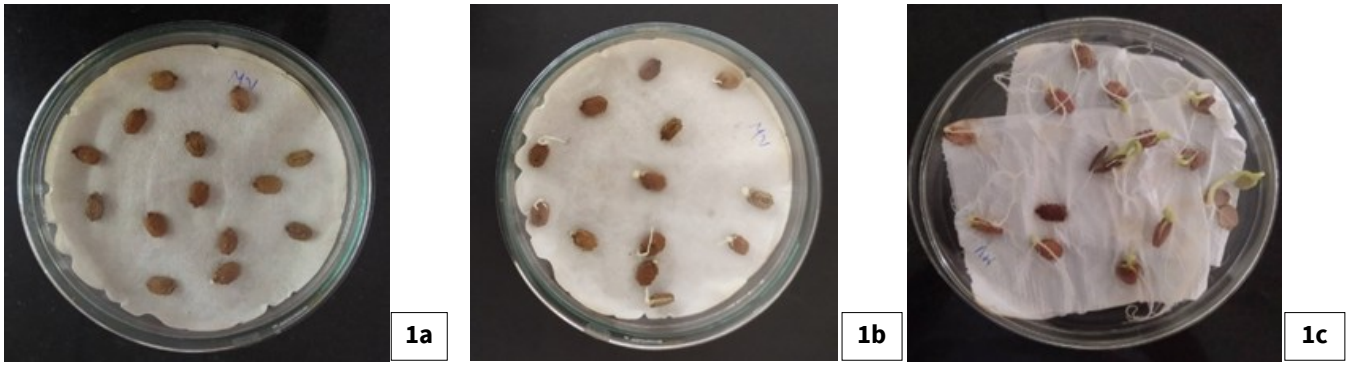

Fig. 1. Germination of seeds of bitter gourd variety 'Preethi' (1a, 1b and 1c indicates germination on day 1, day 2 and day 3).

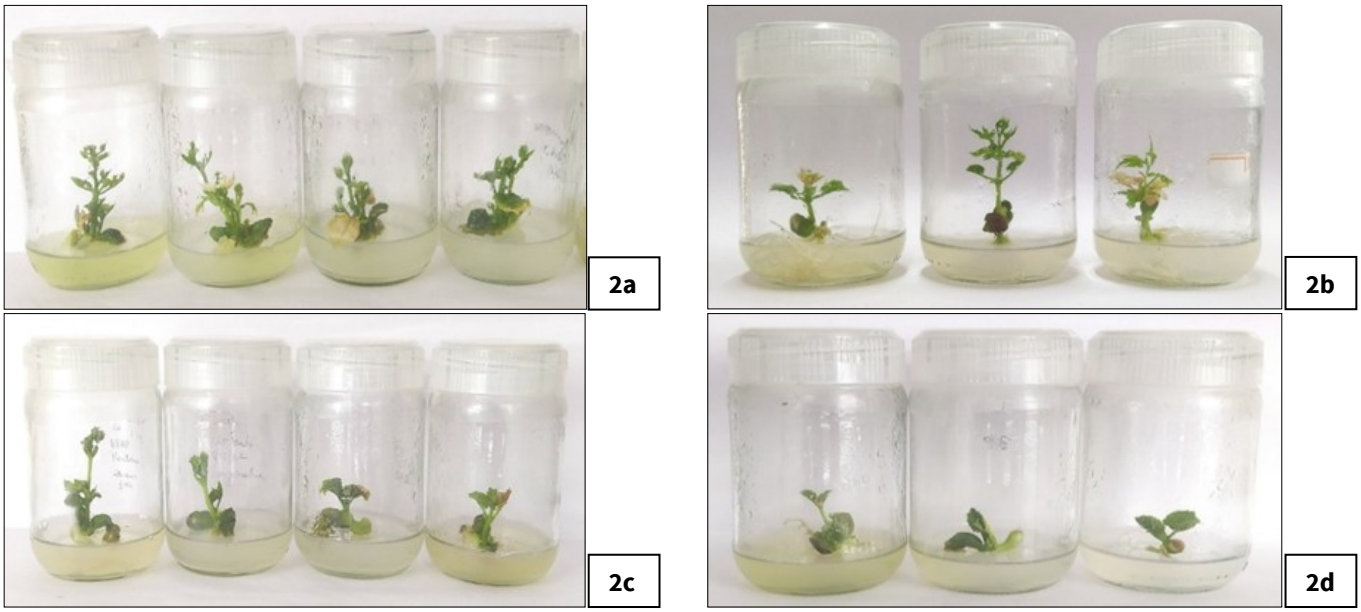

Fig. 2. 20-day-old *in vitro* grown plantlets: (2a) Treatment 1(20 ppm Ribavirin), (2b) Treatment 2 (20 ppm Virus-Ex), (2c) Treatment 3 (20 ppm extract of *B. spectabilis*), (2d) Control.

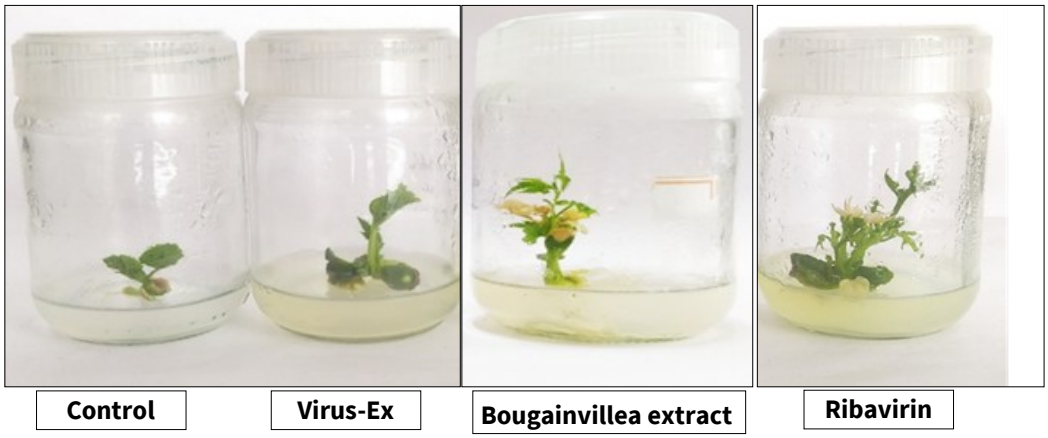

Fig. 3. Comparison of 20 day old *in vitro* grown plantlets under different treatments.

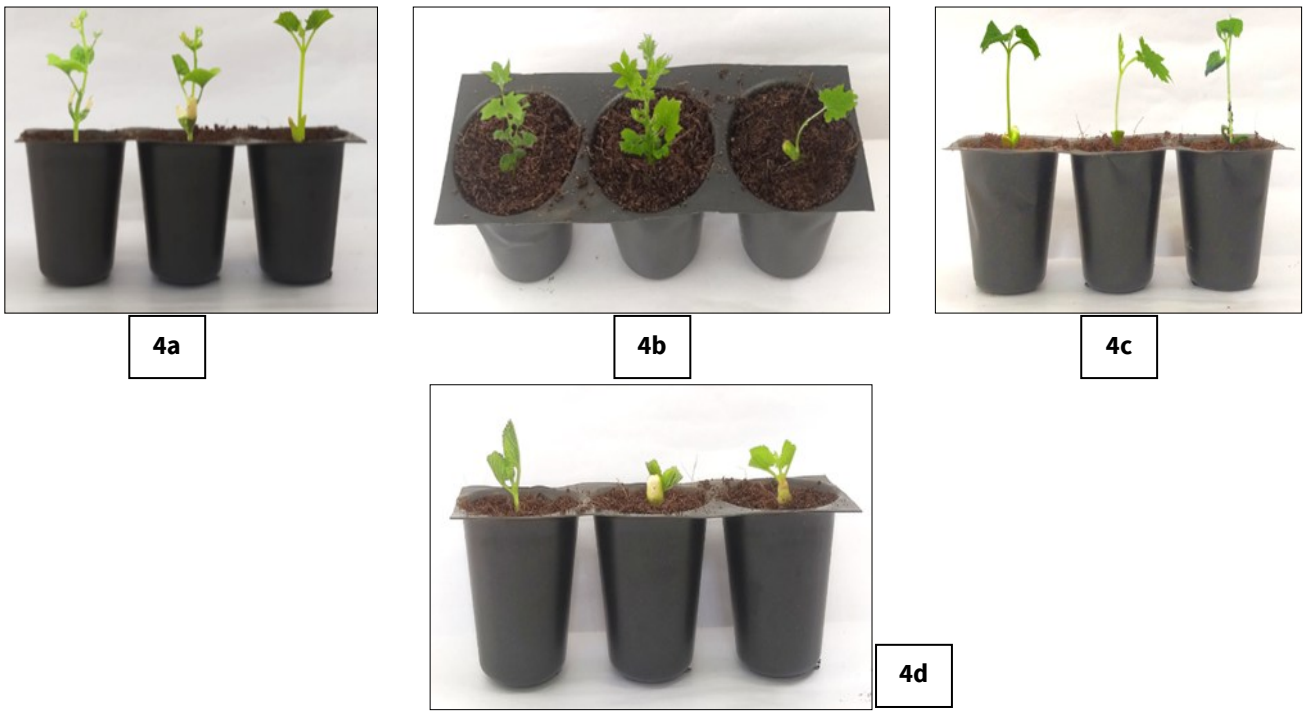

Fig. 4. Hardening of 20-day-old treated plants: (4a) Treatment 1 (20 ppm Ribavirin), (4b) Treatment 2 (20 ppm Virus- Ex), (4c) Treatment 3 (20 ppm Bougainvillea extract), (4d) Control (MS+2 ppm BA).

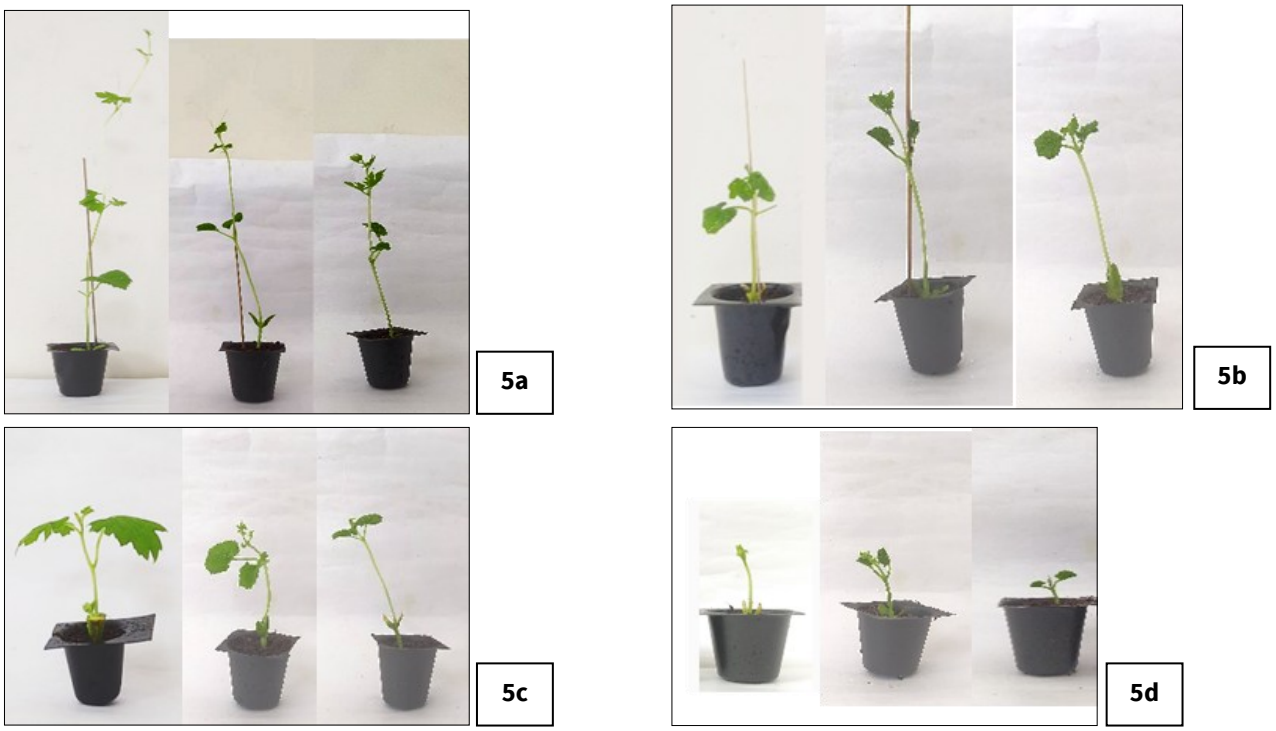

Fig. 5. 35-day-old hardened plants: (5a) Treatment 1 (20 ppm Ribavirin), (5b) Treatment 2 (20 ppm Virus-Ex), (5c) Treatment 3 (20 ppm Bougainvillea extract), (5d) Control.

Table 1. Statistical analysis of biometric observations of 20-day-old *in vitro* treated plants

Treatments	Height of plants (cm)	No. of leaves
T1 (Ribavirin)	4.600[a]	5.333[b]
T2 (Virus-Ex)	4.200[a]	8[a]
T3 (Bougainvillea extract)	4.267[a]	4.667[b]
T4 (Control)	2.400[b]	3.333[b]
SE (d)	0.330	1.115
SE (m)	0.233	0.816
CD (p = 0.05)	0.761	2.663

Table 2. Statistical analysis of biometric observations of 35-day-old *in vitro* treated plants

Treatments	Height plants (cm)	No. of leaves	Leaf area (cm²)
T1 (Ribavirin)	27.900[a]	7.333[a]	24.097[a]
T2 (Virus-Ex)	9.233[b]	6.333[a]	6.943[c]
T3 (Bougainvillea extract)	6.733[c]	6.667[a]	15.160[b]
T4 (Control)	5.100[d]	3.333[b]	1.720[d]
SE (d)	0.481	0.624	1.042
SE (m)	0.34	0.441	0.737
CD (p = 0.05)	1.109	1.438	2.404

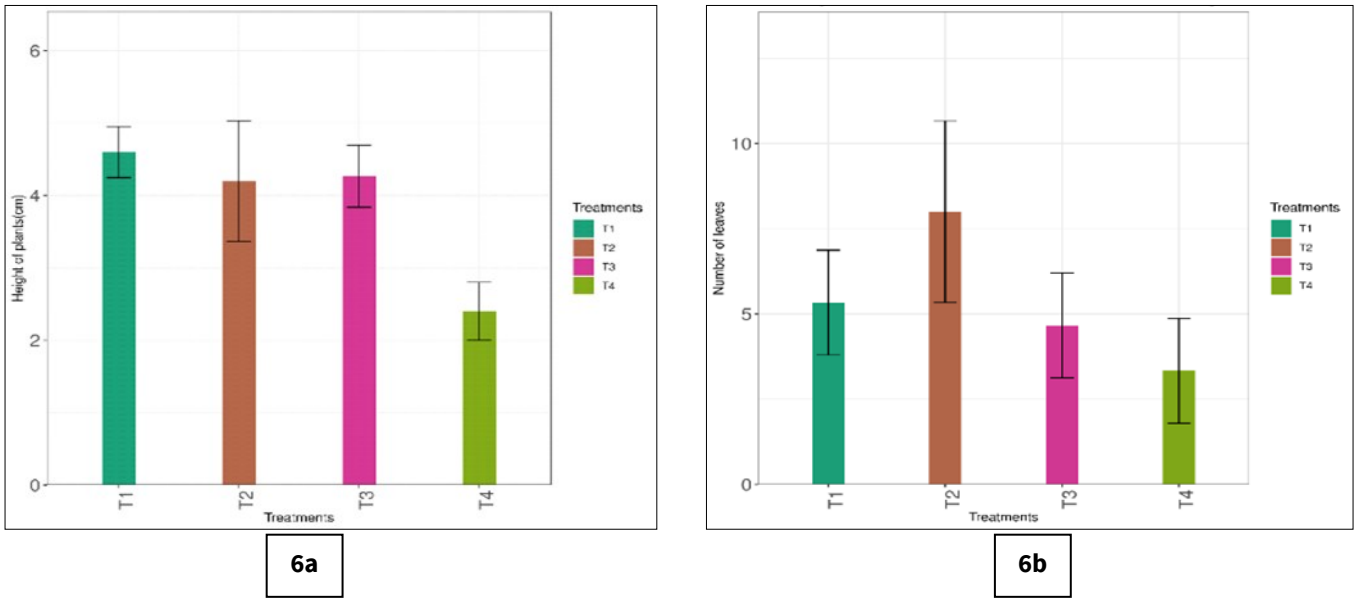

Fig. 6. Statistical analysis of biometric observations of 20-day-old *in vitro* treated plants: (6a) Height of 20-day-old *in vitro* treated plants, (6b) No. of leaves of 20-day-old *in vitro* treated plants.

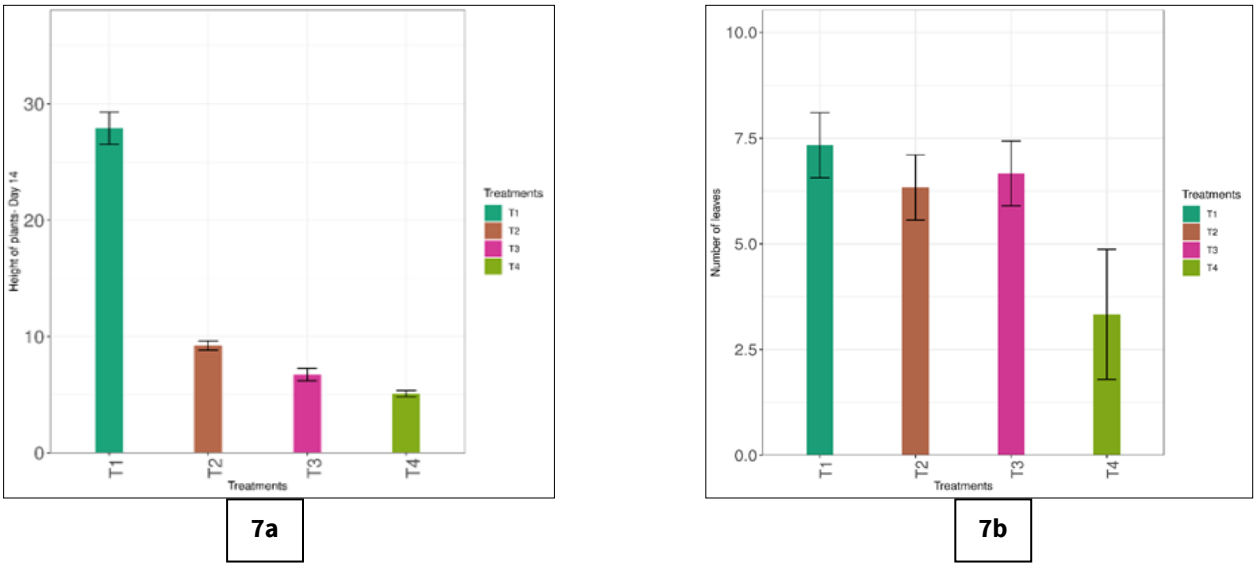

Fig. 7. Statistical analysis of biometric observations of 35-day-old *in vitro* treated plants: (7a) Height of 35-day-old *in vitro* treated plants, (7b) No. of leaves of 35-day-old *in vitro* treated plants.

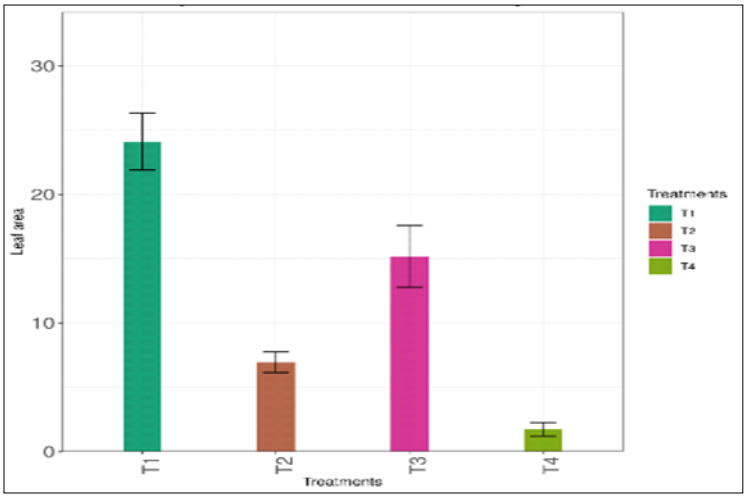

Fig. 8. Leaf area of 35-day-old *in vitro* treated plants.

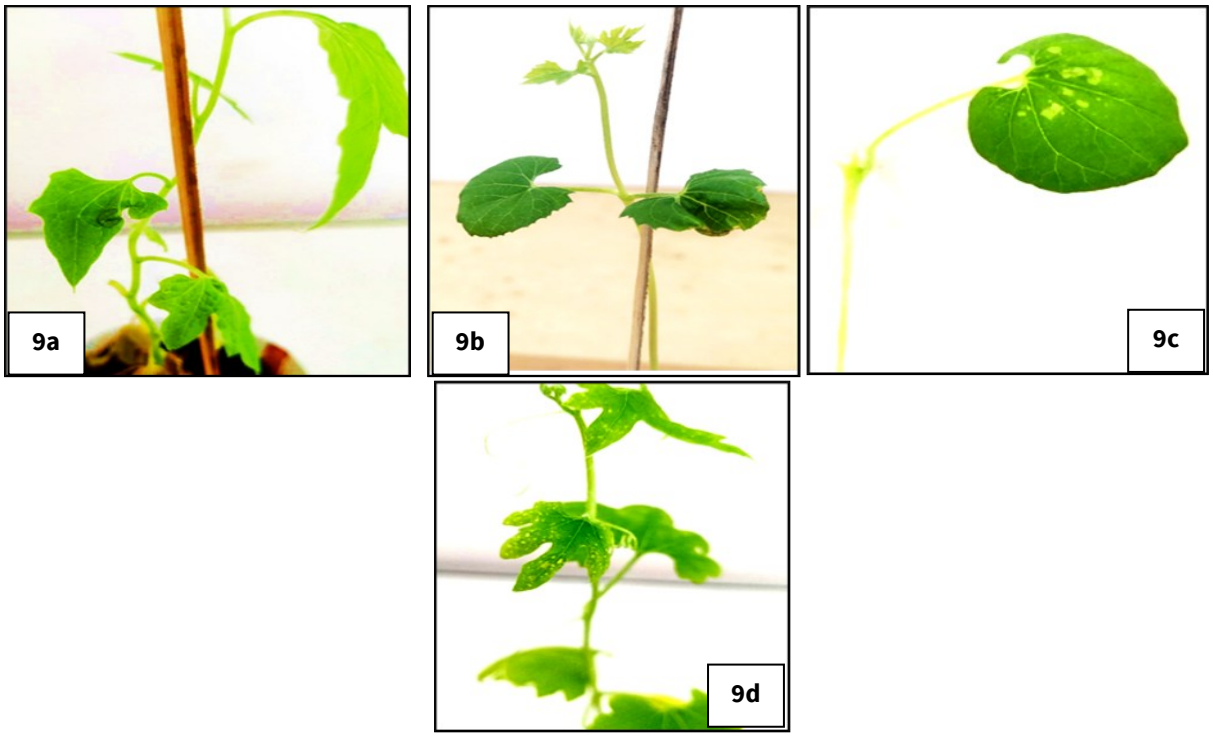

Fig. 9. Symptom development: (9a) Treatment 1 (20 ppm Ribavirin), (9b) Treatment 2 (20 ppm Virus-Ex), (9c) Treatment 3 (20 ppm *B. spectabilis* extract), (9d) Treatment 4 (Control).

PCR gel profile of treatment 1 (ribavirin) and treatment 2 (Virus-Ex) after white fly transmission did not show any amplification and were found negative, which supported the visual observation on symptom development. In contrast, the plants under treatment 3, although lacking visible symptoms, showed an amplicon size of 530 bp in the agarose gel, confirming them as positive. The control plants that developed symptoms also tested positive in PCR (Fig. 10). Peroxidase activity is mentioned in Table 3. A maximum increase in peroxidase activity (3-fold) was noticed in plants treated with 20 ppm ribavirin, followed by bougainvillea extract (2-fold increase) upon whitefly transmission.

Discussion

Bitter gourd is reported to be infected by various begomoviruses, *viz.,* BGYMV, ICMV, PLCBV, SVYV, CLCV and ToLCNDV (2-4). The present study focuses on the infection of the ToLCNDV strain, based on the reports by previous researchers (4, 7, 12-14).

We attempted the molecular confirmation of the virus using polymerase chain reaction, as it is reported to be the most popular detection technique for begomoviruses (15). The present study confirmed the absence of the virus in treatments using ribavirin and Virus-Ex through PCR. The present study used a Deng universal primer, as it is a begomovirus-specific primer set primarily designed on the coat protein (16). Previous researchers had confirmed the presence of ToLCNDV in bitter gourd using Deng primers (6, 14). Among the viral proteins, the coat protein is crucial for the infection of the virus and the coat protein genes are conserved among begomoviruses. Many primers have been designed based on the CP region and utilised for the detection of begomovirus (15).

Ribavirin was selected as a treatment based on earlier research, whereby virus-free plants, viz. tobacco, raspberry and potato were developed *in vitro* using this base analogue (17-19). However, there are no previous reports on *in vitro* chemotherapy using ribavirin in bitter gourd. In the current study, the plants treated using ribavirin did not develop any symptoms of BGYMD upon whitefly transmission and were found negative for begomovirus in PCR analysis, whereas the control plants developed chlorotic symptoms and showed positive results in PCR. The results are in accordance with the previous research, whereby ribavirin at a dose of 20 mg/L successfully eliminated Grapevine fanleaf virus (GFLV) from *in vitro* cultures (20). The result confirms the report of previous researchers, that ribavirin is found to be the most efficient drug against plant viruses *in vitro* (21). The present study found 20 mg/L as the optimum dosage for generating virus-tolerant bitter gourd plants *in vitro*. Similar outcomes were reported by previous researchers and they could generate virus-free plants *in vitro* using ribavirin at a concentration range of 20-50 mg/L (18, 22, 23). In comparison to the control, plants treated with ribavirin showed a significant increase in height, number of leaves and leaf area. Similar responses were noticed under *in vitro* cultures of chrysanthemum and potatoes treated using ribavirin (24, 25). Different modes of action of ribavirin against the virus have been reported like antimetabolites that prevent the replication of the viral genome, mutagenesis effect, interference during the transcription process i.e. during the capping of mRNA etc. (18, 26, 27). The specific mode of action of ribavirin against ToLCNDV in bitter gourd is not elucidated and hence needs to be investigated.

Treatment using Virus-Ex, an eco-friendly, plant-based commercial formulation, in the present study did not result in the development of any symptoms of BGYMD upon whitefly transmission and was found negative for begomovirus in PCR analysis. However, the precise mechanism of action of Virus-Ex remains unknown and there are no reported *in vitro* treatments of Virus-Ex in plants. According to previous study, components of plant extracts can have direct interaction with virus particles in

Table 3. Concentration of peroxidase enzyme and enzyme activity

Treatment	Concentration of peroxidase enzyme (mg/mL)		Enzyme activity (U/mL)	
	Before whitefly transmission	3 days after whitefly transmission	3 days before whitefly transmission	3 days after whitefly transmission
T1 (Ribavirin)	0.218	0.275	0.5435	1.3811
T2 (Virus-Ex)	0.220	0.277	0.6310	0.6840
T3 (Bougainvillea extract)	0.228	0.246	0.5681	1.2279
T4 (Control)	0.213	0.244	0.5319	0.6070

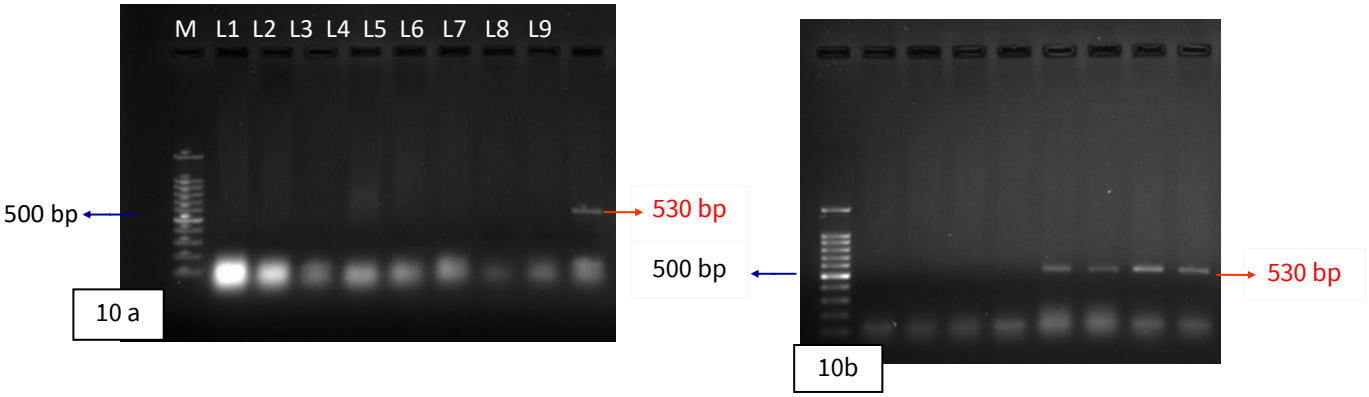

Fig. 10. Gel picture showing PCR amplification of genomic DNA using Deng forward and reverse primer: (10a) Prior to white fly transmission, (10b) Two days after whitefly transmission.

M- 100 bp ladder, Lane 1 & 2- Treatment 1 (MS+ 2 ppm BA+ 20 ppm Ribavirin), Lane 3 & 4- Treatment 2 (MS+ 2 ppm BA+ 20 ppm Virus-Ex), Lane 5 & 6- Treatment 3 (MS+ 2 ppm BA+ 20 ppm Bougainvillea extract), Lane 7 & 8- Control (MS+ 2 ppm BA), Lane 9- Positive control (Virus confirmed sample).

the early stages of infection and can interfere with viral replication (28). This interference could be one of the possible reasons for the tolerance noticed in the present study upon treatment using Virus-Ex, which warrants further investigation.

The treatment using leaf extracts of bougainvillea at 20 ppm concentration did not show any symptoms upon whitefly transmission, however, it was confirmed positive in molecular detection. Reports of symptomless plants as a source of Tomato yellow leaf curl virus (TYLCV) by earlier researchers support the symptomless infection of yellow mosaic virus observed in the present study (29). There are no reports regarding *in vitro* treatment using leaf extracts of bougainvillea for inducing tolerance towards viral infections in bitter gourd. Earlier study reported that spraying its extract showed decreased disease incidence upon challenge inoculation with BGYMV under field conditions (30). Even though the treatment using leaf extract did not show a positive response in the present study, a higher concentration of the extract, more than 50 ppm, can be tried to confirm its effect *in vitro*. Significantly enhanced growth parameters were observed in all treated plants compared to the control, which needs further investigation.

B. tabaci is reported to be a successful vector for the transmission of begomovirus, hence the antiviral activity of all the treatments in the present study was confirmed by the whitefly transmission test (31). In the present study, viruliferous whiteflies were released in an insect-proof cage where 25-day-old *in vitro* treated plants were maintained and allowed to feed for 48 hr, as the acquisition and inoculation access period of *B. tabaci* for begomovirus is reported to be 48-72 hr (32, 33).

The peroxidase activity was observed to increase in both treated and control plants following whitefly transmission. This finding aligns with the previous research, which noted an enhancement of peroxidase activity in black gram after whitefly infestation (34). Furthermore, feeding by whitefly nymphs has been reported to enhance defense-specific peroxidases in tomato and cucumber seedlings (35, 36). Maximum increase in peroxidase activity (3-fold) was noticed in plants treated with ribavirin, followed by bougainvillea extract (2-fold increase) upon whitefly transmission. Earlier study reported a two-fold increase in peroxidase activity in ribavirin-treated faba beans in 24 hr of *B. cinerea* infection compared to control (37). Some other researchers reported that spraying leaf extracts of *B. spectabilis* enhances synthesis of peroxidase and polyphenol oxidase, resulting in a decrease in disease incidence and a rise in growth parameters in bitter gourd (30). These results suggest the possibility of a peroxidise mediated defence mechanism against yellow mosaic virus in the present study.

Conclusion

The present study is the first attempt in bitter gourd to evaluate the effectiveness of *in vitro* chemotherapy against ToLCNDV. The investigation successfully confirmed the antiviral activity of ribavirin and Virus-Ex through whitefly-mediated transmission tests and molecular detection using PCR. Plant height, number of leaves and leaf area of ribavirin-treated plants were found to be higher compared to the control. The results of the present study can be successfully used for the production of virus-free plants in bitter gourd.

Acknowledgements

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Authors' contributions

AMV carried out the research work. SB and AR participated in the design and co-ordination of the experiment. SB and MDD drafted the manuscript. SB, SKB, SA and AR participated in the analysis of result. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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