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Combined infection of a cucumovirus and Potyvirus on ridge gourd (*Luffa acutangula* (L.) Roxb) in Tamilnadu

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Abstract
Ridge gourd (*Luffa acutangula* (L.) Roxb) is an important cucurbitaceous vegetable crop and is being consumed worldwide because of its nutritive value. Field survey was conducted in the major ridge gourd growing areas of Coimbatore district in Tamil Nadu during December 2020. Samples exhibiting virus symptoms like mosaic, mosaic mottling, blistering and puckering of leaves were collected. A total of 10 - 15 samples were collected from each field. The samples were inoculated on several systemic and local lesion hosts. The symptoms *viz*., mosaic, mosaic mottling, blistering, puckering of leaves and stunted growth on systemic hosts especially on different gourds were documented. The chlorotic lesions on *N. plumbaginifolia* and mosaic symptom on *C. amaranticolor* were also observed. The inoculated samples were tested for RNA viruses using the reverse transcription polymerase chain reaction (RT-PCR) method with *Cucumber mosaic virus* (CMV) gene specific and potyvirus universal primers. The results revealed the combined infection of both CMV and potyviruses and the infection of individual viruses were also documented. The severity of disease was evaluated for combined infection and sole infection in ridge gourd. The results revealed that, the combined infection of viruses had a higher severity of disease than single infections.
So, the combination of CMV and potyvirus infection is posing a serious threat to ridge gourd. This is the documented evidence for the occurrence of *Cucumber mosaic virus* and potyvirus in ridge gourd at Tamil Nadu.

Keywords: Combined, cucumovirus, potyvirus, *Luffa acutangula* (L.)

Introduction
Ridge gourd (*Luffa acutangula*) is one of the world's most important cucurbitaceous vegetable crops. It's extensively grown throughout the world in tropical and subtropical climates. It is grown in approximately 11,000 hectares in India, with a production of 1.3 lakh tonnes and a productivity of 15.85 tonnes per hectare. The ridge gourd crop is known for its antioxidant value, iron and fibre content. Ridge gourd is susceptible to a number of diseases. The important fungal diseases on ridge gourd are powdery mildew, anthracnose, *Cercospora* leaf spot, *Fusarium* wilt and downy mildew. *Cucumber mosaic virus* (Nagendran *et al.,* 2018) [15], *Watermelon bud necrosis virus* (Mandal *et al.,* 2003) [22], *Zucchini yellow mosaic virus*, *Cucumber green mottled virus* (Sharma *et al.*,2014), *Tomato leaf curl* New Delhi virus (Nagendran *et al.,* 2017) [14] with yellow mosaic disease (Sohrab *et al.*,2003) [22] were reported on the gourd *Potyvirus* is the second largest genus of plant viruses causing significant economic losses in a wide range of crops (Karthikeyan G *et al.*,2017, Akhtar Ali, 2020) [9]. The genus *Potyvirus* encompasses 175 plant-infecting positive sense RNA viruses (Wylie *et al.*, 2018). *Potyvirus* consists of flexuous, nonenveloped and filamentous virions range from 680 to 900 nm in length and 12-15 nm in diameter (Akhtar Ali, 2020). Potyvirus was primarily spread horizontally by aphids in a non-persistent way in the field, but it was also transferred vertically by seeds in some species (Adams *et al.,* 2012) [2]. There are about 15 potyviruses that cause infections naturally in various farmed or wild cucurbit crops that have been reported so far worldwide. *Cucumber mosaic virus* (CMV) is another notorious virus that infects cucurbits. CMV is the type member of the genus *Cucumovirus*, which belongs to the family *Bromoviridae*. CMV is also transmitted by aphids in a non-persistent manner. CMV virions have icosahedral particles which is 29 nm in dia and the capsid of CMV composed of 180 identical protein subunits (Palukaitis *et al.,* 1992).

There have been numerous reports of mixed viral infections in cucurbits around the world (Nagendran *et al.,* 2017 [14], Bananej and Vahdat (2008) [5], Ali *et al.* (2012) [3, 4], Papayiannis *et al.* (2005) [17], Salvaudon *et al.,* 2013). Bananej and Vahdat (2008) [5] reported the mixed infections of CMV and potyviruses. Similarly, Ali *et al.,* (2012) [3, 4], Papayiannis *et al.,*(2005) [17], Salvaudon *et al.,*2013) reported the mixed infections of potyviruses alone. The mixed infections of viruses from the *Potyvirus* genus and *Cucumber mosaic virus* (CMV) are common in natural environments and they drastically reduce productivity in cucurbits (Barbosa *et al.,* 2016). CMV and potyvirus genus interact synergistically in mixed infections (Wang *et al.,* 2001). Even though more studies were found to report mixed infection of CMV and potyvirus in cucurbits, only very few reports are available in ridge gourd (*Luffa acutangula*). This study deals with the documentation of prevalence of combined infection of CMV and potyvirus in ridge gourd in Tamil Nadu.

Materials and Methods

Survey and sample collection: A systematic field survey was conducted in Coimbatore district during 2020-21 to document the prevalence of virus diseases in ridge gourd. The per cent disease incidence was recorded during the survey. At each stage, totally 60 plants were observed in each field and the percent disease incidence was calculated using the formula Number of plants infected/ Total number of plants observed x 100. Simultaneously, the ridge gourd plants exhibiting symptoms such as mild mosaic pattern, mosaic mottling, mosaic with blistering and puckering were randomly collected from the field. About four to five symptomatic leaves were collected per plant. Samples were taken at various stages of the crop growth, including the vegetative stage, flower initiation, floral initiation to first picking and harvesting.

Symptomatology and mechanical inoculation

The samples collected from the field were inoculated on different selected hosts *viz*., *Trichosanthes cumerina*, *Luffa acutangula*, *Lycopersicon esculentum*, *Nicotiana plumbaginifolia*, *Chenopodium amaranticolor* and *Vigna unguiculata*. The sap inoculation was done at different stages based on the crop growth stages. Infected leaf tissue was used as an inoculum source. Using a sterile ice chilled pestle and mortar, 1g infected leaf tissues was pulverised in a phosphate buffer (pH 7) with 0.1 per cent mercaptoethanol. The leaf surfaces of the host plants were dusted with carborundum 600 mesh and the extracted sap was inoculated on the leaf surface of the host plants by mild abrasion. After a few minutes, the leaves were rinsed with distilled water to remove any excess inoculum and carborundum powder using squeeze bottle. The inoculated plants were kept in an insect-proof glasshouse for observation.

RNA Extraction

The total RNA was extracted using TRIzol reagent according to Chomczynski and Sacchi (2006) [6]. Fresh infected samples (100 mg) were homogenized in a sterile pestle and mortar. The homogenized powder was transferred into micro centrifuge tubes and 1 ml of trizol were added followed by centrifugation at 11000 rpm for 20 min. After centrifugation, the supernatant was transferred to new micro centrifuge tube and 250µl of chloroform was added and centrifuged. Following that, the aqueous layer was added with 250 µl of 2M NaCl and ice-cold isopropanol and incubated over ice for 20 min then centrifuged at 11000 rpm for 20 min. After that, ethanol wash was done and the pellets were dissolved in 30 µl of DEPC water.

Reverse transcription

RNA was quantified using a Nanodrop. The quality of RNA was also assessed by A260/A280 ratio. The first-strand complementary DNA was synthesized using, The reaction mixture (sterile water - 9 µl, 5 × reaction buffer - 4 µl, dNTPs - 2 µl, random primer – 1 µl, reverse transcriptase - 1 µl, RNase inhibitor - 1 µl and total RNA - 2 µg) was prepared and incubated at 42 ºC for 60 min followed by 70 ºC for 5 min (Ramesh and Sreenivasulu 2018) [19]. The resultant cDNA was utilised to perform PCR amplification.

Polymerase Chain Reaction

PCR amplification was performed using the degenerate primer pairs of potyvirus and CMV (Table 2) in the reaction mixture (Master mix - 5 µl, DEPC water – 2 µl, Forward primer – 1 µl, Reverse primer - 1 µl and cDNA – 1 µl) with following condition: initial denaturation of 2 min at 94 °C followed by 35 cycles of denaturation at 94 °C for 30 s, annealing and extension temperature specific for each amplicon and final extension time was 10 min at 72 °C. The PCR product was examined on agarose gel (1.2%) stained with ethidium bromide and viewed in gel documentation unit.

Effect of single and mixed infections of potyvirus and CMV on disease incidence in ridge gourd

The effect of single and mixed infections of potyvirus and CMV on disease incidence in ridge gourd cv. CoH1 was carried out in glass house condition. The molecularly confirmed pure isolate of Potyvirus and CMV in ridge gourd was inoculated in ridge gourd cv. CoH1 through sap transmission. The experiment was carried out with ten plants replicated thrice in a Completely Randomized Design. The combined inoculation of both the virus was also inoculated in the ridge gourd. The effect of single and combined inoculation on disease incidence/severity was calculated at different time intervals *viz*., 14, 28 and 35 DAS and analysed.

Table 1: Standardization of sap inoculation

Crop	Stage of crop	Time of inoculation (DAS)	Reference
Luffa acutangula	2 leaf stage	7	Vinothini *et al.*,2020 [23]
Momordica charantia	2 leaf stage	8	
Chenopodium amaranticolor	2 leaf stage	**7**	
Vigna ungiculata	2 leaf stage	5	
Nicotiana plumbaginifolia	2 leaf stage	9	
Trichosanthes cucumerina	4 leaf stage	8	Nagendran *et al.*,2018 [15]
Lycopersicon esculentum	5-6 leaf stage	14	John *et al.*,2003

Table 2: Primers used for the detection of viruses

Primer	Sequence (5' – 3')	Target virus	Annealing temperature (° C)	Amplicon size (bp)
NIb2F & NIb3R	F: GTITGYGTIGAYGAYTTYAAYAA	Potyvirus	50	~350
	R: TCIACIACIGTIGAIGGYTGNCC			
CMV 1 & CMV 2	F: GATCATCGCCTGAGAATA	CMV	45	~400
	R: TTCCAGAGATGCCTTCG			
	R:TGTTTGTGGAADGGAGCVAGA			

Statistical analysis

The disease incidence data were analysed and grouped using Duncan's multiple range test. The data were statistically analysed using AGRES software. The per cent disease severity was analysed using SPSS software.

Results

Field survey and sample collection

The survey were carried out in ridge gourd growing villages of Coimbatore district *viz.*, Thondamuthur, Madhampatty, Narasipuram, Vedapatti, Devnarpalayam, Devarayapuram, Vadakkipalayam, Poosaripalayam and TNAU Orchard.This was done using MS Excel software and grouping were done using DMRT test in AGRES software. Collectively, the disease incidence ranged from 14 – 33% at vegetative stage. Higher virus disease incidence was recorded in Poosaripalayam village (33.67%) followed by Thondamuthur (26.16%) and lower disease incidence was recorded at TNAU Orchard (14.68%) at vegetative stage as shown in Table 3. During flower initiation, the increase in disease incidence was observed on all the fields. Here also, the highest disease incidence was recorded at Poosaripalayam village (45.57%) followed by Thondamuthur (37.39%) and comparatively low incidence was recorded at Vedapatti village (21.13%). The disease incidence was observed to be ranged from 21 - 45% during flower initiation (Table 2). The disease incidence was found to be increased from 5-10% at first harvest stage. The higher disease incidence was recorded at Poosaripalayam village (54.93%) followed by Narasipuram village (48.96%) and lower incidence was recorded at Vedapatti village (34.54%). At first harvest stage, disease incidence ranged from (34 - 55%). Overall, the disease incidence was observed to be higher during the first picking stage. The leaf samples were also collected at different stages of crop *viz*., vegetative (30-35 days), flower initiation (45-50 days) and first picking stage (60-65 days) for further analysis.

Sap inoculation and symptomatology

The samples collected were inoculated on several systemic and local lesion hosts.The host response upon sap inoculation were observed (Table 4). In *Luffa acutangula*, the systemic mosaic was initiated at 7th day after inoculation and gradually turned to mosaic mottling, blistering, puckering of leaves and even stunted growth during consecutive days (Fig.1c,d & e). In *Trichosanthes cumerina*, mosaic symptom (Fig.1b, 1g) was initiated at 8 th day after inoculation and mosaic mottling (Fig.1a) were observed at 10 th day after inoculation. The systemic mosaic was observed in *Vigna ungiculata* (Fig.1k), *Chenopodium amaranticolor* (Fig.1i), *Momordica charantia* (Fig.1h) and *Lycopersicon esculentum* (Fig.1j) at 4th, 6th, 7th and 10th day after inoculation respectively where as in *Nicotiana plumbaginifolia* (Fig.1l), localized chlorotic lesion was observed at 5th DAI.

Molecular characterization of viruses

RNA was extracted from the inoculated hosts which showed mosiac, mosaic mottling and chlorotic spots and tested by RT PCR using potyvirus (Nib 2F & Nib 3R) and CMV degenerate primers (CMV 1 & 2). The results revealed that the ridge gourd plants with mosaic mottling symptoms amplified the fragment of 350 bp for poty virus (Fig. 4) and 400 bp for CMV (Fig. 5) thus confirming the presence of Potyvirus and CMV in ridge gourd.

Effect of single and mixed infections of potyvirus and CMV on disease incidence in ridge gourd

In ridge gourd, the disease incidence and disease severity was calculated. The combined infection of CMV and *Potyvirus* (62.8%) recorded higher disease incidence followed by potyvirus (58.7%). The least incidence was noticed in plants inoculated by CMV which recorded lower disease incidence (23.3%) as shown in fig 2.

The disease severity were calculated on 14, 28 and 35 days after inoculation (fig 3). At 14 DAI, the disease severity was 45.3% in plants with combined infection, 31% for *potyvirus* infected plants and 29.5% for CMV infected plants. Likewise the disease severity was 51.2%, 36.8% and 34.8% for combined, *Potyvirus* and CMV infected plants respectively on 28 DAI. Similarly at 35 DAI, the disease severity for plants with combined infection, *Potyvirus* and CMV was 58.6%, 41.2% and 38.9% respectively. Collectively, the disease severity were observed to be higher in plants with combined infection of CMV and *Potyvirus* followed by potyvirus. But disease severity was more or less equal for solitary infection of CMV and Potyvirus.

Discussion

In this study, samples were collected at three different stages v.z., vegetative, flower initiation and first picking stage during the survey. The disease incidence were calculated at each stage and higher disease incidence were observed at first picking or first harvest stage. The collected samples were mechanically inoculated into different systemic and local lesion hosts. The symptoms produced by ridge gourd crop upon sap inoculation was systemic mosaic, mosaic mottling, blistering, puckering of leaves and even stunted growth were observed in 7-14 days after inoculation. The snake gourd plants showing mosaic symptom, mosaic mottling were observed at 8 - 10 DAI and the systemic mosaic was observed in cowpea, bitter gourd, and *chenopodium amaranticolor* at 4th, 6th and 7th day after inoculation respectively. The kind of symptoms produced and time taken for symptom expression in different hosts was similar to the report of Vinothini *et al.* (2020) [23]. Furthermore, the symptomatological investigation would not yield a reliable outcome. Hence RT PCR based method was used for the confirmation of viruses. The *potyvirus* degenerate primer (Kumari *et al.,* 2021) detected the presence of *potyvirus* in ridge gourd and snake gourd. Similarly the CMV 1& 2 primer (Kumari *et al.,* 2021) detected the presence of CMV. It's not unusual to discover more than one viruses in the same plant at the same time (Moreno *et al.,* 2020) [11]. The results showed that mixed

infection with potyvirus and CMV was found to be the most common in ridge gourd, accounting for up to 62.8% of cases, followed by Potyvirus infection accounting for up to 58.7% of cases, and finally solitary cucumber mosaic virus infection accounting for up to 23.3% of cases which was consistent with Nagendran *et al.*(2017) [14]. CMV was shown to be less common here, which could be due to combined infection with potyvirus. In combined infection of viruses, one virus's symptom can be concealed or mimiced by another virus which was similar to Vinothini *et al.*(2020) [23]. As per Ruiz *et al.* (2021) [7], the sampling regions may have different environmental circumstances and the collection dates might have played a role in differences in the occurrence of the viruses. With comparison to a single infection, the severity of the disease is higher in mixed infections in ridge gourd under glass house conditions. Eventhough the CMV incidence was lower, its severity was also equal to potyvirus solitary infection. It is clear that in combined infection of viruses in a plant increases the symptom severity because of synergistic interaction (Wang *et al.*,2001, Barbosa *et al.*, 2016). Except for a few well-defined synergistic combinations (Nagendran *et al.*, 2017; Wang *et al.*,2001; Pruss *et al.*, 1997; Murphy *et al.*, 2005, Ruiz *et al.*, 2021) [14, 7] the pathogenic relevance of mixed viral infections in plants may have been underestimated. Until the mixed infection of virus becomes epidemic, more attention was paid to a single infection rather than a group of infections (Moreno *et al.*, 2020) [11]. However, further studies are needed to study the relationship of viruses in infection process and increasing the severities.

Conclusion

The study confirmed the high and prevailing occurences of combined infection of *Cucumber mosaic virus* and *Potyvirus* infection on ridge gourd than single infections of both viruses. *Potyvirus* infection on ridge gourd than single infections of both viruses.

Table 3: Incidence of virus disease in ridge gourd in Coimbatore district of Tamil Nadu

Sl. No.	Village	Disease incidence		
		Vegetative stage	Flower initiation	First picking stage
1.	Poosaripalayam	33.67 [a] (35.47)	45.57 [a] (42.46)	54.93 [a] (47.83)
2.	Thondamuthur	26.16 [b] (30.77)	37.39 [b] (37.70)	48.96 [b] (44.41)
3.	Madhampatty	20[c] (26.57)	27.38 [c] (31.55)	40.93[c] (39.78)
4.	Narasipuram	19.25 [d] (26.02)	24.57 [d] (29.72)	35.54 [f] (36.60)
5.	Vedapatti	16.16[de] (23.71)	21.13[e] (27.37)	34.54 [f] (35.99)
6.	Devnarpalayam	17.83 [de] (24.98)	23.54 [de] (29.03)	36.14[e] (36.96)
7.	Devarayapuram	19.41 [d] (26.14)	24.48 [d] (29.66)	38.56 [d] (38.39)
8.	Vadakkipalayam	21.5 [c] (27.62)	27.55 [c] (31.66)	40.53[c] (39.55)
9.	TNAU Orchard	14.68 [e] (22.53)	21.43 [e] (27.58)	34.57 [f] (36.01)

Mean of three replications.
Figures in parentheses are arcsine transformed values
Means in a column followed by alphabetic superscript letters are significantly different according to DMRT at $P \leq 0.05$.

Fig 1: Complexity of symptoms on different hosts upon sap inoculation of *Cucumovirus* and *potyvirus* from ridge gourd

Fig 1: A. Mosaic mottling, *Potyvirus* (*Trichosanthes cucumerina*) b. mosaic with chlorotic spots, combined infection of CMV and potyvirus (*Trichosanthes cumerina*) c. mosaic, *Potyvirus* (*Luffa acutangula)* d. mosaic mottling, Combined infection of CMV and Potyvirus (*Luffa acutangula)* e. mosaic with blistering, CMV (*Luffa acutangula)* f. mosaic, CMV (*Trichosanthes cumerina*) g. mosaic CMV (Momordica charantia*)* h. mosaic, CMV (*Chenopodium amaranticolor*) CMV (*Luffa acutangula)* i. mosaic, CMV (Lycopersicon esculentum) j. mosaic, CMV (*Vigna ungiculata*) k. localized chlorotic lesion, CMV (*Nicotiana plumbaginifolia*)

Table 4: Host response to sap inoculation on different crops

Test host	Symptoms observed	Time taken (days)
Luffa acutangula	Systemic mosaic, mosaic mottling, mosaic with blistering, mosaic with puckering, stunted growth	7 - 21
Trichosanthes cucumerina	Mosaic	8
Nicotiana plumbaginifolia	Localized chlorotic lesion	5
Lycopersicon esculentum	Systemic mosaic	10
Chenopodium amaranticolor	Systemic Mosaic	6
Vigna unguiculata	Systemic Mosaic	5
Momordica charantia	Systemic mosaic	7
Trichosanthes cucumerina	Mosaic mottling	12
Luffa acutangula	Mosaic mottling	10

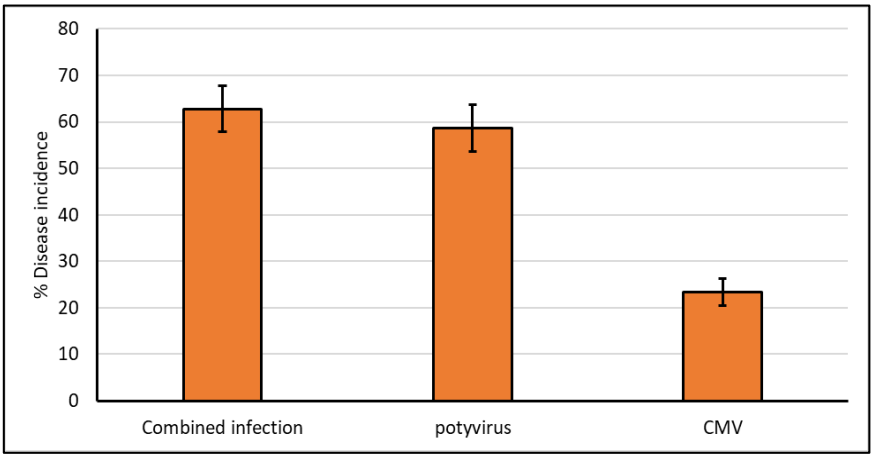

Fig 2: Effect of single and mixed infections of potyvirus and CMV on disease incidence in ridge gourd

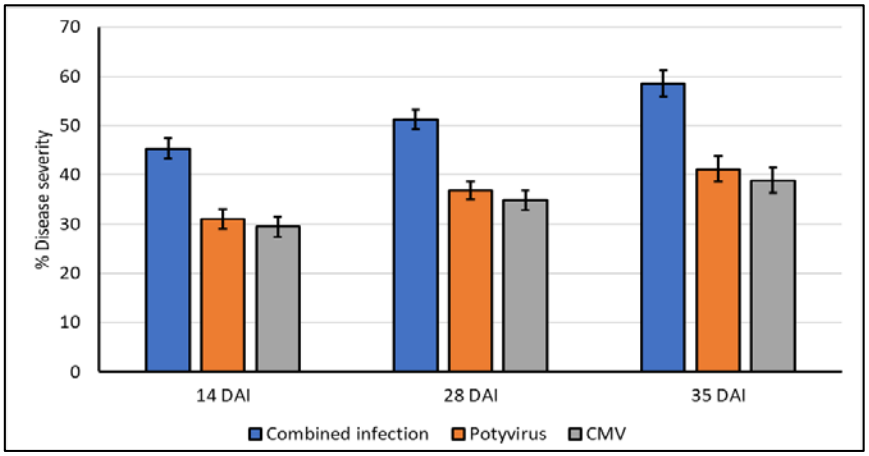

Fig 3: Effect of single and mixed infections of potyvirus and CMV on disease incidence in ridge gourd

Disease severity rating grade for mosaic virus in gourds: 0 =No symptoms, 1=Mild mosaic pattern in young leaves covering<10% area, 2=Mosaic pattern in young leaves covering <25% area, 3=Mosaic pattern in young leaves covering <50%area, blistering and puckering of leaves, 4=Widespread mosaic pattern in young leaves covering <75% area, distortion of leaves, 5=Widespread mosaic pattern in young leaves covering >75% area, distortion of leaves and stunting of the plants

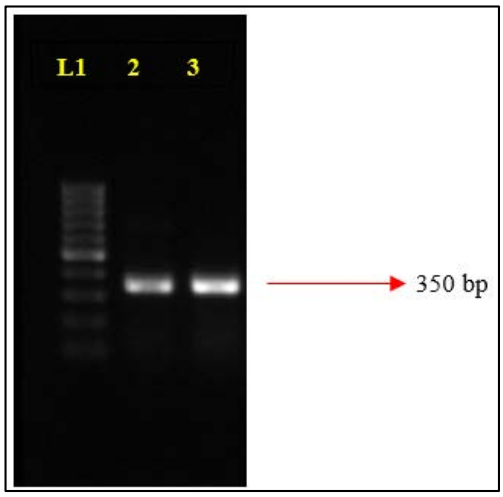

Fig 4: Agarose gel electrophoresis of infected plants using potyvirus degenerate primer Lane 1-100 bp ladder, L2-Ridge gourd (fig 1c), L3- positive control

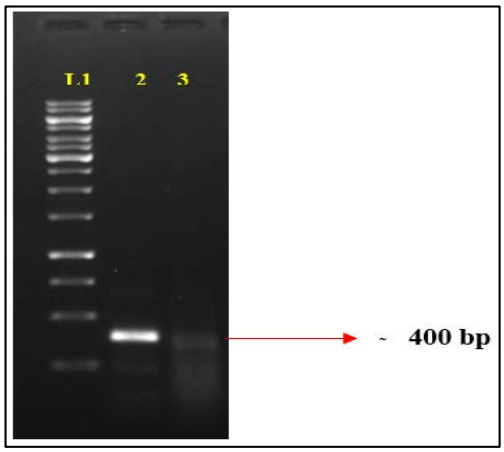

Fig 5: Agarose gel electrophoresis of infected plants using CMV degenerated primer Lane 1- 1Kb ladder, L2- Ridge gourd (fig 1f), L3 – positive control

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