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Studies on Molecular Detection of Cucumber Mosaic Virus and Its Anatomical and Biochemical Changes in *Daucus Carota* L.

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Abstract

The present investigation deals with anatomical and biochemical changes in *D.carota* leaf which was infected with cucumber mosaic virus. CMV was detected from the samples showing mosaic and mottling symptoms with the help of RT-PCR by using cucumovirus detecting primers. Anatomical studies showed that thickness of leaflet, length of palisade cells, breadth of palisade cells were reduced in the infected plant as compared to healthy. The number of stomata per unit area was increased and their length and breadth were decreased in virus infected leaves as compared to healthy. Biochemical studies showed that there was drastic reduction of chlorophyll a, b and total chlorophyll in virus infected leaves. RNA content in the diseased leaves was increased while that of DNA was reduced.

Keywords: Cucumber mosaic virus, RT-PCR, Biochemical changes, Anatomical changes, D.carota.

Introduction

Daucus carota L. belongs to family Apiaceae is a major consumable food crop grown commercially for nutritional value. Carrot is a good source of vitamin A. In India the crop is being grown commercially round the year in the area of 20,124 ha with an annual production of 28, 70,007 tons [1]. Carrot crop growing in the experimental plots, gardens and fields around AMU, Aligarh (Western Uttar Pradesh, India) were found naturally infected, exhibiting mosaic and mottling on entire plant. The crop is reported to be infected by a number of viral and viroid disease; alfalfa mosaic,carrot

latent, carrot mottle, carrot red leaf, carrot virus Y, carrot thin leaf, celery mosaic and cucumber mosaic virus.

Virus particles multiply in the infected plant cells and changes in biochemical compounds of cells such as chlorophyll, organic carbon, nucleic acids, β carotene etc [2]. Similarly Mandahar and Garg [3] reported a decrease in total chlorophyll by 65% followed by 82% reduction in photosynthesis rate in *Luffa aegyptica* due to infection of CMV. Akanda et al [4] reported metabolic changes in tomato leaves due to infection of CMV. Virus infection alters the gross form, arrangement and appearance of cells by disturbing their internal organization. Some anatomical changes also exhibited due to the virus infection in vines like disintegration of tissue, hypertrophy, compactness of tissue and decrease in number of chloroplast in the cells, as reported by various workers [5, 6,]. The present study was undertaken with the objectives to assess the biochemical and anatomical changes in the leaf of *Daucus carota L*. infected with cucumber mosaic virus. To Best of our knowledge this is the first report from India.

Materials and Methods

Molecular detection of CMV in *D.carota*

RNA isolation

Total RNA was extracted from naturally infected mechanically inoculated and healthy leaf samples using RNeasy plant RNA isolation kit (Qiagen, Germany).Viral RNA was isolated from the virus purificate (~ 100µg) by disrupting of virion with 1% SDS followed by extraction with phenol-chloroform, ethanol precipitation and centrifugation at 10,000 rpm for 15 min at 4˚C. The pellet RNA was washed with 70% ehanol, dried and resuspended in RNase-free sterile water.

cDNA Synthesis and RT-PCR

To amplify the complete ORF of coat protein gene of CMV isolate, RT-PCR was performed using viral RNA as template and a pair of cucumber mosaic virus specific primers. First strand cDNA synthesis of CP gene performed using viral RNA (~1 µg) AS template and AMV reverse transcriptase (Pharmacia Biotech Ltd) in a 20µl reaction mixture containing the downstream primer (25pmoles), dNTPs (25pmole each) in a PCR buffer containing 15 mmol/1$MgCl_2$ were employed. The PCR condition for cucumovirus group specific primer was as follows: initial template denaturation at 94˚C for 5 min was followed by 30 cycles consisting of 94˚C/60 sec (denaturation), 52˚C/45 sec (annealing) and 72˚C/90 sec (primer extention) and final extention at 72˚C for 5 min. The amplified products were electrophoresed on 1% agarose gel.

Biochemical and Anatomical studies

For the anatomical studies infected plants of carrot (*D.carota L*.) showing severe symptoms were taken. The leaves which exhibited mosaic and mottling symptoms (fig.1) were fixed for microtomy .In order to study the leaf anatomy, the healthy and virus infected leaves were taken for the preparation of microtome section. Procedure

given by Johnson [7] was followed for paraffin embedding sectioning and staining. For the estimation of biochemical changes leaf samples of apparently healthy and diseased plants were collected when typical symptoms of CMV developed. Chlorophyll content was estimated according to Witham et al [8] using double beam spectrophotometer extracting with 80% acetone. Nucleic acid content was estimated according to Hunter method [9].

Figure 1: Naturally infected carrot plant showing mosaic and mottling in mature leaf.

Results and Discussion

Electrophoresis of RT-PCR products showed positive amplification of the expected sizes DNA bands in all the selected samples of carrot. Approximately 650 bp (fig.2) band was obtained by CMV detecting primers although no such amplicon was obtained in symptomless/ healthy sample. After the confirmation of CMV by the RT-PCR, It is observed that the vascular tissue of the leaves was greatly affected due to viral infection. It is clear from table 1. that there was an increase in the thickness of zone of phloem tissue as compared to the phloem cells of the healthy leaf of carrot. Thickness of xylem and phloem zone was almost equal in diseased leaves, whereas in healthy leaves the thickness of phloem was less as compared to the thickness of xylem zone. The thickness of xylem zone in the midrib region of the leaf reduced in the infected plants whereas the thickness of phloem zone was increased in diseased leaves. Similar observations recorded by Sharma, Pachauri and Chandra [10, 11 and 12]. In the virus infected plants of carrot there was drastic reduction of chlorophyll a (39.7) and chlorophyll b (28.4) and total chlorophyll (68.1) (table-3) as compared to healthy .The ratio of chlorophyll a to b was observed to be higher in all the virus infected plants as compared to healthy. These findings are supported by Haider and Hussain [13] and Akanda et al [4] in the yellow vein mosaic virus infected okra and CMV infected tomato leaves. It has been observed that the thickness of the leaflet, length of palisade cell, breadth of palisade cell was reduced in the infected plant as compared to healthy (table-1). The epidermal peels of healthy and diseased leaves

were stained with phloxin and trypan blue and it was observed that the diseased peel looked dark as compared to healthy, which indicated that virus caused enhancement in the formation of RNA. RNA content in the virus infected leaves was increased (14.28) while that of DNA was reduced (0.31) (table-4). This is similar to the findings of Hossain and Haider [14] and Muqit et al [15]. In the present investigation when the stomata were observed in healthy and diseased leaves it was noticed that the number of stomata per unit area was increased and their length and breadth were decreased in diseased leaves as compared to healthy (table-2). These observations are in accordance with those of Sharma [11], who made similar observation with poppy mosaic virus.

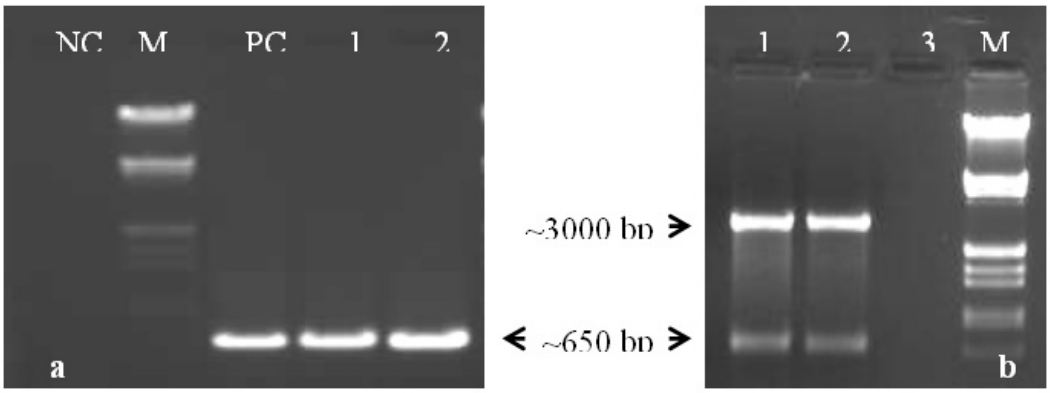

Figure 2: (a) RT-PCR amplification of coat protein (CP) gene. Lane NC= healthy plant, Lane PC= positive control (CMV infected plant), Lane= 1-2 naturally infected carrot samples. (b) Screening of clones by restriction digestion using *EcoR*1 restriction enzyme.Lane1-3Digested plasmids showing CP insert, Lane M= λ-DNA/ *EcoR*1/*Hind*III as DNA marker.

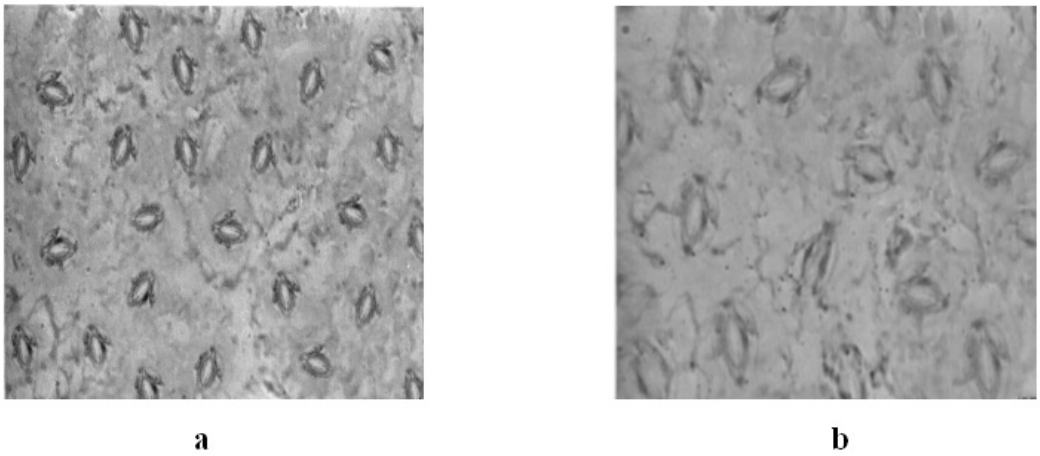

Figure 3: (a) Diseased leaf showing increased number of stomata per unit area (b) Healthy leaf showing decreased number of stomata per unit area.

Table 1: Effect of cucumber mosaic virus on leaf tissue of *D.carota L.*

	Healthy leaf	Diseased leaf
Thickness of leaflet (µ)	163.3	97.8
Thickness of xylem zone	106.3	25.7
Thickness of phloem zone	35.2	47.6
Length of palisade cell	34.6	12.1
Breadth of palisade cell	9.4	5.6

Table 2: Effect of cucumber mosaic virus on stomata in *D.carota* leaf.

	Healthy leaf	Diseased leaf
No. of stomata	15	32
Length of stomata	28	18
Breadth of stomata	21	16

Table 3: Effect of cucumber mosaic virus on total chlorophyll in *D.carota* leaf.

	Healthy leaf	Diseased leaf
Chl a (mg/100g fresh wt)	52.4	39.7
Chl b (mg/100g fresh wt)	46.2	28.4
Total chlorophyll	98.6	68.1
Chl a:b	1.13	1.39

Table 4: Effect of cucumber mosaic virus on total chlorophyll in *D.carota* leaf.

	Nucleic acid contents (mg/g)		Ratio DNA/RNA
	RNA	DNA	
Healthy	72.9	0.66	11.04
Diseased	14.28	0.31	46.06

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