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Full Length Research Paper

Molecular detection of a virus infecting carrot and its effect on some cytological and physiological parameters

Bushra Afreen*, Mohd Gulfishan, Geetesh Baghel, Mehar Fatma, A. Akil Khan and Q. A. Naqvi

Plant Virology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh-202002 India.

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An experiment was conducted to determine the physiological and cytological changes in carrot (*Daucus carota* L.) due to infection of cucumber mosaic virus. The virus isolate causing mottling was detected by reverse transcriptase polymerase chain reaction (RT-PCR) by showing amplification of the expected size of 650 base pair (bp) deoxyribonucleic acid (DNA) band. Regarding the physiological changes, carbon, nitrogen and protein contents was decreased in infected plants as compared to healthy plants while phosphorous content was increased in diseased plant. In cytological changes, the scattered metaphase was observed in the diseased plant cells. Mitotic index of the diseased cells was decreased while N/C ratio was increased. Chromatin bridges were also observed at anaphase I and II due to infection of cucumber mosaic virus.

Key words: Physiological changes, cytological changes, *Daucus carota*.

INTRODUCTION

Carrot (*Daucus carota* Linn), a biennial of Apiaceae family, is a very popular vegetable and have a great importance due to its nutritional value. It is cultivated all over the world. In India the crop is being grown commercially round the year in the area of 20,124 ha with an annual production of 2,870,007 tons (Sindhu, 1998). Northern Uttar Pradesh (UP) contributes the significant production of the total carrot productivity in India. The carrot crop is mostly affected by viruses viz: Carrot thin leaf virus (Howell and Mink, 1976); lucerne mosaic virus (Douine, 1976); celery mosaic virus (Howell and Mink, 1981); carrot mosaic virus (Chod, 1965) and carrot red leaf virus (Stubbs, 1974).

Multiplication of virus particles in the infected plant cells exhibit some physiological and cytological changes such as chlorophyll, carotene, organic carbon, nitrogen, protein and phosphorus due to virus infection (Muqit et al., 2007). Various metabolites of host tissue were altered due to viral infection (Naidu et al., 1986; Mohanty and Sridhar, 1989; Srinivasulu and Jeyarajan, 1990; Chakraborty et al., 1994; Clover et al., 1999; Hemida and Razik, 2002). Similarly, there are several reports on cytological changes due to virus infection. Mirkova et al. (1993) reported abnormalities in meiosis including univalent at diakinesis and metaphase I, lagging chromosome at anaphase I and II in *Lycopersicon esculentum*. Caldwell (1952) found that tomato aspermy virus had a deleterious effect on the prophase of meiosis. Kaul (1968) studied the effect of infection with mosaic virus on the meiotic process of *Datura quarcifolia* and reported complete asynapsis at diakinesis and metaphase I and the presence of 24 univalents. Wilkinson (1953) observed nuclear abnormalities in *L. esculentum* along with

*Corresponding author. E-mail: fauzbushy@gmail.com Tel: +91 9997570636.

Abbreviations: RT -PCR, Reverse transcriptase polymerase chain reaction; **CP,** coat protein; **RNA,** ribonucleic acid; **AMV,** avian myeloblastosis virus; **RT,** root tip treatment; **MI,** mitotic index; **PMCs,** pollen mother cells; **SDS,** Sodium dodecyl sulfate; **ORF,** open reading frame; **CMV,** cucumber mosaic virus; **N/C,** nuclear and cytoplasmic ratio; **PRSV,** papaya ringspot virus; **WMV2,** watermelon mosaic virus 2; **BgMV,** bottlegourd mosaic virus.

Figure 1. Naturally infected carrot plant showing severe mottling in mature leaf and chlorosis in young leaf.

pollen sterility and disruption of reproductive mechanism due to viral infection. It is known that increase in meiotic abnormalities caused an increase of pollen sterility (Reddy and Rao, 1982). Naz et al. (2008) reported some cytological effects in five crops due to virus infection. In view of these reports, this investigation was undertaken to study the physiological and cytological changes due to infection of cucumber mosaic virus in *D. carota*.

MATERIALS AND METHODS

Molecular detection of virus in *D. carota*

Ribonucleic acid (RNA) isolation

Total RNA was extracted from naturally infected, mechanically inoculated and healthy leaf samples using RNase plant RNA isolation kit (Qiagen, Germany). Viral RNA was isolated from the virus purification (~100 µg) by disrupting of the virion with 1% sodium dodecyl sulfate (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% ethanol, dried and resuspended in RNase-free sterile water.

Complementary deoxyribonucleic acid (cDNA) synthesis and reverse transcriptase polymerase chain reaction (RT-PCR)

To amplify the complete open reading frame (ORF) of coat protein gene of cucumber mosaic virus (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 coat protein (CP) gene was performed using viral RNA (~1 µg) as template and avian myeloblastosis virus (AMV) reverse transcriptase (Pharmacia Biotech Ltd) in a 20 µl reaction mixture containing the downstream primer (25 pmoles), dNTPs (25 pmole each) in a PCR buffer containing 15 mmol/L $MgCl_2$. 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 s (denaturation), 52°C/45 s (annealing) and 72°C/90 s (primer extension) and final extension at 72°C for 5 min. The amplified products were electrophoresized on 1% agarose gel with a known DNA marker (Lambda DNA digested with *EcoR* I/ *Hind* III, Bangalore Genei Pvt Ltd.).

Physiological changes due to infection of virus

Due to virus infection, some physiological parameters such as photosynthetic pigment (chlorophyll a, b and carotene), organic carbon, nitrogen, protein and phosphorus were determined in the leaf samples of plants at 12 and 18 days after inoculation with CMV. The carrot plants were grown in 12 inches pots under natural conditions in a glass house at 15 to 25°C. The composition of sand, soil and compost was 1:2:1. Four to five leaf stages of seedlings were selected for different analysis. Mechanical inoculation was done by using virus inoculum, which was prepared by triturating the infected leaves of carrot showing mosaic and mottling symptoms (Figure 1) using sterilized mortar and pestle. For 2 g of leaves, 10 ml of phosphate buffer was added. The crude sap was then centrifuged at 5,000 rpm for 10 min and only the supernatant was used for inoculation. Mechanical inoculation was done by using carborundum (500 mesh) as an abrasive. Plants treated with only buffer were kept as control. Chlorophyll and carotent contents were estimated according to Witham et al. (1986) and Shiraishi (1972), respectively; by using double beam spectrophotometer (model 1200-20 Hitachi) extracting with 80% acetone. Total nitrogen and protein was determined by Kjeldhal. Organic carbon was estimated by Tyurin (1980) methods. Phosphorus was estimated according to Hunter method.

Cytological changes due to infection of virus

Mitotic study

The effects of virus infection on the somatic chromosomes and their mitotic behaviour have been studied by root tip treatment (RT) method. The seeds obtained from healthy and diseased plants of carrot were germinated on moist filter paper in petri dishes. Root tip were cut at the time of maximum meristematic activity having high mitotic index (MI). The treated root tips were washed with distilled water and then fixed in acetic alcohol (90% alcohol + acetic acid in ratio 3:1) solution for 8 to 12 h and then transferred to 70% alcohol after fixation for longer storage. Slides were thoroughly studied to determine the frequency of mitotic index (MI) and was calculated by using the formula:

Mitotic index (MI) = (Total No. of dividing cells/Total No. of cell observered) x 100

Nuclear and cytoplasmic ratio (N/C) was also calculated by taking the help of stage micrometer and occulometer. N/C ratio was calculated by the following formula:

N/C = (Sum of area occupied by the nuclei / Sum of area occupied by cytoplasm) x 100

Five root tips were studied for MI and N/C ratio.

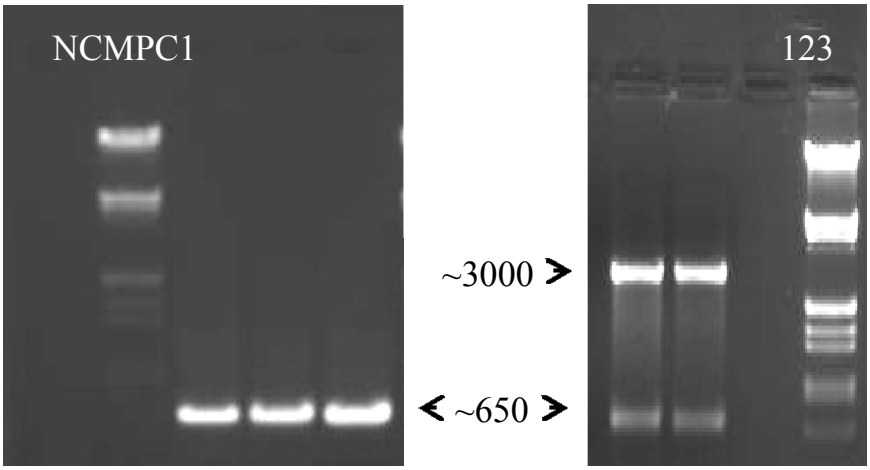

Figure 2. (a): RT-PCR amplification of coat protein (CP) gene. Lane NC = Healthy plant, Lane PC = positive control (CMV infected plant), Lane = 1-2 naturally infected carrot samples; (b): Screening of clones by restriction digestion using *EcoR*1 restriction enzyme. Lane 1-3 = Digested plasmids showing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

Table 1. Physiological parameters due to infection of CMV on *D. carota.*

Physiological parameter	At 12 days		At 18 days	
	Healthy	Infected	Healthy	Infected
Chlorophyll a	68.6	41.6	59.60	18.90
Chlorophyll b	56.6	32.8	61.78	12.8
carotene	15.67	10.3	16.77	7.64
Carbon (%)	38.60	29.61	39.10	26.40
Nitrogen (%)	55.67	0.301	52.70	0.207
Protein (%)	120.10	3.683	112.60	1.206
Phosphorus(ppm)	16.459	19.724	18.624	35.375

Meiotic study

Flower buds from both healthy and virus infected plants were taken from 7.00 to 9.00 am. These were fixed in freshly prepared Carnoy's fluid (absolute alcohol 6: chloroform 3: acetic acid 1) for 24 h, and then stored in 70% alcohol in which few drops of glycerin were added to prevent the material from hardening. Squashes were made permanent in 2% acetocarmine and slides were made permanent though butyl-alcohol series.

RESULTS AND DISCUSSION

Molecular identification of virus

Although a number of viruses have been reported on carrot, the proper identification and characterization of the virus causing mosaic and mottling on carrot at molecular level has not been done. However, in this study; RT-PCR using CMV specific primers revealed an expected size of approximately 650 bp although no such amplicon was obtained in symptomless/healthy samples (Figure 2). Similar amplification was obtained for the confirmation of CMV causing mosaic disease on chilli (Sajid et al., 2006).

Similar amplification of 650 bp confirmed the presence of CMV in the present isolate.

Physiological changes

Some physiological changes were observed due to infection of cucumber mosaic virus on *D. carota* plants. There was a gradual decline in photosynthetic pigments. Chlorophyll contents of the CMV infected plants were found to decreased 18 days after inoculation as compared to 12 days after inoculation. After 18 days, chlorophyll a, b and carotene in infected leaves was 18.90, 12.8 and 7.64, respectively while in healthy leaves it was 59.60, 61.78 and 16.77, respectively (Table 1). It is

Table 2. Mitotic index of the cells of carrot infected with CMV.

S/N	Mitotic index	
	Healthy	Infected
1	24.90	10.20
2	32.61	8.30
3	23.80	8.90
4	27.00	7.50
5	15.50	9.80
Mean	24.76	8.94

Table 3. Nucleus/cytoplasm ratio of the cells of carrot infected with CMV.

S/N	N/C ratio	
	Healthy	Infected
1	0.151	0.204
2	0.126	0.276
3	0.137	0.239
4	0.128	0.162
5	0.099	0.225
Mean	0.128	0.221

Table 4. Meiotic chromosomal abnormalities (%) due to infection of CMV.

S/N	Observations	Healthy	Infected
1	Number of PMCs	50	50
2	Metaphase abnormality	-	7.9
3	Sticky chromosome	3.20	34.60
4	Unequal separation	-	1.60
5	Chromatin bridge	-	5.20

clear from the results that the level of chlorophyll content is decreased in infected leaves as compared to healthy ones. Similar results were obtained by Srivastava et al. (2005). Haider and Hossain (1994) and Akanda et al. (1998) observed similar trends of reduction in chlorophyll and carotene in the yellow vein mosaic virus infected okra and CMV infected tomato leaves, respectively. Similar reduction was observed in the case of carotene. Total organic carbon, nitrogen and protein were decreased in virus infected plants as compared to healthy ones. Increased level of phosphorus content was observed in infected leaves. This might be due to phosphorus containing polypeptide of the virus particles. Muquit et al. (2007) reported that the percentage of carbon, nitrogen and protein was decreased due to the infection of papaya ringspot virus (PRSV), watermelon mosaic virus 2 (WMV2) and bottlegourd mosaic virus

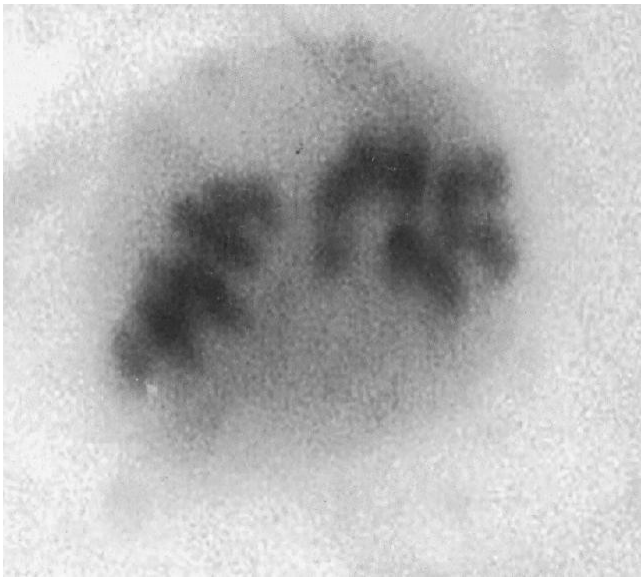

Figure 3. Infected cells showing sticky chromosomes at metaphase.

(BgMV) in ash gourd. Similarly, in this study, the percentage of carbon, nitrogen and protein showed maximum reduction (26.40, 0.207 and 1.206%) after 18 days as compared to healthy plants, but the phosphorus was increased in infected leaves as compared to healthy ones. Similar results were obtained by Matthwes et al. (1963), Haider and Hossain (1994) and Muqit et al. (2007).

Cytological changes

In this study, several changes were observed in the cytology of virus infected plants of carrot. It was evident from Table 2 that, the mitotic index (MI) of healthy and virus infected plant cells of carrot were 24.76 and 8.94, respectively. Thus, the MI of the diseased cells of carrot was decreased in the comparison to healthy plant cells. The N/C was increased (0.221) in diseased plant cells as compared to healthy plant cells (0.128) (Table 3). The nucleus was enlarged due to the infection of CMV. This is similar to the finding of Yadav (2008). According to Kostoff (1933), the effect of mosaic virus infection on tobacco induced some abnormalities in all the stages of meiosis. Similarly, in this investigation, CMV induced some abnormalities at the metaphase and anaphase stage of meiosis. It was clear from Table 4 that, the scattered metaphase was observed in the diseased plant cells while it was absent in healthy plant cells of carrot. The percentage value of metaphase anomalies in virus infected plant cells was 7.9%. The percentage value of appearance of sticky chromosomes in pollen mother cells (PMCs) of healthy plants was 3.20%. However, increased number of sticky chromosome, that is, 34.60% (Figure 3)

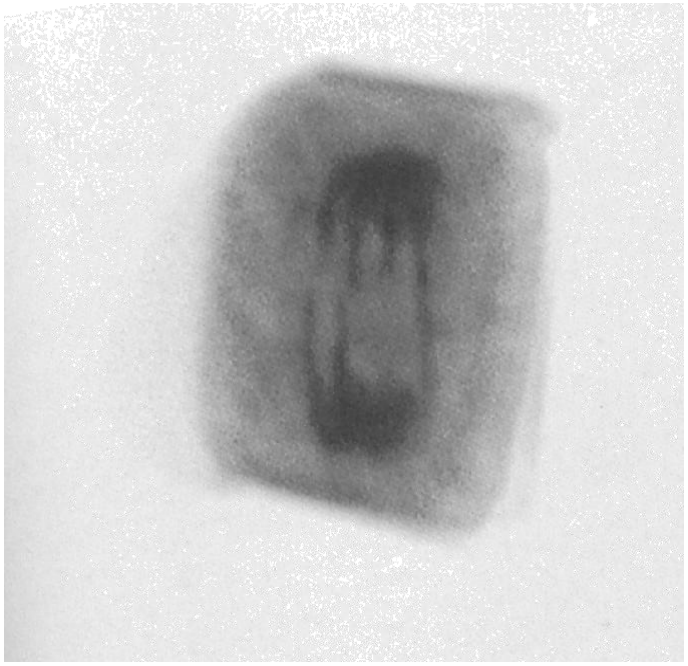

Figure 4. Infected cells showing chromatin bridges at anaphase.

were recorded in the PMCs of infected plants. The PMCs of healthy plants showed equal separations of chromosomes but in the PMCs from infected plants, unequal separation of chromosome was recorded. The percentage value of unequal separation was 1.60%. CMV induced chromatin bridges at anaphase I and II (Figure 4). Chromatin bridges were not found at anaphase of healthy plants; however, the percentage value of chromatin bridges observed in the diseased PMCs was 5.20%. Swaminathan et al. (1959) showed reduced chiasma frequency, irregular anaphase separation and poor seed set the effect of virus on *Capsicum annuum*. However, in this investigation, induced chromatin bridges, unequal separation at anaphase I and II were observed due to virus infection.

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