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Effect of Whitefly Transmitted *Geminiviruses* on the Physiology of Tomato (*Lycopersicon esculentum* L.) and Tobacco (*Nicotiana benthamiana* L.) Plants

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Abstract
Viruses always have profound effect on the growth and development of plants and interact with host defense mechanism and results in altering the physiology of plants. In the present study effect of different whitefly transmitted begomoviruses; (monopartite as well as bipartite) on the contents of soluble sugar and chlorophyll of *Lycopersicon esculentum* and *Nicotiana benthamiana* plants were investigated. It was found that the leaf relative water contents (RWC), total soluble sugars (TSS), fresh and dry biomass, photosynthetic pigments level were more in healthy plants as compared to virus infected plants. Plant infected both with *Tomato leaf curl new dehli virus* and *Cotton leaf curl burewalla virus* associated with betasatelite showed reduced TSS and leaf relative water contents as compared to healthy plants. Among infectious clones *Cotton leaf curl burewalla virus* associated with beta satellite (CLCuV+CLCuB), *Tomato leaf curl new dehli virus* (ToLCNDV) caused more reduction while *Malvestrum yellow vein change manga virus* (MYVCMV) caused less reduction as compared to above mentioned clones. CLCuV+ CLCuB showed significant effect on Chlorophyll *a* (9.60, 8.93mg/g), Chlorophyll *b* (7.2, 5.86 mg/g) and total chlorophyll contents (16.8, 14.8 mg/g) in both plants respectively. While ToLCNDV caused reduction in RWC (80% to 36%) in case of *L. esculentum* while RWC (75 to 30%) in *N. benthamiana*. Among *L. esculentum* TSS was reduced (8.13 to 3.33 mg/g) due to CLCuV+CLCuB infection. In case of *N. benthamiana* TSS was reduced (9.433 to 2.5 mg/g) due to ToLCNDV.
Keywords: Geminivirus, Plant Physiology, TSS, Chlorophyll.

INTRODUCTION

Geminiviruses belong to the family *Geminiviridae*, which is the second largest plant virus family. They cause infection in a wide variety of plants which includes both, monocots and dicots (Briddon *et al*., 2005). Since nineteenth century the typical symptoms of geminivirus infections have been observed in tropical and sub-tropical regions of the world. It is the second class of single stranded DNA viruses. Geminiviruses replicate through an intermediate dsDNA molecule in the infected plant cell nuclei and depend upon the host DNA replication machinery (Jeske, 2007). Geminiviruses are independently replicating viruses, and have smallest genome mostly utilize bidirectional mode of transcription and overlapping genes for competent coding of proteins (Rojas *et al*., 2005). *Begomovirus* the second largest genus of geminiviruses consists of 196 member species, representing the largest genus of family *Geminiviridae* (Brown and Fauquet, 2012). *Begomoviruses* are transmitted by whitefly *Bemisia tabaci* and are widely spread. During the last 30 years, begomoviruses have emerged as important viral pathogens in food, fiber and ornamental crops in the major part of the world. Begomoviruses have been sub-divided into two types of genome either bipartite or monopartite, transmitted by whitefly (insect vector) and mostly infect dicots **.**Now begomoviruses has become destructive group of viruses that infect plants. This is due to a rise in worldwide population and distribution of insect vector and movement of plant materials around the world (Rojas *et al*., 2005; Seal *et al*., 2006). The vast distribution and abundance of begomovirus isolates and continuously newly reporting species as well as high genetic diversity within species suggested that begomoviruses have high mutation rate and they produce highly diverse population in very short time (Patil *et al*., 2010). In some cases virus infection may show no apparently visible symptoms. However, in most of the cases they causes more visible symptoms of disease. These symptoms are induced as a result of hindrance/competition for host resources, which can disturb physiology of the host to cause disease. Infection caused by a specific virus in a host can induce more than 4000 different type genes and are responsible for different changes in host due to infection (Senthil *et al*., 2005).

In recent years, plant diseases are major limiting factor in agricultural production. Therefore, understanding of basic physiological and biochemical changes in relation to disease incidence will be helpful in suggesting economic approaches to control crop damages due to different diseases (Zafar and Athar, 2013). It has been reported earlier that *papaya leaf reduction virus* infection causes changes in carbohydrates contents in course of disease development (Khatri and Chenulu, 1969). Viral diseases of tomato have been progressively more commonly occurring and damaged the crops. The changes in carbohydrates content of leave in virus infected plant tissue has been reported by several workers (Adomalo and Hucheon, 2008; Singh and Shukla, 2009). Phyto-pathogenic infection leads to changes in secondary metabolism based on the activation of defence

programmes, hypersentive response as well as to alter the primary metabolism that affect growth and development of the plant. Therefore, any pathogen attack causes yield losses of crops even in those interactions which do not ended with disease or death of plants (Berger *et al*., 2011).

MATERIALS AND METHODS

The present work was carried out during 2012-2013 at plant virology Lab., Institute of Agricultural Sciences, University of the Punjab, Lahore. The details of the material used and methods followed are elaborated below.

Selection of test plant

Tomato *(Lycopersicon esculentum) and* Tobacco (*Nicotiana benthamiana)* germplasm was selected for study.

Selection of soil, Pot filling and sowing of seeds

Tomato (*L. esculentum*) and Tobacco (*N. benthamiana)* seeds were sown on Mixed soil containing sandy loam and peat moss. Pots were kept under controlled conditions with 16h/day light and the temperature between 22-28 °C (*L. esculentum*) and 20-22 °C (*N. benthamiana*) in growth room for germination of seeds. After 15-20 days of seed germinations, small seedling was transferred in smaller pots and maintained in growth room. Hoagland's nutrient solution was prepared and used as source of nutrient (Hoagland and Arnon, 1950).

Procurement of viral infectious clones

Infectious Clones of *Tomato Leaf Curl New Dehli Virus* (ToLCNDV DNA- A+B) and *Agrobacterium*, *Cotton Leaf Curl Burewalla Virus* (CLCuV+CLCuB), *Malvestrum Yellow Vein Changa Manga Virus* (MYVCMV) were obtained from molecular virology Lab of Institute of Agricultural Sciences, Punjab University, Lahore.

Preparation of *Agrobacterium* Cultures for Agro infiltration

Infectious clones of ToLCNDV, *Agrobacterium*, (CLCuV+ CLCuB*)* and MYVCMV were transformed in competent cells of *A. tumefaciens* strain GV3101 and inoculum was prepared for infectivity analysis, preserved in glycerol stock were taken from -80 ^{O}C freezer. Infectious clone were taken and streaked into LB plates in quadrate pattern. Plates were kept in incubator for 2 days at 28^{O}C. After 48 hours plates were used for culturing and again kept the test tube on shaker at 170 rpm for 48 hours at 28°C. Previous cultures were checked for growth after 2 days of incubation and those cultures having some growth or turbidity were proceeded further for inoculum preparation. About 20µL of kanamycine, 40µL of Rif Ampicilline , 2 µL of acetosyringone were added and mixed in LB broth media.10µL of culture having bacterial growth were taken from test tube and mixed in flask again kept it for 2 days at 28 °C incubator and shaker. 20 ml of culture from flask were transferred to falcon tube under sterilized conditions and centrifuged at 5 °C for 10 minutes at 8000 rpm. Supernatants were discarded and pellet was used for further inoculums preparation. 10ml of $MgCl_2$ (0.1mM) solution, 20µl of acetosyringone were added in falcon and pellet was re suspended in these solutions and incubated at room temperature for 3 hours before infiltration.

Agro Infiltration of Inoculums in Plants

1.5 to 2 ml inoculums having optical density (O.D.) value of 1 was injected in two to three leaves per plant using a syringe. Infiltrations of plants were done on the lower side of younger leaves between two veins. Three spots per leaf were infiltrated and all the treatment plants were infiltrated by same procedure. Plants were kept in a containment glasshouse at 25-28 °C.

STUDY OF PHYSIOLOGICAL PARAMETERS

Biochemical Attributes

For the estimation of biochemical attributes which were disturbed or changed by virus attack following tests were performed.

Measurements of Chlorophyll (*a*), (*b*) and Total Chlorophyll Contents (*a*+*b*):

Leaf chlorophyll contents were estimated by spectrophotometer by adopting methodology described by Arnon, (1949). Absorbance of supernatant was taken at 645 and 663 wavelength by spectrophotometer. 80% acetone was used as a blank. The chlorophyll concentration was calculated as follows;

(Chlorophyll *a*) = Ch *a* (mg/g) = [12.7×A663 – 2.69×A645] ×V/1000×W

(Chlorophyll *b*) = Ch *b* (mg/g) = [22.9×A645 – 4.86×A 663] ×V/1000×W

(Chlorophyll *a*+*b*) = (Ch *a*+*b* mg/g) = [8.02×A663 + 20.20×A645] ×V/1000×W

Total Soluble Sugar Extraction

Total soluble sugar in oven dried leaves of *L. esculentum* and *N. benthamiana* were determined according to the method of Malik and Srivastava, (1985).

AGRONOMIC STUDY OF INFECTED AND HEALTHY PLANTS

Collection of Data at Seedling Stage

After about two weeks morphometric parameters like root and shoot length, fresh as well as dry biomass and leaf relative water content of tomato (*L. esculentum* and *N. benthamiana*) plants were noted with three replications of each treatment.

Leaf Relative Water Content

Data regarding leaf relative water contents were taken. Three weights of leaves were taken. Fresh weight (W1) of leaf samples were noted and were immersed in distilled water and turgid weights (W2) were recorded. Oven dry weights (W3) were also determined. Leaf relative water content was computed using the equation:

$$\text{Relative water content (\%)} = \frac{\text{leaf fresh weight} - \text{leaf dry weight}}{\text{leaf turgid weight} - \text{leaf dry weight}} \times 100$$

Statistical Analysis

Data obtained were subjected to statistical analysis by using more factorial in randomized complete block design with three replicates. Analysis of variance was carried out and means were separated by least significant difference test (LSD). The entire statistical work was done by using the computer package DSTAT ver.1.022 and statistics 8.1.

Aim of the study

This study was aimed to find out the effect of different begomovirus both monopartite (*Cotton Leaf Curl Burewalla Virus associated with beta satellite*, *Malvestrum Yellow Vein Changa Manga Virus*) and bipartite (*Tomato leaf curl new delhi virus*) on the physiology of *L. esculentum* and *N. benthamiana* plants as well as study host and virus interaction and biochemical attributes of plants.

RESULTS Effects of different whitefly transmitted Geminivirus (WTG) were studied on two plants of *L. esculentum* and *N. benthamiana*. After agroinfiltaration different morphometric parameters were recorded (30dpi) viz., root and shoot length, fresh weight, dry biomass, relative leaf water content, root-to-shoot dry weight ratio and root-to-shoot length ratio. Biochemical analysis (Chlorophyll *a*, chlorophyll *b*, Chlorophyll (*a*+*b*), Chlorophyll a/b and total soluble sugars) were performed to check the effect of virus on plants growth.

Maximum mean value of shoot fresh weight (SFW) of *L. esculentum* was 2.76 in healthy plant and lower value was 0.83 in (CLCuV+CLCuB)**.** In case of *N. benthamiana* maximum (RFW) was 1.87 in healthy plant and minimum was 0.6 in (CLCuV+CLCuB). Maximum mean value of root fresh weight (RFW) of *L. esculentum* was 0.543 in healthy plant and lower value was found 0.166 in *Cotton leaf curl burewalla virus* associated with betasetallite (CLCuV+CLCuB).In case of *N. benthamiana* maximum (RFW) was 0.373 in healthy plant and minimum was 0.120 in (CLCuV+CLCuB). The maximum mean (SDW) was 0.51 and 0.39 was observed in healthy plants of *L. esculentum* and *N. benthamiana*. Minimum mean was 0.08 and 0.07 in CLCuV+CLCuB in both plants. RDW was maximum 0.21 and 0.16 in healthy plants as compared to infected plants that was 0.07 and 0.04 in CLCuV+CLCuB.Shoot length (SL) and root length (RL) of both plants explained that maximum RL was 23 and 15 in healthy plants while minimum RL were observed 5.266 in CLCuV+CLCuB, while in case of *L. esculentum* and 5.966 in ToLCNDV in case of *N. benthamiana*. Similarly maximum SL was 27.66 cm and 19 cm among healthy plants, minimum 10.2 cm in CLCuV +CLCuB (*L. esculentum*) and 7.5 cm in ToLCNDV (*N. benthamiana*).Control has maximum value of RDW/SDW ratio which was 34.11 and 26.20 in both plants. MYVCMV has minimum 11.32 in case of *N. benthamiana* ($p > 0.05$) and *Agrobacterium* has minimum value 19.32 for *L. esculentum*. In case of *L. esculentum* water treated plant has maximum RL/SL ratio 1.02 and minimum value has CLCuV+CLCuB which is 0.51. In case of *N. benthamiana* CLCuV+CLCuB has maximum 0.83 while *Agrobacterium* has minimum 0.71 RL/SL ratio.

Data for leaf relative water content of tomato and tobacco after virus infection showed drastically reduced the leaf area in all the treatments. Mean data represents the fact that in case of *L. esculentum* after infection healthy plant was maximum 80% (RWC) while ToLCNDV was showed 36% minimum (RWC) ($p > 0.05$).while in case of *N. benthamiana* healthy control plant has maximum 75% and ToLCNDV has minimum 30% (RWC). The reduction of (RWC) might be due to reduction of leaf area of infected plants.

The changes in the level of total chlorophyll (Chl *a*+*b*) in control and virus infected leaves were measured**.** Chlorophyll a content was more in all healthy leaves sample as compared to virus affected leaves.The maximum mean value was 28.66 (mg/g) in control; while the minimum mean value was 9.60 (mg/g) in (CLCuV+ CLCuB). In case of *N. benthamaiana* Chlorophyll *a* content was higher 23 (mg/g) in healthy plant and minimum 8.93 (mg/g) in (CLCuV+CLCuB).The chlorophyll b content was more in healthy leaves as compared to infected leaves.The maximum mean value of chlorophyll *b* was 23.6 (mg/g) in control and the minimum mean value was 7.2 (mg/g) in (CLCuV+CLCuB). On the other hand in *N. benthamaiana* plants Chlorophyll *b* was maximum 19.5 mg/g in healthy sample as compared to inoculated while the minimum in5.86 (mg/g) in (CLCuV+CLCuB).The maximum mean value in *L. esculentum* plant was 52 (mg/g) in healthy and minimum mean value was 16.8 (mg/g) in (CLCuV+CLCuB) revealed that total chlorophyll content was minimum 14.8 (mg/g) in (CLCuV+CLCuB) and high content was present 42 (mg/g) in healthy tobacco plants. The Chlorophyll *a/b* ratio was more in (ToLCNDV) as compared to infected one. Similarly, also Ch *a/b* ratio slightly increased in virus infected leaves, but there was no significant difference between the control and infected one .The maximum mean value in tomato plant was 1.63 in (ToLCNDV) and minimum mean value was 1.1 in water while in case of *N. benthamiana*. This ratio was minimum 0.96 in water and high level was observed 1.66 in *(*ToLCNDV).

Virus infection affected the TSS effectively, healthy plants accumulates more sugars as compared to infected one. among *L. esculentum* maximum mean value was recorded 8.13 (mg/g) in water and minimum 3.33 (mg/g) in (CLCuV+CLCuB) was present in virus infected plants, meant to say that virus infection significantly reduced (TSS) in both plants (Table 10).Maximum mean value of TSS was 9.43 (mg/g) in Control while minimum 2.5 (mg/g) in (ToLCNDV) in *N. benthamiana*.

DISCUSSION

Begomovirus infections are one of the main biotic stress among the other biotic stresses which the plants encounter with and a fore most limiting factor for plant growth and yield. Although substantial research efforts have been focused concerning plant viruses. Determination of genetics, structure, transport and localization of virus in plant tissues with much less effort aimed at understanding the effect of viral infection on host plant physiology (Zaitlin and Hull, 1987; Balachandran *et al*., 1994; Mohamed, 2010). In addition, viral infections are extremely difficult to control experimentally and their physiological consequences can be highly variable so, leading to a continued lack of understanding of these effects.

Regulation of defence responses has been greatly studied for decades, due to lack of work in this area of research, hardly any information is known about the effects of pathogen infection on primary metabolism (Berger *et al*., 2011). It is evident from the results obtained that virus inoculum caused reduction in plant growth parameters in both *L. esculentum* and *N. benthamiana* plants. Data on growth attributes of these plants clearly reveal the differential response of plants to different viral inoculum and plants produced greater biomass under non stress conditions. Through *Agrobacterium* containing cloned viral DNAs in tomato (*Lycopersicon esculentum*) and tobacco (*N. benthamiana*) plants much reduction was recorded for the infectious clones (ToLCNDV) and (CLCuV+CLCuB) as compared to their non- infected counterparts and control. Whereas, the decrease in growth of plants under simulated viral infectious clones may be due to reduction in photo synthetically active leaf area. Reduction in growth rate of leaf under stress surroundings is an early phenomenon. Growth is attained through cell enlargement, division and differentiation, which are most sensitive physiological processes in stress condition caused by the drop in turgor pressure. Severe viral symptoms have been reported to inhibit plant growth by obstruction in flow of water to the adjacent elongating cells from the xylem (Taiz and Zeiger, 2010). As plant height is a manifestation of inherited biological and environmental aspects. Therefore, under viral attacks roots tend to produce signal like ABA, which is transmitted to the leaves by the xylem thus influencing growth rate of the plant (Sivalingam, 2012).

Through *Agrobacterium* containing cloned viral DNAs in *L. esculentum* and *N. benthamiana* plants, much reduction was recorded for the infectious clones (ToLCNDV) and (CLCuV+CLCuB*)* as compared to control of non-stomatal limitations to photosynthetic rate, some of substantial variables for example chlorophyll *a* and *b*, and concentration of Rubisco, supply of ATP and NADPH to PCR cycle as well as use of products of assimilation are crucial (Sumaira *et al*., 2011).

In the present study from the aformentioned variables only photosynthetic pigments were determined. Furthermore, after treatments with viral inoculum, decrease in photosynthetic pigments was observed in both plants. However, variation in photosynthetic pigments was evident. The maximum concentration of chlorophyll '*a*', chlorophyll '*b*' and total chlorophyll contents was recorded in control plants than the other plants inoculated with infectious clones. Nevertheless, more reduction was recorded in plants inoculated with (ToLCNDV) and (CLCuV+CLCuB). It is probably due to differences in activity of enzymes because pigments biosynthesis has been noted to rely on such certain enzymes or enhanced activity of the cholorophyllase which is an enzyme responsible for chlorophyll deprivation or could be due to the inhibition of chlorophyll synthesis (Lucas, 1998; Marcos *et al*., 2005). This could be due to the relatively faster degradation of chlorophyll than total soluble sugars (Bertamini *et al*., 2010). Chl *a*/*b* ratio was also found to be increased in (ToLCNDV). High Chl *a*/*b* ratio indicates more degradation or lower synthesis of chlorophyll b than a due to virus infection (Muqit *et al*., 2007).Outcomes of the current experiment also revealed that a higher photosynthetic pigment in L. esculentum plants is one of prime factor contributing in photosynthetic capacity and thus growth. However, relationship between amount of photosynthetic pigment and degree of water stress tolerance cannot be easily drawn as (TLCNDV), (CLCuV+CLCuB) and (MYVCMV) were similar in photosynthetic pigments as compared to control. It is generally believed that viral inoculum reduces growth of plants by reducing water potential and cell turgor thereby causing decreased cell expansion and leaf area (Saveetha *et al*. 2010).

The present results suggested that viral inoculum caused a substantial decrease in relative water content of all both plants which is similar to some of previous studies, where it has been documented that water deficit conditions reduced RWC in chives (Yazaki, 2006) and tomato (Harrison, 1999). In the present study, RWC was higher in control, plants treated with *agrobacterium* and water as compared to plants treated with infectious clones. The decrease in soluble sugars under viral infection has also been reported in orchids (Shalitin and Wolf, 2000) and infected plants of *L. esculentum* and *N. benthamiana* after inoculation with (ToLCNDV) (CLCuV+CLCuB) also accumulated minimum amount of soluble sugars.

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Table 1: Severity index based on symptoms severity (Lapidot *et al*., 2006)

Severity Grades	Symptoms
0	no symptoms
1	symptoms were slightly visible only after a careful search
2	slight but more visible symptoms
3	Moderate symptoms over most of the plant
4	Severe symptoms over entire plant

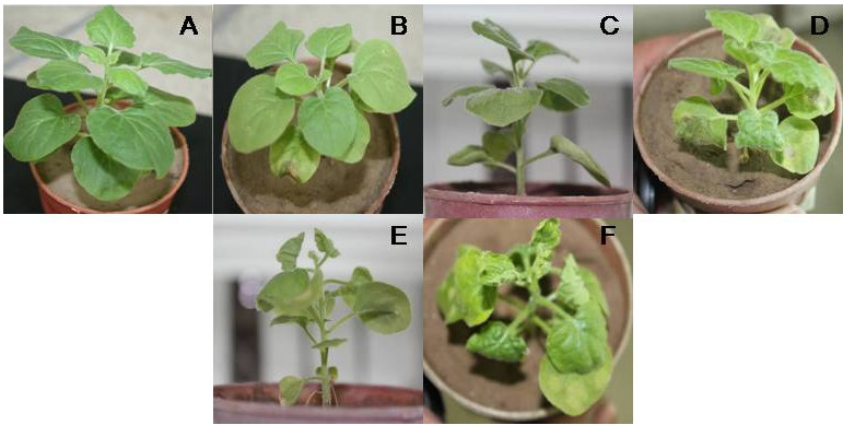


Figure 1: Agroinfiltration of different white fly transmitted virus infectious clones in *Nicotiana benthamiana* a) Healthy Plant b) Plant inoculated with water c) Plant Infected with Infectious Clone of *Agrobacterium d)* Plant Infected with Infectious Clone of *Malvestrum Yellow Vein Change Manga Virus* e) Plant Infected with Infectious Clone of *Cotton Leaf Curl Burewalla Virus* f) Plant Infected with Infectious Clone of *Tomato Leaf Curl New Dehli Virus*.

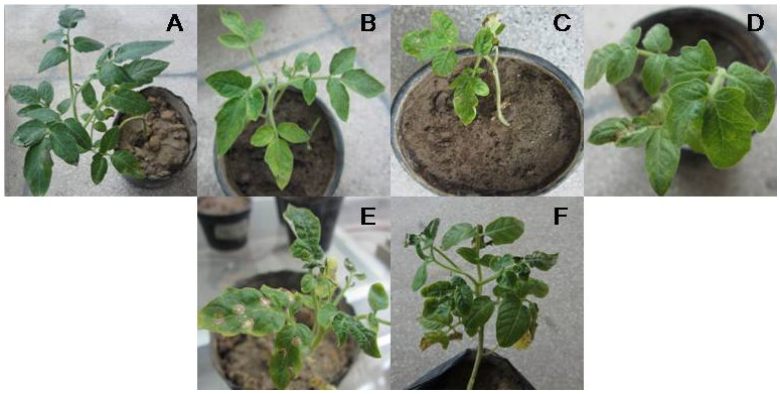
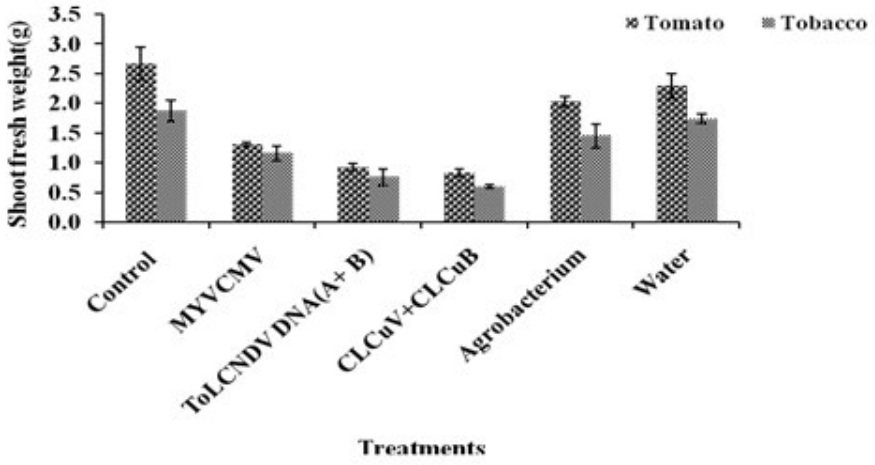
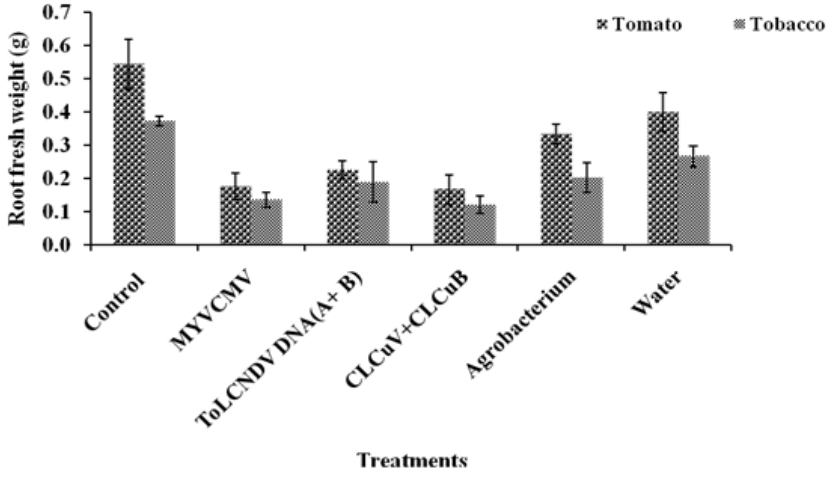


Figure 2: Agroinfiltration of different whitefly transmitted virus infectious clones in *Lycopersicon esculentum* a) Healthy Plant b) Plant inoculated with water c) Plant Infected with Infectious Clone of *Cotton Leaf Curl Burewalla Virus* d) Plant Infected with Infectious Clone of *Agrobacterium* e) Plant Infected with Infectious Clone of *Malvestrum Yellow Vein Change Manga Virus* f) Plant Infected with Infectious Clone of *Tomato Leaf Curl New Dehli Virus* .

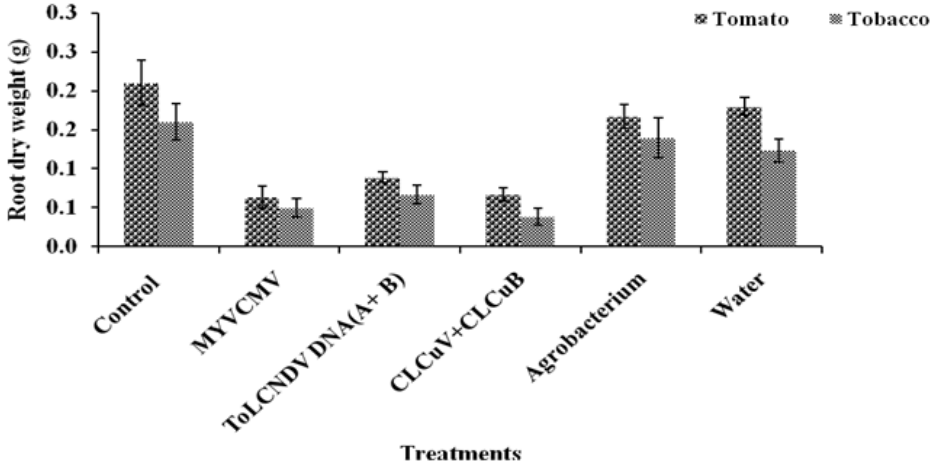
A


Tomato
Tobacco
Shoot fresh weight(g)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
MYVCMV
ToLCNDV DNA(A+ B)
CLCuV+CLCuB
Agrobacterium
Water
Treatments

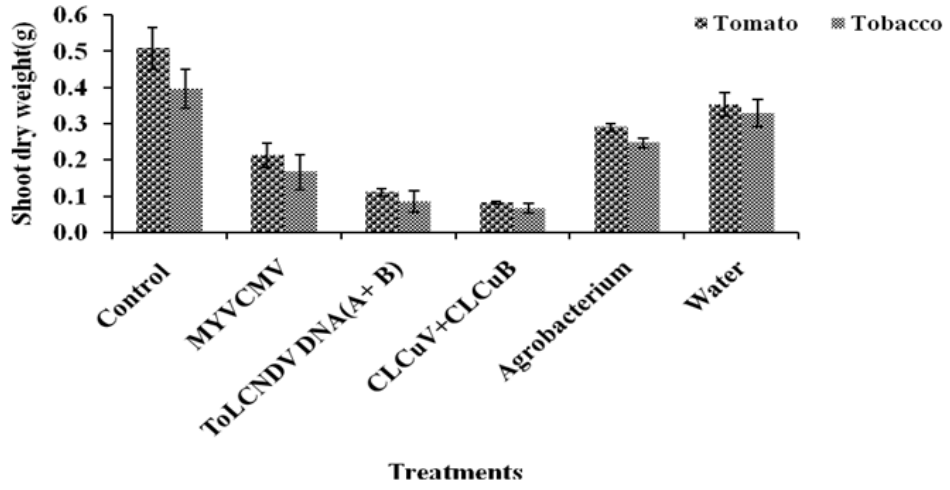
B


Tomato
Tobacco
Root fresh weight (g)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
MYVCMV
ToLCNDV DNA(A+ B)
CLCuV+CLCuB
Agrobacterium
Water
Treatments

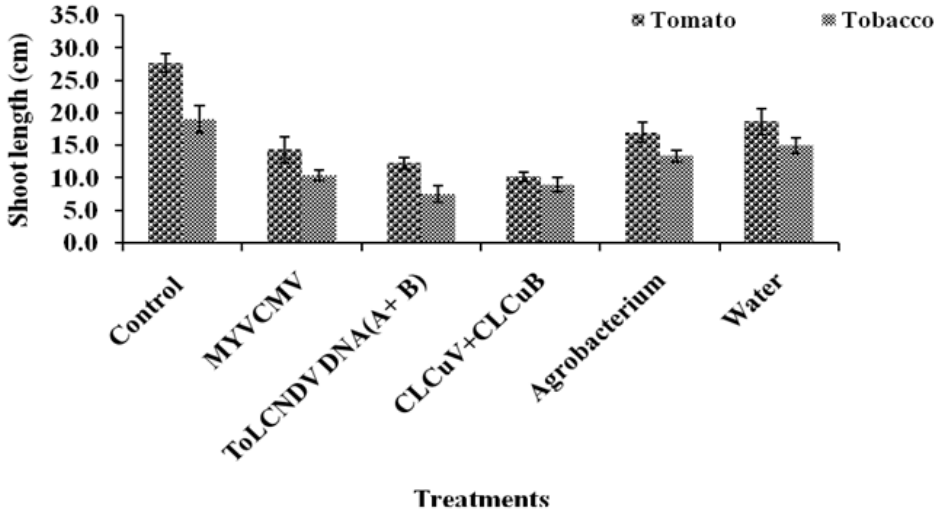
C


Tomato
Tobacco
Root dry weight (g)
0.3
0.3
0.2
0.2
0.1
0.1
0.0
Control
MYVCMV
ToLCNDV DNA(A+ B)
CLCuV+CLCuB
Agrobacterium
Water
Treatments

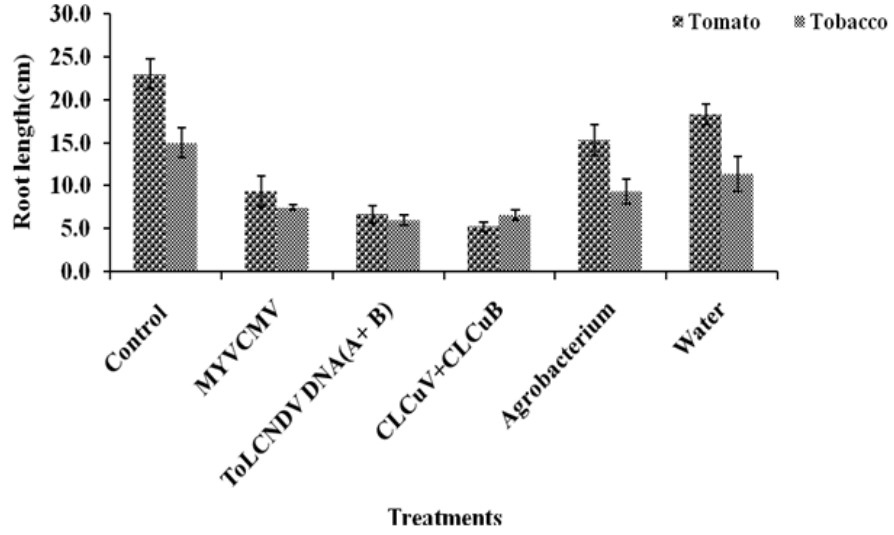
D


Tomato
Tobacco
Shoot dry weight(g)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
MYVCMV
ToLCNDV DNA(A+ B)
CLCuV+CLCuB
Agrobacterium
Water
Treatments

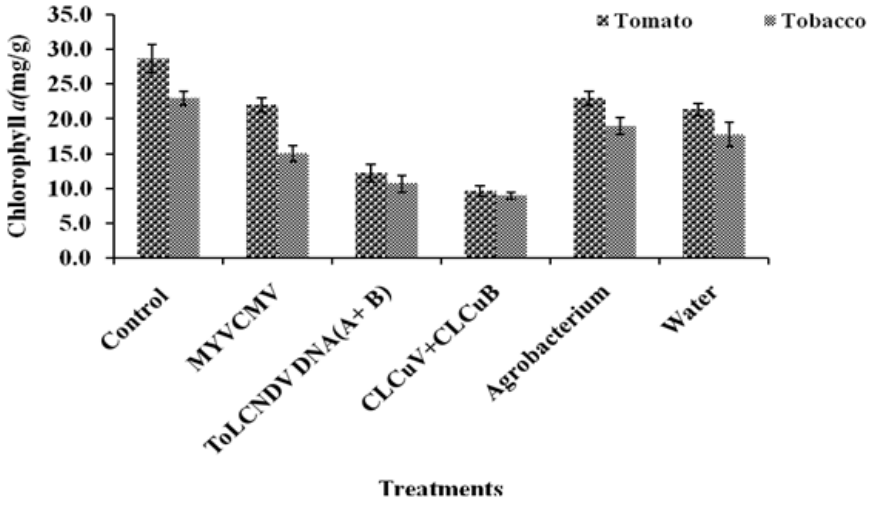
E


Tomato
Tobacco
Shoot length (cm)
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Control
MYVCMV
ToLCNDV DNA(A+ B)
CLCuV+CLCuB
Agrobacterium
Water
Treatments

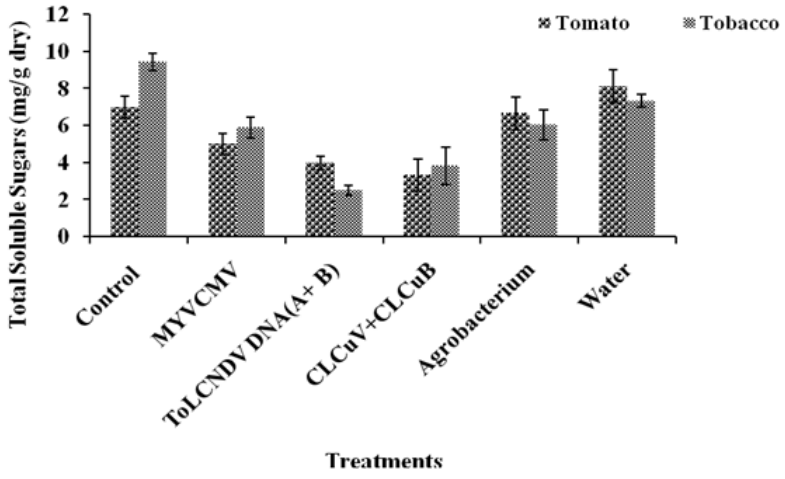
F


Tomato
Tobacco
Root length(cm)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Control
MYVCMV
ToLCNDV DNA(A+ B)
CLCuV+CLCuB
Agrobacterium
Water
Treatments

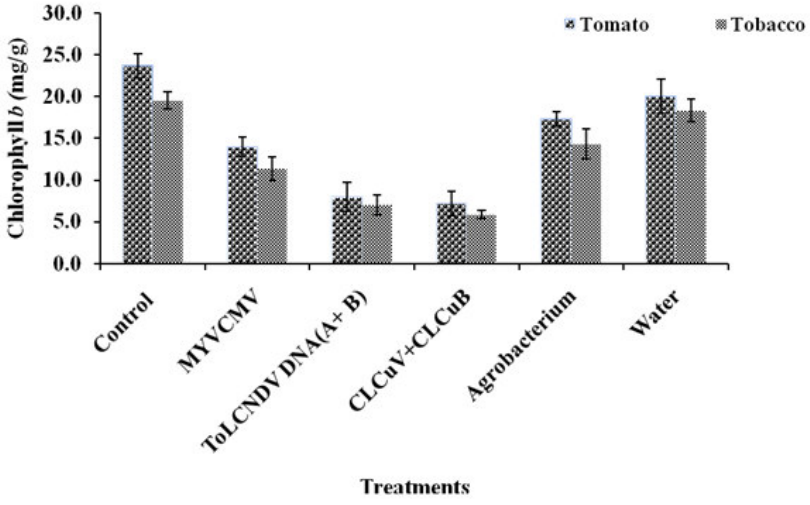
G


Tomato
Tobacco
Chlorophyll a (mg/g)
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Control
MYVCMV
ToLCNDV DNA(A+ B)
CLCuV+CLCuB
Agrobacterium
Water
Treatments

H


Tomato
Tobacco
Total Soluble Sugars (mg/g dry)
12
10
8
6
4
2
0
Control
MYVCMV
ToLCNDV DNA(A+ B)
CLCuV+CLCuB
Agrobacterium
Water
Treatments

I


Tomato
Tobacco
Chlorophyll b (mg/g)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Control
MYVCMV
ToLCNDV DNA(A+ B)
CLCuV+CLCuB
Agrobacterium
Water
Treatments

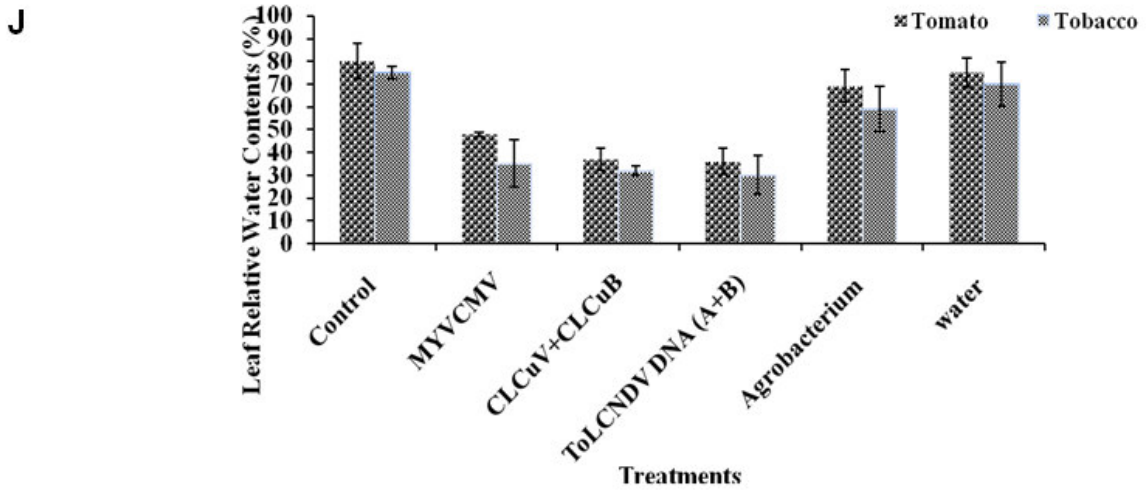


Figure 3: a) Shoot fresh weight b) root fresh weight c) shoot dry weight d) root dry weight e) shoot length f) root length g) chlorophyll a h) chlorophyll b i) Total soluble sugars j) leaf relative water contents ; of Tomato (Lycopersicon esculentum L.) and Tobacco (Nicotiana benthamiana L.) plants at different viral infections.

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