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ROLE OF PLANT EXTRACTS AND SALICYLIC ACID FOR THE MANAGEMENT OF CHILI VEINAL MOTTLE VIRUS DISEASE

^aAmjad Ali, ^bMuhammad A. Zeshan*, ^bYasir Iftikhar, ^aMuhammad Abid, ^cSyeda F. Ehsan, ^dMuhammad U. Ghani, ^bAwais A. Khan

^a Department of Plant Pathology, Bahauddin Zakariya University, Multan, Pakistan.

^b Department of Plant Pathology, College of Agriculture, University of Sargodha, Pakistan.

^c Department of Plant Pathology, University of Agriculture Faisalabad, Pakistan.

^d Institute of Soil and Environmental Sciences, University of Agriculture Faisalabad, Pakistan.

ABSTRACT

Chilli (*Capsicum annuum*), cultivated as spicy crop and has played a vital role in the maintenance of human body. Chilli veinal mottle virus (ChiVMV) is one of the most destructive and fast spreadable disease by vector and mechanically worldwide. The present study confirms the mechanical inoculation of ChiVMV in host plant by leaf inoculation method. Reducing agent carbon tetrachloride (CCl₄) and chloroform (CHCl₃) with ratio 5% and 2.5% reduced the infectivity and longevity of crude virus sap by mixing with them. The exogenous application of chemical inducer (Salicylic acid SA) and botanicals (Eucalyptus and Mint) reduced the disease incidence and severity of ChiVMV in greenhouse study and these were ecofriendly in nature. Salicylic Acid decreased severity (28%) followed by Eucalyptus (11.05%) and Mint (9.66%). The final chlorophyll contents study of healthy and diseased leaf was done with spectrophotometer by using different wavelength absorbance (645nm, 663nm, 652nm and 490nm), the healthy leaf of chilli plant shows more a, b and total chlorophyll as compared to diseased leaves.

Keywords: Mechanical inoculation, Botanicals, Chemical inducers, Salicylic Acid, Spectrophotometer.

INTRODUCTION

The vegetable “chilli (*Capsicum frutescens/Capsicum annuum* L.)’ is one of the most important agricultural crop of Pakistan which are grown in all four provinces of Pakistan with an area of 62 thousand hectares and production 139667 tons (Khan et al. 2019). The climate condition and temperature for the growth of chilli crop is varying, 16-24°C temperature is required for fruit setting with daytime temperature 24-30°C and night 15-18°C. The production and market values of Pakistani chilli is low as compared to other chilli producing countries due to the attacking of a large number of pest (insects), pathogens (fungi, bacteria, nematode, virus), poor seed quality, susceptible cultivars, mal cultural

practices, and some other abiotic factors are included (Hussain and Abid 2011; Hameed et al. 1995). Among all chilli diseases, Chilli veinal mottle virus (ChiVMV) is the most destructive and fast spreadable disease in Pakistan and worldwide. First time in history, the ChiVMV reported from Malaysia (Ong et al. 1979) and then it spread to Asia and Africa (Lee et al. 2013). The yield losses of more than 50 percent have been recorded in Pakistan due to this disease virus (Hameed et al. 1995). It is a ssRNA virus belongs to the family Potyviridae and the genus Potyvirus (Lee et al. 2017; Fei et al. 2019; Jiao et al. 2020; Adediji et al. 2020). This virus is transmitted from one plant to another through a vector aphid (*Aphis gossypii*) in a non-persistent manner, through grafting, mechanically and did not transfer through seed (Shah et al. 2008). The infected chilli plant could not survive happily and have shown the symptoms of vein mottling, stunting in an early stage of infection, dark-green streaks on branches and on the stem, flowers dropping and fruit deforming is the characteristic symptoms of

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* Corresponding Author:

Email: ahmd_1566@yahoo.com

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ChiVMV (Wang et al. 2006; Nono-Womdim et al. 2001; Ahmad and Ashfaq 2018; Gao et al. 2016). To overcome this viral disease in a short time, the production and development of resistant varieties of chilli is a durable strategy. On the other hand, the application of botanicals (Plant extracts) and inducers (Salicylic acid (SA) etc.) is the strong and well-known strategy in past and in present to control the different viral diseases (Petrov 2016; Abad et al. 1997; Andayanie et al. 2020; Murphy et al. 2020). Therefore, we concluded that the uses of botanicals and exogenous application of SAR chemical might be helpful for the eco-friendly management of ChiVMVD. The first objective of this present study is to check the efficacy of exogenous application of SA on chilli plants against ChiVMV and find out the effect of reducing agents on the stability of virus inoculum. In the second objective, evaluate the role of plant extracts against ChiVMV and studied the longevity in vitro (LIV) of the virus on chilli plants.

MATERIALS AND METHODS

Pots preparation and transfer of chilli seedling: Pots preparation was done by using the mixture of Soil, Sand and FYM with ratio 1:1:1. The healthy chilli seedling was transplanted early in the morning. Each pot contains one chilli seedling plant and the total 18 pots were prepared for the whole research works. The treatment of botanicals and the reducing agent was recommended after two weeks of nursery transplanting. To check the presence of virus in healthy transplanted seedling the Enzyme-linked immunosorbent assay was performed and virus free chilli nursery transferred to pots in controlled conditions.

Collection of symptomatic ChiVMV samples and sap preparation for mechanical inoculation: The symptomatic leave samples of ChiVMV were collected from two different regions (Sillanawali and In-service area of UCA) of district Sargodha (Fig.1) for the extraction sap. The sap of chilli leaves was extracted by using the methods of (Hill 1984) and (Noordam 1973). The extraction buffer was prepared by adding 1.42g disodium hydrogen phosphate (Na_2HPO_4) into 50ml of distilled water (solution A) and in next beaker add 1.36g potassium dihydrogen phosphate (KH_2PO_4) in 50ml of distilled water (solution B) and stir carefully. After stirring taken 42.5ml from solution A and 7.5ml solution B and mixed into each other in a beaker. This stock solution is equal to 0.1M phosphate buffer. Surface sterilized the samples with 5% bleach and then wash

with distilled water. Air-dry the specimen on blotter paper, after dryness measured the weight of leaf and crushed it into chilled pestle mortar by adding 0.1M phosphate buffer. After reliable crushing filtered the samples and taken them into the new autoclaved vial.

Confirmation of the presence of ChiVMV by mechanical inoculation: For mechanical inoculation of ChiVMV, washed the plant leaves to remove dust particles and then use of abrasive (carborundum powder) to make injury for the transformation of the virus (Fig.2). Cardboard was also used for the support of chilli leaf during rubbing, inoculate the extracted sap with a cotton swab and cover the leaf with a polythene bag, and waiting for the appearance of the first symptoms on inoculated plant.

Effect of reducing agents on virus infectivity/longevity: To check the infectivity of ChiVMV in extracted sap, crude virus sap was prepared from fresh infected chilli leaves by using the previously discussed method and reducing agents carbon tetrachloride (CCl_4) and chloroform (CHCl_3) was added with the concentration of 5% and 2.5%. The reducing agent CCl_4 used with the ratio of 5ml of 5% and CHCl_3 used with ratio 5ml of 2.5%. The application of these reducing agents was done by every seven (7) days of interval.

Efficacy of exogenous application of Salicylic Acid (SA): Exogenous application of SA act as an inducer in the host plant against virus diseases (Shang *et al.*, 2011; Murphy *et al.*, 1999). During this present study, SA was applied to the healthy chilli plant before 24 hours of inoculation. For the preparation of the SA solution, taken 2g of SA and dissolved it into 1liter of distilled water. Check the results of the SA application every 3-4 days and record the data.

Preparation and application of Plant extracts: Eucalyptus (*Eucalyptus globules*) and Mint (*Menthe L.*) were used as botanicals for the suppression of ChiVMVD during the study. The plant extract was prepared by using the method (Zaidan et al. 2005). Washed the leaves and air-dried on blotter paper for 1-2 hours and stored the leaves in the refrigerator for 24 hours. After 24 hours, grinded the leaves with distilled water in a grinder by using equal weight over volume (w/v). Prepared botanicals applied on pots plant 24 hours before the mechanical inoculation of virus extracted sap. Checked the disease incidence on a daily bases and recorded the data.

Determination of chlorophyll contents in healthy and diseased leaves of Chilli: Take fresh 500mg of diseased chilli leaves and grinded in chilled mortar-pestle with 50ml of acetone solution (Fig.3). After grinding the leaves, measure the volume of the sap with a measuring cylinder. The sap was filtered with a clean muslin cloth and taken in it into new vial. As the

same, the sap of fresh healthy leaves was prepared and taken into new separate vial. The spectrophotometer was used with different wavelengths (645nm, 663nm, 652nm, 490nm) and noticed the reading. Different compounds absorb light of varied wavelengths, so the sample was processed at four wavelengths.



Figure 1. Systematic leaves collected for sap extraction



Figure 2. Mechanically Inoculation of ChiVMV



Figure 3. Both healthy and diseased leaves grinded with 50ml of acetone solution

RESULTS

Mechanical inoculations: During 2016-2017, the experiment was performed at the College of Agriculture (CA), the University of Sargodha (UOS) to control the chilli viral disease Chilli veinal mottle

virus (ChiVMV) by using the botanicals and exogenous application of salicylic acid.

Mechanical inoculation results showed that the ChiVMV is a mechanically transmitted virus and produce a significant loss to chilli plant. The

inoculated plant shown the symptoms of veinal mottling, stunting, and yellowing of leaves (Fig.4). The first symptom was recorded after 14 days of inoculation followed by 21 and 28 days later. Total 18 plants were inoculated in three (03) rows, each row having 6 plant and control plant without any symptom development. The plants in the 1st row exhibited the

lowest disease incidence (50%) and disease severity (12.7%), followed by 2nd row 71% (disease incidence) and 14% (disease severity). The highest disease incidence and severity were noted in 3rd row plants, with values of 75% and 16%, respectively. This result showing that the ChiVMV is mechanically transmitted virus.

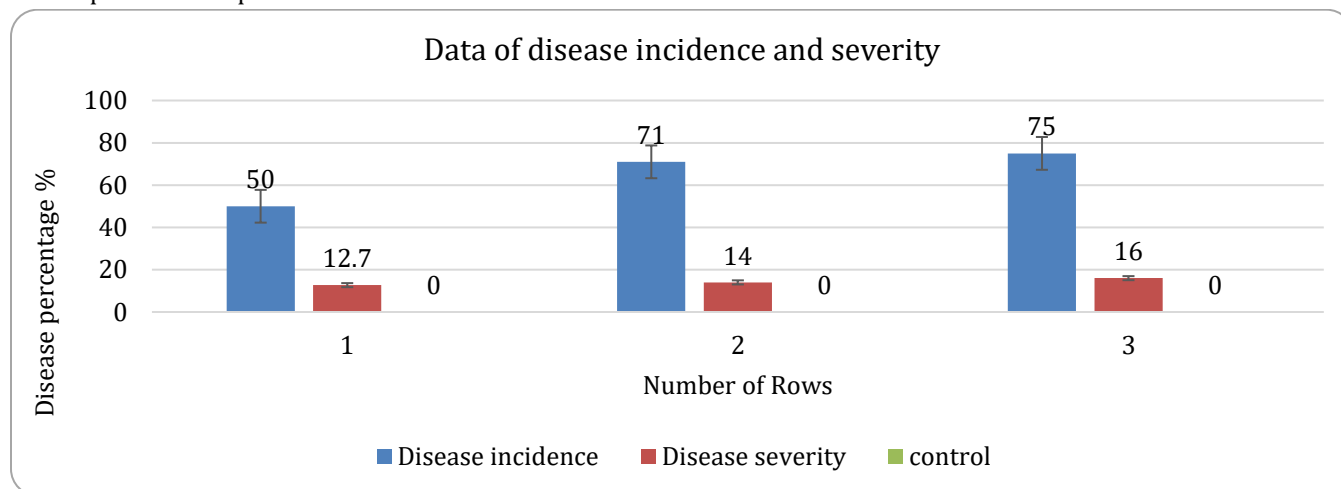


Figure 4. Results of mechanically inoculation of ChiVMV in greenhouse

Effect of reducing agents on virus infectivity: The infectivity of virus was reduced when the reducing agent carbon tetrachloride (CCl₄) and chloroform (CHCl₃) was mixed with crude sap with the ratio of 5% and 2.5%. The reducing agent applied three times in three separate rows with 14 days interval. After 14 days the reducing agent reduces the disease severity with average 14.2% as compared to control 28% in first row. In second row reduce disease

severity 10 % as compared to control 28% and, in third row the reduce disease severity 12% as compared to control 28%. In the other hand, the reducing agent applied separately on each single row and reduces disease severity. The symptoms were checked on daily bases and recorded the data. The reducing agent CCl₄ reduce severity 9.02% as compare to control plant 24.16% and CHCl₃ reduce severity 7.6% as compared to control plant 32.14%.

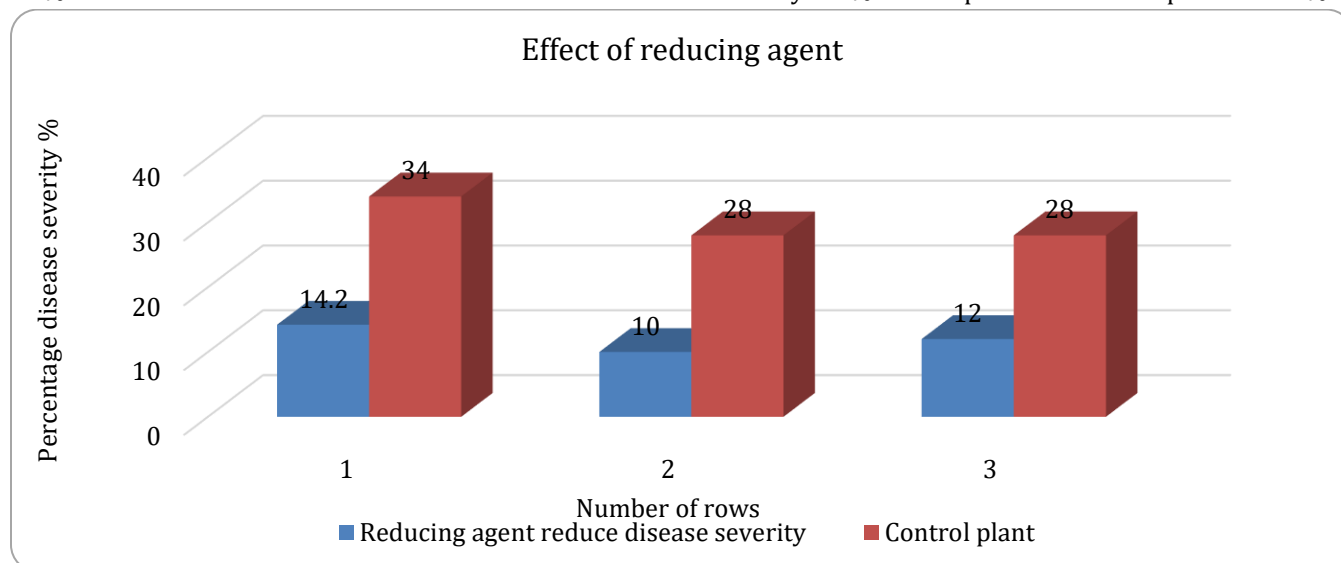


Figure 5. Mixed effect of reducing agent on virus infectivity Separated Efficacy of reducing agent 1:CCl₄ and 2:CHCl₃ on two chilli rows.

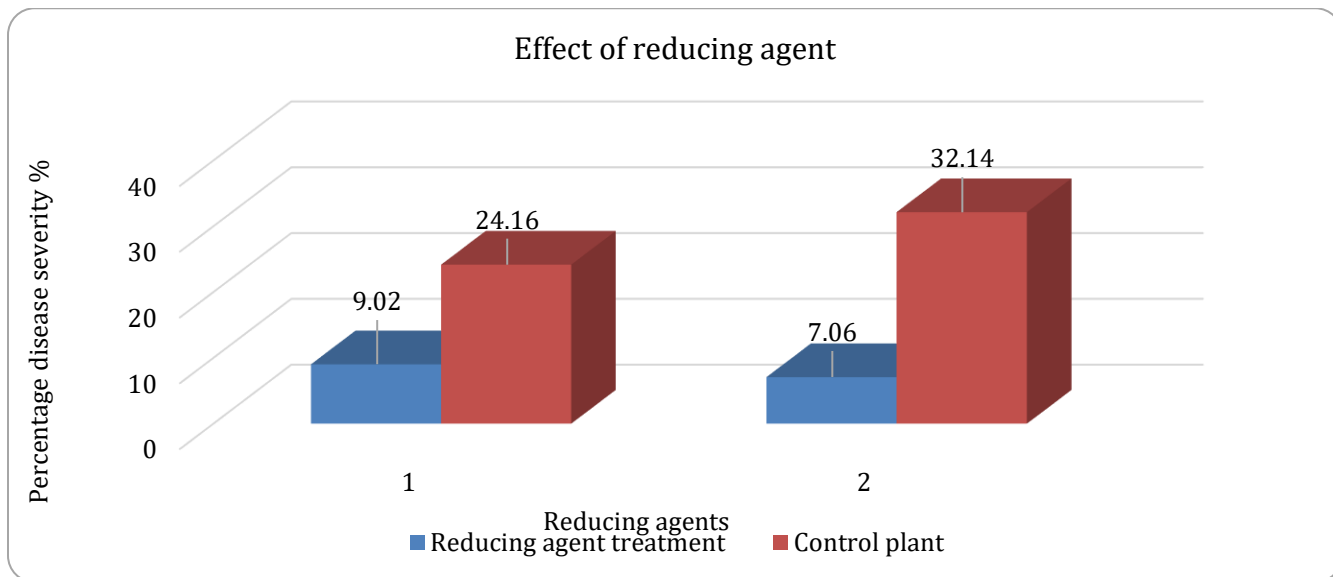


Figure 5. Mixed effect of reducing agent on virus infectivity Separated Efficacy of reducing agent 1:CCl₄ and 2:CHCl₃ on two chilli rows.

Effect of reducing agents on virus longevity: The longevity of infection in crude virus sap was reduced with the mixing of reducing agent CCl₄. The reducing agent mixing sap applied by mechanically inoculation method on healthy plants with the intervals of one month, two months, three months, four months, and five months. The virus longevity reduced with the passage of

time. The control plant treated without reducing agent mixing virus sap. The disease severity of control plant exceeds day by day and the infectivity and longevity of reducing agent mixing sap was reduced day by day. After the fifth application of reducing agent mixing sap, the infectivity of virus become zero, but the control plant shows 100 percent disease severity.

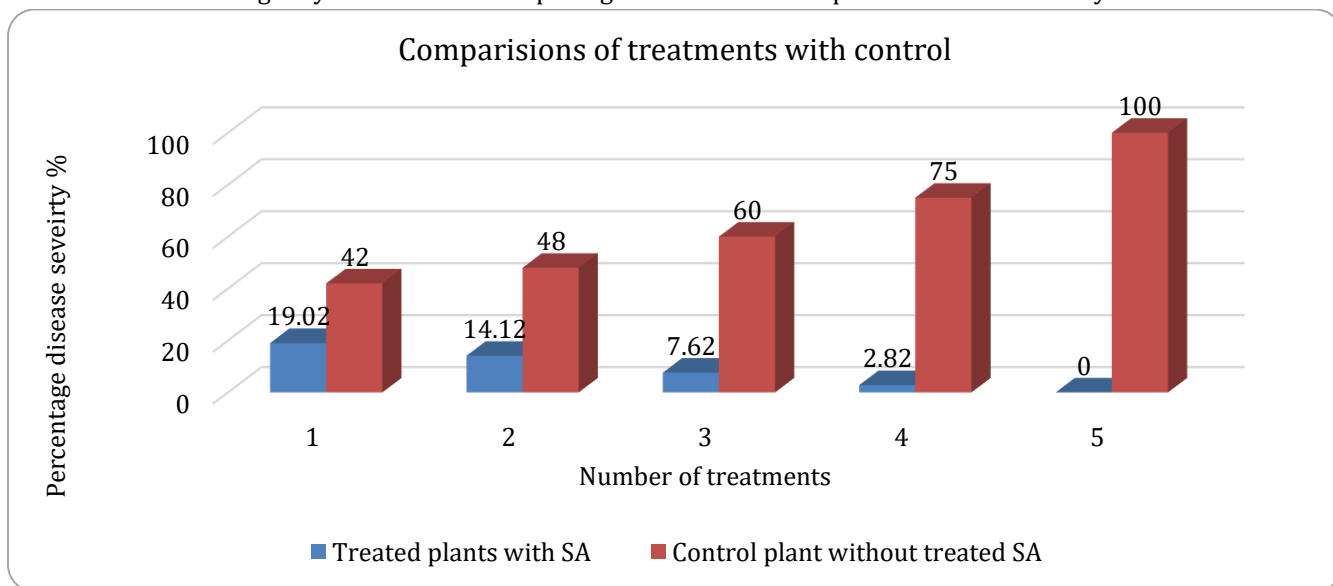


Figure 7. Efficacy of reducing agent against crude virus sap

Efficacy of exogenous application of Salicylic Acid (SA): The prepared solution of SA was applied 24hours before the inoculation of healthy plants with crude virus sap. SA reduce the disease incidence and severity of chilli plant against the ChiVMV in greenhouse. The average incidence and severity

of the 10 plants was recorded after one month of inoculations. The average of incidence percentage was recorded 42% and disease severity reduce 28% as compared to control plant 46%. The control plants not treated with SA and only treated with crude virus sap.

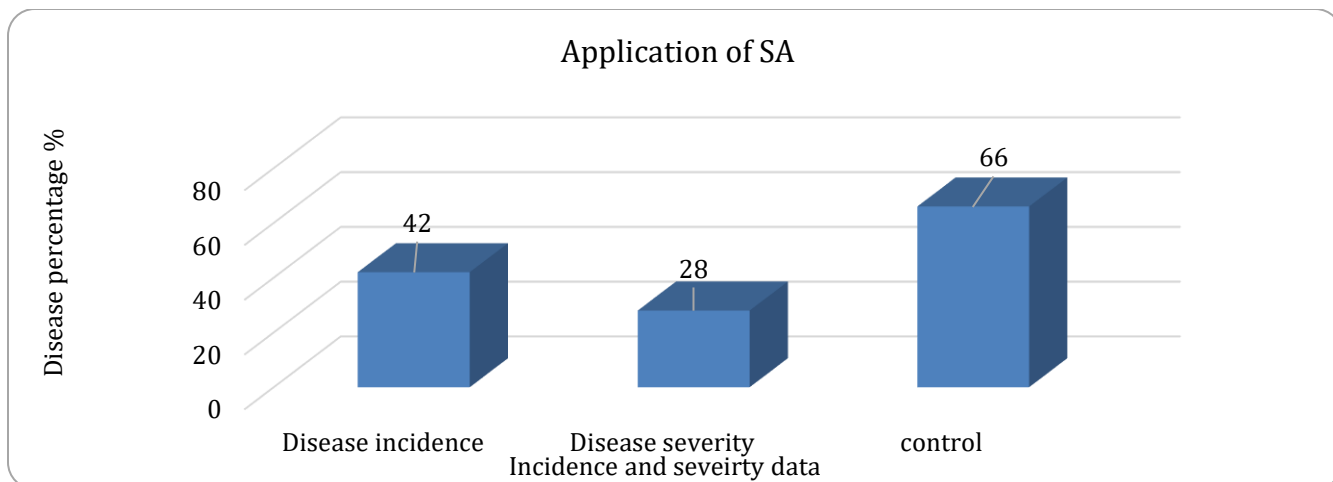


Figure 8. Application of SA and their efficacy in greenhouse

Application of Botanicals or plants extract: The botanicals Eucalyptus and Mint reduce the disease severity of treated plant before the inoculation of virus sap. Eucalyptus and Mint extracts were applied separately on two separate row to check the disease incidence and severity as compared to control plant that

treated with just crude virus sap. The Eucalyptus reduce 11.05% of the average disease severity of ten treated plant as compared to control 30.16% after one month of data recording. The Mint reduce 9.66% of the average disease severity of ten treated plant as compared to control 31.25% after one month of data recording.

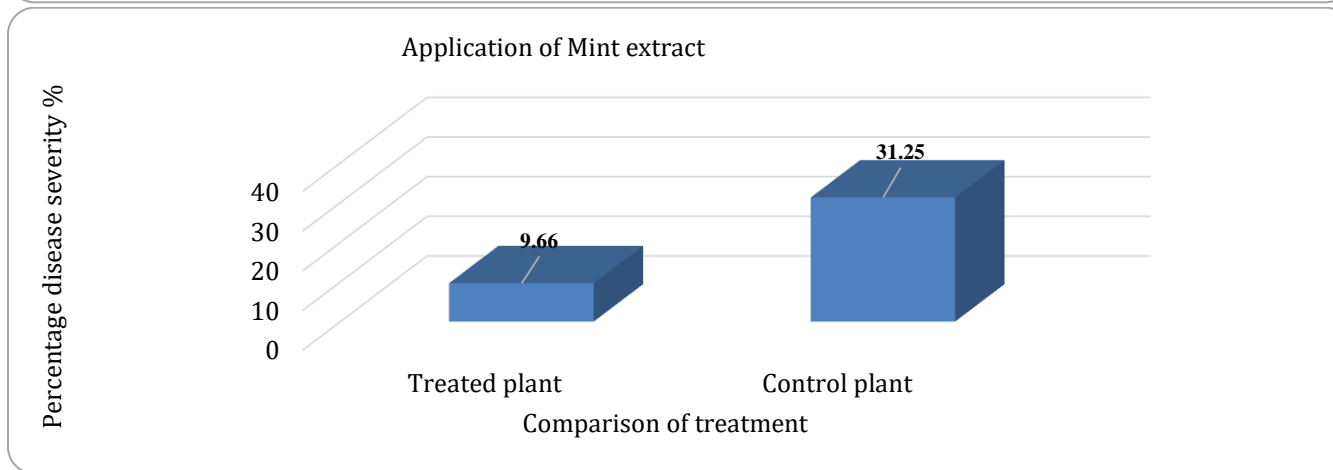
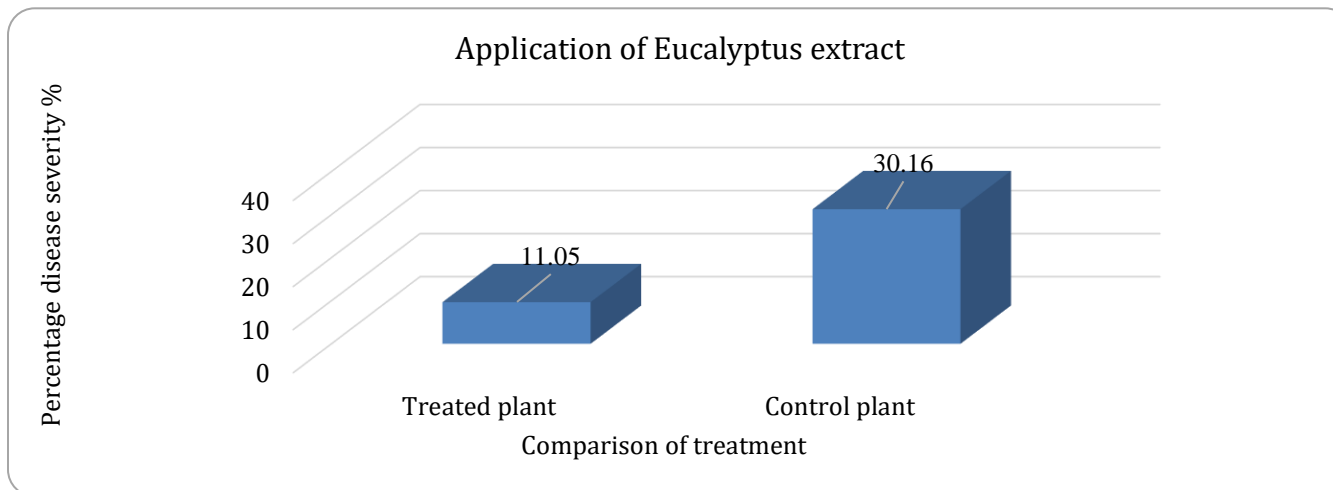


Figure 9 & 10. Efficacy of Eucalyptus extract against ChiVMV Efficacy of Mint extract against ChiVMV

Comparison of all three treatments: The comparison of all three treatments showed that the SA is the best one as compared to both botanicals. The data analysis shown that

the average disease severity of control plant is 42.47% and the SA reduce 28% disease severity of ChiVMV followed by Eucalyptus 11.05% and Mint 9.66%.

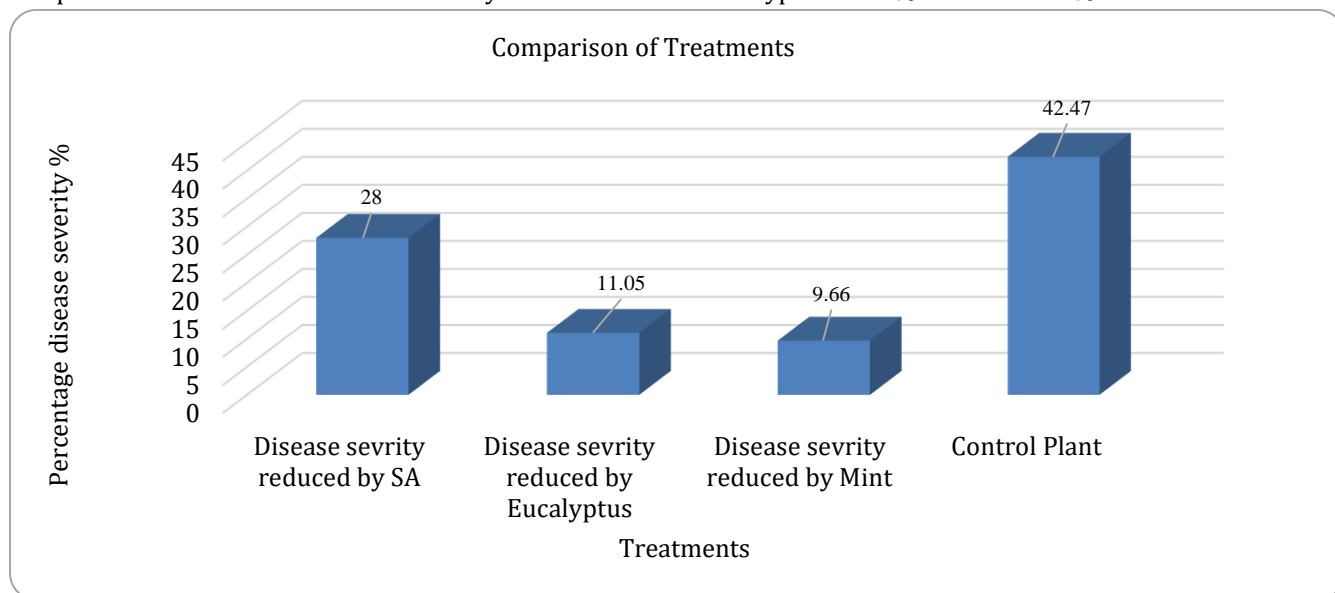


Figure 11. Disease severity of ChiVMV reduced by inducers SA and botanicals Eucalyptus and mint extracts
Determination of chlorophyll contents in healthy and diseased leaves of Chilli plant: To check the chlorophyll contents in leaves of healthy and diseased plant of chilli used spectrophotometer with different wavelength such as 645nm, 663nm, 652nm and 490nm. These wavelengths showed that the healthy leaves have more a, b and total chlorophyll as compared to diseased leaves.

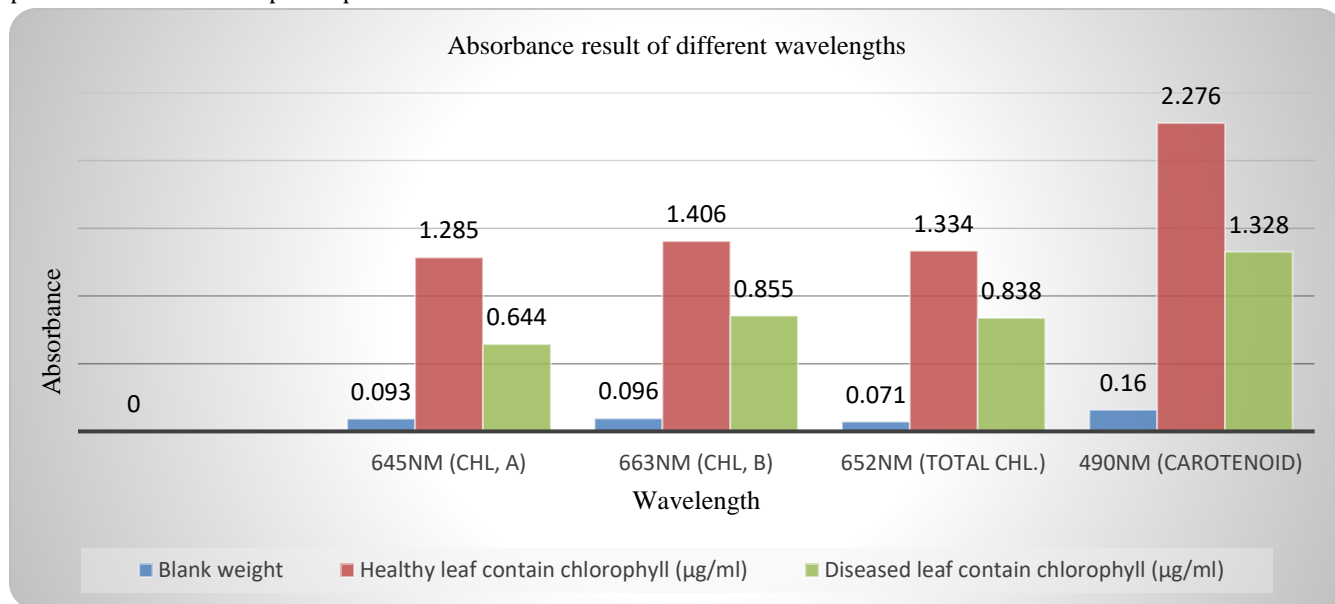


Figure 12. Used equation of Arnon (1949) to determined concentration of chl a, chl b and Total chlorophyll.

Equation for chlorophyll a

- Chl a = $12.7(\Delta_{663}) - 2.69(\Delta_{645})$ (For healthy and diseased leaves)

Equation for chlorophyll b

- Chl b = $22.9(\Delta_{645}) - 4.48(\Delta_{663})$ (For healthy and diseased leaves)

Equation for Total chlorophyll

- Total chlorophyll = $20.2(\Delta_{645}) - 8.02(\Delta_{663})$ (For total chlorophyll)

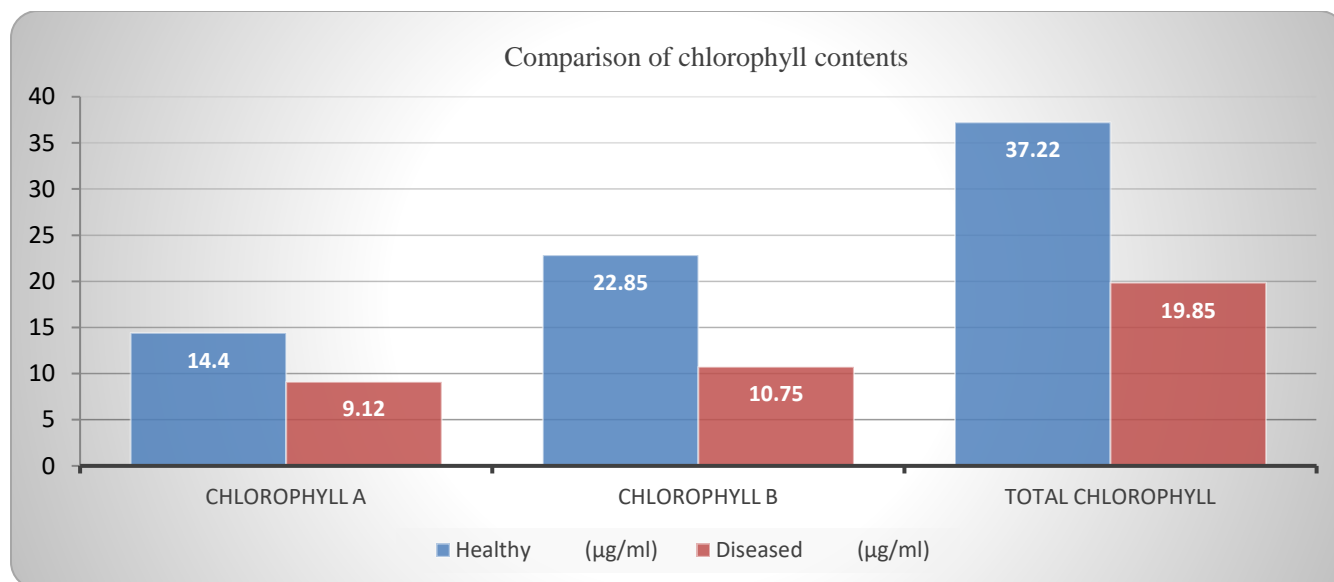


Figure 13. Healthy leaves of chilli plant shows more chlorophyll contents as compared to diseased leaves. After calculation with the equation of Arnon (1949) the results showed that the chlorophyll content in healthy leaf is high as compared to diseased leaf. After reading with spectrophotometer at 645nm wavelength shows the chlorophyll a is 14.4µg/ml in healthy leaf and diseased leaf showed 9.12µg/ml. At 663nm for chlorophyll b, the chlorophyll contents in healthy leaf was 22.85µg/ml and diseased leaf showed 10.75µg/ml chlorophyll contents. At 652nm for total chlorophyll, the healthy leaf showed 37.22µg/mL and diseased leaf show 19.85µg/mL chlorophyll contents.

DISCUSSION

Vegetables are very important for human health as these are sources of vitamins, dietary fiber, nutrients, minerals, and reduced brain stroke and heart diseases (Naseer et al. 2019; Javed et al. 2019). Chilli (*Capsicum annum*) belong to genus *Capsicum*, have third position amongst vegetable of *Solanaceae* family (Moscone et al. 2006). The production and market values of Pakistani chilli is low as compared to other chilli producing countries due to the attacking of a large number of pest, biotic pathogens, poor seed quality and some other abiotic factors (Hussain and Abid 2011; Hameed et al. 1995). Among all chilli diseases, ChiVMVD is the most destructive and fast spreadable disease in Pakistan and worldwide (Moury et al. 2005; Ahmad and Ashfaq 2017). Our result revealed that the ChiVMV is a mechanically transmitted virus and produce a significant loss to chilli plant by causing veinal mottling, stunting and yellowing of leaves. This virus spread in whole plant after the one month of inoculation. The maximum average of disease

incidence 75% and disease severity 16% was recorded after final data reading. The healthy control untreated plant showed zero percent disease in cage. (Shah et al. 2008) conducted an experiment to check the host range and transmission study of Potyvirus ChiVMV through mechanically, grafted and by vector method. They were concluded that the ChiVMV is a mechanically transmitted virus and produce characteristics veinal mottling symptoms on host pepper plant with 50% disease incidence. (HUSSAIN SHAH et al. 2009) were conducted a survey during 2003-04 to find out the prevalence of ChiVMV in four provinces of Pakistan and concluded that this virus has found with 44.7% disease incidence in Pakistani chilli cultivars. Maximum disease incidence during 2003, 51% has found in Punjab as compared to other provinces Sindh 48%, NWFP 41% and Baluchistan 38% respectively. Maximum disease incidence during 2004, 47% has found in Sindh as compared to other provinces Punjab 44%, NWFP 41% and Baluchistan 34% respectively. During survey they were also noticed that the vector of ChiVMV *Aphis gossypii* present in few districts of NWFP.

The combined effect of reducing agents on virus infectivity revealed that the reducing agent CCl_4 and CHCl_3 reduce the virus infectivity. With the passage of time by mixing the reducing agent in sap, reduce the virus infectivity. The reducing agent CCl_4 reduce severity 9.02% as compare to control plant 24.16% and CHCl_3 reduce severity 7.6% as compared to control plant 32.14%. Therefore, we finalized that the reducing agent reduced the disease severity by mixing with crude sap at

the time of mechanical inoculation. As compared to infectivity, the longevity of virus in vitro is also reduced by mixing the reducing agent CCl_4 . The disease severity of control plant exceeded day by day and longevity of reducing agent mixing sap was reduced day by day on treated plant. After the fifth application of reducing agent mixing sap, the infectivity and the longevity of virus became zero but the control plant shows 100 percent disease severity. Therefore, we concluded that the reducing agent not only reduces the virus infectivity but also at the same time reduces the longevity of extracted virus sap.

The current study revealed that the exogenous application of SA in field and in control condition reduces the disease incidence and severity of chilli plant against ChiVMVD. The application of SA before 24 hours of inoculation in control condition reduced 42% of disease incidence and 28% of disease severity as compared to control plant 46%. (Kumari and Vengadaramana 2017) revealed that the exogenous application of chemical inducers activates the defense immune system of plant against different pathogenic microbes. They were also explained that the exogenous application of SA activates and enhances the peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase and chitinase enzymatic activities against viral diseases of chilli plant. (Palmer et al. 2017) studied the effect of SA mediated plant defense and described that how the inducer SA improves plant defense in future against different viral diseases of plant. They were also described the phenomena of SA defense in plant and their useful aspects against the future famines (Zhao et al. 2017). Many researchers concluded that the exogenous application of SA induces resistance in host plant against different viral plant pathogens by the accumulation of PR-proteins and by activating the different antiviral gene expression (Li et al. 2019; Zhang et al. 2020; Wang et al. 2019; Patni and Ansari 2019; Koo et al. 2020; Jahan et al. 2019).

The current study also revealed that the exogenous application of botanicals (Eucalyptus and Mint) reduces the disease severity of treated plant before the inoculation of virus. Eucalyptus and Mint extracts were applied separately on two separate rows to check the disease incidence and severity as compared to control plant that treated with just crude virus sap. Both extracts reduce the severity of ChiVMVD but Eucalyptus extract performs somewhat good as compared to mint extract. The application of botanicals reduces the disease

severity of different plants against the plant viruses (Singh et al. 2020; Narusaka et al. 2020; Awad and El-Helaly 2017). In field condition, the exogenous application of four plant extracts (*Allium cepa*, *Zingiber officinale*, *Calotropis gigantea* and *Azadirachta indica*) were applied against cotton leaf curl viral diseases. The extract *Azadirachta indica* showed good results and reduced disease incidence of CiCuLV, attack of whitefly and egg laying capacity of whitefly as compared to other extracts (Abbas et al. 2020).

To check the chlorophyll contents in leaves of healthy and diseased plants of chilli, a spectrophotometer with different wavelengths such as 645nm, 663nm, 652nm and 490nm. The wavelength 645nm is used to check the chlorophyll A, 663nm for chlorophyll B, 652 for total chlorophyll and 490nm used for carotenoids. These wavelengths showed that the healthy leaves have more a, b and total chlorophyll as compared to diseased leaves. After calculation with the equation of Arnon (1949) the results showed that the chlorophyll content in healthy leaf is high as compared to diseased leaf.

CONCLUSION

The ChiVMV is a mechanically transmitted virus that is easily transmitted by sap inoculation method in suitable host plant (chilli). Reducing agents such as CCl_4 and CHCl_3 reduce the virus infectivity and longevity with the passage of time or multiple applications with few days' intervals. Treatment on inoculated plants with botanicals and application of chemicals showed disease control and these were ecofriendly in nature. Salicylic Acid decreased severity (28%) followed by Eucalyptus (11.05%) and Mint (9.66%). Molecular study is necessary for detection, identification, diagnosis and management of ChiVMV. Salicylic Acid and Eucalyptus extract was the most effective so there is a need to do biochemical analysis to know the exact mechanism of management.

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Contribution of Authors:

Amjad Ali	: Conduct research and write manuscript
Muhammad A. Zeshan	: Conduct research and write manuscript
Yasir Iftikhar	: Supervisor of research and review manuscript
Muhammad Abid	: Analyzed and interpreted data
Syeda F. Ehsan	: Edited the research paper
Muhammad U. Ghani	: Helped in research design and data interpretation, Supervisor of research and review manuscript
Awais A. Khan	: Analyzed the data