Pak. J. Phytopathol., Vol. 33 (02) 2021. 283-292 DOI: 10.33866/phytopathol.033.02.0684

Official publication of Pakistan Phytopathological Society

Pakistan Journal of Phytopathology

ISSN: 1019-763X (Print), 2305-0284 (Online)

http://www.pakps.com

INFECTIVITY ASSAYS FOR SOYBEAN AND COWPEA MOSAIC VIRUSES AND THEIR MANAGEMENT

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A B S T R A C T

Soybean mosaic virus (SMV) and cowpea mosaic virus (CPMV) are the well-known virus pathogens which belongs to the family Potyviridae and Comoviridae, respectively. During 2017, a survey was conducted at Ayub Agricultural Research Institute (AARI) Faisalabad, for the assessment of disease incidence of both viruses in soybean and cowpea fields and collection of symptomatic samples were collected for mechanical inoculation. The research area was prepared at the College of Agriculture, the University of Sargodha, Sargodha (32.0754° N, 72.41168° E. The seeds of susceptible varieties of both cultivars were collected from Ayub Agricultural Research Institute (AARI) and sown in the pots for mechanical inoculation. ELISA test was performed for the confirmation of virus transmission. The effect of reducing agents (chloroform and CCl4) on virus transmission was checked under in in vitro conditions. Aqueous solution of NPK, onion, Salicylic acid (SA), and cow milk was applied for the management of SMV and CPMV. The results showed that the SMV and CPMV are mechanically inoculated by crude sap and reducing agents reduced the efficacy of the virus with the passage of time. The yellow colour appearance of the ELISA plate well indicated the virus presence in inoculated plants. The mean value of disease incidence (38%) indicated that the carbon tetrachloride (CCl4) was known as good reducing agent than chloroform. The NPK controlled the maximum disease incidence 69.23% followed by SA 30.04 %, Onion 8.04% percent, and Milk 6.66%, positive control 100 along with negative control zero percent recorded. The aqueous solution of NPK is the most important eco-friendly approach for the management of plant viral diseases.

Keywords: *Soybean mosaic virus*, C*owpea mosaic virus,* biological assay, mechanical inoculation, NPK, Salicylic acid.

INTRODUCTION

Plant viruses have efficient forms of dissemination, and it is very challenging to combat diseases caused by these mesobiotic factors. They are responsible to induce significant yield losses by limiting the productivity of various economically attractive crops such as beans (Elsharkawy and Derbalah, 2019). It has been studied that mosaic viruses have the potential to affect over 150 types of plants including several vegetables, flowers and fruits. Diseases caused by these viruses are characterized by mottled leaves and mosaic patterns. The most commonly infected plants are soya bean, cucumber, squashes, cowpea, tomato, and cauliflower, but many more plants are susceptible (Tomlinson, 1987).

SMV (Soybean mosaic virus) is a member of the plant virus genus *Potyvirus* and belongs to the family *Potyviridae*. This Virus infects plants that belong to the family Fabaceae but also has been found infecting many other economically valuable crops.

Submitted: June 28, 2021
Revised: November 12, 2021
Accepted for Publication: November 19, 2021
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SMV is the etiological agent of soybean mosaic disease that occurs throughout the soybean-growing regions of the world (Hajimorad *et al.*, 2018). Soybean (*Glycine max* L. Merr.) is one of the most vital sources of proteins including edible oil and pathogenic infections are responsible to degrade yield losses of around 4 billion US dollars annually (Yoon *et al.*, 2018). Among these pathogens, SMV is the most prevalent viral pathogen that adversely affects soybean production across the globe. It reduces yield by approximately 8% to 35% but losses as high as 94% have been estimated (Liu *et al.*, 2016). The latent infection has been reported in several hosts (Chen *et al.*, 2004).

The plants infected with the Soybean mosaic virus express no apparent symptoms of severely deformed and mottled leaves. Mottling is characterized by the appearance of light and dark green patches on leaves. Symptoms are the most obvious on rapidly growing and young leaves. It has been observed that infected leaf blades can become puckered along the veins and curled downward. SMV can cause reduced seed size, reduced pod number per plant and stunting of the plant (Helm *et al.*, 2019; Rehman *et al*., 2021). CPMV (Cowpea mosaic virus) is a member of the genus *Comovirus* that includes 13 additional members in the family *Comoviridae*. According to previous investigations, the CPMV was firstly isolated from an infected cowpea (*Vigna unguiculata*) plant in Pakistan in 1993. Subsequently, this virus has been found to occur in other cowpea cultivating countries such as Pakistan, Nigeria, India, China, Kenya, Tanzania, Japan, Surinam, and Cuba.

CPMV belongs to *Potyvirus* having the family *Potyviridae*. Morphologically, it consists of long elongated particles of length 670-750nm and diameter 12-15nm (Hesketh *et al*., 2017). The single-stranded RNA molecules that are positive sense are found in their genome of nearly 10,000 nucleotides(Junco *et al*., 2021). The viruses of *potyviridae* family destroy the parenchymatous cells and mechanically transmittable by infected plant extract (Banerjee *et al*., 2014). Although the virus transmission also takes place by the vector, *Aphis gossypii*. Plant infected with CPMV exhibit the symptoms of bloating, mosaic, leaf deformation, thickening, and toughening of the pericarp (Freitas *et al*., 2016). Hence, reduced the heathy leaf area and photosynthetic activity reduces and results in deformed, smaller, and hardened fruit that lack commercial value (Yuki *et al*., 2006). There are many hosts of CoPMV and some hosts act as alternate host for their survival and source of inoculum (Hao *et al*., 2003; Shankar *et al*., 2009). CPMV has the ability to infect the *Fabaceace*, and *Passifloraceae* families, but also some infect some species of the *Chenopodiaceae, Amaranthaceae*, *Solanaceae*, as well as vegetables such as soya bean, crotalaria, common bean, peanuts, besides cowpea these are natural hosts (Barros, 2007; Narita, 2007). The present study was performed to determine the effectivity of SMV and CPMV or their virulence as the times passes and reducing agents influence on virus infectivity with mechanical method of inoculation. The usage of reducing agent, NPK solution, onion extract, and cow milk was employed for their management.

MATERIAL AND METHODS

Assessment of SMV and CPMV disease incidence: A survey of infected fields was accomplished in 2017 at *Ayub Agricultural Research Institute, (AARI) Faisalabad (Pakistan) for disease* assessments and sample collections for SMV and CPMV (Fig.1). Three fields were visited during the survey, 20 plats were tagged twenty plants that were used for data collection after 3 days interval for disease assessment. The symptomatic leaves of SMV and CoPMV that show mosaic patterns were collected.

$$\text{Disease Incidence (\%)} = \frac{\text{Number of infected plants}}{\text{Number of total plants}} \text{ X } 100$$

in polyethylene ice bags and bought to the virology laboratory at *College Of Agriculture*, University of Sargodha, Sargodha for inoculation purpose, to perform study sap was prepared. *Incidence* percentage of SMV and CPMV was evaluated by using the formula of Seem, (1984).

Pot experiment for biological assay: The seeds of susceptible variety of soybean and cowpea were collected from AARI and sown in pots that are filled with a mixture of soil, sand, and FYM in the field of research area 32.0754° N and 72.41168° E (Figure 2).

Sap extraction of infected soybean and cowpea leaves: The sap was extracted by using method of (Addy *et al*., 2017). In the chilled mortar, small pieces of SMV and CPMV were mixed with phosphate buffer (0.1M, pH 8.0) by applying liquid nitrogen (Fig.3). Moreover, 2% of

Polyvinyl pyrrolidone (PVP) was added in the buffer and then solution was filtered with muslin cloth. For sap inoculation the leaves surface was injured by dusting carborundum powder and the extracted sap was applied on leaves of 5 week old healthy seedlings. After that, rinsed the leave surface by water in order to remove extra sap inoculum. The leaves were covered with proper polythene sheet in order to avoid attack of insect. The disease symptoms were detected 4-5 weeks later after the inoculation.

Figure.1. Field survey for disease assessment and infected sample collection of SMV and CPMV

Soybean Cowpea

Figure 2. Healthy seedling of soybean and cowpea for biological assay.

A B

Figure 3. A-B Extraction of crude sap for inoculation

ELISA Test for the confirmation of Virus: First of all, the ELISA plate was coated with coating buffer and coating antibody. In the second step, antigen was extracted in a general extraction buffer by taking 0.5g sample and 1ml extraction buffer in a pestle mortar. Poured the mixture in eppendorf and centrifuged it and take the supernatant and pour it into the ELISA plate. Pour 100 ml into each well of the plate. Incubation and washing were performed after every step. In the third step, enzyme conjugate antibody and enzyme conjugate buffer were used. In the last step, two PNPP tablet was used to mix in 10ml PNPP buffer. In order to stop the reaction, 1M NaOH was used. Yellow colour is produced in case of diseased and no colour develop in well that contains the healthy samples.

Effect of reducing agents on the virus transmission: The effect of reducing agents was checked by taking 2.5ml chloroform and carbon tetra chloride in 100ml distilled water in separate beaker and mixed with prepared inoculum sap. The standard protocol of mechanical inoculation was opted afterwards.

Managements of SMV and CPMV: The salicylic acid application (T2), Cow Milk with a 1:10 ratio, and 0.1 % of NPK solution was used to suppress the SMV and CPMV under *in vivo* condition. The 1g of salicylic acid with NPK were mixed in the 1L of water in a beaker. The application was done on mature plants along with immature plants to determine the outcome of these treatments on the disease severity SMV and CPMV.

STATISTICAL ANALYSIS

The crop was sprayed by above mentioned nutrients after 7 days interval. All the data of SMV and CPMV as influenced by nutrients was statistically analyzed, all possible interactions was determined through ANOVA and the treatment was compared by LSD test at 5% level of probability.

RESULTS

Assessment of SMV and CPMV disease incidence: The disease incidence of the selected fields at *AARI, were recorded on a regular basis after 3 days interval. T*he attack of SMV has been found in all selected fields with disease incidence 60%, 100%, and 70%. The maximum CPMV disease incidence was recorded in field 3 with 100% disease incidence followed by field 1 (60%) and field 2 (80%). The incidence of SMV and CPMV has been found in a severe form in Pakistan.

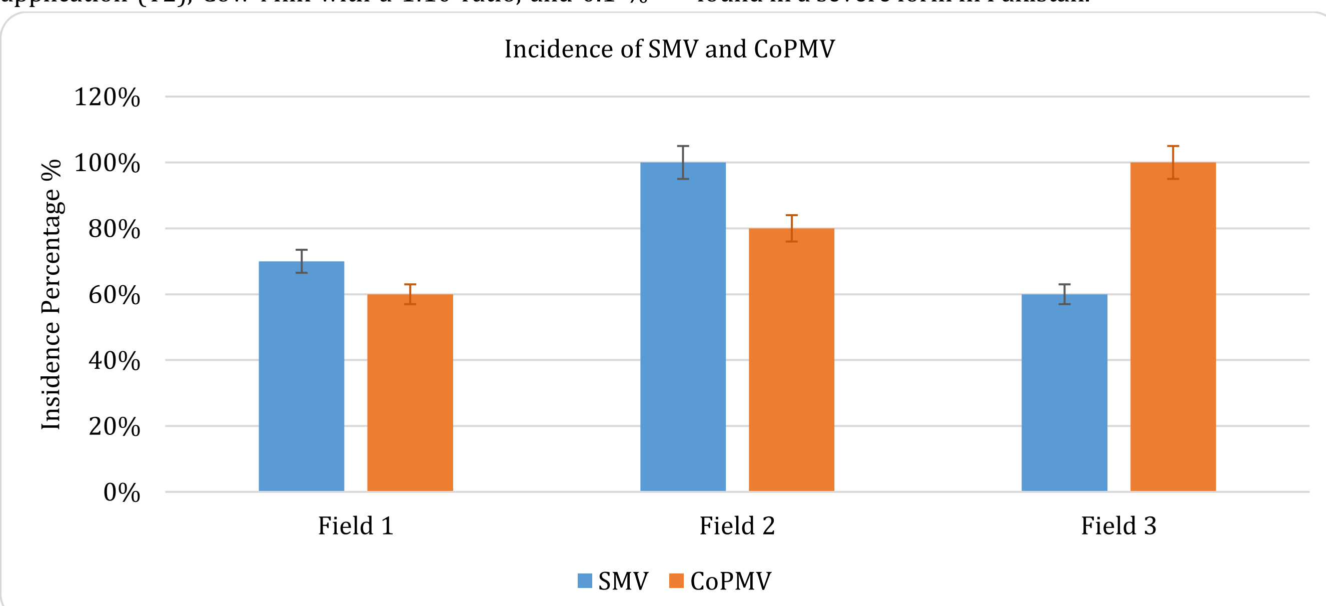

Figure 1. Graphical representation of disease Incidence of SMV and CoPMV.

Infectivity assay: The biological assay for the confirmation of the virus from disease plant to healthy seedling was done by using the method of Addy *et al*. (2017) at the control condition. The extracted crude sap was applied early in the morning on the healthy one and half-month-old Soybean and cowpea seedling by mechanically inoculation method and washed the inoculated leaves with distilled water to remove excess inoculum and coved the leaves with a plastic bag to avoid the insect. After one month of inoculation, the inoculated plant produced the light yellow pattern of mosaic on leaves of both host pants given in figure 4. A severe mosaic pattern appeared on healthy leaves of Cowpea seedling after the inoculation.

Cowpea Soybean

Figure.4. Development of mosaic pattern on both host plant after the mechanical inoculation

Enzyme Linked Immuno Sorbent Assay (ELISA): For the mechanical transmission of the virus through infectivity assay, the prepared inoculum of SMV and CPMV were mechanically inoculated on the soybean and cowpea seedlings, respectively. The mosaic pattern was observed on the leaves of soybean and cowpea after month of inoculation and an ELISA test was performed for the confirmation of SMV and CPMV. The ELISA resulted that the mosaic symptoms produced on soybean and cowpea. The control plant does not show any colour and no symptom were observed on plant whereas, the well of infected plants have shown yellow colour on the ELISA plate and the mosaic pattern was observed on leaves of the plant.

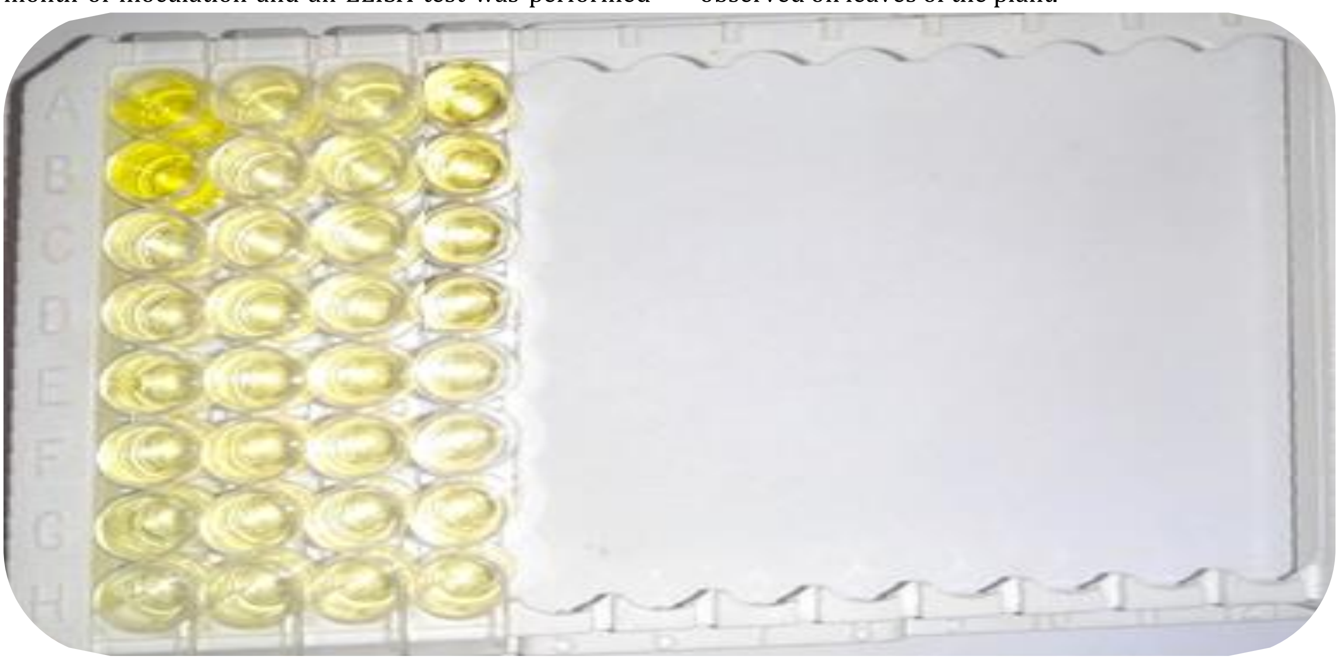

Figure 5. The yellow colour of the ELISA plate indicates the positive result of virus transmission.

Reducing agents: The Carbon tetrachloride (CCl_4) and chloroform are reducing agents and homogenized in the prepared sap with a ratio of2.5:5 percent and determine the infectivity of SMV and CPMV by applying this mixture on the Cowpea and Soybean healthy plant in control condition. Both of these reducing agents are responsible for decreasing the rate of infectivity of SMV and CPMV after the fourth and fifth application in contrast to control plant on which pure SMV and CPMV crude sap was applied. The 80% incidence of chloroform, 60% of carbon tetrachloride (CCl4), 100% of positive control, and 0% of negative control (ddH_2O) was recorded respectively. By applying 4th and 5th times of reducing agents, collaborating crude prepared sap produce regular disease incidence. The mean value of disease incidence indicated that thecarbon tetrachloride (CCl_4) was known as a good reducing agent than chloroform.

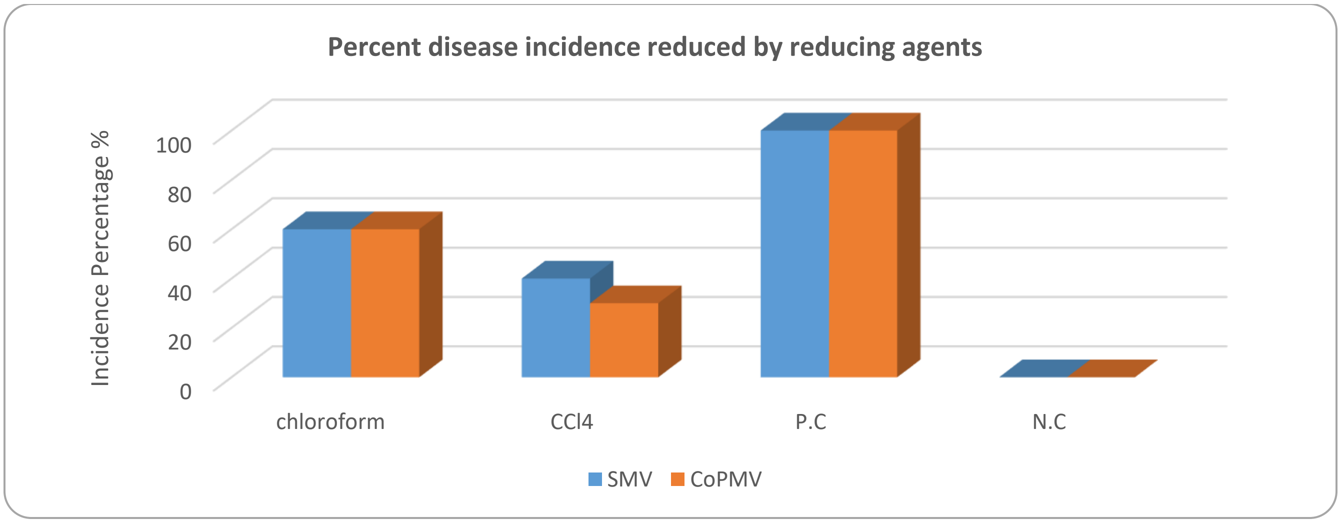

Figure 6. A graphical demonstration cleared the influence of reducing agents in the virus infectivity, that are homogenized in the crude prepared sap of SMV and CoPMV. **P.C**: Positive control (pure crude sap of both viruses) and, N.C: Negative control (ddH_2O).

Management of SMV and CPMV viruses: In vivo, the usage of NPK solution, cow milk and, salicylic acid have given the significant results toward the SMV and CPMV. Every treatment was applied with 4-5 different concentrations on healthy soybean and cowpea nominated plants int his study.

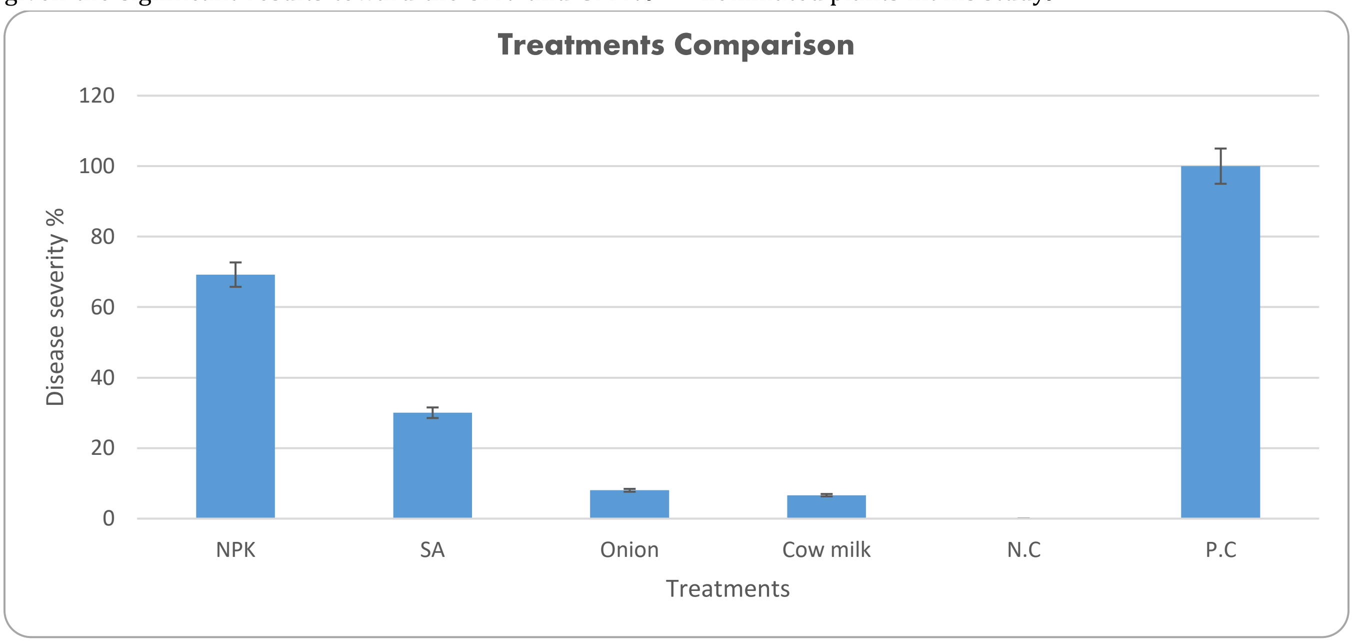

Figure.3. Graphical comparisons of different applied treatments.

In the last, the data recorded was shortened and the treatment mean values was evaluated with the positive control plant (only SMV/CPMV crude sap) and Negative control (ddH_2O). The maximum disease severity on the control plant69% as compared to un-inoculated39% and on the treated plant 32% was recorded. The NPK control the maximum disease severity 69.23% followed by SA 30.04 %, Onion 8.04% percent, and Milk 6.66% than positive control 100 and negative control zero percent as evaluated respectively.

DISCUSSION

Agriculture, being a critical region of Pakistan's economy, at once helps the foremost proportion of the population (Phambra *et al*., 2020; Rehman *et al*., 2021). The agricultural share in GDP is about 22%. Because of the dangerous effect of viral diseases on yield and seed quality of infected crop legumes (Bos *et al*., 1988), the quantity of virus infection discovered through our surveys of commercial crops and experimental plots of soybean and cowpea, is purpose of first-rate situation. This also warrants a reappraisal of the real and ability importance presently assigned to the viral illnesses of soybean and cowpea. The considerable infection of SMV and CPMV in soybean and cowpea growing regions of Punjab, Pakistan offers a measure of economic importance they have already attained (Bashir *et al*., 2006; Rehman *et al*., 2021). Moreover, the occurrence of latest seed-borne viruses of each plant are suggested in experimental plots at research stations of Punjab provides an illustration of capability hazard for industrial plants inside the destiny. The infected experimental plots grow to be source of contamination in the place of business plots both all through developing season and thru sowing of infected seed in subsequent years (Bos, 1992; Jones, 1993). Our survey records additionally illustrate the dangers related to legume crops and new crop genotypes in introducing viral sicknesses, increasing virus occurrence and can cause economic losses in legume vegetation (Bos, 1992). The appearance of yellow vein clearing on new trifoliolate leaves is the first symptom of SMV on the routinely infected plant of soybean on the greenhouse. The downward curving of the leaf margin at the edges and upward curving of the leaf tip additionally arise within the inflamed plant of soybean (Brand *et al*., 1993). The leaves deliver direction and leather look and turn out to be brittle earlier than their maturity stage. The growth is stunted, and a smaller number of poles are decreased infected plant in comparison to the wholesome plant. Mottling also happens all through cold climate conditions and the plant will become masked in the course of warm conditions. The localized lesions are also produced at the leaves(Hajimorad*et al*., 2005; Rehman *et al*., 2021).Same as Soybean, Cowpea is likewise an critical crop, contributing to the fitness and livelihood of millions of human beings in tropical and sub-tropical countries (Singh and Rachie,1985). Young leaves, immature pods, and dry-saved seeds from the plant function food, specifically for rural households. The crop also gives coins earnings and animal forage/fodder, and residue from the crop is valued for soil enrichment. Cowpea crops have also become crucial in southern states of the U.S. and in South America, especially Brazil, as advanced, multi adapted cultivars (Henry *et al*., 1992). Plant infected with CPMV exhibit the symptoms of bloating, mosaic, leaf deformation, thickening, and toughening of the pericarp (de Oliveira *et al*., 2016). Hence, reduced the heathy leaf area and photosynthetic activity reduces and results in deformed, smaller, and hardened fruit that lack commercial value (Yuki *et al*., 2006). There are many hosts of CPMV and some hosts act as alternate host for their survival and source of inoculum). The purpose of the present looks at to test the efficiency and virulence of SMV and CPMV with the passage of time and the impact of reducing agent on virus infectivity by using mechanically inoculation strategies. The application of reducing agent, onion extract, cow milk, and NPK solution was also done for their management. During the study observed that the attack of SMV has been found in all selected fields with disease incidence 70%, 100%, and 60%. The maximum disease incidence was recorded in Field 2 with 100% disease incidence followed by Field 1 (70%) and Filed 3 (60%). Same as SMV, the disease incidence of CPMV was recorded with the Percentage of 60%, 80%, and 100% in all selected three fields. The maximum disease incidence was recorded in Field 3 with 100% disease incidence followed by Field 1 (60%) and Filed 2 (80%). The incidence of SMV and CPMV has been found in a severe form in Pakistan. Ali and Hassan, (1993) reported the incidence of the disease caused by soybean mosaic virus ranged from 7.74 to 72.14% in Northwest Frontier Provinces of Pakistan. Another survey was conducted by Ali, (2017) and concluded the highest percentage rate of SMV in different locations of Pakistan. The biological assay for the confirmation of the virus from disease plant to healthy seedling was done by using the method of Addy *et al*. (2017) at the control condition. After the one month of mechanically inoculation, the inoculated plant produced the light-yellow pattern of mosaic on leaves of both host pants given in figure 4. A severe mosaic pattern appeared on healthy leaves of Cowpea seedling after the inoculation. The same mechanically inoculation process for other virus has been previously reported by (Ali *et al*., 2020; Wei *et al*., 2021). During the present study, the infected samples were collected, then an ELISA test was performed for the confirmation of SMV and CMV. The ELISA plate result cleared that the mosaic symptoms produced on soybean and cowpea inoculated plant after 1 month was due to the symptoms of SMV and CPMV disease. In the ELISA plate, the well of the control plant does not show any colour and no symptom was observed on a plant whereas, the well of infected plants have shown yellow colour on the ELISA plate and the mosaic pattern was observed on leaves of the plant. Mixing of reducing agent in crude sap produce normal disease incidence. The mean value of disease incidence indicated that the carbon tetrachloride (CCl_4) was considered as the best reducing agent as compared to chloroform. Ali *et al*., (2020) evaluated the same reducing agent against the viral disease named *chilli venial mottle viru* and results indicated that the reducing agent hopefully decrease the virus infectivity and virulence. In vivo, the application of Cow Milk, salicylic acid and, NPK solution has shown significant results against the SMV and CoPMV.The NPK control the maximum disease severity 69.23% followed by SA 30.04 %, Onion 8.04% percent, and Milk 6.66% as compared to positive control 100 and negative control zero percent as recorded respectively. Baebler *et al*. (2014) described that Salicylic acid reduces the disease severity and incidence of potato virus Y significantly. Morphological, ultrastructural, and physiological adjustments of faba bean leaves in reaction to bean yellow mosaic virus (BYMV) contamination and SA treatments have been examined. Under BYMV strain, leaves showed signs such as excessive mosaic, mottling, crinkling, size discount, and deformations. Three weeks after utility of SA, photosynthetic charge, pigment contents, and transpiration fee had been drastically decreased in reaction to BYMV contamination. The result of Sseruwagi *et al*. (2003) study showing controversy with the present study, during his study used NPK fertilizer on the symptoms and spread of cassava mosaic virus disease (CMD) and populations of the whitefly vector was investigated in Uganda using three cassava varieties: Migyera, Nase 2 and Ebwanatereka in planting seasons. These results indicate that NPK fertilizer application is not a satisfactory strategy for facilitating the control of CMD.Non-chemical disease management strategy *viz*. NPK solution, Plant extract, and Milk were used on four tomato varieties against *Tomato mosaic virus* (TMV). All the treatments were used at three different concentrations (1%, 2%, and 3%) and replicated three times. Results indicated that the maximum disease reduction was recorded in plants treated with NPK solution (16.35%) and minimum in plants treated with Elephant Ear plant extract (29.65%) Saleem*et al.,* 2021).

Ali and Hassan, (1993) reported the incidence of the disease caused by soybean mosaic virus ranged from 7.74 to 72.14% in Northwest Frontier Provinces of Pakistan. Another survey during 2017 was conducted by Ali, (2017) and discusses the highest percentage rate of SMV in different locations of Pakistan.

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Amjad Ali	:	Conduct research
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Tariq A. Hamid	:	Statistical analysis
Tariq Mahmood	:	Edited manuscript