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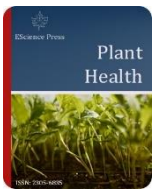

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Exogenous Application of Plant Extracts and Salicylic Acid to Mitigate Cucumber Mosaic Virus and Aphid Population in Cucumber

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ABSTRACT

Twenty cucumber varieties were evaluated for resistance to *Cucumber mosaic virus* (CMV) under greenhouse and field conditions. CMV disease severity was recorded with a disease rating scale and CMV was detected with Enzyme Linked Immunosorbent Assay. The use of salicylic acid (SA) for disease management in the greenhouse and field was also evaluated. Systemic acquired resistance was observed by measuring total phenolics and protein content. The varietal screening results showed that only two lines, Beit-alpha and Durga, were resistant to CMV, with 10% and 13.33% disease severity, respectively. Exogenous application of SA significantly increased total phenolics and protein content compared to control under both greenhouse and field experiments. Higher concentrations of SA proved to be most effective at inhibiting virus replication and disease severity. CMV severity is correlated with the aphid population, and plant extracts and detergent offered an eco-friendly approach to reduce the aphid population and disease severity, with neem extract being the most effective. This study shows that exogenous application of SA and plant extracts are good strategies for reducing CMV severity.

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INTRODUCTION

Cucumber (*Cucumis sativus* L.) is commercially cultivated both in open fields and in high tunnels. In Pakistan, cucumber is cultivated over a total area of 3397 ha with a yearly production of 48535 tones. On average, the world's cucumber yield is very low, largely due to pathogens, particularly viruses. *Cucumber mosaic virus* (CMV) belongs to the genus *Cucumovirus* and infects over 1000 plant species worldwide (Rossinck, 2002). It is one of the most destructive viruses, with a broad host range (Palukaitis and Garcia-Arenal, 2003). There are only a few varieties of flowering plants and vegetables which are resistant to CMV; most commercially grown cucumber cultivars are susceptible. CMV is transmitted through aphids and almost 60 species, particularly *Aphis gossypii* and *Myzus persicae*, transmit CMV through their sap in a non-persistent manner (Harris and Maramorosh, 1977). As there are no direct methods available to control plant viruses, methods for their detection and identification, both in plants and vectors, play a critical role in viral disease management. Enzyme linked immunosorbent assay (ELISA) is a rapid assay for diagnosing CMV, which can be employed both in the laboratory and field.

Management of CMV is not currently possible with a single method due to the rapid spread of the vector, its wide host range, and lack of host resistance. Development of additional management strategies and resistant cultivars are widely thought to be the best possible solution to this problem.

Chemical inducers can be used to increase plant resistance. Inducers such as salicylic acid (SA) may

trigger systemic acquired resistance (SAR), which is characterized by the accumulation of SA and pathogenesis-related (PR) proteins (Ryals et al., 1996). SA is a natural phenolic compound present in many plants. It is an important component of several signal transduction pathways and is implicated in local and systemic resistance to pathogens (Maleck et al., 2000). Salicylic acid may also increase the pathogen-related protein, which in turn may increase the plant's resistance to viral diseases. Exogenous application of salicylic acid may influence several physiological processes, including seed germination, transpiration rate, stomatal closure, membrane permeability, growth, photosynthesis, and fruit yield (Jimenez-Martinez, 2004; Belliure et al., 2005; Lacroix et al., 2005; Lacroix et al., 2007).

Pesticides can be used to control viral vectors. Many pesticides, including Imidacloprid, have been used to control sucking insects, soil insects, termites, and some chewing insects at all feeding stages (Boiteau and Osborn, 1997; Clark et al., 1998). Imidacloprid is effective against *Aphis gossypii* and *Myzus persicae* as it is a systemic pesticide which incorporates itself in the plant xylem and moves between the leaf surfaces. Once it enters the body of a feeding insect, it destroys its nervous system, ultimately resulting in changes in its feeding behaviour, followed by paralysis and death.

As another approach to viral disease management, many medicinal plants possess antimicrobial properties (Nashwa and Abo-Elyousr, 2012; Ashiq et al, 2017) and are applied to control fungal, bacterial, and viral diseases of diverse economically important crops. Additionally, use of plant extracts and soft soap solutions can control most of the important sucking and chewing insects including whiteflies, aphids, thrips, leafminers, armyworm, and small beetles (Elwell and Maas, 1995).

In the case of cucumber, little work has been done to identify resistant varieties and methods for the effective biological control of CMV. Hence, the present investigation was carried out to evaluate whether resistance could be induced in cucumber following exogenous application of SA, to measure changes in total phenolics and protein content in response to SA, and effective treatments for managing the vector population.

MATERIALS AND METHODS

Study Site and Plant Material

The experiment was conducted at the research area of the Department of Plant Pathology, Bahauddin Zakaryia University Multan, Pakistan during 2013 and 2014. Seeds of 20 cucumber varieties were obtained from the Ayub Agricultural Research Institute (AARI) and the *National Agricultural Research Centre Islamabad (NARC).*

Greenhouse Conditions

In the greenhouse, every genotype was sown in 15 cm diameter earthen pots with a sandy loam soil and peat mixture, 25-30°C, 80% humidity, and 16 hours of light. The experiment was repeated twice and conducted in completely randomized design with three replicates.

Field Conditions

The field experiment was conducted in a randomized complete block design (RCBD) with three replicates on a 1.0 m x 5.0 m bed with a 30 cm plant to plant and row to row distance. A single row of a susceptible variety, local kheera, was repeated after every three varieties and all around the field to increase the viral load. Every ridge contained a total of ten plants and four plants were selected for data collection. All normal field practices were applied to maintain a healthy crop.

Virus Maintenance and Mechanical Inoculation

For inoculation, 5 g of cucumber leaves naturally infected with CMV were collected from the field and ground in a pre-chilled mortar with 5 mL 0.01 M phosphate buffer (pH 7.2). For inoculation, completely-expanded seedlings were lightly dusted with carborandum (600 mesh) and rub-inoculated with virus-infected sap (100 µL/leaf) using sponge plugs. Three plants from each pot were selected, tagged, and inoculated on alternate days. Progression of the disease based on visual symptoms was recorded on a weekly basis for 60 days.

Induction of Resistance through Exogenous Application of Salicyclic Acid

Twenty-one days after inoculation, cucumber plants of the local kheera variety were treated with 0.01, 0.1, 1.0, or 10 mM SA and the control was treated with distilled water. Plants with a similar size were selected and divided into 4 groups;

T1: Entire plant leaves sprayed with 0.01, 0.1, 1.0 or 10 mM SA solution without inoculation.

T2: SA+CMV, pre-treated with 0.01, 0.1, 1.0, or 10 mM SA and inoculated with CMV after three days.

T3: Infected leaves, plants inoculated with CMV at the same time as group T2.

T4: Healthy leaves, control was sprayed with distilled water.

Each group contained three replicates. Three weeks after treatment, the youngest fully developed leaves were collected for total protein and phenol content analysis (Radwan *et al*., 2007).

Assessment of CMV Severity

In order to evaluate disease severity, four plants were randomly selected from each row and evaluated using a 0-5 disease rating scale (Bashir, 2005), where 0= No viral symptoms, 1= 1-10 % viral infection, 2= 11-20% viral infection, 3=21-30% viral infection, 4= 31-50% viral infection and 5= More than 50% viral infection. The disease severity index was calculated using the formula (Chaube and Singh, 1990):

$$\text{Disease severity index} = \frac{\text{Sum of numerical ratings}}{\text{Total number of observations}} \times \frac{100}{\text{maximum scale}}$$

The area under the disease progress curve (AUDPC) was calculated by trapezoidal assimilation of disease severity (%) over time for all varieties, considering the whole crop period evaluated (Madden *et al*., 2007).

$$\text{AUDPC} = \sum_{i=1}^{n-1} [(\text{xi} + \text{xi} + 1)/2]\ (\text{ti} + 1 - \text{ti})$$

n represents the total number of dates on which disease severity was recorded; *Xi*, Percent disease severity on the *ith* date; and (t_{i+1}-t_i), the period between two successive assessments.

CMV detection by DAS-ELISA

Young leaves of cucumber plants pre-inoculated with CMV were sampled for virus detection 21 days after establishment of the virus. Three plants of each variety were sampled from the greenhouse and field. The double antibody sandwich ELISA procedure was used to detect CMV with antibodies donated by Agdia Inc. USA (Clark and Adam, 1977). A polystyrene microtiter plate was pre-coated with the coating antibody (capture antibody) and incubated overnight. The virus (antigen) was extracted from leaf tissues collected from the field and greenhouse. 100 µl of antigen in general extraction buffer was loaded in each well of the microtiter plate and incubated overnight. The microtiter plate was then coated with the enzyme conjugate antibody after washing and incubated at room temperature. The plate was then coated with buffer containing 1 mg/ml pNpp and incubated for 30 minutes. The virus titer was then measured on an ELISA reader (Bio Tek, Model ELx 800) at 405 nm.

Measurement of total Phenolic Content

Total phenolic content was estimated with Folin-Ciocalteau reagent (Folin and Ciocalteau, 1927). A 1 g leaf sample was ground in 5 ml of 70% ethanol (Sigma Aldrich-USA) and centrifuged at 14,000 rpm for 5 minutes. 1 ml of supernatant was placed in a test tube and after 3-8 minutes 5 ml of diluted FC reagent was added. 4 ml of 7.5% sodium carbonate solution was added and incubated for 2 hours at room temperature in the dark. 100 mg of pure Gallic acid was added to 1 L water to give a 100 ppm Gallic acid standard for standard curve determination. Color intensity was observed at 765 nm with a spectrophotometer (Model UV, 3000) and total phenolic content was calculated with a standard curve.

Measurement of total Protein Content

Total protein was measured with the Bradford method (Bradford, 1976). A 1 mg/ml BSA (bovine serum albumin) solution was used to prepare a standard curve. 100 mg Coomassie Brilliant Blue G250 was dissolved in 50 ml 95% ethanol and 100ml 85% (w/v) phosphoric acid. This was diluted to 1 liter when the dye was completely dissolved and then filtered through Whatman # 1 paper. 1 g of leaf tissue was ground in 1 ml phosphate buffer (pH 7.4) and centrifuged (Centurion/UK) at 14,000 rpm at 4°C for 15 min. 20 µl of supernatant was added to 780 µL distilled water and 200 µL of dye and incubated for 5 minutes. Absorbance readings were taken with a spectrophotometer at 595 nm and total protein content was calculated with a standard curve.

Determination of Aphid Population

The aphid population was recorded from upper, middle, and lower leaves of four tagged plants from each block. The data was recorded on a weekly basis for eight weeks before and 48 hours after spray treatment (Khan *et al.,* 2011).

Evaluation of Plant Extracts and Detergent

Extracts of different plants, including *Meliaceae* (Neem), *Lamiaceae* (Mint), and *Cactaceae* (Cactus), and a detergent were evaluated in field conditions against aphid insect vectors and CMV severity on local kheera. The experiment was conducted in a randomized complete block design (RCBD) with three replicates. Three plants from each block were selected and tagged for evaluation of aqueous extracts and detergents. To

prepare the extracts, fresh leaves of neem, mint, and cactus were washed thoroughly, disinfected with 1% sodium hypochlorite ($NaOCl_2$), crushed into small pieces, and added to water at a final concentration of 1 and 3% (v/v). Extracts were sprayed three times while the control remained untreated. As a standard of comparison, imidacloprid was sprayed at 5, 6, and 7 g/L (Khan *et al.,* 2011).

Statistical Analysis

Datasets were statistically analyzed by analysis of variance (ANOVA) and treatment means were compared by the least significant difference test (LSD) at ($P \leq 0.05$) for the ELISA (OD_{405nm}) values, phenol content, and total protein under controlled conditions. To determine the disease severity index in the field experiment, Duncan's Multiple Range (DMR) test was used for multiple mean comparisons at ($P \leq 0.05$) with SAS (Statistical software package 8.0 Institute Carry Inc; USA) (Steel *et al.,* 1997).

RESULTS

CMV Disease Severity in Cucumber Varieties

All varieties were affected by CMV and aphids (*Aphis gossypii* and *Myzus persicae)*. Under greenhouse conditions, the varieties showed varying responses to CMV. Three varieties, Proline, Beit Alpha, and Durga, were most resistant. One variety was moderately resistant, six were susceptible, and 10 were highly susceptible. The maximum disease severity (86.67%) was recorded on local kheera and the minimum (13.33%) was on Proline, Beit Alpha, and Durga. The maximum ELISA OD (2.93) was found with local kheera and the minimum (0.89) was recorded for Proline. Similarly, the maximum AUDPC (2776.7) was calculated for local kheera and the minimum (280.0) for Durga. Results from the twenty varieties were roughly similar in the field trial. Beit Alpha and Durga were both found to be resistant in the field while proline, which was resistant to CMV in the greenhouse, showed only a moderately resistant response. Nine varieties were susceptible and eight varieties were highly susceptible. Maximum disease severity (86.67%) was recorded for local kheera and the minimum (3.33%) was found on Beit Alpha. Maximum ELISA OD (2.62) was again noted on local kheera and the minimum (0.21) was in Beit Alpha. The maximum AUDPC (2555.00) was calculated for local kheera and the minimum (81.67) was for Beit Alpha (Table 1). A dendrogram based on the similarity between these varieties showed four groups. Clade one consists of Proline, Durga, and waqas, clade two consists of SR seed, 40 days pak, Captin, F1 hybrid bilal, and Green super, clade three consists of Baran-180, Cucumber 6363, Cucumber F1, Cucumber F1Babu, Akber, Market more 76, and Malaga F1, and clade four consists of Denar 022F1, Beit Alpha, local kheera, and Anmol 227-G (Figure. 1).

Table 1. Detection of virus through ELISA, disease severity (DS) % of *CMV*, and response of different genotypes to disease.

Variety	Greenhouse experiment				Field experiment			
	Disease severity (%)	ELISA OD 405nm	AUDPC	Response	Disease severity (%)	ELISA OD 405nm	AUDPC	Response
Proline	13.33 g	0.89 k	420.00 l	R	15.00 ef	0.81 j	379.17 p	MR*
SR Seed	26.67 fg	1.08 i-k	1073.3 k	MR	31.67 de	1.23 hi	787.50 n	S*
Denar 022F1	40.00 ef	1.21 h-j	1073.3 k	S	33.33 d	1.09 i	700.00 o	S
40 days Pak	80.00 ab	2.41 b	2590.00 b	HS	81.67 a	2.38 b-d	2385.83 c	HS*
Local kheera	86.67 a	2.93 a	2776.7 a	HS	86.67 a	2.62 a	2555.00 a	HS
Baran- 180	66.67 a-d	2.09 c	2426.7 bc	HS	83.33 a	2.45 a-c	2450.00 b	HS
Captain	46.67 d-f	1.35 gh	1423.3 g-i	S	40.00 cd	1.42 gh	1306.67 j	S
Beit Alpha	13.33 g	0.91 k	140.00 m	R	3.33 f	0.21 l	81.67 r	R*
Waqas	53.33 c-e	1.69 ef	1120 jk	HS	43.33 b-d	1.49 fg	1085.00 l	S
Cucumber F1	46.67 d-f	1.38 gh	1516.7 f-h	S	46.67 b-d	1.58 fg	1365.00 i	S
Anmol	66.67 a-d	2.02 cd	2333.3 c	HS	78.33 a	2.31 cd	2070.83 e	HS
Malaga F1	73.33 a-c	2.35 b	2403.3 c	HS	86.67 a	2.59 ab	2240.00 d	HS
Babu	40.00 ef	1.33 g-i	1400.00 hi	S	60.00 b	2.17 d	1248.33 k	HS
227-G	53.33 c-e	1.71 ef	1260.00 ij	HS	33.33 d	1.21 hi	1085.00 l	S
F1 Hybrid Bilal	73.33 a-c	2.52 b	1610.00 ef	HS	43.33 b-d	1.44 f-h	1633.33 f	S
Akbar	60.00 b-e	1.98 cd	1866.7 d	HS	53.33 bc	1.67 ef	1610.00 g	HS

Cucumber 6363	53.33 c-e	1.81 de	1586.7 e-g	HS	55.00 bc	1.83 e	1615.83 g	HS
Market more 76	46.67 d-f	1.48 fg	1190.00 jk	S	41.67 cd	1.39 gh	1032.50 m	S
Green super	40.00 ef	1.26 g-i	1726.7 de	S	45.00 b-d	1.50 fg	1475.83 h	S
Durga	13.33 g	0.96 jk	280.00 lm	R	10.00 f	0.51 k	268.33 q	R
Positive Control	---	1.12 i-k	---	---	---	1.91 e	---	---
Negative Control	---	0.09 l	---	---	---	0.81 j	---	---
Healthy	---	0.01 m	---	---	---	0.06 l	---	---
LSD*	1.78	0.48	4.34	---	18.17	2.02	8.2	---

Means followed by same letter in each column are not statistically different at (P> 0.05); *LSD= Least significant difference, *R= resistant, *MR= moderately resistant, *MS= moderately susceptible, *HS= highly susceptible

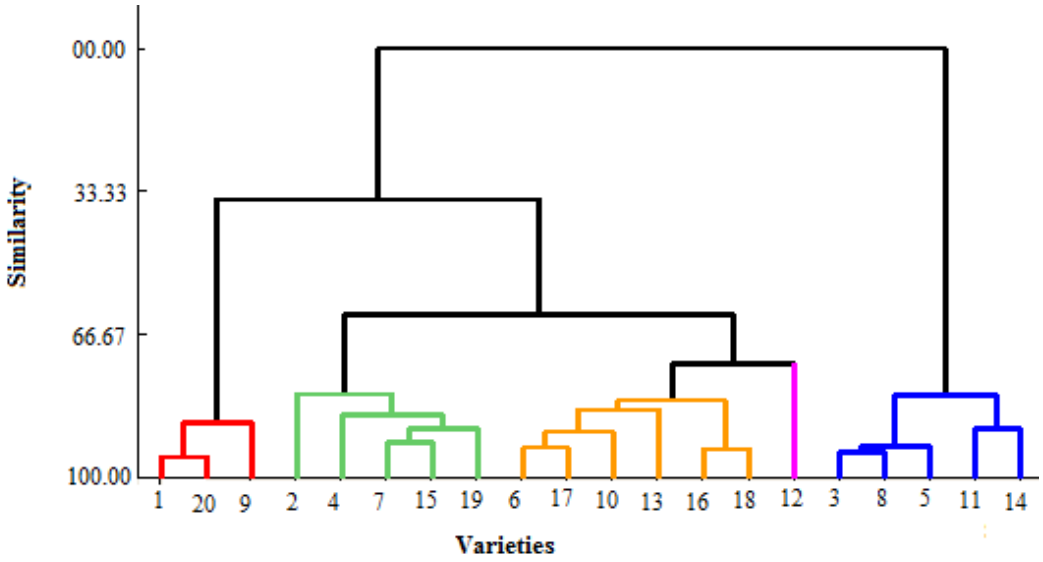

Figure 1. Similarities among the different cuc umber varieties in this study.

Effect of SA on Viral Titer, Phenolics and Protein Content

The maximum viral titer (OD 2.01) was detected in CMV infected leaves of non-treated cucumber, followed by treatment with SA at 0.01 mM and 0.1 mM (OD 1.33 and 1.29, respectively). The lowest OD value (0.08) was observed following treatment with 10 mM SA and this treatment was also the best at controlling disease severity (Table 2). Maximum phenolic content (169.19 mg/g) was observed following CMV infection and treatment with 0.1 mM SA followed by 10 mM SA (161.09mg/g). The minimum phenolic content (4.90 mg/g) was observed under control conditions without infection or SA (Figure 2).

Total protein content increased in inoculated plants compared to healthy plants. The maximum protein content, 36.09 mg/g, was observed following CMV infection and treatment with 10 mM SA, followed by 10 mM SA alone and then 0.1 mM SA + CMV (29.80 mg/g and 29.74 mg/g, respectively).

Table 2. Effect of salicylic acid treatment on CMV titer in cucumber leaves and disease severity.

Extracts	40 Days Pak			Local Kheera			Cucumber F1		
	1%	2%	3%	1%	2%	3%	1%	2%	3%
Mint	2.67 d	2.33 d	2.00 d	2.67 c	2.00 c	1.33 d	2.67 c	1.67 d	2.00 d
Neem	1.67 e	2.33 d	1.33 e	1.67 d	1.33 c	0.67 e	2.33 d	1.33 d	0.67 e
Cactus	3.33 c	3.00 c	2.67 c	3.33 b	2.33 b	2.33 c	2.67 c	3.67 b	2.67 c
Detergent	4.00 b	3.67 b	3.33 b	3.33 b	3.67 b	3.33 b	4.00 b	2.67 c	3.33 b
Control	4.67 a	4.33 a	5.00 a	4.67 a	4.33 a	4.67 a	4.33 a	4.67 a	4.00 a
LSD	0.59	0.98	1.39	1.01	1.03	1.41	1.51	2.35	1.19

Values with the same letter in the columns are not statistically different, LSD= Least significant difference.

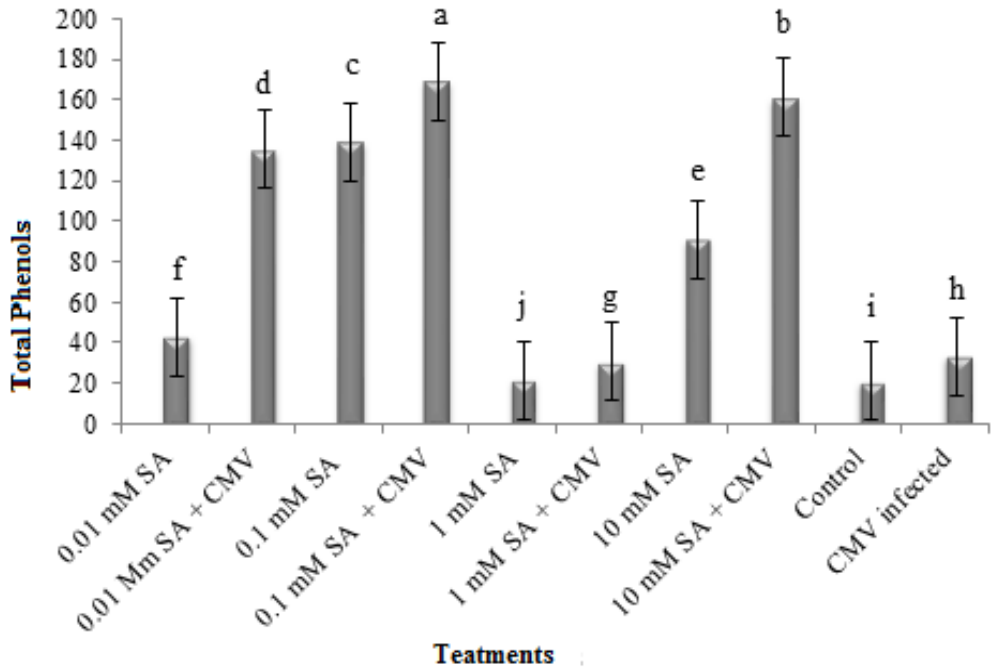

Figure 2. Effect of SA and CMV infection on phenolics content (mg/g fresh weight) of cucumber. The values are means of three replicates ± standard error. An increase in phenolics content is observed as the values are significantly different compared to control using the Duncan's Multiple Range Test at P ≤ 0.05 level.

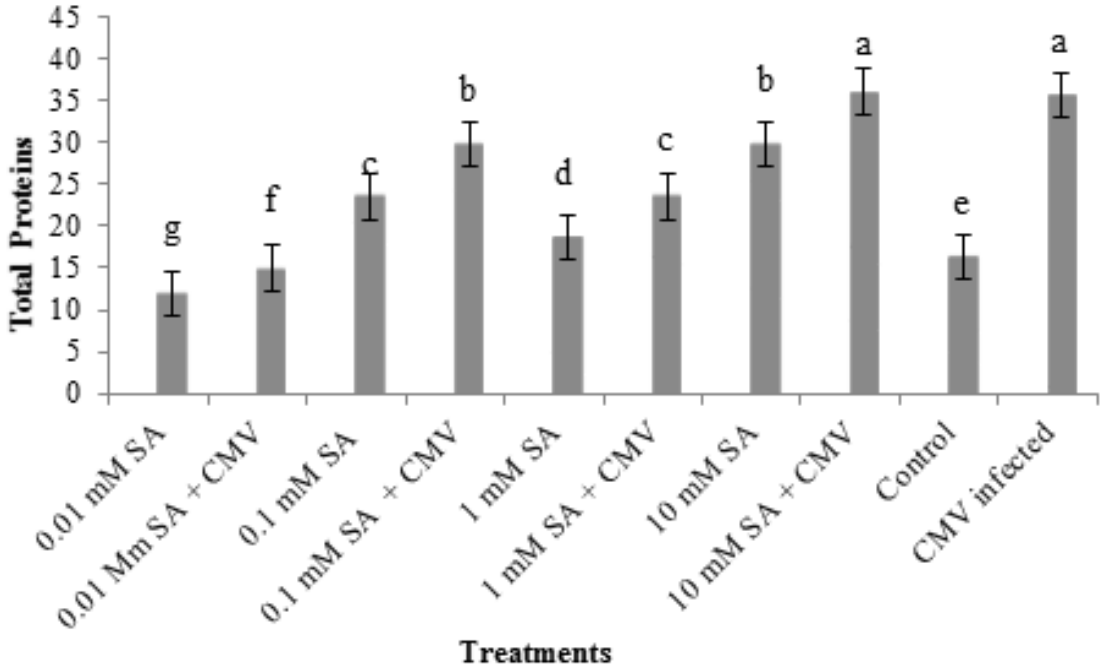

Figure 3: Effect of SA and CMV infection on total protein (mg/g fresh weight) of cucumber. The values are means of three replicates ± standard error. An increase in total protein is observed as the values are significantly different compared to control using the Duncan's Multiple Range Test at P ≤ 0.05 level.

Control plants had 16.34 mg/g. The minimum protein content, 11.88 mg/g, was observed when 0.01 mM SA was applied to non-infected plants (Figure 3).

CMV Management with Plant Extracts

For management with plant extracts and detergent, all treatments on 40 Days Pak showed the best response

when sprayed at a concentration of 3%, showing a 2.00, 1.33, 2.67, and 3.33% viral suppression compared to the control. Similarly, on local kheera, the application of 3% mint, neem, cactus, and detergent showed a mitigating response with 1.33, 0.67, 2.33 and 3.33% suppression when compared to control. Similar results were obtained with Cucumber F1 (Table 3).

Table 3. Effect of different concentrations of plant extracts and detergent on disease severity of CMV on three highly susceptible genotypes. Means followed by the same letter in each column are not statistically different at (P>0.05), LSD= Least significant difference.

Treatments	Virus Concentration ± S.E	Disease Severity % ± S.E
0.01 mM SA + Virus	1.33 ± 0.05 b	79.01 ± 0.31 b
0.1 mM SA + Virus	1.29 ± 0.05 b	83.09 ± 1.14 ab
1 mM SA + Virus	0.29 ± 0.07 b	55.01 ± 1.43 c
10 mM SA + Virus	0.08 ± 0.03 b	2.0 ± 0.31 d
Infected	2.01 ± 0.04 a	87.07 ± 0.48 a
Healthy	0.02 ± 0.15 c	0.00 ± 0.00 e
LSD*	0.31	4.29

Aphid Management

Management of aphid populations plays a vital role in the management of CMV (Figure 4). Aphid populations were successfully reduced following application of mint, neem, or cactus extracts, as well as with detergent. The aphid population was measured to be 10.00 aphids/leaf before treatment with 3% mint extract, and this was reduced to 8.67, 8.67, and 6.00 following the first, second, and third treatment, respectively, showing significant control of the aphid population compared to the control. Similarly, following treatment with 3% cactus extract, the number of aphids decreased from 10.33 to 9.67, 9.67, and 8.00. Spraying with detergent also led to a significant reduction of the aphid population. Treatment with 3% neem, however, had the greatest effect on the aphid population, followed by mint, cactus, and finally detergent. We compared treatment with plant extracts to chemical control through imidacloprid, which was also able to significantly reduce the aphid population.

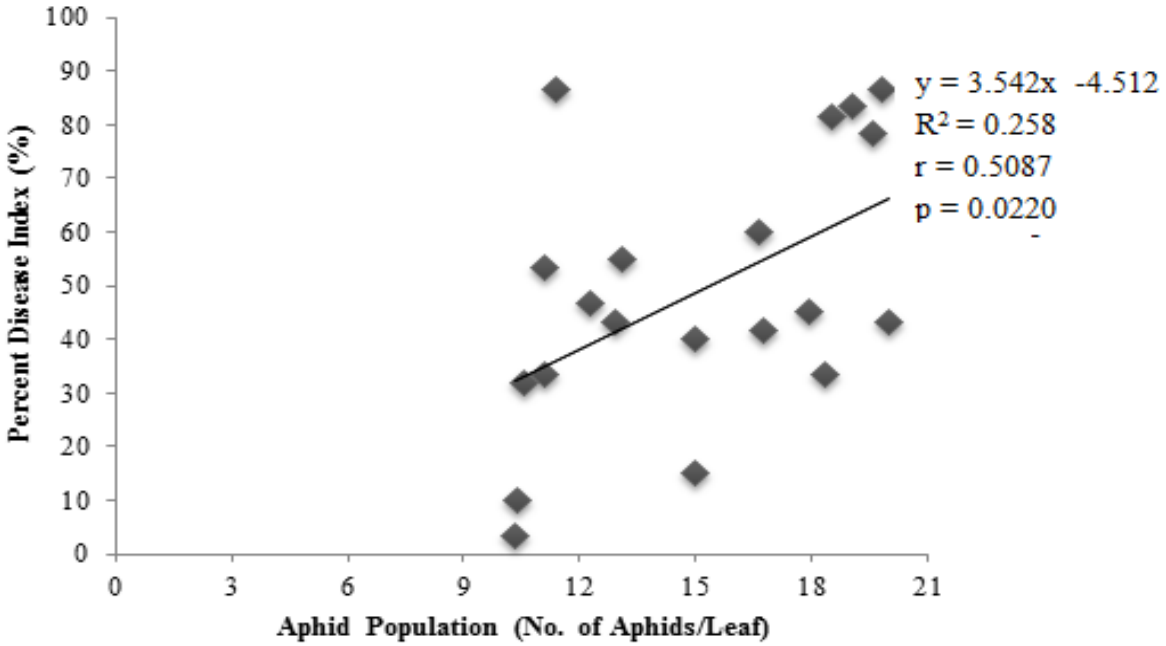

Figure 4. Correlation between the size of the aphid population and CMV disease severity in the field.

Table 4. Control of aphid population on Local kheera through application of different concentrations of plants extracts, detergent, and Imidacloprid under field conditions.

Extracts/ Insecticide	Aphid population recorded upon various sprays on cucumber											
	Before 1st		After 1st		Before 2nd		After 2nd		Before 3rd		After 3rd	
	1%	3%	1%	3%	1%	3%	1%	3%	1%	3%	1%	3%
Mint	11.6a-e	10.00a-c	10.00c-e	8.67c-e	11.67b-e	8.67e-g	11.00a-e	8.67fgh	10.00cd	7.00d-g	8.33fg	6.00c
Neem	8.00de	11.67a-c	7.00e	8.00cde	10.33b-f	7.00fg	6.33f	7.00ghi	10.33cd	7.00d-g	8.33fg	4.00fg
Cactus	15.33ab	10.33a-c	13.33a-c	9.67b-e	12.67bcd	9.33d-g	11.33a-d	9.67c-g	10.67cd	10.00cde	10.00c-g	8.00bc
Detergent	7.33e	10.67a-c	7.67e	9.67b-e	7.33f	10.67a-f	8.67c-f	10.33c-g	11.67bcd	9.00c-f	9.67d-g	9.00b
Control	12.00a-e	11.33a-c	13.00a-c	13.67ab	12.67b-d	14.00ab	13.67ab	14.33ab	12.67bc	13.67ab	12.67bcd	13.67a

Imidachloprid	Before 1st	After 1st	Before 2nd	After 2nd	Before 3rd	After 3rd
5 gm/ L	15.67 d	13.00 b	16.33 c	11.33 b	11.00 c	7.67 c
6 gm/ L	17.33c	8.67 c	17.00 b	9.67 c	14.00 b	8.33 b
7 gm/ L	19.00 b	6.33 d	13.67 d	4.67 d	11.00 c	3.00 d
Control	28.00 a	18.66 a	24.00 a	26.33 a	21.00 a	25.33a

Any two means not sharing a letter in common differ significantly at (P>0.05), Economic Threshold Level = 8-10 aphid adults or nymphs per leaf.

Three sprays of 7 g/L imidacloprid provided the most effective control of the aphid population, reducing the population from 19 to 6.33, 4.67, and 3.00 aphids/ plant after three sprays. As treatment with neem was nearly as effective as imidacloprid, plant extracts may be a good replacement for insecticides (Table 4).

DISCUSSION

Evaluating germplasm for disease resistance is the first step in a genetic improvement breeding program and resistant cucumber varieties could potentially form the basis of sustainable disease management strategies. Here, we screened several cucumber varieties for resistance to CMV. Our results showed that three varieties of the twenty lines we tested, Proline, Beit Alpha, and Durga, showed resistance under greenhouse conditions. Two varieties, Beit Alpha and Durga, also showed resistance under field conditions. Only these two varieties were resistant in both greenhouse and field conditions; the rest of the cucumber lines were either susceptible or highly susceptible to CMV infection. This dearth of genetic resistance to CMV in cucumber is likely because resistance to CMV is recessively inherited in cucurbit crops (Devi *et al*., 2012). There are two sources of resistance against CMV, one of which is temperature dependent, while the other is not temperature dependent and confers a high level of resistance against CMV. Our results showed non-viral race-specific and temperature-dependent resistance, which demonstrated variability in response from moderately resistant to susceptible against CMV in greenhouse and field conditions. Development of resistant varieties has been considered the most effective means of controlling plant diseases, particularly those caused by viruses (Tewari and Ramanjam, 1974).

The present study revealed that no variety is highly resistant to CMV. Beet alpha and Durga showed moderate resistance, similar to results shown by Khan *et al*. (2011). The incidence of different strains of CMV was recorded in 20 genotypes of *C. annuum* under both field and controlled conditions. It is reported that CMV is spreading with a relative incidence of 44.7% throughout Pakistan (Iqbal *et al*., 2012).

Disease severity and virus titer of some varieties were different under greenhouse and field conditions. For example, SR Seeds was moderately resistant in the greenhouse while appeared to be susceptible in the field. Similarly, Proline was resistant in the greenhouse but showed only moderate resistance in the field. These differences may be due to environmental factors, vectors, or alternate hosts. Our results showed that resistance to CMV in cucumber is very rare as only two varieties showed resistance in both greenhouse and field conditions. These two varieties may be a good source for developing CMV resistance in a crop breeding program.

As there was a lack of genetic resistance to CMV, we examined other strategies for managing the disease. We first examined the induction of resistance prior to pathogen attack by exogenous application of salicylic acid. It has been reported that SA is able to induce resistance to virus infection in crop plants (Radwan *et al*., 2007) and is a major signalling molecule in response to pathogen invasion (Radwan *et al*., 2007b). Application of SA promotes the accumulation of secondary metabolites at the site of infection. In our experiments, we also observed many changes in the morphology and metabolism of cucumber leaves, and these changes might be related with the production of secondary metabolites. We observed that virus inoculated plants showed symptoms when SA was applied even at low concentrations of 0.01 mM, 0.1 mM, and 1 mM as virus titer gradually decreased. At a higher concentration of 10 mM viral symptoms did not appear and the virus was not detectable through ELISA. These results demonstrate that application of SA 3 days before inoculation of CMV in cucumber leaves prevented the appearance of symptoms and also reduced the concentration of the virus. SA application increased phenolic and total protein content, inhibited viral replication and movement, and resulted in the suppression of viral symptoms. Similar reports have shown that CMV infected Arabidopsis leaves pre-treated with 0.1 mM SA and 0.06 mM jasmonic acid showed less severe symptoms than controls (Luo *et al*., 2011). Elbadry *et al*. (2006) also reported that exogenous application of SA led to systemic resistancess against BYMV in fava bean plants. Similar results were obtained by Meena *et al*. (2001) who reported that foliar application of 1 mM salicylic acid significantly reduced disease intensity. Lacroix et al (1982) suggested that application of salicylic acid at high concentrations may induce the full set of systemic acquired resistance (SAR) genes. Naylor *et al*. (1998) showed that replication of *Tobacco mosaic virus* (TMV) was inhibited when SA was applied soon after viral inoculation in tobacco plants and suggested that SA might inhibit long distance viral movement and interfere with viral exit from the leaf.

Phenolic compounds are a highly diverse class of secondary metabolites that are widely distributed among plants (Matern and Kneusal, 1988). Different physiological roles have been attributed to phenolics, including protecting plants from abiotic and biotic stresses (Dicke and Loon, 2000; Abid *et al* 2008). Our study showed that, after SA application, phenolic contents increased with increasing concentrations of SA. The maximum amount of phenolic compounds were produced following treatment with 10 mM SA. Furthermore, the plants did not show any symptoms and the virus was also not detected by ELISA, which was not the case for lower concentrations of SA. Matern and Kneusal (1988) demonstrated that, upon pathogen attack, phenols are the primary metabolites that accumulate at the site of infection. Francki *et al*. (1979) suggested that phenolics accumulate at sites of infection as a response to host-pathogen interactions.

The current study showed that total protein content increased in inoculated plants when treated with SA plants as compared to the control. Similarly, Devi et al. (2012) demonstrated that total protein content increased in virus inoculated plants compared to healthy controls. Loon (1989) reported that plants accumulate specific proteins, known as pathogenesis related proteins (PRs), which possess physicochemical properties that enable them to defend against proteolytic cleavage and harsh environments. Clarke *et al*. (1998) also reported that SA treatments induced accumulation of defense-related proteins at the site of pathogen infection. It was previously reported that when barley seedlings were treated with low concentrations of SA, protein content did not increase significantly compared to high concentrations (Popova *et al.,* 2003). We obtained similar results as protein contents were much lower (11.88mg/g) when treated with 0.01 mM SA compared to 10 mM SA when maximum protein content (36.09 mg/g) was observed. In our opinion, while viral proteins increase following inoculation, application of SA induces pathogen related proteins which mitigate the production of viral proteins, limiting viral viral replication and movement and keeping the plant free of

viral symptoms.

We observed that as the aphid population increased so did CMV disease severity, demonstrating that an important step in controlling CMV is to control the aphid population. Similar studies were carried out by Labonne *et al.* (1982) which showed that CMV is transmitted in a non-persistent manner by aphids and that *Aphis gossypii* is the most effective vector. The only way to control CMV is to use virus-free seed and to control aphids, as discussed by Balogun et al. (2007) suggesting that the use of virus-free seeds together with the eradication of viral reservoirs can be effective in controlling CMV. In this study, treatment with a 3% concentration of different plants extracts, neem, mint, and cactus, or with detergent significantly minimized the disease severity of CMV concentration, as was described earlier by Ali et al. (2005). We conclude that neem extract can effectively control the aphid population at a 3% concentration while 7 g/L imidacloprid is more effective at controlling the aphid population compared to 5 and 6 g/L. While imidacloprid has been found to be effective against *Aphis gossypii* and *Myzus persicae* (Ngumbi et al., 2007), based on the residual toxicity of imidacloprid, neem extracts may be more environmentally friendly.

CONCLUSION

This study concludes that exogenous application of salicylic acid induces CMV resistance in cucumber plants, which, together with plant extracts, offers a good management strategy for CMV as they can also minimize the aphid population following repetitive application.

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