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RESEARCH

Suppression of *Cucumber mosaic virus* by wind processing and *Fusarium equiseti* GF19-1

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ABSTRACT

Background: Cucumber mosaic caused by *Cucumber mosaic virus* (CMV) is a serious threat to crop production all over the world.
Objective: The aim of the current investigation was to evaluate the potential of fan-forced wind and the growth promoting fungus, *Fusarium equiseti* GF 19-1, as novel and safe management strategies against the invasion of CMV in cucumber plants.
Methods: The fan was operated two times per day (8:00 am and 18:00 pm, each time for 30 min). Moreover, cucumber plants were treated with cell free filtrate (CF) of GF 19-1 at 1 day before virus inoculation.
Results: The wind velocity (2.6 m/s) resulted in decreased virus severity and titer compared with the control. However, the potential of wind velocity lower or higher than 2.6 was less effective on CMV. Additionally, the severity and titer of CMV were significantly reduced in GF19-1 treated plants in comparison with the control. Quantitative real-time PCR results exhibited increased transcription levels of pathogenesis related genes, chitinase, PAL1 and LOX1.
Conclusion: In cucumber, treatments with artificially generated wind and root colonizing *F. equiseti* GF19-1 or its CF elicited induced resistance against CMV infection, leading to a restriction of pathogen propagation and disease development.

Keywords: *Cucumber mosaic virus*; Induced resistance; Plant growth promoting fungi; Wind processing; Cucumber; qRT-PCR

BACKGROUND

Cucumber mosaic virus (CMV) is a very important and destructive pathogen affecting vegetable production worldwide (Palukaitis *et al.*, 1992 and, Roossinck 1999). CMV (Genus Cucumovirus) is transmitted by several species of aphids and natural mechanical inoculation with a wide host range, thus management of CMV infection is difficult. Both greenhouse and field grown cucumber were severely affected by CMV infection causing stunted seedlings with deformed and yellow leaves (Roossinck 1999 and, Mayers *et al.*, 2005). Although the use of resistant cucumber cultivars can increase the protection against the pathogens, the appearance of new virus strains overcomes the resistance of the cultivar. The available method of controlling CMV is to control aphids by using pesticides. However, the use of pesticides becomes unacceptable because it causes several environmental and human health problems. Complicated mechanisms are involved in plant response to pathogen infection (Meera *et al.*, 1994, 1995; Shivanna *et al.*, 1996a, b; and Van Loon *et al.*, 1998). Several elicitors of systemic resistance were examined against plant pathogens including both biotic and abiotic elicitors (Hassan *et al.*, 2014, 2015; El-Kazzaz *et al.*, 2015 and; El-Naggar *et al.*, 2015). The biotic elicitors include the biological sources such as beneficial microorganisms and pathogens while the abiotic elicitors include non-biological sources such as physical and environmental stimuli (Hyakumachi, 1994 and; Koike *et al.*, 2001). Different defense responses are triggered in plants after exposure to the

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appropriate stimuli (Hahlbrook *et al.,* 1989; Hassan *et al.,* 2014). Wind stress is considered one of the most important environmental stresses that influence plant growth (Elsharkawy *et al.,* 2015). Systemic resistance was initiated in plants after local infection with *Tobacco mosaic virus* (TMV) and then the mechanisms of action against several plant viruses were explored (Ryu *et al.,* 2004; Elsharkawy *et al.,* 2012, 2013 and; Elsharkawy and Mousa, 2015).

Wind stress can cause different changes in the thickness of cuticle layer and stem (Todd *et al.,* 1972). Additionally, defense response was stimulated by wind due to friction or contact between plants. Control of rice blast disease by wind processing was first recorded in rice (Taguchi *et al.,* 2014). The severity of rice blast disease was reduced due to wind processing at a wind velocity 3 ~ 5 m / s. Wind processing increased the activity of defense enzymes such as peroxidase in kidney bean compared with control plants (Donald and Cipollini, 1998). Moreover, the symptoms of anthracnose disease, caused by *Colletotrichum lindemuthianum,* were reduced in kidney bean leaves treated with wind at speed of 3 m / s (Donald and Cipollini, 1997).

Recently, attention has been attracted to the role of beneficial microrganisms in management strategies of plant diseases. *Fusarium equiseti* is a root associated fungus (Horinouchi *et al.,* 2007). It was reported that *F. equiseti* treatments promoted plant growth and resistance to plant pathogens (Saldajeno and Hyakumachi, 2011 and; Horinouchi *et al.,* 2007). Colonization of plant roots with barley grain inoculum (BGI) of *F. equiseti* GF18-3 improved disease resistance to anthracnose disease in cucumber (Saldajeno and Hyakumachi, 2011). Interestingly, the severity and titer of *Papaya ring spot virus* (PRSV) were significantly reduced in cucumber plants treated with the fungus *P. simplicissimum* GP17-2 (Elsharkawy and Mousa, 2015). Reverse transcription PCR results showed increased transcription levels of resistance genes in cucumber leaves due to culture filtrate treatment of the fungus.

Although the efficiency of wind processing in control of plant pathogens was previously reported, the phenomenon was evaluated in a limited number of plants. In this study, cucumber plants were used to study the induction of resistance by wind and *F. equiseti* against CMV and the mechanisms involved in disease resistance by exploring the transcription levels of defense associated genes.

MATERIALS AND METHODS

Plant

Cucumber seeds cv. Hisham, which is a popular cultivar grown in greenhouses in Egypt and is susceptible to *Cucumber mosaic virus* (CMV), was used in all experiments. Seeds were sterilized with ethanol (70%) for 1 min followed by sodium hypochlorite (1%) for 5 min and washed in sterile distilled water (SDW) for 3 times before planting. Seeds were pre-germinated for 48 hours. Sterilized plastic pots (10cm in diameter) were filled with peat moss soil. Cucumber seeds were cultivated at 24°C for 21 days in greenhouse under 12 h light: 12 h dark conditions. These cucumber plants were utilized as a host plant in all experiments.

Pathogen and induction treatments

Cucumber mosaic virus was maintained in tobacco (cv. Xanthi-nc) and was used as a pathogen. *Fusarium equiseti* GF19-1 was a personal gift from Prof. Mitsuro Hyakumachi, Plant Pathology Laboratory, Gifu University, Japan. GF19-1 was cultured on potato dextrose agar (PDA) medium for 6 d in the dark at 24°C. Fifteen disks (5 mm in diameter) of this fungus mycelia were taken from the edges of 6-d-old cultures and transferred to 300 mL Erlenmeyer flasks containing 100 g sterilized barley grains in 100 mL distilled water to prepare barley grain inoculum (Elsharkawy *et al.,* 2013). Similarly, fifteen disks were transferred to 300-ml

Erlenmeyer flasks containing 100 ml potato dextrose broth (PDB) and incubated for 10 d at 24°C followed by filter sterilization to prepare culture filtrate of GF19-1 (Elsharkawy *et al., 2012*).

Electric fan (blade diameter of 25 cm) was utilized for blowing air at the required speed. Cucumber seedlings were exposed to wind processing for 4 days 2 times per day and each time 30 min. Barley grain inoculum of GF19-1 was amended to soil in a concentration of 2 % (w/w) before sowing cucumber seeds, while induction treatments with CF of GF19-1 and wind processing were done at 1 day before virus inoculation. Cucumber seedlings were inoculated with CMV by mechanical inoculation with prepared virus inoculum (CMV was obtained from Faculty of Agriculture, Kafrelsheikh University) using sterilized and pre-chilled pestles and mortar (Elsharkawy and Mousa, 2015). Treatment groups were: A, different wind speed (processing time: 30 min, processing period: 4 days) at 0 m/s (control), 2.1 m/s and 2.6 m/s B, treatment with barley grain inoculum (BGI) of GF19-1; C, treatment with culture filtrate (CF) of GF19-1. Cucumber plants were grown at 24°C for 14 days in the greenhouse after virus inoculation.

Disease severity evaluation

Symptoms developments were assessed after CMV inoculation. Severity of symptoms was evaluated at 7 and 14 days after virus inoculation following the scale (0-10) with 0 = no disease symptoms and 10 = severe stunting and deformed plants (Ryu *et al.*, 2004).

Virus titer in the leaves of cucumber was estimated at 7 and 14 days after CMV inoculation using Enzyme-Linked Immunosorbent Assay (ELISA) following the technique described by Clark and Adams (1977) and adopted by Elsharkawy *et al.*, (2013). The ELISA experiment was carried out three times with 10 plants per treatment.

Analysis of defense related genes expression

Cucumber seedlings were treated and CMV was inoculated as described previously. RNA was extracted from cucumber leaves at 24 h after induction treatments with wind processing or CF of GF19-1 and 1 DPI. Total RNA was extracted using RNA Purification Kit following the manufacturer protocol (Thermo Scientific, Fermentas, #K0731). Synthesis of cDNA was done using Reverse Transcription Kits following the manufacturer protocol (Thermo Scientific, Fermentas, #EP0451). Quantitative RT-PCR (qRT-PCR) with SYBR Green was used to measure expression of mRNAs of target genes (*Chitinase, PAL* and *LOX1*), with *EF 1-a* (Table 1) as an internal reference following the manufacturer protocol (Thermo scientific, USA, # K0221). The quantities critical thresholds (Ct) of target genes were normalized with quantities (Ct) of housekeeping gene (*EF1-a*) by used the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 1: List of primers used in RT-PCR analysis.

Gene	Forward primer (/5 ------ /3)	Reverse primer (/5 ------ /3)
EF 1-a	CTGTGCTGTCCTCATTATTG	AGGGTGAAAGCA AGAAGAGC
CHIT1	TGGTCACTGCAACCCTGACA	AGTGGCCTGGAATCCGACT
PAL1	ATGGAGGCAACTTCCAAGGA	CCATGGCAATCTCAGCACCT
LOX1	CTCTTGGGTGGTGGTGTTTC	TGGTGGGATTGAAGTTAGCC

Data analysis

All the data were expressed as means ±S.E. The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS, 18.0 software, 2011 and the individual comparisons were obtained by Duncan's multiple range test (DMRT). Values were considered statistically significant when $P \leq 0.05$.

RESULTS

Effect of *F. equiseti* GF19-1 and wind processing on systemic protection against CMV

Leaves of cucumber seedlings whose roots were inoculated with BGI or CF of GF19-1 showed significant reduction in disease severity and virus symptoms at 7 and 14 days post inoculation (DPI). Typically, non-treated control seedlings exhibited severe mosaic and deformed leaves. Cucumber seedlings were subjected also to wind processing at different wind speed 2.1 and 2.6 m/s for 4 days treatment period and 30 min treatment time. Interestingly, pre-treatment of cucumber seedlings with wind velocity 2.1 and 2.6 m/s resulted in significantly decrease of disease symptoms at 7 and 14 DPI relative to the control (Fig.1). The obtained results confirmed that the potential of CF treatment was even better than BGI of GF19-1.

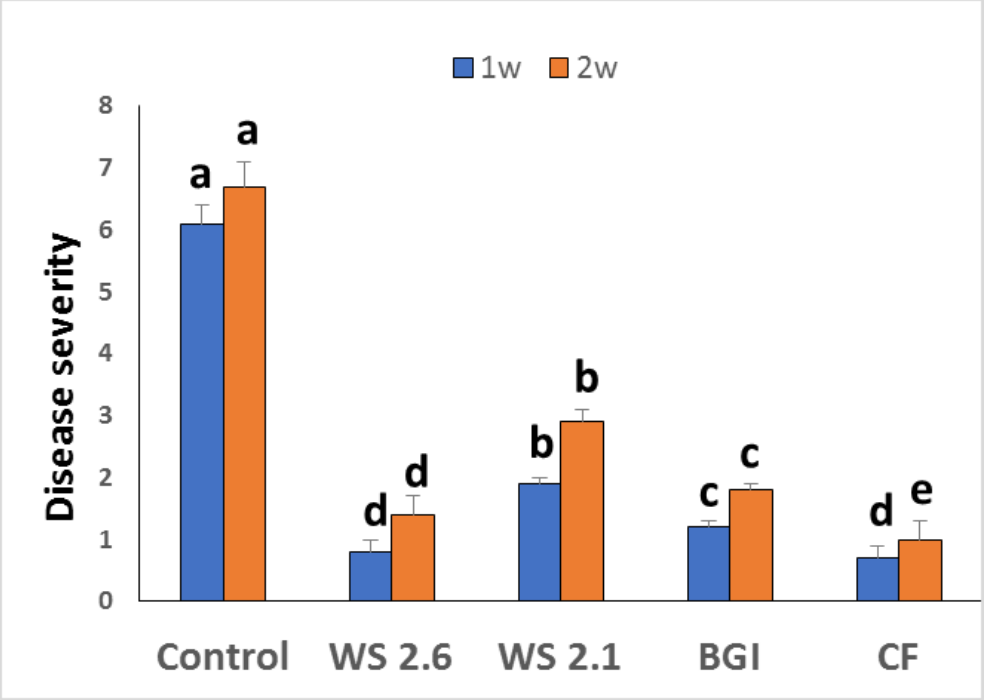

Fig. 1: Disease severity of Cucumber mosaic virus-inoculated cucumber plants treated with wind speed (WS 2.6 or 2.1 m/s) or barley grain inoculum (BGI) and culture filtrate (CF) of *F. equiseti* GF 19-1 relative to non-treated control plants at 7 and 14 days post-inoculation.

Effect of *F. equiseti* GF19-1 and wind processing on CMV titer in cucumber

Based on ELISA results, CMV titer in cucumber leaves were significantly reduced in all treatments compared with the control. CMV titer was significantly decreased in cucumber seedlings pre-treated with the BGI and CF of GF19-1 compared to the control (Fig. 2). The effect of CF treatment was more effective than BGI treatment. The tendency of virus titer reduction by the wind processing was observed. Among wind treatments, the most effective treatment was obtained by wind velocity of 2.6 m/s followed by 2.1 m/s treatment (Fig. 1). CMV titer in BGI

treatment was almost similar to virus titer in wind processing (2.6 m/s) treatment. Re-isolation frequency of BGI treated plants were 94% from cucumber roots at 6 weeks after planting.

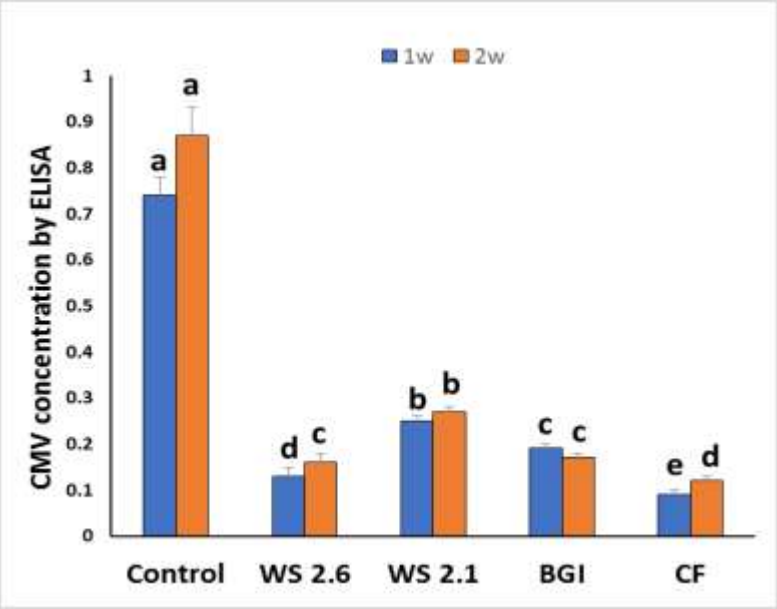

Fig. 2: *Cucumber mosaic virus* concentration in leaves of cucumber plants treated with wind speed (WS 2.6 or 2.1 m/s) or barley grain inoculum (BGI) and culture filtrate (CF) of *F. equiseti* GF 19-1 relative to non-treated control plants at 7 and 14 days post-inoculation.

Expression of defense -related genes

In cucumber plants, BGI treatment induced the highest transcription levels of *Chitinase* gene followed by CF treatment, wind velocity 2.6 m/s and wind velocity 2.1 m/s. Expression levels of *Chitinase* gene were higher at 2 DPI than immediately before virus inoculation (Fig. 3).

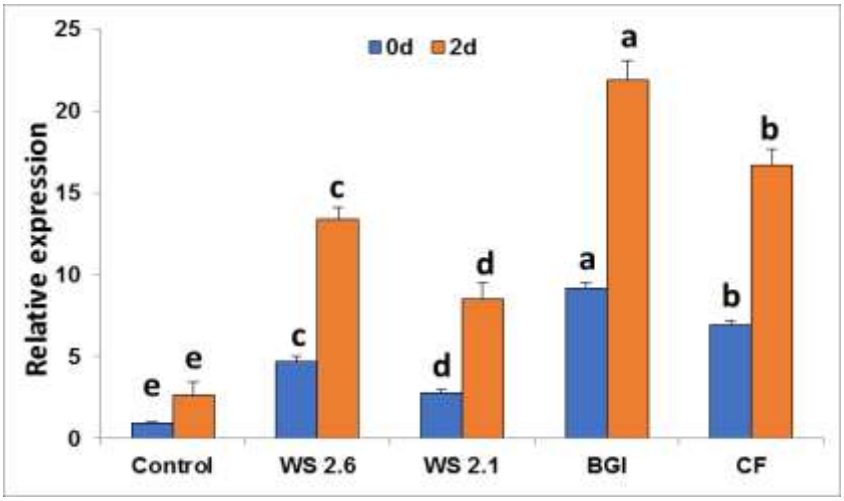

Fig. 3: Expression of defence-related gene *Chitinase* in leaves of cucumber plants treated with wind speed (WS 2.6 or 2.1 m/s) or barley grain inoculum (BGI) and culture filtrate (CF) of *F. equiseti* GF 19-1 relative to non-treated control plants at 0 and 2 days post-inoculation.

The expression levels of *PAL1* gene was strongly elevated in CF treated plants and wind. processing treatments especially at wind speed 2.6 m/s. No significant difference was found between BGI treatment and the control in the expression of *PAL1* gene (Fig. 4).

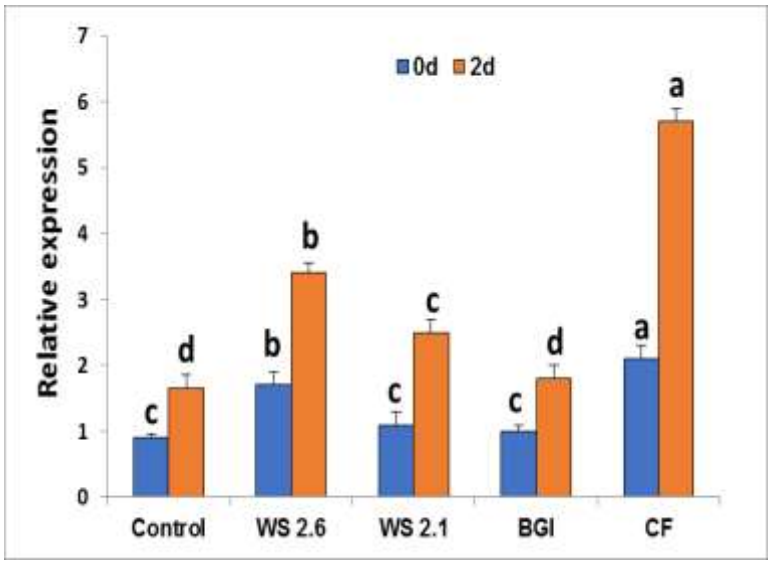

Fig. 4: Expression of defence-related gene *PAL1* in leaves of cucumber plants treated with wind speed (WS 2.6 or 2.1 m/s) or barley grain inoculum (BGI) and culture filtrate (CF) of *F. equiseti* GF 19-1 relative to non-treated control plants at 0 and 2 days post-inoculation.

Among all treatments, CF treatment resulted in the highest transcription levels of *LOX1* gene followed by wind velocity 2.6 m/s and wind velocity 2.1 m/s. *LOX1* expression levels in response to BGI treatment was almost similar to the expression levels in the control (Fig. 5).

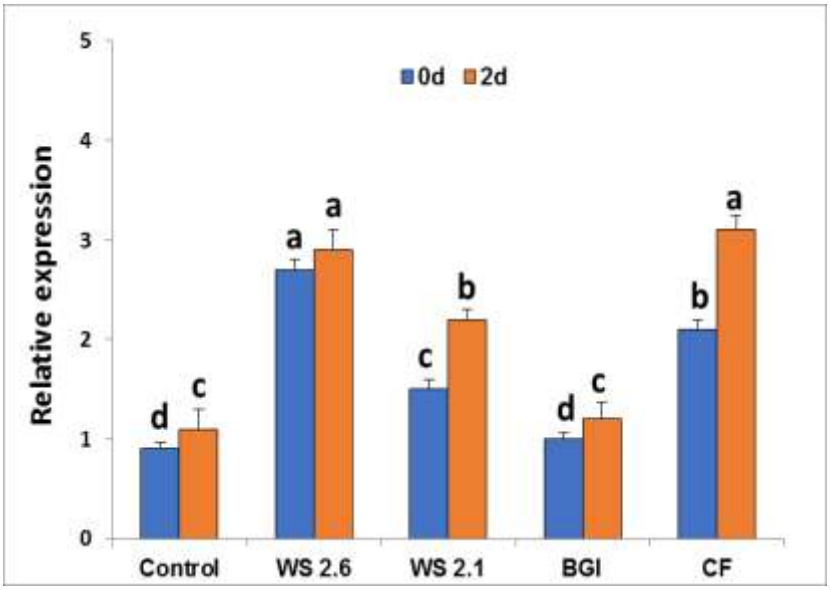

Fig. 5: Expression of defence-related gene *LOX1* in leaves of cucumber plants treated with wind speed (WS 2.6 or 2.1 m/s) or barley grain inoculum (BGI) and culture filtrate (CF) of *F. equiseti* GF 19-1 relative to non-treated control plants at 0 and 2 days post-inoculation.

DISCUSSION

Cucumber mosaic disease caused by CMV considers the most common viral pathogen with a very wide host range and various means of transmissions (Roossinck, 1999; Ryu *et al.*, 2004 and; Mayers *et al.*, 2005). Management options of CMV are limited and restricted to control the aphid vectors. Urgent need to alternatives of pesticides is increased day by day. Induced resistance was suggested to be a promising alternative to chemical pesticides (Mayers *et al.*, 2005 and; Hossain *et al.*, 2007).

Induction of resistance against plant pathogens by wind processing was reported in tomato (Elsharkawy *et al.*, 2015). The results showed that fan-forced wind at 2.6 m/s for 4 days and 30 min per each time effectively reduced CMV severity and titer. Disease suppression was stable at 7 and 14 DPI by wind processing at 2.6 m/s. Similarly, disease incidence of rice blast was reduced by wind processing at wind velocity 3 ~ 5 m/s (Taguchi *et al.*, 2014). Additionally, the symptoms of anthracnose on kidney bean were suppressed by wind processing at a wind speed 3 m/s (Donald and Cipollini, 1997). Treatment with wind at a speed of 2.6 m/s was more effective than wind speed 2.1 m/s in disease suppression at 7 and 14 DPI. The highest disease suppression effect of rice blast disease was obtained using strong wind speed (Taguchi *et al.*, 2014). The results demonstrated that CMV titer in cucumber leaves was decreased by operating fan-forced wind. The potential of wind processing at speed 2.6m/s was higher than wind speed 2.1 m/s in reduction of CMV titer. High correlation between disease severity and virus titer in the plant was found (Wang et al. 2009). CMV severity and titer were also suppressed in GF19-1 treatments. The results are consistent with Elsharkawy *et al.*, (2013) who reported that *Trichoderma asperellum* SKT-1 inhibited virus growth and reduced disease severity of CMV in *Arabidopsis* plants.

The suppression of *Fusarium* crown and root rot in tomato by blast processing was suggested to be due to induced systemic resistance (Elsharkawy *et al.*, 2015). The accumulation of phytoalexins in the roots could be the main reason of disease suppression. The activity of peroxidase was increased after wind processing in kidney bean plants (Donald and Cipollini, 1997). The mechanism of resistance induction by wind processing could be due to stimulation of protein kinase signaling pathways (Knight *et al.*, 1992). Physical stimulation due to wound induced resistance might be responsible for induced resistance (Elsharkawy *et al.*, 2015). Injury due to shaking between tomato leaves, without causing severe damage to the whole plant, was highly correlated with wound response. Wound response was highly correlated with the expression of jasmonic acid (JA) responsive genes. The results of this study showed increased transcription levels of SA and JA- responsive genes in the leaves of BGI and CF of GF19-1 and wind processing treatments. Similarly, wind processing at speed of 4 m/s enhanced the expression levels of *PAL* and *Chitinase* genes in tomato resulting in suppression of root rot disease (Elsharkawy *et al.*, 2015). Additionally, JA and salicylic acid (SA) signaling pathways were induced by the fungus *P. simplicissmum* GP 17-2 resulting in systemic resistance in tobacco against CMV. Synthesis of phytoalexins depends on the expression of phenylalanine ammonia lyase (*PAL*) gene. Elevated expressions of JA- responsive genes explain the role of JA in CMV disease resistance.

CONCLUSION

In conclusion, this study showed the potential of wind processing and both BGI and CF of GF19-1 to control CMV infection in cucumber plants. Wind processing at a wind velocity of 2.6 m/s was effective against CMV. Moreover, pre-treatment with the rhizosphere fungus GF19-

1 suppressed the infection with CMV in cucumber plants. To the best of our knowledge, this is the first report to control CMV by wind processing. Fan-forced wind could be considered as a promising management strategy of CMV without utilizing any pesticides in order to reduce the environmental pollution.

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