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Foliar application of liquiritin protects Chinese flowering cabbage against cucumber mosaic virus and increases health-promoting compounds

Waheed Akram a,b, Aqeel Ahmad a, Sabin Fatima a, Tehmina Anjum b, Basharat Ali d, Shakeel Ahmed e,f, Mario Juan Simirgiotis e, Hafiz Muhammad Khalid Abbas a, Muhammad Aslam c, Juxian Guo a, Wenlong Luo a, Mei Fu a and Guihua Li a

aGuangdong Key Laboratory for New Technology Research of Vegetables/Vegetable Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, People’s Republic of China; bDepartment of Plant Pathology, Faculty of Agricultural Sciences, University of the Punjab, Lahore, Pakistan; cPest Warning and Quality Control of Pesticides Punjab, Lahore, Pakistan; dInstitute of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan; eInstituto de Farmacia, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile; fFood Laboratory, Institute of Food Science Research (CIAL-CSIC-UAM), Madrid, Spain

ABSTRACT
Decades of research have revealed notable similarities between the immune systems of the plant and animal kingdoms. Liquiritin has long been used to stimulate the body immunity in animals against an array of diseases. Considering the homology of some induced immune responses between animals and plants, we examined the effects of exogenously applied liquiritin to stimulate defense responses in Chinese flowering cabbage plants against cucumber mosaic virus (CMV) infection under greenhouse and field conditions. Foliar application of liquiritin (200 ppm) effectively suppressed the development of CMV symptoms by not less than 40% compared with the control in cabbage plants in both greenhouse and field trials along with the significant increases in the marketable yield and nutritional quality of cabbage. Liquiritin application enhanced the production of phenolic compounds and different defense-related enzymes in treated plants. Moreover, quantitative real-time PCR analysis revealed that liquiritin significantly up-regulated the expression of different defense-related genes upon pathogen inoculation, indicating an induction of the salicylic acid-mediated defense system. Collectively, the findings of this study indicate that liquiritin can effectively control CMV in cabbage plants.

Introduction

Brassica rapa is an important vegetable crop cultivated worldwide to meet requirements for oil and fodder, whereas the fresh leaves are used in salads and as garnishes (Li et al. 2020). In addition, it has contributed the Brassica A genome to the amphidiploid crop species Brassica napus and Brassica juncea (Lowe et al. 2004), and also been demonstrated to contain a range of phenolics, vitamins, and glucosinolates with anticarcinogenic and antioxidative effects in humans (Cheng et al. 2012).

Diseases are important factors limiting the production of brassica vegetables, which are known to be affected by 10–20 different viruses (Wilson et al. 2012). Cucumber mosaic virus (CMV) is among the most economically damaging pathogens in Brassica crops (Moreno et al. 2004). CMV is the type member of the genus Cucumovirus in the family Bromoviridae (Scholthof et al. 2011). Infected plants exhibit symptoms that include strong leaf mosaic patterns and leaf distortion, stunted growth, reduced flower production, and fruit lesions (Shi et al. 2018). The decreases in plant yield being closely associated with the stage at which crop plants are attacked by CMV. Plants infected at the seedling stage are incapable of developing appropriate curd or seeds, development of the disease in mature plants can result in reduced crop yields. Currently, there is a limited availability of appropriate treatments for the management of viral diseases in infected plants. Control of CMV in the field by controlling its vector is not very effective. Some resistance genes have been utilized to manage losses caused by CMV. At several instances, immunity caused by resistance genes is overcome by different strains of CMV (Palukaitis and García-Arenal 2003).

Numerous plants have developed mechanisms of systemic resistance to restrict pathogens at the sites of infection. This resistance enhances the defense status of the non-exposed plant parts against pests (Daisy et al. 2012), whereas the up-regulated expression of defense-related genes modulates phytohormone biosynthesis (Durrant and Dong 2004). In this regard, the application of biotic and abiotic primers can be used to induce resistance through the enhancement of signaling proteins within plant cells, and subsequent increases in the synthesis of pathogenesis-related proteins can contribute to amplifying plant immunity (Ahmad et al. 2020; Ahmad et al. 2021).

Both plants and animals are endowed with a conserved innate immune system able to neutralize pathogens and to contain the infection (Roudaire et al. 2021). The essence of both plant and animal immunity is the recognition and protection against the foreign. Similarities or analogous
mechanisms seem surprising when immunity to pathogen infections is considered in the case of plants and animals (Maekawa et al. 2011). The defense-related activities of plants are initiated by the detection of signaling molecules also termed as elicitors that function as signaling molecules in plants (Nürnberger and Brunner 2002). The elicitor molecules are called antigens in the course of immune reactions in animal receptors. Interestingly, animals or plants nucleotide-binding receptors likely detect effector molecules injected into the host cell by the pathogen to hijack the immune signaling cascade (Nürnberger and Brunner 2002). All these facts refer to the possibility that recognition of animal antigens or elicitor molecules by plants can also trigger immune responses to hinder the invasion of pathogens inside plant body. Liquiritin is a flavanone glycoside that plays a significant role in boosting the immune system of mammals (Chedd et al. 2010), and in our recent research, we observed that this compound also has a beneficial effect on the growth of Chinese flowering cabbage plants (Akram et al. 2020). Furthermore, we found that a foliar spray of 750 ppm liquiritin enhanced the contents of phenolics and glucosinolates in addition to acylated and glycosylated flavonoids in cabbage plants Akram et al. (2020).

On the basis of aforementioned similarities between animal and plant defense mechanisms and our previous findings with respect to liquiritin (Akram et al. 2020), we postulated that the application of this compound might have the effect of eliciting defense responses against the cucumber mosaic virus (CMV) in Chinese flowering cabbage plants. In the present study, we accordingly sought to examine the protective effects of exogenous application of liquiritin on Chinese flowering cabbage plants infected by CMV. To the best of our knowledge, this is the first study that has sought to elucidate the effects of exogenously applied liquiritin on the activation of plant defense responses to reduce the damage caused by a viral disease. The findings of this study will contribute to the identification of other synthetic, non-toxic, fit-for-human consumption elicitor molecules capable of up-regulating plant defense responses against viral diseases.

Materials and methods

Plants, virus, and chemicals

For the purposes of the present study, we used the Caixin variety of Brassica rapa L. ssp. parachinensis as host plants. The virus used was the GSS-073 strain of CMV isolated from B. rapa plants. Commercial-grade liquiritin of 99% purity was obtained from RitoTo Botanicals (China), and Benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH), used as a positive control, was obtained from Sigma-Aldrich (St. Louis, MO, United States).

Plant growth conditions

The greenhouse experiment conducted in the present study entailed growing plants in pots containing sterilized Tref Jiffy (USA) media, which were placed in an insect-proof greenhouse at 20/25 ± 3°C (night/day) under a 16-h photoperiod.

Elicitor pre-treatment and virus application

Different concentrations (0, 50, 100, 150, and 300 ppm) of liquiritin were sprayed over the foliage of Chinese flowering cabbage plants at the four-leaf stage (spraying until run-off; approx. 18 mL suspension plant⁻¹). Inoculation of CMV was performed 2 days after the application of the elicitor (Elsharkawy 2019). As a positive control for the activation of systemic acquired resistance, we used 100 mM BTH (Beris et al. 2018). Plants inoculated with sterilized distilled water served as control or mock-inoculated treatment as suggested by Tungadi et al. (2017). The entire experiment was conducted using a randomized complete block design, with 10 plant replicates in each treatment, and the study was repeated twice.

Disease severity analysis

The level of resistance to CMV induced by liquiritin in Chinese flowering cabbage plants was evaluated both morphologically and serologically. Disease severity was rated on a scale from 0 to 10, as described by (Ghandi and Anfoka 2000), and the disease severity index was analyzed using the equation proposed by Raupach et al. (1996). Virus concentrations were estimated using enzyme-linked immunosorbent assays (ELISAs) according to Elsharkawy et al. (2013).

Assessment of induced resistance

An additional independent experiment was conducted to evaluate the efficacy of liquiritin in triggering systemic resistance in supplemented plants. All treatments were the same as those described for the previous experiment, with the one exception that only a single concentration of liquiritin (200 ppm) was used, and we performed the analyses described in the following sections.

Evaluation of defense-related biochemicals

Changes in the activities of phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), and peroxidase (PO) and the contents of phenolic compounds in treated plants were observed over a time course from the first day of virus inoculation. Plant leaf samples used for analyses were collected at different time points and stored in liquid nitrogen. Phenolic contents were estimated according to Zieslin and Ben Zaken (1993). For extraction of defense-related enzymes, 1 g of pre-washed leaf sample was vortexed in an ice-chilled mortar in the presence of 5 mL ice-cold 100 mM sodium phosphate buffer at pH 7.0. The mixture was subjected to centrifugation at 4000×g for 15 min at 4°C. The activities of PO, PPO, and PAL in the resulting supernatant were estimated spectrophotometrically. PO activity was analyzed using 0.5 mL of 1% hydrogen peroxide, 0.05 M pyrogallol, and 1 mL of enzymatic extract (Fu and Huang 2001). For estimates of PPO activity, 60 mM catechol was mixed with 1 mL of enzyme extract and 50 mM sodium phosphate buffer (pH 6.5) (Mayer et al. 1966), whereas PAL activity was determined using a mixture of 0.4 mL of 25 mM Tris-HCl (pH 7.5), 1 mL of enzyme extract, and 0.5 mL of l-phenylalanine (Burrell and Rees 1974).
**Evaluation of changes in defense-related genes**

Changes in the expression levels of some pathogenesis-related (PR) genes in response to exogenous elicitor were quantified 1 week after pathogen inoculation. Total RNA was extracted using Trizol reagent (Thermo Fisher Scientific, MA, US) according to the manufacturer’s instructions. Approximately 1 μg of total RNA was reverse transcribed to single-stranded cDNA, and RT-qPCR was thereafter performed to examine gene expression, as described by Abe et al. (2011), using a GoTaq qPCR Master Mix Kit (Promega, USA) and a Bio-Rad CFX96 Real-Time PCR System. EF1-α was used as a housekeeping gene for normalization of the transcription levels of observed genes, based on the cycle threshold method. The primers were designed using the Primer3Plus interface (http://frodo.wi.mit.edu/). The primers specificity and efficiency were also determined in RT-qPCR. Details of primers are provided in supplemental table 2.

**Field experiments**

To evaluate the potential of foliar application of liquiritin (200 ppm) and its effect on the yield and quality of Chinese flowering cabbage, we performed field experiments during 2018 and 2019 in agricultural fields in the vicinity of Guangzhou City, China, which had a previous history of viral disease. Foliar elicitor was sprayed three times under field conditions (Kong et al. 2018), starting from the six-leaf stage and repeated at 10-day intervals. The study was arranged in a randomized block design, and for all treatments, we assessed five replicate plots, each containing 50 Chinese flowering cabbage plants cultivated on raised beds in a single row. Similar amounts of BTH and water were applied to the BTH and water-treated control plants, respectively. At 60 days after elicitor application, disease suppression was examined both morphologically and molecularly, as described previously. To assess disease occurrence, 15 leaf samples were randomly selected from all plants and stored in liquid nitrogen until used for analysis.

**Plant quality assessment**

Field-grown plants at final harvest were also analyzed for improvement in nutrient quality and marketable yield under the influence of exogenous elicitor. The marketable value of plants was calculated using the following formula:

\[
\text{Marketable value} (\%) = 100 - (100 \times \text{percentage of or diseased plants}/\text{percentage healthy plants})
\]

Ultra-fast liquid chromatography-quadrupole time-of-flight tandem mass spectrometry (UFLC-QToF-MS/MS) analysis, based on the MRM method, was employed to determine the amounts of different glucosinolates and total phenolics in treated plants. For this purpose, 30 leaf samples were randomly collected from each treatment plot and pooled to give composite samples. Prior to lyophilization, the samples were cooled to −80°C by maintaining on dry ice. The levels of glucosinolates and phenolic acids in leaf powder preparations were determined as described by Francisco et al. (2009). Likewise, alkaline hydrolysis was performed according to Francisco et al. (2009) prior to analysis of glucosinolates and phenolics using an API 4000 QTRap mass spectrometer equipped with a ‘TurboIonSpray’ probe (AB Sciei, Foster City, CA, USA) linked to a UFLC system (Shimadzu, Kyoto, Japan). Optimization of the experimental conditions was based on the parameters recommended by Cataldi et al. (2007). Details regarding the separation of chromatograms and investigation situations have been described previously by Akram et al. (2020). Supplemental table 1 shows the experimental conditions used to quantify glucosinolates and phenolics using sinigrin (Sigma) and sinapic acid as standards for glucosinolates and phenolics, respectively (Moreira-AraÚJo et al. 2018).

**Statistical analysis**

Experimental trials were repeated twice, and mean values are presented. Data were inspected for normality by employing the Shapiro–Wilks test. Data were analyzed by performing ANOVA, and differences among treatments were analyzed using Duncan’s new multiple range test. The average of data representing yield factors for each field trial plot was analyzed according to Moore and Dixon (2015).

**Results**

The protective effects of exogenously applied liquiritin against CMV in Chinese flowering cabbage

Twenty days after virus inoculation, plants were examined for symptom development and disease severity. Compared with the pathogen control, we found that the disease severity index was reduced in plants sprayed with liquiritin. As shown in Figure 1, application of liquiritin at concentrations greater than 200 ppm significantly reduced disease severity, with reductions of up to 63.9% being recorded for Chinese flowering cabbage plants treated with 200 ppm liquiritin. The results of ELISAs presented in Figure 1 indicate trends consistent with the disease severity index scores, with the application of liquiritin significantly reducing viral antigens in a dose-dependent manner. However, the data indicated that applying elicitor at concentrations in excess of 200 ppm did not appear to provide any further enhancement of the protective effect against CMV. BTH, which was used as a positive control, was similarly found to result in reductions in CMV severity and titer in treated cabbage plants (Figure 1).

**Assessment of induced resistance**

Analysis of changes in defense-related compounds

To elucidate the biochemical basis of liquiritin (200 ppm) mediated induced resistance, quantification of total phenolics and different enzymes of phenylpropanoid biosynthesis was performed at different time points post liquiritin application, and was compared to water treated (control) plants and BTH treatment. Exogenous application of liquiritin significantly elevated total phenolic contents in Chinese flowering cabbage plants at different time points, we observed the most pronounced responses in plants receiving the application of both liquiritin and pathogen (Figure 2(A)). Total phenolic contents were peaked at 24hpi interval where these were 2.3- fold higher in plants receiving liquiritin +
CMV as compared to the plants receiving pathogen alone (Figure 2(A)). Total phenolic contents were likewise significantly increased in BTH-supplemented plants (positive control treatments).

PO and PPO activities in Chinese flowering cabbage plants treated with liquiritin+ CMV were significantly increased up to 2.6 and 1.7 folds, respectively, as compared to the control (water treated) plants for 48 dpi interval (Figure 2(B,C)). Whereas, the same values ranged at 1.3- and 0.7- folds respectively, in CMV alone inoculated plants (Figure 2). PAL activity peaked at 24 dpi and highest activity was seen in the plants treated with liquiritin+ CMV (Figure 2 (D)). PAL activity was 1.8-, 1.1- and 1.3- fold higher in CMV + liquiritin treated plants as compared to the control, liquiritin alone, and CMV alone treatments, respectively, on 24 dpi (Figure 2(D)).

**Changes in the expression of defense-related genes**

To determine whether liquiritin (200 ppm) affects the SA mediated signaling pathway during pathogen infection, defense-related genes were quantified by RT-qPCR 7 days after inoculating plants with CMV. These PR genes are widely used as molecular markers for resistance response against pathogens (Seo et al. 2008). The induced expression of these genes showed significant differences (P< 0.05) among liquiritin alone, liquiritin + CMV, CMV alone and control (water treated) plants (Figure 3).

Liquiritin treatment followed by CMV challenge resulted in elevated levels of PR1 transcript levels up to 2.3- and 1.7- folds respectively, compared to both non-treated control and CMV alone inoculated plants (Figure 3). Similarly, expression levels of PR-3 and PR-5 increased up to 5.1- and 3.9- folds in liquiritin + CMV inoculated plants respectively, compared to that of control plants. Whereas, expression levels of PR-3 and PR-5 increased up to 3.4- and 3.1-folds respectively, in liquiritin + CMV inoculated plants as compared to that of CMV alone treatment (Figure 3). In case of PR4, expression levels in liquiritin + CMV inoculated plants increased up to 1.5 and 0.8 folds respectively, as compared to the CMV alone and water-treated control plants (Figure 3). This indicated the activation of SA mediated signaling in Chinese flowering cabbage plants under influence of liquiritin treatment.

**Field experiment**

To evaluate virus resistance under field conditions, Chinese flowering cabbage plants were treated with liquiritin three times at 10-day intervals, starting from the six-leaf stage. In the field trials conducted in both 2018 and 2019, we found that the disease index of plants sprayed with liquiritin was significantly reduced by up to 43.8% compared with the control plots (Table 1). Consistently, ELISA absorbance values were significantly lower in the liquiritin-supplemented plants than in the controls across both field trials (Table 1).
Plant quality assessment

The data obtained from field experiments indicated that the foliar application of liquiritin effectively increased the marketable yield of Chinese flowering cabbage by an average of 39.6% over both field trials (Table 1). Moreover, the exogenous application of liquiritin significantly enhanced the synthesis of different phenols and glucosinolates in plants under field conditions, with contents of the glucosinolates neoglucobrassin, glucoalyssin, glucobrassicin, and 4-methoxyglucobrassicin increasing on average basis up to 2.3-, 1.9-, 0.7-, 1.3-, and 2.1-folds, respectively, compared with the control plants across both field trials (Figure 4). Likewise, higher inducible quantities of phenolic acids were detected in Chinese flowering cabbage plants receiving foliar elicitor (Figure 4), with sinnapic acids and caffeic acid increasing by more than 3-fold under the same conditions (Figure 4).

Discussion

Liquiritin is a major flavonoid found in the roots of licorice plants (Glycyrrhiza spp.), the medicinal properties of which, as well as its activation of immune responses in humans, have been well studied (Uto et al. 2019). In the current study, we, therefore, sought to assess the potential of liquiritin, applied as a foliar spray, in suppressing disease caused by CMV in Chinese flowering cabbage plants. We accordingly found that foliar application of liquiritin significantly reduced the severity of CMV in cabbage plants grown under both greenhouse and field conditions. It is worth mentioning here that Benzo-(1,2,3)-thiadiazole-7-carbothioic

**Figure 2.** Time course study of the levels of (A) total phenolics, (B) PO, (C) PPO and PAL activity in potato plants. The levels were measured in the leaves of Chinese flowering cabbage plants after the following treatments: Water-treated control (C), CMV alone (T1); liquiritin (200 ppm) alone (T2); BTH alone (T3); liquiritin (200 ppm) pretreatment followed by pathogen infection (T4); BTH pretreatment followed by pathogen infection (T5). Vertical bars show standard error between different replicates of the same treatment. Leaves of the plants were used for quantifications of phenolic compounds and PO, PPO and PAL enzymes. Quantifications were performed at different time points. The experiment was repeated twice and mean values are presented here.

**Figure 3.** Effect of liquiritin application on the expression levels of different defense-related genes of Chinese flowering cabbage plants. The levels were measured after one week of pathogen flowering cabbage plants under the following treatments: Water-treated control (C), CMV alone (T1); Liquiritin (200 ppm) alone (T2); BTH alone (T3); Liquiritin (200 ppm) pretreatment followed by pathogen infection (T4); BTH pretreatment followed by pathogen infection (T5). Vertical bars show standard error between different replicates of the same treatment whereas, small letters show level of significance among different treatments as governed by ANOVA and DNMRT at $p = 0.05$. Leaves of the plants were used for RT-qPCR analysis. Analysis was performed at 7 dpi of CMV application. The experiment was repeated twice and mean values are presented here.
acid S-methyl ester (BTH) was used as a positive control in this study. This chemical has been reported to induce systemic resistance in tomato against cucumber mosaic virus (Ghandi and Anfoka 2000). During the initial phase of the study, we set out to standardized the dose of liquiritin for field application and established that foliar spraying with liquiritin at a concentration of 200 ppm or more effectively suppressed CMV disease symptoms in cabbage plants under greenhouse conditions. Hence, 200 ppm was selected for subsequent field application, and was found to be similarly effective in suppressing disease symptoms in field trials conducted in both 2018 and 2019. Consistently, the CMV titer was observed to be significantly reduced and the ELISA results indicated reduced virus load in treated cabbage plants compared with control plants. These results are consistent with those obtained in some previous studies, in which exogenous elicitors were found to provide protection against viral diseases in plants (Mushtaq et al. 2020; Shah et al. 2020). Additionally, we demonstrated that liquiritin elicitation induced systemic resistance by upregulating certain defense responses in Chinese flowering cabbage. Thus, we speculate that liquiritin-mediated disease (CMV) suppression in Chinese cabbage is likely a consequence of an induction of defense/resistance responses. Overall, we determined the efficacy of the exogenously applied elicitor by evaluating the reduction in disease severity and virus titer and expression patterns of defense-related genes, and by doing so, characterized the role of different factors contributing to the induction of resistance.

Elicitor molecules trigger disease resistance in plants, and it is well established that elicitors are perceived by corresponding arrays of receptors molecules. This detection in turn activates associated signaling cascades, which promote changes in the physiological state of affected plants. To validate the hypothesis of induced resistance, in the present study, we assessed elicitor-triggered immunity against plant pathogens, and accordingly detected elevated levels of different defense-related compounds in treated plants, which peaked on the 2nd day after elicitor treatment. Notably, it was found that the defense-related biochemicals triggered by liquiritin are comparable to those induced by treatment with the positive control BTH. In this regard, the upregulated production of phenolic compounds and enzymes that participate in phenylpropanoid pathways, such as PAL, PO, and PPO, has been recognized as a hallmark event of induced resistance mediated by exogenous elicitors (Conrath et al. 2001; Vallad and Goodman 2004). Subsequent to CMV infection, we found that plants pre-treated with exogenously applied liquiritin accumulated higher levels of total phenolics and defense-related enzymes compared with control plants, thereby signifying that liquiritin triggers an enhancement of resistance by stimulating different defense mechanisms in plants of Chinese flowering cabbage.

It has been reported that the defense responses of plants are closely associated with the salicylic acid and/or jasmonic acid signaling pathways (Pieterse et al. 2001; Kmiciek et al. 2016). Furthermore, it has been demonstrated that liquiritin priming results in the up-regulation of defense-related genes, thereby enhancing pathogen resistance (Niu et al. 2011). In the present study, we also observed that the expression of PR-related genes was enhanced in Chinese flowering cabbage plants treated with exogenous elicitors, which is consistent with the findings of previous studies and thus provides compelling evidence that PR-related genes also play roles in mediating certain defense responses against plant pathogens. The likelihood that liquiritin activates the salicylic acid-dependent signaling pathway was also reinforced by the similar expression pattern profiles of PR-related genes in CMV-infected plants pre-treated with liquiritin and BTH (positive control).

In addition to the beneficial effects of liquiritin with regards to CMV disease resistance, we also observed increases in the marketable yield and nutritional quality of Chinese flowering cabbage plants grown under field conditions, with the former being significantly higher than that of the control plants. Furthermore, UFLC-QToF-MS/MS analysis in the MRM mode revealed significant increases in the levels of certain glucosinolates in response to treatment with the foliar elicitor. Moreover, in leaf samples of cabbage plants treated with elicitors, we also detected higher levels of phenolic acids, which can contribute to reducing plant susceptibility to pathogenic agents (Mandal et al.
2010), as well as similar significant increases in bound phenolic acids (Figure 4).

Conclusion
In this study, we have shown for the first time that exogenous application of liquiritin can induce CMV resistance in Chinese flowering cabbage plants. Pre-treatment with liquiritin promoted significant increases in the concentrations of different defense-related biochemicals and PR genes, which were well correlated with induced resistance in response to this foliar elicitor. Our findings also indicate that levels of valuable nutritional and medicinal compounds in the leaves of Chinese flowering cabbage plants can be enhanced by the foliar application of liquiritin.

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All data generated or analyzed during this study are included in this article. WA, TA, and BA conceived and designed the experiments. WA, SF, SA, AA, SF, MA, JG, LW, SA performed the experiments. NAY, WA, BA, MIS, and GL analyzed the data. WA, GL, and NAY wrote and revised the manuscript.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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Notes on contributors
Dr. Waheed Akram received the Ph.D. degree in Agricultural Sciences, from the University of the Punjab, Pakistan, in 2015. He currently works as an assistant professor at the Faculty of Agricultural Sciences, University of the Punjab, Pakistan. His current research interests include biocontrol of plant diseases. The author has got many publications dealing with induced systemic resistance and plant stress physiology.

Dr. Ageel Ahmad received the Ph.D. degree in Agricultural Sciences, from the University of the Punjab, Pakistan, in 2015. He currently works as an assistant professor at the Guangdong Academy of Agricultural Sciences, China. He has published numerous research articles on plant stress management and induced resistance.

Dr. Sabin Fatima received the Ph.D. degree in the field of Agricultural Sciences, from the University of the Punjab, Pakistan, in 2016. Currently she is serving as Assistant Director of Agriculture, Directorate General of Pest Warning and Quality Control of Pesticides Punjab, Pakistan. Her research work mainly deals with induced systemic resistance.

Prof. Dr. Tehmina Anjum is currently serving the Department of Plant Pathology of the Faculty of Agricultural Sciences, University of the Punjab, Pakistan. Her research work mainly deals with plant biochemistry, induced systemic resistance and plant stress physiology.

Dr. Basharat Ali is serving the Institute of Microbiology and Molecular Genetics of the University of the Punjab, Pakistan. He mainly deals with microbiology, biocontrol and characterization of plant growth promoting bacteria.

Dr. Shakeel Ahmad is serving as adjunct professor in Instituto de Farmacia, Facultad de Ciencias, Universidad Austral de Chile, Chile. The author has completed PhD from Huazhong Agricultural University, China. His areas of specialization include plant biochemistry and stress physiology.

Dr. Mario Juan Simirgiotis is director of the Instituto de Farmacia, Facultad de Ciencias, Universidad Austral de Chile, Chile. The author is specialized in the area of plant biochemistry. He has got a number of publications in peer reviewed journals. He has been involved in different research projects with plant biochemistry, medicinal botany, pharmacology.

Dr. Hafiz Muhammad Khalid Abbas is a post doctorate fellow in Guangdong Academy of Agricultural Sciences, China. He completed PhD from Huazhong Agricultural University, China, in Plant Pathology. His core expertise in plant pathology and biotechnology.

Muhammad Aslam is serving as Director General of Pest Warning and Quality Control of Pesticides Punjab, Pakistan.

Miss Juxian Guo is working as researcher at the Vegetable Research Institute of the Guangdong Academy of Agricultural Sciences, China. Her research areas include quality improvement of vegetables and vegetable propagation.

Dr. Luo Wenlong is working as associate researcher at the Vegetable Research Institute of the Guangdong Academy of Agricultural Sciences, China. His research areas include plant genetics, QTL findings, and plant breeding.

Dr. Mei Fu is working as post-doctorate fellow researcher at the Vegetable Research Institute of the Guangdong Academy of Agricultural Sciences, China. Her expertise are in the field of plant genetics and plant biotechnology.

Prof. Dr. Guihua Li is serving the Vegetable Research Institute of the Guangdong Academy of Agricultural Sciences, China, as director Brassica research group. She has made a lot of publications in plant genetics and plant breeding. Currently, she is hosting different research projects mainly dealing with the plant breeding and plant biotechnology.

ORCID
Waheed Akram http://orcid.org/0000-0003-3811-6677

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