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Enhancing Citrus Resistance against Huanglongbing using Plant Activators and Copper Acetate

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Citrus greening (Huanglongbing or HLB) is one of the most destructive citrus diseases. It is caused by the phloem-limited bacterium *Candidatus Liberibacter* spp. and is difficult to control. Therefore, this study was conducted to evaluate the effect of plant activators (Benzoic acid, Citric acid, Calcium chloride dihydrate, Di-Potassium hydrogen phosphate (K_2HPO_4), Potassium Di Hydrogen Phosphate KH_2PO_4, Salicylic acid) and copper acetate against the citrus greening. For this purpose, one-year-old citrus plants of a susceptible variety were collected from citrus research institute Sargodha (CRI) and planted (under RCBD) in the research field area of the department of Plant Pathology, University of Agriculture Faisalabad. All plant activators and copper acetate were applied at three concentrations (0.5, 0.75, and 1%) and inoculum of citrus HLB was applied after one day after application of activators and copper acetate. Among all plant activators and copper acetate, salicylic acid expressed the most significant results at all concentrations against greening disease. So, it is concluded that the application of salicylic acid is a meaningful control strategy against citrus greening.
Keywords: *Candidatus liberibacte*, benzoic acid, systemic acquired resistance, copper acetate, Huanglongbing, *Citrus sinensis*.

INTRODUCTION

Citrus crop is prone to attack by a number of diseases like canker, scab, melanose, withertip, gummosis, Tristeza virus, decline, brown leaf spot, and greening (Huanglongbing). These diseases, particularly greening, pose a global threat to citrus production (Batool et al., 2007). However, all over the world, the most destructive disease of citrus is greening which is also known as Huanglongbing (Das et al., 2019). In Asia and Africa, it has been estimated that, at least 60 million trees have been discarded due to citrus greening (Timmer et al., 2003). It is caused by a bacterial pathogen, Candidatus *liberibacter* which parasites vascular bundles of citrus. There are three different strains of *Candidatus liberibacter* bacteria, named as Africanus (Laf), Americanus (Lam), and Asiaticus (Las) (Teixeira et al., 2005a; Teixeira et al., 2005b; Teixeira et al., 2005c; Wang, 2019). Visual symptoms of citrus greening develop on leaves and fruits. One or more shoots become yellow and show HLB symptoms. Infected leaves exhibit irregular yellow to green areas, often described as a blotchy mottle appearance. In advanced stages of the infection, symptoms like zinc deficiency can be observed followed by leaf and twig drop (Jin, 2017). The infected fruits remain small, asymmetrical, the stylar end remains green when they ripen, and that is why it is called "greening" (Bove, 2006). On cutting the fruit half, dark ended seeds can be observed and vascular bundles in the fruit axis are discolored (Bove, 2006). Citrus greening transmission is an important issue in controlling the disease. It can be spread through vector and grafting of the healthy plant with an infected plant (Batool et al., 2007). There are two species Asiatic Psylla (*Diaphorina citri*) and African Psylla (*Triozaerytreae*) which are responsible for the transmission of citrus greening in persistent means (Jin, 2017).

In recent years, Systemic acquired resistance (SAR) is becoming an effective strategy to control different diseases of cultivated crops with highly promising results. So, it can be used for controlling those diseases in which a conventional

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control strategy is less effective (Barros et al., 2010). There is a natural protection mechanism in plants that can be activated by the attack of pathogens or by resistance inducers (Durrant & Dong, 2004; Van Loon et al., 2006; Oliveira et al., 2016). Resistance inducer activates a different type of resistance in plants, some activate Systemic induced resistance (SIR) while some induce Systemic acquired resistance (SAR). SAR is involved in systemic nonspecific defense mechanism which is the most effective against pathogens (Bagio et al., 2016). The mechanism of SAR includes a particular defense signaling pathway in which signal molecule, salicylic acid is involved and is allied with the accumulation of Pathogenesis related (PR) Proteins (Vallad & Goodman, 2004; Aranega-Bou et al., 2014). Plant activators are eco-friendly compounds that are responsible for inducing resistance in plants against many plant pathogens by activating the defense genes in plants by transmitting signals through the signal transduction pathway which is facilitated by salicylic acid (Sreeja, 2014; Hu et al., 2018). Their injurious effects on human health and the environment are minimal because they lack direct antibiotic or pesticidal activity (Haq et al., 2021). Moreover, the resistance of plant pathogens to these plant activators. is unlikely to develop because they don't interact directly with the pathogens (Huang & Hsu, 2003). New strategies need to be adopted to combat pathogen aggressive and virulent strains (Jalali et al., 2006), which exhibit physiological and biochemical changes like lignin formation, Pathogen related proteins, and phytoalexins production in plants after application. It is assumed that plant activators have incredible effects against pathogens. Therefore, in the present study different plant activators were applied against citrus greening to evaluate their efficacy against this disease.

MATERIALS AND METHODS

Collection of Citrus plants**:** Citrus plants of 1-year age of a susceptible variety (Grapefruit) were collected from citrus research institute Sargodha and planted in the filed area of the University of Agriculture Faisalabad under RCBD design, having 3 feet row to row and 3 feet plant to plant distance. All the horticultural practices were done at the proper time to keep the plants in good condition.

Preparation and application of treatments**:** Three different concentrations (0.5, 0.75 and 1%) of plant activators (Benzoic acid, Citric acid, Calcium chloride dihydrate, Di-Potassium hydrogen phosphate (K_2HPO_4), Potassium Di Hydrogen Phosphate KH_2PO_4, Salicylic acid) and Copper acetate were made by adding 5, 7.5 and 10g into each bottle of an activator and copper acetate, having 1 liter distilled water and then applied on plants early in the morning. Distill water as control was also applied to plants for comparison.

Inoculum preparation and pathogen inoculation**:** Meanwhile, citrus leaves which were showing symptoms of citrus greening were collected in plastic bags from the different fields of district Sargodha, Pakistan, and stored in the refrigerator at 4℃. Symptomatic leaves were grinded and passed through a muslin cloth to remove any material other than sap and then stored. Sap obtained from citrus greening infected leaves were used to inoculate plants. Plants were inoculated by spraying sap on plants and by injecting sap into leaves and trunk early in the morning after 24 hours of application of plant activators and copper acetate.

Statistical analysis**:** An experiment was performed under Randomized Complete Block Design (RCBD). Weekly basis data were recorded to evaluate the efficacy of plant activators and copper acetate against citrus greening.

RESULTS

Evaluation of plant activators and copper acetate against citrus greening disease under field conditions**:** Significant results were expressed by all the treatments (T), concentrations (C), Weeks (W), and their interactions (Treatments × Concentrations, Treatments × Weeks, Concentrations × Weeks, and Treatments × Concentrations × Weeks). Minimum disease incidence was expressed by salicylic acid (30.96) followed by Potassium-Di-Hydrogen Phosphate (32.44), Citric acid (38.22), Calcium Chloride Dihydrate (42.22), Benzoic acid (44.00), Di Potassium Hydrogen Phosphate (45.56) and Copper acetate (56.82) as compared to control (Table 1). The interaction between Treatments (T) × concentrations (C) showed that salicylic acid exhibited minimum disease incidence (41.33, 32.00 and 19.56 percent) at three concentration (0.5, 0.75 and 1 percent) respectively while potassium-di-hydrogen phosphate showed 41.33, 34.66, 21.33 percent, citric acid 52.00, 34.66 and 28.00 percent, Calcium chloride dihydrate 56.00,38.66 and 32.00 percent, benzoic acid 54.66, 44.00 and 33.33 percent, Di Potassium Hydrogen Phosphate 57.33, 44.01 and 35.33 percent and copper acetate 59.80, 58.66 and 52.00 percent disease incidence when applied at 0.5, 0.75 and 1 percent concentrations respectively (Figure 1). The interaction between treatments (T) and weeks (W) showed that all treatments viz; salicylic acid (12.61), Potassium Dihydrogen phosphate (13.33), Citric acid (13.33), Calcium Chloride Dihydrate (13.33), Benzoic acid (13.33), Di Potassium Phosphate (15.55) and copper acetate (20.00) as compared to control expressed minimum disease incidence at 1st week followed by 2nd week (22.22, 22.22, 20.00, 26.66, 24.44, 28.91 and 37.77), 3rd week (31.11, 31.11, 37.77, 40.00, 42.22, 42.22 and 57.778), 4th week (40.00, 42.22, 53.33, 57.77, 60.00, 60.00 and 75.55), and 5th week (48.88, 53.33, 66.66, 73.33, 80.00, 81.11 and 93.00) respectively (Figure 2). However, the interaction between treatments (T) × concentrations (C) × Weeks (W) exhibited that minimum disease incidence was shown by salicylic acid in 1st (20.00, 13.33 and 4.5 percent), 2nd (26.66, 26.66 and 13.33 percent), 3rd (40.00, 33.33 and 20.00 percent), 4th (53.33, 40.00 and

26.66 percent) and 5th (66.66, 46.66 and 33.33 percent) week, when applied @ 0.5, 0.75 and 1% concentration respectively, followed by Potassium Di Hydrogen Phosphate showed disease incidence in 1st (20.00, 13.33 and 6.66 percent), 2nd (26.66, 26.66 and 13.33 percent), 3rd (40.00, 33.33 and 20.00 percent), 4th (53.33, 46.66 and 26.66 percent) and 5th (66.66, 53.33 and 40.00 percent) week, citric acid in 1st (20.00, 13.33 and 6.66 percent), 2nd (33.33, 13.33 and 13.33 percent), 3rd (53.33, 33.33 and 26.66 percent), 4th (66.66, 46.66 and 46.66 percent) and 5th (86.66, 66.66 and 46.66 percent) week, Calcium chloride dihydrate in 1st (20.00, 13.33 and 6.66 percent), 2nd (40.00, 20.00 and 20.00 percent), 3rd (53.33, 33.33 and 33.33 percent), 4th (73.33, 53.33 and 46.66 percent) and 5th (93.33, 73.33 and 53.33 percent) week, Benzoic acid in 1st (20.00, 13.33 and 6.66 percent), 2nd (33.33, 26.66 and 13.33 percent), 3rd (53.33, 40.00 and 33.33 percent), 4th (73.33, 60.00 and 46.66 percent) and 5th (93.33, 80.00 and 66.66 percent) week, Di Potassium Hydrogen Phosphate in 1st(26.66, 13.33 and 6.66 percent), 2nd (40.00, 26.66 and 20.00 percent), 3rd (53.33, 40.00 and 33.33 percent), 4th (73.33, 60.00 and 46.66 percent) and 5th (93.33, 80.00 and 70.00 percent) week and Copper acetate in 1st (20.00, 20.00 and 20.00 percent), 2nd (40.000, 40.00 and 33.33 percent), 3rd (60.00, 60.00 and 53.33 percent), 4th (80.00, 80.00 and 66.66 percent), 5th (99.00, 93.33 and 86.66 percent) week, when applied at 0.5%, 0.75% and 1% concentrations respectively, as compared to control (Figure 3).

Table 1. Evaluation of different plant activators and copper acetate against citrus greening under field conditions.

Treatments	Manufacturer	Disease incidence (%)
Salicylic acid	Henan Xingfa Bio-Technology Co., Ltd. Henan, China	30.967h
Potassium Di Hydrogen Phosphate	Shaanxi Iknow Bio-Technology Co., Ltd. Shaanxi, China	32.444g
Citric Acid	GoodchemTechnology Co., Ltd. Shanghai, China	38.222f
Calcium Chloride Dihydrate	Weifang Longhong Chemical Co., Ltd. Shandong, China	42.222e
Benzoic Acid	A.M Food Chemical Co., Ltd. Shandong, China	44.000d
Di Potassium Hydrogen Phosphate	Shaanxi Iknow Bio-Technology Co., Ltd. Shaanxi, China	45.561c
Copper Acetate	WujiangWeishida Copper S&T Co., Ltd. Jiangsu, China	56.822b
Control		61.233a
LSD	0.2249	

*Mean values in a column sharing similar letters do not differ significantly as determined by the LSD test (P ≤ 0.05).

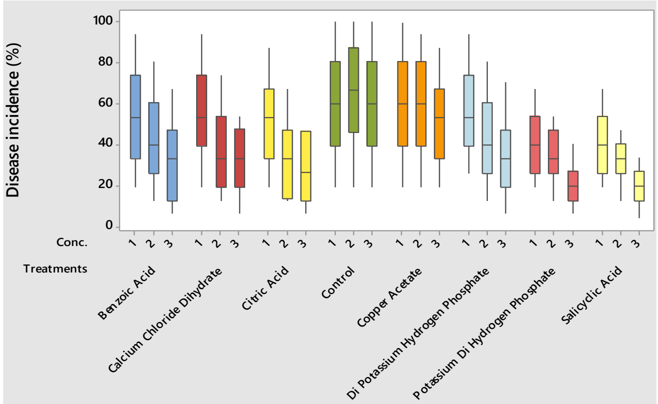

Figure 1. Impact of interaction between treatments and concentrations (0.5, 0.75 and 1%) against citrus greening disease under field conditions.

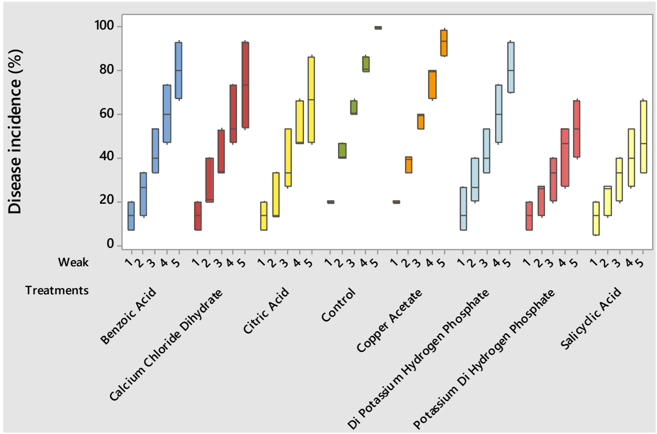

Figure 2. Impact of interaction between treatments and weeks against citrus greening disease under field conditions.

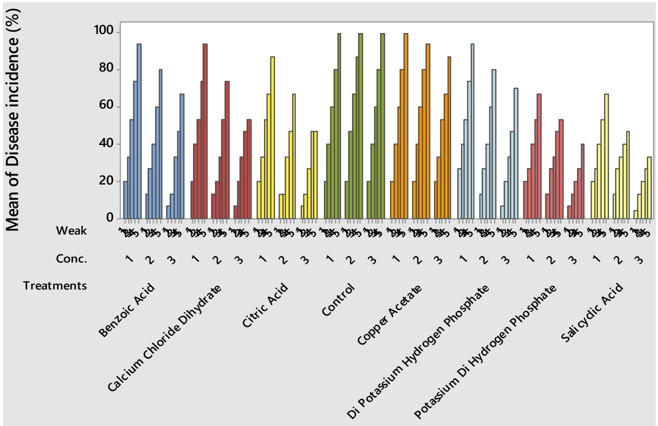

Figure 3. Impact of interaction between treatments, concentrations and weeks against citrus greening disease under field conditions.

DISCUSSION

***Evaluation of plant activators and copper acetate against citrus greening disease*:** There is a natural protection mechanism in plants that can be activated by the attack of pathogens on plants (Oliveira et al., 2016; Durrant & Dong, 2004; Van Loon *et al*., 2006; Usman *et al*., 2024; Matloob *et al*., 2025). Delayed response or inactivation of plant defense mechanisms leads to the development of the disease. Activation of the plant defense system and producing incompatibility with a pathogen, generally depends on the resistant genes present in the plant.

Six plant activators (Salicylic acid, benzoic acid, KH2PO4, calcium chloride dihydrate, K2HPO4, and citric acid) and copper acetate were evaluated against citrus greening disease and the minimum disease incidence was exhibited by salicylic acid. Plant activators activate the plant defense system and former susceptible become resistant when applied exogenously at a concentration that is non-toxic to plant (Jalali et al., 2006; Elwan & El-Hamahmy, 2009; Yaqoob et al., 2024). Salicylic acid is one of the key phytohormones which enhances the growth and resistance of plants because of the accumulation of IAA (Indole acetic acid). Moreover, a suitable quantity of salicylic acid can increase the activity of resistant genes (Howard et al., 2000; Ye & Ng, 2002; Khan et al., 2003). These results were supported by the work of Hu et al. (2018), who observed that the application of salicylic significantly controls the citrus greening disease through mediating plant systemic acquired resistance (SAR). Injecting salicylic acid in citrus greening infected plants induced the expression of SAR related PR (pathogen-related) proteins such as PR1, PR2, PR3, and PR15. Moreover, Bacteria causing citrus greening disease degrades the salicylic acid present in plants by encoding a functional salicylic acid hydroxylase (Li et al., 2017). However, the application of salicylic acid seems to be able to overcome the degradation of Salicylic acid through an enzyme released by *Candidatusliberibacter* (Hu et al., 2018). During MAMP-triggered Immunity or Effector-triggered immunity, the level of the plant hormone salicylic acid becomes elevated (Iwai et al., 2007, Nobuta et al., 2007; Garcion et al., 2008; Palmer et al., 2017). In fact, studies have shown that salicylic acid is both required and sufficient to activate plant defense against biotrophic and semi-biotrophic pathogens. In salicylic acid biosynthesis mutants lacking salicylic acid accumulation, the plant is severely limited in its ability to withstand infection by biotrophic and semi-biotrophic pathogens (Fu & Dong, 2013). Exogenous application of salicylic acid or one of its active analogs is sufficient to upregulate plant defense against biotrophic and semi-biotrophic pathogens (Lu, 2009). Besides functioning in systemic acquired resistance, salicylic acid has also been shown to interfere with quorum sensing of bacterial pathogens (Joshi et al., 2016; Gilani *et al*., 2025). Also, salicylic acid reduces the production of virulence factors and inhibits the type III secretion system. For example, salicylic acid can significantly inhibit three known virulence factors in *Pseudomonas aeruginosa*: pyocyanin, proteases, and elastase (Prithiviraj et al., 2005; Bandara et al., 2006). Salicylic acid and its derivatives were also found to inhibit the expression of the type III secretion system in *Erwinia amylovora* and *Chlamydia* pneumonia (Bailey et al., 2007; Felise et al., 2008). The promoter activity of the *E.amylovorahrpA*gene, which encodes a type III pilus, could be severely inhibited by salicylic acid *in vitro* (Khokhani et al., 2013).

***Conclusion*:** The present study demonstrates that the use of plant activators, particularly salicylic acid, offers a promising and eco-friendly approach to manage citrus greening disease (HLB). Among all tested compounds, salicylic acid exhibited the highest efficacy in reducing disease incidence across all concentrations and time intervals, indicating its potential to activate systemic acquired resistance (SAR) in citrus plants. This strategy not only minimizes environmental risks but also reduces the likelihood of pathogen resistance development. Therefore, integrating salicylic acid into citrus disease management programs could significantly enhance the resilience of citrus crops against greening disease and contribute to sustainable citrus production.

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***Availability of data*:** The data is available with the corresponding author which can be made available on request.

***Consent to participate*:** All participants consented for this research study.

***Informed consent*:** The participants signed informed consent regarding publishing their data and photographs.

***Consent for publication*:** All authors submitted consent to publish this research article in JGIAS.

***SDGs addressed*:** No poverty; Zero hunger; good health and well-being.

***Policy referred*:** Biochemical-Based Disease Control Strategy; Integration into Citrus Disease Management Programs

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