Research Article

Evaluation of Organic Acids to Determine Antifungal Potential against Green Mold of Citrus (Kinnow Mandrin) Caused by Fungus *Penicillium Digitatum* (Pers. Fr.) Sacc

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Abstract | Present study was designed to develop alternative methods to synthetic fungicides, currently used to control green mold of citrus caused by the *Penicillium digitatum*. Antifungal activities of organic elicitors were evaluated and all the elicitors expressed significant results. Salicylic acid was the most effective to control the disease and showed minimum mycelial growth diameter (35mm) followed by benzoic acid (44mm), jasmonic acid (45.5), and ascorbic acid (50mm) as compared to control (92mm) treatment. Organic elicitors activated the defense system, by inducing the biochemical alterations. These treatments effectively enhanced the activities of peroxidase (POD), Superoxide dismutase (SOD), and total phenolic contents. Salicylic acid showed highest POD activity (8.4 U/min g protein), SOD (6.96 U/ min g protein) and TPC (3.21 mg GAE/ 100g of sample) as compared to control where POD (1.13 U/min g protein), SOD (1.46 U/ min g protein), and TPC (1.14 mg GAE/ 100g of sample). Amount of hydrogen peroxide (H_2O_2), the signaling molecule was also significantly increased (30.33 mmol/ mg FW) and catalase (CAT) was higher (91 U/min g protein) in fruit treated with ascorbic acid as compared to control (22.67 U/min g protein) treatment. These results indicated that application of organic elicitors could increase the disease resistance by H_2O_2 elevation and by induction of antioxidant enzymes. This study was planned to evaluate the organic elicitors and to determine the biochemical changes in citrus fruit.

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Introduction

Citrus is one of the most important world fruit crop as well as in Pakistan. Several diseases such as, blue mold, green mold, alternaria rot, anthracnose and stem end rot negatively effect citrus fruits during postharvest phase. Green mold of citrus caused by *Penicillium digitatum* is potential threat, among all diseases (Shi et al., 2018). In Pakistan, due to postharvest diseases, 40% of the total citrus is wasted during storage (Naseer, 2010), mainly due to the attack of fungal pathogens (Liu et al., 2007) *P. digitatum* affects the fruits in orchards during packing, storage and marketing (Palou et al., 2008). The pathogen is necrotrophic, enter the fruit through injuries in rind caused during transportation (Ballester et al., 2010; Palou, 2009). Due to attack of *P. digitatum* soft watery spots followed by white mycelium production on the surface of lesion and then diameter of lesion increases and olive green spores produced on the surface of

infected fruit (Boubaker et al., 2009). In this alarming situation the use of chemical based fungicides, is main factor for the management of citrus diseases during storage (Hao et al., 2011). However, the use of synthetic fungicides has harmful effect on human health, environment and development of resistant strains of fungus against fungicides (Khamis et al., 2012), therefore chemical based method is highly discouraged these days.

So it is the need of the hour to develop an alternative control measures to control postharvest disease like green mold. The demands of eco-friendly organic produce with minimal residual effect is gaining importance in market. Natural resistance of plants against pathogens is activated through physical, biological and chemical elicitors. (Zafar et al., 2012). Currently many studies have revealed that induction of resistance in plants by using abiotic and biotic elicitors is an efficient method to control the fungal diseases spread. (Gianfranco et al., 2016; Droby et al., 2000; Soylu et al., 2003). Resistance of plants against pathogens is based on inducible defense mechanism and pre-existing antimicrobial compounds. Application of abiotic and biotic treatments to induced disease resistance in plants against pathogens is very attractive approach against diseases (Droby et al., 2002; Qin et al., 2003).

Salicylic acid (SA) is the resistance inducer and have the antifungal potential against a number of pathogens on citrus, pear and mango (Zainuri et al., 2001; Shaat and Galal, 2004; Cao et al., 2006). The signal molecules such as jasmonic acid (JA) and SA play a vital role in plant development and growth with responses to abiotic and biotic stresses (Yao and Tian, 2005).

Defense compounds are formed by particular enzymes which catalyze biosynthetic reactions which is due to the signal molecules involved in signal transduction system (Fu and Dong, 2013). This triggers the resistance in plants known as induced resistance which activates the defense mechanism and gives protection to plants against pathogens (Pieterse et al., 2012; Walters et al., 2013; Pieterse et al., 2014). Such compounds, move systemically when applied exogenously and induces resistance against pathogens and rapid defense response expression are produced by these compounds (Conrath et al., 2002; Fu and Dong, 2013).

Quick and massive production of reactive oxygen species (ROS) within host cells, referred to as an oxidative burst, have an important role in defense mechanism of host. In number cases the first response of a plant to a pathogen comprises the production of hydrogen peroxide (H_2O_2), which act as a substrate for the oxidative cell wall protein cross-linking and is involved in lignification. In addition, the toxicity of H_2O_2 may directly inhibit pathogen growth. Due to infection caused by *P.* digitatum, production of reactive oxygen species is retarded which elicits the defense mechanism (Macarisin et al., 2007). Antioxidant enzymatic activities of superoxide dismutase (SOD) and catalase (CAT) maintains the ROS homeostasis, which directly plays a role in conferring resistance to an extensive range of pathogens (Mittler, 2002). The antioxidant enzymatic activities of SOD and CAT in fruits infected with *P. digitatum* shows a significant drop after infection (Torres et al., 2011).

The objective of this study was to investigate antifungal activities of postharvest application organic elicitors against *P. digitatum* and to monitor the changes in activities of defense enzymes peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), total phenolic contents (TPC) and hydrogen peroxide (H_2O_2) production.

Materials and Methods

Plant material

Kinnow mandarin (*Citrus reticulata* B.) were harvested from an orchard (8 year old) growing in Chak No. 104, Silanwali road, District Sargodha, Punjab, Pakistan (31.8162° N, 72.5382° E). After harvesting, fruit were transferred to laboratory, Department of Plant Pathology, College of Agriculture, University of Sargodha, Punjab, Pakistan, and washed with tap water. Mature fruit with uniform size and without wounds were stored in growth room at 25± 2 °C.

Fungal cultures

P. digitatum was isolated from diseased kinnow mandarin, obtained from orchard grown in Bhalwal, District Sargodha, Punjab, Pakistan (32.2751° N, 72.9047° E). A seven days old culture of fungus was grown on Potato Dextrose Agar (PDA) media. This culture was used as a source of inoculum. The cultures were stored at 8± 0.5°C for future work in refrigerator (PEL), Pakistan (Pak Elektron Limited).

Postharvest dip application of different concentrations of salicylic acid (SA), jasmonic acid (JA), ascorbic acid (AA) and benzoic acid (BA) on disease development

Mature fruit having uniform size, free from visible disease symptoms were harvested from unsprayed orchard. Fruit were surface sterilized with 1% (v/v) sodium hypochlorite for 2min, washed with sterile water and then those fruit were air dried in laminar hood. The fruit were dipped into an aqueous solution of SA (3mM, 5mM, and 7mM), JA (3mM, 5mM, and 7mM), AA (3mM, 5mM, and 7mM) and BA (3mM, 5mM, and 7mM) for 10min. The control fruit were dipped into sterilized distilled water for 10min. The experiment was performed under completely randomized design with four treatments, three concentrations and 3 replications**.** Sterilized cork borer protruding 3 mm was used to make two holes in the rind of the fruit. A conidial suspension of *P. digitatum* (10^7 conidia ml^{-1}) was prepared and with the help of micropopette (100 µl) 10 µl was injected into each hole (Janisiewicz et al., 2000). The treated fruit were put into corrugated boxes and stored for 7 days at 90± 2% relative humidity and 25±1 ° C temperature in growth room. Diameter of mycelial growth was measured after 4 and 7 day of inoculation.

Determination of superoxide dismutase (SOD), peroxidase (POD), total phenolic contents (TPC), catalase (CAT) and hydrogen peroxide activity (H_2O_2)

Sample preparation: Buffer solution was prepared which contained 100ml distilled water, Na_2HPO_4 (2.82 g /100 ml of water and NaH_2PO_4 (3.3 g/100mlof water) and pH was maintained 6.8. One gram of sample was grinded in 20µl buffer solution with pestle and mortar and transferred into eppendorf tube. (Alici and Arabaci, 2016).

Superoxide dismutase (SOD) activity: The superoxide dismutase activity was determined as described previously (Giannopolitis and Ries, 1997). The solution was prepared containing 0.222g of methionine in distilled ionized water (15 ml), 0.2 molar buffer, distilled ionized water (17.5 ml) containing 0.013g of Riboflavin, 17.5 ml (DI water) having 0.015g of NBT and 0.0375 ml of Triton-X in 17.5 ml of distilled ionized water. Eppendorf tubes which contains the reaction mixture was retained under UV lamp for 15 minutes then riboflavin was added and absorbance was noted at 560 nm in spectrophotometer (96 micro well plate reader Bio Tek, model, H-Quant™, Winooski, VT, USA.).

Catalase (CAT) and peroxidase (POD) activity: Activities of CAT and POD was calculated by following the procedure of Chance and Maehly (1995) with some amendments. For CAT activity, reaction solution contained phosphate buffer of pH= 7 (50 mM), gum solution (0.1 mL) and hydrogen peroxide 5.9 mM. The reaction started by addition of test sample. This solution was poured in 96 well plate and was placed in spectrophotometer at 240 nm and reading of absorbance was observed. Reaction solution to observe the activity of POD contain phosphate buffer pH=5 (50 mM), H_2O_2 40mM, guaiacol (20mM) and 0.1 mL gum solution. Sample was added in this reaction mixture and then changes in absorbance was determined at 470nm, three replicates were used.

Total phenolic contents (TPC): Total phenolic contents were measured by following Folin-Ciocalteus reagent procedure, as described by (Ainsworth and Gillespie, 2007). In F-C reagent of 200 µL was added to each sample (100 µL) and vortexed it carefully. After this, 800 µL of Na_2CO_3 (700mM) was poured into each sample and at room temperature it was incubated for two hours. Then 200 µL was taken and poured into 96 well plate, placed in spectrophotometer (96 micro well plate reader BioTek, model, H-Quant™, Winooski, VT, USA.) at 765nm absorbance was noted. TPC was measured by using Gallic acid curve of calibration.

$$Y = 0.0055x + 0.0987$$
$$R^2 = 0.9968$$

Determination of H_2O_2: Samples of fresh weight of 1g was standardized in trichloroacetic acid (0.1%) and was placed in centrifuge machine at 12000 rpm at 4° C for 15 min. Potassium phosphate buffer pH=7.0 (1.3 mL) and potassium iodide solution of 1M (1 ml) was mixed with 0.3 ml supernatant. This solution was incubated for 5 min at room temperature and then placed in spectrophotometer at 390nm and absorbance was measured. The amount of H_2O_2 was calculated from a standard curved obtained from recognized concentration of H_2O_2 (Velikova et al., 2000).

Statistical analysis

Data was statistically analyzed in Statistix 8.1 software. Performed ANOVA and statistical significance was assessed at p=0.05, LSD test was used to separate means Steel et al. (1997).

Results and Discussion

In vitro evaluation of organic elicitors to control green mold of citrus

In vitro antifungal effect of organic elicitors at different concentrations was evaluated against *P. digitatum* and mycelial growth diameter was determined. The concentration of all organic elicitors applied as postharvest treatment significantly reduced mycelial growth diameter. application of SA showed lowest mycelial growth diameter after seven days i.e. (35 mm) at 7mM, followed by benzoic acid (44 mm), Jasmonic acid (45.5 mm) and ascorbic acid (50 mm) at 7mM concentration as compared to control which was (92mm) after 7 days as shown in Figure 1.

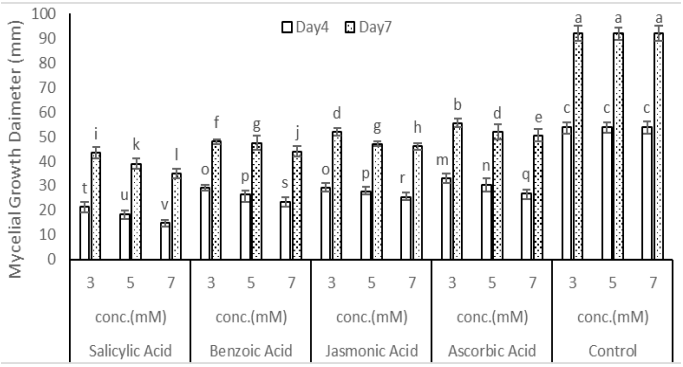

Figure 1: *Effect of organic acids on mycelial growth diameter on Kinnow mandrin fruit inoculated with P. digitatum. Statistical significance determined at p ≤0.05 according to the LSD test.*

Changes in defense related enzyme activity in treated fruit

Effect of organic elicitors on peroxidase (POD) and catalase (CAT) activity: Postharvest treatment of fruit with organic elicitors significantly induced the activities of peroxidase in fruit during storage. POD activity was significantly higher in fruit treated with SA (8.4 U/min g protein) at 7mM, (7.7 U/ min g protein) at 5mM and (7.03 U/min g protein) at 3mM concentration followed by BA (6.53 U/ min g^{-1} protein) at 7mM concentration as compared to control which showed minimum activity of POD (1.13 U/min g protein). All the treatments significantly ($p \leq 0.05$) increased the defense related enzyme POD as shown in Figure 2. Catalase activity was observed minimum in control fruit as compared to treated fruit. Among all treatments Salicylic acid showed minimum CAT concentration (45 U/min g protein) at 3mM concentration. Fruit treated with Ascorbic Acid showed maximum concentration of CAT 91 (U/min g protein) at 3mM concentration as shown in Figure 3.

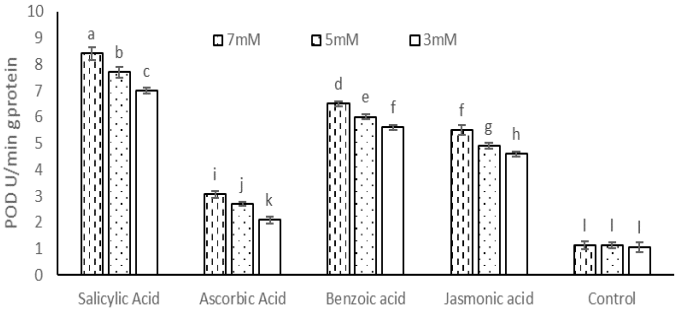

Figure 2: *Effect of organic acids on the level of POD in Kinnow mandarin fruit treated with P. digitatum. Statistical significance determined at p ≤ 0.05 according to the LSD test.*

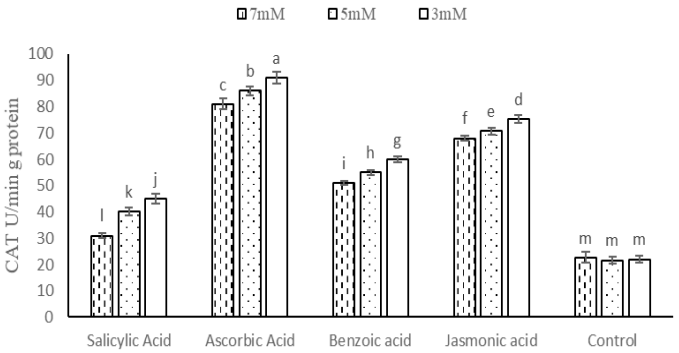

Figure 3: *Effect of organic acids on the level of CAT in Kinnow mandarin fruit treated with P. digitatum. Statistical significance determined at p ≤ 0.05 according to the LSD test.*

Superoxide dismutase (SOD) activity: Application of organic elicitors increased the defense related enzyme SOD. All the fruit treated with organic elicitors showed the increased level of SOD as compared to control. In control fruit minimum SOD activity was observed as compared to treated fruit. Fruit treated with ascorbic acid at 3mM concentration showed minimum SOD activity 2.23 (U/ min g protein), 2.46 and 2.66 at 5 and 7mm concentration respectively followed by BA, JA and SA, as shown in Figure 4.

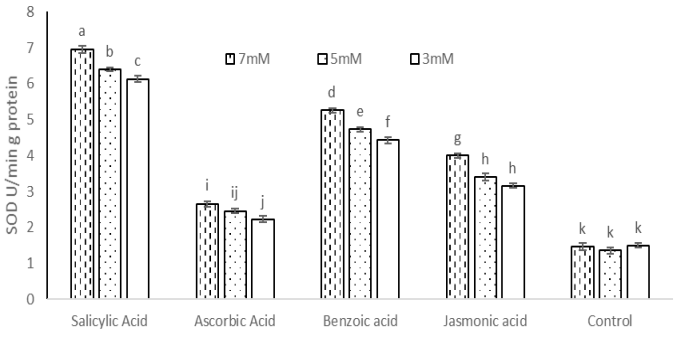

Figure 4: *Effect of organic acids on the level of SOD in Kinnow mandarin fruit treated with P. digitatum. Statistical significance determined at p ≤ 0.05 according to the LSD test.*

Hydrogen peroxide activity: Organic elicitors significantly increased the level of H_2O_2. H_2O_2 contents in fruit treated with SA was 34.66, 32.66,

30.66 at 7, 5 and 3mM concentration, followed by benzoic acid 30.33, 28.33, 26.67 (mmol/ mg FW) at 7,5 and 3mM concentration. All the treatments showed high level of H_2O_2 at all concentration as compared to control 9.66 (mmol/mg FW). Figure 5.

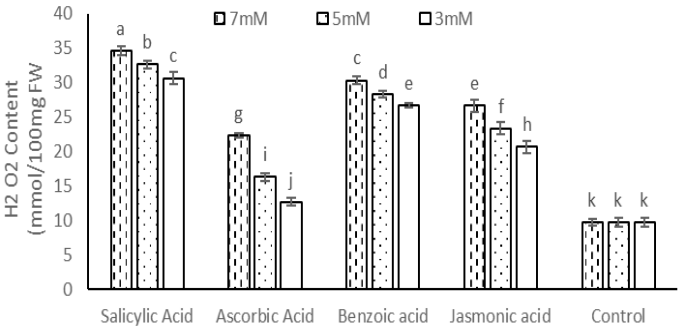

Figure 5: *Effect of organic elicitors on the level of H_2O_2 in Kinnow Mandrin inoculated with P. digitatum. Statistical significance determined at $p \leq 0.05$ according to the LSD test.*

Total phenolic contents (TPC): Total phenolic contents in fruit treated with organic elicitors was higher as compared to control. TPC in fruit treated with SA was 3. 21, 3.01 and 2.92 (mg GAE/ 100g of sample) at 7, 5 and 3mM concentration, followed by benzoic acid 2.82, 2.62 and 2.42 (mg GAE/ 100g of sample) at 7, 5 and 3mM concentration. All the treatments showed high level of TPC at all concentration as compared to control, as shown in Figure 6.

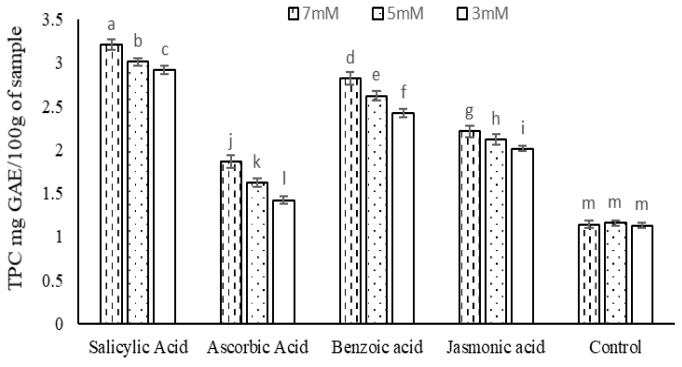

Figure 6: *Effect of organic acids on the level of TPC in Kinnow mandarin fruit treated with P. digitatum Statistical significance determined at $p \leq 0.05$ according to the LSD test.*

In current situation postharvest diseases are managed through the use of fungicides, but these have negative impacts on environment human and biodiversity. So, there is dire need of time to develop the alternative control measures for the management of green mold of citrus. To ensure food safety, organic elicitors can be used as an alternative of synthetic chemicals. However, it is necessary to evaluate different concentrations of organic acids, to determine the best concentration (Asghari and Aghdam, 2010). Therefore, in the current study organic elicitors were evaluated against *P. digitatum* causing green mold of citrus.

Results of present study showed that all the elicitors exhibited their efficacy against mycelial growth of *P. digitatum*. The treatments at 7mM were more effective in reducing the growth of pathogen as compared to other concentrations. SA was the most effective elicitor at all concentrations. All the organic elicitors showed significant results, it may be due to the direct fungitoxic and fungistatic effect of Salicylic acid, Jasmonic acid, Benzoic acid and Ascorbic acid. Fungitoxic effect of organic elicitors has previously been reported against many pathogens such as *Monilinia fructicola, Alternaria alternata* and *P. expansumm* in sweet cherry (Qin el al., 2003; Yao and Tian, 2005; Wang and Li, 2008). In present study the control of *P. digitatum*, SA showed best results by exhibiting less minimum mycelial growth diameter (35mm) followed by benzoic acid (44mm), Jasmonic acid (45.5 mm) and ascorbic acid (50 mm) at 7mM concentration. Results of present studies were similar with studies of Zafar et al., 2012. Strobel and Porter (2005), studied the effect of SA and observed 50% reduction of fungal growth at 2.0- 5.0 mM concentration and also reported that at concentration 0.5mM or lower had minimum effect on many plant pathogenic fungi. In plant growth regulation, in the responses to abiotic or biotic attack and interaction with other microorganisms SA has been found to play an important role (Yalpani et al., 1994; Senaratna et al., 2000). It has been proved to be a vital signaling molecule against many pathogenic organisms and plays an important role in disease resistance (Alverez, 2000). Jasmonic acid also showed the good results to control the fungus, which was also reported by Droby et al., 1999. Increasing the concentration of organic elicitor plays a vital role to enhance the systemic acquired resistance in plants (Verbene et al., 2000). Application of salicylic acid is responsible for the induction of resistance against pathogens. (Volt et al., 2009).

In addition to direct fungitoxic and fungistatic effect of organic elicitors, they are also related to involve the defense related mechanisms. Rapid generation of H_2O_2, has been reported as one of the earliest events correlated with resistance to plant pathogen (Baker and Orlandi, 1995) and is related to disease resistance in mango (Zeng et al., 2006), peach (Liu et

al., 2005) and pear fruit (Cao and Jiang, 2006). Fruit treated with salicylic acid showed increased level of H_2O_2 contents 34.66 (mmol/mg FW) followed by benzoic acid (30.33 mmol/mg FW) which was greater as compared to control. Therefore, reactive oxygen species (ROS) generation is the important part of organic elicitors- induced disease resistance mechanism in kinnow fruit. Although the production of ROS is involved in resistance to plant disease, high level production of ROS induce lipid peroxidation which is harmful for membrane integrity, which leads to the loss of membrane integrity of plant tissues. ROS are scavenged out by CAT which is an antioxidant enzyme and prevents the injurious effects of hydrogen peroxide on plant organs (Lamb and Dixon, 1997). The enzymes such as Super oxidase dismutase (SOD) and Peroxidase (POD) are also important in such action, SOD can defend cells from oxidant stress by dismutating SOD anion (O^-) to water which has a vital role in disease resistance of fruit. In addition, for defense reaction, oxidation of phenols may be due to the H_2O_2 generation (Chittoor et al., 1999), which is generally catalyzed by the enzyme POD. Induction of SOD by application of organic elicitors contribute to enhance H_2O_2 contents in tissues of fruit. It was also indicated that POD activity was increased by application of organic elicitors which may partly be involved in protecting the tissues from injury which is caused due to high level of ROS incited by application of these organic acids. Similar phenomena was also observed in the studies relevant to it on other fruits such as Peach, Pear and Mango (Liu et al., 2005; Cao and Jiang, 2006; Zeng et al., 2006). Production of POD is related with resistance to disease and helpful in phenolic cross link synthesis which involves in connecting biopolymer chains (Mohammadi et al., 2002). This indicates that increase in activity of POD is related to resistance of orange fruit against microorganisms. Plants having resistance against pathogen have increased level of POD (Percival, 2001). Toxic H_2O_2 is removed potentially with peroxidase (Passardi et al., 2004) and hydrogen peroxidase is highly toxic to many organisms (Wang et al., 2014). Total phenolic contents were higher in fruit treated with organic elicitors as compared to control fruit. Phenolic compounds may cause resistance to disease by limiting the growth of pathogen (Isaac, 1991).

Future directions

There is need of time to know the specific genes which are involved in the production of primary or secondary metabolites/ proteins and to study the mechanisms which are involved in the suppression of disease.

Conclusions and Recommendations

Organic elicitors activate the defense system of fruit by induction of H_2O_2, SOD, POD, CAT and TPC. It is concluded that organic elicitors could be used as alternative to synthetic fungicides, against *P. digitatum* causing green mold of citrus. Farmers should use organic elicitors instead of synthetic fungicides; which are toxic for humans and animals.

Author's Contribution

All authors contributed in this study. Dr. Zafar Iqbal designed the studies and helped in performing the experiment. Dr. Muhammad Atiq helped in performing the biochemical analysis. Awais Ahmed Khan performed the experiments and prepared the manuscript.

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