

# Potential of Resistance to *Penicillium* Spp. In Valencia Sweet Orange Through Use of Salicylic Acid and Methyl Jasmonate

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## Research Article

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# Abstract

Citrus green and blue moulds cause postharvest losses worldwide. Therefore, the potential of preharvest foliar application of salicylic acid (SA) and methyl Jasmonate (MeJ) was investigated to control infection of *Penicillium* spp. An aqueous solution containing different concentrations of SA (3, 6, 9 mM) or MeJ (3, 4, 5 mM) and 'Tween 80' (0.05%) as a surfactant were sprayed onto whole trees seven days before harvest. Among the treatments, the pre-harvest spray of SA (9 mM or MeJ 5 mM) showed good efficacy reducing colony growth, wound rotting (rotting of peel around the wound and spore mass density of *Penicillium* spp. when compared with control. A pre-harvest spray of SA (9 mM) reduced colony growth by 71.02% and 68.69% on fruit inoculated with *P. digitatum* and *P. italicum*, respectively. The activity of fruit softening enzymes markedly increased following fungal infection. The decay in the fruit was found to be associated with the upregulation of activities of exopolygalacturonase (exo-PG), endopolygalacturonase (endo-PG) and Endo-1,4- $\beta$ -D-glucanase (e-gase). However, in un-inoculated fruit, negligible enzyme activity was observed. Contrarily the SA-treated fruit showed less activity of exo-PG, endo-PG and e-gase enzymes. These findings clued to develop natural control of *Penicillium* spp through use of organic elicitors in sweet orange fruit.

## Introduction

Sweet orange is grown in tropical and subtropical regions of the world and also an economically important fruit crop in Australia. Sweet orange fruit is a rich source of vitamins and minerals. Sustainable citrus production is vulnerable to green mould [*P. digitatum* (Pers. Fr.) Sacc.] and blue mould (*P. italicum* Wehmer) which are serious pathogens causing pre- and postharvest losses [1, 2]. Both fungi produce peculiar symptoms such as fruit softening and white mycelium on the lesion surface. As the infection progresses, the fruit is fully covered with blue or olive-green spores which can be disseminated through air currents. These fungi cause enormous economic losses which tend to increase sharply during high infection years. Green mould alone has been reported to cause huge losses in Citrus worldwide [3]. Synthetic chemicals like imazalil, sodium ortho-phenyl phenate (SOPP), thiabendazole (TBZ) or mixtures of these chemicals have been recommended to control infections of blue and green moulds [4]. Some new chemicals such as fludioxonil, pyrimethanil, azoxystrobin and trifloxystrobin have also been widely used to control these pathogens [5, 6]. The use of these chemicals has environmental concerns and chemicals residues have also been detected in the fruit. Moreover, overuse of fungicide induces resistance in *Penicillium* strains. Therefore, alternate strategies may be developed which may be safe for the environment and could also overcome the resistance to fungicides.

One way to reduce the use of pesticides is to induce resistance in fruit against the pathogen which may be invoked through the use of physical [8, 9], chemical or biological means [10, 11]. Host-pathogen interaction was dictated by a number of factors including natural compounds which induced resistance against the pathogens. Organic elicitors are known to be environmentally safe and can induce resistance reaction. SA or MeJ treatments have been used to induce resistance against pathogens in various plant species [12]. However, little efforts have been carried out to determine their efficacy against fruit softening

enzymes in Valencia sweet oranges. Previously, a study for the optimization of SA or MeJ was conducted which showed its potential against regulation of disease [13]. It is generalized that treatment of Valencia orange with SA and MeJ induces resistance to *Penicillium* spp. including decreased levels of cell wall degrading enzymes. However, systematic and comprehensive studies on the role of SA and MeJ to reduce the levels of cell wall degrading enzymes on sweet orange peel are still lacking. So the major objectives of this study were: (1) assess the efficacy of pre-harvest application of SA or MeJ to Valencia orange to control citrus green and blue moulds; (2) compare the activities of softening enzymes such as exopolygalacturonase (exo-PG), endopolygalacturonase (endo-PG) and Endo-1,4-β-D-glucanase (E-Gase) in pre-harvest treated and untreated fruit (3) standardize the best concentrations of elicitors for commercial use in future.

## Materials And Methods

### Fungal cultures

Fungal cultures i.e. *P. digitatum* and *P. italicum* were collected from diseased 'Valencia' sweet oranges (*Citrus sinensis* L. Osbeck) at Gingin, Western Australia (Latitude 31 ° 21' S, Longitude 155 ° 55' E). The collected species were isolated and purified by the method already described by Iqbal et al. [13]. The cultures were purified on citrus peel agar (CPA) and stored at 25 ± 1 ° C.

### Experiment No. 1: Application of different concentrations of SA and MeJ as Pre-harvest spray

#### Plant material

Treatments were carried out on twelve-year-old 'Valencia' sweet orange trees. Planting density of the trees was 7.5 m between rows and 2.7 m between trees with north-south row direction. The soil of the orchard was sandy loam and uniform agronomic and horticultural practices were adopted for all the experimental trees during the entire experimental period.

#### Treatments and experimental design

Aqueous solutions containing SA (3, 6, 9 mM), MeJ (3, 4, 5 mM) and 'Tween 80' (0.05%) as a surfactant were sprayed onto the whole tree one week before harvest. Untreated trees were considered as control. Sprayer manufactured by Selecta Trolley Pak Mk II, Victoria, Australia was used to give complete coverage till running off with standard pressure (250 KPa). The experiment was arranged in a randomized block design with three replications. Singletree was treated as an experimental unit.

Blemish free Valencia orange fruit were selected based on uniform size. After one week, the fruit were picked and punctured with two holes in the rind (equatorial region) by using a sterile nail (3 mm wide). The concentration of conidial suspension of *P. digitatum* and *P. italicum* was adjusted to 10<sup>7</sup> conidia ml<sup>-1</sup> with the help of a haemocytometer (Biolab Heilbronn, Germany). Each hole received a concentration of 10 µl suspension [13]. Each treatment comprised of three replications and there were 10 fruit within each

replication. The fruit following treatment were placed in corrugated cartons and kept at 25 °C temperature and (95%) humidity and for the entire duration of the experiment. All fruit were subjected to evaluation of diseases symptoms such as wound rotting incidence, fungal colony growth and green or blue mass density. The disease scoring was done when symptoms manifestation started on control (inoculated) fruit and recorded on daily basis after 4<sup>th</sup>, 5<sup>th</sup> and 6<sup>th</sup> day of inoculation till the control fruit were fully covered with fungal mass. The density of fungus was determined as described in [13].

## **Experiment No. 2. Determination of activities of softening enzymes**

### **Protein determination**

The treatments described under experiment No. 1 were used for the determination of enzymes causing softening of the fruit. The protein content of the fruit rind was determined as per [14]. Protein contents were calculated with bovine serum standard curve and values were given in mg.

### **Determination of the activity of Exo-PG, Endo-PG, and E-Gase in the rind**

The activities of enzymes (exo-PG, endo-PG, and e-Gase) in the rind of the fruit were determined by the method dictated by [15] Dong *et al.* (2001) and further modification suggested by [16]. The activities of exo-polygalacturonase in the rind of the fruit were expressed as  $\mu\text{g galacturonic acid mg protein}^{-1}\text{h}^{-1}$ . Whilst, the activities of endo-polygalacturonase and endo-1,4- $\beta$ -D-glucanase in the rind of the fruit were expressed as viscosity changes  $\text{mg}^{-1}\text{ protein hr}^{-1}$ .

### **Statistical analyses**

The data were subjected to the two-way ANOVA using Genstat release 8, Agricultural Trust, Rothamsted Experimental Station, Rothamsted, UK. Significance of the treatments and interactions were compared with Fisher's LSD following significant  $P \leq 0.05$  F test.

## **Results**

### **Disease suppression with an application of elicitors as a pre-harvest spray**

All the pre-harvest spray treatments of SA and MeJ reduced colony growth and wound rotting incidence significantly ( $P \leq 0.05$ ) on the treated fruit as compared to control (Table 1–3; Fig. 1). Both the elicitors applied as a pre-harvest spray proved much effective up to 3rd day after inoculation. Mean inhibition of colony growth of *P. digitatum* was 56.24% and 71.02% on Valencia orange fruit received a preharvest spray of SA (6 and 9 mM), respectively, which was significantly higher as compared to MeJ and control (Table 1). Wound rotting incidence was also reduced by 71.50% and 76.53% with the preharvest spray application of SA (6 and 9 mM), respectively which was significant as compared to control and MeJ treatments. Similarly, the preharvest spray application of SA (6 and 9 mM) has also reduced the colony

growth of *P. italicum* by 53.66% and 68.69% and wound rotting incidence by 68.23% and 69.89% on the treated fruit over control (Table 2).

Table 1

Disease incidence and severity (colony growth) of *P. digitatum* on Valencia orange treated pre-harvest with different concentrations of salicylic acid (SA) and methyl jasmonate (MeJ)

Treatment	Colony growth (mm)			Mean Conc. (mm)	GI* (%)	Wound rotting (%)	WI (%)†
	Day-4	Day-5	Day-6				
SA-3 mM	6.95	38.83	80.80	42.19	43.61	86.7	12.68
SA-6 mM	5.15	28.40	64.67	32.74	56.24	28.3	71.50
SA-9 mM	3.85	19.48	41.73	21.68	71.02	23.3	76.53
MeJ-3mM	13.03	49.98	82.60	48.53	35.14	88.3	11.03
MeJ-4mM	11.83	46.95	77.27	45.35	39.39	78.3	21.14
MeJ-5mM	9.83	38.18	68.50	38.83	48.10	46.7	53.37
Control	46.17	77.23	101.10	74.83	-	99.3	-
Mean (time)	13.83	42.72	73.81	43.45		64.41	
LSD ( $P \leq 0.05$ ) : Treatment = 0.923; Conc = 1.305; Time = 1.13; Treatments x Conc.=1.84; Treatments x time = 1.59; Conc. x time = 2.26; Treatments x Conc. x time = 3.19; Wound rotting: Treatment = 9.92; Conc = 14.03; Treatment x Conc = 19.84							
Conc = concentration; *GI = Growth inhibition; †Wound rotting inhibition							

Table 2

Disease incidence and severity (colony growth) of *P. italicum* on Valencia orange treated pre-harvest with different concentrations of salicylic acid (SA) and methyl jasmonate (MeJ)

Treatment	Colony growth (mm)			Mean Conc. (mm)	GI* (%)	Wound rotting (%)	WIt (%)
	Day-4	Day-5	Day-6				
SA-3 mM	9.15	40.93	83.0	44.36	42.18	88.33	11.36
SA-6 mM	6.98	31.02	68.67	35.55	53.66	31.66	68.23
SA-9 mM	4.88	21.28	45.90	24.02	68.69	30.0	69.89
MeJ-3 mM	14.53	51.73	83.10	49.78	35.12	90.0	9.69
MeJ-4 mM	13.63	48.82	79.57	47.34	38.29	83.33	16.38
MeJ-5 mM	11.67	40.55	70.23	40.81	46.81	50.0	49.82
Control	48.03	80.18	102	76.73	-	99.66	-
Mean (time)	15.55	44.93	76.06	45.51		67.56	
L SD ( $P \leq 0.05$ ) = Colony growth: Treatment = 1.08; Conc = 1.53; Time = 1.32; Treatments x Conc.=2.16; Treatments x time = 1.87; Conc. x time = 2.65; Treatments x Conc. x time = 3.75;Wound rotting: Treatment = 7.50; Conc = 10.61; Treatment x Conc = 15 Conc = concentration; *GI = Growth inhibition; †Wound rotting inhibition							

Table 3

Green and blue mass density produced by *P. digitatum* and *P. italicum* on pre-harvest treated Valencia fruit with different concentrations of salicylic acid (SA) and methyl jasmonate (MeJ)

Treatment	<i>P. digitatum</i>			<i>P. italicum</i>		
	Day-4	Day-5	Day-6	Day-4	Day-5	Day-6
SA-3 mM	0 (None)	3 (Sparse)	4 (Moderate)	2 (Exiguous)	3 (Sparse)	5 (Max)
SA-6 mM	0 (None)	2 (Exiguous)	3 (Sparse)	0 (None)	2 (Exiguous)	3 (Sparse)
SA-9 mM	0 (None)	2 (Exiguous)	2 (Exiguous)	0 (None)	2 (Exiguous)	2 (Exiguous)
MeJ 3 mM	1 (Slight tint)	3 (Sparse)	5 (Max)	2 (Exiguous)	4 (Moderate)	5 (Max)
MeJ-4 mM	1 (Slight tint)	3 (Sparse)	4 (Moderate)	1 (Slight tint)	3 (Sparse)	4 (Moderate)
MeJ-5 mM	0 (None)	3 (Sparse)	4 (Moderate)	1 (Slight tint)	3 (Sparse)	4 (Moderate)
Control	3 (Sparse)	5 (Max)	6 (Full green)	4 (Moderate)	5 (Max)	6 (Full blue)
*Rating of blue and green mass density was done on 0–6 disease rating scale: 0 = No colour development; 1 = slight tint; 2 = Exiguous; 3 = Sparse; 4 = Moderate; 5 = Maximum coloration; 6 = Full green colour						

### The activity of Exo-PG, Endo-PG, and E-Gase in the rind

The pre-harvest spray application of SA (9 mM) significantly reduced pathogen induced rind softening in Valencia fruit inoculated with *P. digitatum* and *P. italicum* and stored at  $25 \pm 1$  °C as compared to MeJ (5 mM) and inoculated control. Rind of the fruit treated with SA (9 mM) and MeJ (5 mM) exhibited significantly lower exo-PG activity after 24, 72 and 120 hr of inoculation compared to the inoculated control (Fig. 2). The un-inoculated fruit initially displayed enhanced activity of exo-PG and galacturonic acid level increased from 10.93 µg after 24 hr to 20.67 µg after 72 hr but declined sharply (5.01 µg) after 120 hr. SA treated fruit showed reduced Exo-PG activity with galacturonic acid levels (11.40, 69.68 and 57.76 µg) and MeJ (15.03, 89.16 and 79.04 µg) after 24, 72 and 120 hr of inoculation, respectively. Overall, SA treated fruit exhibited decreased activity of exo-PG enzyme in the rind as compared to control and methyl jasmonate treated fruit (Fig. 2).

SA and MeJ significantly ( $P \leq 0.05$ ) reduced the activity of endo-PG as compared to control. SA treated fruit showed decreased activity of Endo-PG giving viscosity changes of 0.32, 32.08 and 24.08 mg<sup>-1</sup> protein hr<sup>-1</sup> followed by methyl jasmonate which exhibited 0.87, 43.9 and 31.34 mg<sup>-1</sup> protein hr<sup>-1</sup> after 24, 72 and 120 hr of inoculation, respectively (Fig. 3). Endo-PG activity in the rind of inoculated control fruit sharply increased from 1.82 mg<sup>-1</sup> protein hr<sup>-1</sup> after 24 hr to 52.99 mg<sup>-1</sup> protein hr<sup>-1</sup> after 72 hr but

slightly declined after 120 hr ( $40.42 \text{ mg}^{-1} \text{ protein hr}^{-1}$ ). In the MeJ treated fruit, the activity of endo-PG in the rind showed a quick rise from 24 hr to 72 hr but declined after 120 hr. Fruit treated with SA (9 mM) proved to be the best treatment in reducing the levels of endo-PG in Valencia fruit and showed an average of  $18.83 \text{ mg}^{-1} \text{ protein hr}^{-1}$  as compared to inoculated control (31.74) after 24, 72 and 120 hrs.

Pre-harvest SA application suppressed the e-gase activity in the rind tissue as compared to inoculated control and MeJ pre-harvest spray (Fig. 4). The control fruit inoculated with *P. digitatum* exhibited a peak in e-gase activity after 72 hr ( $34.58 \text{ mg}^{-1} \text{ protein hr}^{-1}$ ) and continued to increase after 120 hr ( $46.57 \text{ mg}^{-1} \text{ protein hr}^{-1}$ ) of inoculation with no decline.

Viscosity changes in SA treated fruit after 24 and 72 hr were 1.10 and  $22.63 \text{ mg}^{-1} \text{ protein hr}^{-1}$  which declined to  $20.14 \text{ mg}^{-1} \text{ protein hr}^{-1}$  after 120 hr of inoculation.

## Discussion

The results demonstrated that the spray application of SA and MeJ one week before harvest significantly ( $P \leq 0.05$ ) reduced blue and green mould development on Valencia fruit as compared to the untreated control (Table 1–3). Pre-harvest spray application of SA (6 and 9 mM) showed significant inhibition of fungal growth, wound rotting, and green or blue mass density on artificially inoculated 'Valencia' orange fruit which might have curtailed chances of further conidial dissemination [15].

Preharvest spray application of SA (6 and 9 mM) inhibited colony growth of *P. digitatum* by 56.24% and 71.02% and *P. italicum* by 53.66% and 68.69%, respectively, on the fruit as compared to control (Table 1 & 2). Both the treatments showed reduction in wound rotting incidence of *P. digitatum* by 71.50% and 76.53% and of *P. italicum* by 68.23% and 69.89%, respectively as compared to control which is significantly higher than control and treatments of MeJ. The role of SA in the induction of the disease resistance had been identified and it was noticed that SA made a remarkable increase in the hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and superoxide anion, phenylalanine ammonia-lygase and expression of pathogenesis-related protein in tomatoes and thus increased the resistance against the pathogen [16]. SA reduces soft rots caused by *Penicillium* spp. by the induction of resistance through elevated antioxidant enzymes activity [17]. SA also delays senescence which helps to control fruit decay. This necessitates commercial use of SA as a pre-harvest spray in disease management strategies.

MeJ treatment (5 mM) followed by SA in its positive effect on pre-harvest treated Valencia fruit and showed significant results as compared to control (Table 1–3). MeJ has been demonstrated to upregulate the defence-related proteins and phenolics [18]. Exogenous application of MeJ resulted in reduced decay incidence and restricted lesion diameter improving vigour in different fruits like tomato, sweet cherry, peach and loquat [18–20]. Induction and activation of immune response provide fruit protection which checks the spread of pathogens safely [21].

# Activities Of Exo-pg, Endo-pg And E-gase

It was demonstrated that pre-harvest spray of SA (9 mM) or MeJ (5 mM) onto Valencia orange fruit was effective in reducing rotting accelerated by fruit softening enzymes. SA-treated fruit showed reduced exo-PG activity with galacturonic acid levels of 11.40, 69.68 and 57.76  $\mu\text{g}$  while MeJ-treated fruit exhibited comparatively enhanced exo-PG activity with galacturonic acid levels of 15.03, 89.16 and 79.04  $\mu\text{g}$  after 24, 72 and 120 hr of inoculation, respectively. Similarly decreased viscosity changes were exhibited by endo-PG and e-gase in the rind of SA treated fruits. The reduction in citrus rind softening and lesion development with the preharvest spray of SA (9 mM) may be due to reduced activities of cell wall hydrolysis enzymes in the rind [22]. Recently, Ennab et al. [23] also reported that salicylic acid and putrescine effectively reduced fruit softening in Murcott Mandarin fruit treated postharvest as compared to control in 2018 and 2019 seasons.

A marked increase of galacturonic acid levels in the inoculated control treatment (33.06  $\mu\text{g}$  (24 hr), 137.57  $\mu\text{g}$  (72 hr), 131.96  $\mu\text{g}$  (120 hr) as compared to SA and MeJ treated fruit may be associated with the hydrolysis of host pectic substances by exo-PG. An exo-PG has been reported to be the sole enzyme catalysing hydrolytic cleaving of pectic chains in decayed peels of orange infected with *P. digitatum* [24]. Earlier, Achilea et al. [25] reported that high galacturonic acid levels occurred in the albedo before the flavedo of grapefruit and the extent of maceration depended on the host response. Polygalacturonases catalyse a hydrolytic cleavage in *P. italicum* [24]. The capacity to reduce tissue cohesiveness has also been described previously for *P. expansum* in potato tissue and *P. paxilli* in cucumber tissue [25]. The presence of higher exo-PG activity in infected tissue suggests that it may be responsible for the excessive accumulation of galacturonic acid levels which are produced during blue and green mould infections. The initial stages of symptom development are linked to increased levels of D-galacturonic acid in tissue infected with *P. digitatum* and *P. italicum* [26]. Galacturonic acid accumulation and its diffusion into healthy tissue are considered an essential factor in pathogenicity of these two moulds [27]. Fruit softening in the present study occurred only in response to pathogen infection and there was no development of softening zones on either wounded or un-inoculated fruits. These observations confirmed the endo and exo-PGs to be of fungal origin. This is supported by the finding of Barmore et al. [28] who found that endo-PG was not detected in injured-uninfected rind, but was produced by the *P. Italicum* during growth in vitro on Valencia oranges. Exo-PG, endo-PG and e-gase activities were remarkably less in fruit treated with SA as compared to inoculated control and MeJ. There is a similarity in the decay mechanism by the green and blue mould. This finding led Barmore and Brown [27] to conclude that the type of PG produced did not cause any obvious histopathological differences during the pathogenesis of two organisms.

The cell wall degrading enzymes are involved in fruit softening, lesion formation and decay. Changes in polymerization and sugar composition of polysaccharides are related to structural changes of the cell wall in fruit tissue [29]. Polygalacturonase (PG) has been reported as the most important enzyme for fruit softening [30]. Activities of softening enzymes induced by various organisms have been reported in apple, avocado, cherry, papaya, pear and tomato [31, 32].

*P. italicum* is transmitted to an uninfected fruit by contact and the exudate contains high endo-PG activity while *P. digitatum* spores penetrate and infect only wounded peel turning into typical green mould lesions later [33]. Pectolytic enzymes produced by *Penicillium* spp. soften the peel and facilitate the hyphal penetration. The citrus fruit rotting in the present study was found associated with the production of exo-PG and e-gase in *P. digitatum* and endo-PG in *P. italicum*. Endo-PG causes sufficient pectin degradation to permit intercellular penetration by hyphae of *P. italicum*.

Our findings demonstrate that the pre-harvest application of both SA and MeJ has the potential to reduce damage caused by green and blue moulds in Citrus. This is possibly ascribed to the reduced activity of cell wall degrading enzymes in *P. digitatum* and *P. italicum* induced by the two elicitors. Although the effectiveness of SA and MeJ may not be comparable to that of synthetic fungicides they have implications in terms of providing safe substitutes for chemicals. Compounds which increase host defense system promise eco-friendly alternatives for postharvest disease control [34]. Organic elicitors along with other natural products may replace the conventional and toxic fungicides or could be incorporated into integrated disease management strategies of postharvest diseases in future.

## Declarations

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### Conflict of Interest

The authors declare no conflict of interest that negatively or positively influence the manuscript.

### Ethical statement

It is certified that any part of manuscript does not violate copy right. Author(s) are fully aware of publication guidelines outlined by COPE and ethical responsibilities of authors outlined by European Journal of Plant Pathology Journal.

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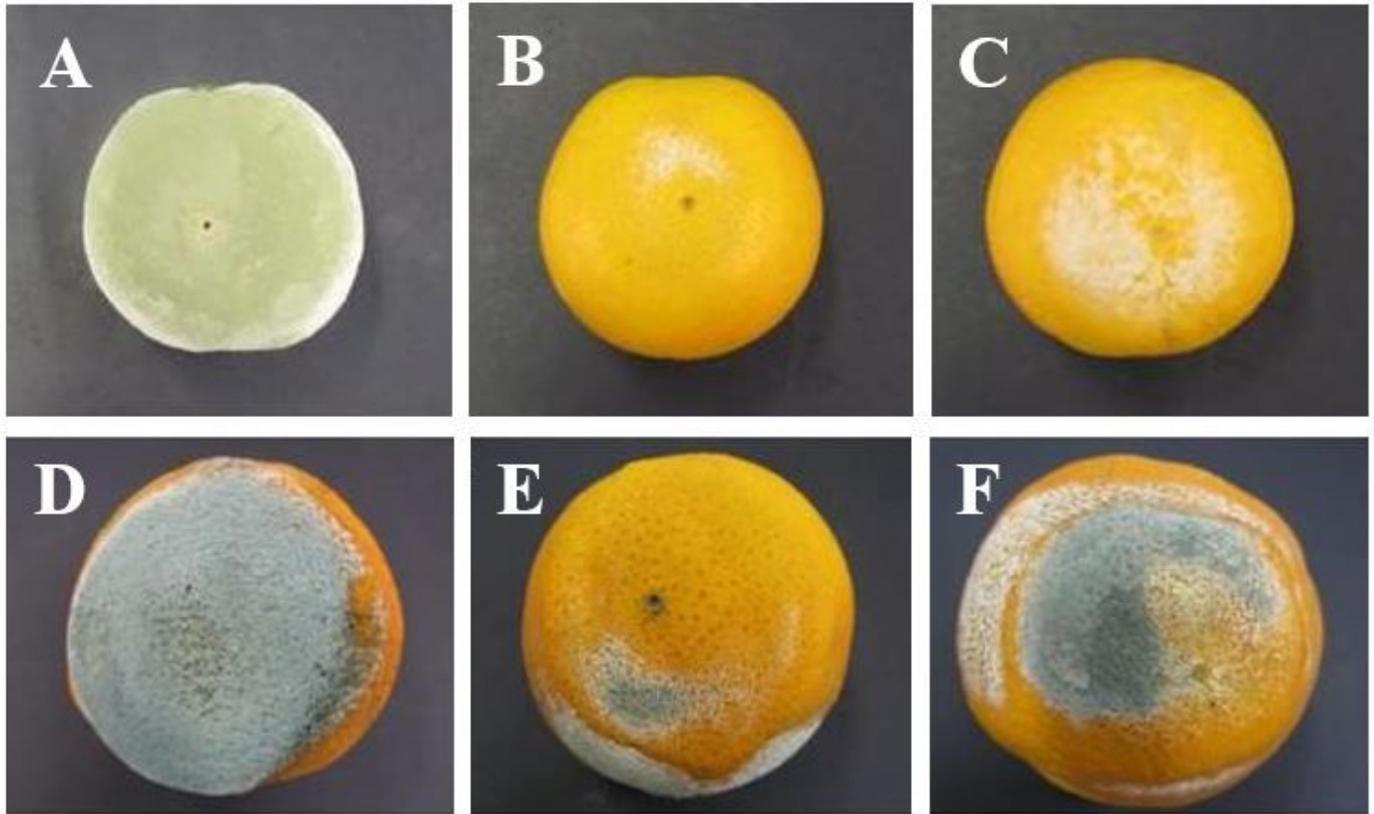
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## Figures



**Figure 1**

Colony growth and green mass density on Valencia orange treated pre-harvest with tested concentrations of salicylic acid and methyl jasmonate *Penicillium digitatum*= (A) Control pre-harvest; (B & C) SA 9 & MeJ 5 mM pre-harvest *Penicillium italicum*= (D) Control pre-harvest; (E) SA 9 mM pre-harvest; (F) MeJ 5 mM pre-harvest

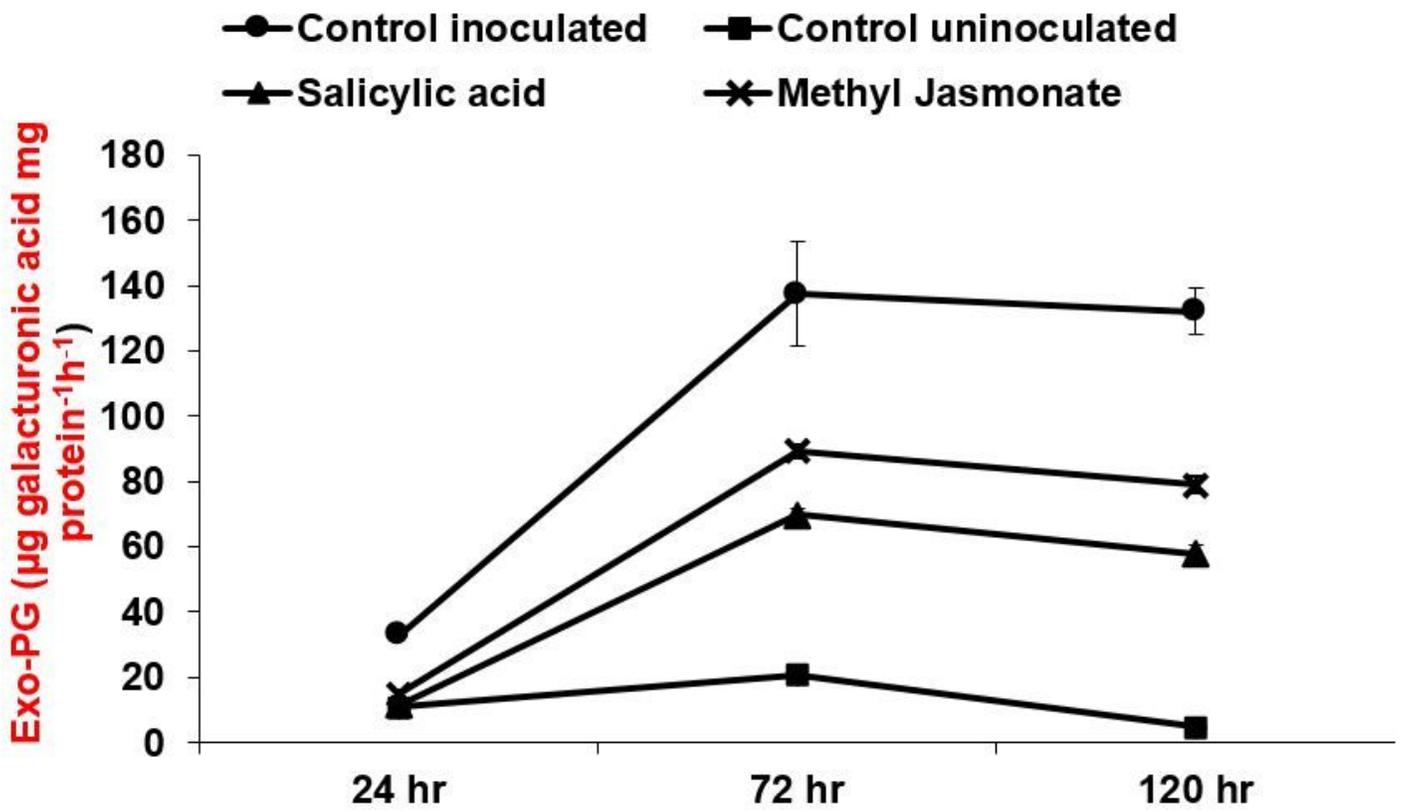


Figure 2

Changes in the activity of exo-polygalacturonase ( $\mu\text{g galacturonic acid mg protein}^{-1}\text{h}^{-1}$ ) in the rind of Valencia orange fruit following pre-harvest spray with SA (9 mM) or MeJ (5 mM) and inoculated with *P. digitatum*

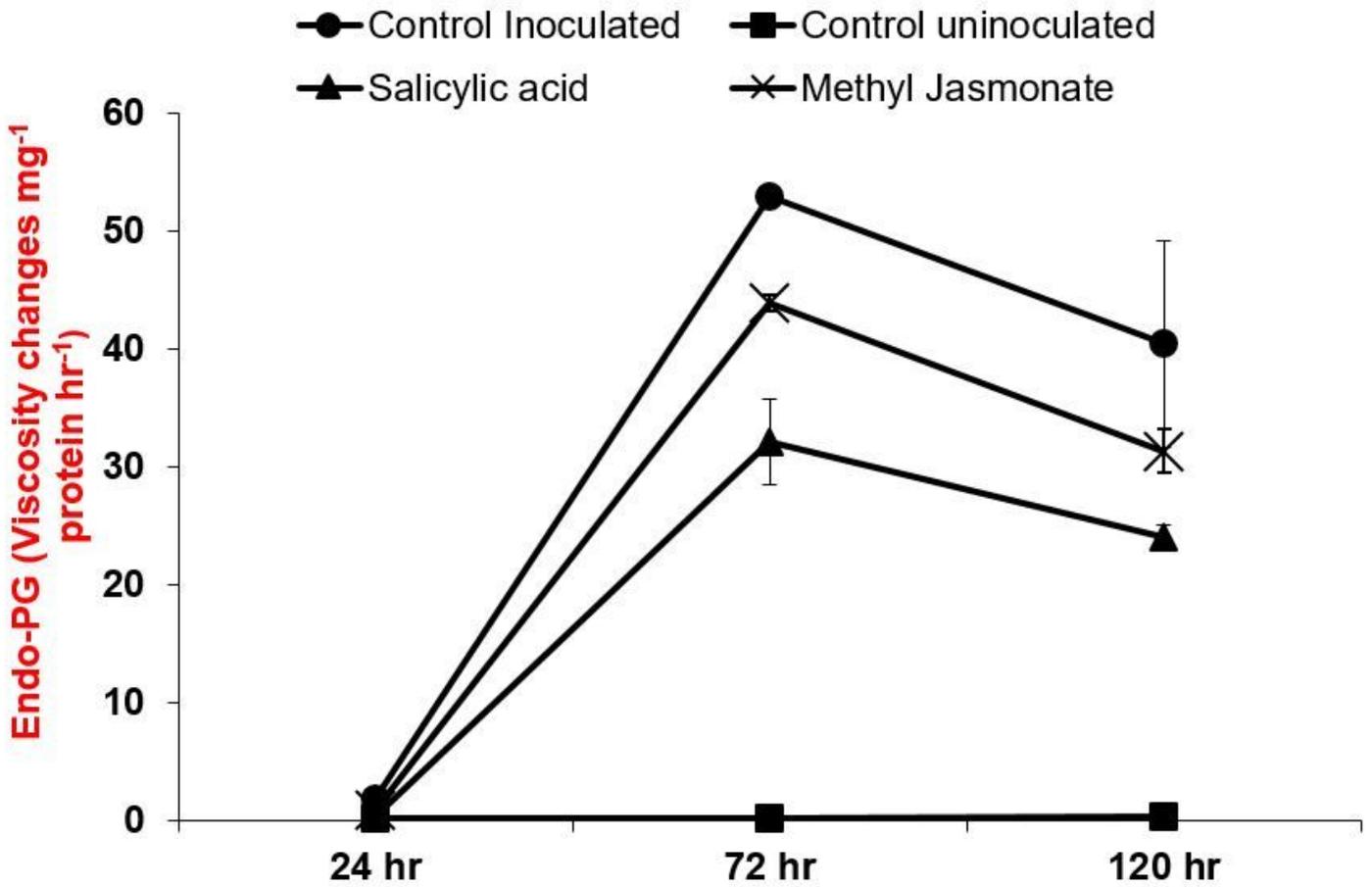


Figure 3

Changes in the activity of endopolygalacturonase (endo-PG, viscosity changes mg<sup>-1</sup> protein hr<sup>-1</sup>) in the rind of Valencia orange fruit following pre-harvest spray with SA (9 mM) or MeJ (5 mM) and inoculated with *P. italicum*

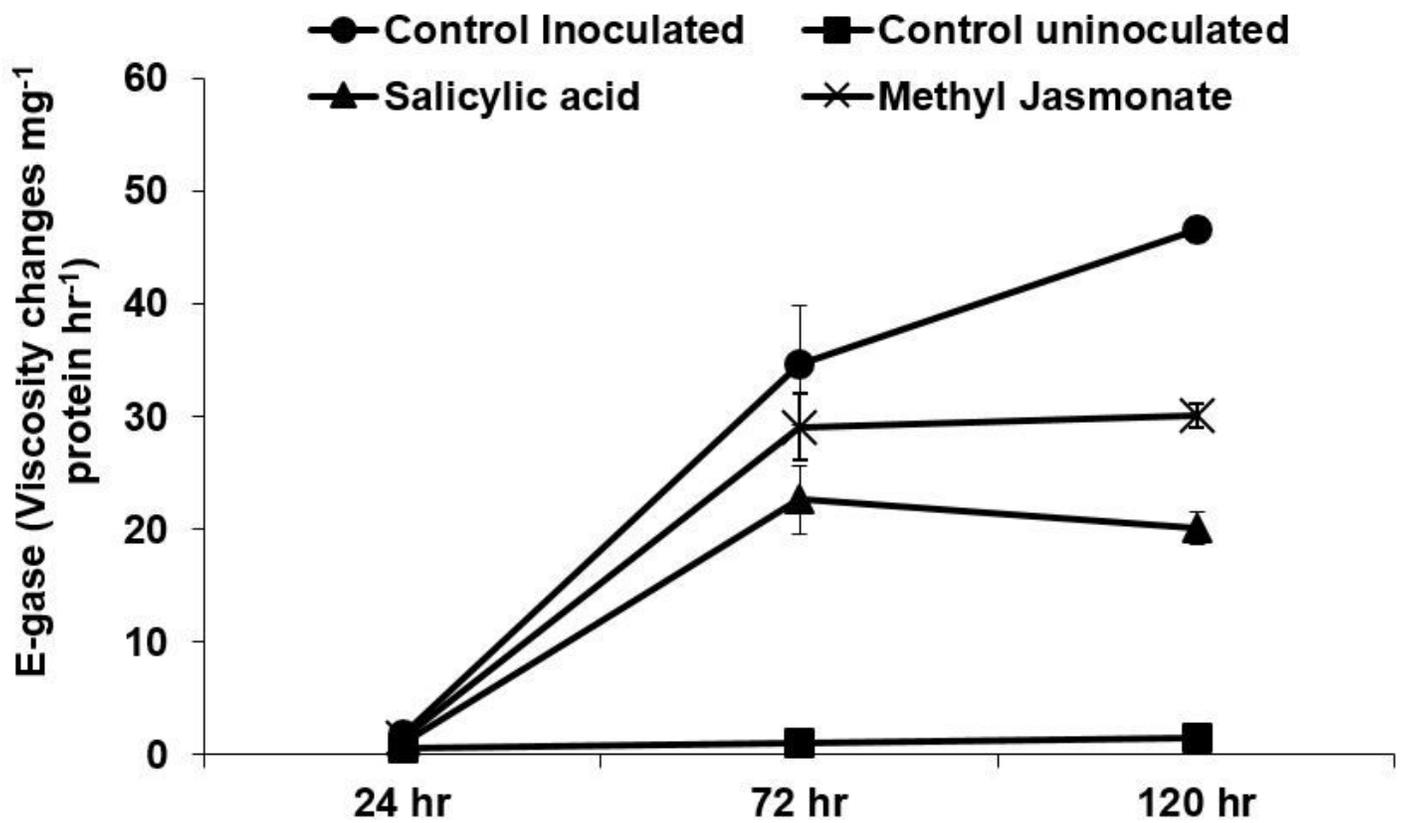


Figure 4

Changes in the activity of Endo-1,4-β-D-glucanase (E-gase, viscosity changes mg<sup>-1</sup> protein hr<sup>-1</sup>) in Valencia orange sprayed pre-harvest with SA (9 mM) or MeJ (5 mM) and inoculated with *P. digitatum*.