ISSN 2537_ 0715
IJSRSD (2021): Volume 4, Issue 2, July 2021
Received: May 2021, Accepted: June 2021

International Journal of Scientific
Research and Sustainable Development

Effects of Salicylic Acid in The Normal and Nano Form Against Selected Fungi That Infect Citrus Trees (*Citrus sinensis)*.

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Abstract

Evaluation of the potential antifungal activity of salicylic acid in the normal and nano form on the pathogenic fungi (*Alternaria alternata* and *Penicillium digitatum*) causing citrus leave spots and fruit decay was studied under *in vitro* and *in vivo* conditions. Characterization of prepared salicylic acid nanoparticles (SA NPs), *i.e*., size distribution and the stability showed that, size distribution range mainly within 8-40 nm as well as hydrodynamic size for produced nanoparticles is 8-40 nm. Salicylic acid nanoparticles and bulk material at 0.5 and 1 mM, *in vitro* significantly reduced the mycelial growth for *Alternaria alternata* and *Penicillium digitatum*, whereas there was not a significant difference between salicylic acid nanoparticles and bulk material, however significant changes of the linear growth were noticed between the low and the high concentrations. Examinations using transmission electron microscopy (TEM) showed hyphal and conidial deformations in addition to changes in cellular structure of *A. alternata* and *Penicillium digitatum* when treated with salicylic acid nanoparticles at 1mM concentration. In case of *Alternaria alternata*, an increasing of the spinulose conidial cell wall and cell membrane showed and appearance of numerous vacuoles in the cytoplasm with undefined cytoplasmic organelles as well as well-defined ultrastructure organelles of the mycelium, meanwhile in case of *Penicillium digitatum* salicylic acid nanoparticles exhibited a disrupted cell wall (CW) and several invaginations in the cytoplasmic membrane; shrinkage and decrease in thickness with completely cytoplasmic organelles leakage and accumulation of salicylic acid nanoparticles. The effect of salicylic acid in the normal and nano form were evaluated under field conditions against phytopathogenic fungi *Alternaria alternata* and *Penicillium digitatum* on Valencia orange in Ismailia governorate during growing season 2019/2020, salicylic acid in the normal and nano form, significantly reduced the percentages of disease incidence for both *Alternaria alternata* and *Penicillium digitatum* compared with the untreated control under field conditions.

Keywords: Citrus, *Citrus sinensis*, *Alternaria alternata*, *Penicillium digitatum*, salicylic acid.

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Introduction

Citrus sinensis (L.) is one of the major commercial fruit plants which, due to its high content of vitamin C and potential antioxidant, is widely eaten as fresh fruit and juice **(Gorinstein *et al.*, 2001).** The cultivar in over 137 countries on six continents is primarily grown in tropical or subtropical regions of the world **(Ismail and Zhang, 2004)**. Sweet orange is an important fruit crop that demands excellent quality and shelf life in international trade. Regrettably, several pathogens are known to attack which affect the quality of the fruit. In developing countries, where fresh fruit is adequately protected and managed properly, losses during transit and storage may be over 50% **(Eckert and Ogawa, 1985).** Spoilage microorganisms may be introduced in the cultivation on the seed itself, during field growth, harvesting, handling and storage and distribution **(Barth *et al.*, 2009)**.

Most citrus fruits are infected with a variety of bacteria and fungi, but only a small proportion of them are resistant to plant pathogenic microorganisms due to a specific environmental condition. Extensive applications of agrochemicals to maintain yield and crop protection from plant pathogenic microorganisms have sparked thoughtful brainstorming topics. Researchers and policymakers are anticipating resolutions to protect the environment by reducing pesticide consumption while reaching preferred agricultural yield to feed the world's ever-growing population **(Tilman *et al.*, 2002)**. Agrochemicals used extensively in agriculture contribute to global warming and enhance the likelihood of harmful bacteria developing resistance **(Jang *et al.*, 2014; Hahn *et al.*, 2014)**. To deal with the forthcoming multi-dynamic stressors, we need to devise a novel method for controlling phytopathogens, increasing plant immunity, strengthening the signalling network, and promoting plant development **(Kushalappa *et al.*, 2016; Andolfo *et al.*, 2016)**.

Salicylic acid (SA) is a naturally occurring phenolic molecule found in plants that plays an important role in the signalling pathways that lead to the beginning of systemic acquired resistance (SAR). Up-regulation of seed germination, respiration, photosynthesis, vegetative development, flower formation, senescence, thermogenesis, and cellular redox homeostasis are just a few of the pathways in plants where SA plays a vital role **(Malamy *et al.*, 1992; Raskin *et al.*, 1990; Vlot *et al.*, 2009; Khan *et al.*, 2015; Rivas-San and Vicente, 2011; Kumar, 2014).**

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External use of SA in different parts of the plant stimulates many metabolic pathways and can be an alternative approach to treating diseases and increasing plant productivity. The bioactivity of external use of SA in plants can be affected by various factors, such as concentration, duration of treatment, age of plants, species, and target areas **(Revas San and Vicente, 2011; Kumar Kumar, 2014)**. SA plays a dual role in plant protection, leading to different pathways of resistance, and can also have a positive effect on phytopathogens.

Nanotechnology is taking the world to a new era in the management and diagnosis of plant pathogens. External use of various natural nanomaterials is an important magical solution to many environmental problems **(Shoala, 2018)** to fight plant pathogens, enhance plant protection and produce plants without harming the environment. Thus, our current research focuses on the use of nano- and general-form salicylic acid (SA) to increase activity against plant pathogens and enhance plant protection and production using small amounts of SA. To fill a major research gap on SA in-vitro use to achieve its dosage release for a long-term effect on a permanent basis.

Material and Methods

***Source of the tested fungal isolates*:**

The tested fungi *Alternaria alternata* and *Penicillium digitatum* were previously examined for their pathogenic ability, as all the tested fungi were pathogenic, with varied degree to citrus leaves and showed also different level of disease symptoms on fruits, **(Abdelmalek and Salaheldin, 2016)**.

Antifungal activity of salicylic acid in the nano and normal form

In this study the fungal pathogens *Alternaria alternata* and *Penicillium digitatum* were grown on potato dextrose agar (PDA) and incubated at 28 ± 2^{o}C for 7 days. In vitro assay was carried out on PDA treated with 0.5 and 1 mM of salicylic acid in the nano and normal form. Five mL of it was poured into the media before plating into each 90 × 15 mm Petri dish. The media containing salicylic acid nanoparticles was incubated at room temperature. After 48 hr of incubation, an agar plugs of 5 mm diameter containing fungi was inoculated simultaneously at the center of each Petri dish and incubated at 28 ± 2^{o}C. After 2 wk of incubation, inhibition zones were measured. The test was repeated twice, and each treatment replicated three times **(Kabir *et al.*, 2011)**.

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The inhibition rate (%) was calculated by using the following formula

$$\text{Inhibition rate (\%)} = \frac{R - r}{R} \times 100$$

That:

R = radial growth of fungi in control plate

R = radial growth of fungi in salicylic acid in the nano and normal form treated plates.

Salicylic acid nanosynthesis

Salicylic acid was acquired from Sigma-Adrich (CAS number: 20283-92-5) and dissolved in 10 ml 100% ethanol before being sonicated for an hour at ambient temperature (25°C) with an ultrasonic power and frequency of 50 kHz (XUBA3Analogue Ultrasonic Bath, Grant Company).

Dynamic light scattering (DLS)

Salicylic acid nanoparticle distribution and size were measured at room temperature using a dynamic light scattering method with a Zetasizer Nano ZS (Malvern Instruments, UK). 30µl of the nanoparticle was diluted with 3ml of water at 25°C prior to measurement. The mean of the Z-average of three independent batches of nanoparticles was used to calculate particle size.

Transmission Electron Microscopy of salicylic acid nanoparticles (SA NPs):

For TEM analysis, a drop of the solution was placed on the carbon coated copper grids (CCG) and dried by allowing water to evaporate at room temperature. Electron micrographs were obtained using JEOL GEM-1010 transmission electron microscope at 70 kV at The Regional center for Mycology and Biotechnology, (RCMB) Al- Azhar University **(Amin and El-Sharkawy, 2019).**

Electron microscopy ultrastructure examination

For TEM preparation, the samples were fixed in 3% glutaraldehyde, rinsed in phosphate buffer, and post-fixed in potassium permanganate solution for 5 min. at room temperature. The samples were dehydrated in an ethanol series ranging from 10% to 90% for 15 min in each alcohol dilution and finally with absolute ethanol for 30 min. Samples were infiltrated with epoxy resin and acetone through a graded series till finally in pure resin. Ultrathin sections were collected on copper grids. Sections were then double stained in uranyl acetate followed by lead citrate. Stained sections were observed with a JEOL - JEM 1010 transmission electron

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microscope at 70 kV at The Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University **(Amin, 2016; Elsherbiny *et al.*, 2020).**

Field trials and data analysis

To determine the efficacy of salicylic acid normal and nanoparticles formula against some disease on Valencia orange in the field, an experiment was carried out in Om Qamar Village Cairo Ismailia Desert Road. during growing season 2019/2020, salicylic acid normal and nanoparticles were used at 0.5 and 1.0 mM simultaneously. It was applied on the tree beginning in February every fifteen days the treatment was repeated three times. Results were obtained monthly from February to December. Distilled water was used as a negative control.
Disease incidence (%) of leaf spots was calculated by counting the numbers of infected leaves out of 150 leaves among the treated plants in field trials. Each experiment was repeated three times. To assess disease incidence of fruit rots, a total of 102 plants were randomly selected from the orchards. A sample size of ten (10) plants and fifty (50) fruits were selected from orchard of Citrus to record disease incidence. Computations were achieved with the help of the following formulae (Safdar *et al*., 2010).

$$\text{Disease Incidence (\%)} = \frac{\text{No. of infected fruits}}{\text{Total No. of fruits}} \times 100$$

Statistical analysis

To determine significant differences between treatments, the data was subject for the Variance Analysis (ANOVA) and the MultiRange Test by Duncan was used. The scan was carried out using Windows (IBM Inc., Chicago, IL) SPSS Statistical Data Editor 23.0 and $P < 0,05$ was considered significantly differently.

Results

Characterization of SA nanoparticles

Dynamic light scattering technique was performed to understand the size distribution and the stability of prepared SA nanoparticles (Fig 1). Dynamic light scattering technique was performed to understand the size distribution and the stability of prepared SA NPs has a size distribution range mainly within 9.96-27.3 nm as shown in (Fig 2, Fig 3). An average hydrodynamic size for produced nanoparticles is 9.96-27.3 nm.

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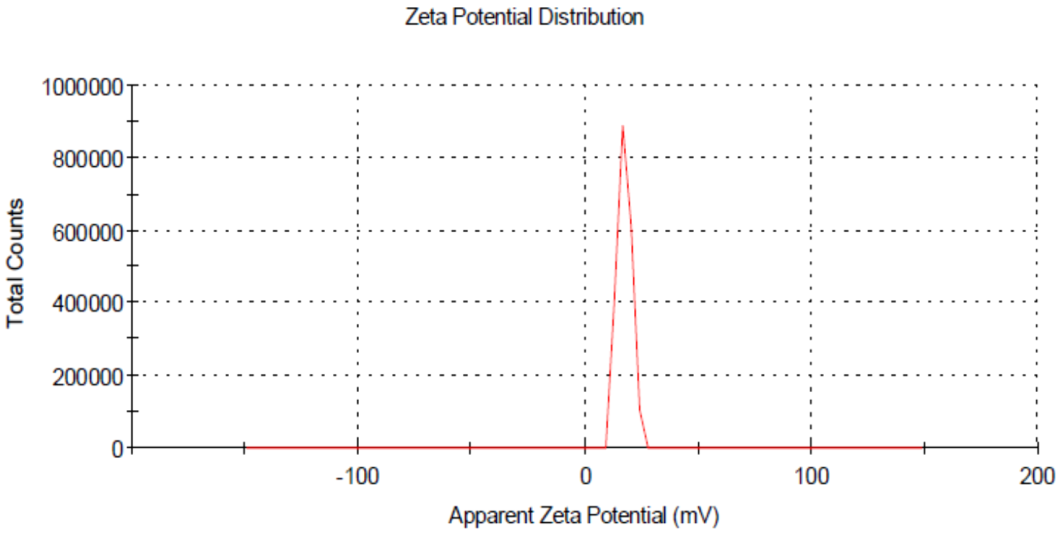

Fig. 1. Zeta potential values of salicylic acid nanoparticles.

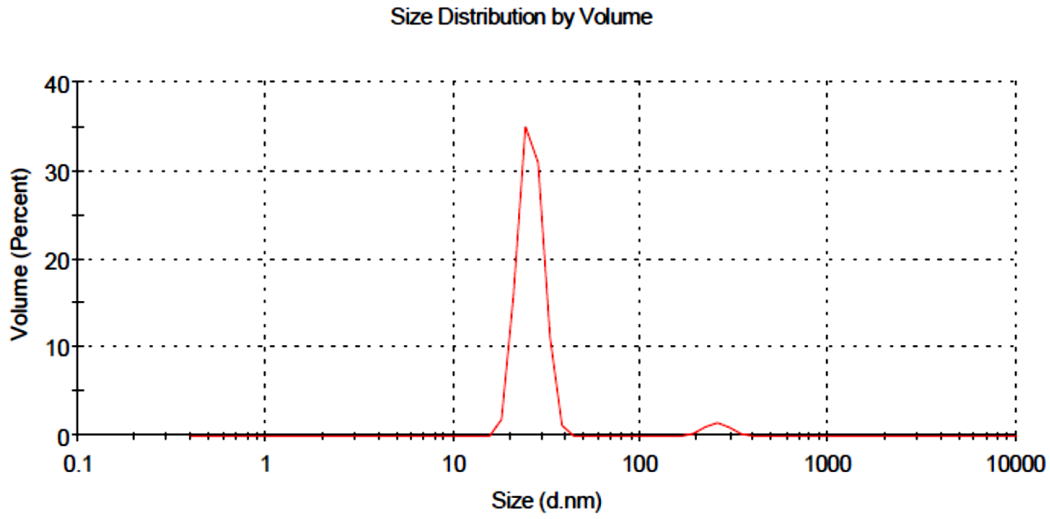

Fig 2. Z average size of SA NPs, 40 nm.

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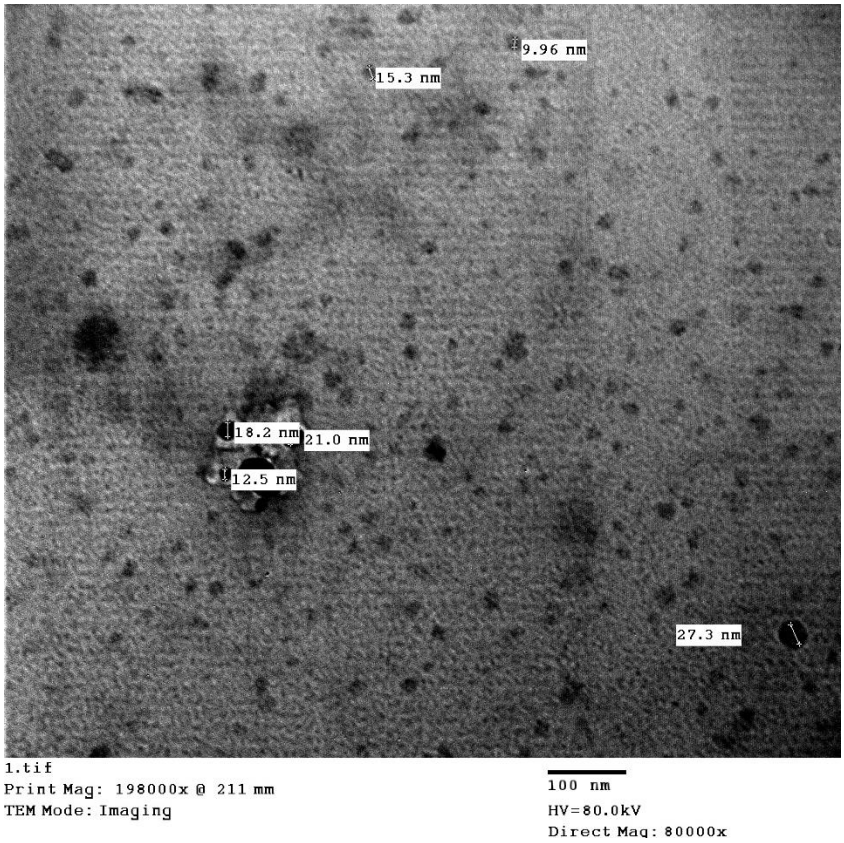

Fig 3. Transmission electron microscopy (TEM) view of formulated SA nanoparticles.

Antifungal effect of salicylic acid on A. alternata growth in vitro:

When compared to the control, all concentrations of SA nanoparticles and bulk material suppressed *A. alternata* mycelial growth on PDA medium. However, the treatments at 0.5 and 1 mM had varying effects on *A. alternata* growth (Table 1). SA nanoparticles at 1 mM were more effective than other tested concentrations in inhibiting *A. alternata* mycelial growth. Meanwhile, SA at 0.5 mM inhibited *A. alternata* only slightly. Furthermore, at 0.5 mM, there were no significant differences between SA nanoparticles and bulk material. While significant discrepancies in linear growth of *A. alternata* were detected between the low and the high concentrations of the two SA forms.

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Table 1. Antifungal effect of salicylic acid on the growth (mm) of *A. alternata in vitro.*

Treatments	Conc. mM	Linear growth	
		Mm	Ef %
Salicylic acid Bulk material	0.5	79.5 b	11.66
	1	52.5 c	41.66
Salicylic acid nanoparticles	0.5	71.00 b	21.11
	1	46.75 c	48.05
Control		90.00 a	

Within each column, same letter/s indicates no significant difference among treatments at (P<0.05). mm= millimetres. Ef % = Efficacy Control (un-treated).

Antifungal effect of salicylic acid on P. digitatum growth in vitro:

The mycelial growth of *P. digitatum* on PDA medium has been suppressed compared to the control by all concentrations of SA nanoparticles and bulk material. Nevertheless, different suppression effects were observed with *P. digitatum* growth at 0,5 and 1 mM. (Table 2). Treatment for SA nanoparticles at 1 mM were more effective than other concentrations tested in inhibiting *P. digitatum* mycelial growth. In the meantime, SA with 0,5 mM has shown low inhibition of *P. digitatum*. Furthermore, the data showed that the SA nanoparticles and bulk material were recorded at 0.5mM without any significant differences. However significant changes in linear growth of *P. digitatum* were noticed between the low and the high concentrations of the two SA forms.

Table 2. Antifungal effect of salicylic acid on the growth (mm) of *P. digitatum in vitro.*

Treatments	Conc. mM	Linear growth	
		Mm	Ef %
Salicylic acid Bulk material	0.5	81.5 b	9.44
	1	60.5 c	32.77
Salicylic acid nanoparticles	0.5	78.00 b	13.33
	1	54.75 c	39.16
Control		90.00 a	

Within each column, same letter/s indicates no significant difference among treatments at (P<0.05). mm= millimetres. Ef % = Efficacy Control (un-treated).

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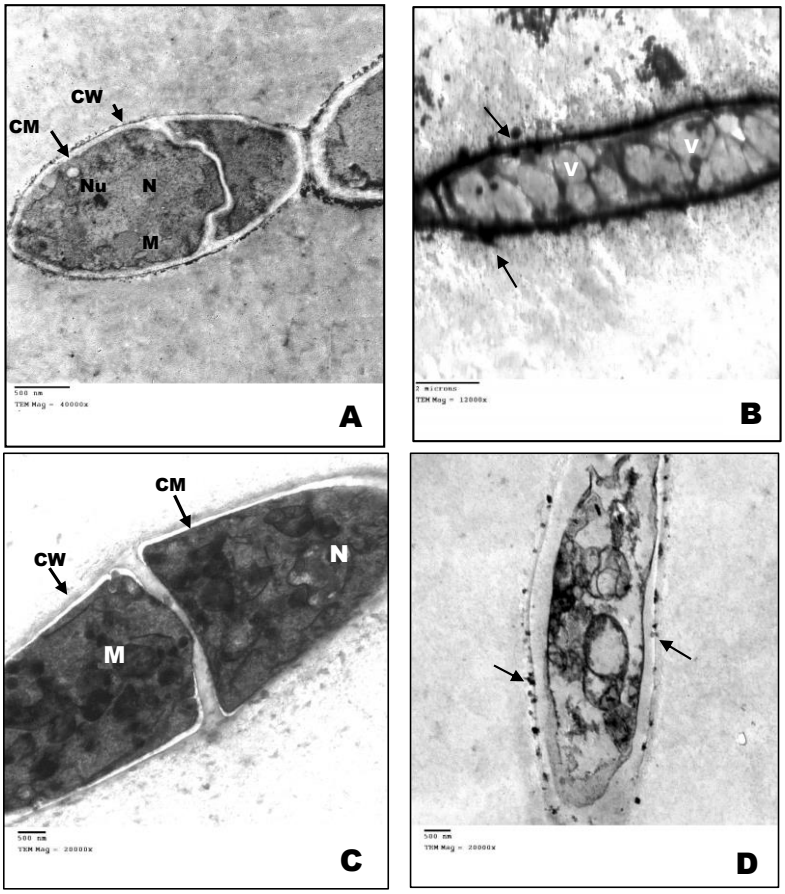

Fig 4. TEM micrograph of *Alternaria alternata.* A; control, B; treated with salicylic acid nanoparticles and *Penicillium digitatum* C; control, D; treated with salicylic acid nanoparticles. CW: Cell Wall, CM: Cell membrane, M: Mitochondria, N: Nucleus.

In *Alternaria alternata* ultrathin sections at 7 days of growth, there were clear changes according to the treated with salicylic acid nanoparticles at 1mM concentration; the spinulose conidial cell wall and cell membrane showed increasing in thickness with accumulation of salicylic acid nanoparticles (arrows) and appearance of numerous vacuoles (V) in the cytoplasm with undefined cytoplasmic organelles (Fig 4B) compared with the control (without treatments); mycelium that showed well-defined ultrastructure organelles; nucleus (N) with its nucleolus (Nu), mitochondria (M) and an even cell wall (CW) and cell membrane (CM); (Fig 4A).

The untreated (control) *Penicillium digitatum* ultrathin sections exhibit a compact cell wall (CW), continuous cytoplasmic membrane (CM), homogeneous and electron-dense cytoplasm with normal organelles appearance; (M), nucleus (N) (Fig 4C).

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By contrast, *Penicillium digitatum* ultrathin sections cultured with 1 mM concentration salicylic acid nanoparticles exhibited a disrupted cell wall (CW) and several invaginations in the cytoplasmic membrane; shrinkage and decrease in thickness with completely cytoplasmic organelles leakage and accumulation of salicylic acid nanoparticles (arrows) (Fig 4D).

Table 3. Effect of salicylic acid in the normal and nano form against phytopathogenic fungi *Alternaria alternata* and *Penicillium digitatum* on Valencia orange in Ismailia governorate.

Treatments	Concentrations mM	% Disease incidence	
		Alternaria alternata (Leaf spot)	*Penicillium digitatum* (Fruit rot)
Nano salicylic acid SA NPs	0.5	7	11
	1	5	8
Normal Salicylic acid Bulk	0.5	13	14
	1	9	10
Control	---	28	27

Table (3) showed that nano and normal salicylic acid decreased the percentage of disease incidence for both *Alternaria alternata* and *Penicillium digitatum* compared to the control (Table 3).

Discussion

The main purpose of this study was to assess the effectiveness of SA treatment for leaf spot and fruit rot diseases, incidence of diseases, morphological and fungal organelles in two forms (nanoparticles and bulk materials). SA NPs are produced and afterwards analyzed by the dynamic method of light diffusion. The results showed the development of large and average nanoscale dispersed particles. In this work we investigated the improved activity of SA NPs in the suppression of fungal growth, changes in fungal property, reduced disease severity, incidence, and increased plant defense response compared with their bulk equivalents. In the improved action mode of SA NPs nanosize effects, which are clearly different from those of their large counterparts, might be explained **(Aslani *et al.*, 2014)**. The increased surface area and the tiny size of the nanoparticles enhance their solubility and dispersion into biodiversity samples **(Chen *et al.*, 2020)**. In vitro results have shown that the linear growth of *A. alternata* and *Penicillium digitatum* has been significantly suppressed by both forms of SA therapy. A significant inhibition occurred when the level of SA is increased. This finding is well supported by several previous studies that show that SA can prevent fungal growth directly in vitro **(Panahirad *et al.*, 2014; Qi *et al.*, 2012)**.

The linear growth of *A. solani*, with an increase in salicylic and citric acid concentrations, was significantly decreased. SA treatment has suppressed mycelial growth and conidial sprouting of *Fusarium mangiferae* **(Kumar & Bains, 2018)**. SA treatment at 2 mM showed direct fungal toxicity on *Monilinia Fructicola* and considerably inhibited mycelial growth and in vitro spore germination **(Yao & Tian, 2005)**. Several potential mechanisms proposed by **Dieryckx et al. (2015)** represented that the fungal growth inhibition following SA applications. These include (i) accumulation of Reactive Oxygen Species (ROS) and suppressing enzymes involved in ROS detoxifications, (ii) alterations in mitochondrial respiration by disturbing the the Krebs cycle, and (iii) damage to fungal cell wall integrity by reducing the fungal cell volumes of Cerato-platanine-related protein (CPP). CPP are small, produced proteins produced by the fungi and play a major role in fungal growth **(Gaderer *et al.*, 2014).** PR-1 proteins may be involved in cell wall thickening and resist pathogen spread in the apoplast, according to one proposed function **(Chmielowska *et al.*, 2010)**. Some research has shown that β-1,3-glucanase proteins can act directly by degrading the pathogen cell wall, or indirectly by generating signal molecules that act as a defence elicitor.

Conclusion

This study showed that the exogenous application of SA in nanoparticles could increase the defence response of navel orange, which interpreted the application to decrease the severity of leaf spot and fruit rot. Implementing SANPs in agricultural practises may minimise the scope of chemical controls and that finding is intended to pave the way for a better and more sustainable method of disease control and reduction in losses of yield.

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