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Salicylic acid impact on *Penicillium digitatum* and *Alternaria alternata in vitro* and post-harvest lemon diseases

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Abstract: Lemons are susceptible to the post-harvest decay caused by *Penicillium digitatum* and *Alternaria alternata*, causing the green mold and black rot disease, respectively. The current study aimed to investigate the potential impact of salicylic acid (SA) as a natural defense inducer on radical growth, spore germination, and disease development afflicted by *P. digitatum* and *A. alternata*. Antifungal activities of SA were determined *in vitro* by plating fungal cultures on medium supplemented to various SA concentrations (0, 1, 2, 4, 6, 8, and 16 mM). Our *in vitro* experiments demonstrated that SA significantly reduced conidial germination and the radial growth of both pathogens in a dose-dependent manner. Moreover, *in vivo* assays confirmed that SA remarkably reduced lesion diameter on the lemon fruits treated by 8- and 16-mM SA before inoculation by both tested pathogens. To sum up, we suggested a potential implication of SA as a post-harvest treatment to control *P. digitatum* and *A. alternata* on a lemon at commercial scales.

Key words: Salicylic acid, Radical growth, conidial germination, Fungal pathogens

INTRODUCTION

Citrus is one of the most extensively cultivated fruit crops worldwide, distributed over the tropical, subtropical, and Mediterranean regions (Liu et al. 2012). Lemons are among the most commonly consumed citrus worldwide since this fruit contains high levels of vitamin C, antioxidants, and flavonoids, beneficial for human health (Yan et al. 2010). Lemon fruits are susceptible to various post-harvest diseases, accounting for imposing severe economic losses in these fruit crops. Fungal pathogens cause nearly 30%-50% citrus damages at different storage phases after harvest (Ladanyia 2010). Green mold and black rot caused by *Penicillium digitatum* and *Alternaria alternata*, respectively, are considered as economically destructive fungal pathogens, leading to severe quality and quantity losses during transportation and storage procedures (Aminifard et al. 2013; Babalar et al. 2007; Ismail & Zhang 2004; Palou 2014; Timmer et al. 2003). Both agents are necrotrophic pathogens, penetrating the fruit through rind wounds caused during pre-and post-harvest phases, including transportation (Cheng et al. 2020; Marcet-Houben et al. 2012; Saito & Xiao 2017). Previous reports demonstrated that any mechanical and physiological injury during pre and post-harvest handling practices facilitates penetration and contamination with these fungi (Martinez-Romero et al. 2006). Besides the economic losses, mycotoxins produced by these fungi impose various acute and chronic effects on humans and animals' health (Barkai-Golan & Paster 2008).

Applications of synthetic chemical fungicides such as prochloraz and imazalil are the most effective measure to control post-harvest disease in citrus fruits (Ismail & Zhang 2004; Kinay et al. 2007). Nevertheless, the use of chemical fungicides causes long-term health problems and environmental concerns because of the chemical residing in the food chain (Zubrod et al. 2019).

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Moreover, this has led to the systematic development of fungicide-resistant strain, often cross-resistant to different types of fungicides (Jurick et al. 2017; Kinay et al. 2007). Hence, there are many restrictions on producing chemical fungicides in developed countries, and governments are trying to limit their usage in agricultural ecosystems (McGrath 2004).

The best alternative strategies for managing post-harvest diseases are biological and physical practices (Spadaro & Gullino 2004). Additionally, applying safe low-toxicity chemicals such as natural compounds and inducers has been considered a sustainable approach to control post-harvest disease by prompting plant resistance against fungal pathogens. It is documented that these strategies are environmental-friendly, economical, and sustainable for plant protection (Da Rocha & Hammerschmidt 2005; Panebianco et al. 2014; Patel et al. 2020; Terry & Joyce 2004). Exogenous application of biological and physical elicitors, inducing defense mechanisms against fungal pathogens is a potential approach for controlling post-harvest disease. This strategy is considered vital component of an integrated post-harvest disease management program. Pre- or post-harvest application of salicylic acid (SA) reduces the citrus post-harvest disease by inducing the accumulation of H_2O_2 as well as primary metabolites and declines the lesion diameter caused by *P. expansum* on sweet cherry fruit through activating the antioxidant enzymes (Xu & Tian 2008; Zhu et al. 2016). SA is an important plant hormone, extensively involved in the biosynthesis of different defensive compounds in plants and triggers multiple defensive reactions against diverse biotic stresses (Ali et al. 2018; Palmer et al. 2017).

There are accumulating reports demonstrating that SA enhances the resistance of fruits, including citrus, pears, apples, cherry, strawberry, and tomato, against diverse fungal pathogens (da Rocha Neto et al. 2015; Spletzer & Enyedi 1999; Yao & Tian 2005; Yu et al. 2007; Zainuri et al. 2001). Furthermore, various studies indicated that SA possesses antifungal activity *in vitro*. It is shown that this natural inducer could significantly inhibit the mycelial growth of *Rhizopus stolonifer*, *Botrytis cinerea*, and *Colletotrichum gloeosporioides* (He et al. 2017; Shen & Yang 2017; Zhang et al. 2010). Similarly, other research was conducted to investigate the antimicrobial impacts of SA against various fungi causing rot, e.g., *B. cinerea* in strawberries (Babalar et al. 2007), *B. cinerea* in kiwifruits (Fatemi et al. 2013), *P. digitatum* in blood oranges (Aminifard et al. 2013).

The efficacy of SA as an antifungal agent and the inducer of resistance to manage green mold and black rot disease of lemon fruits has never been investigated. We hypothesize treatment of lemon fruits by SA culminating in eliciting defense reactions and reducing the post-harvest disease caused by *P. digitatum* and *A. alternata*. This idea encouraged us to investigate the inhibitory impact of SA, as a natural defense inducer, on the growth and conidial germination of *P. digitatum* and *A. alternata*, causing green mold and black rot disease in vitro, (b) to evaluate the efficacy of SA as a preharvest treatment on controlling the investigated post-harvest disease *in vivo*.

MATERIALS AND METHODS

Plant material

Healthy, blemish, and freshly harvested lemon fruits were obtained from a commercial orchid located in Guilan province, Iran. The harvested fruits were transferred to the laboratory of Plant Pathology at the University of Tehran and were stored at room temperature (20 °C, 85–95% relative humidity).

Pathogen inoculation

We obtained *P. digitatum* and A. *alternata* from the fungal collection of the Plant Protection department located at the University of Tehran. They were initially isolated from lemon fruits kept in cold storage rooms in Mazandaran and Alborz provinces, Iran. Fungal cultures were maintained on potato dextrose agar (PDA) (Sigma-Aldrich Chemie, Steinheim, Germany) at 25 °C. The conidial suspensions of the targeted pathogens were prepared from 6-day-old cultures in sterile distilled water. Spore concentration was calculated by a hemocytometer tool and adjusted as needed with sterile distilled water. Stock solutions of 100 mM SA were prepared in ethanol, and subsequently, 2 µl of this solution was added to 1 ml of the water to gain 200 µM solution of SA. The working solutions were obtained from diluting the 200 µM SA in distilled water.

Mycelial growth assays

To evaluate the effect of different SA concentrations on mycelial growth of the *P. digitatum* and A. *alternata*, both fungal isolates were inoculated on PDA medium and maintained for five days at 25 °C to produce new fungal cultures. Furthermore, fresh PDA plates amended with various SA concentrations (0, 1, 2. 4, 6, 8, 10, 12, 14, and 16 mM) were prepared. Subsequently, PDA plugs excised from actively growing edges of fungal colonies were placed in the center of the media supplemented with various SA concentrations. The inoculated plates were kept for seven days at 25 °C under darkness. Finally, fungal colony diameters were recorded by seven-days post-inoculation. Experiments were repeated twice with three biological replications per treatment.

Germination frequency assay

PDA plates amended with 0,1, 2, 4, 6, and 8 mM SA were made and cut into one cm^2 plug positioned on glass slides, were subsequently inoculated with 10 µl of a spore suspension (10^4 spores/mL) that were then covered with a coverslip. The samples were kept in Petri plates containing a piece of wetted cotton wool to maintain high relative humidity and were incubated at 25 ºC for three days.

The germination frequency of each isolate was measured based on the number of germinated spores of 100 randomly selected spores through a light microscope (Zeiss, Munich, Germany) at 40 x magnification. The experiments were conducted in three replicates, and the percentage of germinated spores was recorded, as shown in Fig. 1.

RESULTS

Impact of SA on mycelial growth

Impacts of various SA concentrations (0, 1,2,4, 6, 8, and 16 mM) on the colony diameter formation of *P. digitatum* and *A. alternata* seven days post-inoculation were shown in Fig. 1.

We observed a negative correlation between SA concentration and colony diameter formation. As we expected, the highest colony diameter was recorded on the control medium (without SA), whereas the minimum colony diameter formation was found on a medium supplemented with 16 mM SA. In both treatments, an increase in SA concentration led to a significant reduction in colony diameter formation of both targeted pathogens, indicating that SA significantly influences the mycelial growth of both fungal pathogens in a dose-dependent manner (Fig. 2).

Impact of SA on conidial germination

Impact of various SA concentrations (0, 1, 2, 4, 6, and 8 mM) on conidial germination of *P. digitatum* and *A. alternata* 24, 48, and 72 hours post-inoculation displayed in Fig. 2. Our results indicated that SA triggered conidial germination of *P. digitatum* at low concentrations in such a way that the highest germination frequency of 90-98% was recorded at 1 and 2 mM, whereas other applied concentrations (6 and 8 mM) blocked the conidial germination of the *P. digitatum*. Additionally, *A. alternata* conidial germination was significantly reduced at 1 and 2 SA mM compared with that of control (0 mM), whereas SA at high concentrations (6 and 8 mM) inhibits conidial germination of *A. alternata* (Fig. 3).

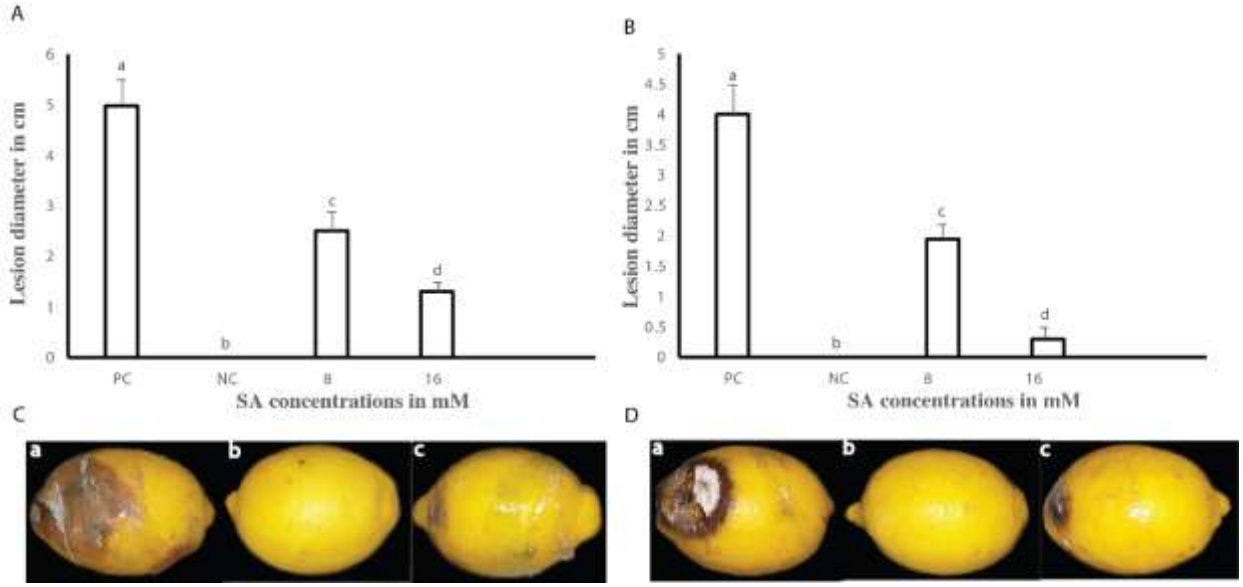

Fig. 1. Lesion diameter formed on lemon fruits treated by 8 and 16 mM SA prior to inoculation by (A) *Penicillium digitatum* and (B) *Alternaria alternata*, (C-a) Disease development on lemon fruits inoculated by *P. digitatum* (Positive control: PC), (C-b) Lemon fruit treated by distilled water (Negative control: NC) and Lemon fruits treated by 16 mM SA for 1 min before inoculation by *P. digitatum*. (D-a) Disease development on lemon fruits inoculated by *A. alternata*. (Positive control (PC)), (D-b) Lemon fruit treated by distilled water (Negative control: NC) and Lemon fruits treated by 16 mM SA for 1 min before inoculation by *A. alternata*.

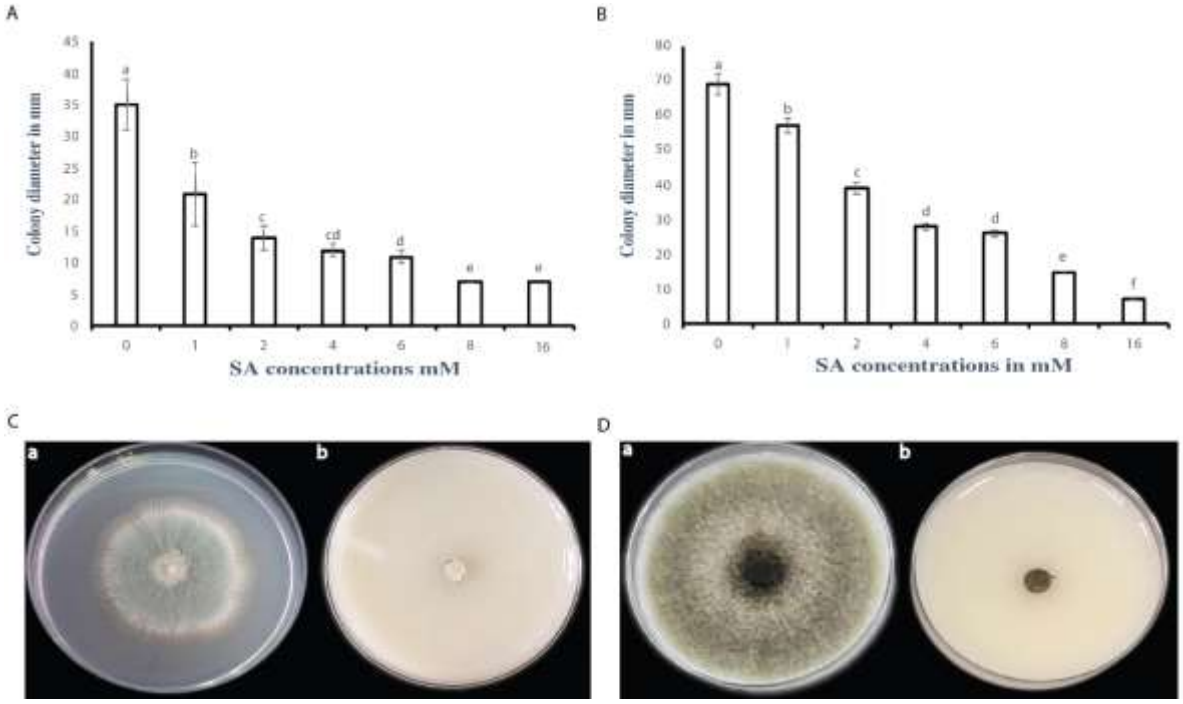

Fig 2. SA significantly impacts colony diameter formation of (A) *Penicillium digitatum* and (B) *Alternaria alternata* in a dose-dependent manner PDA plugs cut from the actively growing edge of both fungal pathogens were placed in the center of the PDA medium supplemented with 0, 1, 2, 4, 6, 8, and 16 mM SA, and the colony diameter formation was recorded seven days post-inoculation. (C-a and b) Radial growth of *P. digitatum* on PDA medium amended by 0 and 16 mM SA, (D-a and b) Radial growth of *A. alternata* on PDA medium supplemented with 0 and 16 mM SA.

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SA reduces disease development

We monitored the disease development of green mold and black rot on the lemon fruits inoculated by *P. digitatum* or *A. alternata*. We measured the lesion diameter formed on the inoculated fruits 25 days post-inoculation. Our results demonstrated that SA remarkably reduced lesion diameter on the inoculated lemon fruits treated by 8 and 16 mM SA.

The highest lesion diameters were observed on the positive control. No lesions were formed on the fruit inoculated by distilled water (negative control). The highest applied SA concentration (16mM) provides the maximum inhibition of both targeted post-harvest diseases.

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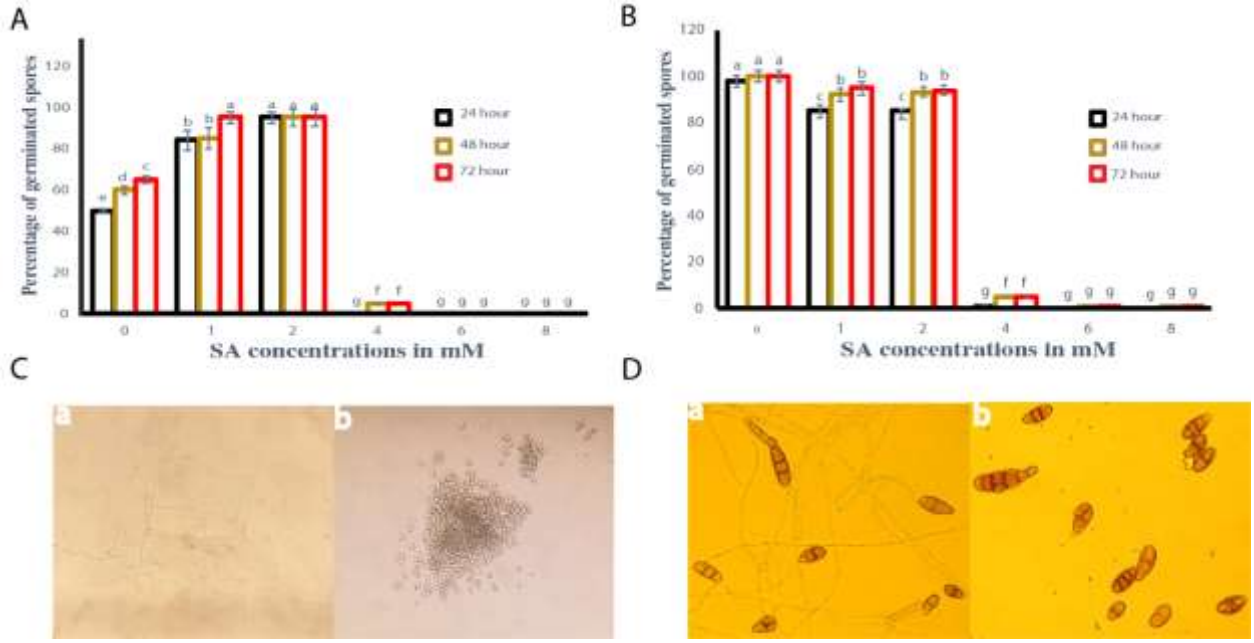

Fig 3- Spore germination frequencies of (A) *Penicillium digitatum* and (B) *Alternaria alternata* after treatments with SA. Spores of the targeted fungal pathogens were plated on PDA plugs amended with 0, 1, 2, 4, 6 and 8 mM SA, and the percentage of the germinated spores were determined at 24, 48, and 72 hours post-inoculation. For each strain, 50 spores were analyzed in three biological replicates. (C) Microscopic visualization of spore germination of *P. digitatum* placed on a PDA plug supplemented with 0 (a), and 8 (b) mM SA, (D) Microscopic visualization of spore germination of *A. alternata* spotted on a PDA plug supplemented with 0 (a) and 8 (b) mM SA.

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DISCUSSION

In the previous decade, induced resistance in citrus fruits through applications of organic and natural substances such as SA has increasingly emerged as a promising and alternative measure compared to the synthetic fungicides to control post-harvest fungal pathogens (Aminifard et al. 2013; Fatemi et al. 2013; Iqbal et al. 2012; Zhu et al. 2016). Presently, SA with *essential* antimicrobial and elicitor activities provides valuable potential in controlling the citrus post-harvest disease as a natural and biodegradable compound (Benhamou 1996). SA as a phytohormone plays crucial role in enhancing various physiological functions such as plant growth and eliciting plant defense mechanisms towards biotic and abiotic stresses (Arif et al. 2020).

There is accumulating evidence demonstrating that exogenous application of SA as spraying or dipping is an efficient approach, to manage citrus post-harvest pathogens (Iqbal et al. 2012; Shi et al. 2018). Numerous studies indicated a positive impact of SA treatment on the preservation of physical and chemical fruit properties such as the weight loss of strawberries (Shafiee et al. 2010), total soluble solids, and titratable acidity of grapes (Qin et al. 2015) and different physicochemical characteristics of apples (da Rocha Neto et al. 2016). Therefore, SA plays a vital role in maintaining the fruit quality during the post-harvest handling practices along with its capacity to manage post-harvest pathogens due to its potential as an antifungal agent.

Our results indicated that SA possesses a potential impact in reducing the colony growth, conidial germination of *P. digitatum* or *A. alternata*, causal agents of green mold, and black rot diseases. Furthermore, we observed that high SA concentration (16 mM) significantly reduced the disease development on lemon fruits inoculated by the investigated fungal pathogens even with a low pre-treatment time of 1 min. Exogenous treatments of SA (≥6 mM) were more effective in decreasing the measured parameters, including mycelial growth, spore germination, and disease development of both tested fungal pathogens. Complete inhibitions of spore germination through applications of high SA concentrations (6 and 8 mM) were noticed, demonstrating SA's potential in reducing the virulence of the applied post-harvest phytopathogens. Remarkable reductions of measured parameters here might be attributed to the fungitoxic impact of the SA (Iqbal et al. 2012; Qin et al. 2015; Yao & Tian 2005).

Our study corroborated the fungistatic or fungitoxic impact of SA treatment on the radial growth and spore germination of two economically damaging post-harvest fungal pathogens (*P. digitatum* or *A. alternata*) in a dose-dependent manner as previously documented (da Rocha Neto et al. 2015, 2016; Yu et al. 2007). The results showed the potential impact of SA as fungistatic agent at low SA concentrations where the radial growth and conidial

germination with lower frequencies compared with the control. The *in vitro* results (treatment by SA at high concentrations) were in agreement with results of Iqbal et al (2012) where it was shown that SA (≥6 mM) has a direct fungitoxic effect to completely inhibit the radial growth and conidial germination of *P. digitatum* and *P. italicum* (Iqbal et al. 2012). On the other hand, this finding was in disagreement with research conducted by Panahirad et al (2012). They demonstrated that five mM SA concentration was able to completely block the *Rhizopus stolonifer* growth under *in vitro* conditions (Panahirad et al. 2012). However, the complete fungal inhibition through treatment with a higher SA concentration (≥10.0 mM) than reported here documented in other plant-pathogenic fungi such as *Botrytis cinerea* (Strobel & Porter 2005). Additionally, Strobel & Porter (2005) documented that 2-10 mM SA concentrations had a moderate inhibitory impact on *B. cinerea and C. graminicola in vitro*. Therefore, they recommended adding synergistic antifungal materials such as cupric chloride to achieve complete growth inhibition of the mentioned phytopathogens combined with two mM SA (Strobel & Porter 2005). Numerous reports support the data obtained in the current study on the antifungal activity of SA. Indeed, there is a positive correlation between the antimicrobial activity of SA and its concentration, as reported by others (Aminifard et al. 2013; Babalar et al. 2007; da Rocha Neto et al. 2015, 2016; Fatemi et al. 2013; Iqbal et al. 2012; Strobel & Porter 2005; Yu et al. 2007). Interestingly, we found that SA stimulated the conidial germination of *P. digitatum* up to 95% at low concentrations (1 and 2 mM) compared to the control treatment (up to 50%)a and was not observed in the case of *A. alternata*.

In the current study, we showed that short time dipping of the lemon fruits (1 min) into the high SA concentrations (8 and 16 mM) could significantly restrict disease development caused by both phytopathogens. This result was in agreement with that of Iqbal et al (2012) whose employed two methods (spraying and dipping) to evaluate the efficacy of various SA concentrations in controlling citrus blue and green molds (Iqbal et al. 2012). It was demonstrated that eight mM SA could substantially reduce colony/lesion diameter, wound rotting, and spore mass density of *P. digitatum* compared to post-harvest treatment. Nevertheless, the application of 0.5 mM SA led to a significant reduction of decay incidence and lesion size on sweet cherry fruit inoculated by *P. expansum*. Furthermore, two mM SA applied as a preharvest treatment reduced remarkably lesion diameters on sweet cherry fruit caused by *Monilinia fructicola* (Yao & Tian 2005). Aminifard et al (2013) reported that treatment of blood orange fruits by five mM SA decreased, weight loss percentage, promoting the life storage of fruits, and leads to the lowest decay caused by *P. digitatum* (Aminifard et al. 2013). Additionally, Fatemi *et al.* (2013) confirmed that five mM SA employed, as a post-harvest treatment provided a lower decay caused by *B. cinerea* on kiwi fruit (Fatemi et al. 2013). It is worth mentioning that SA can trigger the activities of some antioxidant enzymes, such as playing a pivotal role in providing resistance response towards post-harvest phytopathogens (Tian et al. 2007).

Taken together, we have demonstrated the best threshold of SA concentration to reduce the viability of *P. digitatum* and *A. alternata* and to lose their virulence on lemon fruits. This claim is probably attributed to the fungitoxic impact of SA in reducing the spore germination and mycelial growth of both examined fungal pathogen that, eventually, leads to the virulence reduction of these post-harvest fungi. These findings suggest a potential implication of SA as a preharvest treatment to control *P. digitatum* and *A. alternata* at commercial scales. Indeed, the exogenous application of SA could be included as an essential component of the integrated program management of post-harvest disease as previously suggested. However, this idea required further and extensive investigations under long-term cold storage of lemon fruit to obtain novel insight into how SA induce defense mechanism such as defensive enzymes in the treated fruit. We recommend determining activities of the antioxidant enzymes in the treated lemon fruits by SA in the subsequent studies.

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مطالعه تاثیر سالیسیلیک اسید بر بیمارگر های *Penicillium digitatum* و *Alternaria alternata* در محیط کشت و پس از برداشت میوه لیمو

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چکیده : لیمو میوه‌ای حساس به پوسیدگی‌های پس از برداشت ناشی از *Penicillium digitatum* و *Alternaria alternate* است، که به ترتیب عامل بیماری‌های قارچی کپک سبز و پوسیدگی سیاه می‌باشند. مطالعه حاضر با هدف بررسی توانایی ساسلیک اسید (salicylic acid (SA)) بعنوان یک محرک دفاعی طبیعی برای کنترل *P. digitatum* و *A. alternate* در نظر گرفته شد. فعالیت ضد قارچی SA در شرایط آزمایشگاه با کشت جدایه‌های قارچی روی محیط‌های حاوی غلظت‌های مختبف SA (۰، ۱، ۲، ۴، ۶، ۸ و ۱۶) مورد بررسی قرار گرفت. آزمایشات آزمایشگاهی ثابت کردند که SA به طور معنی داری رشد شعاعی و جوانه زنی کنیدیوم هر دو بیماگر را در رفتار وابسته به دز کاهش می‌دهد. همچنین سنجش‌های برون آزمایشگاهی نیز نشان دادند که تیمار میوه‌های لیمو با غلظت‌های ۸ و ۱۶ میلی مولار SA قبل از تلقیح با جدایه‌های هر دو بیمارگر، به طور قابل ملاحضه‌ای قطر لکه‌های موجود در سطح میوه را کاهش می‌دهد. در مجموع، استفاده از SA بعنوان یک عامل بلقوه در جهت تیمار پیش از برداشت، به منظور کنترل بیمارگرهای *P. digitatum* و *A. alternate* در مقیاس تجاری پیشنهاد می‌شود.

کلمات کلیدی: سالسیلیک اسید، رشد قارچی، جوانه زنی، بیمارگرهای قارچی

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