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EVALUATION OF SALICYLIC ACID SOLUTION ON FUNGUS *BOTRYTIS CINEREA* THAT CAUSED STRAWBERRY GRAY MOLD

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Abstract

The current search aim to detect botrytis fruit rot or gray mold that consider one of the most important serious diseases of strawberry, cause significant loss of pre- and post-harvest fruit due to the ability of disease to develop not only in the field, but also during storage and transit. Using natural phenolic compound Salicylic acid (SA), in hope to control strawberry diseases by understanding it's action on fungus and plant. It has been found that exposure strawberry to (SA) less than 24 hours, able to protect and enhance growth of plant. While, exposure strawberry seedling to (SA) more than 24 hours, show negative effect on leaf anatomy by increase starch grain and reduce formation of vascular tissue. In addition, to make change in mycelium of *B. cinerea* after treatment with salicylic acid at 2 mg/ml concentration, by accumulate vacuole, and prevent growth of fungus.
Keywords: Strawberry gray mold, Salicylic acid, *Botrytis cinerea*, Antifungal activity

Introduction

Gray mold disease of strawberry fruits (*Fragaria x ananassa*) caused by necrotrophic fungus *Botrytis cinerea*, that belong to Sclerotiniaceae family (Ascomycota), consider serious problem in cover culture and during storage crops due to suitable environmental condition such as warm temperatures, poor air circulation and light ,lead to rapid sporulation (Williamson *et al*., 2007 and Ciliberti *et al.,* 2015). Numerous approaches were used to control these disease achieved by different fungicides, soil fumigants and bio agents (Kim *et al*., 2007 Deising *et al*., 2008 and Boutin *et al.,* 2014) Unfortunately, most these approaches have unpleasant effect due to the increase resistant strains of pathogens against various chemical fungicides (Forster *et al.,* 2004; Deising *et al*., 2008 and Zafra *et al.,* 2015). Salicylic acid is monohydroxybenzoic acid consider one of the most famous natural phenolic compound (Arberg, 1981; Hayat *et al.,* 2010 and Alam *et al.,* 2013) that, isolated from plant White willow (*Salix alba*) that, affects a variety of biochemical and molecular events associated with induction of disease resistance ,its present as fungi toxic and antibacterial activities (Chandra & Bhatt, 1998 : Eraslan *et al*., 2010 and Liu *et al.,* 2016). Therefore, the main reasons for using salicylic acid as antifungal agents is their natural origin and limited chance of pathogens developing resistance compared to their synthetic alternatives, being plant products are easily convertible into known eco-friend materials (Gnanamanickam, 2002).

Strawberry's Gray Mold

Botrytis blight disease not only caused infection on strawberries, but also infects a wide array of herbaceous annual and perennial plants. Airborne fungus *Botrytis cinerea* (saprophyte) consider unspecialized necrotrophic fungus that a bearing as grayish masses of mycelium conidiophores, and conidia on the surface of rotted tissues and can survive as small hard nodules known as sclerotia or "resting structures" that able to growth into a mycelium which produces conidia (spores) .These spores are the agents of the primary infection of the plants. Not all species of Botrytis readily form sclerotia, so they may not be observed on all plants (Ciliberti *et al.,* 2015).

At warm weather, a masses of silver-gray spores on the dead or dying tissue, Figure (1). These spores are readily liberated, and may appear as a dust coming off of heavily infected on strawberries. This disease is a serious problem in all strawberry production during most year and the incubation period of *Botrytis* is very short and easily wind-dispersed (Kim *et al*., 2007 & Deising *et al*., 2008).

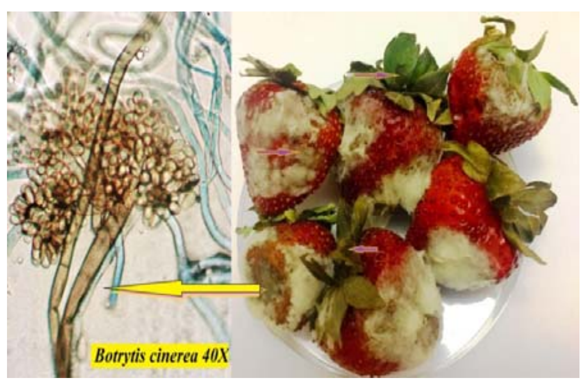


Fig. 1 : Gray mold on strawberry fruits. Photo: D.Y.M. Yousif

In the middle Winter and beginning Spring, with harmony prolonged periods of raining cloud and cool temperatures. Gray mold disease could be found during all stages of strawberry fruit development. The general diagnostic symptom are: fruit start rotted with smooth gray mold growth. Usually at the stem end of the fruit appear with light brown lesions because the flower infection or occur on the sides of fruit where soil, besides that, the infected berries, flower petals or standing water are in contact. Infected berries may remain firm, yet become covered with gray spores and mycelium. Some fruit die before maturity and other failure to developed, under high humidity due to suitable condition to the moldy growth and obvious as white to gray cottony mass and lesions may develop slowly. When gray mold completely propagation on fruit, it appear so tough, dry and mummified (Fernandes *et al.,* 2011).

Under high humid, and wet conditions, the gray mold fungus will produce silver masses of conidia that are spread via wind and splashing water onto the strawberry crop. For infection to take place, spores require free water on the plant tissues. Therefore, after rains gray mold infection can be widespread and transport in to other plants and prolonged leaf wetness. The most suitable temperature for spore germination was ranged from 59 to 72° F (15 to 25° C) Figure (2).

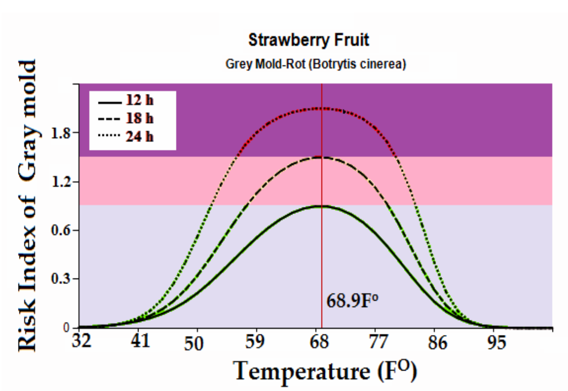


Fig. 2 : Gray mold disease risk on strawberry fruit at different temperatures

Generally, different way of disease cycle can be introduced in to the fields such as (1) transplants often are already contaminated with *Botrytis* spores or colonized by *Botrytis* mycelium growing in senescent and dead leaves and other tissues; (2) over wintering structures (sclerotia) survive in the soil or on plant residues in and around the field; (3) over wintering mycelium is present in dead and decayed residues from the previous crop; (4) active spore-producing *Botrytis* growth is present on adjacent crops, which can include nearby vegetable and second year strawberry plantings.

Many agronomic and horticultural practices were applied using resistant varieties, chemical and physical methods to control strawberry diseases (Fernandes *et al.,* 2011). Regrettably, most these ways have side effect and create new problems, due to the development resistant strains of pathogens against various chemical fungicides, residual toxicity in addition to the harmful result from the up natural uses (unfair hunts) which cause large effect on the environmental situation, the health and economic development. Continuously with random used of chemical fungicides has consider to be hazardous to different. Due to all these reasons, numerous searcher try to test many natural compounds to control disease and reduce economic losses.

Salicylic acid (SA)

SA is a ubiquitous monohydroxybenzoic acid (**$C_7H_6O_3$**) and its molecular weight 138.12 consist of an aromatic ring with one hydroxyl-OH group and is synthesized by plants and first isolated in 1829 by Leroux (Hayat *et al.,* 2010 and Alam *et al.,* 2013). That, consider some product of secondary metabolism and enters the defense mechanism owned by the plant against the pathogens SA is peel reagent (Arberg, 1981) which caused scabrous ness the surface that make change on surface properties (Yalpani, 1993; Mauch & Metraux, 1998 and Vicente &, Plasencia, 2011) figure (3). The salicylic acid has many effects, such as stimulating the plant to form protein-related with pathogenesis, and increasing the flowering period, inhibits the formation of ethylene, germinating the seeds and closing wounds. It reflects the effect of Abscisic acid (Vicente &, Plasencia, 2011).

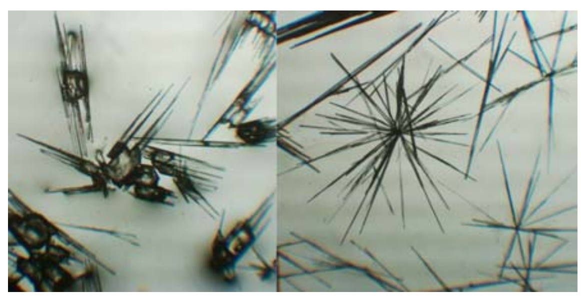


Fig. 3 : Dissolved Salicylic acid with ethanol 96%. Note that appear needles- like. Photos: D.Y.M. Yousif.

They major function of SA are: in lignin biosynthesis, regulation of plant responses to abiotic stimuli, pigmentation, growth, reproduction, resistance to pathogens, also in cell growth, seedling establishment, respiration, stomata closure, senescence-associated gene expression, basal thermos tolerance, nodulation in legumes, and fruit yield and many other functions (Vicente &, Plasencia, 2011; Zargarianetal, 2016; Dina, 2017 and Baraa & Dina, 2018). SA was thought to be the insignia due to (1) prompt, defense responses when added to plants, (2) transport systemically to other plant parts, (3) it is found in phloem exudates of infected leaves, and (4) it is required in systemic tissue for SAR signal development. However, later studies using grafting experiments showed that SAR response in wild type scions was triggered by infected SA deficient rootstocks (NahG) implying that SA is not the mobile signal (Kessmann & Ryals, 1994 and Park *et al*., 2007). SA in plants is synthesized via 2 pathways, one mediated by phenylalanine ammonia lyase (PAL) while other is mediated by isochorismate synthase (ICS) (Meuwly, 1995). In the PAL-mediated pathway, phenylalanine serves as precursor. Phenylalanine also serves as a precursor for the biosynthesis of other plant compounds such as phytoalexins, phenolics, and flavonoids that may provide physico-chemical barriers, hence preventing pathogen invasion (Yildirim, 2008). In tobacco, PAL converts phenylalanine to cinnamic acid, which is further converted to benzoic acid which serves as a precursor of SA (Figure 4). Through the shikimate pathway, chorismate is converted to isochorismate by ICS. Isochorismate is then converted to SA by isochorismatepyruvatelyase (Achuo *et al*., 2004 and Vlot *et al.,* 2009).

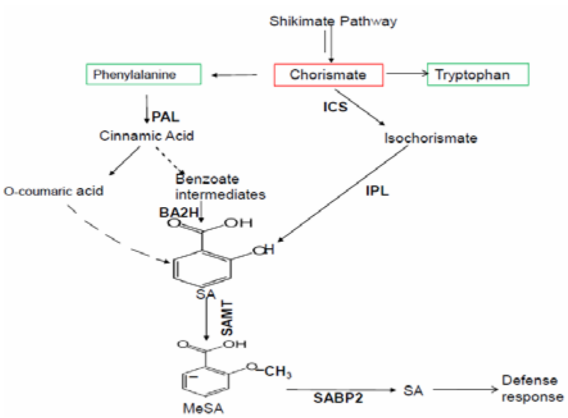


Fig. 4 : Pathway for SA Biosynthesis and Disease Resistance. Enzymes in this pathway are shown in bold. Abbreviations: PAL, phenylalanine ammonia lyase; ICS, isochorismate synthase; IPL, isochorismate pyruvate lyase; BA2H, benzoic acid-2- hydroxylase; SAMT, SA methyltransferase; (Figure adapted from Vlot *et al*., 2009).

Salicylic Acid Pathway and Disease Resistance

SA is involved in both local and systemic induced disease resistance responses. Advances in our understanding of plant defense signaling have shown that a network of signal transduction pathways are employed by plants some of which are dependent while others are independent of salicylic acid (Amin *et al*., 2007 and Vlot *et al*., 2009). Examples include signal transduction pathways mediated by phytohormones such as auxins, ethylene, cytokinin, gibberellins, jasmonates, and peptide hormones. SA is a key signal in thermogenesis regulation and disease resistance. SA also activates defense responses against biotrophic and hemi-biotrophic pathogens as well as plays a role in systemic acquired resistance (SAR) development (Kessmann & Ryals, 1994; Mauch & Metraux, 1998 and Achuo *et al*., 2004)

When a plant survives infection by a pathogen at one site, it often develops increased resistance to subsequent attacks at sites throughout the plant and enjoys protection against a wide range of pathogenic species. This phenomenon, called Systemic Acquired Resistance (SAR) (Figure 5), develops over several days following initial infection (Vicente &, Plasencia, 2011; Heil & Bostock, 2002; Gunesetal, 2007). Systemic acquired resistance appears to result from increased levels of certain PR proteins that we have already mentioned, including chitinases and other hydrolytic enzymes. Although the mechanism of SAR induction is still unknown, one of the endogenous signals involved is likely to be salicylic acid (Vicente & Plasencia, 2011; Zargarian *et al*., 2016 and Dina, 2017). This benzoic acid derivative accumulates dramatically in the zone of infection after the initial attack, and it is thought to establish SAR in other parts of the plant (Johansson, 2015). Another compound that accumulates at the site of infection and may play a role in SAR is H_2O_2. However, like salicylic acid, $\mathbf{H_2O_2}$ is unlikely to function as a long-distance signal.

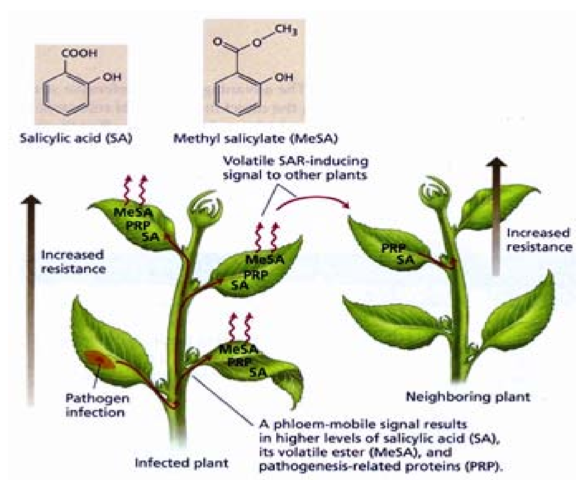


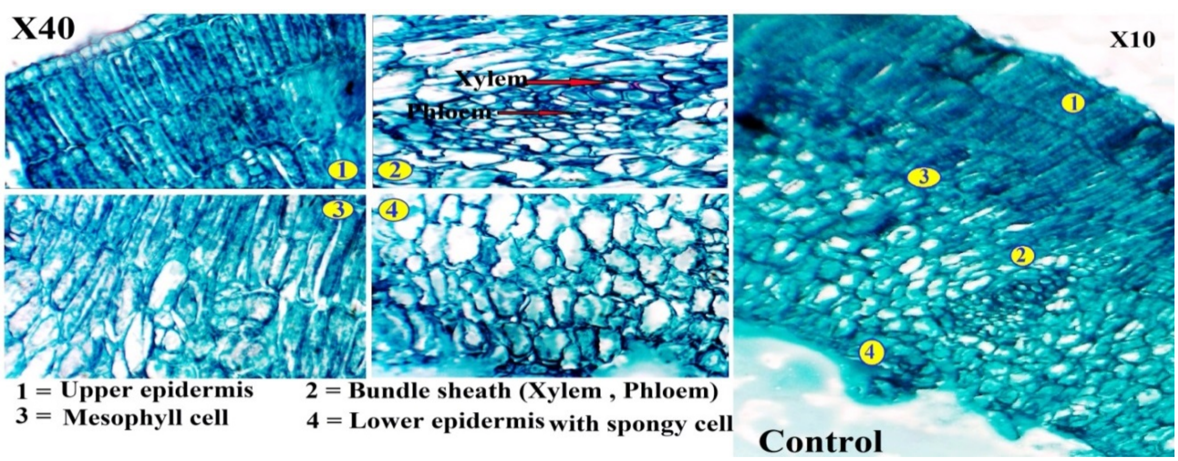
Fig. 5 : Disease Resistance. Initial pathogen infection may increase resistance to future pathogen attack through development of systemic acquired resistance (SAR) source: Taiz & Zeiger (2010)

Effect Salicylic Acid on strawberry and Gray mold disease

SA is a endogenous plant hormone (Raskin, 1992) that consider one of the most great agronomic potential regular growth and development of plant, that play an important role of antioxidant enzymes and increase plant tolerance to biotic and abiotic stresses (Hayat *et al.,* 2010 and Alam *et al.,* 2013). It is a regulator factor of photosynthesis process, due to their ability to caused change in leaf and chloroplast structure (Vicente &, Plasencia, 2011) Biosynthesis of proteins and soluble sugars by added SA, will increase in photosynthetic pigments. Accumulation of soluble sugar due to reduction the activity of Glucokinase, considered one of the important aspect in SA treatment condition (Arberg, 1981 and Yildirim *et al*., 2008).

Observations were recorded that present higher concentration of exogenous SA where decrease the thickness of plant leaf and accumulate carbohydrate products, that resulting from photosynthesis process as starch form (Xie *et al*., 2007 and Yildirim *et al.,* 2008) and that lead to slow down and stop the photosynthesis process completely which caused plant death (Zargarian *et al*., 2016 and Baraa & Dina, 2018). While, pretreatment with low concentration of SA had no effect on sugars under control condition (Figure 6). SA at (2mg/ml) caused changes in the anatomy of the leaf structure in strawberry seedling. This change could affect the growth of the plant. Nevertheless, showed a positive effect of SA at (1 mg/ml) on the composition of structures such as increase number of xylem vessels, mesophyll cells size, tissue of epidermis (Türkyilmaz *et al*., 2005 and Nina & Meruert, 2017). Many investigating focus on role of salicylic acid at concentration more than hundred lM (Rajjou, *et al*., 2005 and Xie *et al*., 2007). It is mention that SA have physiological concentration. Furthermore, increase in concentrations of SA have toxic effects on growth of plant and development, due to stimulate ROS biosynthesis (Singh & Usha, 2003).

Spry seedling for more than 24 hours negatively affected leaf anatomy by showed a positive effect of SA on the composition of structures such as xylem vessels, Kranz anatomy, mesophyll cells size (Hramtsova *et al*., 2003) tissue of epidermis, leaf blades and chloroplast development. Finally, the presence of salicylic acid in small amount has beneficial effects on the morphology of leaf (Türkyilmaz *et al*., 2005 and Nina & Meruert, 2017). Pretreatment of seedling with salicylic acid increase starch and soluble sugar content in seedling leaves Figure (6) (Orzechowski, 2008). Accumulation of soluble sugar due to reduction the activity of Glucokinase, considered one of the important aspect in SA treatment condition (Poór *et al.,* 2010). The duplex stimulation impact of SA on the biosynthesis of soluble sugars in strawberry leaf was note. Soluble sugars accumulated in leaf plants for osmotic organizing in response to present large amount of phenolic compound (toxic impact). Nevertheless, results concerning the effect of salicylic acid on carbohydrate accumulation differ (Durango *et al*., 2013 and Ning Zou *et al*., 2014).



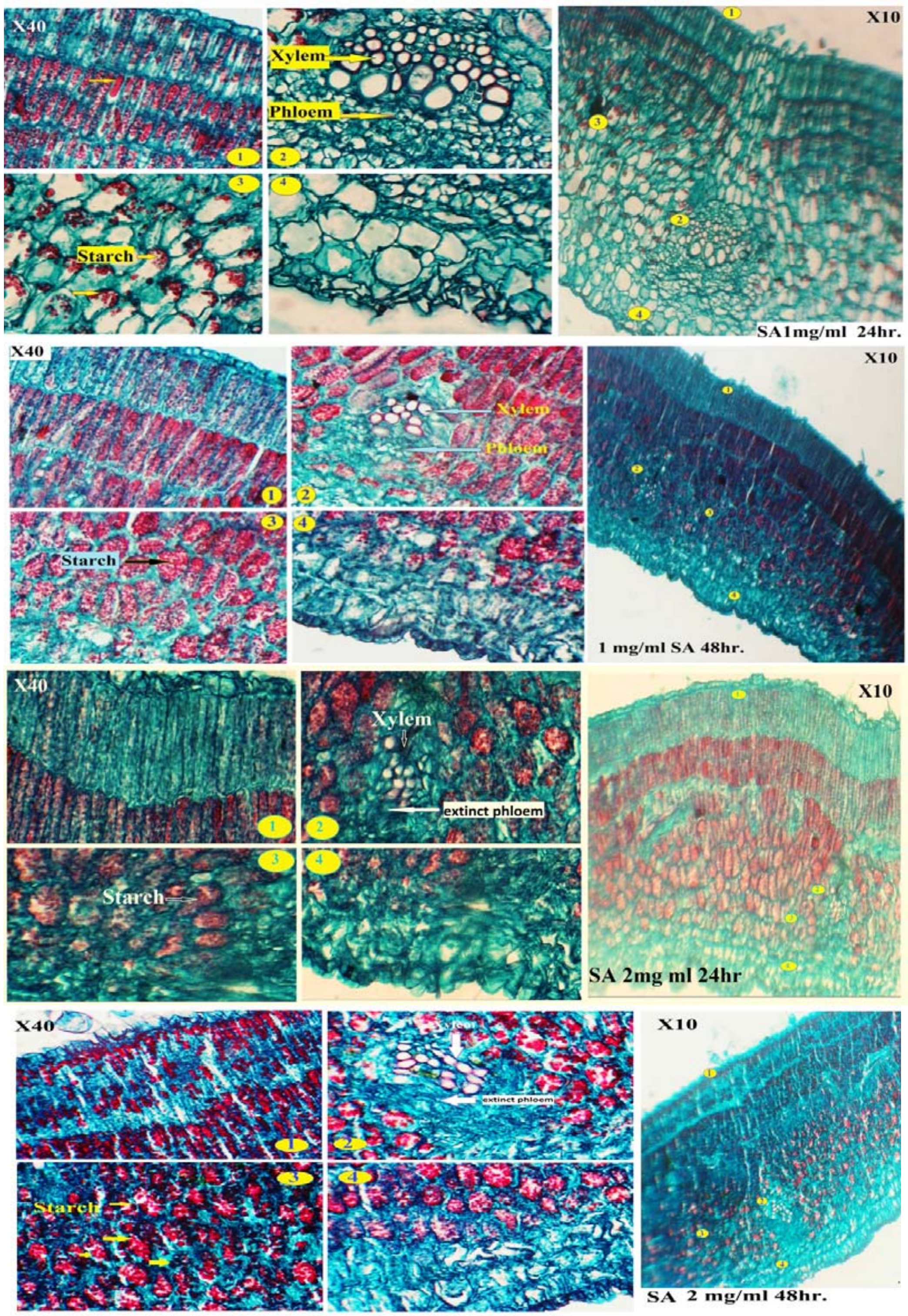


Fig. 6 : Light micrographs of the cross sections in strawberry leaf that spry with SA at different concentration for different time. Photos: D.Y.M. Yousif

SA is a endogenous plant hormone (Arberg, 1981; Hayat *et al.,* 2010), consider one of the most great agronomic potential regular growth and development of plant, that play an important role of antioxidant enzymes and increase plant tolerance to biotic and abiotic stresses. It is a regulator factor of photosynthesis process, due to their ability to caused change in leaf and chloroplast structure (Vicente & Plasencia, 2011).

Fariduddin and other (2003), reported that exogenous salicylic acid able to enhanced the photosynthetic rate, CO_2 assimilation and water use efficiency in *Brassica juncea*. Salicylic acid (2-hydroxybenzoic acid) consider as endogenous and exogenous elicitor ,plays important role in plant growth and development because it's plant hormone (Raskin, 1992) and serves as an endogenous signal to activate certain immune responses and to establish disease resistance by induction processes of Systemic Acquired Resistance (SAR) (Heil & Bostock, 2002; Gunesetal, 2007). Various defense-related stimuli have been shown to trigger enhanced SA levels in local and systemic plant tissues. Exogenous application of SA can stimulate particular enzymes catalyzing biosynthetic reactions to produce defense compounds (Achuo *et al*., 2004) and induce reactive oxygen intermediates (ROI) production, pathogenesis-related (PR) gene expression and immunity against various pathogens with bio trophic or hemi bio trophic lifestyles (Mauch & Metraux, 1998 and Durango *et al*., 2013). In another words, it's can be affirmed that SA acts in two ways, by inducing resistance in sweet green pepper, and also by the fungi toxic action on the pathogen (Singh & Usha , 2003). SA is known as an antioxidant compound which is involved in prohibition of the activity of reactive oxygen species (Amin *et al*., 2007). Interestingly, It was found that SA was able to significantly reduce mycelia growth at 2 mg/ml concentration completely, because SA is a natural phenolic compound contain monohydroxybenzoic acid with ortho and para position of OH- group (Türkyilmaz *et al*., 2005), that have inhibitory effect on microbial and that the reason to toxic effect on fungus (Fariduddin *et al.*, 2003) While, the less concentration was less (limited) effective for reducing phytopathoginc fungus, that make microscopic changes in the mycelium, maybe due to low concentration of salicylic acid make fungus utilize tiny amount of SA and storage in vacuole (Figure 7). The possible mechanisms action of SA, including: Heterogeneous morphology of the outer covering of the mycelium and conidia from thin mycelium to rosary shape and phenomenon Vacuolization. Besides that, SA could cause shrinking as seen in Figure (7) central vacuole loses water, that make large gaps between the cell wall and wall membrane due to osmosis (Tsekova *et al*., 2013). Vacuolization is rare phenomenon present external agents make fungi adaptive to physiological responses.

In another words, vacuole is believed to be involved in fungus defense due to accumulates a variety of hydrolytic enzymes, like lysosomes and stored foreign molecular as a disposal site for waste and poisons for fungus defense strategies to protect fungal cell from death (Zafra *et al.,* 2015) Different studies insure that appearing vacuoles inside fungus play very imported role to control & balance the cellular function, including storage of metabolites, degradation different molecular and regulation of cytosolic concentrations of phenol and detoxifies potentially toxic compound (Fariduddin *et al*., 2003 and Ansari *et al.,* 2013). This rare phenomenon may match to adaptive physiological responses to external agents.

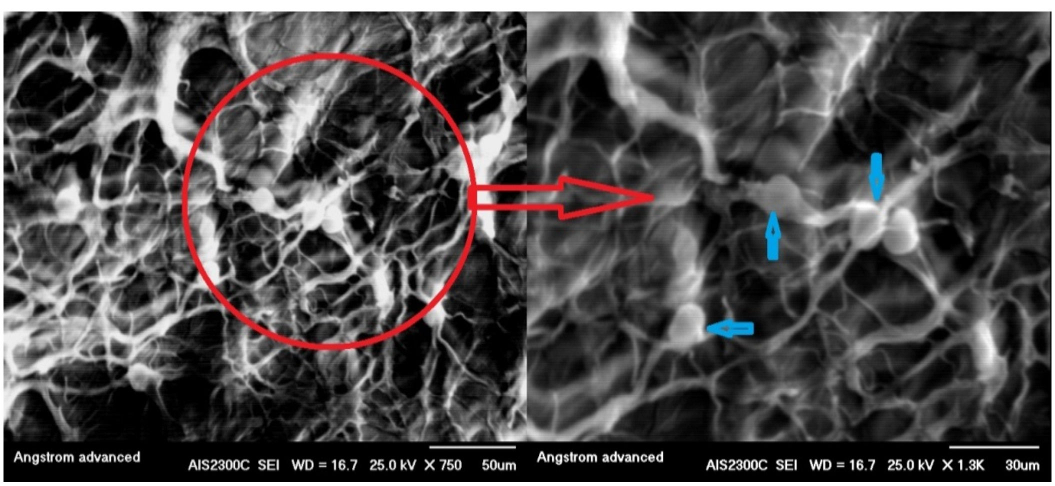


Fig. 7 : Scan electron microscope (SEM) image mycelium of *B. cinerea* after treatment with salicylic acid at 2 mg/ml concentration. Note rosary shape. Photo: D.Y. Yousif.

Exogenous application of SA promote defense mechanisms and production of antioxidants in fruits during storage that leads to a increases firmness of the fruit and decrease in lipid peroxidation of the cell membrane and results in maintained cell membrane structure (Alaea *et al.*, 2005 and Alaey *et al.*, 2011) and add acetyl salicylic acid (ASA) during storage for three months at refrigerator temperatures, will an excess the total anthocyanin concentration of pomegranate fruit (Shafiee *et al.*, 2007). By increase quality of strawberry plant salicylic acid consider a endogenous growth regulator, that stimulates flowering in a range of plants, increasing flower life, and controlling ion uptake by roots and stomatal conductivity (Zafari *et al.*, 2015 and Baninaiem *et al.*, 2016).

A previous studies reported that salicylic acid promotes flowering by acting as a chelating agent. In addition, salicylic acid induced local and systemic resistance to fungal pathogens. The advantageous impact of spry salicylic acid on the seedlings of strawberry noted a significantly reduced gray mold on seedling and improve seedlings development compared with control (Feliziani, *et al.*, 2015). It was shown that the using of phenolic compound will protected the seedling by disease resistance stimulated growth of seedlings, increase plant tolerance to environmental stresses (Zafari *et al.*, 2015 and Ezzat *et al.*, 2017) and enhance plant growth and yield (Sayyari *et al.*, 2011 and Ezzat *et al.*, 2017).

Numerous publications have mention that SA was able to reducing weight loss effective and retarding the senescence of fruit tissue, delaying the ripening process and evolution of ethylene to enhance flesh firmness for instance of sweet cherry (Yao & Tian, 2005) kiwifruit (Bal & Celik, 2010) and banana (Srivastava & Dwivedi, 2000) during cold storage SA and other derivatives are widely used to enhance pre- and postharvest quality of fruit by controlling strength of harvested different fruit softening such as peaches and strawberry during storage Babalar and other 2007 and Shafiee and other 2010 showed pre harvest and postharvest treatments of sweet cherry fruit with SA found significantly reduce disease percentages in storage at room temperature. While, at freezing temperature the spoiler effects of pre harvest SA treatments on postharvest disease were better than those of the postharvest treatment.

The hydroponic systems are very useful and proved to have several advantages in comparison with conventional agricultural systems (Geoponic). Under the hydroponic system, growth rate of strawberry seedlings are increase after spray with SA and faster than geoponic system that grown under the same conditions (Kläring, 2001 and Bridgewood, 2003). Generally, there was an inverse relationship between salicylic acid concentration and flowering time in both cultivars, where the increased concentration of the solution reduced the flowering time. While there is a positive relationship between salicylic acid concentration and the number flowering clusters, crown per plant, leaves, leaf area for each seedlings and total yield as see in table (1). Numerous searcher and scientists believe that there are several reasons for the vast differences between hydroponic and soil plants agriculture. Whereas increase oxygen level in the hydroponic growing mediums aid to stimulate root growth. Strawberry seedling with abundance oxygen in the root system also absorb nutrients faster (Kläring, 2001 and Bridgewood, 2003). Besides that, the nutrients in a hydroponic system are mixed with the water and deliver directly to the root system. The plant does not have to search in the soil for the nutrients that it requires due to close the nutrients to the root system. Those nutrients are being delivered to the plant several times per day, with very little energy to find and break down food. The seedling then uses this saved energy to grow faster and to produce more fruit. Hydroponic plants also have fewer problems with funguses infection and disease. In general, plants grown hydroponically are healthier and happier plants (Thongkamngam & Jaenaksorn, 2013 and Thongkamngam, & Jaenaksorn, 2017).

Table 1: Some component characteristics of *Strawberry cultivars* that spry with SA at different concentration .

Strawberry cultivars	SA treatments	Characteristics of growth rate											
		2015/2016						2016/2017					
		Days to flowering	No. flowering clusters	No. crowns/ plant	No. leaves/ plant	Leaf area/ plant	Total Yield (kg/plant	Days to flowering	No. flowering clusters	No. crowns/ plant	No. leaves/ plant	Leaf area/ plant	Total Yield (kg/plant)
In Geoponic	0 mg/ml(water)	56.20	5.89	4.82	23.23	353.58	0.543	55.22	5.83	5.15	22.53	364.40	0.607
	1mg/ml	53.74	7.24	5.99	25.29	372.68	0.615	53.69	7.66	6.23	24.98	362.45	0.643
	2mg/ml	49.13	7.46	6.01	27.96	394.04	0.621	51.28	7.82	5.74	28.28	390.04	0.629
	4mg/ml	46.37	8.52	6.21	30.08	405.25	0.716	47.69	9.12	5.96	31.67	410.28	0.715
In Hydroponic	0 mg/ml(water)	53.40	6.79	5.82	30.23	642.58	1.347	53.40	6.66	5.76	30.12	642.58	1.542
	1mg/ml	50.44	8.43	6.26	33.29	684.78	1.575	50.32	8.45	6.31	33.33	684.78	1.678
	2mg/ml	46.23	9.88	7.00	37.96	699.05	1.621	46.57	9.91	7.06	37.89	699.05	1.728
	4mg/ml	42.12	10.23	7.51	39.08	821.25	1.783	42.25	10.12	7.48	39.10	821.25	1.872

Conclusion

Without doubt, many management strategy used to protection strawberry plant either pre or post harvesting from gray mold. Although, side effect of using fungicide spraying, but it's still the faster way to reduce or stop the disease progress. However, it is important to note that using natural compound such as salicylic acid to control strawberry's gray mold, consider one of the most safety way with less environmental hazards to improve the postharvest life and maintaining quality of strawberry and reducing the rate of rachis browning during postharvest storage. With notes that SA have physiological concentration.

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