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### **Research paper**

# Salicylic acid is associated with improved growth and resistance of olives (*Olea europaea* L.) to Verticillium wilt

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### Abstract

To improve the growth of olive and find a suitable approach for controlling *Verticillium dahlia* in this plant, treatment of different cultivars by salicylic acid (SA) was evaluated. Nine-month-old seedlings of Koroneiki, Marry, Rowghani, and Zard cultivars were pre-treated with 0, 5, and 10 mM SA at 15-days intervals, and then roots were inoculated by dipping into a defoliating isolate of *V. dahliae*. The dry weight of different tissues was measured separately at the end of the trial (14 weeks after inoculation). The response was assessed by grading the severity of symptoms on the 0-4 scale and calculating the area under the disease progress curve. Also, the concentration of phenolic compounds in the root tissues, the activity of the superoxide dismutase enzyme, the final intensity of symptoms, and the percentage of dead plants were measured. Foliar spray of 10 mM SA increased the vegetative growth of the olive cultivars. The results showed that treatment with SA decreases the severity of Verticillium wilt. The root phenol level and the activity of superoxide dismutase were significantly higher in the plants treated with 10 mM SA as compared to the control plants. Disease progression had a negative relationship with the superoxide dismutase activity and total phenols of the roots in olive cultivars.

Keywords: Olive cultivars; Salicylic acid; Superoxide dismutase; Total phenol; Verticillium dahlia

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### Introduction

Olive growth and productivity are limited by various diseases and pests. *Verticillium dahliae* Kleb. is one of the most devastating diseases of olive in Iran (Sanei *et al.* 2008) and the world, which reduces the quality and yield of olive cultivars (Montes-Osuna and Mercado-Blanco 2020). The defoliating pathotype of *V. dahliae* with high optimal growth temperature (27 °C) causes higher disease severity in olive cultivars (Sanei *et al.* 2008; Xu *et al.* 2012).

Plants have physical barriers and inducible defense mechanisms to limit pathogen development, such as induction of hypersensitive response (Maleck and Dietrich 1999), cell wall fortification, and production of pathogenesisrelated molecules like phytoalexins with antipathogen activities (Ferreira *et al.* 2007). Induced resistance is defined as the increased expression of plants' defense mechanisms against pathogens, caused by elicitors and other factors (Mishra *et al.* 2012). Due to the negative effects of fungicides on the ecosystem, using elicitors is becoming an alternative method for the protection of plants (Vimala and Suriachandraselvan 2009).

Oligosaccharides, peptides, and lipids are some of the compounds that can act as elicitors (Mott *et al.* 2014). They participate in the

induction of oxidative burst at the plasma membrane, activation of protein phosphorylation cascades, and changes in transcription profiles (Nuhse et al. 2003). Salicylic acid (SA), a plant phenol, is used extensively as an elicitor, which regulates plant growth and development (Al-Absi et al. 2009) and contributes to the plant defense against pathogens (Raskin 1992). SA has been shown to have a wide range of impacts on the growth and photosynthesis of plants (Pancheva et al. 1996). Based on Abo-Elyousr et al. (2009), the severity of onion leaf blight induced by Stemphylium vesicarium was considerably decreased when SA was sprayed on the leaves. Chaudhry et al. (2001) have reported the reduction of Ascochtya rabiei by applying SA on the susceptible chickpea cultivar C-727. Chitra et al. (2008) indicated the role of SA in developing resistance to Alternaria alternata in groundnut.

SA has been shown to alter bio productivity and crop growth (Fathi *et al.* 2019; Hayat *et al.* 2010). It may also generate several metabolites in plants, as well as changes in photosynthetic indices (Fariduddin *et al.* 2003; Hassannejad and Porheidar Ghafarbi 2018). Arfan *et al.* (2007) reported the stimulating effect of SA on the growth of olive plants under salinity. SA pre-treatment has decreased Verticillium wilt severity and suppressed the *V. dahliae* pathogenicity in olive plants (Gharbi *et al.* 2016).

The goal of this study was to investigate the effect of foliar SA spraying on the development of Verticillium wilt symptoms and progression of the disease severity and the role of polyphenols in the plants' defence in four olive cultivars.

# Materials and Methods Plant and fungal material

Nine-month-old olive cultivars Koroneiki, as resistant (Sanei and Razavi 2017b), and Marry, Rowghani, and Zard as susceptible (Sanei and Razavi 2017a) were used in the experiment. The fungal material was a highly virulent (defoliate, VCG1) strain of *V. dahliae* which was collected from the Zard cultivar in Golestan region by the Plant Pathology Laboratory of the Plant Protection Department, Gorgan University of Agricultural Sciences and Natural Resources, Iran.

### Pathogen inoculum and inoculation

The inoculum was prepared by growing colonies of V. dahliae on potato dextrose agar (PDA) cultures for seven days at  $25 \pm 0.5$  °C in the dark. The inoculum concentration was adjusted to 4  $\times$ 10<sup>6</sup> conidia per milliliter with sterile water (Colella et al. 2008). Plants were inoculated by dipping their bare root system into the inoculum for 30 minutes. The plants then were transferred into sterile plastic containers comprising 1000 ml of prior autoclaved silty-loam soil (Table 1) and incubated at  $24 \pm 4$  °C (Lopez-Escudero *et al.* 2004). The experiment was carried out in a greenhouse with fluorescent tubes providing supplemental light for 16 hours per day at the air temperature ranging from 20 °C to 26 °C. Control plants that had not been inoculated were given the same treatment as the inoculated plants, but instead, the distilled sterile water was used. The seedlings were grown under well-watered irrigation conditions at five-day intervals from March to July.

### Application of SA and experimental design

Olive plants were treated with SA (Sigma-Aldrich, Lyon, France) solutions (0, 5, and 10 mM) before inoculation at 15-days intervals. The experiment was arranged as factorial with three factors (SA, inoculation, cultivar) based on randomized complete blocks with four replications.

## Dry weight

The tips of all shoots of each plant were marked with an indelible marker at the final node to evaluate the influence of SA on the vegetative growth of olive plants. The fresh weight of the whole plants was measured with a balance, while the plant roots were naked and removed from the soil for dipping. Thirteen weeks following inoculation, the fresh weight of new green leaves and shoots grown from marked points at shoot tips and the whole root were recorded separately. These parts were placed individually in paper envelopes, dried at 70 °C for 48 hours, and then their dry weight was measured (Birem. *et al.* 2016).

### **Disease assessment**

Two weeks after inoculation, the severity of the disease was assessed every week for 14 weeks. Wilt resistance was graded according to Lopez-Escudero *et al.* (2004) on a scale of 0 to 4 based on the percentage of plant tissue affected by chlorosis, leaf and shoot necrosis, or defoliation (0 = healthy plant or plant without symptoms; 1 = 1 - 33%, 2 = 34 - 66%, and 3 = 67 - 99% affected tissue; 4 = dead plant). To determine the severity

of reactions, the percentage of dead plants (Lopez-Escudero and Blanco-Lopez, 2001) as well as other signs, such as marginal patches on the leaves and uneven twig growth, were observed (Lopez-Escudero et al. 2004). The area under the disease progress curve (AUDPC) was calculated for each cultivar in the period of 14 weeks as follows (Campbell and Madden 1990): AUDPC=  $[(t/2 \times (S_2 + 2S_3 + \bullet \bullet + 2S_{i-1} + S_i)/4 \times n] *100 (t$ = time between observations in days;  $S_i$  = final mean severity of the ith observation; 4 = maximaldisease severity; n = number of observations). During the study, the fungus was isolated from the infected shoots or leaf petioles of affected plants to determine the plant infection (Lopez-Escudero et al. 2004). The frequency of V. dahliae reisolation was determined by disinfecting stem segments with 1 percent NaOCl for five minutes and culture of xylem chips on C'zapeck Dox Agar (supplemented with 100 ppm streptomycin sulfate) for 14 days at 25  $\pm$  1 °C (Tsror *et al.* 2001). The degree of resistance of olive cultivars was determined according to Table 2 (Lopez-Escudero et al. 2004).

# Total phenols and superoxide dismutase enzyme

The content of total phenols in roots was measured using a colorimeter 100 days after inoculation by *V. dahlia* and its unit was milligrams of tannic acid equivalents per dry weight (Sofo *et al.* 2004). The superoxide dismutase (SOD; EC 1.15.1.1) enzyme activity was measured according to Giannopolitis and Ries (1977). Each assay was repeated four times.

### Data analysis

The data for severity of Verticillium wilt, frequency of *V. dahliae* reisolation from the olive

xylem, vegetative growth of olives, total phenols, and SOD activity were subjected to analysis of variance and the means were compared by the

Table 1. Physica	l and chemical	properties of	f the soil in the	e experiment
1 4010 11 1 11 10104		properties of		enperment.

Texture	N (%)	Organic carbon (%)	P (ppm)	K (ppm)	pН	EC (dS.m -1 )
Silty loam	0.21	2.39	14.7	275	7.55	1.24

Table 2. Resistance categories and disease parameters for the reaction of olive cultivars to *Verticillium dahliae* (Lopez-Escudero *et al.* 2007).

Resistance category	AUDPC <sup>a</sup>	<b>FMS</b> <sup>b</sup>	PDP <sup>c</sup>			
Highly resistant	0-10	0.0-1.5	0			
Resistant	11-30	0.0-1.5	0			
Moderately resistant	31-50	1.5-2.5	0-30			
Susceptible	51-70	2.5-3.0	31-50			
Extremely susceptible	71-100	3.0-4.0	51-100			
ALIDDC: and an the discourse and an an an and a second an						

<sup>a</sup>AUDPC: area under the disease progress curve; <sup>b</sup>FMS: final mean severity of symptoms; <sup>c</sup>PDP: percentage of dead plants.

Fisher's protected LSD at  $p \le 0.05$ . Statistical analyses were performed by the R.3.3.1 program.

### Results

Based on analyses of variance, effects of SA, inoculation, cultivar, and their interactions were significant for all characters under study, except for the main effect of inoculation on SOD (data not shown). At all concentrations of SA, inoculation with *V. dahliae* did not significantly reduce the dry weight of shoots, new green leaves, and roots in the Koroneiki cultivar as compared to non-inoculated plants, except for the dry weight of shoots at 10 mM SA (Table 3). For the sensitive cultivars (Marry, Rowghani, Zard), inoculation with *V. dahliae* decreased, in most cases, the dry

weight of shoots, new green leaves, and roots at all SA concentrations as compared to the respective controls (Table 3). However, foliar spray of 10 mM SA significantly improved these characters on the inoculated sensitive cultivars as compared with the inoculated olive plants at 0 mM SA (Table 3).

There was a decrease in wilt incidence due to the foliar application of SA at 10 mM concentration (Table 4). The inoculated untreated plants showed 91% wilt incidence. By contrast, the inoculated SA-treated plants showed a significant reduction in disease severity, and the wilt incidence was around 29.33%.

The resistant cultivar Koroneiki had significantly higher root total phenols and SOD

activity than all sensitive cultivars in both inoculated and non-inoculated olive plants at all SA concentrations. The application of 10 mM SA significantly increased the total phenol accumulation and SOD activity of inoculated and non-inoculated sensitive and resistant olive cultivars as compared with the respective controls when no SA was applied (Table 3). Total phenols and SOD activity had a negative relationship with AUPDC (Figure 1).

Table 3. Dry weight of the roots and new green leaves and shoots, SOD activity, and root phenol content of olive plants 100 days after inoculation with *Verticillium dahliae* and treated with salicylic acid

Factors		Dry weight (g)			Root total phenols	SOD activity	
		Leaves	Roots	Shoots	(mg/g DW)	(units per mg protein)	
SA (0 mM)							
<sup>a</sup> Inoculation	Variety						
С	Koroneiki	2.41	6.38	2.71	25.82	54.2	
	Marry	2.48	5.80	2.87	16.76	47.0	
	Rowghani	2.56	5.71	2.87	16.45	45.4	
	Zard	2.43	5.73	2.78	17.30	48.2	
Ι	Koroneiki	2.40	6.40	2.68	25.12	52.0	
	Marry	1.44	3.29	1.24	16.79	48.6	
	Rowghani	1.35	4.04	1.53	16.33	47.6	
	Zard	1.30	3.87	1.00	16.59	44.4	
SA (5 mM)							
С	Koroneiki	2.44	6.20	2.80	25.64	55.8	
	Marry	2.30	5.41	2.70	16.68	46.8	
	Rowghani	2.44	5.74	2.87	16.68	46.4	
	Zard	2.46	6.18	2.78	16.2	45.4	
Ι	Koroneiki	2.63	6.36	2.70	25.90	56.2	
	Marry	1.38	3.17	1.38	16.54	46.2	
	Rowghani	1.28	4.09	1.61	22.16	51.8	
	Zard	1.21	3.47	1.20	16.59	46.2	
SA (10 mM)							
С	Koroneiki	2.96	6.42	3.12	33.96	70.8	
	MarryC	2.84	5.42	2.87	24.78	54.4	
	Rowghani	3.02	6.25	3.08	24.78	55.8	
	Zard	2.92	5.78	2.91	24.42	54.4	
Ι	Koroneiki	2.85	6.30	2.73	31.16	63.0	
	Marry	2.52	5.32	2.85	22.84	57.2	
	Rowghani	2.52	5.70	2.84	25.20	55.0	
	Zard	2.53	5.66	2.76	22.22	52.0	
LSD (0.05)		0.303	0.825	0.329	1.844	3.688	

<sup>a</sup>Inoculation status: C = non-inoculated control, I = inoculated plants; SA: salicylic acid: SOD: superoxide dismutase

### Discussion

The ability of a plant to prevent or restrict pathogen growth and multiplication is known as disease resistance (Chaudhary *et al.* 2001). Verticillium wilt resistance/tolerance has been

reported in olive rootstocks and commercial cultivars (Bubici and Cirulli 2012; Erten and Yildiz 2011) and also in wild olives (Sanei and Razavi 2017a). Either resistant or susceptible plants respond to pathogens by inducing several strategies. Exogenous application of systemic elicitors can be a valuable alternative to the conventional methods of controlling pathogens (Desender *et al.* 2007). In this study, the foliar spray of 10 mM SA reduced the disease incidence in all cultivars, however, the reduction was much

more pronounced in the sensitive cultivars. Hayat *et al.* (2010) in a review about the efficiency of SA foliar application on plants indicated the effects of this hormone on providing protection and tolerance against various biotic stresses, and alleviation of the toxic effects of abiotic stresses.

Table 4. Mean disease parameters assessed in the olive cultivars inoculated with the defoliating isolate of *Verticillium dahlae* and treated exogenously with salicylic acid.

		Severity of Verticillium wilt external symptoms AUDPC <sup>a</sup> 100 DAI <sup>b</sup>		Frequency of V. dahliae	Resistance level	Percentage of dead plants
Factors				reisolation		
1 400015				from the olive		
			(0-4 scale)	xylem (%)		
Cultivar	SA			•		
Marry						
	0	60.04	3.00	1.70	S	40
	5 mM	53.78	2.70	1.70	S	0
	10 mM	42.84	1.00	1.50	MR	0
Rowghani						
e	0 mM	65.88	3.10	1.75	S	0
	5 mM	64.76	3.00	1.75	S	0
	10 mM	45.48	2.00	1.10	MR	0
Koroneiki						
	0	9.14	0.80	0.60	HR	0
	5 mM	11.88	0.80	0.50	HR	0
	10 mM	6.34	0.60	0.15	HR	0
Zard						
	0	69.92	3.20	2.75	S	40
	5 mM	63.90	2.80	2.75	S	0
	10 mM	39.94	2.00	1.35	MR	0
LSD (0.05)		3.464	0.224	0.141		

<sup>a</sup>AUDPC: area under the diseas e progress curve; <sup>b</sup>DAI = days after inoculation; S = susceptible, MS = moderately susceptible, R = resistant, HR = highly resistant

Gharbi *et al.* (2016) reported the effect of foliar spraying of SA on induced resistance in olive.

The susceptibility level of the evaluated olive cultivars was linked to the growth reduction in this report and inoculation with *V. dahliae* reduced the dry weight of leaves, shoots, and roots of the susceptible olive varieties. On the other hand, resistant genotypes showed few symptoms and produced new leaves and shoots after inoculation. Birem *et al.* (2016) used the fresh and

dry weights of plants as the main indicators to determine the differences in vegetative growth of olive cultivars with varying levels of resistance to *V. dahlia.* Several authors have shown the growth reduction induced by *V. dahliae* in olive (Birem *et al.* 2016), pepper (Goicoechea 2006), sunflower (Sadras *et al.* 2000), tomato, and eggplant (Karagiannidis *et al.* 2002). According to Sadras *et al.* (2000), the reduction in the growth of sunflower plants due to *V. dahliae* infection was attributed to the reduction in the leaf area, which was caused by the reduced leaf expansion early in the season and faster leaf senescence in older plants.

Our study showed that the application of 10 mM SA alleviated the adverse effects of inoculation with *V. dahliae* on new green leaves,

shoots, and roots of the sensitive cultivars of olive. Hayat *et al.* (2010) indicated the effects of this hormone on enhancing the growth and productivity in plants. The influence of SA on carboxylation efficiency, photosynthesis, nitrate reductase activity, and seed yield has been reported in *Brassica juncea* (Fariduddin *et al.* 

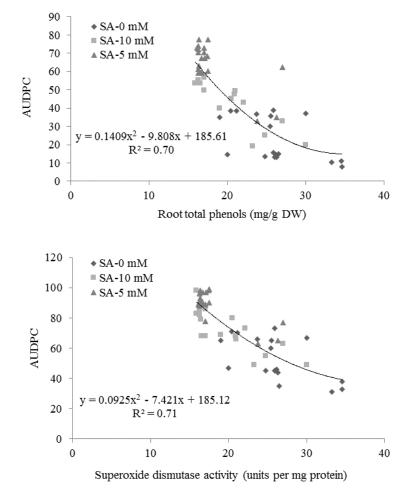


Figure 1. Relationship of root total phenols and superoxide dismutase activity with the area under the disease progress curve (AUDPC) of the olive cultivars infected with a defoliating isolate of *Verticillium dahlae*.

*et al.* 2003). The enhancement in photosynthetic activity in wheat (Arfan *et al.* 2007) and diminishing the inhibiting effect of salinity on the growth of olive plants (Al-Abasi 2009) have been

attributed to the stimulating effect of SA treatment under salinity conditions.

The application of 10 mM SA increased the total phenol content and SOD activity in all

cultivars at both inoculated and non-inoculated conditions. Resistance to pathogens increases the level of certain compounds, resulting in disease protection (Del Rio et al. 2003). Enhanced phenolic compounds buildup in plant tissue following pathogen attacks has been reported by many authors (e.g. Roussos et al. 2002; Del Rio et al. 2003; Baidez et al. 2007; Markakis et al. 2010). Baidez et al. (2007) reported that quercetin and luteolin aglycons followed by rutin, oleuropein, luteolin-7-glucoside, tyrosol, pcoumaric acid, and catechin have an antifungal effect against V. dahliae at in vitro conditions. Elicitor compounds trigger a cascade of defense responses such as the production of defense enzymes like chitinases and glucanases, and cell wall fortification (Desender et al. 2007; Ferreira et al. 2007). SA is engaged in activating several compounds to defend plants against pathogen attacks (Chitra et al. 2008). Response to SA results in the production of secondary metabolites such as phytoalexins (Ahuja et al. 2012). Antioxidant defense enzymes, including SOD, are also important in the response of plants to various stresses and are induced by SA. For example, Xi

*et al.* (2021) demonstrated that the clubroot incidence rate and disease index declined and the activities of antioxidant enzymes such as SOD, ascorbic acid-peroxidase, catalase, and glutathione reductase increased after application of 0.6 mM exogenous SA on the plants.

### Conclusion

This study showed the positive effect of SA on the growth and incidence of *V. dahliae* in olive cultivars under investigation. The substantial effect of SA on the root phenolic content and SOD activity was also observed in this study, which can protect the olive plants from the *V. dahliae* attack.

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### **Conflict of Interest**

The authors declare that they have no conflict of interest with any people or organization concerning the subject of the manuscript.

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# ار تباط اسید سالیسیلیک با بهبود رشد و تحریک مقاومت زیتون (*Olea europaea* L.) به پژمردگی ور تیسیلیومی

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چکیدہ

به منظور بهبود رشد و پیدا کردن یک روش مناسب برای کنترل Verticillium dahliae، عامل پژمردگی ورتیسیلیومی، تیمار ارقام زیتون توسط اسید سالیسیلیک (SA) مورد مطالعه قرار گرفت. در این بررسی، نهالهای نُه ماهه زیتون شامل کورونایکی، ماری، روغنی و زرد پس از تیمار توسط SA با غلظتهای ۰، ۵ و ۱۰ میلیمولار با جدایه برگریز dahliae .V، جدا شده از زیتون از منطقه گرگان، بهروش غوطهور کردن ریشه مایهزنی شدند. محلول پاشی گیاهان زیتون هر ۱۵ روز یکبار توسط SA انجام شد. در پایان آزمایش، ۱۴ هفته پس از مایهزنی، وزن خشک بخشهای مختلف گیاه بعلور جداگانه اندازه گیری شد. مقاومت با تعیین شدت علائم با استفاده از مقیاس ۴-۰ و محاسبه سطح زیر منحنی پیشرفت بیماری ارزیابی شد. درصد گیاهان خشک شده، میانگین شدت علائم نهایی، فراوانی جداسازی Adhliae از مقیاس ۴-۰ و محاسبه سطح زیر منحنی پیشرفت بیماری ارزیابی شد. درصد گیاهان خشک شده، میانگین شدت برگها با غلظت ۱۰ میلیمولار SA با افزایش رشد رویشی همراه است. SA شدت بیماری ارزیابی شد. گیاهان تعان داد که محلول پاشی برگها با غلظت ۱۰ میلیمولار SA با افزایش رشد رویشی همراه است. SA شدت بیماری را کاهش داد. گیاهان تعان دند که معلول پاشی سوپراکسید دیسموتاز بیشتری نسبت به گیاهان شاهد داشتند. پیشرفت بیماری با کاهش داد. گیاهان تیمار شده میزان فنل ریشه و فعالیت سوپراکسید دیسموتاز بیشتری نسبت به گیاهان شاهد داشتند. پیشرفت بیماری با میزان فنل ریشه و فعالیت سوپراکسید دیسموتاز رابطه منفی داشت.

**واژههای کلیدی:** اسید سالیسیلیک؛ ارقام زیتون؛ پژمردگی ورتیسیلیومی؛ سوپراکسید دیسموتاز؛ فنل کل