J. Crop Prot. 2022, 11 (2): 253-267

Research Article

Efficacy of salicylic acid and acetylsalicylate in enhancing faba bean resistance against *Orobanche crenata* parasite

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Abstract: *Orobanche crenata*, an obligate holoparasite, causes severe damage to faba bean grown in the Mediterranean region. A greenhouse experiment was conducted to determine and compare the potential of salicylic acid (SA), and acetylsalicylate (AcSA) used as seed soaking (0.5-1.5 mM, each) or foliar spray (2-6 mM, each) to increase faba bean tolerance to *Orobanche crenata* infection. Under the challenge of *Orobanche* infection, the application of SA or AcSA enhanced the growth of faba bean plants by improving their viability, height, and fresh and dry weight. SA and AcSA reduced the growth of parasite tubercles and retarded their development to emerged spike, the most harmful stage. AcSA was more effective than SA in increasing faba bean tolerance to *O. crenata* infection, and seed soaking showed the greatest effect. At 110 days of sowing, soaking seeds in 0.5 or 1 mM AcSA completely prevented the death of infected plants and increased the weight of plants by 22 and 67%, respectively, and pod weight/plant by 512 and 442%. Moreover, these two treatments greatly reduced *Orobanche* growth, and complete inhibition of tubercles and emerged spikes occurred by soaking seeds in 0.5 mM AcSA. *Orobanche* infection greatly increased phenolic content and antioxidant activity in the host tissues, but their levels tended to reduce by all salicylate treatments. The results suggest the great potential of soaking host seeds in AcSA, the inexpensive commercial form of SA, can enhance plant resistance against *Orobanche* parasite.

Keywords: *Vicia faba*, salicylate compounds, *Orobanche*, tolerance

Introduction

Faba bean *Vicia faba* L. sometimes referred to as a broad bean, horse bean, or field bean, is a major food and feed legume because of the high nutritional value of its seeds, which are rich in protein and starch (Rubiales, 2010). Egypt is considered one of the largest consumers of faba bean in the world, which is consumed as a vegetable in many ways (El-Mergawi and Taie, 2014). The obligate holoparasite *Orobanche crenata* causes severe damage to faba beans grown in the Mediterranean region (El-Dabaa *et al*., 2021). *O. crenata* plant produces thousands of seeds that can remain viable in the soil for more than 10 years and germinate in response to chemical signals from faba bean roots. After seed germination, the parasite attaches to the host root by a haustorium through which it absorbs water, minerals, and organic compounds, producing a tubercle from which a shoot arises and emerges from the soil to flower

Handling Editor: Ali Mokhtassi-Bidgoli

* Corresponding author: ragabelmergawi05@hotmail.com
Received: 18 November 2021, Accepted: 24 July 2022
Published online: 10 September 2022

[DOR: 20.1001.1.22519041.2022.11.2.7.0]

and set seeds (Brun *et al*., 2017). Several control strategies have been implemented to manage *Orobanche*, but without complete success (Triki *et al*., 2018).

Many factors, such as prior pathogen attacks and various chemical and environmental stimuli, may act on plants to induce systemic acquired resistance (SAR) to subsequent pathogen attacks. SAR has proven to be a tool for controlling plant pathogens, including fungi, bacteria, viruses, and parasitic weeds (Triki *et al*., 2018). Applications of chemicals to induce SAR and activate the plant's defense system were considered a new technology for plant pathogen control. Induction of systemic resistance is associated with gene induction, the activation of a wide range of resistance mechanisms, and the production of a wide range of defense compounds (Sillero *et al*., 2012). Salicylic acid (SA) is a phenolic plant hormone that regulates growth. It acts as a signaling molecule in activating the plant defense responses against many stress factors (Wani *et al*., 2017). The natural resistance of plants to pathogens and parasitic weeds is based on the combined effects induced mechanisms, among which is the SAR which is controlled by a signaling pathway and depends on endogenous accumulation of SA (Yang *et al*., 2010). SA is considered a plant signal molecule and involved in the induction of SAR, which activates many defense compounds, including phenolic acids, coumarins, flavonoids, and lignin (Al-Wakeel *et al*., 2013). SA was the first synthetic compound to induce enhanced activation of various defense responses against major pathogens on multiple crops. Induced resistance of host plants by SA can be used to control agronomically important *Orobanche* species (Abbes *et al*., 2014; Perez-de-Luque *et al*., 2004; Sauerborn *et al*., 2002). Several studies on different plant species such as pea (Perez-de-Luque *et al*., 2004), sunflower (Sauerborn *et al*., 2002), and oilseed rape (Veronesi *et al*., 2009) indicate that the application of SA or its synthetic functional analog, benzo (1,2,5) thiadiazole - 7 – carbothioic acid S-methyl ester (BTH) can trigger SAR against broomrapes. In clover, root application of SA significantly reduced the number of established *O. minor* parasites by more than 75% (Kusumoto *et al*., 2007). Also, Madany *et al*. (2020) found that applying SA at 1 mM as seed-priming mitigated the adverse effects of *O. ramosa* stress in tomato plants, especially at oxidative stress levels.

Since faba bean cultivation is common in Mediterranean areas, where *Orobanche* is frequent, it is essential to establish an effective and safe method that makes faba bean more tolerant to *Orobanche* infection. There is a lack of information on the differences between SA and its inexpensive commercial form (AcSA) in alleviating oxidative stress induced by the Orobanche parasite. A commercially manufactured form of SA is acetylsalicylate (AcSA), and in an aqueous solution, AcSA is hydrolyzed completely to SA (Matysiak *et al*., 2020). We found that exogenous application of AcSA at different concentrations caused higher effects on six plant species and produced more endogenous SA than those of corresponding SA treatments (El-Mergawi and Abd El-Wahed, 2020). Therefore, this study has aimed to determine and compare the potential of SA and AcSA used as seed priming or foliar spray in increasing faba tolerance to *O. crenata* infection. Moreover, the effect of the *Orobanche* parasite on the oxidative and redox status of faba bean plants and the impact of salicylates upon mitigating the infection threats and antioxidant defense markers, including the antioxidant metabolites, were also studied.

Materials and Methods

An experiment was conducted during the growing season (November-April) of 2019/2020 at the greenhouse of the National Research Centre, Giza, Egypt. Seeds of faba bean (*Vicia faba*) Sakha 4 cultivar were obtained from Agricultural Research Centre, Giza, Egypt. Analytical standard of SA and AcSA were purchased from S D Fine-Chem Limited (India) and applied to seeds and plants in two different methods; (1) seed soak treatments and (2) foliar spray treatments. Six presoaking treatments were conducted by incubating seeds in SA or AcSA solutions (200 ml/100 seeds) at 0.5, 1.0, and 1.5 mM each for 24 hrs. Nonsalicylate-treated seeds were incubated in distilled water for 24 h. The seeds were sown in each 30-cm diameter plastic pot containing about 5 kg sandy loam soil (48.4% sand, 41.1% silt, and 10.5% clay; pH 8.0). About 200 mg of *O. crenata* seeds were mixed with the soil of the infested pot. Commercial rhizobia and 8 g of superphosphate (15%, P2O5) were incorporated into the top 30 mm of the soil at sowing. The pots were placed in a greenhouse (25 ± 3 °C, 12 h photoperiod). After 20 days from sowing, seedlings were thinned to three uniform seedlings per pot. Plants were supplied with nitrogen (ammonium sulfate, 21% N, 6 g/pot) three times at 4, 6, and 8 weeks after sowing. Pots were divided into fourteen groups with five replications; thirteen *O. crenata*-infested groups and one non-infested group (control healthy) and arranged in a randomized complete block design. As for the thirteen infested groups, one group served as infested control, six groups for presoaking treatments, and six groups for foliar spraying treatments. Foliar spray treatments were applied to 30-day–old plants with a hand-held sprayer with either SA or AcSA solutions at three concentrations (2, 4, and 6 mM, each). Foliar spray treatments were repeated after 15 days after the first spray. Samples from all tested treatments were collected at 75 and 110 days after sowing, and growth parameters of faba bean and *Orobanche* were determined. The plant growth parameters were evaluated in terms of plant height (cm), number of branches, number of leaves, fresh plant weight (g), plant dry weight (g), number of pods, and weight of pods (g). Also, the total number and weight (g) of attached *Orobanche,* tubercles, and emerged spikes per pot were determined. Fresh 4^{th} leaf of 75-days-old plants was collected, fast cleaned with distilled water to remove the salicylate residues, and used to determine the antioxidant metabolites.

Determination of total phenolics

Total phenolics in faba bean leaves were extracted with 70% acetone and determined according to the method previously mentioned by El-Mergawi and Taie (2014) using the Folin-Ciocalteu reagent. In brief, 0.1 ml of extract was added to 7.9 ml of distilled water, 0.5 ml of Folin-Ciocalteu reagent, 1.5 ml of sodium carbonate solution (200 g/L), and mixed vigorously. The mixture was allowed to stand for 1 h at room temperature, and then the absorbency was measured at a wavelength of 765 nm. Gallic acid was used as a standard, and the results were expressed as mg gallic acid g^{-1} fresh sample.

Determination of antioxidant capacity

Antioxidant capacity or free radical scavenging activity was determined according to Brand-Williams *et al*. (1995) using 1,1-diphenyl-2-picryl-hydrazil (DPPH) reagent. In brief, 1.5 ml of freshly prepared methanolic DPPH solution (0.02 mg/ml) was added to 0.75 ml of 70% acetone extract and then stirred. The de-colorizing was recorded after 5 min of reaction at a wavelength of 517 nm and compared with a blank control. The DPPH radical scavenging activity of the extracts was measured using the Trolox standard curve. Results were expressed as μmol Trolox g^{-1} fresh sample.

Determination of lipid peroxidation

Lipid peroxidation was estimated in vitro after the formation of malondialdehyde (MDA), a by-product of lipid peroxidation that reacts with thiobarbituric acid (TBA). Briefly, 0.3 g of fresh leaf tissue was ground in 5 mL of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000 g for 10 min, and 0.5 mL of the supernatant was mixed with 2 mL of 0.5% TBA in 20% TCA. The mixture was heated at 90 °C for 20 min. The resultant mixture was centrifuged at 10,000 g for 5 min. The absorbance of the supernatant was measured at 532 nm. The values were corrected for non-specific absorption by subtracting absorbance read at 600 nm. The level of lipid peroxidation was calculated by using the extinction coefficient of 155 mM cm^{-1} (Sima *et al*., 2012).

Statistical analysis

A comparison of means (LSD, 5% level) was performed using MsTATC Version 2.1. Analyses were performed in triplicate. We found that the obtained data were normally distributed for

ANOVA, and then their analyses of variance were calculated according to Gomez and Gomez (1984).

Results

Effect of salicylate on 75-days-old-plants

The effect of SA and AcSA treatments at two applied methods on growth parameters of infected faba bean plants compared with infected plants alone or healthy non-infected plants are shown in Table 1. By contrast with healthy plants, *O. crenata* infection reduced the number of branches (8.3%), plant height (3.4%), number of leaves (25.5%), fresh biomass (61.8%), and dry biomass (59.9%) of host plants. But, applications of SA or AcSA greatly improved plant height, fresh biomass, and dry biomass of infected plants. The greatest enhancement effect on the fresh and dry weight of infected plants was by AcSA treatment. On the other side, SA and AcSA treatments failed to produce significant effects on a number of branches and number of leaves when compared with infected plants alone. Comparing the two application methods, it can be observed that number of branches and number of leaves did not show any significant differences (Table 1). The seed soaking method produced a significant increase in fresh and dry biomass of infected plants accompanied by a significant decrease in plant height when compared with the foliar spray method. As shown in Table 2, except non-significant effect on a number of branches, all growth parameters of faba bean plants showed significant variations in response to the application of different concentrations of SA or AcSA at the two applied methods. Among tested treatments, soaking faba bean seeds in 1.5 mM of SA and foliar application of AcSA at 6 mM produced the tallest plants. Whereas the highest values of the number of leaves, plant fresh, and dry weight was observed when the host seeds were soaked in AcSA at 1 mM and 1.5 mM.

Effect of salicylate on 110-days-old-plants

The obtained data indicated that *O. crenata* infection had a suppressive effect on all growth parameters of faba bean plants when measured after 110 days from sowing (Table 3). It caused the death of 70% of the host plants and reduced plant height (14.9%), number of leaves (37.4%), fresh weight (48.9%), dry weight (48.3%), number of pods (67.5%) and weight of pods (85.6%). Improvement in all growth parameters was observed due to the exogenous application of either SA or AcSA compounds (Table 3). The greatest effect occurred with AcSA; it decreased plant death (78.6%) and increased the number of leaves (16.9%), the weight of plant (38.1%), number of pods (92.3%), and weight of pods (303%) when compared with non-treated plants. The two applied methods varied in their effects on growth parameters, and seed soaking showed the greatest impact (Table 3). This method greatly decreased plant death (82.9%) and produced the highest values of growth parameters in plant height, number of leaves, fresh weight, dry weight, and number and weight of pods/plant. Whereas using salicylates as foliar application produced the tallest plants (75.2 cm), when compared with infected (57 cm) and healthy non-infected plants (67 cm).

Application of different concentrations of SA or AcSA at two applied methods varied in their effects on tested growth parameters of infected faba bean plants (Table 4). All tested treatments caused a decrease in plant death, and complete prevention of host death occurred by seed soaking in either SA at 0.5 mM or AcSA at 0.5 and 1 mM treatments. Moreover, all the applied treatments improved various growth parameters of infected plants compared to non-treated infected plants. Applied salicylate treatments produced great increases in plant height as compared with infected and healthy non-infected plants, and foliar application with AcSA at 4 mM and SA at 2 or 4 mM produced the tallest plants. With few exceptions, all salicylate treatments produced a significant increase in the number of leaves per plant. The highest number was obtained by soaking seeds either in SA at1.5 and 0.5 mM or in AcSA at 1mM as when spraying plants with AcSA at 4 mM. As shown in Table 4, a great increase in plant fresh and dry weight occurred with AcSA treatments. The heaviest

[DOR: 20.1001.1.22519041.2022.11.2.7.0]

plants were obtained with soaking seeds in either AcSA at 1 mM or SA at 1.5 mM. Among tested treatments, the highest number and weight of pods were observed for seed soaking in AcSA at one of the three tested concentrations. Pods weight of these treatments was between three to five times those produced by infected plants alone.

Table 1 Effect of SA and AcSA applied by seed soaking or foliar spray on growth of *Orobanche* infected faba bean plants, 75 days after sowing.

Entries	Criteria	No of branches/plant	Height (cm)	No of leaves/ plant	Fresh weight (g/plant)	Dry weight (g/plant)
Salicylate compounds	SA	1.0 a	70.8 a	18.9 b	29.8 c	4.41 c
	AcSA	1.0 a	69.1 a	20.3 b	37.1 b	5.48 b
	Infected plant	1.1 a	63.7 b	20.7 b	20.7 d	3.06 d
	Healthy plant	1.2 a	66.0 b	27.8 a	54.2 a	7.64 a
Applied methods	Soaking	1.0 a	68.4 b	20.4 b	35.2 b	5.22 b
	Spraying	1.0 a	71.6 a	18.8 b	31.6 c	4.68 c
	Infected plant	1.1 a	63.7 c	20.7 b	20.7d	3.06 d
	Healthy plant	1.2 a	66.0 bc	27.8 a	54.2 a	7.64 a

SA: Salicylic acid, AcSA:
Acetyl salicylate. Data within a column followed by the same letter are not significantly different (LSD at $P \leq 0.05$).

Table 2 Effect of salicylate treatments on growth of *Orobanche* infected faba bean plants, 75 days after sowing.

Method	Treatments	No of branches/plant	Height (cm)	No of leaves/plant	Fresh weight (g/plant)	Dry weight (g/plant)
Soak	0.5 mM SA	1.1 a	66.7 c	19.7 bc	28.0 e	4.14 e
Soak	1.0 mM SA	1.0 a	73.0 b	19.7 bc	29.8 de	4.41 de
Soak	1.5 mM SA	1.0 a	77.0 a	19.7 bc	33.3 cde	4.93 cde
Spray	2 mM SA	1.0 a	71.8 b	19.7 bc	36.0 cd	5.33 cd
Spray	4 mM SA	1.0 a	72.0 b	19.0 bc	30.7 de	4.54 de
Spray	6 SA	1.0 a	64.2 c	15.7 d	20.8 f	3.08 f
Soak	0.5 mM AcSA	1.0 a	67.2 c	20.3 bc	35.3 cde	5.25 cde
Soak	1.0 mM AcSA	1.0 a	63.2 c	22.0 b	45.7 b	6.76 b
Soak	1.5 mM AcSA	1.0 a	62.8 c	21.0 bc	39.3 c	5.82 c
Spray	2 mM AcSA	1.0 a	71.3 b	19.7 bc	37.5 cd	5.55 cd
Spray	4 mM AcSA	1.0 a	71.8 b	18.0 c	30.7 de	4.54 de
Spray	6 mM AcSA	1.0 a	78.3 a	20.8 bc	34.0 cde	5.03 cde
	Infected plant	1.1 a	63.7 c	20.7 bc	20.7 f	3.06 f
	Healthy plant	1.2 a	66.0 c	27.8 a	54.2 a	7.64 a

SA: Salicylic acid, AcSA: Acetyl salicylate.
Data within a column followed by the same letters are not significantly different (LSD at $P \leq 0.05$).

Table 3 Effect of SA and AcSA applied by seed soaking or foliar spray on growth and yield of *Orobanche* infected faba bean plants, 110 days after sowing.

Entries	Criteria	% Dead plants	Plant height (cm)	No of leaves/plant	Fresh weight (g/plant)	Dry weight (g/plant)	No of pods /plant	Weight o f pods/plant (g)
Salicylic compounds	SA	32 b	73.8 a	24.5 b	29.1 c	4.92 c	1.9 c	7.3 c
	AcSA	15 c	72.5 a	24.9 b	34.1 b	5.76 b	2.5 b	13.3 b
	Infected	70 a	57.0 c	21.3 c	24.7 d	4.17 d	1.3 d	3.3 d
	Healthy	0 d	67.0 b	34.0 a	48.3 a	8.07 a	4.0 a	22.8 a
Applied methods	Soaking	12 c	71.2 a	26.1 b	33.5 b	5.66 b	2.3 b	13.1 b
	Spraying	35 b	75.2 a	23.4 c	29.6 c	5.01 c	2.1 bc	7.5 c
	Infected	70 a	57 b	21.3 d	24.7 d	4.17 d	1.3 c	3.3 d
	Healthy	0 d	67 ab	34.0 a	48.3 a	8.07 a	4.0 a	22.8 a

SA: Salicylic acid, AcSA: Acetyl salicylate.
Data within a column followed by the same letters are not significantly different (LSD at $P \leq 0.05$).

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Table 4 Effect of salicylate treatments on growth and yield of infected faba bean plants, 110 days after sowing.

Method	Treatments	%Dead plants	Plant height (cm)	No of leaves/plant	Fresh weight (g/plant)	Dry weight (g/plant)	No of pods/plant	Weight of pods/plant (g)
Soak	0.5 mM SA	0	72.7abcd	27.0 b	28.0 ef	4.73 ef	2.4 bc	10.4e
Soak	1.0 mM SA	8	67.7cde	26.3 bc	26.8 f	4.53 f	1.7 bc	11.8cde
Soak	1.5 mM SA	46	75.0abcd	28.0 b	39.0 b	6.59 b	2.4 bc	11.1de
Spray	2 mM SA	50	78.0a	21.0 f	30.7 de	5.19 de	1.3 c	3.03f
Spray	4 mM SA	42	72.3abcd	21.7 ef	24.3 f	4.11 f	1.3 c	3.3f
Spray	6 mM SA	46	77.3ab	23.0 def	25.7 f	4.34 f	2.7 bc	4.1f
Soak	0.5 mM AcSA	0	62.0d	23.0 def	30.3 de	5.12 de	3.0 b	16.9b
Soak	1.0 mM AcSA	0	73.7abcd	27.3 b	41.3 b	6.98 b	2.0 c	14.6bcd
Soak	1.5 mM AcSA	18	76.0abc	24.7 cd	35.7 c	6.03 c	2.7 bc	13.9bcde
Spray	2 mM AcSA	15	76.0abc	23.7 de	32.7 cd	5.53 cd	2.7 bc	13.5bcde
Spray	4 mM AcSA	25	78.3a	27.0 b	33.3 cd	5.63 cd	2.4 bc	5.7f
Spray	6 mM AcSA	33	69.0bcd	23.7 de	31.0 de	5.24 de	2.0 bc	15.2c
	Infected plant	70	57.0e	21.3 ef	24.7 f	4.17 f	1.3 c	3.3f
	Healthy plant	0	67.0de	34.0 a	48.3 a	8.07 a	4.0 a	22.8a

SA: Salicylic acid, AcSA: Acetyl salicylate.
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Effect of salicylate on *O. crenata* parasite

Growth parameters of *O. crenata* were measured 75 and 110 days post sowing (Table 5). At the earlier recording, SA significantly increased the number of attached.

Orobanche and a significant decrease in their weights when compared with infected plants alone. Meanwhile, AcSA was considered the most effective treatment; it suppressed the number and weight of attached *Orobanche* by 21.9 and 49.8%, respectively. The increased number of attached *Orobanche* resulted from using salicylate compounds as a foliar spray (Table 5). But, the significant effect of seed soaking was observed only on the weight of attached *Orobanche*.

At 110 days of sowing, the attached *Orobanche* were collected from each pot and divided, depending on the stage of development, into tubercles (stages1-3) and emerged spikes (stages 4 and 5). Both salicylate compounds showed a significant decrease in the number and growth of tubercles and emerged spikes compared with infected plants alone. AcSA reduced number and weight of tubercles per pot by about 29 and 35%, respectively, compared with about 21 and 40% for SA. Exposing infected faba bean plants to SA or AcSA retarded the development of attached tubercles to emerged spikes. The greatest effect on emerged spikes occurred with AcSA; it inhibited the number and weight of spikes per pot by 82.2% and 77.4%, respectively, corresponding with 46.7 and 51.2%, respectively, for SA. The inhibition in the growth of tubercles and emerged spikes were observed under the two applied methods used, and seed soaking was considered the most effective one (Table 5). Soaking faba bean seeds in salicylate reduced number and weight of tubercles by about 41%, as well as the number and weight of spikes by about 70%, compared with infected plants alone.

Different SA and AcSA concentrations used as seed soaking or foliar spray varied in their effects on number and growth of attached *Orobanche* after 75 days of sowing (Table 6). Application of various SA concentrations as seed soak or foliar spray tended to significantly increase the number of attached *Orobanche* and varied in their effects on the weight of attachments. The most effective treatment was soaking seeds in AcSA at one of the three tested concentrations. These treatments reduced number of attachments between 78-95% and their weights between 78-88%.

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Table 5 Effect of SA and AcSA applied by seed soaking or foliar spray on *Orobanche crenata* parasite, after 75 and 110 days from faba bean sowing.

Entries	Treatments	75 days after sowing		110 days after sowing			
		Attached (*Orobanche*/pot)		Underground (tubercles/pot)		Emerged (spikes/pot)	
		No	Weight (g)	No	Weight (g)	No	Weight (g)
Salicylate compounds	SA	39.5 a	70.5 b	7.9 b	8.1 c	4.8 b	20.7 b
	AcSA	19.3 c	41.0 c	7.1 c	8.9 b	1.6 c	9.6 c
	Infected	24.7 b	81.7 a	10.0 a	13.6 a	9.0 a	42.4 a
Applied methods	Soaking	23.2 b	43.6 c	5.9 b	8.0 c	2.3 c	12.9 c
	Spraying	35.7 a	67.8 b	9.1 a	9.1 b	4.1 b	17.5 b
	Infected	24.7 b	81.7 a	10.0 a	13.6 a	9.0 a	42.4 a

SA: Salicylic acid, AcSA: Acetyl salicylate.
Data within a column followed by the same letters are not significantly different (LSD at $P \leq 0.05$).

Table 6 Effect of salicylate treatments on *Orobanche crenata* parasite, after 75 and 110 days from faba bean sowing.

Method	Treatments	75 days after sowing		110 days after sowing			
		Attached *Orobanche*/pot		Underground tubercles/pot		Emerged spikes/pot	
		No	Weight (g)	No	Weight (g)	No	Weight (g)
Soak	0.5 mM SA	40.0 c	60.0 c	4.0 e	5.4 e	1.3 f	5.0 g
Soak	1.0 mM SA	56.0 b	84.1ab	5.3 de	8.7 d	2.8 de	10.0 f
Soak	1.5 mM SA	34.7 d	79.2 b	9.3 bc	11.6 c	4.7 c	27.0 b
Spray	2 mM SA	41.7 c	93.1 a	12.3 a	10.5 c	13.3 a	39.8 a
Spray	4 mM SA	39.7 c	79.2 b	8.0 c	6.5 e	4.0 cd	23.3 c
Spray	6 mM SA	25.0 e	27.1 e	8.7 c	6.0 e	2.7 de	19.0 d
Soak	0.5 mM AcSA	1.2 g	9.8 f	0.0 f	0.0 g	0.0 f	0.0 h
Soak	1.0 mM AcSA	1.5 g	10.6 f	11.0 ab	16.0 a	1.7 ef	5.0 g
Soak	1.5 mM AcSA	5.5 g	18.1 f	6.0 d	6.0 e	3.3 d	30.1 b
Spray	2 mM AcSA	18.3 f	39.2 d	3.7 e	4.0 f	1.0 f	7.4 fg
Spray	4 mM AcSA	62.3 a	86.2 ab	12.0 a	14.0 b	2.7 de	14.2 e
Spray	6 mM AcSA	27.0 e	82.2 ab	10.0 bc	13.3 b	0.7 f	0.7 h
	Infected plant	25.0 e	82.4 ab	10.0 bc	13.6 b	9.0 b	42.4 a

SA: Salicylic acid, AcSA: Acetyl salicylate.
Data within a column followed by the same letters are not significantly different (LSD at $P \leq 0.05$).

different concentrations from SA and AcSA at two applied methods produced a significant decrease in number and weight of tubercles and emerged spike. Generally, SA and AcSA treatments tended to produce more severe effects on an emerged spike than on tubercles. Whereas tested treatments varied in their effects on tubercle growth, most SA and AcSA treatments produced significant decreases in number and weight of emerged spikes. Among tested treatments, the most suppression effect was produced by soaking seeds in 0.5 mM AcSA; it caused a complete inhibition on attached tubercles and emerged spikes. Also, soaking seeds in 0.5 mM SA greatly reduced number and weight of tubercles (60%) and emerged spike (88%) relative to control plants. On the contrary, among salicylate treatments, foliar application of 2 mM SA produced the highest number and the heaviest weight of emerged spikes.

Effect of salicylate on antioxidant agents

The effect of salicylate treatments on phenolic content, antioxidant activity, and lipid peroxidation (MDA) are presented in Tables 7 & 8. The obtained results revealed that levels of phenolics and antioxidant activities in *Orobanche*-infected plants showed a significant increase compared to healthy ones (Table 7). Meanwhile, exposing infected plants to SA or AcSA as seed soaking or foliar spray resulted in a great decrease in phenolic contents and antioxidant activity compared with infected plants alone. Whereas levels of MDA did not show any significant change due to *Orobanche* infection or using two salicylate compounds either as seed soaking or foliar spray (Table 7).

[DOR: 20.1001.1.22519041.2022.11.2.7.0]

Table 7 Effect of SA and fig AcSA applied by seed soaking or foliar spray on phenolic content, antioxidant activity and malondialdehyde (MDA) content in infected faba bean leaves.

Entries	Criteria	Total phenolics (mg GAE g^{-1} FW)	Antioxidant activity (μM trolox/100 g FW)	MDA (nM g^{-1} FW)
Salicylate compounds	SA	9.32 b	79.7 b	15.7 b
	AcSA	8.94 b	81.7 b	16. 8 a
	Infected plant	12.92 a	172.2 a	16.8 a
	Healthy plant	10.67 b	115.0 b	16.8 a
Applied methods	Soaking	10.28 b	99.0 b	16.0 a
	Spraying	7.99 c	62.3 c	16.6 a
	Infected plant	12.92 a	172.2 a	16.8 a
	Healthy plant	10.67 b	115.0 b	16.8 a

SA: Salicylic acid, AcSA: Acetyl salicylate.
Data within a column followed by the same letters are not significantly different (LSD at $P \leq 0.05$)

Table 8 Effect of salicylate treatments on phenolic content, antioxidant activity and malondialdehyde (MDA) content in infected faba bean leaves.

Method	Treatments	Total phenolics (mg GAE g^{-1} FW)	Antioxidant activity (μM trolox/100 g FW)	MDA (nM g^{-1} FW)
Soak	0.5 mM SA	10.45 d	88.2 e	16.1 cd
Soak	1.0 mM SA	9.46 e	72.8 fg	17.1 bc
Soak	1.5 mM SA	11.28 c	104.0 d	14.1 de
Spray	2 mM SA	8.75 ef	65.3 gh	12.8 ef
Spray	4 mM SA	8.28 fg	77.8 f	17.6 bc
Spray	6 mM SA	7.72 fg	69.9 fg	16.5 c
Soak	0.5 mM AcSA	14.15 a	179.3 a	15.7 cd
Soak	1.0 mM AcSA	8.20 fg	78.6 f	16.1 cd
Soak	1.5 mM AcSA	8.12 fg	71.1 fg	16.8 bc
Spray	2 mM AcSA	7.43 g	49.5 i	22.5 a
Spray	4 mM AcSA	7.39 g	49.9 i	18.7 b
Spray	6 mM AcSA	8.37 fg	61.6 h	11.3 f
	Infected plant	12.92 b	172.2 b	16.8 bc
	Healthy plant	10.67 d	115.0 c	16.8 bc

SA: Salicylic acid, AcSA: Acetyl salicylate.
Data within a column followed by the same letters are not significantly different (LSD at $P \leq 0.05$).

Data displayed in Table 8 indicate that except for the significant increase in phenolic content and antioxidant activity by soaking seeds in 0.5 mM of AcSA, decreases in the levels of two agents were observed for all salicylate treatments, as compared with infected plants alone. Whereas tested treatments caused great variations in the levels of MDA in host tissues, a remarkable increase was obtained by spraying plants with 2 and 4 mM of AcSA.

Discussion

O. crenata parasite is considered the most devastating constraint for faba bean cultivation in the Mediterranean and Middle East regions (Rubiales *et al.*, 2010). Using eco-friendly chemicals such as elicitors and resistance inducers for strengthening plant health and defense mechanisms is being sought (Briache *et al.*, 2020). SA is a natural phenolic hormone reported as a chemical defense-inducer that enhances plants resistance against biotic attackers and induces SAR gene expression (Kusumoto *et al.*, 2007). AcSA is a synthetic compound (trade name: aspirin) that easily degrades to SA and has the same effect as the latter. Its undoubted advantage is its easy availability and low price (Matysiak *et al.*, 2020). In the present study, the ability to use SA and its derivative AcSA as seed soaking or foliar spraying in inducing faba bean resistance against the *O. crenata* parasite was examined at 75 and110-days of post-sowing. *O. crenata* infection caused high mortality of infected faba bean plants and significantly reduced their growth parameters in plant height, number of leaves, and fresh and dry weight. These results agree with the results obtained by Sillero *et al*. (2012) and Abbes *et al*. (2014). The presence of the root parasite can negatively influence the host biomass by acting as a competing sink for assimilation and reducing the host plant's photosynthetic efficiency (Briache *et al*., 2020).

Previous studies reported that exogenous application of SA on the faba bean plant induced resistance by strengthening plant health and plant defense mechanism against Orobanche parasite (Abbes *et al*., 2014; Triki *et al*., 2018). Under the challenge of Orobanche infection, we found that application of SA or AcSA compounds tended to enhance faba bean growth by improving plant viability, height, fresh weight, dry weight, and with less extended number of leaves. The chemical inducers, including SA and its analogs, were reported to activate SAR in plants by increasing the induction of pathogenesis-related proteins, diminishing the damage caused by *Orobanche* spp. (Perez-de-Luque *et al*., 2004; Kusumoto *et al*., 2007; Sillero *et al*., 2012). Moreover, Hayat *et al*. (2009) suggested that exogenous application of SA modified physiological, biochemical, and molecular processes in plants, including anti-oxidative enzyme activities, and regulated the components of its signaling pathway besides getting overlapped with other pathways mediating resistance.

According to results, tested treatments varied in their effects on faba bean growth depending on salicylate compound, concentration, and method of application. We noticed that both SA and its analog AcSA were effective in enhancing plant viability as well as fresh and dry weight of the host plant, but AcSA was more effective than SA. Although AcSA was considered the inexpensive commercial form of SA, there is little information in the literature on its effect on plant resistance against *Orobanche* parasite. Kabiri and Naghizadeh (2015) found that applying AcSA at low concentrations can increase plant resistance against stress conditions by maintaining cell membrane integrity and neutralizing or scavenging reactive radicals. Variations between SA and AcSA in their effects on host growth may reflect the differences in the chemical structure of the two SA sources that lead to producing variations in the capability of plants to absorb and accumulate salicylate compounds (Ozpinar *et al*., 2017). In a previous study, we found that exogenous AcSA treatments tended to cause higher effects on the growth of six plant species, accompanied by an increased capability of tested plants to absorb and accumulate more SA from AcSA solutions than from SA solutions (El-Mergawi and Abd El-Wahed, 2020). The results follow those of Perez-de-Luque *et al*. (2004). They reported that SA was less effective than its benzo (1, 2, 5) thiadiazole - 7 – carbothioic acid S-methyl ester (BTH) derivative in enhancing pea resistance against *O*. *crenata* parasite.

In this experiment, seed soaking was shown to be more effective in enhancing faba bean growth than foliar spraying. It produced a significant increase in plant survival, number of leaves, fresh and dry weight when compared with the foliar application method. A similar result was reported by Triki *et al*. (2018) on faba bean infected with *O*. *foetida* parasite. On the contrary, we observed that foliar application treatments produced the tallest plants when compared with seed-soaking treatments and even with healthy non-treated plants. Following this result, Matysiak *et al*. (2020) showed a great increase in plant height due to the foliar application of SA and AcSA.

Among tested SA and AcSA treatments, soaking faba bean seeds at the concentration of 1 or 0.5 mM AcSA was considered the most effective treatment in alleviating the harmful effect of *Orobanche* parasitism on faba bean growth. These two treatments completely prevented the death of infected plants. They increased their weights by about 120 and 70%, respectively, 75 days post sowing, and with about 67 and 22%, 110 days post sowing. Moreover, the weight of the pods of these two treatments was more than four to five times the weight in infected plants alone. The literature about the effect of AcSA is limited. This result is indirectly in accordance with those reported by Kabiri and Naghizadeh (2015). They found that seed priming with AcSA at 1 mM was considered the most effective treatment in increasing the tolerance of barley to water stress conditions via maintaining cell membrane integrity and neutralizing or scavenging reactive oxygen species.

In this study, the effect of using SA and AcSA at two application methods on the growth of *O. crenata* parasite was determined after 75 and 110 days of sowing. Except for the significant increase in several attached *Orobanche* by SA at 75-days, both SA and AcSA compounds reduced the number of attached *Orobanche* and inhibited the growth of tubercles and emerged spikes. The beneficial effect of SA on activating and enhancing natural faba bean resistance against *O. crenata* parasite was observed by many investigators (Abbes *et al*., 2014; Triki *et al*., 2018; Madany *et al*., 2020). Kusumoto *et al.* (2007) suggested that the reduction of *O. minor* tubercles in red clover by SA treatments was due to the inhibited elongation of parasite radicles and the activation of defense responses in the host root, including lignification of the endodermis. Lignification has been reported as a defense reaction against *Orobanche* penetration, connection to the vascular system, and tuber development (Goldwasser *et al*., 1999); it helped the plant strengthen the cell wall against physical and enzymatic breakdown (Yang *et al*., 2016).

At the first time (75 days of sowing), SA produced a great increase in the number of attached Orobanche compared with non-treated plants. Based on this result, it can be postulated that SA did not have any reduced effect on *Orobanche* seeds germination and their attachments to the host roots (Kusumoto *et al*., 2007; Véronési *et al*., 2009). Hence, it can be supposed that SA reduced *Orobanche* infestation by inhibiting tubercles growth and development by activation of defense responses in the host root (Kusumoto *et al*., 2007; Pérez-de-Luque *et al*., 2004; Sauerborn *et al*., 2002; Véronési *et al*., 2009).

We found that SA and AcSA produced the greatest effect on emerged spike formation. These compounds retarded Orobanche development from the first growth stages (tubercles) to the advanced stage (emerged spike). In line with these treatments, Perez-de-Luque *et al*. (2004) noticed that SA analog BTH caused a reduction in the number of *O. crenata* attachment and most of them being arrested at the first stages, and the number of those reaching emerged spike stage was significantly reduced. These results suggested that host defense activation by the two salicylate compounds could prevent root tissue penetration, connection to the vascular system, and/or tubercle development (Goldwasser *et al*., 1999).

To the best of our knowledge, there is little information in the literature on the effect of AcSA on Orobanche parasitism. Obtained results indicated that the growth and development of *O. crenata* varied greatly depending on salicylate compound, applied methods, and tested concentrations. Comparing SA and AcSA, the greatest reductions in parasite growth occurred with AcSA treatment. However, variations between SA and its derivatives in their effects on growth and development of *O. crenata* grown in pea plant was observed by Perez-de-Luque *et al*. (2004). They showed that SA treatment was less effective on *O. crenata* grown in pea plants than its derivative BTH.

Meanwhile, the two applied methods tended to reduce *O. crenata* infestation, but the most effective method was obtained by seed soaking. As compared with the foliar application method, seed soaking significantly reduced the weight of attached Orobanche at 75-days and the number and weight of tubercles and emerged spikes at 110-days. In line with these results, Triki *et al*. (2018) reported that a pre-treatment of seed with SA at 1 mM was more effective in controlling O. *foetida* infestation in faba bean compared to foliar spraying at the same concentration. Whereas, Briache *et al*. (2020) found that foliar application of SA on faba bean plants did not significantly affect O. *crenata* infestation.

In the present study, different SA or AcSA treatments varied in their effects on *Orobanche* growth and development. This confirms the results of Perez-de-Luque *et al*. (2004). At 75 days of sowing, soaking seeds in AcSA at 0.5 or

[DOR: 20.1001.1.22519041.2022.11.2.7.0]

1 or 1.5 mM resulted in a great reduction in number of attached *Orobanche* (78-95%) and their weights (78-88%) relative to control. The effect of these treatments may be related to a reduction of *Orobanche* seeds germination by reducing the production of germination stimulants or increasing inhibitors released by the host plant roots, as well as to the reduction of the *Orobanche* attachment to the host roots (Abbes *et al*., 2014). Among tested treatments, soaking faba bean seeds in 0.5 mM AcSA was considered the most effective treatment in controlling the *Orobanche* parasite; it produced the lowest values of number and weight of attachment after 75 days and completely prevented tubercles and emerged spike formation after 110 days. To a lesser extent, seed soaking in 0.5 mM SA and foliar application of 2 mM AcSA reduced *Orobanche* infestation by decreasing tubercle and spike numbers. Reduced biomass of *O. cumana* by treating sunflower seeds with low SA doses (0.5 and 1 mM) was observed by Yang *et al*. (2016).

Phenolic compounds are metabolic products that plants synthesize during development and respond to various biotic and abiotic stresses (Stalikas, 2007). Our results showed that *O. crenata* infection caused a great increase in phenolic contents and antioxidant activity in faba bean leaves. Accumulating phenolics in the infected plants supports the significant role of these compounds in increasing plant resistance to *Orobanche* parasite (Briache, *et al*., 2020). We found that application of various SA and AcSA concentrations as seed soaking or foliar spraying tended significantly decrease phenolic content and antioxidant activity relative to infected non-treated plants. In line with these results, Yang *et al*. (2016) showed that the increase in phenolics content in sunflower due to *O. cumana* infection was not reversed by soaking host seeds in 0.5 or 1 mM of SA. But, we found that the most effective treatment in controlling *Orobanche* (soaking seeds in 0.5 mM AcSA) achieved the maximum value of phenolics and antioxidant activity. Many investigators have previously observed a positive effect of the most effective SA treatments on the level of phenolics and antioxidant activity in Orobanche-infected plants (Ahmed *et al*., 2012; Briache *et al*., 2020; Madany *et al*., 2020).

Malondialdehyde (MDA) content represents the damage of reactive oxygen species (ROS) to membrane lipids (Xi *et al*., 2021). Our results indicated that the level of MDA in *Orobanche* infected plants and SA and AcSA treated plants did not produce any significant changes compared with healthy non-infected plants. In line with this result, Yang *et al*. (2016) found that treating sunflower seeds with 0.5 or 1 mM SA did not cause significant changes in MDA content compared to the respective control.

Conclusions

Generally, the present study indicated the efficacy of using SA and AcSA at different concentrations by two applied methods to induce faba bean resistance against *O. crenata* parasite. The two salicylate compounds tended to increase the growth of infected plants by reducing the growth and development of *Orobanche* parasite. The most effective treatments were recorded for AcSA, especially when used as seed soaking. Soaked faba bean seeds in aqueous solutions of AcSA at 0.5 and 1 mM were considered the most effective treatments in improving faba bean yield, reducing *Orobanche* attachment, and retarding their development to the harmful advanced stage (emerged spike). To our knowledge, this is the first study that sheds light on the crucial effect of AcSA, the commercially inexpensive form of SA, in alleviating the stress induced by the *Orobanche* parasite.

Conflict of interest

R. A. El-Mergawi, Mahmoud A. T. El-Dabaa, and Ebrahim R. El Desoki declare that they have no conflict of interest.

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[DOR: 20.1001.1.22519041.2022.11.2.7.0]

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اثربخشی اسید سالیسیلیک و استیل سالیسیلات در افزایش مقاومت باقلا در برابر گل جالیز *Orobanche crenata*

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چکیده: گل جالیز *Orobanche crenata*، یک هولوپارازیت اجباری است که باعث آسیب شدید به باقلا که در منطقه مدیترانه می‌شود. آزمایش گلخانه‌ای برای تعیین و مقایسه پتانسیل اسید سالیسیلیک (SA) و استیل سالیسیلات (AcSA) مورد استفاده به‌عنوان خیساندن بذر (هر کدام ۰/۵- ۱/۵ میلی‌مولار) یا محلول‌پاشی (۲-۶ میلی‌مولار، هر کدام) برای افزایش تحمل لوبیا انجام شد. تیمارها توسط SA یا AcSA باعث افزایش رشد گیاهان باقلا با بهبود زنده ماندن، قد، وزن تر و خشک آنها شد. SA و AcSA رشد توبرکل‌های انگل را کاهش دادند و رشد آن‌ها را تا ظهور سنبله‌ها به تأخیر انداختند. AcSA در افزایش تحمل لوبیا به گل جالیز مؤثرتر از SA بود و خیساندن بذر بیشترین تأثیر را نشان داد. در ۱۱۰ روز کاشت، خیساندن بذرها در ۰/۵ یا ۱ میلی‌مولار AcSA به‌طور کامل از مرگ گیاهان آلوده و افزایش وزن گیاهان به‌ترتیب ۲۲ و ۶۷ درصد و هم‌چنین وزن غلاف/بوته به میزان ۵۱۲ و ۴۴۲ درصد جلوگیری کرد. علاوه بر این، این دو تیمار تا حد زیادی رشد گل جالیز را کاهش دادند و با خیساندن بذر در ۰/۵ میلی‌مولار AcSA، مهار کامل غده‌ها و خوشه‌های پدید آمده رخ داد. گل جالیز به میزان زیادی محتوای فنلی و فعالیت آنتی‌اکسیدانی را در بافت میزبان افزایش داد، اما سطوح آن‌ها با تمام تیمارهای سالیسیلات کاهش یافت. نتایج حاکی از آن است که خیساندن بذر میزبان در AcSA در افزایش مقاومت گیاه در برابر گل جالیز بسیار کارایی دارد.

واژگان کلیدی: باقلا، سالیسیلات، گل جالیز، تحمل