

ORIGINAL ARTICLE

Induction of systemic resistance to *Orobanche crenata* in lentil by exogenous application of salicylic acid and indole acetic acid

Fatima Zahra Briache^{1,2}, Majda El Amri^{1,2} , Mounia Ennami³, Moez Amri⁴,
Zine El Abidine Triqui², Rachid Mentag^{1*} 

¹ Biotechnology Research Unit, Regional Center of Agricultural Research of Rabat, National Institute of Agricultural Research, Rabat, Morocco

² Department of Biotechnology and Plant Physiology, Faculty of Sciences, Mohammed V University, Rabat, Morocco

³ Department of Crop Production, Protection and Biotechnology, Institute of Agronomy and Veterinary Medicine Hassan II, Rabat, Morocco

⁴ Agro-sciences (AgBS), University Mohammed VI Polytechnic (UM6P), Benguerir, Morocco

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*Corresponding address:
rachidmentag@yahoo.ca

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Abstract

Orobanche crenata parasitism on lentil (*Lens culinaris* Medik) is one of the most destructive factors for this crop in Morocco. Field and pot assays were performed to study the mitigation of *O. crenata* stress on two lentil genotypes, Bakria (partially resistant to *O. crenata*) and Zaaria (susceptible), using salicylic acid (SA) and indole acetic acid (IAA). These two hormones were applied separately at concentrations of 1 mM and 0.09 mM, respectively, using seed pre-treatment and/or foliar spray methods. SA and IAA seed pre-treatment for the susceptible genotype Zaaria and foliar spray for the resistant genotype Bakria gave the best control of *O. crenata* under field and controlled conditions. This control reached ~91% in Zaaria and 83% in Bakria and was sometimes accompanied by an increase in plant growth and seed yield compared to the untreated plants. Biochemical assays showed that SA and IAA reduced *O. crenata* infestation in lentil through induction of systemic acquired resistance characterized by increasing activities of phenol metabolizing enzymes (phenylalanine ammonia-lyase, peroxidase, and polyphenol oxidase) implicated in natural defense systems of plants. Treatment of plants with SA or IAA could be an alternative strategy of crop protection with more satisfactory preservation of the environment.

Keywords: indole acetic acid, lentil, *Orobanche crenata*, salicylic acid, systemic acquired resistance

Introduction

Lentil (*Lens culinaris* Medik) plays an important agro-ecological, nutritional and socio-economic role worldwide. Lentil grain is a good source of proteins and several other essential elements for human nutrition. It also plays an important role in improving soil fertility and structure thanks to its root system and ability to fix atmospheric nitrogen through symbiosis with rhizobia (Laskar *et al.* 2019). This lentil-rhizobium symbiosis reduces the supply of nitrogen fertilizers, protects the environment against mineral pollution and improves

the yield of the subsequent crop (Sijilmassi *et al.* 2021). In 2019, the average lentil production in the world was estimated to be around 5.7 million tonnes, out of which around 3.5% were produced in Africa. Ethiopia is the first producer of lentils in Africa, with a contribution of more than 50% of production, followed by Morocco with around 20%. In Morocco, lentils are the third most important food legume crop after faba bean (*Vicia faba* L.) and chickpea (*Cicer arietinum* L.). The cultivated area is estimated to be 40,000 ha while

production reached an average of 26,000 tons over the last 10 years (Faostat 2020). Despite the importance of lentil, its harvested area, production and yield are low and unstable because of the impact of many abiotic and biotic constraints (Mbasani-Mansi *et al.* 2019). In Mediterranean regions, the parasitic plant *O. crenata* is one of the main biotic factors limiting the development of lentil (Amri *et al.* 2019; Ennami *et al.* 2020). This parasitic plant devoid of chlorophyll totally depends on the host plant for water and nutrients and causes considerable losses that can wipe out the entire yield of a crop season (Abbes *et al.* 2014; Briache *et al.* 2019). Many methods have been investigated to control *O. crenata* including agronomic practices, biological and chemical methods but none of them has resulted in successful control of the parasite (Abdel-Kader *et al.* 2009; Razavifar *et al.* 2017; Briache *et al.* 2020; Amri *et al.* 2021). Furthermore, various other technical, economic and environmental constraints hinder the adoption of the majority of these control methods (En-nahli *et al.* 2021). So far, genetic resistance combined with chemical options remain the best and most effective way to control *Orobanche* in farmers' fields. Among the chemical products used there are glyphosate (belonging to the phosphonoglycines family), imazethapyre (belonging to the imidazolinones family) and rimsulfuron (belonging to the sulfonylureas family) (Mesa-Garcia and Garcia-Torres 1985; Jacobsohn and Eldar 1992; Eizenberg *et al.* 2004). However, these herbicides have harmful effects on the environment and human health (Andreotti *et al.* 2018; Elsakhawy *et al.* 2020). To control *Orobanche* in lentil, the doses of glyphosate used prove to be phytotoxic and its reduction decreases the effectiveness of treatment (Pala 2019). The use of other solutions preserving the crop and the environment are being sought.

Plant growth regulators, elicitors and other environmentally friendly compounds, have been used to enhance plant health, plant defense mechanisms and improve the resistance against various pathogens including fungi, bacteria, viruses and parasitic weeds (Šindelářová *et al.* 2002; Achuo *et al.* 2004; Sillero *et al.* 2012; Abbes *et al.* 2014; Lavanya *et al.* 2017; Triki *et al.* 2018; Almas *et al.* 2019; Briache *et al.* 2020). Exogenous application of these compounds can put the plant in an activated state and cause a defensive response similar to that against invasion by pathogens (Qamar *et al.* 2015). This defense response is often characterized by induction of certain key enzymes of secondary metabolism such as peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), and superoxide dismutase (SOD) leading to increased phenolic compounds production. Phenolic compounds can play several roles in plant defense against pathogens, such as phytoanticipins, phytoalexins, structural barriers and activators for plant defense genes

(Katoch *et al.* 2005; Briache *et al.* 2020). Salicylic acid (SA) and indole acetic acid (IAA) are two plant growth regulators that play an important role in many plant physiological processes. SA is considered to be a signal molecule involved in the induction of systemic acquired resistance (SAR) in plants. It activates the synthesis of numerous defense compounds including pathogenesis related proteins, phenolic acids, coumarins and flavonoids (Katoch *et al.* 2005). The exogenous application of SA to plants has been proposed to be an applicable strategy to control *Orobanche* parasitism through stimulation of natural defense mechanisms of host plants, leading to a systemic acquired resistance (SAR) (Abbes *et al.* 2014; Triki *et al.* 2018; Briache *et al.* 2020). Thus, soaking seeds in SA solution can reduce *O. crenata* infestation in faba bean (Briache *et al.* 2020) and *O. ramosa* in tomato (Al-Wakeel *et al.* 2013). Foliar spray or seed pre-treatment using SA can reduce *O. foetida* infestation in faba bean (Abbes *et al.* 2014; Triki *et al.* 2018). Treatment of red clover roots by SA was also reported to reduce the number of *O. minor* attachments (Kusumoto *et al.* 2007). IAA was reported to have a positive effect on defense induction through the activation of glucanase and phytoalexin synthesis (Beckman 2000). Indeed, the exogenous application of IAA can enhance resistance against pathogens including fungi and parasitic plants (Ueno *et al.* 2011; Al-Wakeel *et al.* 2013; Briache *et al.* 2020). The aim of this study was to (i) evaluate the effect of SA and IAA exogenous applications on lentil infestation by *O. crenata* under field and controlled conditions and (ii) investigate the induction of major biochemical processes involved in the defense response of the host plant.

Materials and Methods

Plant material

Two Moroccan lentil genotypes were used in this study, Bakria, partially resistant to *O. crenata*, and Zaaria that was reported to be susceptible to *O. crenata* (Mbasani-Mansi *et al.* 2019; En-nahli *et al.* 2021). *O. crenata* seeds used in the controlled conditions experiments were collected on faba bean plants grown in the Zaer region, Morocco.

Pot experiment

Plant growth and treatments

The assay was monitored in 6 l plastic pots. Pots were filled with sterilized soil inoculated or not inoculated with *O. crenata* seeds (20 mg · kg⁻¹ of soil). For each genotype, the pots were divided into two sets; infested (inoculated) and non-infested (non-inoculated). Seven different treatments were adopted for each set,

control (untreated) and six SA and IAA treatments. Two treatments involved soaking lentil seeds for 6 hours either in 1 mM SA or in 0.09 mM IAA before sowing. Another two treatments included spraying lentil plants at flowering stage with 1 mM SA or 0.09 mM IAA and two treatments with a combination of both previous applications. All pots were arranged in a randomized complete block design with five replications. This experiment was carried out in a greenhouse under natural day-light conditions.

Orobanche crenata infestation and host agro-morphological measurements

Four months after planting, evaluation of the effects of different treatments on *O. crenata* infestation and host plant development was conducted. After removing lentil plants from pots, roots were gently washed with water and the number of *O. crenata* infestation events per plant was determined by counting the developmental stages as described by Briache *et al.* (2019) from 3 to 8 (S3 – *O. crenata* tubercles >2 mm without root formation; S4 – tubercles with crown-root; S5 – stage spider; S6 – sprout already visible remaining underground; S7 – spike stage; S8 – spike flowering and setting of seeds). Total host shoots, roots, and *O. crenata* tubercle dry weights were measured after leaving samples at 70°C for 48 h. Fresh root samples were stored at –80°C for biochemical assays.

Investigation of antioxidant enzymes activity

Extraction and determination of phenylalanine ammonia-lyase (PAL) activity (EC 4.3.1.5)

PAL enzyme in roots of infested and non-infested plants was extracted and its activity was determined according to Solecka and Kacperska (2003). PAL activity was determined by measuring the absorbance at 290 nm and expressed as EU · mg⁻¹ root fresh weight · h⁻¹ (One unit of enzyme activity equals the amount of PAL that produced 1 μM of trans-cinnamic acid in 1 h).

Extraction and determination of peroxidase (POX) activity (EC 1.11.1.7)

POX activity in roots of non-infested and infested treated plants was assayed according to Polle *et al.* (1994). The absorbance was measured at 470 nm and the enzyme activity was expressed as EU · mg⁻¹ root fresh weight · min⁻¹ (U – μM tetragaiacol produced per minute).

Extraction and determination of polyphenol oxidase (PPO) activity (EC 1.14.18.1)

PPO activity in roots of non-infested and infested treated plants was measured by following the protocol of Al-Wakeel *et al.* (2013). The absorbance was measured at 410 nm and the PPO activity was expressed as EU · mg⁻¹ root fresh weight · min⁻¹.

Field evaluation trial

The trial was conducted during the cropping season 2016/17 at INRA-Marchouch research in a high *O. crenata* infested field. Planting was performed at the end of November 2016 according to a randomized complete block design with five replications. Each genotype was planted in eight rows of 2 m length, with 0.5 m inter-row spacing and a plant density of 10 seeds per m². One untreated row was kept as the control and the other rows were subjected to seven different treatments. One treatment involved spraying plants at flowering stage, with glyphosate at a dose of 36 g · ha⁻¹ and in the other six treatments SA or IAA solutions were applied as described in the pot experiment.

At crop maturity, agro-morphological parameters (dry weight of host shoot and seed yield) and *O. crenata* infestation parameters were recorded. The infestation level was estimated by determining the number and dry weight of emerged *O. crenata* spikes, the incidence (percentage of host plants showing emerged spikes), the percentage of *O. crenata* infestation reduction calculated using the formula: % of *O. crenata* infestation reduction = (*O. crenata* spike number in infested control – *O. crenata* spike number in treated plant)/*O. crenata* spike number in infested control × 100), and the severity (provides information on the impact of the parasite on host vigor using an 1–9 scale (Abbes *et al.* 2007)).

Statistical analysis

Statistical analyses were performed using the SPSS software (IBM SPSS Statistics 23, NY, USA). Analyses of variance ANOVA were conducted to assess whether there was a significant variation for each studied variable. Treatment means were compared using Duncan's test at $p = 0.05$.

Results

Effects of hormonal treatments on *Orobanche crenata* infestation in the pot experiment

Results showed a significant difference ($p \leq 0.05$) for *O. crenata* numbers and dry weights between the two tested genotypes. The numbers and dry weights of *O. crenata* events developed in Zaaria untreated plants were 11.9 and 4.54 g, respectively, against 5.11 and 0.61 g recorded for the resistant Bakria untreated plants. Application of SA (1 mM) or IAA (0.09 mM) treatments significantly decreased *O. crenata* infestation for both lentil genotypes (Table 1). For the

Table 1. Effects of salicylic acid (SA) (1 mM) or indole acetic acid (IAA) (0.09 mM) treatments on *Orobanche crenata* infestation levels in the two lentil genotypes assessed in pot assay

Geno- type	Treatments	Number of <i>O. crenata</i> infestation events/developmental stage						Total number of <i>O. crenata</i> infestation event · plant ⁻¹	Total <i>O. crenata</i> dry weight [g]
		S3	S4	S5	S6	S7	S8		
Zaaria	untreated	1.57 a	3.00 a	2.67 a	2.00a	1.33a	1.33a	11.90 a	4.54 a
	SA (seed soaking)	0.75b	0.44b	0.63 b	0.38b	0.06b	0.00b	2.25 b	0.63 b
	IAA (seed soaking)	0.00b	0.83b	1.00 b	0.17b	0.00b	0.00b	2.00 b	0.02 b
	SA (foliar spray)	0.33b	1.13b	1.38 b	1.25a	2.25a	0.25a	6.58 a	2.34 a
	IAA (Foliar spray)	1.75 a	0.46b	1.98 a	0.33b	0.50a	0.00b	5.02 a	0.69 b
	SA (seed soaking + foliar spray)	1.75 a	0.31 c	1.28 b	0.25b	0.00b	0.00b	3.60 b	0.32 b
	IAA (seed soaking + foliar spray)	1.00 a	0.36b	0.43 b	0.22b	0.15b	0.00b	2.15 b	0.40 b
Bakria	untreated	0.11b	0.67b	2.50 a	0.50b	0.39a	0.00b	5.11 a	0.61 a
	SA (seed soaking)	0.00b	0.31 c	1.66 ab	0.42b	0.00b	0.00b	2.39 b	0.14 b
	IAA (seed soaking)	0.00b	0.05 c	0.25 b	0.15b	0.00b	0.00b	0.45 c	0.09 b
	SA (foliar spray)	0.00b	0.00 c	0.33 b	0.48b	0.00b	0.00b	0.81 c	0.22 b
	IAA (foliar spray)	0.00b	0.69b	0.59 b	0.37b	0.00b	0.00b	1.64 bc	0.05 b
	SA (seed soaking + foliar spray)	0.00b	0.10 c	0.37 b	0.40b	0.00b	0.00b	0.87 c	0.29 b
	IAA (seed soaking + foliar spray)	0.00b	0.50b	0.28 b	0.46b	0.00b	0.00b	1.24 bc	0.10 b

Values are presented as mean of five replications. Data in a column followed by different letters are significantly different at the 0.05 levels by Duncan's test

resistant genotype Bakria, all treatments significantly decreased *O. crenata* numbers and dry weights compared to control plants. Soaking seeds in IAA was the most effective treatment with 91% decrease of the *O. crenata* events number against 84 and 83% observed for SA foliar application alone and combined with seed pre-treatment, respectively. Decreases of 68 and 75.7% in *O. crenata* numbers per lentil plant were recorded with IAA foliar spray alone and combined with seed pre-treatment, respectively. Seed soaking in SA was slightly less effective with only 53% decrease of *O. crenata* number. All *Orobanche* attachments observed in treated plants were at underground stages (S3 to S6) with no presence of emerged spikes (S7 and S8). For the susceptible genotype Zaaria, seed pre-treatment with SA or IAA alone or in combination with foliar application significantly reduced *O. crenata* infestation compared to control plants (Table 1). Such decreases were observed for both underground and emerged *O. crenata* tubercles. The same results were also observed for *O. crenata* dry weight. A maximum decrease of 83% in *O. crenata* numbers was observed for Zaaria plants subjected to IAA pre-treated seeds. No significant difference was observed for *O. crenata* numbers in plants sprayed with IAA and untreated control but total *Orobanche* dry weight was significantly different (0.69g in IAA treated plant against 4.54 g in control).

Effects of hormonal treatments on lentil growth in the pot experiment

For the Zaaria genotype, *O. crenata* infestation resulted in a significant decrease of shoot and root biomass in untreated plants (Fig. 1). In response to SA or IAA treatments, no significant effect was observed on shoot biomass in both infested and non-infested plants compared to their respective controls (Fig. 1A). On the other hand, SA or IAA foliar spray and SA seed pre-treatment significantly increased root biomass of infested plants compared to the untreated control. The maximum increase (two times higher than infested control) was observed in SA sprayed plants (Fig. 1B).

Orobanche crenata infestation did not affect shoot dry weight of untreated Bakria plants. Total shoot dry weights of 0.59 g and 0.65 g were recorded in infested and non-infested control plants, respectively. However, root biomass of control plants decreased by 25% because of *O. crenata* parasitism (Fig. 2). Hormonal treatments, with the exception of IAA seed soaking, significantly decreased shoot biomass of both infested and non-infested plants compared to their respective controls (Fig. 2A). SA seed soaking treatment generated the minimum values of shoot dry weight reaching 0.05 g and 0.2 g in both infested and non-infested plants, respectively.

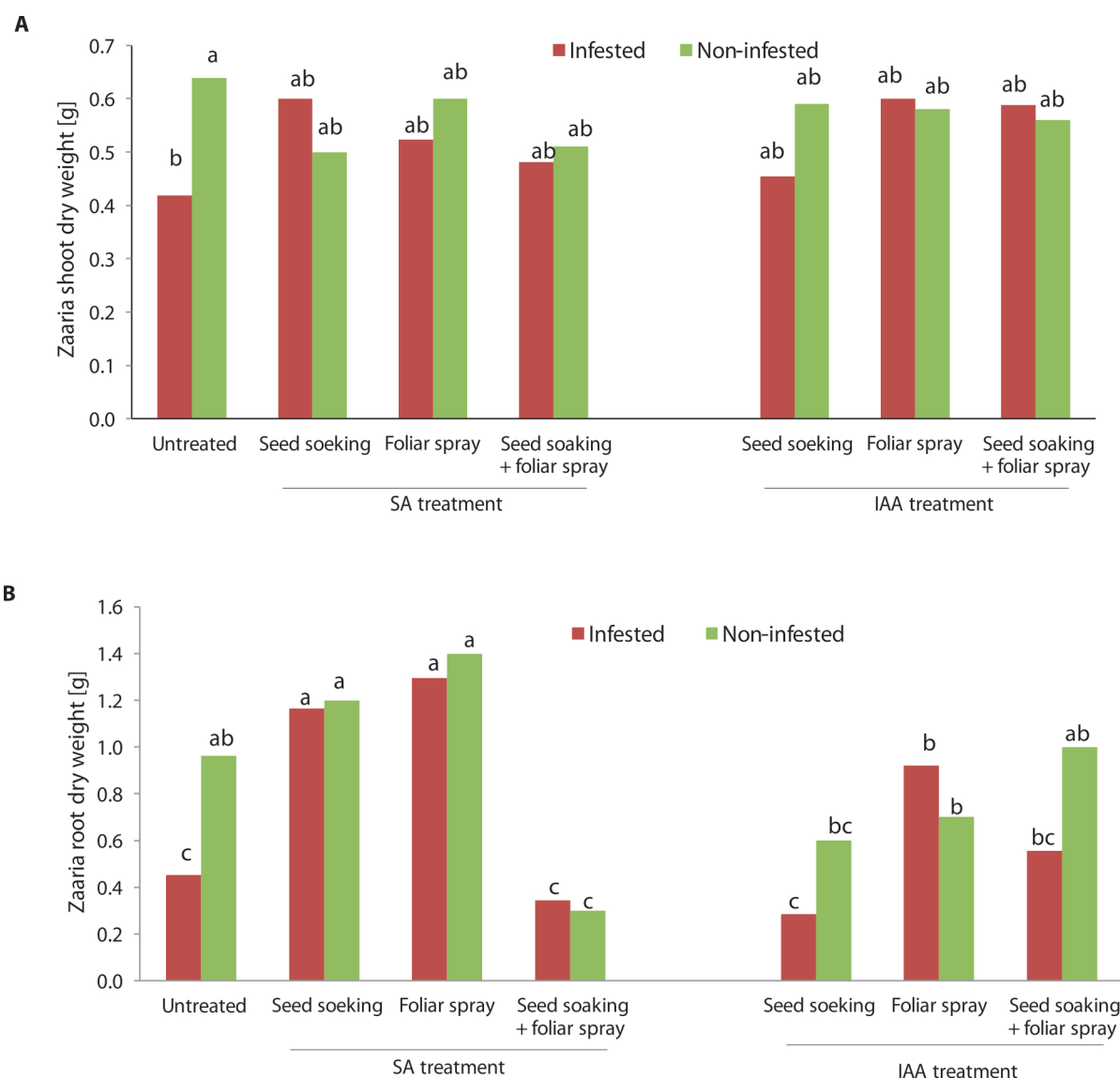


Fig. 1. Effects of salicylic acid (SA) (1 mM) and indole acetic acid (IAA) (0.09 mM) treatments on shoot (A) and root (B) dry weight (g) of Zaaria genotype assessed in pots. Data are means of five replications. Different letters above the bars indicate significantly different means between treatments at the 0.05 level by Duncan's test

IAA treatments using seed soaking or foliar application methods significantly increased root biomass of both infested and non-infested plants (Fig. 2B). IAA foliar spray generated the maximum increase of root biomass in infested and non-infested plants that was 6 and 3.5 times higher than respective controls. SA foliar spray alone or combined with seed soaking also increased the root biomass of infested plants. The respective increase was 3 and 0.5 times higher than infested control.

Effect of hormonal treatments on PAL, POX and PPO activities in host roots

Analysis of the enzymatic activity in lentil roots showed a significant variation ($p \leq 0.05$) of PAL, POX, and PPO activities depending on experimental

conditions (non-infested/infested) and the applied treatments (Fig. 3 and 4). Under infested conditions, Zaaria control plants (untreated) showed a significant increase of PAL and POX activities (Fig. 3). Thus, PAL activity in infested and non-infested controls was 19.3 and 11.3 UE/mg root fresh weight/h, respectively, and POX activity was 43.5 and 17.2 UE · mg⁻¹ root fresh weight · min⁻¹, respectively. However, no significant effect of infestation was observed in PPO activity of control plants (Fig. 3). After application of hormonal treatments, some changes in the activity of evaluated enzymes were observed in the Zaaria genotype. IAA treatments resulted in a significant increase of PAL activity in roots of both infested and non-infested plants compared to untreated controls while SA treatments did not generate any significant effect. PAL activity in IAA treated plants ranged from 25.6 to 28.5 UE · mg⁻¹

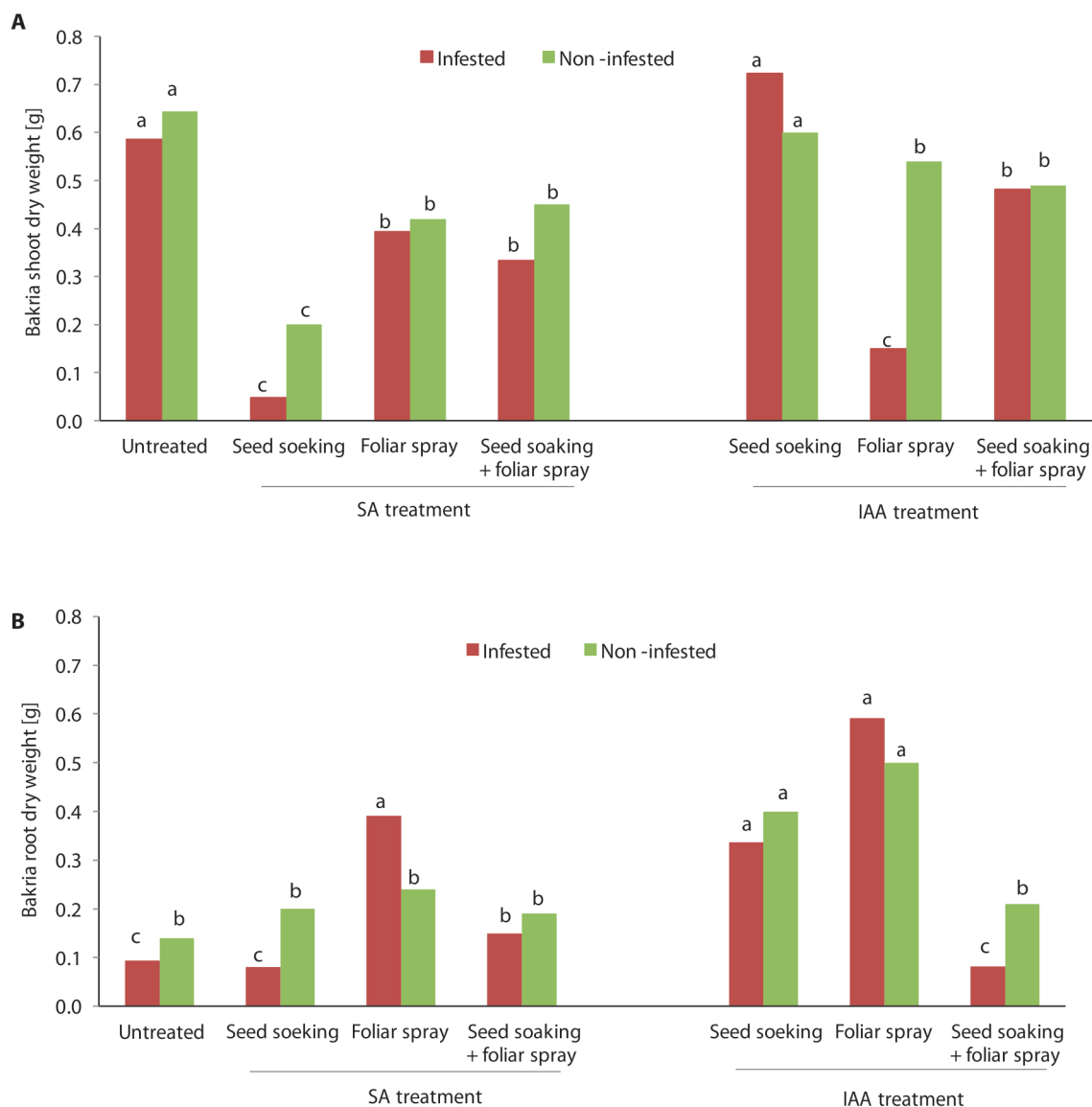


Fig. 2. Effects of salicylic acid (SA) (1 mM) and indole acetic acid (IAA) (0.09 mM) treatments on shoot (A) and root (B) dry weight (g) of Bakria plants assessed in pots. Data are means of five replications. Different letters above the bars indicate significantly different means between treatments at the 0.05 level by Duncan's test

root fresh weight $\cdot \text{min}^{-1}$ under infested conditions and from 19.5 to 25 $\text{UE} \cdot \text{mg}^{-1}$ root fresh weight $\cdot \text{min}^{-1}$ under non-infested conditions. The maximum PAL activity (28.5 $\text{UE} \cdot \text{mg}^{-1}$ root fresh weight $\cdot \text{min}^{-1}$) was observed in infested plants subjected to IAA seed pre-treatment combined with foliar spray (Fig. 3A). POX enzyme activity did not show any increase in response to treatments; rather, a significant decrease was recorded in infested plants in response to SA seed pre-treatment and IAA foliar spray (Fig. 3B). PPO enzyme activity in Zaaria roots significantly increased in response to all SA and IAA treatments under infested and non-infested conditions. IAA seed soaking induced higher PPO activity in both infested and non-infested plants by 4.64 and 3 $\text{UE} \cdot \text{mg}^{-1}$ root fresh weight $\cdot \text{min}^{-1}$, respectively (Fig. 3C).

For Bakria untreated plants, *O. crenata* infestation increased the PAL, POX, and PPO activities (Fig. 4) and these increases were more pronounced than the susceptible check Zaaria (Fig. 3 and 4). Thus, PAL activity in infested and non-infested Bakria controls was 26.1 and 13.4 $\text{UE} \cdot \text{mg}^{-1}$ root fresh weight $\cdot \text{min}^{-1}$, respectively, POX activity was 15.6 and 4.5 $\text{UE} \cdot \text{mg}^{-1}$ root fresh weight $\cdot \text{min}^{-1}$, respectively, and PPO activity was 2.4 and 0.8 $\text{UE} \cdot \text{mg}^{-1}$ root fresh weight $\cdot \text{min}^{-1}$, respectively. SA and IAA treatments applied on infested plants did not generate any significant variation in PAL enzyme activity while a significant increase was observed in non-infested plants compared to untreated controls (Fig. 4A). PAL activity in non-infested treated plants ranged from 16.4 to 20 $\text{UE} \cdot \text{mg}^{-1}$ root fresh weight $\cdot \text{min}^{-1}$. POX activity in both infested

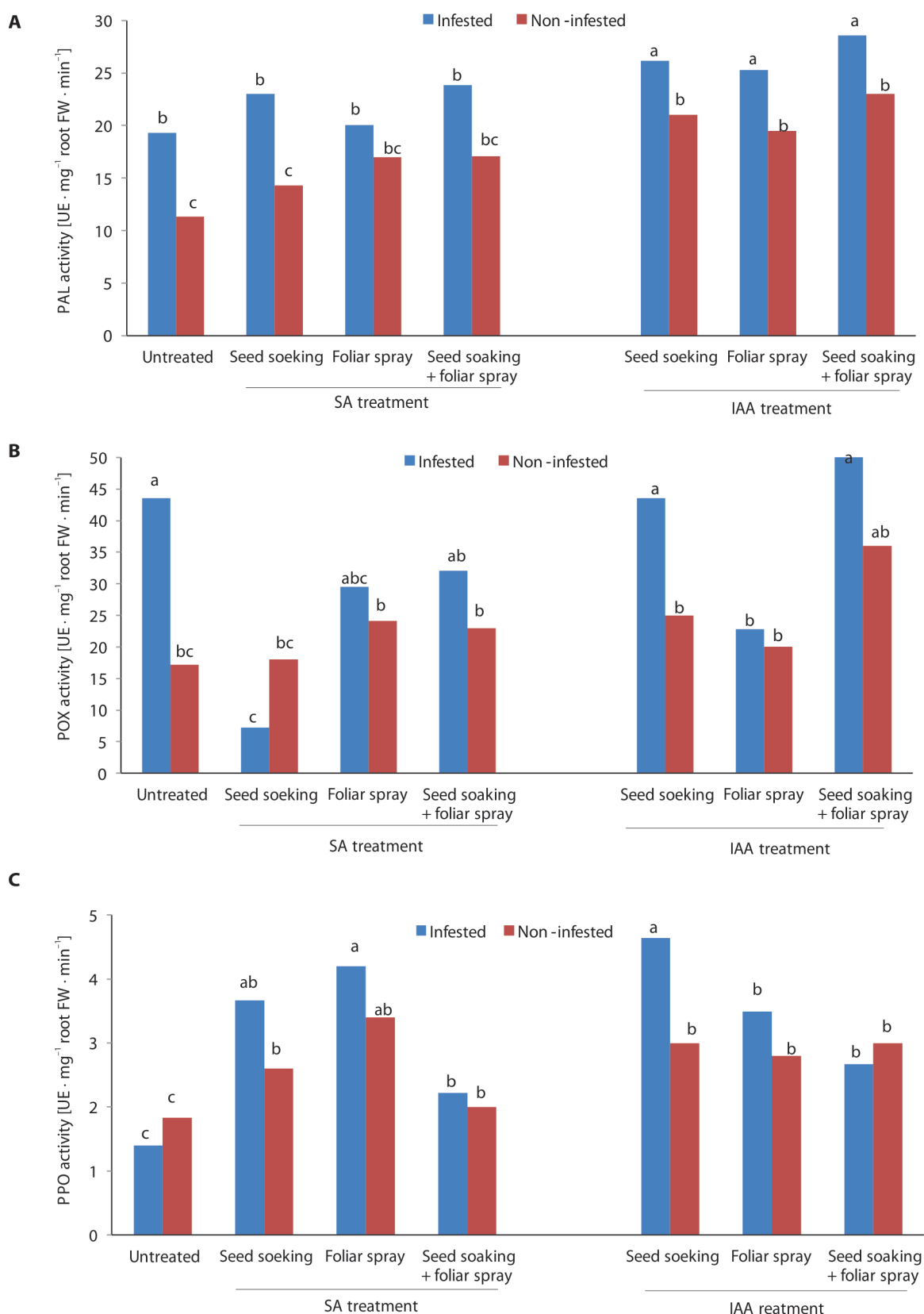


Fig. 3. Effects of salicylic acid (SA) (1 mM) and indole acetic acid (IAA) (0.09 mM) treatments on phenylalanine ammonia-lyase (PAL) (A), peroxidase (POX) (B), and polyphenol oxidase (PPO) (C) activities in roots of Zaria plants. Data are means of three replicates for each treatment. Different letters above the bars indicate significantly different means between treatments at the 0.05 level by Duncan's test

and non-infested plants significantly increased in response to all hormonal treatments. Compared to untreated plants, SA seed pre-treatment combined with

foliar spray induced higher POX activity where it was 45 UE · mg⁻¹ root fresh weight · min⁻¹ in infested plants and 34.7 UE · mg⁻¹ root fresh weight · min⁻¹

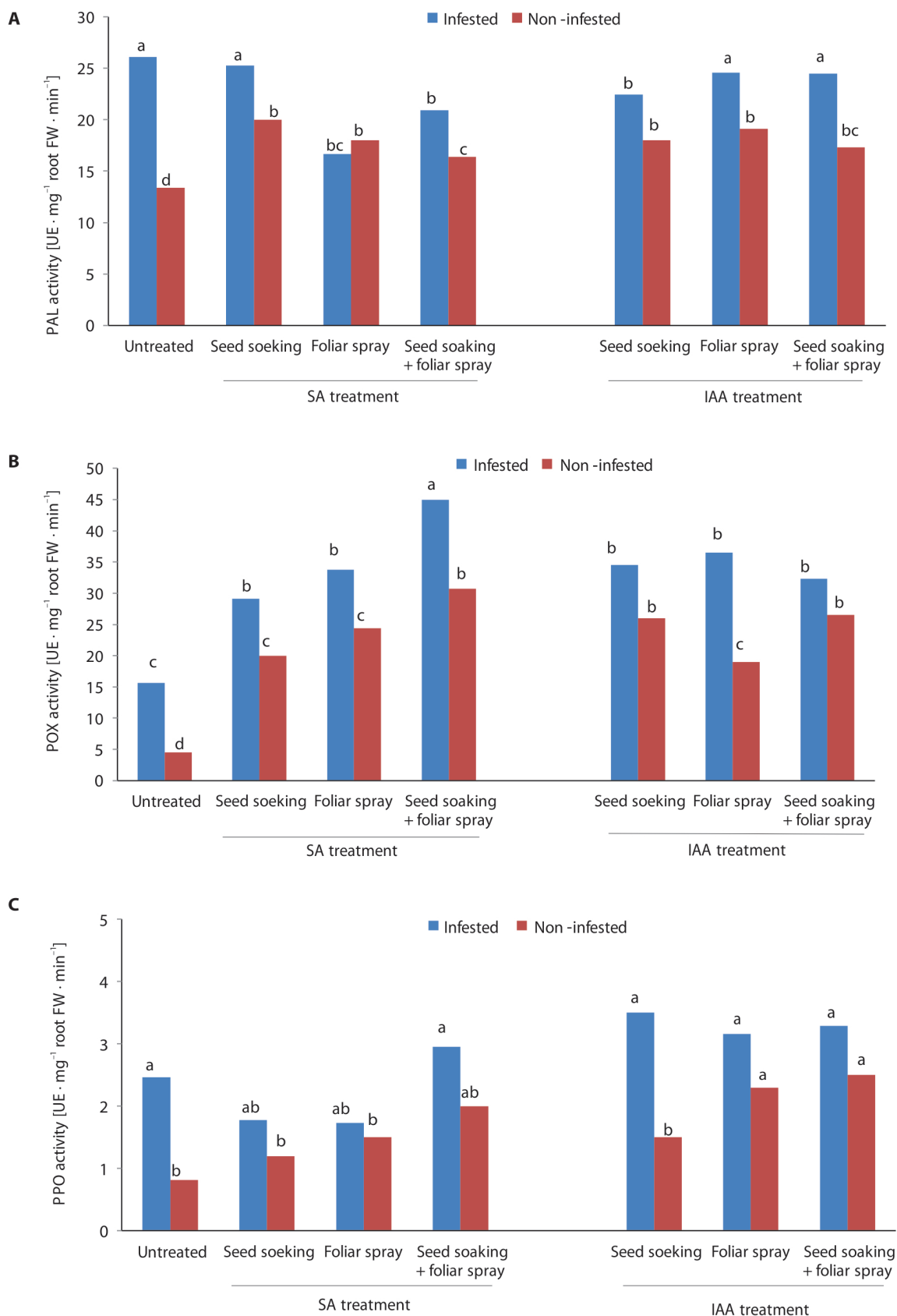


Fig. 4. Effects of salicylic acid (SA) (1 mM) and indole acetic acid (IAA) (0.09 mM) treatments on phenylalanine ammonia-lyase (PAL) (A), peroxidase (POX) (B), and polyphenol oxidase (PPO) (C) activities in roots of Bakria plants. Data are means of three replicates for each treatment. Different letters above the bars indicate significantly different means between treatments at the 0.05 level by Duncan's test

in non-infested plants (Fig. 4B). Concerning PPO enzyme activity, no significant effects of treatments

were observed in Bakria infested plants while its activity in non-infested plants increased only in

response to IAA foliar spray alone ($2.3 \text{ UE} \cdot \text{mg}^{-1}$ root fresh weight $\cdot \text{min}^{-1}$) or combined with seed pre-treatment ($2.5 \text{ UE} \cdot \text{mg}^{-1}$ root fresh weight $\cdot \text{min}^{-1}$) (Fig. 4C).

Assessment of the effects of different treatments on *Orobanche crenata* infestation under open field conditions

During the cropping season 2016/17, high *O. crenata* infestation levels occurred due to favorable environmental conditions ($10\text{--}12^\circ\text{C}$ average air temperature and sufficient water supply (150 mm) during winter (Fig. 5). For the susceptible genotype Zaaria, untreated plants showed high infestation level with 91.07% incidence, a severity level of 5.6, and 1.47 emerged *O. crenata* spikes with a dry weight of 1.6 g. Compared to this genotype, untreated plants of the resistant genotype Bakria showed low *O. crenata* infestation level with 54.28% incidence, a severity level of 2.33, and

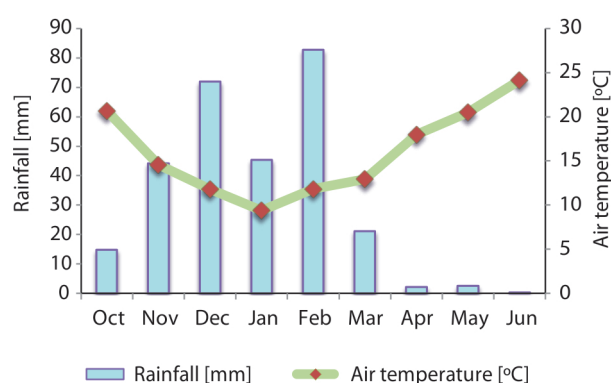


Fig. 5. Average air temperature ($^\circ\text{C}$) and rainfall (mm) during the cropping season 2016/17 at the Marchouch experimental station of INRA-Morocco

1.13 emerged *O. crenata* spikes per plant with a dry weight of 0.43 g (Table 2). Application of glyphosate reduced *O. crenata* infestation by 100% and 85% for

Table 2. Effects of salicylic acid (SA) (1 mM) and indole acetic acid and (IAA) (0.09 mM) treatments on *Orobanche crenata* infestation levels in the two lentil genotypes assessed in the field

Geno-type	Host plant treatment	Incidence [%]	Severity**	<i>Orobanche crenata</i> infestation reduction [%]	Number of emerged <i>Orobanche crenata</i> spikes per host plant	<i>Orobanche crenata</i> spikes dry weight per host plant [g]
Zaaria	untreated	91.07 a	5.6 a	0 e	1.47 a	1.60 a
	glyphosate (foliar spray)	0 c	1 b	100 a	0 d	0 c
	SA (seed soaking)	39.99 bc	1 b	57.1 bc	0.63 bcd	0.46 b
	IAA (seed soaking)	39.16 bc	1.2 b	50.3 c	0.73 bcd	0.99 b
	SA (foliar spray)	72.30 a	3.8 a	42.8 c	0.84 bc	0.4 b
	IAA (foliar spray)	56.62 abc	3 a	38.7 d	0.90 abc	1.24 a
	SA (seed soaking + foliar spray)	29.62 bc	1.4 b	70 b	0.44 bcd	0.44 b
	IAA (seed soaking + foliar spray)	43.12 bc	2 ab	55.1 bc	0.66 bcd	0.74 b
Bakria	untreated	54.28 a	2.33 a	0 e	1.13 a	0.43 b
	glyphosate (foliar spray)	6.52 c	1 b	85 b	0.17 d	0.14 c
	SA (seed soaking)	35.71 b	1.6 b	33.6 d	0.75 bcd	0.22 c
	IAA (seed soaking)	53.23 a	1.5 b	51.3 c	0.55 bcd	0.15 c
	SA (foliar spray)	33.95 b	1 b	63.7 b	0.41 cd	0.16 c
	IAA (foliar spray)	43.06 b	1.8 b	22.1 de	0.88 bc	0.49 b
	SA (seed soaking + foliar spray)	50.44 a	1.5 b	0 e	1.13 a	0.51 b
	IAA (seed soaking + foliar spray)	56.27 a	2.6 a	15 de	0.96 abc	0.57 b

Incidence: percentage (%) of lentil plants presenting emerged *O. crenata* spikes per row

**Severity scale: 1 – absence or few emerged spikes without any *Orobanche* seed production; 3 – sporadic emerged spikes (no more than two per plant) but with few *Orobanche* seed production; 5 – more than two emerged spikes per host plant with average *Orobanche* seed production and an almost normal plant growth; 7 – many emerged spikes (three to five) per plant, with normal *Orobanche* growth and seed production and significant growth and grain yield reduction of the host; 9 – very important number of emerged spikes per plant, abundant *Orobanche* seed production and serious reduction of host grain yield or complete destruction of the host). Values are presented as mean of five replications. Data in a column followed by different letters are significantly different at the 0.05 levels by Duncan's test

both susceptible Zaaria and resistant Bakria genotypes, respectively, compared to untreated plants. Different application methods of both hormones SA and IAA resulted in different effects on *O. crenata* infestation (Table 2). For the susceptible genotype Zaaria, hormonal treatments significantly reduced *O. crenata* spike numbers and dry weights with decreases ranging from 70% to 38.7%. Compared to control plants, the reduction of *O. crenata* spike numbers recorded for Zaaria plants reached a maximum of 70% with the combined treatment of both SA seed pre-treatment and foliar application. SA seed pre-treatment reduced *O. crenata* spike numbers by 57% against 50.3% with IAA seed pre-treatment alone and 55.1% in combination with foliar spray. IAA foliar spray was less effective and reduced *O. crenata* spike numbers by 38% with no significant effect on dry weight. For the resistant genotype Bakria, SA or IAA seed pre-treatments and SA foliar treatment significantly reduced *O. crenata* spike numbers and dry weights compared to untreated plants. Such reductions were 33.6, 51.3, and 63.7%, respectively. However, SA and IAA seed pre-treatments combined with foliar spray as well as IAA foliar spray did not generate any significant reduction neither in *O. crenata* number nor in dry weight.

Assessment of the effects of different treatments on lentil growth under open field conditions

Development and yield of both lentil genotypes under hormonal treatments were also assessed (Table 3). Results showed that glyphosate, and SA and IAA treatments did not have any significant influence on shoot biomass of both genotypes compared to untreated plants. Glyphosate treatment of Zaaria plants resulted in a negative effect on seed yield ($0 \text{ g} \cdot \text{plant}^{-1}$) in contrast to a seed yield increase of 89% observed with IAA seed pre-treatment compared to untreated plants. Regarding other SA and IAA treatments, no significant effect on seed yield was observed in treated plants. Concerning the resistant genotype, increases of 98.6% and 65.2% in seed yield were recorded in response to SA seed soaking or foliar treatments, respectively (Table 3).

Discussion

Glyphosate was classified as the most widely used herbicide against *Orobanche* worldwide (Johal and Huber 2009). Recently, it has been found that it poses many human and environmental problems. In 2015, the IARC (International Agency for Research on Cancer, Lyon, France), an organization referred to as the

Table 3. Effects of salicylic acid (SA) (1 mM) or indole acetic acid (IAA) (0.09 mM) treatments on shoot dry weight and seed yield (g) of both genotypes assessed in field

Geno- type	Host plant treatment	Shoot dry weight · plant ⁻¹ [g]	Seed yield · plant ⁻¹ [g]
Zaaria	untreated	3.04 a	0.62 b
	glyphosate (foliar spray)	0.49 a	0 c
	SA (seed soaking)	2.51 a	0.44 b
	IAA (seed soaking)	2.61 a	1.17 a
	SA (foliar spray)	2.96 a	0.43 b
	IAA (foliar spray)	3.21 a	0.91 ab
	SA (seed soaking + foliar spray)	3.14 a	0.87 ab
	IAA (seed soaking + foliar spray)	1.82 a	0.63 b
Bakria	untreated	2.71 a	0.73 b
	glyphosate (foliar spray)	2.66 a	0.72 b
	SA (seed soaking)	4.06 a	1.45 a
	IAA (seed soaking)	1.87 a	0.37 bc
	SA (foliar spray)	2.36 a	1.20 a
	IAA (foliar spray)	1.73 a	0.54 b
	SA (seed soaking + foliar spray)	3.91 a	0.51 b
	IAA (seed soaking + oliar spray)	2.22 a	0.66 b

Values are presented as mean of five replications. Data in a column followed by different letters are significantly different at the 0.05 levels by Duncan's test

specialized cancer agency of the World Health Organization (WHO, Geneva, Switzerland), classified glyphosate as "likely carcinogenic" to humans (Andreotti *et al.* 2018; Elsakhawy *et al.* 2020). This encouraged research to find ecofriendly chemicals in order to develop herbicides with new chemical formulas and modes of action. The use of ecofriendly products such as hormones and elicitors, for enhancing natural plant defense rather than directly attacking the pathogens, has been reported in many previous studies (Šindelářová *et al.* 2002; Sillero *et al.* 2012; Abbes *et al.* 2014; Kukawka *et al.* 2018; Triki *et al.* 2018; Kaczmarek *et al.* 2020; Spsychalski *et al.* 2021). The current research describes the effect of two phytohormones, salicylic acid (1 mM) and indole acetic acid (0.09 mM), on improving lentil resistance to *O. crenata* in field and under controlled conditions (in pots). The choice of SA and IAA concentrations came from the literature where they were frequently reported as optimal concentrations for inducing plant resistance to various pathogens (Sillero *et al.* 2012; Abbes *et al.* 2014; Briache *et al.* 2020). The use of different culture conditions made it possible to obtain complementary information on the parasitism process and on treatment effects.

Pot experiment results showed that both hormones significantly reduced *O. crenata* infestation in the two lentil genotypes. Infestation decreases ranged from 44 to 83% in Zaaria and from 53 to 91% in Bakria in response to SA (1 mM) and IAA (0.09 mM) applications. Similar results have been reported in previous studies using IAA and SA to control *O. crenata* in faba bean (Briache *et al.* 2020) and *O. ramosa* in tomato (Al-Wakeel *et al.* 2013). Briache *et al.* (2020) reported that SA or IAA as seed pre-treatments were more effective in controlling *O. crenata* infestation in both resistant and susceptible faba bean genotypes than foliar spraying. Triki *et al.* (2018) also reported better effects of SA seed pre-treatment than foliar spraying to control *O. foetida* infestation in faba bean. However, Abbes *et al.* (2014) confirmed the effectiveness of foliar application of SA on reducing *O. foetida* infestation in faba bean. In our study, the three treatment methods (seed soaking and/or foliar spraying) resulted in a good reduction of *O. crenata* infestation in the resistant genotype Bakria. However, SA or IAA seed pre-treatment alone or combined with foliar application were more efficient than foliar spray to control *O. crenata* in the susceptible genotype Zaaria. This difference of the effects of treatment methods observed in the two lentil genotypes could be explained by the activation of different resistance mechanisms in resistant and susceptible genotypes. The reduction of infestation observed in both genotypes in response to SA and IAA treatments was characterized by a reduced number of both underground and emerged tubercles with a significant change in *O. crenata* dry weight compared to untreated controls. This could be explained by the delayed tubercle establishment and development. Several studies reported the involvement of phytohormones such as salicylic acid and jasmonic acid in host defense responses against *Orobanche* at initial stages of the holoparasitic plant-host interaction (Gutjahr and Paszkowski 2009; Torres-Vera *et al.* 2016; Casadesús and Munné-Bosch 2021). Previous studies performed on faba bean under *O. foetida* infestation reported that SA treatments significantly decreased *Orobanche* seed germination resulting in low infestation (Abbes *et al.* 2014; Triki *et al.* 2018). Other authors did not find a significant reduction of seed germination demonstrating that there were no toxic effects of hormones on *O. minor* (Kusumoto *et al.* 2007), *O. crenata* (Pérez-de-Luque *et al.* 2004), *O. Cumana* (Sauerborn *et al.* 2002), and *P. ramosa* seeds (Véronési *et al.* 2009).

Several authors mentioned that application of resistance-inducing agents resulted in a reduction of *Orobanche* number concomitant with observable growth reduction in host plants (Bigirimana and Höfte 2002; Lopez and Lucas 2002; Perez *et al.* 2003; Abbes *et al.* 2014; Triki *et al.* 2018). Similar results were observed in response to SA treatment with significant

decreases of shoot dry weight in Bakria plants. The biomass reduction was explained by Heil *et al.* (2000) as an allocation cost of treated plants, which is a result of a metabolic competition between biomass production and defense. Bakria plants subjected to IAA seed pre-treatment did not show any decrease of shoot biomass against a significant increase of root biomass in addition to a significant reduction of *O. crenata* parasitism. Plants sprayed with IAA also showed a significant increase of root biomass in addition to a significant reduction of *O. crenata* parasitism. These results confirm the role of IAA as a growth hormone and also as an inducer of systemic acquired resistance (SAR) in plants (Wang and Fu 2011). The agro-physiological behavior of the susceptible genotype Zaaria under treatments was different than those observed in the resistant Bakria with no significant decrease in shoot growth and a significant increase of root growth. Indeed, plants sprayed with SA and IAA showed shoot and root biomasses similar to those of non-infested plants despite heavy orobanche infestation. Similar results were reported in tomato infested by *P. ramosa* where elicitors' exogenous applications significantly improved tomato growth (Al-Wakeel *et al.* 2012). This could be explained by a strengthening of plant health under infested conditions in response to treatments following appropriate timing and number of treatments as suggested by Buschmann *et al.* (2005).

Phenolic compounds and related oxidative enzymes are generally considered to be some of the important biochemical parameters for disease resistance (Pradeep and Jambhale 2002). They differentiate resistant and susceptible genotypes (Ojha and Chatterjee 2012). The results presented in Figures 3 and 4 indicate that as a consequence of *O. crenata* parasitism, the phenylalanine ammonia-lyase (PAL), peroxidase (POX), and polyphenol oxidase (PPO) activities in the resistant genotype Bakria were found to be much higher than in the non-infested plants. In the susceptible genotype Zaaria, *O. crenata* infestation increased the activity of PAL and POX only with lower levels than those observed in the resistant genotype, which could be explained by the activation of different resistance mechanisms in the two tested genotypes. Similar results were observed in wheat in response to spot blotch infection (Das *et al.* 2012), in *Saraca Asoca* in response to anthracnose disease (Ojha *et al.* 2005), in *Trichoderma harzianum* in response to *Furarium solani* (Chakraborty and Chatterjee 2007), and in onion in response to stemphylium leaf blight disease (Kamal *et al.* 2008). Activation of these enzymes results in the development of an antioxidant defense system leading to plant protection against oxidative stress damage by either partial suppression of reactive oxygen species production or the scavenging of reactive oxygen species (Hossain and Dietz 2016). Application of SA and

IAA treatments resulted in different effects on PAL, POX, and PPO activities in lentil roots depending on genotypes. In the susceptible genotype Zaaria, all hormonal treatments significantly increased PPO enzyme activity while PAL activity increased only under IAA treatments and POX did not express any additional activation. However, in the resistant genotype Bakria, POX activity was increased in response to all hormonal treatments. These results confirm the effectiveness of SA and IAA exogenous application in induction of lentil defense and also the difference of activated mechanisms in both resistant and susceptible genotypes. Similarly, it was reported in a previous study that certain biochemical changes, including the increase of phenols production and antioxidant enzyme activities, can occur after application of SA and IAA on faba bean infested by *O. crenata*. These biochemical changes can act as markers for induced systemic resistance (Briache *et al.* 2020). Furthermore, Al-Wakeel *et al.* (2013) stated that PAL, POX, and PPO might be some of the elements of the tomato defense system in response to *P. ramosa*.

IAA and SA were tested under field conditions to confirm the evaluated effectiveness of treatments during the pot experiment. In the field, treatment of Zaaria and Bakria genotypes with glyphosate resulted in 100 and 85% of *O. crenata* parasitism reduction, respectively, but there was also a negative effect on seed yield. SA and IAA treatments overcame this limitation. In fact, these treatments did not influence yield and at the same time it reduced parasitism. SA or IAA seed pre-treatment alone or combined with foliar application were more efficient in Zaaria plants than foliar spray. Similar results were observed in pots but with high effectiveness of treatments. This difference in treatment efficiency between field and pot assays may be due to several factors, such as environmental influence, inoculum density and plant growth. For the resistant genotype Bakria, SA foliar application was the most efficient treatment on controlling *O. crenata* infestation in the field with a decrease of 63.7% compared to untreated plans. Such a positive effect was reflected in increased seed yield. These results confirmed the important role of the timing of the first treatment and the number of treatments applied. In previous studies, Mbasani-Mansi *et al.* (2019) reported that the Zaaria genotype is characterized by a high susceptibility to *O. crenata* and the possibility of an early attack. This could explain the selection of SA seed pre-treatments combined with foliar application as a suitable control method. Thus, the parasitism process started when the lentil seed has a reserve of the signaling molecule (SA) accumulated during its soaking in SA solutions which can trigger the defense mechanisms. The same study reported that the Bakria genotype is characterized by a partial resistance to *O. crenata* expressed by a weak

and late attack. This may explain the effectiveness of the foliar spray method that coincides with the attack. On the other hand, these results confirm the systemic effect of the two tested hormones. The treatment was applied at a foliar level and then the effect was seen on the roots by reducing the parasite attachment and development. This confirms the systemic translocation of the induction signal from the aerial part to the roots. Translocation of the defense induction signal have also been reported by Sillero *et al.* (2012) in faba bean infested by *O. crenata* after spraying with SA.

Conclusions

The present study confirmed that the harmful influence of *O. crenata* infestation on lentil could be attenuated by the application of hormones such as, salicylic acid and indole acetic acid. The use of these two hormones has a qualitative advantage that distinguishes it from herbicides of chemical origin. Thus, SA and IAA work to improve vegetative characteristics of lentil as well as enhancing its ability to enhance resistance to *O. crenata*, in contrast to the chemical pesticide glyphosate that has a negative effect on the host under certain conditions and on the environment. The effectiveness of hormonal treatment depends on several factors including the genotype, treatment methods, number, dose and time of application. The adjustment of doses and treatment methods, depending on the genotype, could make this approach an efficient way to improve lentil plant growth and resistance to *O. crenata*.

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