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Effect of salicylic acid on growth, nodulation and N_2-fixation in water stressed chickpeas using ^{15}N and ^{13}C

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Key words: chickpea, N_2-fixation, salicylic acid, water stress, ^{15}N, $\Delta^{13}C$.

Abstract: A pot experiment was conducted to determine the impact of foliar spraying of salicylic acid (SA) on dry matter (DM), carbon isotope discrimination ($\Delta^{13}C$), nitrogen uptake (NY) and N_2-fixation (using ^{15}N) by chickpea plants subjected to three soil moisture regimes (high stress FC1, mild stress FC2 and well-watered FC3). Water stress drastically affected nodulation, DM, NY, N_2-fixation. However, plants responded positively to SA as a means of enhancing growth and overcoming the stress conditions, particularly under FC2 where the measured growth criteria (DM and NY) were relatively similar to those of the FC3. Salicylic acid significantly enhanced amounts of fixed N_2 by 32, 30 and 19% in FC1, FC2 and FC3, respectively. Water stress caused a decrease in $\Delta^{13}C$ values. However, SA increased $\Delta^{13}C$ in water stress treatments, implying that a maximization of DM may occur via an enhancement of CO_2 uptake due to stomatal opening and carboxylation activity. In conclusion, the beneficial effect of SA in enhancing plant performance (growth, N-uptake and N_2-fixation) was affected by soil water content. SA application may be considered an important agricultural practice for the better symbiotic performance in water stressed as well as in well watered chickpeas plants.

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All relevant data are within the paper and its Supporting Information files.

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1. Introduction

Chickpea (*Cicer arietinum* L.) is an important pulse legume crop widely grown across the Mediterranean basin where water availability is probably the most limiting factor for crop quality and productivity, comprising economical output and human food supply (Kurdali, 1996; Krishnamurthy *et al*., 2013). Water deficit is a multidimensional stress affecting plants at morpho-physiological, biochemical and molecular levels including inhibition of growth, accumulation of compatible organic solutes, changes in phytohormones endogenous contents, modifications in expression of stress responsive-genes among others (Vasanthaiah and Kambiranda, 2011). In the semi-arid areas of the Mediterranean region, this grain legume is cultivated on a large scale under rain-fed conditions, where water stress occurring during the post-flowering period is considered the major limiting abiotic stress, reducing growth and N_2-fixation (Kurdali, 1996; Kurdali *et al.,* 2002). Thus, increasing N_2-fixation is considered of a great importance to improve yield and performance of chikpea in the the agricultural systems. Increasing the efficiency of legumes to fix N_2 may be addressed by several approaches including selection of the best plant-microbial combinations and appropriate agricultural practices and managements (Hardarson, 1993; Kurdali *et al.*, 2005). A better plant nutrition (e.g., K, P, Si, etc.) can effectively alleviate the adverse effects of drought (Waraich *et al.*, 2011) and hence enhance N_2-fixation (Kurdali and Al-Shammaa, 2010; Kurdali *et al.,* 2013).

Salicylic acid (SA) is one of the endogenous growth regulators that are involved in a range of physiological and metabolic responses in plants (Hayat *et al.*, 2010). It coordinates growth and development with plant responses to the environment in a complex signal-transduction network (Aimar *et al.,* 2011). In recent years, SA has been the focus of intensive research due to its function as an endogenous signal mediating local and systemic plant defense responses against pathogens (Rivas-San Vicente and Plasencia, 2011). Moreover, it has, also, been reported that SA is a potential tool in reducing or alleviating the adverse effects of abiotic stress in plants (Khan *et al.*, 2015; Amirinejad *et al.,* 2017). Exogenous application of SA has been shown to be beneficial for plants either in optimal or stress environments. Salicylic acid is involved in regulatating various plant metabolic processes and modulating the production of varied osmolytes and secondary metabolites, as well as maintaining plant-nutrient status. Hence, it protects plants under abiotic stress conditions (Khan *et al*., 2015). The effectiveness of SA in inducing stress tolerance depends upon plant species, method of addition, time of application and the concentration (Hayat *et al*., 2010; Gharbi *et al*., 2018). Low concentrations of exogenous SA provides tolerance against damaging effects of stresses on plants, whereas, higher concentrations of SA did not show the same effects (Senaratna *et al*., 2000). Recent evidence highlighted the importance of SA as a regulator of photosynthesis due to its effect on stomatal conductance and the activity of enzymes such as RuBisCO and carbonic anhydrase (Rivas-San Vicente and Plasencia, 2011). Extensive studies have been conducted on the effect of SA application on physiological and biochemical parameters (e.g., gas exchange, stomatal conductance, chlorophyll, photosynthetic rate measurements and enzyme activity estimations) in relation to abiotic stresses (Khan *et al.,* 2003; Khodary, 2004; Hayat *et al.,* 2010; Ghasemzadeh and Jaafar, 2013; Lee *et al*., 2014; Miura and Tada, 2014). During photosynthesis, C3 plants discriminate against the heavy isotope of carbon (^{13}C) leading to a depletion of the plant dry matter in ^{13}C. Carbon isotope discrimination ($\Delta^{13}C$) positively correlates with C_i/C_a (i.e., the ratio of internal leaf CO_2 concentration to ambient CO_2 concentration) and thus provides an integrated measurement of the photosynthesis efficiency in response to environmental conditions prevailing during the plant growth cycle (Farquhar *et al*., 1989). $\Delta^{13}C$ has been intensively studied as a selection criterion for drought tolerance in several C3 species (Farquhar *et al*., 1989). Water stress can alter $\Delta^{13}C$ as a result of its effects on the balance between stomatal conductance and carboxilation (i.e. RuBisCO), (Farquhar *et al.,* 1989). Because of the correlation between $\Delta^{13}C$ and gas exchange values (i.e., C_i/C_a), the isotopic methods represent an alternative to gas exchange measurements. (Farquhar *et al.,* 1989). Carbon isotope discrimination was also used for studying the impact of agricultural practices including fertilizer applications such as nitrogen (Iqbal *et al.,* 2005), potassium (Kurdali and Al-Shammaa, 2010) and silicon (Kurdali *et al*., 2013) on crop performance enhancements under water stress conditions. Accordingly, we hypothesize that $\Delta^{13}C$ can be affected by SA application. Available literature on the relationships between SA and carbon isotope discrimination is very scarce and, to our knowledge, only in one case, the effect of SA on plant water relationships was studied (Barkosky and Einhellig, 1993). On the other hand, the ^{15}N isotope dilution is one amongst several available methods to quantify plant-associated N_2-fixation and provides a valuable mean for evaluating factors affecting N_2-fixation such as drought (Kurdali *et al*., 2002) and salinity (Kurdali and Al-Ain, 2002). Therefore, the objective of this study was to determine the effect of SA on the performance of chickpea plants (growth, nitrogen uptake and N_2-fixation) grown under various soil moisture levels using stable isotopes (i.e., ^{15}N isotopic dilution and ^{13}C isotope discrimination).

2. Materials and Methods

Soil properties and plant materials

The experiment was conducted in pots, each one containing 5 Kg of thoroughly mixed soil collected from Deir AL-Hajar agricultural experiment station, located south east of Damascus, Syria (36° 28'E, 33° 21' N; altitude 617 m). Some climatic data of the experimental site during the growing period is shown in Table 1. The main physical and chemical soil properties were: pH 7.80, EC_e 0.31 dSm^{-1}, Soil bulk density was 1.20 g cm^{-3}, organic matter 1.25 per cent, cations (Ca^{++} 2.25, Mg^{++} 0.97, K^+ 0.14 and Na^+ 1.27 mmol L^{-1}), anions (SO_4^{--} 1.27, HCO_3^- 1.07 and Cl^- 0.55 mmol L^{-1}), available P (Olsen) 13.40 mg g^{-1}; total N 0.12 per cent, NO_3^- 33.6 mg g^{-1}, NH_4^+ 28.1 mg g^{-1}. The soil is classified as a clay loam, with an average 57.89% clay, 39.47% silt, and 2.63% sand.

Seeds of chickpea (*Cicer arietinum* L.), and barley as a non-fixing plant were sown. After germination, plants were thinned to two plants per pot. The pots were set outdoors under natural climatic conditions. All pots were protected from rainfall by manually operated shelter equipped with movable sheet of transparent flexible plastic. Since abundant nodules had already been observed on the roots of chickpea plants grown in the area, from which the soil was collected and used for this experiment, the seeds were not inoculated.

Table 1 - Some climatic data during the growing season of the experimental site

Variable	February	March	April	May
Minimum temperature (°C)	4.9	7.7	11.5	14.8
Maximum temperature (°C)	19.5	21.2	29.2	30.5
Relative air humidity (%)	74	67	59	58
ET0 (mm day^{-1})	2.7	3.2	6.3	8.4

Experiment design and treatments

The pots were arranged in a split plot design, with salicylic acid treatments (SA) being the main plots and the irrigation regimes are the sub-main. Salicylic acid $C_6H_4(OH).COOH$ had the following specification: assay min. 99%, melting point 157-162°C, maximum limits of Impurities: chloride 0.01%, sulphate 0.03%, iron 0.002%, heavy metals 0.001%.

Two SA treatments were used: (SA^-, control without SA and SA^+,10^{-5}mol L^{-1}). Within each of the SA treatments, three irrigation regimes, expressed as percent of field capacity(FC), were applied (FC1, high stress 45-50%; FC2, mild stress 55-60% and FC3, well-watering 75-80%). All treatments were replicated four times.

Soil water content in all pots was maintained at around 75% of field capacity from planting up to bud flower initiation (5 weeks after planting). Thereafter, plants were subjected to the above-mentioned soil moisture regimes. Foliar spraying of the plants with salicylic acid (SA) was initiated at the same time of applying water regimes and performed 6 times at 10 days intervals. For non-SA treated chickpeas, plants were sprayed with distilled water set as control (SA^-). Pots were weighed every three days, and water was added to maintain the soil moisture regimes as previously described. The pots were kept weed-free and any drainage was prevented.

^{15}N-Application

An equivalent rate of 25 kg N ha^{-1} of ^{15}N labeled urea (5% ^{15}N atom excess) was applied to chickpea and barley plants to estimate the fractional contribution of nitrogen derived from air (Ndfa, i.e N_2-fixation), soil (Ndfs) and from fertilizer (Ndff), using the isotopic dilution method (Fried and Middelboe 1977). Two equally split applications of N fertilizer (12.5 kg N ha^{-1} for each application) were applied at 2-week intervals starting from complete seedling emergence. This procedure was followed to stabilize the ^{15}N enrichment of the N pool and to minimize N immobilization. Barley was used as a non-fixing reference crop for estimating the N fraction derived from the atmosphere (%Ndfa) in chikpeas, and was similarly treated with the above mentioned treatments (i.e., both of watering regimes and SA application).

Plant sampling and isotopic composition analysis

Plants were harvested twelve weeks after planting. Shoots and nodules were dried at 70°C for 72 h, weighed for dry matter determinations. Shoots were then ground to a fine powder. Total nitrogen was determined by Kjeldahl procedure, and $^{15}N/^{14}N$ isotope ratio was measured using an emission spectrometry (Jasco-150, Japan). The nitrogen fraction derived from the atmosphere (%Ndfa) was calculated using the equation of Fried and Middelboe (1977):

$$\%Ndfa = \left(1 - \frac{atom\ \%\ ^{15}N\ excess_{\ Chikpea}}{atom\ \%\ ^{15}N\ excess_{\ Barley}}\right) \times 100$$

The percent N derived from fertilizer (%Ndff) was calculated using the following equations:

$$\%Ndff = \frac{atom\ \%\ ^{15}N\ excess_{\ plant}}{atom\ \%\ ^{15}N\ excess_{\ fertilizer}} \times 100$$

The percent N derived from soil (%Ndfs) was calculated as follows:

$$\%Ndfs = 100 - (\%Ndfa + \%Ndff)$$

Amounts of nitrogen (mg N $plant^{-1}$) derived from N_2-fixation (Ndfa), soil (Ndfs) and from fertilizer (Ndff) were calculate by multiplying the fractional contribution of each source (%) by nitrogen yield.

The $^{13}C/^{12}C$ ratio ($\delta^{13}C$‰) was determined on subsample of shoots using the continuous-flow isotope ratio mass spectrometry (Integra-CN, PDZ Europea Scientific Instrument, UK). Carbon isotope discrimination (Δ^{13}C‰) values were estimated using the equation of Farquhar *et al.* (1982):

$$\Delta^{13}C = (\delta\ ^{13}C_{air} - \delta^{13}C_{sample})/(1 - \delta^{13}C_{sample}/1000)$$

where $\delta^{13}C_{air}$ is the δ^{13}C value in air (-8‰) and $\delta^{13}C_{sample}$ is the measured value in the plant.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) test, and means were compared using the Least Significant Difference (Fisher's PLSD) test at the 0.05 level of probability ($P<0.05$). Moreover, correlation coefficients (r) between Δ^{13}C and the studied parameters (e.g., DM, NY, per cents & amounts of Ndfa, Ndff and Ndfs) were estimated.

3. Results

Effect of salicylic acid on dry matter and nitrogen yield

Dry matter yield (DM) of chikpea plants was significantly affected by water stress (Table 2). The lowest dry matter value (5.57 g pot^{-1}) was observed in non-SA treated plants grown under the highest water stress level (FC1). Increasing soil moisture from FC1 to FC2, from FC1 to FC3, and from FC2 toFC3 resulted in significant increases in DM by 40%, 64%, and 17%, respectively. The foliar spray of SA had produced appreciable results by increasing dry matter yield under stress conditions and the effect was more pronounced in plants subjected to a mild water stress. At each irrigation regime, the SA increased DM by 13%, 17%, and 6% in FC1, FC2, and FC3, respectively. It is worth mentioning, also, that DM in SA-treated plants grown under moderate water stress (FC2, 9.06 g pot^{-1}) was relatively similar to that of well-watered plants without SA treatment (FC3, 9.12 g pot^{-1}). However, DM of well-plants (FC3) was not significantly enhanced by SA as compared to non-treated SA plants.

The pattern of total nitrogen yield (NY) was relatively similar to that of dry matter yield (Table 2). Water stress significantly reduced the NY and the highest reduction was recorded under the highest stress level (FC1). In non-SA treated plants, the lowest value of NY (129 mg N pot^{-1}) was in FC1. Increasing soil moisture from FC1to FC2, from FC 1to FC3 and from FC2 to FC3 resulted in significant increases in NY by 38%, 58%, and 15%, respectively. The exogenous supply of SA significantly enhanced nitrogen accumulation by 14%, 20%, and 10% in FC1, FC2, and FC3, respectively. The highest amount of TN was observed in well-watered plants (FC3) treated with SA application (223.4 mg N pot^{-1}) representing a 73% increment over the control (i.e., non-treated SA plants in FC1). It is evident from DM and NY data that water stress adversely affected the production of dry matter and nitrogen yield of chikpeas, and exogenous application of SA at 5 mM L^{-1} was successful in alleviating the adverse effect of water stress.

Effect of salicylic acid on nodule dry matter

Effects of SA on nodule dry weight of chickpea plants grown under different water stress conditions are given in figure 1. The lowest nodule dry weight was noted in the highest water stressed plants (FC1). For the non-SA treated plants, increasing soil moisture from FC1 to FC2, from FC1 to FC3, and from FC2 to FC3, resulted in increases in nodule dry weight by 31%, 62%, and 24%, respectively. The exogenous supply of SA significantly enhanced nitrogen accumu-

Table 2 - Total dry matter yield (g pot^{-1}) and nitrogen yield (mg N pot^{-1}) of chickpea plants grown under different water regimes as affected by salicylic acid (SA)

Salicylic acid	Irrigation treatments			
	FC1	FC2	FC3	LSD $_{0.05}$
DM (g pot^{-1})				
SA^{-}	5.57±0.12 B c	7.77±0.41 B b	9.12±0.31 A a	0.48
SA^{+}	6.29±0.41 A b	9.06±0.47 A a	9.68±0.40 A a	0.68
LSD $_{0.05}$	0.52	0.76	0.62	
N-uptake (mg N pot^{-1})				
SA^{-}	129.06±4.68 B c	177.45±2.95 B b	203.33±10.53 B a	10.98
SA^{+}	146.78±5.11 A c	213.09±4.51 A b	223.41±4.17 A a	7.37
LSD $_{0.05}$	8.47	6.59	13.85	

Means ±SD within a column (capital letter) and within a row (small letter) followed by the same letter are not significantly different ($P<0.05$). FC: water regimes, expressed as % of field capacity (FC1, high stress 45-50%; FC2, mild stress 55–60% and FC3, well-watering 75–80%). SA-: control without SA, SA+ 10-5 Mol l^{-1}.

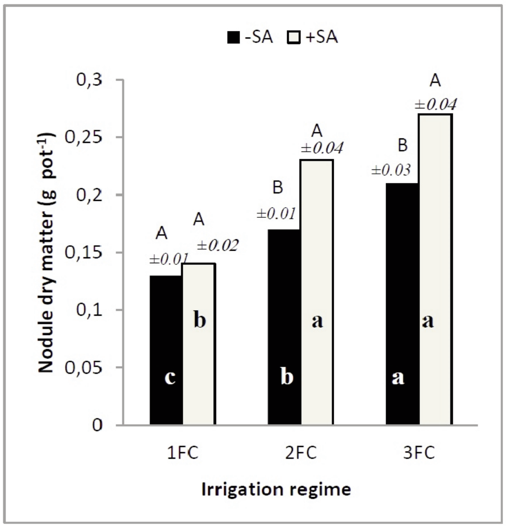

Fig. 1 - Nodule dry matter (g pot^{-1}) of chickpea plants grown under different water regimes as affected by salicylic acid (SA). Columns followed by the same letter are not significantly different (P<0.05); Capital letters (effect of SA in each irrigation regime); Small letters within a row (comparison among irrigation regimes either for SA+ or SA-).

lation by 41% in FC2 and 29% in FC3 watering treatments. However, the enhancement of nodule dry weight in FC1 by SA was not significant (8%). For the SA treated plants, increasing soil moisture from FC1 to FC2, from FC1 to FC3, and from FC2 to FC3, resulted in increases in nodules dry weight by 71%, 93%, and 13%, respectively. These results illustrate the importance of foliar spray of SA in enhancing nodules dry weight of chickpea grown under the different water regimes.

Effect of salicylic acid on nitrogen uptake from the available sources

Nitrogen derived from fertilizer (Ndff), soil (Ndfs) and atmosphere (Ndfa, i.e., N_2-fixation) in chickpeas grown under various watering regimes as affected by SA application are given in Table 3. Regardless of SA application, the proportions of Ndff and Ndfs (%) in chickpea plants significantly decreased as soil field capacity increased. However, the opposite was true regarding the %Ndfa which showed a higher value under optimal irrigation conditions (FC3) compared to water stressed treatments (FC1 and FC2). For the non-SA treated plants, the observed values of %Ndfa were 33, 40, and 43.8% in FC1, FC2, and FC3, respectively. Chickpea plants significantly enhanced their nitrogen fixation (i.e., 39, 43.3 and 47.3% in FC1, FC2, and FC3, respectively) in response to SA application

Table 3 - Proportions (%) and amounts (mg N pot^{-1}) of nitrogen derived from fertilizer (Ndff), soil (Ndfs) and atmosphere, i.e. N_2-fixation (Ndfa) in chickpea plants grown under different water regimes as affected by salicylic acid (SA)

Salicylic acid	Irrigation treatments			$LSD_{0.05}$
	FC1	FC2	FC3	
%Ndff				
SA-	12.62±0.22 A a	11.40±0.18 A b	10.69±0.27 A c	*0.36*
SA+	11.59±0.08 B a	10.77±0.21 B b	10.02±0.19 B c	*0.27*
$LSD_{0.05}$	*0.29*	*0.34*	*0.40*	
Ndff (mg N pot^{-1})				
SA-	16.30±0.74 A b	20.22±0.24 B a	21.73±1.45 A a	*1.52*
SA+	17.01±0.67 A b	22.96±0.84 A a	22.38±0.82 A a	*1.25*
$LSD_{0.05}$	*1.22*	*1.07*	*2.04*	
%Ndfs				
SA-	53.83±0.94 A a	48.60±0.77 A b	45.55±1.14 A c	*1.54*
SA+	49.42±0.36 B a	45.94±0.90 B b	42.71±0.82 B c	*1.17*
LSD 0.05	*1.24*	*1.45*	*1.72*	
Ndfs (mg N pot^{-1})				
SA-	69.48±3.1 A b	86.22±1.0 B a	92.66±6.2 A a	*6.49*
SA+	72.54±2.9 A b	97.91±3.6 A a	95.44±3.5 A a	*5.31*
LSD 0.05	*5.18*	*4.56*	*8.71*	
%Ndfa				
SA-	33.55±1.2 B c	40.01±0.8 B b	43.76±1.4 B a	*1.90*
SA+	39.00±0.5 A c	43.29±1.1 A b	47.28±1.0 A a	*1.45*
$LSD_{0.05}$	*1.53*	*1.79*	*2.13*	
Ndfa (mg N pot^{-1})				
SA-	43.29±1.8 B c	71.01±2.6 B b	88.94±4.2 B a	*4.92*
SA+	57.22±1.7 A c	92.23±1.9 A b	105.6±1.2 A a	*2.58*
$LSD_{0.05}$	*3.06*	*3.97*	*5.38*	

Means ±SD within a column (capital letter) and within a row (small letter) followed by the same letter are not significantly different (P<0.05). FC: water regimes, expressed as % of field capacity (FC1, high stress 45-50%; FC2, mild stress 55-60% and FC3, well-watering 75–80%). SA-: control without SA, SA+ 10-5 Mol l^{-1}.

in all watering regimes. Inversely, both %Ndff and %Ndfs values were decreased by SA treatments. However, amounts of Ndff and Ndfs (mg) increased due to the foliar application of SA in all watering regimes. Likewise, the exogenous supply of SA significantly enhanced the amounts of fixed N_2 by 32%, 30%, and 19% in FC1, FC2, and FC3, respectively. Regardless of SA application, it can be noticed that the amount of fixed N_2 in the well-watered plants (FC3) was almost doubled as compared to those subjected to high stress (FC1). It is, also, worth mentioning that amount of Ndfa in SA-treated plants grown under moderate water stress (FC2) was close or even higher than that of well-watered (FC3) chickpea without SA. These results may illustrate the importance of SA in saving irrigation water and alleviating water stress influences to ensure appropriate yield and N_2-fixation.

Effect of salicylic acid on carbon isotope discrimination

Effect of SA on carbon isotope discrimination (‰$\Delta^{13}C$) in shoots of chickpea plants subjected to different water stress conditions is given in figure 2. Water stress caused a considerable decrease in $\Delta^{13}C$ compared to well irrigated conditions (Fig. 2). For the non-SA treated plants, the mean $\Delta^{13}C$ value in well-watered chickpea (FC3) was 21.29‰, while it significantly decreased to 20.41‰ and 19.88‰ in mild (FC2) and high water stressed (FC1) plants, respectively. The exogenous application of SA increased $\Delta^{13}C$ particularly in high and mild water stressed plants. The lowest $\Delta^{13}C$ value was obtained in FC1 (20.43‰) and increased to 21.34‰ in FC2 and 21.50‰ in FC3, with no significant difference being obtained between the latter two values. On the other hand, correlations between $\Delta^{13}C$ and the studied parameters showed positive relationships with DM (0.953**), NY (0.949**), nodule dry weight (0.92*), %Ndfa (0.948**), and amounts of Ndfa, Ndfs and Ndff (0.957**, 0.91* and 0.91*, respectively). However, negative relationships between $\Delta^{13}C$ and %Ndff and %Ndfs (-0.948**) were observed.

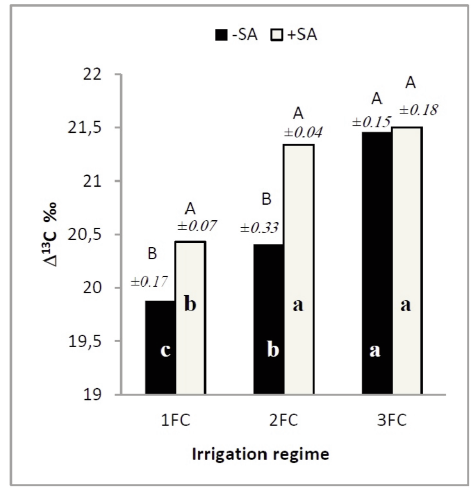

Fig. 2 - Carbon isotope discrimination (Δ13C) in chickpea plants grown under different water regimes as affected by salicylic acid (SA). Columns followed by the same letter are not significantly different (P<0.05); Capital letters (effect of SA in each irrigation regime); Small letters within a row (comparison among irrigation regimes either for SA+ or SA-).

4. Discussion and Conclusions

The results of this study showed that water stress occurring during the flowering period of chickpea plants considerably affected their growth and N_2-fixation. Total dry weight and nitrogen yield decreased significantly in plants as field capacity decreased. Salicylic acid-treated plants exhibited an increase in tolerance to water stress, particularly under mild-stress conditions where the measured growth criteria (i.e., dry matter and nitrogen yield) were relatively similar to those of the non-stressed plants regardless of whether the plants treated with or without SA applications. Gutierrez-Coronado *et al.* (1998) reported a similar increase in the growth of soybean plants in response to salicylic acid treatment. Afshari *et al*. (2013), also, indicated that SA increased the growth and physiological attributes of cowpea under water stress. Moreover, foliar application of SA in drought stressed chickpeas significantly enhanced plant biomass through increasing proline content of leaves (Farjam *et al.,* 2014). Correspondingly, the beneficial role of SA to adverse effects of other abiotic stresses (e.g., heavy metals, salinity and cold..) has been examined in various legumes such as chickpea (Hayat *et al.,* 2014), medic (Palma *et al.,* 2013), lentil (Misra and Saxena, 2009), and common bean (Torquato de Agostini *et al*., 2013).

Water stress is known to decrease nodulation by affecting the establishment of the symbiosis between the host and rhizobia, thereby decreasing the number and the mass of nodules and reducing N_2-fixation (Kurdali *et al.,* 2002). The undertaken study showed that nodulation and N_2-fixation were reduced as soil water content decreased. Such reductions could be attributed to the limitation of photosynthesis and the decrease of photosynthates supply to nodules (Kurdali, 1996). Other physiological factors such as O_2 availability in nodules could be involved with the decline of N_2- fixation. Guérin *et al*. (1991) suggested that O_2 supply to bacteroids, in water stressed nodules, is restricted either by a limitation of diffusion or by a degradation of leghaemoglobin, and therefore hinders respiration and ATP production, with the final result of reduction of N_2-fixation (Patterson and Hudak, 1996). However, application of SA is found to be beneficial for nodulation and N_2-fixation in plants either grown under optimal or water stress conditions. Several factors are well known to be involved in nodulation and N_2-fixation of grain legumes including the occurrence of effective rhizobium strains, the availability of photosynthesis, nitrogenas activity and its protection against O_2 via increasing of leghemoglobin contents, and the effectiveness of enzymes mediating the export of fixed nitrogen from nodules to plant (e,g., GS, GOGAT, and GDH).

The occurrence of N_2-fixation under various soil water regimes could be explained by the presence of effective indigenous rhizobia strains. On the other hand, it is well known that the high dry matter yield of a given plant species may imply a higher photosynthetic rate (Kurdali and Al-Shammaa, 2010). In a study carried out on soybean, foliar application of salicylic acid enhanced the water use efficiency (WUE) and photosynthetic rate (Kumar *et al*., 2000). Also, Hayat *et al.* (2010, 2012) reported that the exogenous application of SA to chickpea increased the net photosynthetic rate, favoring the production of photosynthates and the nodules obtained a significant proportion of the carbohydrates. The development of healthy nodules in SA-treated plants, as expressed in terms of an increase in nodule dry matter (Fig. 1), is expected to be accompanied by leghemoglobin content increment and consequently increased nitrogen fixation via stimulation of nitrogenase activity (Hayat *et al*., 2012). Moreover, higher leghemoglobin content in SA-treated plants (Balestrasse *et al*., 2004; Hayat *et al*., 2014) might be a consequence of the decrease generation of ROS. Thereby preventing oxidative damage to plants in general and nodules in particular, for cadmium (Cd) stressed chickpea (Hayat *et al.*, 2014) and soybean plants (Balestrasse *et al*., 2004). In this context, Palma *et al.* (2013) reported that SA alleviated the negative effect of salt stress in the *Medicago sativa* - *Sinorhizobium meliloti* symbiosis through the increased level of nodule biomass and the induction of the nodular antioxidant metabolism under salt stress. Similarly, Misra and Saxena (2009) showed that SA could be used as a potential growth regulator to improve salinity tolerance of lentil plants by enhancing proline metabolizing system.

Hayat *et al*. (2012, 2014) reported that, the exogenous application of SA to chickpea plants enhanced the activities of the enzymes involved in nitrogen fixation and assimilation (e,g., NR, GS, GOGAT, GDH and nitrogenase) regardless of whether the plants are grown in the presence or absence of cadmium. On the light of the aforementioned studies, it can be suggested that the beneficial effect of SA on N_2-fixation in chickpea plants might be resulted from enhancing nodulation, leghemoglobin contents and the activity of enzymes involved in nitrogen fixation and assimilation.

In a review paper, Hayat *et al.* (2010) reported that, during the early stages of nodulation, exogenous SA inhibited the growth of Rhizobia and the production of Nod factors by them and also delayed the nodule formation, particularly in plants producing indeterminate nodules, thereby decreasing the number of nodules per plant (Van-Spronsen *et al*., 2003; Mabood and Smith, 2007). In this study, however, since SA was supplied prior to flowering stage of chickpeas (i.e. indeterminate nodule type), it is most likely that nodule formation was established. Therefore, the further benefits of SA were most probably resulted from nodule growth development (DM) and functioning (N_2-fixation).

Carbon isotope discrimination values ($\Delta^{13}C$) in chickpea's shoots were affected by soil water content and SA applications. Water stress significantly decreased $\Delta^{13}C$ values as field capacity decreased. However, the exogenous application of SA increased $\Delta^{13}C$ particularly in high and mild water stressed plants. It has been reported that $\Delta^{13}C$ can reflect the integrated response of physiological processes to environment. Water stress can alter $\Delta^{13}C$ as a result of effects on the balance between stomatal conductance and carboxilation (Farquhar *et al.*, 1989). The lower $\Delta^{13}C$ value in the stressed plants compared to the non-stressed plants implies that C_i/C_a ratios were lower under stress. A lower C_i/C_a ratio could result either from stomatal closure induced by stress or from higher rates of photosynthetic capacity or a combination of both (Condon *et al.*, 2002; Kurdali *et al.*, 2013). Because of the lower dry matter yield in the water stressed plants (FC1-SA$^-$) compared with FC2-SA$^-$ or with FC3-SA$^-$, it was unlikely that a higher photosynthetic capacity occurred in FC1. Therefore, the lower $\Delta^{13}C$ value in the high and mild water stressed chickpeas compared to well-watered plants (i.e. SA$^-$)resulted mainly from stomatal closure induced by stress. The principal components of photosynthesis that influence discrimination are diffusion of CO_2 through stomata and the carboxylation process mediated by Rubisco (O'Leary, 1988). In SA treated plants, the higher ‰$\Delta^{13}C$ value in high and mild water stressed plants comparing with non-SA treated plants were associated with higher dry matter yield (i.e. higher photosynthetic activity). Therefore, the higher $\Delta^{13}C$ following SA applications imply that a maximization of yield may occur via a maximization of CO_2 uptake activity due to stomatal opening (Condon *et al.*, 1987). Janda *et al.* (2014) concluded that the effect of SA on the photosynthetic machinery is indirect, originating from its influence on stomatal conductivity. Moreover, it has been reported that the increases in photosynthetic rates of soybean (Khan *et al.*, 2003) and ginger (Ghasemzadeh and Jaafar, 2013) plants following SA applications were the result of increased CO_2 uptake activity at the chloroplast level (i.e. Rubisco activity), rather than simple increase in stomatal opening, i.e., reduced the resistance to entry CO_2 in the leaves. In stressed plants, Lee *et al.* (2014) reported that the content of rubisco was increased by SA in tobacco plants treated with NaCl. Likewise, Khodary (2004) concluded that SA treatment of salt stressed maize could stimulate their salt tolerance via accelerating their photosynthesis performance (i.e. Rubisco activity) and carbohydrate metabolism. Recently, Gharbi *et al*. (2018) reported that combination of applied SA as a priming agent or concomitantly with NaCl were required to maintain a good water use efficiency in salt-treated tomato plants using carbon isotope discrimination. According to Farquhar *et al.* (1982), a higher $\Delta^{13}C$ values is caused by a higher C_i/C_a ratio due to higher stomatal conductance which leads to higher photosynthetic rate and hence higher biomass, (i.e, positive correlation between $\Delta^{13}C$ and DM, $r=0.95^{**}$). Accordingly, it can be suggested that the beneficial effect of SA in enhancing dry matter yield of high and mild water stressed chickpeas could be resulted from higher CO_2 uptake activity by Rubisco (i.e., photosynthetic activity) in addition to higher CO_2 uptake at stomatal level (i.e., higher C_i). On the other hand, in FC3, no significant effects of SA were obtained neither on $\Delta^{13}C$ nor on dry matter yield, indicating that the field soil capacity (75-80%) is an optimal watering regime to ensure a good biomass production. Consequently, it can be concluded that SA application can alter $\Delta^{13}C$in water stressed plants (e.g., FC1 and FC2) as a result of effects on the balance between stomatal conductance and carboxylation.

In addition to the positive relationship between $\Delta^{13}C$ and dry matter yield of chickpeas, higher $\Delta^{13}C$ values were also associated with enhancements of %Ndfa (0.948**), amount of N_2-fixed (0.957**) and nodule dry matter yield (0.92*). Hence, it can be suggested that carbon isotope discrimination could be used as an indicator for nodulation and N_2-fixation efficiency. This observation is in harmony with that of Knight *et al.* (1993) who reported a positive correlation between $\Delta^{13}C$ and the amount of N_2-fixed in lentil inoculated with different strains of rhizobia, and with results of Kurdali and Al-Shammaa (2010) in potassium fed lentil grown under water stress conditions.

Salicylic acid can be involved in the regulation of uptake of several plant-beneficial elements (Khan *et al.*, 2015). Exogenously supplied SA can improve plant growth under stresses by stimulating the accumulation of mineral elements including nitrogen (Gunes *et al.*, 2005; Yildirim *et al.*, 2008). In this study, exogenously applied SA to mild water-stressed chickpea resulted in increments of soil nitrogen uptake (Ndfs) as well as amount of N derived from fertilizer (Ndff) and its use efficiency (%NUE). Such increments may support the idea that low SA concentrations (10^{-5} M) can induce nitrite reductase synthesis, which plays a key role in nitrogen metabolism, by mobilizing intracellular NO^{3-} and can provide protection to nitrite reductase degradation (Ghasemzadeh and Jaafar, 2013). Consequently, our finding indicates that the application of SA is beneficial to get improvement in nitrogen uptake and metabolism in plants grown under stressed conditions. Such improvements enhance the protein/enzyme/growth hormone synthesis, developing the metabolic activity and resulting in increasingthe growth and plant productivity (Akhtar *et al.,* 2013). On the other hand, the positive correlations between $\Delta^{13}C$ and total N uptake, amounts of Ndfs or Ndff may illustrate a relationship between photosynthesis and nitrogen metabolism. In addition, the negative relationship between $\Delta^{13}C$ with %Ndfs or %Ndff (0.91^{*}) is also reported by Iqbal *et al.* (2005) in wheat plants grown under rain-fed conditions implying that $\Delta^{13}C$ could be used to predict these parameters. Consequently, it can be suggested that carbon isotope discrimination could be used as an indicator for nitrogen uptake from the available sources. A priority for future research should be elucidation of relationships between carbon isotope discrimination and nutrient use efficiency in various plant species and genotypes, and how these vary in response to environmental conditions and agricultural practices.

The ability of SA to increase chickpea growth, nitrogen uptake and N_2- fixation, ameliorating the adverse effect of water stress, may have significant implications in improving the plant performance and overcoming the growth barrier arising from conditions of limited water availability. A simplified scheme representing the beneficial effects of exogenous application of salicylic acid on growth and N_2-fixation as reported in this study and in the literatures (i.e. Hayat *et al.,* 2010, 2012 and 2014) is shown in figure 3. Further field investigations are required to illustrate the role of salicylic acid in terms of growth, yield, and the time course of N_2 fixation of legumes grown under rain-fed conditions in the semi-arid areas**.**

To the best of our knowledge, the present study is the first report on the relationship between SA application and carbon isotope discrimination ($\Delta^{13}C$) in N_2 fixing plants (e.g., chickpea). Salicylic acid increased $\Delta^{13}C$ values in water stressed plants, implying that a maximization of dry matter yield may occur via an enhancement of CO_2 uptake due to stomatal opening and carboxylation activity. Moreover, $\Delta^{13}C$ could be used as an indicator for nitrogen uptake, nodulation and N_2 fixation.

Although application of SA is found to be beneficial for plants either in optimal or water stress conditions, its main effect in enhancing plant performance (growth and N-uptake from the available sources) was affected by soil water content as follows:

Under high water stress conditions (FC1), the main effect of SA was on plant growth, nodulation

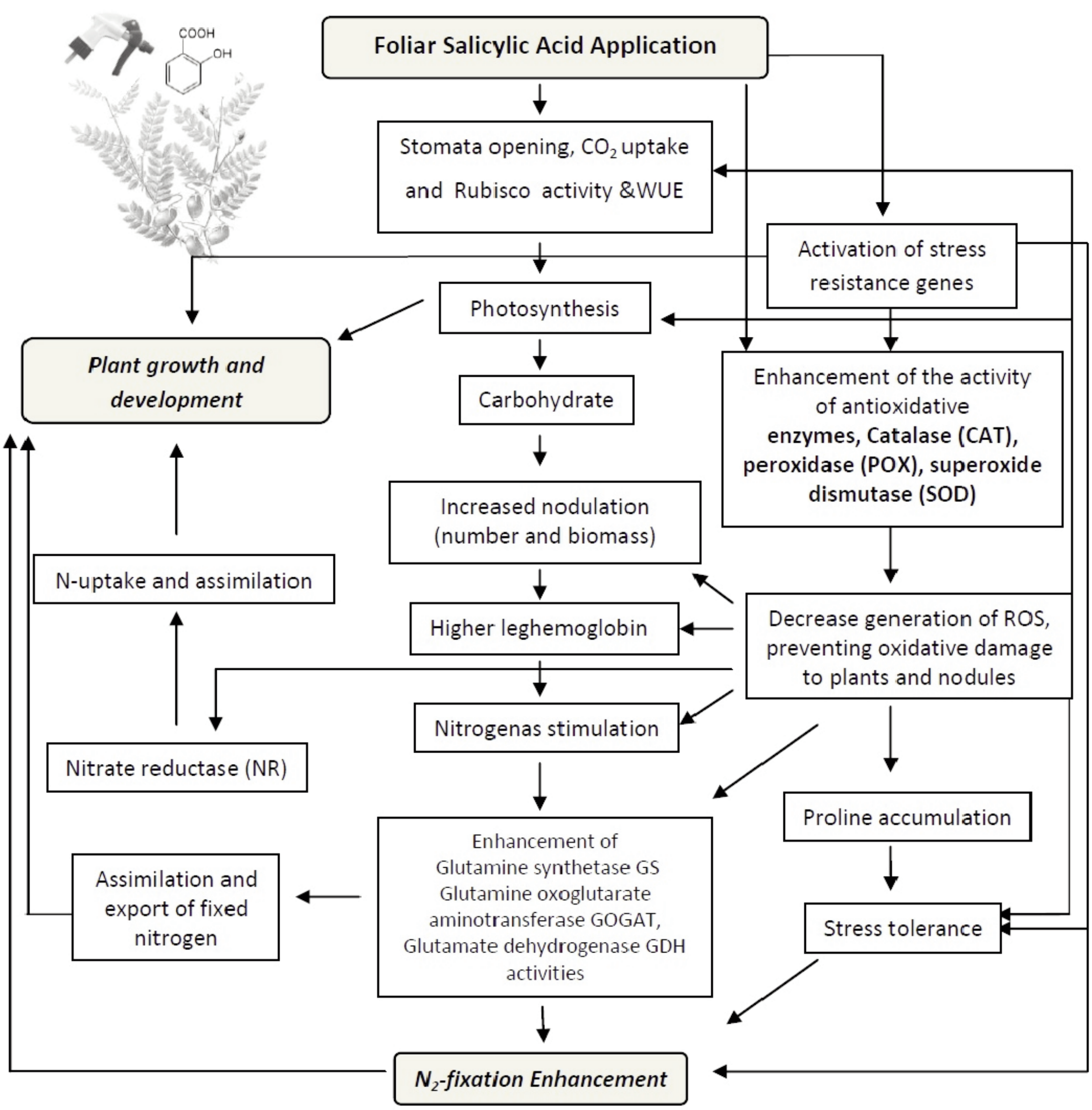

Fig. 3 - Simplified schemes representing the beneficial effects of exogenous application of salicylic acid on growth and N_2-fixation as presented in this study and in the literatures (i.e. Hayat *et al.*, 2010, 2012 and 2014).

and N_2 fixation.

Under mild water-stressed conditions (FC2), SA ameliorated plant growth, nodulation, soil and fertilizer N uptake in addition to fixed N_2.

Under optimal watering conditions (FC3), the beneficial effect of SA was on nodulation and N_2 fixation.

Overall, SA application may be considered as an important agricultural practice for the symbiotic performance (i.e., nodulation and N_2 fixation) in water stressed as well as in well-watered chickpeas plants.

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