Study of Salicylic Acid Influence on Seedling Growth and Nitrogen Metabolism in Watermelon (*Citrullus lanatus* L.)

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Abstract: Salicylic acid is involved in the regulation of metabolic activity and defense mechanism in plants under various stress conditions. Present study was conducted to determine the effects of salicylic acid (10 to 500 μM) on seedling growth, development and nitrogen use efficiency in watermelon (*Citrullus lanatus* L.) plants with or without nitrogen nutrient. Salicylic acid increased contents of chlorophyll, total non-structural carbohydrate and total nitrogen, as well as nitrate assimilation through the induction of nitrate reductase (EC 1.6.6.1) activity in isolated watermelon cotyledons. Accumulation of salicylic acid was two-fold higher in cotyledons without nitrate supply in comparison to that with nitrate supply. Further 50 μM of SA induced enhancement in seed germination and growth characteristics. However higher salicylic acid concentrations inhibited above physiological characteristics. Results show that, field application of salicylic acid need optimum physiological concentration (e.g., 50 μM) to increase nitrogen use efficiency particularly during germination and seedling growth.
Keywords: Watermelon (*Citrullus lanatus* L.); Watermelon cotyledons; Nitrate-nutrition response; Nitrate reductase activity; Salicylic acid.

1. Introduction

Phenylpropanoids are increased or it may be de-novo synthesized in response to adverse environmental conditions, which play an important role in regulation of biochemical, physiological and molecular responses in plants[1]. These include effects on nitrate (NO_3^-) assimilation, ion uptake, enzyme regulation, membrane organization, photosynthetic carbon dioxide assimilation and nutrient deficiency in plants[2,3,4,5].Levels of some compounds related to secondary metabolism show a sensitive response to nutrient deficiency in plants[6,7]. Accumulation of phenolic compounds is a symptom of nutrient-stress, while production of different classes of phenolics depends on the nature of stress[8]. Higher levels of phenolics explain diagnosis of nutrient disorders and the visual symptoms caused by nutrient deficiency in shoot culture of organo[5]. However, effect of secondary metabolites on growth and development of plants under limited availability of nitrogen (N) nutrient is not clear.

To attain optimal growth and development plants tend to maintain constant levels of essential nutrients, despite their limited availability in most soils. These limitations are usually due to low nutrient concentration or accessibility[9]. To cope with reduced nutrient availability, plants trigger physiological and developmental responses aimed to increase nutrient acquisition that, in many cases, alter the whole plant morphology and metabolism [10]. Plants use adaptive mechanisms to stimulate growth in the organs that directly participate in nutrient acquisition[11,12]. Relative availability of soil ammonium and nitrate to most plants will become increasingly important in determining their productivity as well as their quality as food[13]. This is the case of plants grown under low N-supply, which triggers proliferation of lateral roots, resulting in increased amount of surface availability for N-uptake[11]. These responses to maintain N-supply for plants may be helpful at maturity but, during germination and seedling growth morphological alteration cannot be sufficient. Therefore, phenolic acids (PAs) based regulation of N-metabolism due to environmental constraints requires more study to understand germination and seedling growth under N deficiency. Recently, salicylic acid (SA) received attention after it was determined that it can induce resistance to pathogens as well as abiotic stress tolerance in plants[14,15,16]. Analyzing the role of secondary compounds, such as PAs may provide a method for the diagnosis of nutrient disorder in plants. Therefore, effects of exogenous SA on growth, development and nutrient metabolism in watermelon is determined to understand physiological responses to N-nutrition.

The objectives of this study were to investigate role of SA in regulation of NR (nitrate reductase) activity, chlorophyll synthesis, carbohydrate content, total N-content, NO_3^- assimilation; percent seed germination, seedling development and dry mass of watermelon (*Citrullus lanatus* L.) plants.

2. Materials and methods

2.1 Plant materials and culture conditions

Seed of watermelon (*Citrullus lanatus* L.) cv. "WN-8" were obtained from Institute of Crops, Wuhan Academy of Agricultural Sciences. Seeds were sterilized with 0.01% $HgCl_2$ for about 10 min, washed thoroughly with tap water followed by distilled water. Seeds were placed on moist Whatman No. 1 filter paper in acid washed Petri dishes (15x15 cm) for germination in an incubator at 25℃ ± 2℃ for 48 h. After this period, cotyledons of uniform size were isolated and allowed to green and expand under constant illumination for 72 h in culture room, temperature maintained at 25 ± 20C. Cotyledons were transferred to Petri dishes containing SA treatment (10, 50, 100 and 500 µM) with or without NO_3^-. Treated tissues were exposed to continuous illumination with light intensity of 100 µW $m^{-1}s^{-2}$ in the culture room for 48 h, after which cotyledons were subjected to biochemical analyses. Controls were incubated either in distilled water (without NO_3^-) or with 20 mM KNO_3.

2.2 Analysis of growth parameters

Dynamics of growth analysis of *Citrullus lanatus* L. cv WN-8 were started from 7 days old seedlings raised in sterilized Petri-dishes after a 6 h treatment of pre-soaked seeds with different (10, 50, 100, 500) µM SA in presence as well as absence of NO_3^- (20 mM KNO_3). Percent germination was recorded for 7 days and seeds were considered germinated when the radical became visible. Analyses were at 7 and 14 days to determine root and shoot lengths. Dry weight of 14 days old seedlings was determined after they were placed in oven at 600C until a constant weight was obtained. Other seedlings were transferred to pots containing black soil as a growth medium. Pots were provided only tap water.

2.3 Estimation of chlorophyll content

To determine chlorophyll content 72 h old fully expanded cotyledons weighing 100 mg (Precision Balance, Model No. CB-125) were collected after 24 h after start of treatment with SA, placed in 80% acetone and homogenized to extract the chlorophyll. The resulting solution was extracted through pre-weighted filter paper using a Buchnner funnel. The volume of the remaining acetone-chlorophyll solution was measured; solutions were kept in dark tubes in ice to minimize chlorophyll degradation. Absorbance of solutions was measured at 645 nm and 663 nm using a digital spectrophotometer (Perkin-Elmer) for chlorophyll a and b, respectively, and chlorophyll contents were calculated using Arnon's equation[17].

2.4 Estimation of NR activity

In vivo NR activity was determined by the method of Hageman and Hucklesby [18]with slight modification. For determination of NR activity 100 mg of shredded cotyledons were placed directly into 10 ml of incubation medium (300 mM KNO_3 as substrate in 1% isopropanol). The reaction was performed in the dark for 30 min in a water bath maintained at 300C with constant shaking. NR activity was calculated as the amount of enzyme, which produced micromoles of nitrite g^{-1} fresh weight in 1 h. The amount of nitrite was determined spectrophotometrically at 540 nm.

2.5 Determination of SA content using HPLC

Content of SA in watermelon cotyledons was determined by Daayf et al [19]. 1.0 gm of watermelon cotyledons from each treatment were macerated in pestle and mortar with 80% aqueous ethanol (80:20, 10 ml) and homogenate was centrifuged at 1500 rpm for 15 minutes. Supernatant was treated with light petroleum ether and filtered through Whatman paper no. 1. Clear supernatants were evaporated under vacuum at room temperature.The residue was dissolved in 1 ml HPLC grade methanol, filtered through membrane filter (Millipore, 0.45µ) and stored at 40 C for HPLC analysis. Further analysis were performed using (Shimadzu Corporation, Kyoto Japan) comprising LC-20 ATVP reciprocating pumps, a variable SPD-20A UV-VIS detector at 280 nm, C-18 reverse HPLC column 250x4.6 mm I'd. Particle size 5µC-18, (Phenomenex USA) at 360 C. Concentration of SA was calculated by comparing peak areas of reference compounds with that in the sample. Amount of SA (mg of sample) = Peak area of sample × Amount of standard × 20 / Peak area of standard.

2.6 Analytical methods

One hundred mg of dried cotyledons were used for N-analysis. The N-content was determined by a modified micro-Kjeldahl method after digestion with concentrated H_2SO_4 [20]. Total non-structural carbohydrates (TNC) in cotyledons were assayed for total soluble sugars and starch. Total sugar content was analyzed with the method of Scheible *et al.*[21]. The starch content was measured as glucose content, following an enzymatic hydrolysis of starch residues[22].

2.7 Statistical analysis

The experiment was arranged in a complete block design with five replications. Tests of significance between treatments were done using analysis of variance (ANOVA) and Duncan's multiple range tests[23].

3. Results

3.1 Growth analysis

SA induced several affects depending on the concentration applied and high doses were required to observe inhibitory action in watermelon plants. Percent seed germination was highest at 50 µM of SA with or without NO_3^- and effect of SA was more significant in absence of NO_3^- than in the presence of NO_3^- (Table 1). However, 500 µM SA caused reduction in germination by 30.2% in respect to the control. Studies were performed for 14 days to determine the influence of SA on seedlings growth. Results indicated that 20 µM of NO_3^- in conjugation with 50 µM of SA increased root and shoot length, while higher doses of SA were inhibitory with or without NO_3^- (Table 1). Growth parameters determining effect of SA in watermelon have been influenced by the specific concentration of treatment rather than the supply of external NO_3^-. To overcome this complication, all concentrations were plotted against total plant dry mass. 50 µM of SA exhibited highest dry matter (g per plant) in 14 days old seedlings, while higher doses of treatment reduced plat dry matter even in presence of external NO_3^-(Table 1).

3.2 Chlorophyll content

Total chlorophyll content was recorded as the sum of chlorophyll a and b. The 50 µM SA produced the highest chlorophyll content, which gradually declined thereafter at higher concentrations (100-500 µM) in the absence and presence of NO_3^- (Table 2). 50 µM SA increased near about 5 times higher chlorophyll content in cotyledonary tissues in comparison to aqueous control whereas total chlorophyll content reduced significantly at 500 µM SA treatment both in presence and absence of NO_3^- (20 mM KNO_3).

3.3 Non-structural carbohydrates

The content of soluble sugar increased at 50 µM SA compared to the aqueous control as well as NO_3^-control and decreased at higher concentrations (Table 2). The effect of SA was more significant in absence of external NO_3^-. The 50 µM of SA without NO_3^- produced 3 folds increases in the content of sugars, the least being in plants treated with 500 µM SA. In the presence of external NO_3^-, only 50 µM of SA produced increases in sugar content compared to the control, while 500 µM SA reduced sugar levels. Similar trends were observed for starch content, except at the 100 and 500 µM concentrations SA where, the starch content was sharply reduced comparison to the control (Table 2). TNC status in the cotyledons did not respond at higher supply of NO_3^- nutrition, indicates counter action of exogenous NO_3^- to SA whereas, in the absence of exogenous NO_3^-, increase in TNC was due to increasing concentration of soluble sugars at 50 µM SA treatment.

3.4 Nitrogen content

PA induced changes in the level of N-content were analyzed on dry weight basis in 7 days old watermelon cotyledons. N-content increased significantly by treating with 50 µM SA in comparison to aqueous control, while the N-level declined sharply at higher concentrations (100-500 µM SA) (Table 2). Concentration based SA response of SA was more significant in absence of exogenous NO_3^- in comparison to with NO_3^-. In absence of exogenous nitrate, highest level of N was observed at 50 µM SA (64.14 mg g^{-1} of dry weight) whereas, in the presence of exogenous nitrate it was 52.40 mg g^{-1} of dry weight (Table 2). External NO_3^- interactive properties with SA may be due to inhibition of NO_3^- uptake at higher concentrations of SA.

3.5 NR activity

To see the effect of SA on possible correlation between the NO_3^- assimilation and NR activity, SA treated watermelon cotyledons were demonstrated for NR activity in absence as well as presence of NO_3^- nutrition (Table 3). In absence of exogenous NO_3^-, SA increased 5 fold of NR activity (µM NO_2^- h^{-1} g^{-1} fresh weight) at 50 µM and then significantly reduced at higher concentrations of the SA (Table 3). While, in presence of exogenous NO_3^-, increase in NR activity was observed maximum at 50 µM of SA with gradual reduction at higher doses of SA. In an attempt to check the effect of SA on the rate of enzyme action, NR activity was calculated in terms of percent control and was found that 50 µM of SA (without NO_3^-) increased NR activity by 369% however, higher concentration (500 µM) reduced it by 16% of aqueous control.

3.6 SA content in absence and presence of NO^{3-}

SA content was determined in 7 days old watermelon cotyledons by a reverse phase HPLC to investigate the effect of exogenous NO_3^- (20 mM KNO_3) supply on SA accumulation. N-deficiency showed significant accumulation of SA in cotyledons. SA content was 2 fold high in 7 days old watermelon cotyledons without NO_3^- in comparison to with NO_3^- in control (Table 3). SA accumulation was reduced (18%) under the supply of exogenous NO_3^- at 50 µM SA. Data presented by table 3 showed a correlation between accumulation of SA and exogenous supply of NO_3^-.

Table 1. Differential effect of pre-soaking seed treatment of SA on percentage seed germination, root length, shoot length and plant dry-weight (DW) of watermelon seedlings in absence and presence of 20mM exogenous KNO_3.

SA(µM)		Germination (%)	Root length (cm)		Shoot length (cm)		Plant DW (g plant-1)
		48 Hrs	7 Days	14 Days	7 Days	14 Days	14 Days
Control	(-Nitrate)	65.2±0.078e	2.96 ± 0.009g	6.0 ± 0.172c	10.1 ± 0.008e	15.5 ± 0.102f	6.5 ± 0.088c
	(+Nitrate)	69.3±0.081d	3.00 ± 0.179e	6.23 ± 0.013b	14.6 ± 0.178b	17.7 ± 0.092e	6.7 ± 0.178d
10	(-Nitrate)	72.9±1.408b	4.2 ± 0.178c	7.5 ± 0.172a	13.5 ± 0.017c	19.1 ± 0.089c	6.9 ± 0.282g
	(+Nitrate)	71.2±0.171c	3.76 ± 0.017d	5.9 ± 0.102c	11.4 ± 0.282d	18.7 ± 0.179d	6.8 ± 0.423h
50	(-Nitrate)	79.3±0.213a	7.2 ± 0.268a	7.4 ± 0.014a	15.3 ± 0.424a	22.8 ± 0.018a	7.7 ± 0.007i
	(+Nitrate)	78.7±0.139a	5.1 ± 0.141b	6.4 ± 0.214b	14.7 ± 0.102b	21.7 ± 0.214b	7.8 ± 0.171j
100	(-Nitrate)	50.8±0.171f	2.5 ± 0.282f	4.8 ± 0.017d	9.25 ± 0.014f	13.1 ± 0.283g	4.9±0.101f
	(+Nitrate)	30.4±0.139i	1.86 ± 0.014h	4.6 ± 0.021e	7.6 ± 0.042g	12.8 ± 0.424h	4.7±0.091e
500	(-Nitrate)	46.8±0.101g	2.2 ± 0.424g	3.25 ± 0.282f	6.5 ± 0.141h	11.2 ± 0.008i	4.8±0.017a
	(+Nitrate)	43.2±0.138h	1.9 ± 0.282h	2.9 ± 0.424g	5.3 ± 0.268i	10.9 ±0.172j	4.6±0.213b
*CD		1.878	0.215	0.178	0.168	0.105	0.205

Footnote: Each value represented as mean ±SE (n=5), mean values followed by same letter (s) are not significantly different ($P < 0.05$) CD: critical difference.

Table 2. Biochemical changes in cotyledonary tissue-content of chlorophylls, carbohydrates and total nitrogen in watermelon cotyledons in response to SA in absence and presence of nitrate 20 mM KNO_3.

SA(µM)		Total Chlorophyll (mg g^{-1} F.W.)	Total Sugars (mg g^{-1} D.W.)	Total Starch (mg g^{-1} D.W.)	Nitrogen (mg g^{-1} D.W.)
Control	(-Nitrate)	0.027±0.001b	4.46± 0.576g	17.49 ± 1.101e	15.1 ± 0.050g
	(+Nitrate)	0.064±0.004ab	6.99 ± 0.600f	21.74 ± 0.602d	30.01 ± 0.200f
10	(-Nitrate)	0.131±0.001ab	8.19 ± 0.070b	31.24 ± 0.570b	25.3 ± 0.056d
	(+Nitrate)	0.105±0.004ab	8.99 ± 0.150c	22.49 ± 2.800c	47.9 ± 0.056b
50	(-Nitrate)	0.156±0.001ab	13.4± 0.500a	36.24± 5.700a	64.14 ± 0.010a
	(+Nitrate)	0.141±0.004ab	9.49 ± 0.701c	21.49 ± 0.501d	52.40 ± 0.280b
100	(-Nitrate)	0.091±0.001a	7.99 ± 0.800d	11.99 ± 0.200f	36.1±0.035c
	(+Nitrate)	0.076±0.005ab	7.19± 0.151e	11.74 ± 1.100g	34.24±0.004e
500	(-Nitrate)	0.071±0.004ab	2.19 ± 0.153h	9.69± 1.501h	9.08±0.020h
	(+Nitrate)	0.070±0.001ab	1.74± 0.570i	2.44 ± 0.502i	10.93±0.051i
*CD		0.0118	0.931	0.711	0.105

Footnote: Each value represented as mean ±SE (n=5), mean values followed by same letter (s) are not significantly different ($P < 0.05$) . CD: critical difference.

Table 3. Effect of SA on nitrate reductase activity in watermelon cotyledons grew with distilled water in absence and presence of nitrate (20 mM KNO_3).

SA (µM)	Without NO_3^-	% of control	With NO_3^-	% of control
Control	118.00 ± 0.4d	100	508.00 ±1.0c	100
10	271.00 ± 2.7b	224	651.00 ± 0.9d	126
50	571.00 ± 2.7a	469	709.00 ± 5.6a	137
100	259.00 ±2.9c	228	441.00 ± 1.4d	85
500	102.00 ± 2.7e	83	275.00 ± 1.9e	53

Footnote: Each value represented as mean ±SE (n=5), mean values followed by same letter (s) are not significantly different($P < 0.05$).CD: critical difference.

4. Discussion

Plants have evolved adaptive responses to grow in soils with low amount of one or several nutrients. These responses implicate complex metabolic changes generated by nutrient deficiency. SA induced several affects depending on the concentration applied. Higher doses of SA were required to observe inhibitory action in

watermelon plants. Percentage of seed germination was found significantly higher at 50 μM of salicylic acid and sharply reduced at higher doses both in absence as well as presence of exogenous nitrate. Higher levels of SA may inhibit nitrate uptake system and cause retardation in growth and development. Glass [24] observed concentration based inhibitory potency of PAs on ion-uptake in barley (*Hordeum vulgare* L. cv. Karlsberg). Rajjou *et al.* [25] have also been reported similar observations on seed germination and seedling establishment of *Arabidopsis thaliana*. SA might be involved in mobilization of internal tissue NO_3^- and chlorophyll biosynthesis to increase the functional state of the photosynthetic machinery in plants [26], or it may induce accumulation of α-amino levulinic acid (α-ALA) in cotyledons. Ananiev *et al.* [27] reported increases in chlorophyll biosynthesis in excised cotyledons of *Cucurbita pepo* L. (zucchini), cv. *Cocozelle* in response to growth regulator. This induction may be due to the interaction of PAs with light [28,29] producing higher rates of carbohydrate synthesis through photosynthetic activity. This is possibly due to changes in membrane organization at higher SA level or to chelation of some important elements of cellular and organeller membrane [30]. It is not clear why N-content increased, when NO_3^- was not applied. However; internal nitrate may provide an inductive concentration to NR activity at lower concentrations of SA and/or SA induced modulation of nitrogen use efficiency (NUE) in cucumber cotyledons [31]. It may be that increase in NO_3^- assimilation was dependent on the physiological concentration (e.g. 50 μM) of SA when NO_3^- was absent.

The imbalance between demand and N-supply in crops can result in either sub-optimal yield or the addition of environmentally damaging excesses of fertilizer. The uptake and assimilation of N by roots is known to change with supply in a manner that suggests that the N status of plants is somehow sensed and can feedback to regulate these processes with interaction of phytohormones[32]. Limited N-availability reduces the growth and plant productivity and induces secondary metabolism[5,6]. The results from our HPLC analysis support the hypothesis that SA favored growth and development by increasing NUE in cucumber. In absence of NO_3^-, accumulation of SA in cucumber play protective role for nutritional disorder. Previous results support exogenous application of 50 μM SA was beneficial for growth and development in comparison to high doses (500 μM) of SA[33].

The possible explanation for the concentration based effect of SA on NR activity is that NR activity was induced and/or prevention of enzyme degradation was prevented. Results indicated that concentrations of SA at 10 to 50 μM might induce NR synthesis by mobilization of intracellular NO_3^-, and provide protection to *in vivo* NR degradation in absence of NO_3^- [34]. Fariduddin *et al.* [35]reported increased NR activity due reduced concentrations of SA while higher concentrations were observed to be inhibitory to NR activity in *Brassica juncea* Czern & Coss cv. Varuna.

Effect of SA on carbon and N-metabolism:

In higher plants, NO_3^- assimilation is dependent on the supply of carbon skeletons, indicating a close interaction between carbon and N-metabolism. Increase in the level of PAs in plants under stress of N-nutrition has been reported[36]. NO_3^- assimilation proceeds at a low rate in plants with low carbohydrate levels[37]. Certain sugars increase N-assimilation rate and amino acid synthesis[38].Studies with mustard (*Brassica juncea* Czern & Coss cv. Varuna) and wheat (*Triticum aestivum* L.) reported direct relationships between photosynthetic CO_2 assimilation and NO_3^- assimilating enzymes in response to SA[35,39]. In these studies, plants were treated by foliar application of SA; however, in this work we tested pre-soaking seed with SA in absence and presence of NO_3^-. The rate of NO_3^- assimilation in cotyledons increased in response to 50 μM SA, with increases in amounts of soluble sugars and starch at same SA concentrations (Table 2), though accumulation of starch content is low compared to that of total N in watermelon (Table 2). The effect of exogenous SA on physiological characteristics of plants may depend on its concentration as well as nutritional conditions of the plants.

Present study indicates a positive correlation between chlorophyll content and total N in watermelon cotyledons. Moreover, it seems that effect of SA was more significant in absence of NO_3^- than in presence of nitrate. Increases in N-content, and chlorophyll content at lower concentration of SA, indicates that the acid plays a regulatory role during the biosynthesis of active photosynthetic pigments. Although the direct effect of SA on chlorophyll biosynthesis in plants is not clearly understood, α-ALA mediated enhancement in chlorophyll biosynthesis by benzyladenine (synthetic SA) [27]. Reduction in level of total N and chlorophyll content at 500 μM SA may be due to the breakdown/degradation of chlorophyll or inhibition of foliar proteins required for production of photosynthetic pigments.

5. Conclusions

SA response against nutrient stress is a new study in the field of crop physiology. Excessive use of chemical fertilizers in agriculture industries has appeared as a threat to soil health and yield. Results indicated that seed imbibition with SA affected physiological processes related to growth and development in cucumber plants. At lower concentrations, SA significantly increase rate of seed germination and plant dry mass even if added NO3- was 20 μM. Plants treated with 10 and 50 μM SA had higher chlorophyll levels and NO3- assimilation through the induction of NR activity. However 100 and 500 μM were detrimental to plant health. SA, a natural

endogenous growth regulator, if used exogenously, may improve plant growth and yield of cucumber.

6. References

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