

Effect of Salicylic Acid and Gallic Acid on the Growth Responses and
Physiological Changes of *Vicia faba* (cv. Giza 402) Seedlings.

II- Nucleic acid contents and nitrogen metabolism.

By

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Abstract

Treatment with SA and GA at 1 and 5mM, mostly elevated the values of nitrate-, nitrite-, ammonia- and amide-N in *Vicia faba* seeds and seedlings compared with those of the untreated ones, during the whole experimental period. *Vicia faba* seedlings imposed to 1 and 5mM SA and GA resulted in progressive increases in the contents of total free amino acids. Treatment with GA at 1mM, resulted in the highest level of total free amino acids throughout the experimental period. Conversely, using both types of phenolic acids at 1 and 5mM, mostly reduced the protein-N and total-N amounts during the different ages of germination and seedling growth as compared to the control ones, but unlikely, total soluble-N was variably changed due to these phenolic acid treatments.

The higher concentration (5mM) of phenolic acids employed comparably reduced the amounts of DNA and RNA than the control amounts while the low concentration, of these acids (1mM) increased them especially at the later ages of seedling growth (7 and 14 days). A completely reversed situation was realized regarding the activities of the nuclease enzymes DN- ase and RN - ase i.e raising the concentration of SA and GA from 1 to 5mM raised the activities of these hydrolytic enzymes.

Nitrate reductase activity (NRA) was markedly elevated above the control activity especially at the later ages of seedling growth (7 and 14 days) due to treatment with SA and GA. On the other hand, nitrite reductase activity (NiRA) was either generally reduced as a consequence of treatment with 5mM of SA and GA or highly accelerated due to application of 1mM of the same acids as compared to the control activities at all ages of germination and seedling growth, although 5mM GA increased this enzyme activity at the latest age of growth (2 weeks).

Key words: *Vicia faba*, Gallic acid at 1mM: GA1, Gallic acid at 5 mM: GA5, Salicylic acid at 1mM: SA1, Salicylic acid at 5mM: SA5, Nitrate reductase activity: NRA and Nitrite reductase activity: NiRA.

Introduction

Phenols and phenolic acids are of considerable importance in the regulation of plant growth and metabolism (Wain and Taylor, 1965 and Jain and Srivastava, 1981 a,b). Although their role as a plant morphogenetic regulator is well established (Asthana and Srivastava, 1978), there has been little study of the mechanism at the molecular level.

Phenolic derivatives of benzoic acid and cinnamic acid are commonly found in soils and many of these compounds are considered allelochemicals (Rice, 1984). Phenolic allelochemicals are released by plants into soils as leaf leachates, root exudates, and by plant tissue decomposition (Guenzi and McCalla, 1966; Tukey, 1970 and Rice, 1984).

The results of Glass (1974) and Glass and Dunlop (1974) showed that phenolics have nonspecific effects on root cell membranes and therefore on ion uptake, and that the degree of inhibition of ion uptake caused by a phenolic compound is correlated with its lipid solubility and its ability to depolarize the membrane potentials of root cells. Many phenolic compounds are known to possess chelating properties (Clemetson and Anderson, 1966). In fact, SA accelerates leaching of soluble nitrogen from maize endosperm (Jain and Srivastava, 1981 a). In addition, SA may induce NRA by interacting with the NR specific inhibitor which has been shown in many systems (Srivastava, 1980). It was found that incubation of both roots and shoots of maize with nitrate and low concentration of SA increased nitrate reductase activity (NRA) but did not affect nitrite reductase activity (NiRA), while using high concentration of SA decreased NR and NiR activities (Jain and Srivastava, 1981 a,b).

This study was carried out to investigate the changes in the nitrogen and nucleic acids (DNA and RNA) contents and DN ase, RN ase, NR and NiR activities in *Vicia faba* (cv. Giza 402) seedlings imposed to low and high concentrations (1 and 5mM) of salicylic acid (SA) and gallic acid (GA).

Materials and Methods

Plant material and growth conditions:

Detailed growth conditions for the germination of *Vicia faba* seeds (cv. Giza 402) and their treatments with phenolic acids (gallic acid and salicylic acid) have been previously described (Roushdy *et al* under press) and are only briefly outlined here.

Seeds of *Vicia faba* were germinated in 5 groups of plastic pots, each group consisted of 8 pots and each pot contained 15 equal seeds and received 15 cm³ of the following solutions: The 1st group received distilled water to represent the control treatment, the 2nd group received salicylic acid (SA) solution at 1mM, the 3rd group received SA at 5mM, while the 4th and 5th groups received gallic acid (GA) at 1 and 5mM respectively. After 1,2,7 and 14 days, germinated seeds and seedlings were harvested and quickly oven - dried at 80°C for nitrogen estimation, air - dried for nucleic acid determination or kept frozen for the assay of enzyme activities. (During the experimental period, all pots were maintained under constant climatic conditions; day / night temperature 22-18 °C, relative air humidity 60-65%, 12 h day length).

Methods of analysis.

Nitrogen determination:

Total-N and total soluble-N were determined by micro- kjeldahl after digestion in sulphuric acid (Strauch, 1965). Subtracting the total soluble-N from the total-N gave the value of protein-N. Total free amino acids were estimated colourimetrically by ninhydrin method as adopted by Muting and Kaiser (1963). Estimations of NO₃-N, NO₂-N, NH₄-N and amide-N were carried out according to methods described by Snell and Snell, 1949; Snell and Snell, 1939; Delory, 1949 and Naguib, 1964 respectively.

Determination of DNA and RNA

DNA and RNA were extracted and determined according to the methods described by Schneider (1945) and Ogur and Rosen (1950).

Enzyme assays:

Enzymes studied in this work were estimated in the crude extracts that were prepared by homogenizing 1-5g fresh plant tissues with Tris-HCl buffer at pH 7.4 (Guerrier and Strullu, 1990). The homogenates were centrifuged at 7000g for 30min. and the supernatants were directly used for the enzyme assays.

Ribonuclease (RNase) and deoxyribonuclease (DN ase) were assayed by the method of Wilson (1968) using RNA and DNA as the substrates, respectively. Activities of nitrate reductase (NR) and of nitrite reductase (NiR) were assayed in vitro and NO_2 concentrations were measured colourimetrically (Stevens and Oaks, 1973 and Losada and Paneque, 1971).

The activity of each enzyme was expressed as $(A - A_0) / tv$, where A is the absorbance of the sample after incubation minus the absorbance at zero time, TV is the total volume of the supernatant, t is the time (in minutes) of incubation with substrate and v is the total volume of the supernatant taken for incubation (Fick and Qualset, 1975).

Results and Discussion

Changes in DNA and RNA contents and DN ase and RN ase activities:

Results presented in table 1 show that there are marked and progressive increases in the relative contents of DNA and RNA correlated with the increase in seedling age from 1-14 days. Throughout the experimental period, *Vicia faba* seedlings imposed to GA1 contained the maximum level of nucleic acids while seedlings imposed to SA5 contained the minimum level. The order of increase in these contents in seedlings grown with SA1 and GA5 and untreated seedlings were as follows: control > GA5 > SA1 after 1 and 2 days and SA1 > control > GA5 after 7 and 14 days.

The pattern of changes in DN ase and RN ase activities, in response to the different treatments applied, was, more or less, the reverse of the DNA and RNA

behaviour. The rate of DNA and RNA hydrolysis in seedlings imposed to the low and high concentration of SA and GA were successively less and more than that in the control seedlings. These increases and decreases in the nucleic acid contents are parallel to the changes in the growth rates of *Vicia faba* seedlings grown with 1 and 5mM SA and GA (Abdalla, *et al.*, underpress).

In this connection, Kovacs and Sirokman (1969), Kefeli (1978) and Hassanein *et al.* (1987) stated that, the natural phenolic inhibitors declined the nucleic acid (RNA and DNA) content in the treated plants. It is known that phenolic acids reduce plant water utilization (Blum and Dalton, 1985 and Booker *et al.*, 1992), turgor and osmotic pressures and close stomata (Einhellig *et al.*, 1985 and Booker *et al.*, 1992). According to Kessler *et al.* (1964), Sheoran and Garg (1974) and Hassanein and El-Telwany (1989) water stress induced by phenolic treatments strongly suppressed the RNA and DNA content. A decrease in RNA was attributed to intensified activity of cytoplasmic RNase, whereas reduced DNA content was attributed to impaired synthesis and / or due to enhanced DNase activity under water stress condition. On the other hand, Rauser and Hanson (1966) showed that saline treatments caused leakage of divalent cations (Ca^{++} and Mg^{++}) which normally stabilize ribosomes against endogenous nucleases. Many phenolic compounds are known to possess chelating properties (Clemetson and Anderson, 1956). Chelation of some important elements of cellular and organellar membranes, thereby increases their permeability.

Changes in NR and NiR activities:

Table 2 shows that the activities of NR and NiR measured in *Vicia faba* seedlings exposed to the different treatments showed marked progressive increases towards the experimental end (except NRA of the control that sharply declined at 7 days). The pattern of increases in NRA was: GA5> SA1> control> SA5> GA1 at 1 and 3 days whereas it was: GA5> GA1> SA1> SA5> control at 7 and 14 days. On the other hand, NiRA was in the order of: SA1> GA1> control> SA5> GA5 after 1, 3 and 7 days while it was: GA1> GA5> SA1> control > SA5, at the final experimental time (14 days after germination) and NiRA by

5mM SA as observed in this experiment is consistent with the report of inhibition of NRA and NiRA by higher concentrations of phenolic acids (Schrader and Hageman, 1967 and Jain and Srivastava, 1981 a,b).

In addition, Jain and Srivastava (1981 a,b) demonstrated that in maize seedlings a low concentration of SA increased NRA while had no effect on nitrite reductase activity (NiRA). The increase in NRA in response to 5mM GA is supported by the data obtained by Knypl (1974) who found that 10mM chlorogenic acid induces NRA in cucumber cotyledons. The recorded influence of the used phenolic acids on the activities of both NR and NiR may be based on the following factors: a- Phenols may interact with specific inhibitors which has been shown in many systems (Srivastava, 1980). b-SA may allow the free access of metabolites involved in the induction of NRA (Jain and Srivastava, 1981 a). c- Phenols may affect the balance between synthesis / activation and degradation / inactivation processes of NR as reported by Jain and Srivastava (1981 a). d- Alternatively the effects of phenolic acids especially polyphenolic acids (GA) on the activities of NR and NiR may be mediated *via* phytohormones. Many phenolic compounds are known to provide protection to auxins against oxidation (Schneider and Whitman, 1974; Wilkins, 1979 and El-Telwany *et al.*, under press).

Changes in the nitrogen contents:

When comparing the values of nitrate-, nitrite-, ammonia - and amide-N detected in untreated *Vicia faba* seeds and seedlings during the whole experimental period, these fraction were remarkably elevated in SA and GA treated - plants (at concentrations of 1 and 5mM) although during the first 48 hours, slight reductions were determined in response to both GA treatments (1mM) in nitrate-N and (5mM) in amide-N and SA treatment (5mM) in amide-N (table3).

Treatment of *Vicia faba* seeds with 1 and 5mM, SA and GA resulted in progressive increases in the amount of total free amino acids. Its content was either increased (during the first 48 h) or decreased (at 7 and 14 days) above and below those determined in the control seedlings, whereas seedlings imposed to GA1 contained the highest level of total free amino acids throughout the experimental period.

Conversely, using both types of phenolic acids at 1 and 5mM, mostly reduced the protein-N and total-N amounts during the different ages of germination and seedling growth as compared to the control ones.

As regard to the total - soluble-N fraction estimated in both phenolic acids - treated plants, it either markedly increased due to 1mM GA (at 2,7 and 14 days), 5mM GA (at 7 and 14 days) and 1 and 5mM SA (at 7 days only) or mostly decreased due to 1 and 5mM SA (at 1,2 and 14 days), 1mM GA (at 1 day) and 5mM GA (at 1 and 2 days) above and below the control.

Generally speaking, GA (1 and 5mM) applied to *Vicia faba* seeds, considerably increased both total-N and all soluble-N fractions as compared to SA.

Nitrogen is a crucial element in determining the rates of CO₂ fixation and plant growth (Winter, 1985 and Nobel, 1988). Several studies have found a lower mineral content, including nitrogen element, in plants treated with phenolic acids (Jain and Srivastava, 1981 a; Balke, 1985; Kobza and Einhellig, 1987, Mersic and Singh, 1988; Klein and Blum, 1990 and Booker *et al.*, 1992). The total nitrogen, organic nitrogen and protein contents of the maize embryonic axis (root + shoot) from seedlings raised with 10mM Ca (NO₃)₂ were substantially higher than those from the control when low concentration of SA was applied, whereas the nitrate contents at higher concentration of SA in both minus and plus nitrate seedlings were significantly lower than in their respective controls (Jain and Srivastava, 1981 a,b). In addition, Ahmed, (1987) found that Na-salicylate at 4000 and 8000ppm resulted in significant increases in grain protein content in wheat at beginning of maturation and non-significant changes later on.

The general decline in protein, DNA and RNA contents with concomitant marked and progressive increases in total free amino acids and amide-N contents in *Vicia faba* seedlings imposed to SA and GA may be attributed either directly to the increased activity of the proteolytic enzymes or/and the increased activities of DN

ase and RN ase or indirectly to the induced water deficiency caused by these phenolic acids at least in seedlings imposed to 5mM SA and GA (Kessler *et al.*, 1964; Sheoran and Garg, 1978 and Booker *et al.*, 1992). The general progressive increases in the nitrate and nitrite contents accompanied by a remarkable decline in protein and total-N observed in the present study are in accordance with results of both Kessler and Oesterheld (1970) who observed nitrate generation and NRA in N-starved algae and Funkhouser and Garay (1981) who suggested that reduced nitrogen compounds are oxidized to nitrate in seedlings of higher plants and algae. A correlation between NRA and NiRA and organic nitrogen as observed in several systems (Johnson *et al.*, 1976 and Peuke and Tischner, 1991) suggests that these enzymes are operative in the assimilation of nitrate and nitrite. These conclusions support the results obtained in our work, where the progressive increases in nitrate and nitrite nitrogen contents were accompanied with increased NR and NiR activities.

The progressive decreases in the total-N contents in *Vicia faba* seedlings imposed to the used phenolic acids, as compared with the results of the untreated seedlings, may be attributed to the accelerated efflux of some soluble metabolites in response to the applied phenolic acids or/and to the increased demand of soluble N as a prerequisite for intensive growth rate at this period (Clemetson and Anderson, 1966; Jain and Srivastava, 1981 a ; Hassanein *et al.* , 1987; Bergmark, 1990 and Abdalla, *et al.*, under Press).

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Table (1) Effect of salicylic acid and gallic acid on the changes of DNA and RNA contents and DNase and RNase activities during seed germination and early seedling growth of *Vicia faba*. Each value is the average of three replicates (\pm sd).

Age/ day	Concentration mM	DNA	RNA	DN - ase	RN ase
		mg g ⁻¹ dry weight			
1	Control (H ₂ O)	0.62 \pm 0.2	1.68 \pm 0.3	19.8 \pm 1.8	48.5 \pm 4.5
	SA1	0.48 \pm 0.1	1.03 \pm 0.2	21.5 \pm 1.5	51.3 \pm 3.6
	SA5	0.41 \pm 0.1	0.65 \pm 0.2	25.3 \pm 2.3	63.6 \pm 3.1
	GA1	0.78 \pm 0.2	2.11 \pm 0.3	20.9 \pm 2.2	43.6 \pm 3.4
	GA5	0.58 \pm 0.2	1.18 \pm 0.3	23.6 \pm 1.9	56.5 \pm 2.8
2	Control (H ₂ O)	0.98 \pm 0.2	2.31 \pm 0.3	24.8 \pm 2.0	59.2 \pm 6.4
	SA1	0.72 \pm 0.2	1.68 \pm 0.1	23.6 \pm 1.8	63.3 \pm 3.5
	SA5	0.58 \pm 0.1	1.37 \pm 0.2	27.3 \pm 1.8	66.5 \pm 3.1
	GA1	1.10 \pm 0.3	2.66 \pm 0.2	22.6 \pm 1.5	54.7 \pm 3.7
	GA5	0.82 \pm 0.3	1.98 \pm 0.2	26.1 \pm 1.6	64.8 \pm 5.1
7	Control (H ₂ O)	1.16 \pm 0.2	2.66 \pm 0.3	23.2 \pm 2.2	69.7 \pm 5.3
	SA1	1.37 \pm 0.2	3.40 \pm 0.3	21.3 \pm 0.3	62.7 \pm 4.3
	SA5	0.83 \pm 0.2	2.14 \pm 0.2	25.6 \pm 1.7	76.8 \pm 3.8
	GA1	1.78 \pm 0.3	3.87 \pm 0.1	19.8 \pm 2.0	58.4 \pm 2.6
	GA5	1.01 \pm 0.3	2.33 \pm 0.2	24.3 \pm 2.3	74.9 \pm 3.6
14	Control (H ₂ O)	1.60 \pm 0.3	3.84 \pm 0.2	19.3 \pm 0.9	48.5 \pm 6.3
	SA1	1.92 \pm 0.2	4.05 \pm 0.3	16.8 \pm 1.1	40.6 \pm 5.0
	SA5	1.30 \pm 0.2	3.23 \pm 0.2	24.4 \pm 1.5	54.6 \pm 4.4
	GA1	2.29 \pm 0.2	4.68 \pm 0.3	15.6 \pm 2.0	36.7 \pm 3.3
	GA5	1.48 \pm 0.1	3.63 \pm 0.3	23.1 \pm 1.6	52.4 \pm 4.1

Table (2) Effect of salicylic acid and gallic acid on the activities of NR and NiR enzymes during seed germination and early seedling growth of *Vicia faba*. Each value is the average of three replicates (\pm sd).

Age/ day	Concentration mM	NR mg NO ₂ h ⁻¹ g ⁻¹ fr.wt.	NiR mg NO ₂ h ⁻¹ g ⁻¹ fr.wt.
1	Control (H ₂ O)	60 \pm 4	45 \pm 3
	SA1	66 \pm 4	54 \pm 3
	SA5	50 \pm 4	36 \pm 3
	GA1	42 \pm 3	50 \pm 4
	GA5	83 \pm 3	33 \pm 4
2	Control (H ₂ O)	70 \pm 5	60 \pm 5
	SA1	78 \pm 5	74 \pm 5
	SA5	54 \pm 2	54 \pm 5
	GA1	48 \pm 2	66 \pm 4
	GA5	94 \pm 5	45 \pm 4
7	Control (H ₂ O)	33 \pm 3	66 \pm 5
	SA1	87 \pm 8	94 \pm 9
	SA5	78 \pm 8	60 \pm 4
	GA1	100 \pm 10	81 \pm 6
	GA5	124 \pm 10	56 \pm 6
14	Control (H ₂ O)	84 \pm 6	78 \pm 8
	SA1	114 \pm 10	98 \pm 8
	SA5	94 \pm 9	74 \pm 8
	GA1	125 \pm 9	144 \pm 10
	GA5	164 \pm 11	110 \pm 15

Table (3) Effect of salicylic acid and gallic acid on the changes in the nitrogenous constituents during seed germination and early seedling growth of *Vicia faba*. Each value is a mean of three replicates (\pm sd) and expressed as mg g^{-1} dry weight.

Age/ day	Concentration mM	Total free amino acids	Nitric -N	Nitrite -N	Ammonia -N	Amino -N	Total Soluble -N	Total -N	Protein -N
1	Control (H_2O)	0.8 \pm 0.15	0.36 \pm 0.08	0.26 \pm 0.03	0.09 \pm 0.01	0.86 \pm 0.2	30.0 \pm 3.0	72.6 \pm 5.1	42.6 \pm 3.5
	SA1	4.1 \pm 0.31	0.73 \pm 0.10	0.27 \pm 0.03	0.15 \pm 0.02	0.98 \pm 0.1	10.2 \pm 1.2	59.7 \pm 4.5	49.5 \pm 3.3
	SAS	1.6 \pm 0.18	0.51 \pm 0.10	0.29 \pm 0.03	0.28 \pm 0.02	0.45 \pm 0.1	7.6 \pm 0.5	44.6 \pm 4.2	37.0 \pm 3.3
	GAI	5.1 \pm 0.41	0.15 \pm 0.01	0.28 \pm 0.03	0.34 \pm 0.02	1.18 \pm 0.3	21.6 \pm 1.9	66.9 \pm 3.6	45.3 \pm 4.1
2	Control (H_2O)	2.4 \pm 0.41	1.20 \pm 0.2	0.32 \pm 0.02	0.46 \pm 0.06	0.63 \pm 0.2	20.1 \pm 2.6	62.0 \pm 3.0	41.9 \pm 4.1
	SA1	1.7 \pm 0.11	0.48 \pm 0.05	0.24 \pm 0.02	0.06 \pm 0.01	0.81 \pm 0.2	25.2 \pm 5.0	66.4 \pm 3.3	41.2 \pm 4.5
	SAS	5.1 \pm 0.33	0.90 \pm 0.04	0.26 \pm 0.02	0.11 \pm 0.03	1.22 \pm 0.2	16.8 \pm 2.5	49.6 \pm 3.1	32.8 \pm 2.8
	GAI	2.4 \pm 0.31	0.62 \pm 0.03	0.28 \pm 0.02	0.21 \pm 0.03	0.63 \pm 0.1	12.6 \pm 2.5	36.0 \pm 2.0	24.2 \pm 2.5
7	Control (H_2O)	6.5 \pm 0.42	0.90 \pm 0.08	0.27 \pm 0.02	0.26 \pm 0.03	1.53 \pm 0.3	30.2 \pm 3.3	60.3 \pm 4.5	30.1 \pm 2.5
	SA1	3.8 \pm 0.34	1.35 \pm 0.1	0.30 \pm 0.02	0.38 \pm 0.03	1.00 \pm 0.2	24.0 \pm 3.3	52.2 \pm 4.1	28.2 \pm 3.2
	SAS	8.1 \pm 0.44	0.23 \pm 0.03	0.24 \pm 0.02	0.12 \pm 0.02	0.72 \pm 0.1	19.4 \pm 2.6	57.3 \pm 5.3	37.9 \pm 3.6
	GAI	6.3 \pm 0.50	1.15 \pm 0.09	0.25 \pm 0.02	0.18 \pm 0.02	1.29 \pm 0.3	29.6 \pm 3.1	39.8 \pm 3.6	10.2 \pm 1.6
14	Control (H_2O)	3.0 \pm 0.36	0.90 \pm 0.09	0.27 \pm 0.02	0.18 \pm 0.02	0.98 \pm 0.2	27.6 \pm 3.3	32.3 \pm 3.6	4.7 \pm 0.5
	SA1	12.0 \pm 0.55	1.65 \pm 0.11	0.26 \pm 0.02	0.36 \pm 0.03	1.62 \pm 0.3	40.2 \pm 4.0	49.3 \pm 3.8	9.1 \pm 0.4
	SAS	4.8 \pm 0.37	2.33 \pm 0.18	0.30 \pm 0.02	0.44 \pm 0.03	1.17 \pm 0.2	33.6 \pm 4.1	41.2 \pm 4.0	7.6 \pm 0.9
	GAI	16.0 \pm 1.80	0.58 \pm 0.15	0.27 \pm 0.02	0.18 \pm 0.01	1.41 \pm 0.3	27.2 \pm 2.4	54.6 \pm 3.1	27.4 \pm 2.3
14	Control (H_2O)	14.4 \pm 1.31	1.80 \pm 0.28	0.28 \pm 0.02	0.28 \pm 0.01	1.8 \pm 0.3	24.0 \pm 2.4	36.6 \pm 3.0	12.6 \pm 1.1
	SA1	10.5 \pm 0.86	1.10 \pm 0.14	0.30 \pm 0.02	0.36 \pm 0.01	1.53 \pm 0.2	21.6 \pm 1.8	28.4 \pm 2.8	6.8 \pm 1.1
	SAS	17.5 \pm 1.45	2.10 \pm 0.15	0.29 \pm 0.02	0.56 \pm 0.05	2.7 \pm 0.5	33.8 \pm 2.0	45.0 \pm 2.5	11.2 \pm 1.5
	GAI	11.9 \pm 2.3	2.63 \pm 0.35	0.33 \pm 0.02	0.80 \pm 0.08	1.62 \pm 0.3	31.0 \pm 3.3	40.3 \pm 3.5	9.3 \pm 0.8