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Does foliar application of salicylic acid protects nitrate reductase and enhances resistance in virus infected maize?

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The present study was conducted to assess whether exogenous applied salicylic acid (SA) as a foliar spray could ameliorate the adverse effects of virus infection in two maize cultivars (maize cv. sabaini and maize cv. Nab El-gamal). The plants were grown under normal field conditions for two weeks in sand clay soil, and then sprayed with either 2 or 4 mM SA. Two weeks later, plants were subjected to infection with two different concentrations of virus (TMV1 and TMV2), and were harvested 10 days later. Fresh and dry matter, shoot and root lengths, proline, soluble protein, soluble sugars as well as nitrate reductase activity were measured. Both fresh and dry matter were decreased under virus infection however, SA enhanced the fresh and dry matter production in both cultivars regardless the type of virus or SA concentration used. In roots, both fresh and dry matters were not affected. The shoot length was enhanced by salicylic acid than root length regardless the concentration used or virus treatment. The water content was much higher in shoots than roots especially in maize cultivar sabaini. Proline was accumulated in SA virus infected plants than reference control especially in cv sabaini. Soluble proteins and soluble sugars were accumulated in SA virus infected plants and in cv sabaini more than Nab El-gamal as compared with reference control. NRA was reduced in virus infected cultivars and cv sabaini was dramatically affected than Nab El-gamal. Treatment of plants with SA had a positive effect on preserving the activity of NR but was still less than the reference control regardless the cultivar used.

Key words: Nitrate reductase, proline, protein, salicylic acid, sugars, viruses.

INTRODUCTION

Salicylic acid (SA) is a component of the signal transduction pathway needed for induction of systemic acquired resistance, a plant-wide enhancement of resistance against a broad spectrum of pathogens (Murphy et al., 2001).

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Abbreviations: SA, Salicylic acid; **SAR,** systemic acquired resistance; **FW,** fresh weight; **DW,** dry weight; **NRA,** nitrate reductase activity; **TMV,** tobacco mosaic virus.

The trigger for SA synthesis and induction of systemic acquired resistance is the recognition of an invading microorganism by a product of a resistance gene (Baker, 1997). Often, this recognition is accompanied by the hypersensitive response a form of rapid programmed host cell death in a region around the point of pathogen entry (Hammond-kosack and Jones, 1996).

Salicylic acid, which is naturally synthesized by plants, plays an important role as a signal molecule that induces the tolerance mechanisms under the influence of both biotic and abiotic stresses such as virus, bacteria, fungi infections, freezing, drought, heat and heavy metals (Yalpani et al., 1994; Dat et al., 1998 Senaratna et al., 2000, Hussain et al., 2011). When plants are exposed to salt stress, they adapt their metabolism in order to cope with the changed environment.

Survival under these stressful conditions depends on the plant's ability to perceive the stimulus, generate and transmit signals and instigate biochemical changes that adjust the metabolism accordingly (Hasegawa et al., 2000). Moreover, under salt stress, SA applications were found to enhance the biosynthesis of proline, photosynthetic pigments, enzymatic and non-enzymatic antioxidants which all are stated as related to plant stress tolerance (Shakirova et al., 2003). Several studies also supported a major role of SA in modulating the plant response to several a biotic stresses including salt and water stress (Yalpani et al., 1994; Senaratna et al., 2000). In maize plants, pre-treatment with SA induced the production of antioxidant enzymes, which in turn increased chilling and salt tolerance (Janda et al., 1999). The objective of the present study was to assess the role of exogenous salicylic acid applications for tolerance in maize subjected to virus infection as well as to investigate the effects of SA individually and accompanied with virus infection on two maize cultivars.

MATERIALS AND METHODS

Two maize cultivars (maize Sabaini and Nab El-gamal) were brought from agronomy department, Faculty of Agriculture, Minia University, El Minia, Egypt. The kernels were left to germinate in the following manner: 1) Group A without any treatment (virus or salicylic reference control); 2) Group B treated with virus TMV1 (50%); 3) Group C treated with virus TMV2 (75%); 4) Group D combination of TMV1 + 2 mM salicylic; 5) Group E combination of TMV2 + 2 mM salicylic; 6) Group F combination of TMV1 + 4 mM salicylic, and 7) Group G combination of TMV2 + 4 mM salicylic.

Kernels of two maize cultivars were germinated in pots with sand clay soil at normal field conditions. Plants were left to grow for two weeks and then sprayed by salicylic acid (2 or 4 mM). Two weeks later plants were also treated with viruses (TMV, 50 and 75% 100 µl each) by making injurious infections in leaves by carborandum (10 days later plants were harvested). To determine the dry matter, the freshly harvested organs (shoots and roots) were dried in an aerated oven at 105°C for 24 h. The soluble proteins were determined according to the method adopted by Lowery et al. (1951). Free proline was determined according to Bates et al. (1973), soluble sugars by anthrone suphuric acid method which was carried out by Fales (1951) and Schlegel (1956) and adopted by Badour (1959).

NR-activity

Leaves (1 g fresh weight) were ground with mortar and pestle in liquid nitrogen. Two (2) ml extraction buffer (100 mM Hepes-KOH pH 7.6; 20 mM $MgCl_2$, 10 µM FAD, 5 mM DTT, 1 mM Pefabbloc, 0.2 mM PMSF, 1% polyvinyl pyrrolidone (PVP) and 0.05% casein) were added to the still frozen powder and grinding continued to thaw. The suspension was then centrifuged for 12 min (4°C, 12000 rpm) and the supernatant was removed and kept on ice. The reaction medium contained (total volume 1 ml) 50 mM HEPE pH 7.6, 10 µM FAD, 1 mM DTT, 5 mM KNO_3, 0.2 mM NADH and either 20 mM $MgCl_2$ or 20 mM EDTA. The reaction was started by addition of 100 µl extract and terminated after 5 min by addition of 125 µl zinc acetate solution (0.5M). After a short centrifugation (4°C, 5 min, 12000 rpm), 10 µl PMS was added to 950 µl of the supernatant in order to oxidize excess NADH. After 20 min in the dark, formed nitrite was measured colorimetrically by adding 750 µl of 1% sulfanilamide in 3 M HCl, and 750 µl of 0.02% N-naphthyl-ethylene diamine hydrochloride, and absorption was determined at 546 nm. For each series, blank and a nitrite standard (20 µM KNO_2) was included.

The data of all experiments were subjected to one way analysis variance and means were compared using the least significant difference test (L.S.D.) using statistical program (Sta. Base. Exe.) on computer.

RESULTS

Salicylic acid is an endogenous growth regulator of phenolic nature, which participate in the regulation of physiological processes in plants such as growth, photosynthesis, nitrate metabolism and also provide protection against biotic and a biotic stresses. The use of salicylic acid (2 and 4 mM) had different effects on both maize cultivars treated with different viruses (TMV1 and TMV2) during the vegetative growth. In maize Nab El-gamel, both fresh and dry weight of plants were not affected by virus TMV1, however treatments with TMV2 enhanced both fresh and dry matter over control by 107.2 and 107.6%, respectively (Table 1). The pretreatment of plants with salicylic acid 2 or 4 mM had a significant effect on both fresh and dry matter especially plants treated with 4 mM salicylic acid and TMV1 which have 151.1 and 131.5% fresh and dry matter, respectively of virus treated plants.

In roots, both fresh and dry matter were not affected with both treatments (salicylic or viruses) except for 2 mM SA treated with TMV1 which had 125.5 and 120% of virus treated plants only (Table 1). The shoot length was significantly enhanced by salicylic acid more than root length regardless the concentration of SA used or virus treated. The relative water content of both shoots and roots of maize cultivar was increased in plants treated with salicylic and infected with viruses regardless the concentration used or virus treated (Table 1). The highest values of relative water content were obtained (88.4 and 82.9% of control plants, respectively) in both shoots and

Table 1. Effect of virus treatment on fresh and dry matter (gm), relative water content (RWC) shoot and root lengths (cm) of maize Nab El-gamal treated with different concentrations of SA.

Treatment	Shoot				Root			
	FW	DW	RWC	Length	FW	DW	RWC	Length
Reference	9.6 ± 0.32	1.3 ± 0.1	86.4	45.2 ± 1.1	2.03 ± 1.1	0.43 ± 0.3	78.8	20 ± 9.5
V 1	9.8 ± 4.8	1.3 ± 0.6	86.7	49.6 ± 5.6	1.96 ± 0.8	0.40 ± 0.3	79.5	17.6 ± 3
V2	10.3 ± 1.0	1.4 ± 0.1	86.4	46.2 ± 1.8	2.0 ± 0.4	0.52 ± 0.05	74.0	18.3 ± 6
V1+2 mM SA	14.1 ± 4.7	1.7 ± 0.6	87.9	53 ± 3.8	2.46 ± 0.4	0.48 ± 0.02	80.5	19.8 ± 3
V2+ 2 mM SA	11.3 ± 2.2	1.41 ± 0.4	87.6	43.9 ± 0.6	1.66 ± 0.1	0.41 ± 0.02	75.3	15.3 ± 3
V1+ 4 mM SA	14.8 ± 3.2	1.71 ± 0.4	88.4	49.6 ± 6.3	1.86 ± 0.6	0.42 ± 0.1	77.4	19.2 ± 2.8
V2 +4 mMSA	13.8 ± 2.6	1.75 ± 0.5	87.3	45.2 ± 2.3	2.1 ± 1.0	0.36 ± 0.3	82.9	16.5 ± 4.1
LSD at 5%	3.9	0.52		8.4	1.16	0.19		6.12

RWC, Relative water content; FW, fresh weight; DW, dry weight. Data means of 3 replications ±SD.

Table 2. Effect of virus treatment on fresh and dry matter (gm), relative water content (RWC) shoot and root lengths (cm) of maize Sabaini treated with different concentrations of SA.

Treatment	Shoot				Root			
	FW	DW	RWC	Length	FW	DW	RWC	Length
Reference	8.3 ± 1.8	1.3 ± 0.32	84.3	38.7 ± 6.0	2.5 ± 1.3	0.38 ± 0.08	84.8	16.7 ± 0.7
V 1	9.03 ± 3.4	1.33 ± 0.2	85.3	37.8 ± 5.6	2.4 ± 0.78	0.44 ± 0.17	81.7	18.7 ± 8.0
V2	8.2 ± 2.8	1.1 ± 0.37	86.6	38.9 ± 9.5	1.5 ± 0.44	0.25 ± 0.01	83.3	17.4 ± 3.2
V1+2 mM SA	6.3 ± 1.2	0.92 ± 0.2	85.4	34 ± 3.1	1.7 ± 0.6	0.26 ± 0.12	84.7	18.4 ± 2.7
V2+ 2 mM SA	7.7 ± 2.3	1.1 ± 0.3	85.7	38.8 ± 5.1	1.8 ± 0.3	0.26 ± 0.04	85.3	14.6 ± 2.0
V1+ 4 mM SA	9.9 ± 2.4	1.31 ± 0.4	86.9	39.9 ± 2.3	1.66 ± 0.7	0.28 ± 0.03	83.1	18.1 ± 1.3
V2 +4 mM SA	7.9 ± 2.9	1.1 ± 0.3	86.1	34.9 ± 6.2	3.2 ± 1.1	0.39 ± 0.4	87.8	17.8 ± 2.1
LSD at 5%	3.14	0.38		7.82	1.33	0.20		6.12

Data means of 3 replications ±SD.

roots.

In maize cultivar Sabaini, the plants responded differently to both salicylic and virus infection compared with cultivar Nab El-gamal. Both fresh and dry matter of shoots and roots were slightly enhanced by salicylic acid (Table 2). The most obvious increase in fresh and dry matter over control plants were obtained with V1+4 mM SA (119.2 and 100.7%, respectively). However, in roots, the most enhanced effect of salicylic acid was obtained with V2+4 mM SA (128 and 102.6%) of control plants. Both shoot and root lengths were slightly affected by both treatments regardless the virus or salicylic acid used. The values of water content were obvious in roots than shoots in all treatments; the highest water content was obtained with virus and 4 mM SA (86.1 and 87.8%) in both shoots and roots (Table 2).

Plants adapt to stress by changes in cellular metabolism. A number of these adaptive responses are associated with the accumulation of osmolytes like proline and sugars. The proline concentration in maize cultivar Nab El-gamal was increased under virus treatment compared with control (Table 3). The treatment of maize with exogenous application of SA had a marked and significant effect on proline accumulation in both shoots and roots. It is worth to mention that, shoots accumulated proline more than roots. Maize sabaini (shoots) accumulated proline around two folds higher than reference control (153.5%), however in roots; proline was decreased with salicylic acid treatment.

The soluble proteins were markedly increased in both maize cultivars regardless the organ analyzed or treatment used (Table 4). It is worth to mention that, plants infected with virus and treated with 4 mM SA significantly enhanced the accumulation of soluble proteins about two folds higher than reference control (273.3 and 260%) in shoots of both cultivars.

Virus infection reduced nitrate reductase activity in shoots of both maize cultivars (Table 5). The reduction was obvious in maize Nab El-gamal than maize sabaini. The activity of nitrate reductase in maize sabaini was sensitive compared to maize Nab El-gamal. The treatment with any of the two viruses' concentrations resulted in reduction of activity. SA treatments had a protective effect on nitrate reductase activity but the

Table 3. Effect of virus treatment on proline concentration (mg/gDW) for both maize cultivars (Nab El-gamal and Sabaini) treated with different concentrations of SA.

Treatment	Nab El-gamel		Sabaini	
	Shoot	Root	Shoot	Root
Reference	0.21 ± 0.02	0.08 ± 0.02	0.28 ± 0.05	0.12 ± 0.01
V 1	0.23 ± 0.01	0.19 ± 0.02	0.15 ± 0.01	0.15 ± 0.02
V2	0.26 ± 0.04	0.09 ± 0.04	0.42 ± 0.01	0.2 ± 0.02
V1+2 mM SA	0.27 ± 0.01	0.15 ± 0.02	0.27 ± 0.01	0.14 ± 0.01
V2+ 2 mM SA	0.24 ± 0.03	0.09 ± 0.03	0.32 ± 0.05	0.06 ± 0.01
V1+ 4 mM SA	0.24 ± 0.02	0.12 ± 0.03	0.24 ± 0.04	0.07 ± 0.01
V2 + 4 mM SA	0.25 ± 0.02	0.08 ± 0.02	0.43 ± 0.05	0.08 ± 0.01
LSD at 5%	0.044	0.047	0.064	0.027

Data means of 3 replications ±SD.

Table 4. Effect of virus treatment on soluble protein concentration (mg/g DW) for both maize cultivars (Nab El-gamal and Sabaini) treated with different concentrations of SA.

Treatment	Nab El-gamel		Sabaini	
	Shoot	Root	Shoot	Root
Reference	10.9 ± 1.6	12.8 ± 0.98	20.9 ± 2.8	17.6 ± 0.91
V 1	13.2 ± 1.3	11.7 ± 0.7	19.9 ± 1.1	41.4 ± 5.8
V2	19.9 ± 1.1	10.9 ± 0.98	23.3 ± 1.7	16.1 ± 0.96
V1+2 mM SA	13.7 ± 0.63	20.5 ± 3.1	40.8 ± 3.7	13.3 ± 1.2
V2+ 2 mM SA	17.43 ± 1.8	19.3 ± 1.1	36.5 ± 1.8	32.7 ± 0.84
V1+ 4 mM SA	13.7 ± 0.6	28.5 ± 4.5	54.5 ± 2.4	30.3 ± 2.8
V2 + 4 mM A	29.8 ± 1.1	7.7 ± 0.8	34.5 ± 2.6	26.5 ± 1.7
LSD at 5%	2.08	3.17	4.20	4.43

Data means of 3 replications ±SD.

Table 5. NR activity (µmol g^{-1} FW h^{-1}) and soluble sugars (mg/g Dw) in shoots of both maize cultivars infected with virus and treated with different concentrations of SA.

Treatment	Nab el- gamal		Sabaini	
	Shoot NR	Soluble sugars	Shoot NR	Soluble sugars
Reference (NRA)	15.6 ± 3.2	29.8 ± 3.2	6.8 ± 0.35	32.8 ± 3.3
V 1 (NRA)	7.5 ± 0.8	36.3 ± 4.2	4.9 ± 0.7	38.7 ± 4.4
V2 (NRA)	4.2 ± 0.5	35.4 ± 2.5	3.8 ± 0.4	39.4 ± 2.8
V1+2 mM SA (NRA)	9.9 ± 0.2	45.2 ± 1.8	4.6 ± 0.8	59.2 ± 5.1
V2+ 2 mM SA (NRA)	8.4 ± 0.5	42.5 ± 4.2	4.4 ± 0.23	55.4 ± 3.2
V1+ 4 mM SA (NRA)	7.5 ± 0.9	31.6 ± 5.4	3.9 ± 0.5	44.6 ± 4.3
V2 + 4 mM SA (NRA)	8.7 ± 0.3	33.8 ± 3.3	4.2 ± 0.9	46.3 ± 7.1
LSD at 5%	1.3	5.02	1.11	7.95

Data means of 3 replications ±SD.

activity still lower than reference control.

DISCUSSION

Maize is widely cultivated throughout the world and a greater weight of maize is produced each year than any other grain. The mitigation effect of SA to a biotic stress was investigated through SA application by foliar spray of maize (Khodary, 2004). For last two decades, SA has received much attention because of its involvement in plant defense mechanisms against both biotic and abiotic stresses. Biotic stresses have been demonstrated recently; Sakhanokho and Kelley (2009) recorded that SA typically showed the salt tolerance under *in vivo* conditions in two botanical medicinal *Hibiscus* species. SA functions in plants as a key component of the signal transduction pathway leading to the induction of SAR and plays a role in resistance to all microbial pathogens, including fungi, bacteria, and viruses (Delaney et al., 1994).

From the results of the present study, it is obvious that virus treatments decreased the fresh and dry matter of both maize cultivars. The reduction was pronounced in maize sabaini than maize Nab El-gamal. However, exogenous application of SA often improved the plant growth in virus infected plants in both cultivars. Many studies supports that SA induced resistance of maize to salinity and osmotic stress (Tuna et al., 2007) and in wheat (Mutlu et al., 2009). Exogenous application of SA stimulated N and relative water content (Shirasu,et al., 1997) and this is in accordance with our results. SA-induced increase in growth could be related to enhancement of antioxidants that protect the plants from oxidative damage El-Tayeb (2005) or enhanced photosynthetic capacity in maize (Khan et al., 2003). The increase in fresh weight and water content under SA treatment in our results could be attributed to the conservation of plants to water under virus and SA treatments (Barkosky and Einhelling, 1993). The observed increase in plant height, shoot biomass and in root biomass in both maize cultivars treated with SA may be related to the ability of SA to induce antioxidant responses that protect them from damaging (Senaratna et al., 2000).

Accumulation of proline is a mechanism by which plants adapt to water stress and create anti stress defense. Plants that produce higher levels of proline are able to survive under stress (Delauney and Verma 1993; Szepesi et al., 2005). The protective action of SA during virus stress was demonstrated by enhanced proline production particularly in shoots of maize sabaini treated with (V2+4 mM SA) which reached ~153.3% of control plants.

The production of soluble proteins under virus infection in both maize cultivars especially cultivar sabaini treated with (V1+4 mM SA) which reached around (260.7%) of control plants, supported by increased relative water content (RWC) in that cultivar, proved that the accumulation of osmolytes including sugars also allows additional water to be taken up from environment which reduce water shortage within the plants and help to stabilize protein structure (Low, 1985). This is in accordance with the results obtained by Zahra et al. (2010) working with tomato with observed increase in leaf protein levels.

Nitrate reductase (NR, EC1.6.6.1) is localized mainly in the cytosol and its expression at the transcriptional levels is affected by nitrate, light and plant hormones. Stress provokes either increase, decrease as well as no effect on nitrate reductase activity (Abd El- Baki et al., 2000). Virus treatments inhibited nitrate reductase activity in both maize cultivars. The activity of NR was lower in maize sabini than maize Nab El-gamal, however the drop in activity in sabaini under virus stress was lower than Nab El-gamal. An efficient N assimilation is said to be favored by a high rate of CO_2 assimilation (Ferrario et al., 1998). SA induced conservation of water in stressed plants and also accumulated sugars which favored NR activity and protection in SA virus treated plants, this probably reflects the maintenance or even induction of root elongation at virus infected plants, which can be considered as an adaptive response to stress (Balibrea et al., 2000). The induction of NR activity may be also due to liberation of nitrate from vacuole under SA which favor NR activity since nitrate affects NR- mRNA (Abd El- Baki et al., 2000).

In conclusion, SA may probably induced resistance to TMV infected maize in large part by inhibiting virus replication (Chivasa et al., 1997) or resist cell to cell movement. We also conclude that cell and tissue development exerts a powerful influence over the design of the defensive signaling pathway and the resistance mechanisms that they trigger. The future application of this plant hormone holds a great promise as a management tool for providing tolerance to our agricultural crops against stress agents (biotic or abiotic) aiding to improve crop yield in near future.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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