Abstract of Ph.D. thesis

Improving effect of salicylic acid pre-treatments on the acclimation of tomato to salt stress: the role of reactive oxygen species and NO

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INTRODUCTION

Salinity is one of the major stresses which can change the ion homeostasis of plant tissues and causes hyperosmotic stress. Tomato plants are usually grown in greenhouse in hydroponic culture. These plants are more sensitive to different biotic and abiotic stresses than the plants grown in soil. In tomato producing countries, for example in Spain or Israel, irrigation water has high salt content which can affect fruit quality. Therefore it is very important to investigate the mechanism of salt tolerance and discover some processes to increase salt tolerance of these plants. Pre-treatment with exogenous salicylic acid (SA) can be an efficient procedure to improve salt stress tolerance.

According to our earlier results, protective effect of exogenously applied SA against salt stress depends on the mode of application, the developmental phase of plants and the applied concentrations. Lower concentrations of SA can induce hardening process which can improve the tolerance to salt stress in tomatoes (*Solanum lycopersicum* Mill. L. cvar Rio Fuego) grown in hydroponic cultures. Long term treatment with $10^{-4}$ M SA can improve the salt tolerance of tomatoes, $10^{-7}$ M SA is not effective and higher concentrations of SA ($>10^{-3}$ M) induce cell death.

The comparison of the biochemical and physiological background of different SA concentrations in time dependent manner during the “priming” has not been performed until now.

Therefore the main goal of my study was to investigate the most important biochemical and physiological factors during the “priming” and after 100 mM NaCl exposure in tomato (*Solanum lycopersicum* Mill. L. cvar Rio Fuego) at wide concentration range of SA ($10^{-7}$-$10^{-2}$ M).

AIMS

In my thesis the following questions have been raised about the SA-induced priming:

1. **Effects of SA pre-treatment applied in a wide concentration range during “priming”:**
   - How can SA affect the stomatal conductance during long term pre-treatment?
   - What is the effect of SA-induced stomatal closure on the photosynthetic activity, on the primary photochemical processes, on the contents of photosynthetic pigment, and on the total sugar during SA pre-treatments? Is there any change in the photosynthetic activity during SA-induced priming?
   - Can SA promote the accumulation of different polyamines during pre-treatment? Is there any relationship between the accumulation of polyamines and ethylene production? What is the role of these processes in “priming”?
   - How does SA influence the level of reactive oxygen species (ROS) and nitric oxide (NO) in tomato root tissues as a function of time? We are also interested in what is the role of ROS or NO in the hardening process. Is there any difference between the importance of ROS or NO in the induction of defence reactions?
• How can long term pre-treatment of SA affect the enzymatic antioxidant mechanisms like the activities of SOD and CAT as a function of time?

2. **Effect of SA pre-treatment on the improvement of acclimation against 100 mM NaCl:**
• Are there any differences in the photosynthetic activity between control and SA pre-treated plants during salt stress?
• How can long term SA pre-treatment modify markers of stress resistance, chlorophyll *a* fluorescence induction parameters, photosynthetic pigment contents and the total sugar contents in the plants during salt stress?
• How does long term SA pre-treatments affect the accumulation of various growth-regulating compounds such as ethylene production during salt stress? How can this process contribute to the successful acclimation against salt stress?
• How can long term pre-treatment with SA influence ROS and NO levels during salt stress?
• How can SA influence SOD and CAT activity in plants exposed to high salinity?

3. **Interaction of ROS and NO in mesophyll protoplasts:**
• Mesophyll protoplasts were prepared to investigate the effect of SA, 100 mM NaCl and their combination in a model system, at cell level. Using this model system, it was easy to detect the intracellular changes inside the cells. I investigated the effect of different concentrations of SA on the generation of intracellular ROS and NO and on the viability of cells exposed to various concentration of SA and 100 mM NaCl. The effect of different growth-regulating compounds, Put, Spd, Spm, ABA and ACC on the viability of protoplasts and the relationship between ROS and/or NO accumulation and cell viability were also revealed. We were also interested in whether plasma membrane (PM) localized NADPH oxidase contributed to the generation of ROS.
MATERIALS AND METHODS

1. Experiments with intact plants:

1.1 Plant material, applied treatments
Tomato (*Solanum lycopersicum* Mill. L. cvar Rio Fuego) seeds were germinated at 26°C for 3 days in the dark. Seedlings were placed into perlite for 2 weeks. The plants were placed into hydroponic culture and were grown under controlled conditions in greenhouse. Prior to being subjected to 100 mM NaCl, the plants were pre-treated with $10^{-7}$-$10^{-2}$ M SA for 3 weeks and then they were exposed to high salinity for 1 week. Samples were prepared 1 day, 2 days, 1 week, 2 weeks and 3 weeks after SA exposure and 1 week after 100 mM NaCl treatment.

1.2 Determination of photosynthetic parameters
1.2.1 Measurement of stomatal conductance
Stomatal conductance was measured on abaxial and adaxial surfaces of the leaves with steady state porometer (PMR-2, PP systems, UK).

1.2.2 Measurement of CO$_2$ assimilation and chlorophyll a fluorescence
Chlorophyll a fluorescence and the net photosynthetic rate were measured with a portable photosynthesis system (LI-6400, LI-COR, Lincoln, Nebraska, USA) with an infra red gas analyzer.

1.2.3 Determination of photosynthetic pigment contents
Determination of photosynthetic pigment contents were performed by the method of Sims et al. (2002). The extraction of chlorophyll was carried out with acetone in 2 phases. Optical density was measured with a KONTRON Double-Beam spectrophotometer at 470, 534 and 661 nm.

1.2.4 Measurement of total sugar content
Total sugar content was determined by the phenol-sulphuric acid method (Dubois et al. 1956). Optical density was measured with a KONTRON Double-Beam spectrophotometer at 490 nm. Total sugar contents were calculated on fresh weight basis.

1.3 Determination of growth regulating compounds
1.3.1 Determination of ethylene production
1 g of plant tissues were collected and placed into ethylene sampling tubes. Samples were incubated for 1 hour in these tubes in the dark. Ethylene production was measured with gas chromatography (Hewlett Packard 5890 Series II.).
1.3.2 Analysis of polyamines
Polyamines were determined as described by Flores and Galston (1982). Polyamines were separated by HPLC (JASCO, HPLC system, Japan) with a 45/55 (v/v) mixture of acetonitrile/water on a reverse-phase column (Apex octadecyl, 5 µm; 250 mmx4.6 mm) and monitored with UV detector at 254 nm.

1.4 Determination of the activities of antioxidant enzymes
1.4.1 Measurement of superoxide dismutase (SOD) (EC 1.15.1.1) activity
Plant tissues were homogenized in 50 mM phosphate buffer, pH 7.0, containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 1% polyvinyl-polypirrolidone and the homogenate was centrifuged. Reaction mixture contained enzyme extract, nitro blue tetrazolium (NBT) and riboflavin. SOD was measured with a KONTRON Double-Beam spectrophotometer at 560 nm.

1.4.2 Measurement of catalase (CAT) (EC 1.11.1.6) activity
Plant tissues were homogenized in 50 mM phosphate buffer, pH 7.0. Then the homogenate was centrifuged. Reaction mixture contained enzyme extract, phosphate buffer (50 mM, pH 7.0) and 1% H2O2. CAT was measured with a KONTRON Double-Beam spectrophotometer by following the decrease in absorbance between 1 and 2 minutes at 240 nm.

1.5 Determination of ROS and NO and viability
1.5.1 Detection of O2^- production
For the determination of O2^-, samples were stained with 3 mg/ml NBT. For the detection of O2^-, a Zeiss Axiowert 200M-type fluorescence microscope equipped with a high-resolution digital camera (Axioacam HR, HQ CCD camera, 1300x1030 dpi, Carl Zeiss, Jena, Germany) was used.

1.5.2 Determination of H2O2 content
H2O2 was measured spectrophotometrically according to Velikova et al. (2000). Fresh leaf or root tissues were homogenized in 0.1% trichloroacetic acid (TCA). After centrifugation 10 mM phosphate buffer and 1 M KI were added to the supernatant. The absorbance was measured with a KONTRON Double-Beam spectrophotometer at 390 nm.

1.5.3 Detection of ROS and NO and the viability
For the detection of ROS, the root segments were stained with 2,7-dichlorofluoresceine diacetate (H2DCF-FDA). NO was detected with 4,5-diamonofluoresceine diacetate (DAF-2 DA) and fluorescein diacetate (FDA) was used as a probe for viability. For the detection of fluorescence intensity a Zeiss Axiowert 200M-type fluorescence microscope equipped with a high-resolution digital camera (Axioacam HR, HQ CCD camera, 1300x1030 dpi, Carl Zeiss, Jena, Germany) was used.
2. Experiments with mesophyll protoplasts as model system:

2.1 Protoplast isolation and treatment

Protoplasts were prepared from the young, terminal leaves of untreated tomato plants. Leaf strips were digested with a solution containing 2% cellulase (Onozuka R-10) and 0.5% macerozyme (Onozuka R-10). After isolation protoplasts were resuspended in a buffer containing 525.6 mM mannitol, 12.5 mM sodium acetate and 5 mM CaCl₂. The protoplasts were then treated with different concentrations of SA ($10^{-7}$-$10^{-3}$) and or/with 100 mM NaCl. Growth regulating compounds were applied at 2.5 mM (Put, Spd and Spm) or at $10^{-5}$ M (abscisic acid (ABA) and 1-aminocyclopropane-1-carboxylic acid (ACC)).

2.2 Detection of ROS, NO and viability

For the detection of ROS, the root segments were stained with 2,7-dichlorofluoresceine diacetate (H₂DCF-FDA). NO was detected with 4,5-diamonofluoresceine diacetate (DAF-2 DA) and fluorescein diacetate (FDA) was used as a probe for viability. For the detection of fluorescence intensity a Zeiss Axiovert 200M-type fluorescence microscope equipped with a high-resolution digital camera (Axiocam HR, HQ CCD camera, 1300x1030 dpi, Carl Zeiss, Jena, Germany) was used.
RESULTS

Our aim was to investigate the changes in the biochemical and physiological parameters of tomato plants treated with SA during pre-adaptation period. We were also interested in how $10^{-4}$ M SA pre-treatment can induced tolerance to salt stress and how $10^{-3}$ and $10^{-2}$ M SA pre-treatment resulted in a decrease in the viability of the plants.

Our results can be summarized as follows:

1. **Effect of SA pre-treatment on tomato plants during „priming”**
   
   1.1 *Role of photosynthetic activity in the SA induced hardening process*
   
   SA applied at $10^{-3}$ M concentration decreased the stomatal conductance and resulted in a reduction of photosynthetic activity. Total sugar content in roots treated with $10^{-3}$ M SA was significantly reduced and in the absence of osmotically active carbohydrates the water retaining capacity of tissues declined. Lower concentrations of SA did not cause any difference in the contents of photosynthetic pigments, and in the primary photochemical processes of photosynthesis ($F_v/F_m$, $\Phi_{PSII}$, $q_p$) compared to the control plants. Although lower concentrations of SA decreased stomatal conductance at the beginning of pre-treatment, after 3-week-long pre-incubation stomatal conductance of SA treated leaves did not differ significantly from those of control plants. Pre-treatment with $10^{-4}$ M SA enhanced total sugar content of root tissues during the „priming” suggesting that sugar accumulation contributed to the osmotic adaptation in the root tissues of $10^{-4}$ M SA-treated plants.

   1.2 *Effect of ethylene in the acclimation*
   
   $10^{-3}$ M SA caused considerable increase in ethylene production of tomato roots but the ethylene synthesis was inhibited by $10^{-2}$ M SA. Significant enhancement in ethylene production could lead to the death of root tissues at $10^{-3}$ M SA treatment. SA applied at $10^{-2}$ M, triggered the cell death, but in this case the PCD did not depend on the ethylene production. Lower concentrations of SA caused a moderate ethylene production of the leaves, however, after three weeks $10^{-4}$ M SA did not result in change of the ethylene production of root and shoot tissues compared to the control.

   1.3 *Accumulation of polyamines during pre-treatment*
   
   Higher concentrations of SA ($10^{-3}$ M and $10^{-2}$ M SA) affect the synthesis of growth-regulating compounds differently. In $10^{-2}$ M SA treated plants putrescine (Put) content was 5-fold greater and spermine (Spm) was 25-fold greater than in control plants. $10^{-3}$ M SA also diminished the ethylene production of leaf tissues. We can state that accumulation of polyamines also occurred at $10^{-2}$ M SA treatment.
10^{-3} M SA significantly enhanced the ethylene production and the accumulation of Put and Spd in root tissues. We can conclude that 10^{-3} M SA treatment induced the biosynthesis of polyamines and ethylene and this could result in a fast and considerable accumulation of reactive oxygen species causing oxidative stress. In contrast with higher concentrations of SA, 10^{-4} M SA induced a transient increase in ethylene production and enhanced the concentration of Put in the leaves and Spd and Spm in the roots resulting a polyamine spectrum which characterizes halophytes.

1.4 ROS or NO, or both

It was demonstrated that higher concentrations of SA (10^{-3} M and 10^{-2} M) decreased O_2^- level considerably increased the activity of SOD and inhibited the activity of CAT resulting in high levels of H_2O_2 in the leaves and the accumulation of ROS in the roots. Both of 10^{-3} M and 10^{-2} M SA concentrations could induce significant increase in NO production in the apical root tissues. Enhancement of ROS and NO production of root apices caused a fast decrease in the cell viability. In root tissues 10^{-4} M SA did not increase SOD and inhibited CAT activity and enhanced ROS production in root apices. However, there were no differences in NO production between control and 10^{-4} M SA pre-treated plants during the hardening process. The small increases in ROS accumulation in root apices did not decrease the viability, on the contrary, it increased the antioxidant capacity of the plants and induced the defense mechanisms.

2. Improvement of salt stress acclimation by SA pre-treatment

2.1 Changes in photosynthetic activity in SA pre-treated plants during salt stress

Decrease in stomatal conductance was considerable after exposure to 100 mM NaCl in tomato. Salt stress reduced the CO_2 assimilation rate, the contents of chlorophyll a, chlorophyll b, carotenoids and anthocyanin of tissues. The primary photochemical processes of photosynthesis (Φ_{PSII}, q_p) of control plants also declined after exposure to high salinity. In contrast with untreated plants, 10^{-7} and 10^{-4} M, increased stomatal conductance and photosynthetic activity during salt stress. 10^{-4} M SA pre-treatment enhanced maximal CO_2 assimilation rate (A_{max}) and carboxylation efficiency (CE) resulting in more efficient CO_2 fixation. Lower concentrations of SA, (e. g. 10^{-4} M) increased the content of photosynthetic pigments and improved the primary photochemical processes of photosynthesis (Φ_{PSII}, q_p) compared to the salt stressed control.

In control and 10^{-7} M SA pre-treated roots, total sugar content did not decline during 100 mM NaCl exposure, however, in the roots of 10^{-4} M SA pre-treated plants an enhanced sugar content could be detected under high salinity. Increase in soluble sugars of root under salt
stress could contribute to the osmotic adaptation resulting successful acclimation during salt stress condition.

2.2 Changes in ethylene production of SA pre-treated plants during salt stress
Salt stress enhanced the ethylene synthesis of the leaves, however SA pre-treatments, mainly $10^4$ M decreased the ethylene production during 100 mM NaCl exposure. In root tissues, $10^4$ M SA decreased the ethylene emanation after 3 weeks of pre-treatment. During salt stress $10^4$ M SA reduced further the ethylene synthesis contributing to a successful acclimation.

2.3 Role of ROS and NO production in salt stress acclimation
Salt stress enhanced SOD and diminished CAT activity inducing a high level of $H_2O_2$ in the leaf tissues and the generation of ROS in root apices. Salt stress significantly induced the production of NO in root tissues. A high amount of ROS ($H_2O_2$) and NO considerably decreased the viability of root cells. SA pre-treatments also increased SOD activity in leaves during 100 mM NaCl exposure, but increased CAT activity in roots contributing to the elimination of salt stress inducing ROS ($H_2O_2$). During salt stress the level of NO and ROS decreased in the roots of SA pre-treated plants compared to the salt stressed controls improving the vitality of root cells.

3. Interaction between ROS and NO in mesophyll protoplasts
Both $10^{-3}$ M SA and 100 mM NaCl considerably increased the intracellular ROS and NO levels of the protoplasts and caused oxidative damage. Viability of the protoplasts decreased in response to $10^{-3}$ M SA treatment and salt stress, respectively, in parallel with high intracellular production of NO and ROS, which were effectively blocked by DPI, an inhibitor of NADPH oxidase. Salt stress induced ROS and NO were prevented in the presence of $10^{-7}$ M and $10^{-4}$ M SA and viability did not change significantly compared to control protoplasts. In intact tomato plants we could demonstrate that changes in the concentrations of different growth-regulating compounds can contribute to successful acclimation or to cell death. To investigate the effect of these compounds on ROS and NO production and cell viability we used protoplasts as a model system. Spd and Spm enhanced the levels of ROS and NO and caused a significant decrease in the viability of the cells. This suggests that polyamines may increase ROS production not only in the apoplast, though the activity of apoplastic polyamine oxidase (PAO), but also intracellularly. Since PAO may be localized in different compartments inside in the cells, for example in peroxisome, and these compartments could contribute to the generation of ROS. Put also increased the accumulation of NO, but it was smaller compared to the effects of Spm and Spd and did not decrease the viability of protoplasts. These results correspond with our earlier findings observed in plant tissues. ABA is a very important stress
hormone that controls the acclimation to high salinity. ABA enhanced both ROS and NO levels and decreased the viability in 2.5 hours however, after 5 hours of incubation ABA reduced ROS production and improved the cell viability. In contrast with ABA, ACC, the immediate precursor of ethylene significantly increased the accumulation of ROS and NO causing a significant decrease in the cell viability.

Summarizing the results, we can conclude that higher concentrations of SA could decrease the efficiency of photosynthesis and cause oxidative damage. Both the levels of ROS and NO considerably increased by $10^{-3}$ and $10^{-2}$ M SA and resulted in desorganization of the root system. However $10^{-4}$ M SA could improve the efficiency of photosynthesis during salt stress. $10^{-4}$ M SA using in long term experiments, could increase total sugar content in the roots contributing to the osmotic adaptation, could decrease the ethylene production and could modify the polyamine spectrum during the”priming”. $10^{-4}$ M SA during the hardening process could enhance the ROS production of root apices. However during salt stress decreased ROS levels can be observed compared to the salt stressed control root tissues. In $10^{-4}$ M SA pre-treated root apices ROS production could contribute to a successful acclimation to oxidative stress, however NO was not involved in SA induced priming. At $10^{-3}$ and $10^{-2}$ M SA application parallel accumulation of ROS and NO caused the death of cells.

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LIST OF PUBLICATIONS
(Present thesis is based on articles marked by an asterisk)


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**Posters**


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