

## INTERACTION BETWEEN SALT STRESS AND ANGULAR LEAF SPOT (*PSEUDOMONAS SYRINGAE* PV *LACHRYMANS*) IN CUCUMBER

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### Summary

We studied the effects of sequentially applied salt stress and *Pseudomonas syringae* pv *lachrymans* (*Psl*) infection in cucumber (*Cucumis sativus* L.). Infection development, shoot and root growth potential, the concentrations of chlorophyll and proline as well as electrolyte leakage, lipid peroxidation and H<sub>2</sub>O<sub>2</sub> production were determined. Cucumber plants were first exposed to salt stress and irrigated for seven days with 50 or 100 mM NaCl and thereafter inoculated by *Psl*. Abiotic stress compromised the defence response to pathogen and disease severity was the highest in 100 mM NaCl-treated plants. The reduced performance of salinized plants under biotic stress could be related to salt stress-induced plant growth inhibition with leaf expansion being the most sensitive to salinity, decreased chlorophyll content, increased electrolyte leakage and prolonged H<sub>2</sub>O<sub>2</sub> accumulation in leaves implying perturbations in redox homeostasis. The response of NaCl-treated and control plants to bacterial infection differed in terms of H<sub>2</sub>O<sub>2</sub> generation and lipid peroxidation. This study confirmed that proline is an important component of local and systemic responses to salt stress and infection. The results contribute to our knowledge of the nature of plant response to a combination of abiotic and biotic stresses.

key words: salinity, *Pseudomonas syringae* pv *lachrymans*, combination stress, cucumber

### INTRODUCTION

In nature, plants are often exposed to a combination of different stress factors. Among a wide variety of abiotic and biotic stressors, salinity

and pathogens are important factors affecting plant health and productivity. Salt stress presents an increasing threat to worldwide agriculture. The harmful effect of high salinity on plant growth is attributed to the combination

of: (1) low osmotic potential of soil solution (osmotic stress), (2) nutritional imbalance, (3) ion toxicity (salt stress) and (4) oxidative stress (Parvaiz & Satyawati 2008). *Pseudomonas syringae* pv *lachrymans* (*PsI*), causing the angular leaf spot disease, is an important biotrophic bacterial pathogen of *Cucurbitaceae* crops, especially cucumber. Increased occurrence of *PsI* has caused significant losses in cucumber yield in Poland in recent years (Olczak-Woltman *et al.* 2008).

The available data indicates that the response of plants to a combination of two different stresses is unique and cannot be extrapolated from the reaction of plants to each of the different stresses applied individually (Mittler 2006, Atkinson & Urwin 2012). It is known that exposure to one stressor can alter plant response to the subsequent stress and both positive and negative interactions between abiotic and biotic stresses have been reported (Knight *et al.* 1998, Desprez-Loustau *et al.* 2006). Although the impact of individual stressors e.g. drought, salinity, chilling, pathogen infection have been extensively studied, little is known about how a combination of different stresses, applied simultaneously or sequentially, affects plants.

In a few studies on salinity-pathogen interactions contrasting results have been obtained indicating that the negative effects of abiotic stress and pathogens can be additive or plant resistance to pathogens may be enhanced by abiotic stress. Enhanced resistance of barley (*Hordeum vulgare*) against barley powdery mildew (*Blumeria graminis* f.sp. *hordei*) was induced by salt stress (Wiese *et*

*al.* 2004). However, the resistance of tomato to *Pseudomonas syringae* pv *tomato* was not affected by salinity (Thaler & Bostock 2004) and in *Arabidopsis* stress-triggered increased concentration of abscisic acid induced susceptibility to *P. syringae* pv *tomato* (Mohr & Cahill 2007).

To dissect how salinity interacts with pathogen stress we determined the effects of sequentially applied salt stress and *PsI* infection in cucumber (*Cucumis sativus*). Infection development, shoot and root growth potential, the concentrations of chlorophyll and proline as well as electrolyte leakage, lipid peroxidation and H<sub>2</sub>O<sub>2</sub> production were studied.

## MATERIALS AND METHODS

### Plant material and the experimental design

Cucumber plants cv. Cezar were grown in plastic pots (400 cm<sup>3</sup>) filed with a peat-based substrate, in a growth chamber under irradiance of about 350  $\mu\text{E m}^{-2}\cdot\text{s}^{-1}$ , photoperiod 16/8h (day/night) and temperature 23°C. Three week-old plants were first exposed to salt stress and irrigated for seven days with 50 or 100 mM NaCl and thereafter infected with *PsI* (strain No IOR 1990 obtained from the Bank of Plant Pathogens of the Institute of Plant Protection in Poznań, Poland). Bacteria for inoculation were cultured for 24 h on King B medium at 28°C with vigorous shaking and centrifuged at 3500 g for 10 min. The bacterial pellet was washed twice and resuspended in sterile water, and adjusted to 10<sup>7</sup> cfu/ml. Three fully expanded leaves of cucumber were inoculated

with bacteria or sterile distilled water (control) using a needle-less hypodermic syringe. Analyses were made on the day of inoculation, i.e. after seven days of salt treatment (T0) and two (T2), five (T5) and seven (T7) days after inoculation. The development of infection as well as plant growth under stress conditions (length of shoots and roots, leaf size and dry weight) and the degree of leaf cell damage recognized by Evans blue staining (Yamamoto *et al.* 2001) were analyzed. Plant height and the central root length were measured with a metric ruler. Roots were carefully removed from soil, cleaned with water, spread along a ruler and the length of the root system of each plant was measured along the main root. Furthermore, the concentrations of chlorophyll (Porra *et al.* 1989), proline (Bates *et al.* 1973), electrolyte leakage (Masood *et al.* 2006) and lipid peroxidation based on the formation of thiobarbituric acid reactive substances (TBARS, Yagi 1982) were determined. Hydrogen peroxide was detected histochemically by using 3,3'-diaminobenzidine (DAB) according to Thordal-Christensen *et al.* (1997). Infection development and DAB-stained area, given as percentage of leaf area affected, were automatically quantified by an algorithm run in MATLAB. The leaf surface were also analysed using this computer software.

Each data point was the mean of six independent experiments ( $n=6$ ) and one plant per treatment was analysed in each experiment. Data were statistically analysed using Kruskal-Wallis one-way analysis of variance

and followed up by comparisons of mean ranks with P-values  $<0.05$  were considered significantly different.

## RESULTS AND DISCUSSION

### **Plant growth, chlorophyll content and infection development**

Inhibition of vegetative growth is one of the primary negative effects of salt stress and most annual crops are salt sensitive during vegetative development (Munns & Tester 2008, Haghighi *et al.* 2012). We found that seven-day salt stress reduced shoot elongation growth by 15% and by 20.5% ( $p>0.05$ ) for 50 mM NaCl and 100 mM NaCl, respectively. Leaf expansion decreased with salinity and in 100 mM NaCl-treated plants the leaf area was reduced by 43% when compared with control. In the case of roots, application of severe salt stress (100 mM NaCl) reduced the elongation growth by 25% ( $p>0.05$ ). (Table 1). These results confirm that leaf growth is usually more affected by salinity than root growth (Munns 2002). The subsequent biotic stress did not change shoot, leaf and root growth. Moreover leaves from NaCl-treated plants were denser (leaf area/leaf dry weight) with lower chlorophyll content (Table 1). The first symptoms of angular leaf spot of cucumber in the form of chlorotic spots were observed two days after inoculation on both NaCl-treated and non-treated plants. With ongoing pathogenesis these spots became larger and necrotic. Salinity favored the development of *PsI* infection (Fig. 1).

Table 1. Changes in vegetative growth and chlorophyll content in cucumber plants exposed to salt stress and *Pseudomonas syringae* pv *lachrymans* infection

| Treatment       | Shoot length<br>(cm) | Root length<br>(cm) | Leaf surface<br>(cm <sup>2</sup> ) | Leaf dry weight<br>(g) | Total<br>chlorophyll<br>(mg·g <sup>-1</sup> FW) | Chlorophyll a<br>(mg·g <sup>-1</sup> FW) | Chlorophyll b<br>(mg·g <sup>-1</sup> FW) |
|-----------------|----------------------|---------------------|------------------------------------|------------------------|-------------------------------------------------|------------------------------------------|------------------------------------------|
| Control         | 33.3±7.2a            | 54.0±24.0a          | 106±14.5a                          | 0.12±0.01a             | 2.33±0.28ac                                     | 1.67±0.32a                               | 0.66±0.13a                               |
| Psl             | 36.5±8.9a            | 54.1±17.7a          | 102.9±13.3a                        | 0.13±0.01ab            | 1.65±0.07abc                                    | 1.24±0.31ab                              | 0.41±0.12b                               |
| 50 mM NaCl      | 28.3±3.9a            | 54.3±10.9a          | 73.6±10.1ab                        | 0.12±0.01ab            | 1.68±0.03abc                                    | 1.20±0.25ab                              | 0.48±0.13ab                              |
| 50 mM NaCl+Psl  | 29.8±5.3a            | 60.4±15.0a          | 77.5±6.7ab                         | 0.14±0.02ab            | 1.32±0.07b                                      | 0.92±0.06b                               | 0.40±0.03b                               |
| 100 mM NaCl     | 26.5±9.6a            | 40.6±5.4a           | 60.7±10.9b                         | 0.12±0.01a             | 1.71±0.05ac                                     | 1.22±0.20ab                              | 0.49±0.10ab                              |
| 100 mM NaCl+Psl | 26.3±2.9a            | 39.9±12.2a          | 59.1±10.9b                         | 0.16±0.01b             | 1.47±0.10b                                      | 1.14±0.09ab                              | 0.33±0.07b                               |

Measurements were performed on the seventh day after the inoculation. Data represent the mean values (±SD) of six replicates (n=6). Means for each trait denoted by different letters are significantly different at  $P<0.05$ .

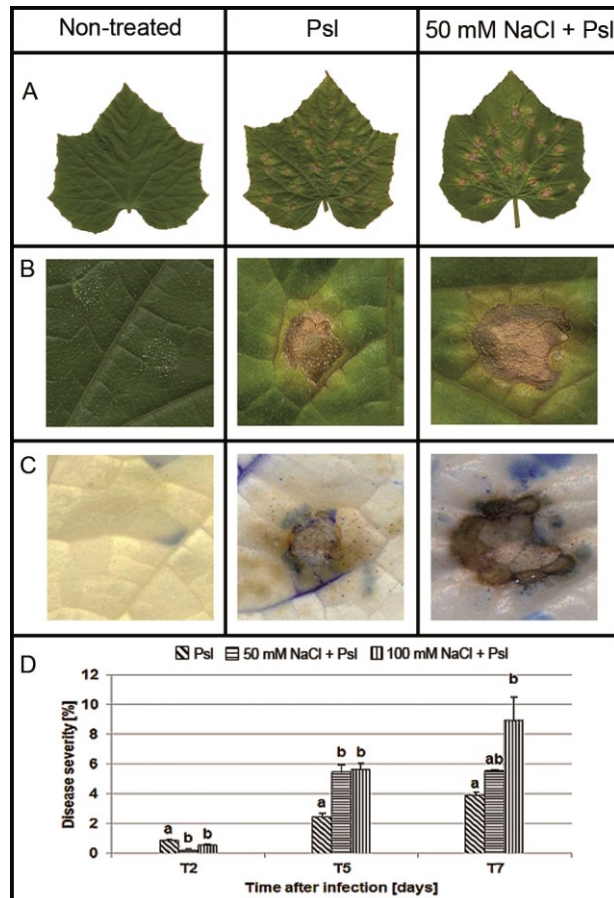


Fig. 1. Angular leaf spot symptoms on cucumber leaves recorded 7 days after inoculation (A, B) and the concomitant loss of plasma membrane integrity evaluated by Evans blue staining (C). The pictures show representative leaves. Similar results were obtained routinely. Severity of angular leaf spot caused by *Pseudomonas syringae* pv *lachrymans* on the second leaf of cucumber plants (D). Disease severity is expressed as the percentage of leaf area affected by lesions. Data represent the mean values ( $\pm$ SD) of seven replicates ( $n=7$ ). The bars with different letters for each time point after inoculation are significantly different from each other ( $P<0.05$ ).

Seven days after inoculation (T7) in 50 mM NaCl and 100 mM NaCl treated plants the necrotic lesions took up 5.54% and 8.95% of total leaf area, respectively and were more intense than in non-salinized plants (3.89%). Evans blue staining confirmed that in salinized plants the pathological

changes were not restricted to the inoculation site but also appeared in the surrounding tissues (Fig. 1).

Moreover, chlorophyll content in infected cucumber plants showed a general decreasing trend (Table 1) but the changes were significant only for chlorophyll b concentration in

infected non-salinized plants (62% of control) and for total chlorophyll in plants previously treated with 100 mM NaCl and then infected (86% of control).

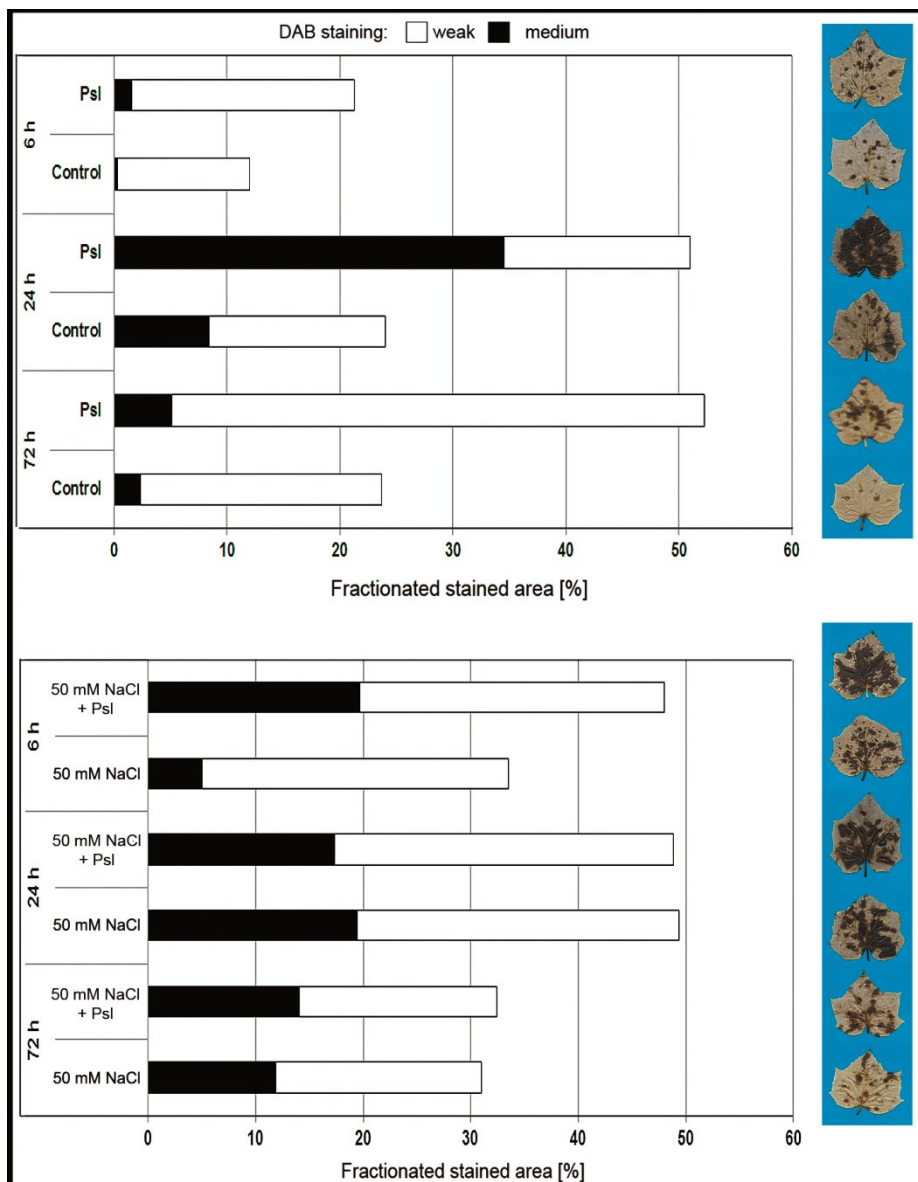


Fig. 2. Histochemical detection of  $H_2O_2$  with DAB staining in cucumber leaves. Representative scanned images of DAB-stained leaves (right) were analysed by a computer method to automatically quantify the  $H_2O_2$  staining patterns in leaves. The quantification is given as a percentage of stained area partitioned according to the weak and medium staining intensity (left).

These data might reflect a shift of redox balance towards prooxidative conditions placing a metabolic burden on plants exposed to salt. Infection induced an increase in  $H_2O_2$  production, however the time-course profiles differed in non-salinized and salinized plants. In non-salinized plants the upward trend was maintained for up to 72 hours after infection when the DAB-stained surface accounted for 52% of total leaf area. In NaCl-treated plants, biotic stress application induced a more pronounced generation of  $H_2O_2$  only 6 h after inoculation (Fig. 2). The unsuccessful attempt by infected NaCl-treated plants to suppress necrotic disease symptoms may be related to a compromised expression of  $H_2O_2$ -dependent signalling pathways critical to defence response

to biotrophic pathogens (Glazebrook 2005). Moreover, the negative interaction between abscisic acid-dependent signalling pathway activated under salt stress and salicylic acid-mediated defence against *Psl* could also contribute to this effect (Mohr & Cahill 2007).

### Lipid peroxidation

*Psl* infection caused TBARS accumulation in all plants, although with different intensity. In infected non-salinized plants a prolonged increase in TBARS content ranging from  $76.64 \text{ nmol} \cdot \text{g}^{-1} \text{ FW}$  (T2) to  $116.6 \text{ nmol} \cdot \text{g}^{-1} \text{ FW}$  (T7) was found. In salt-treated plants, a significantly increased concentration of TBARS was observed five days after inoculation in cucumber that previously received 50 mM NaCl (Fig. 3).

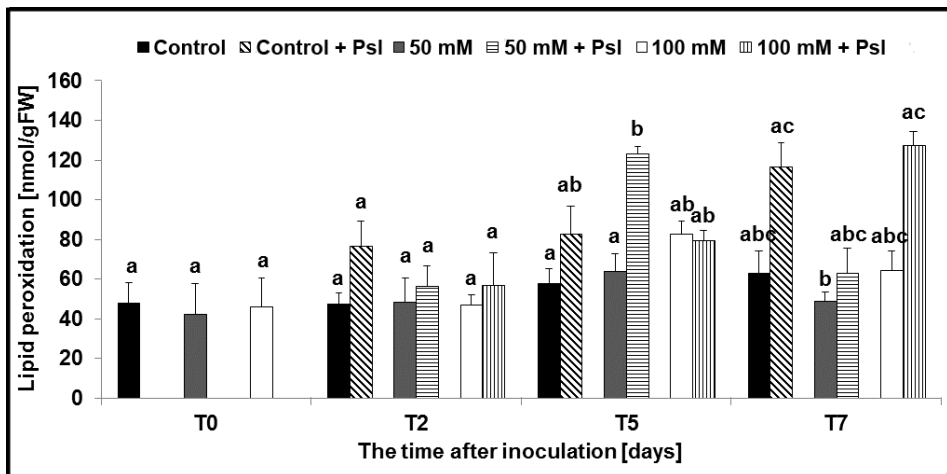


Fig. 3. Changes in lipid peroxidation in leaves of cucumber plants exposed to salt stress and *Pseudomonas syringae* pv *lachrymans* infection.

Data represent the mean values ( $\pm$ SD) of six replicates ( $n=6$ ). The bars with different letters for each time point after inoculation are significantly different from each other ( $P<0.05$ ).

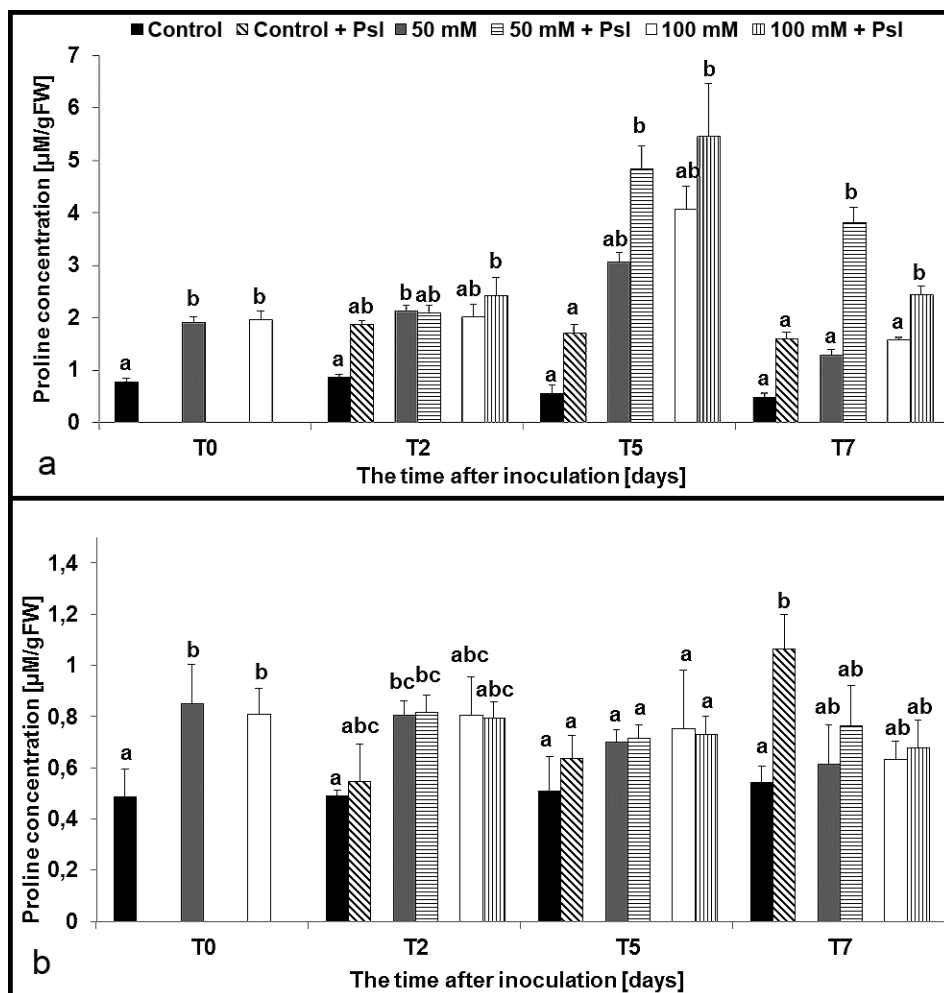


Fig. 4. Changes in proline content in leaves (A) and roots (B) of cucumber plants exposed to salt stress and *Pseudomonas syringae* pv *lachrymans* infection. Data represent the mean values ( $\pm$ SD) of six replicates ( $n=6$ ). The bars with different letters for each time point after inoculation are significantly different from each other ( $P < 0.05$ ).

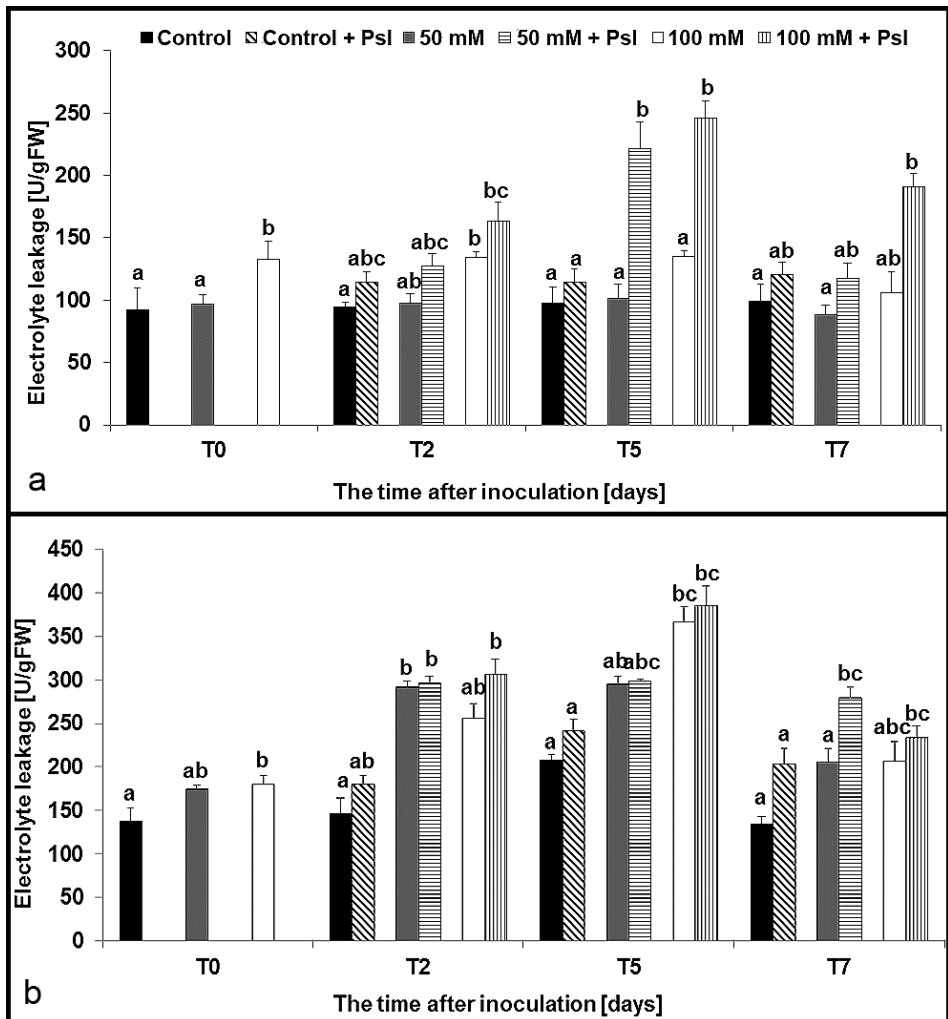


Fig. 5. Electrolyte leakage in leaves (A) and roots (B) of cucumber plants exposed to salt stress and *Pseudomonas syringae* pv *lachrymans* infection. Data represent the mean values ( $\pm$ SD) of six replicates ( $n=6$ ). The bars with different letters for each time point after inoculation are significantly different from each other ( $P<0.05$ ).

Keppler and Novacky (1985) also reported an intensive increase in TBARS concentration in *Psl*-infected cucumber plants. Although in our study lipid peroxidation, a well-known biochemical marker of oxidative stress, was not induced in cucumber leaves by NaCl treatment ( $T_0$ ), the pattern of infection-induced changes in TBARS content in salinized plants suggest that they were more prone to the oxidative degradation of membrane lipids.

### **Proline concentration**

The beneficial effects of proline accumulation during stress, related mainly to its osmoprotective, antioxidant and signalling roles, has been widely confirmed (Verbruggen & Hermans 2008). In our study salt stress ( $T_0$ ) increased the free proline concentration by approximately 60%. However, its content was always higher in leaves compared to roots. In infected plants proline content tended to increase in leaves along with the development of necrotic spots. The most pronounced effect was found at  $T_5$  in 50 mM and 100 mM NaCl-treated plants where its concentration was about 1.6- and 1.3-times higher than in leaves of non-salinized plants, respectively. Moreover, a significant increase in proline content was noted at  $T_7$  in roots of infected non-salinized plants (Fig. 4). Accumulation of proline could be an indication of disturbed physiological conditions triggered by abiotic and biotic stressors and confirm its role in local and systemic stress responses.

### **Electrolyte leakage**

Salt stress is known to modify the fluidity and permeability of mem-

branes (Mansour *et al.* 2002, Hasegawa *et al.* 2000). In this study an increase in relative electrolyte leakage, representing membrane damage caused by salt stress, was observed in leaves and roots of NaCl-treated plants (Fig. 5). Roots, being the first target of salt stress, were characterized by higher levels of electrolyte leakage than leaves, especially in  $T_5$ . Electrolyte leakage was also strongly enhanced after *Psl* infection in leaves of plants previously exposed to salinity stress and the maximum effect (100% of control) was found at  $T_5$ . These results suggest an increased degree of membrane disorganization in plants exposed to salinity and biotic stress.

## **CONCLUSIONS**

1. The negative contribution of salt stress to cucumber defence against *Pseudomonas syringae* pv *lachrymans* could be related to: (a) inhibition of plant growth with leaf expansion being the most sensitive to salinity, and reduction of chlorophyll content deteriorating plant performance under stress and (b) increased electrolyte leakage indicating cell membrane injury and prolonged  $H_2O_2$  accumulation in leaves implying perturbations in redox signalling involved in defence response to pathogen.
2. Proline and membrane disorganization, manifested by electrolyte leakage, are important components of local and systemic responses to both salt stress and infection.

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## INTERAKCJA MIĘDZY STRESEM SOLNYM A BAKTERYJNĄ KANCIASTĄ PLAMISTOŚCIĄ (*PSEUDOMONAS SYRINGAE* PV *LACHRYMANS*) U OGÓRKA

### Streszczenie

W pracy badano efekty sekwencyjnego działania stresu solnego i infekcji *Pseudomonas syringae* pv. *lachrymans* (Psl) u ogórka (*Cucumis sativus*). Analizowano rozwój infekcji, wzrost pędu i korzeni, stężenie chlorofilu i proliny oraz wyciek elektrolitów, peroksydację lipidów i generowanie  $H_2O_2$ . Rośliny ogórka poddawano stresowi solnemu, podlewając je przez siedem dni roztworem NaCl o stężeniu 50 mM lub 100 mM, a następnie zakażano zawiesiną bakterii Psl. Stres abiotyczny osłabiał odpowiedź obronną ogórka na infekcję. Największe nasilenie choroby stwierdzono u roślin traktowanych wcześniej 100 mM NaCl. Słabsze funkcjonowanie roślin zasolonych NaCl w warunkach stresu biotycznego mogło być spowodowane negatywnymi skutkami stresu solnego w postaci zahamowania wzrostu, a zwłaszcza rozwoju blaszki liściowej, obniżenia stężenia chlorofilu, zwiększenia wycieku elektrolitów i wydłużonego w czasie gromadzenia  $H_2O_2$  w liściach, wskazującego na zaburzenia homeostazy redoks. Różnice w odpowiedzi roślin kontrolnych i traktowanych NaCl na infekcję bakteryjną dotyczyły generowania  $H_2O_2$  i peroksydacji lipidów. Badania potwierdziły, że prolina jest ważnym elementem lokalnej i systemicznej odpowiedzi na stres solny i infekcję. Uzyskane wyniki poszerzają naszą wiedzę na temat odpowiedzi roślin na stres abiotyczny i biotyczny, działające w połączeniu.