INTERACTION BETWEEN SALT STRESS
AND ANGULAR LEAF SPOT

(PSEUDOMONAS SYRINGAE PV LACHRYMANS) IN CUCUMBER

Joanna CHOJAK¹, Elżbieta KUŹNIAK¹, Urszula ŚWIERCZ¹,
Joanna SEKULSKA-NALEWAJKO², Jarosław GOCŁAWSKI²

¹ Department of Plant Physiology and Biochemistry, Faculty of Biology and
Environmental Protection, University of Lodz, Banacha 12/16, 90-237 Lodz, Poland
² Computer Engineering Department, Technical University of Lodz, Stefanowskiego
18/22, 90-924 Lodz, Poland

Received: September 24, 2012; Accepted: November 2, 2012

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 H₂O₂ 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 H₂O₂ accumulation in leaves implying perturbations in redox homeostasis. The response of NaCl-treated and control plants to bacterial infection differed in terms of H₂O₂ 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* (*Psl*), causing the angular leaf spot disease, is an important biotrophic bacterial pathogen of *Cucurbitaceae* crops, especially cucumber. Increased occurrence of *Psl* 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 *Psl* 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$_2$O$_2$ production were studied.

**MATERIALS AND METHODS**

**Plant material and the experimental design**

Cucumber plants cv. Cezar were grown in plastic pots (400 cm$^3$) filled with a peat-based substrate, in a growth chamber under irradiance of about 350 μE m$^{-2}$·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 *Psl* (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 $10^7$ 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, Haghhighi 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 Psl 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 (cm) | Root length (cm) | Leaf surface (cm²) | Leaf dry weight (g) | Total chlorophyll (mg g⁻¹ FW) | Chlorophyll a (mg g⁻¹ FW) | Chlorophyll b (mg g⁻¹ FW) |
|------------------|-------------------|------------------|--------------------|---------------------|-------------------------------|----------------------------|----------------------------|
| Control          | 33.3±7.2a         | 54.0±24.0a       | 106±14.5a          | 0.12±0.01           | 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±0.01           | 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 (±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$_2$O$_2$ 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$_2$O$_2$ 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 $\text{H}_2\text{O}_2$ 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 $\text{H}_2\text{O}_2$ 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 $\text{H}_2\text{O}_2$-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 nmol·g$^{-1}$ FW (T2) to 116.6 nmol·g$^{-1}$ 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 (±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).](image-url)
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 (±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 (±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 (T₀), 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 (T₀) 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 T₅ 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 T₇ 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 membranes (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 T₅. 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 T₅. 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 H₂O₂ 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.
REFERENCES

Atkinson N.J., Urwin P.E. 2012. The interaction of plant biotic and abiotic stresses: from genes to the field. J. Exp. Bot. 63: 3523-3543. [DOI:10.1093/jxb/ers100]

Bates L.S., Waldren R.P., Teare I.D. 1973. Rapid determination of free proline for water-stress studies. Plant Soil 39: 205-208. [DOI: 10.1007/BF00018060]

Desprez-Loustau M.D., Marçais B., Nageleisen L.M., Piou D., Vannini A. 2006. Interactive effects of drought and pathogens in forest trees. Ann. For Sci. 63: 597-612. [DOI: 10.1051/forest:2006040]

Glazebrook J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Ann. Rev. Plant Physiol. Plant Mol. Biol. 51: 463-499. [DOI: 10.1146/annurev.phyto.43.040204.135923]

Haghighi M., Afifipour Z., Mozafarian M. 2012. The alleviation effect of silicon on seed germination and seedling growth of tomato under salinity stress. Veget. Crops Res. Bull. 76: 119-126. [DOI: 10.2478/v10032-012-0008-z]

Hasegawa P.M., Bressan R.A., Zhu J-K., Bohnert H.J. 2000. Plant cellular and molecular responses to high salinity. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51: 463-499. [DOI: 10.1146/annurev.arplant.51.1.463]

Knight H., Brandt S., Knight M.R. 1998. A history of stress alters drought calcium signalling pathways in Arabidopsis. Plant J. 16: 681-687. [DOI: 10.1046/j.1365-313x.1998.00332.x]

Keppler D.L., Novacky A. 1987. Involvement of membrane lipid peroxidation in the development of a bacterially induced hypersensitive reaction. Phytopathology 76: 104-108. [DOI: 10.1094/Phyto-76-104]

Mansour M.M., Salama F.K.H.A., Al-Mutawa M.M., Abou Hadid A.F. 2002. Effect of NaCl and polyamines on plasma membrane lipids of wheat roots. Biol. Plant. 45: 235-239. [DOI:10.1023/A:1015144607333]

Masood A., Shah N.A., Zeeshan M., Abraham G. 2006. Differential response of antioxidant enzymes to salinity stress in two varieties of Azolla (A. pinnata and A. filiculoides). Environ. Exp. Bot. 58: 216-222. [DOI: 10.1016/j.envexpbot.2005.08.002]

Mittler R. 2006. Abiotic stress, the field environment and stress combination. Trends Plant Sci. 11: 15-19. [DOI: 10.1016/j.tplants.2005.11.002]

Mohr P.G., Cahill D.M. 2007. Suppression by ABA of salicylic acid and lignin accumulation and the expression of multiple genes, in Arabidopsis infected with Pseudomonas syringae pv tomato. Funct. Integr. Genomics 7: 181-191. [DOI: 10.1007/s10142-006-0041-4]

Munns R. 2002. Comparative physiology of salt and water stress. Plant Cell Environ. 25: 239-250. [DOI: 10.1046/j.0016-8025.2001.00808.x]

Munns R., Tester M. 2008. Mechanisms of salinity tolerance. Ann. Rev. Plant Biol. 59: 651-81. [DOI: 10.1146/annurev.arplant.59.032607.092911]

Olczak-Woltman H., Schollenberger M., Mădăry W., Niemirowicz-Szczytt K. 2008. Evaluation of cucumber (Cucumis sativus) cultivars grown in Eastern Europe and progress in breeding for resistance to angular leaf spot (Pseudomonas syringae pv. lachrymans). Eur. J. Plant Pathol. 122: 385-393. [DOI 10.1007/s10658-008-9304-3]

Parvaiz A., Satyawati S. 2008. Salt stress and phyto-biochemical responses of plants – a review. Plant Soil Environ. 54: 89-99. [DOI: 10.1093/jpe/rt017]
Porra J.R., Thompson W.A., Kriedemann P.E. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim. Biophys. Acta 975: 384-394. [DOI: http://dx.doi.org/10.1016/S0005-2728(89)80347-0]

Thaler J.S., Bostock R.M. 2004. Interactions between abscisic-acid-mediated responses and plant resistance to pathogens and insects. Ecology 85: 48-58. [DOI: 10.1890/02-0710]

Thordal-Christensen H., Zhang Z., Wei Y., Collinge D.B. 1997. Subcellular localization of H$_2$O$_2$ in plants: H$_2$O$_2$ accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction.

INTERAKCJA MIĘDZY STRESEM SOLNYM A BAKTERYJNĄ KANCIASTĄ PLAMISTOŚCIĄ (**Pseu**domonas syringae pv **lachrym**ans) U OGÓRKA

**Streszczenie**

W pracy badano efekty sekwencyjnego działania stresu solnego i infekcji **Pseudomonas syringae** pv **lachrym**ans (Psl) u ogórka (**Cucumis sativus**). Analizowano rozwój infekcji, wzrost pędu i korzeni, stężenie chlorofilu i proliny oraz wyciek elektrolitów, peroksydację lipidów i generowanie H$_2$O$_2$. 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$_2$O$_2$ w liściach, wskazującego na zaburzenia homeostazy redoks. Różnice w odpowiedzi roślin kontrolnych i traktowanych NaCl na infekcję bakteryjną dotyczyły generowania H$_2$O$_2$ i peroksydacji lipidów. Badania potwierdziły, że proлина 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.