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Original scientific paper

The effect of cholic acid treatment on the oxidative status of soybean plants

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Abstract: The objective of this work was to study the effect of treatment of young soybean plants with cholic acid of different concentrations on their oxidative status. Young soybean plants, grown hydroponically for two weeks, were treated by adding cholic acid to the nutrient solution at the concentrations 20, 40, 60 and 80 mg/L, the control being without cholic acid. After one week, several parameters of the oxidative status were determined in the leaves and roots of the plants: contents of superoxide ($O_2^{\bullet-}$), hydroxyl radicals ($^{\bullet}OH$) and glutathione (GSH), lipid peroxidation (LP), the superoxide dismutase (SOD) activity and the soluble protein accumulation, as well as the contents of chlorophylls and carotenoids. Treatments with cholic acid increased $O_2^{\bullet-}$, LP, $^{\bullet}OH$ and GSH in the leaves of the treated plants, while only the OH content increased in the roots at higher cholic acid concentrations. The obtained results support the idea that cholic acid, as an elicitor of defence responses in plants, might act through the generation of an oxidative burst.

Keywords: cholic acid; soybean; oxidative status.

INTRODUCTION

Plants have evolved efficient mechanisms to combat pathogen attacks. One of the earliest responses to an attempted pathogen attack is the generation of an oxidative burst, which can trigger hypersensitive cell death. This is called a hypersensitive response (HR) and is considered a major element of plant disease resistance. The HR is thought to deprive the pathogens of their food supply and confine them to the initial infection site.[1] It occurs when a plant can specifically recognize the pathogen during an "incompatible" interaction.[2] The cell death is manifested as a rapid collapse of tissue and shows some typical morphological

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features: membrane blebbing, nucleus condensation, fragmentation of DNA, shrinkage of the cell, *etc*. Cell death is also a feature of disease symptoms but it occurs very late in the infection process during a 'compatible' interaction. The HR is not due to the action of pathogen virulence factors that kill the plant cells, but rather appears to be a form of programmed cell death (PCD) in plants.[3]

Recently, it was established that cholic acid is an elicitor of hypersensitive cell death, pathogenesis-related protein synthesis and phytoalexin accumulation and could induce defence responses in rice plants.[4] Elicitor molecules, beside inducing accumulation of phytoalexins, trigger a plant defence response called an oxidative burst, which involves the production of reactive oxygen species.[5,6] Bile acids can also promote the generation of reactive oxygen species and the increase in reactive oxygen species caused by bile acids is well documented only in mammalian tissues.[7,8] The effect of bile acids on plants, especially on the antioxidative status, has hitherto not been studied. Antioxidant systems are produced during interactions between pathogens and plant hosts.[9,10] The susceptibility of a plant to oxidative stress may depend on the overall balance between factors that increase oxidant generation and those cellular components that exhibit an antioxidant capability.[11]

The aim of this work was to study the effect on their oxidative status of the treatment of young soybean plants with cholic acid of different concentrations.

EXPERIMENTAL

All employed chemicals were of reagent grade, purchased from Merck (Darmstadt, Germany) or Sigma Aldrich.

Plant material and treatment

Soybean seeds, genotype Bečejka, were obtained from the Institute of Field and Vegetable Crops, Novi Sad, Serbia. Prior to germination, the seeds were surface sterilized by soaking in a 5 % solution of commercial bleach for 20 min and washed with distilled water. The seeds were sterilized again by dipping in 70 % ethanol for one minute, followed by soaking in commercial bleach for ten minutes and then rinsed three times with sterile distilled water. The seeds were germinated on wet paper towels in a thermostat for 3 days at 25 °C. Subsequently, the seedlings were transferred to pots with full nutrient solution (1 mmol/L $MgSO_4$, 3 mmol/L $Ca(NO_3)_2$, 0.19 mmol/L KH_2PO_4, 0.31 mmol/L $NH_4H_2PO_4$, 46 μmol/L B, 9 μmol/L Mn, 0.8 μmol/L Zn, 0.3 μmol/L Cu, 0.8 μmol/L Mo and 75 μmol/L Fe as Fe–EDTA) and grown in a controlled environment (temperature 25 °C, relative humidity 60 % and light intensity 16000 lux) for two weeks. The treatments with cholic acid were realised by adding cholic acid (as sodium cholate) to the nutrient solution at concentrations of 20, 40, 60 and 80 mg/L, the control plants being grown in nutrient solution without the addition of cholic acid. Samples of roots and leaves were taken for biochemical analyses 7 days after treatment.

Biochemical assays

For the determination of the oxidative status parameters, 1 g of fresh plant material was homogenized with 10 cm^3 0.10 M K_2HPO_4 at pH 7.0. After centrifugation at 15000 *g* for 10 min at 4 °C, aliquots of the supernatant were used for the biochemical assays.

A UV/visible spectrophotometer model 6105, Jenway, Dunmon, UK was used for the spectroscopic measurements.

The superoxide radical was measured by the inhibition of adrenalin auto-oxidation.[12] The hydroxyl radical was assayed by the inhibition of deoxyribose degradation.[13] The superoxide-dismutase (SOD; EC 1.15.1.1) activity was measured by monitoring the inhibition of nitroblue tetrazolium (NBT) reduction at 560 nm.[14] Lipid peroxidation (LP) was measured spectrophotometrically as malonyldialdehyde (MDA) production at 532 nm with thiobarbituric acid (TBA), as described by Placer *et al*.[15] and Gidrol *et al*.[16]. The chlorophyll and carotenoid contents were estimated according to Sairam *et al*.[8] and soluble protein content was determined according to Bradford.[17] The results of the oxidative status parameters were normalized per milligram of homogenate soluble protein; the chlorophyll and carotenoid contents are expressed per gram of fresh leaf matter (f.m.).

Statistical analyses

All determinations were made in triplicate and the values are expressed as the mean± ±standard deviation. The statistical significance was tested by one-way Anova, followed by comparisons of the means by the Duncan multiple range test ($p < 0.05$).

RESULTS AND DISCUSSION

One of the earliest biochemical changes observed after pathogen recognition is an increased production of reactive oxygen species (ROS), *i.e*., the so-called "oxidative burst". Numerous reports showed a rapid production of ROS in response to various infections or elicitor treatments.[18–20] The superoxide radical and H_2O_2 play various roles in the signal transduction pathway leading to HR cell death. They can act directly as antimicrobial compounds or induce a rapid cross--linking of proteins in the cell wall. They can also act as messengers, triggering, for example, modification of ion fluxes or the production of secondary messengers such as salicylic acid.[10] In this work, a similar response was observed in the leaves of soybean after treatments with cholic acid (Figs. 1 and 2). This is in agreement with the findings of Delledonne *et al*.[20] that inoculation of soybean cell suspensions with *Pseudomonas syringae* stimulated a strong oxidative burst.

ROS are known to be produced very locally and at high levels during the HR. Hence, in addition to their predicted signalling role, they can also act in a direct way. Cytochemical studies showed that the accumulation of ROS is related to rapid death of infected cells and is correlated with a rapid loss of membrane integrity.[10] The superoxide content in the leaves of the investigated plants increased significantly compared to the control with increasing cholic acid concentration. At the same time, this effect was not observed in the root tissue (Fig. 1). This is probably because the photosynthetic apparatus is absent from this plant organ. Photosynthesis is one of the most important metabolic pathways in which the generation of ROS occurs.[21] In addition, the activity of the antioxidant enzyme SOD was increased in the roots and the increase was higher compared to that observed in the leaves (Fig. 3). SOD is of utmost importance since it catalyzes the dismutation of the superoxide radical to molecular oxygen and hydro-

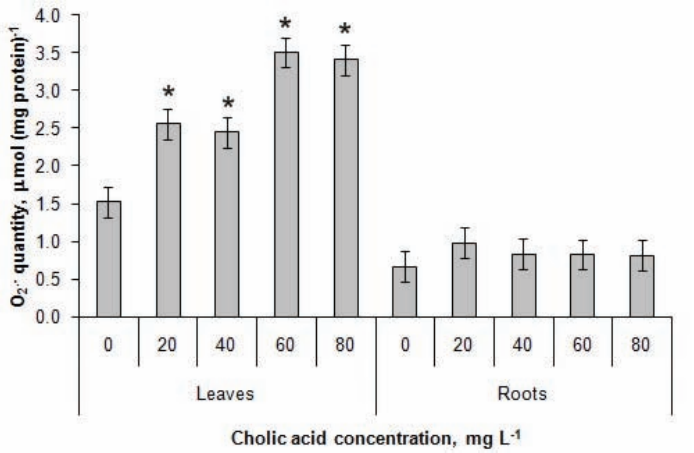

Fig 1. Superoxide content in leaves and roots of young soybean plants treated with cholic acid of different concentrations. Bars represent the standard deviation. Columns labelled with an asterisk are significantly different (Duncan test, $p < 0.05$) from the corresponding control.

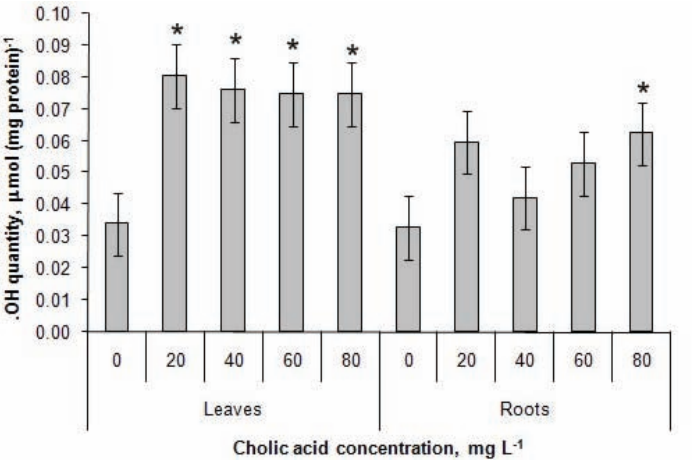

Fig. 2. •OH content in the leaves and roots of young soybean plants treated with cholic acid of different concentrations. Bars represent the standard deviation. Columns labelled with an asterisk are significantly different (Duncan test, $p < 0.05$) from the corresponding control.

gen peroxide,[14] thus preventing the formation of other, more toxic oxygen species, such as the •OH radical. Some other authors also reported an increase in the SOD activity in plants under oxidative stress.[22,23] Aggressive oxygen radicals are thought to cause lipid peroxidation (LP) and membrane damage that might be directly responsible for the collapse of the cells. This is in agreement with the present findings for the quantities of •OH and MDA, the main end product of LP (Figs. 2 and 4). The quantity of •OH increased significantly for most of the treatments with cholic acid, which was particularly pronounced in the

soybean leaves and to a smaller extent in the roots (only treatment with highest cholic acid concentration, 80 mg L^{-1}, had an effect). These results suggest that the most intensive process of destruction of cell membranes occurred in the leaves, due to higher quantity of the most reactive oxygen species – OH, thus confirming the hypothesis that the accumulation of ROS is in positive correlation with the peroxidation of membrane lipids.

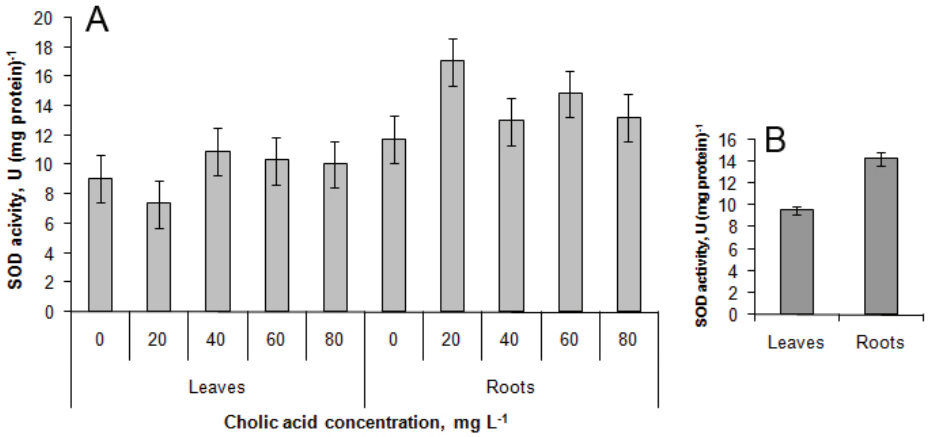

Fig. 3. A) SOD activity in leaves and roots of young soybean plants treated with cholic acid of different concentrations. Bars represent the standard deviation. B) Averages of SOD activity for all treatments in the leaves and roots of young soybean plants. Bars represent standard deviation. Values are significantly different (Duncan test, $p < 0.05$).

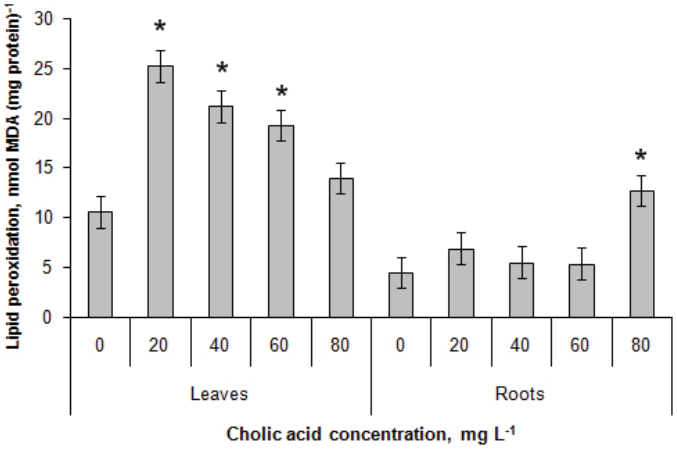

Fig. 4. Lipid peroxidation in the leaves and roots of young soybean plants treated with cholic acid of different concentrations. Bars represent the standard deviation. Columns labelled with an asterisk are significantly different (Duncan test, $p < 0.05$) from the corresponding control.

The tripeptide glutathione (γ-Glu–Cys–Gly) is involved in many aspects of metabolism: removal of hydroperoxides, protection from ionizing radiation, maintenance of the sulphhydryl status of proteins, complexation of xenobiotic or endogenous reactive compounds, aiding in their detoxification and excretion, *etc*.[24] Many of these functions are accomplished by reactions at the cysteinyl sulphhydryl group, catalyzed by glutathione-requiring enzymes. The obtained results showed that the glutathione (GSH) content increased significantly in soybean leaves on cholic acid treatment (Fig. 5). Even the lowest applied concentration of cholic acid (20 mg L^{-1}) caused a significant increase in the GSH content in soybean roots compared to the control. It seems that GSH plays an active role in the leaves in the detoxification and removal of ROS produced by cholic acid treatment.

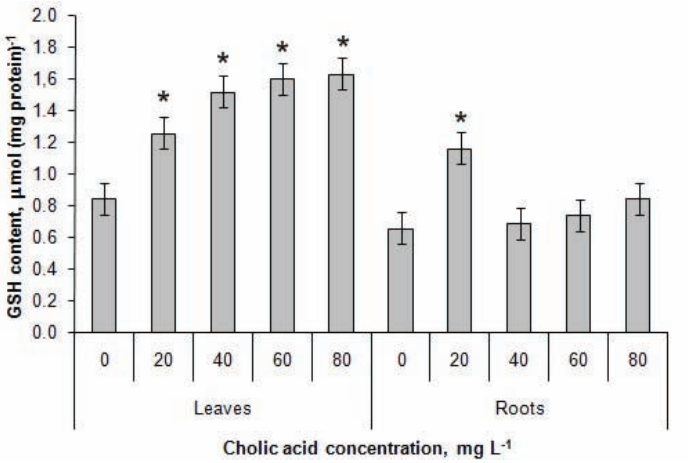

Fig. 5. GSH content in the leaves and roots of young soybean plants treated with cholic acid of different concentrations. Bars represent the standard deviation. Columns labelled with an asterisk are significantly different (Duncan test, $p < 0.05$) from the corresponding control.

The chlorophyll and carotenoid contents in soybean leaves increased due to cholic acid treatment, being highest with the treatment of 80 mg L^{-1} (Fig. 6). Some other authors[8,25] reported a decrease in the content of plant pigments under oxidative stress conditions. It seems that under the present experimental conditions, a *de novo* biosynthesis of chlorophylls and carotenoids occurred in the leaves of soybean as a response to cholic acid treatment. These mechanisms should be further investigated since there are no previous studies in this area.

CONCLUSIONS

Increased quantities of $O_2^{\bullet -}$, LP and $^{\bullet}OH$ in leaves of treated plants support the idea that cholic acid, as an elicitor of defence responses, could act through the generation of an oxidative burst. The GSH content increased significantly in soy-

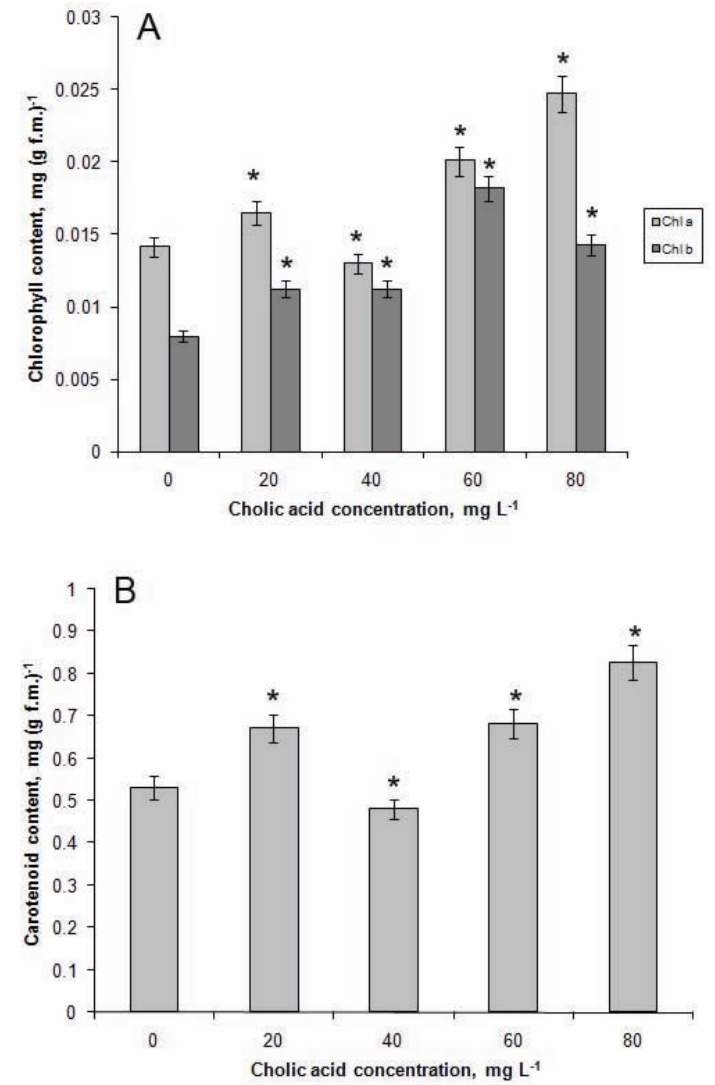

Fig. 6. Chlorophyll *a* and *b* (A) and carotenoid (B) content in the leaves of young soybean plants treated with cholic acid of different concentrations. Bars represent the standard deviation. Columns labelled with an asterisk are significantly different (Duncan test, $p < 0.05$) from the control.

bean leaves as affected by cholic acid treatments, which could mean that GSH plays an active role in leaves in the detoxification and removal of ROS produced because of the treatments.

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ИЗВОД

ЕФЕКАТ ТРЕТМАНА ХОЛНОМ КИСЕЛИНОМ НА ОКСИДАТИВНИ СТАТУС БИЉАКА СОЈЕ

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Циљ овог рада је био да се испита ефекат третмана различитим концентрацијама холне киселине на оксидативни статус младих биљака соје. Младе биљке соје, узгајане хидропонски две недеље, третиране су додавањем холне киселине у хранљиви раствор у концентрацијама од 20, 40, 60 и 80 mg/L, док код контролних биљака није додавана холна киселина. Недељу дана након почетка третмана одређено је неколико показатеља оксидативног статуса у листовима и корену биљака: количина супероксида ($O_2^{\bullet-}$) и хидроксил радикала ($^{\bullet}OH$), као и глутатиона (GSH), липидна пероксидација (LP), активност супероксид-дисмутазе (SOD), садржај растворљивих протеина, хлорофила и каротеноида. Третмани холном киселином довели су до повећања $O_2^{\bullet-}$, LP, $^{\bullet}OH$ и GSH у листовима биљака, док је у корену утврђен повећани садржај $^{\bullet}OH$ у третманима са већим концентрацијама холне киселине. Добијени резултати говоре у прилог претпоставци да холна киселина као молекул покретач може деловати путем стварања реактивних кисеоничних врста код биљака.

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REFERENCES

1. E. M. Govrin, A. Levine, *Curr. Biol*. **10** (2000) 751
2. D. Pontier, C. Balague, D. Roby, *C. R. Acad. Sci Paris* **321** (1998) 721
3. J. T. Greenberg, *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **48** (1997) 525
4. J. Koga, H. Kubota, S. Gomi, K. Umemura, M. Ohnishi, T. Kono, *Plant Physiol.* **140** (2006) 1475
5. K. A. Blee, S. C. Jupe, G. Richard, A. Zimmerlin, D. R. Davies, G. P. Bolwell. *Plant Mol. Biol.* **47** (2001) 607
6. T. Kawano, R. Pinontoan, N. Uozumi, Y. Morimitsu, C. Miyake, K. Asada, S. Muto, *Plant Cell Physiol*. **41** (2000) 1259
7. R. J. Sokol, B. M. Winklhofer-Roob, M. W. Devereaux, J. M. McKim Jr., *Gastroenterology* **109** (1995) 1249
8. C. M. Palmeira, A. P. Rolo, *Toxicology* **203** (2004)1
9. S. I. Kwon, A. J. Anderson, *Physiol. Mol. Plant Pathol*. **58** (2001) 73
10. Dj. Malenčić, J. Miladinović, M. Popović, *Cent. Eur. J. Biol*. **3** (2008) 155
11. R. K. Sairam, D. V. Singh, G. C. Srivastava, *Biol. Plant*. **47** (2003/4) 61
12. H. P. Misra, I. Fridovich, *J. Biol. Chem*. **247** (1972) 3170
13. K. H. Cheesman, A. Beavis, H. Esterbaurer, *Biochem. J*. **252** (1988) 649
14. C. N. Giannopolitis, S. K. Ries, *Plant Physiol*. **59** (1977) 309
15. Z. A. Placer, L. L. Cushman, B. C. Johnson, *Anal. Biochem*. **16** (1966) 359
16. H. Gidrol, H. Sergihini, B. Nobhani, B. Mocquot, P. Mazilak, *Physiol. Plant*. **76** (1987) 591
17. M. M. Bradford, *Anal. Biochem*. **72** (1976) 248
18. N. Doke, *Physiol. Plant Pathol*. **23** (1983) 359

19. C. Lamb, R. A. Dixon, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48** (1997) 251
20. M. Delledonne, J. Zeier, A. Marocco, C. Lamb, *Proc. Natt. Acad. Sci. USA* **98** (2001) 13454
21. B. Halliwell, *Chloroplast Metabolism*, Oxford University Press, New York, 1984, p. 160
22. S. Gupta, R. P. Webb, A. S. Holaday, R. D. Allen, *Plant Physiol.* **103** (1993)1067
23. H. M. Kang, E. Saltveit, *Plant Cell Environ.* **25** (2002) 1233
24. M. Arias, W. B. Jakoby, *Glutathione: Metabolism and Function*, Raven Press, New York, 1976, p. 103
25. D. Štajner, M. Popović, M. Štajner, *Biol. Plant.* **47** (2003/4) 575.