BIOLOGIA PLANTARUM 45 (2): 303-306, 2002

BRIEF COMMUNICATION

Reddening of cotton (*Gossypium hirsutum* L.) leaves

A. EDREVA*,[1], A. GÜREL**, E. GESHEVA* and H. HAKERLERLER***

*D. Kostoff Institute of Genetics, Bulgarian Academy of Sciences, BG-1113 Sofia, Bulgaria**
*Ege University, Faculty of Agriculture, Department of Field Crops, 35100 Bornova - Izmir, Turkey***
*Ege University, Faculty of Agriculture, Department of Plant Nutrition, 35100 Bornova - Izmir, Turkey****

Abstract

Reddening of leaves is a physiological disorder in cotton induced by different abiotic stresses. Dramatic biochemical changes occurred in reddening leaves: strong accumulation of anthocyanins and drop of chlorophyll content, important increase of proline content and peroxidase activity. The lipid peroxide content indicative of membrane fragmentation was decreased. In this way a multicomponent system encompassing anthocyanins, proline, and peroxidase may act coordinately to overcome abiotic stress in cotton.

Additional key words: abiotic stress, anthocyanins, chlorophylls, lipid peroxides, peroxidase, proline.

Reddening of cotton leaves is a physiological disorder observed in some regions of India and Sudan (Dastur *et al.* 1960, Ishag *et al.* 1987) and in the last ten years also in the Aegean region of Turkey. It leads to disturbance of plant growth and development and finally too important economic losses. The phenotypic expression of reddening has a phase-dependent pattern. The first symptoms – appearance of reddish shades in the normal green colour of leaves – are observed already in an early phase of cotton growing; maximal expression of red colouring of both bottom and upper leaves is observed in adult plants at the end of vegetation period. According to observations of Gürel *et al.* (unpublished) reddening of cotton leaves in some fields of the Aegean region is provoked by high soil salinity resulting in osmotic stress in plants. Other abiotic factors, such as mineral deficiency, temperature, and irradiation, may also be of importance.

The abnormal red colouring of leaves may be due to accumulation of red pigments from the flavonoid (C_6-C_3-C_6) group, namely anthocyanins, accompanied by chlorophyll (Chl) degradation. Increased synthesis of anthocyanins in vegetative plant parts as a response to various stress stimuli and their protective role was reported by Dixon and Paiva (1995). The involvement of other plant components (proline, peroxidase, lipid peroxides) may also be hypothesised. Regulation of osmolarity and cell wall plasticity, stabilisation of membranes and proteins, antioxidant, antiradical and signal functions are accomplished by these molecules, important in stress responses of plants (Gaspar *et al.* 1986, Low and Merida 1996, Hare *et al.* 1998, Cosgrove 1998, Verma 1999).

Thus, the aim of the present work was to assess the activity of peroxidase (PO; E.C.1.11.1.7) and the contents of anthocyanins, Chls, proline, and lipid peroxides in cotton leaves exhibiting symptoms of reddening in comparison with green leaves of plants of the same age.

Cotton (*Gossypium hirsutum* L.) plants cv. Nazilli 84 were grown in two locations of the Aegean region (Söke and Menemen) under conditions appropriate for the culture. High soil salinization is characteristic for some fields of these locations. Samples (upper leaves) were taken from 50-60 uniformly developed plants at the end of vegetation period (middle of September) when leaf

Received 3 April 2001, *accepted* 6 June 2001.
Abbreviations: Chl - chlorophyll, d.m. - dry mass, IEU - International enzyme units, PO - peroxidase, UV - ultra-violet.
Acknowledgements: We thank Tübitak and Izmir Itas-Technopark (Turkey) as well as the Bulgarian Academy of Sciences for financial support of a Joint research and development project (2000 - 2002).
[1]Fax (+359) 2 757087, e-mail: ttsonev@obzor.bio21.bas.bg

reddening is fully expressed. Two stages of reddening were assayed: light symptoms (presence of small, slightly red colored spots taking no more than 1/10 of leaf lamina) and severe symptoms (formation of large, diffuse, deeply red coloured spots covering about 1/2 of leaf lamina). In each stage the areas of leaves bearing symptoms of reddening were cut out and analysed. Upper leaves of plants of the same age without symptoms of reddening were used as controls.

Total anthocyanin content was determined as described by Hodges and Nozzolillo (1996). One g fresh material was extracted with 1 % HCl in methanol (m/v). The absorbance was read at 536 nm, a correction for phaeophytin absorbance at 600 nm being introduced. Anthocyanin content [mmol kg^{-1}(d.m.)] was calculated using the molar extinction coefficient of 33 000. Absorption spectra of the extract in the 400 - 800 nm light region were also taken. Total Chl content was estimated in the same extract (Hodges and Nozzolillo 1996). The absorbance was read at 660 nm, and the Chl content [mmol kg^{-1}(d.m.)] was calculated using the molar extinction coefficient of 90 100. For determining peroxidase activity, fresh material (1 g) was homogenised with 3 cm^3 of 0.05 M Tris-glycine buffer, pH 8.3, containing 17 % sucrose (m/v) in the presence of *Dowex 1X8* (200 - 400 mesh) to remove the phenols. The homogenate was centrifuged for 50 min at 20 000 *g* and the supernatant was saved. The protein content was estimated according to Lowry *et al.* (1951). The enzyme activity was determined by the method of Herzog and Fahimi (1973) using 1 mM 3,3'-diaminobenzidine tetrahydrochloride as substrate. The concentration of H_2O_2 in the reaction mixture was 1.3 mM. The kinetics of the reaction were monitored at 465 nm using a UV-VIS recording spectrophotometer. The PO activity was expressed as international enzyme units (IEU) where one unit of peroxidase catalyzed the decomposition of 1 μM H_2O_2 per minute at 25 °C. To determine proline content, the method of Bates *et al.* (1973) was used. Fresh material (0.5 g) was grinded with 10 cm^3 3 % solution (m/v) of sulfosalicylic acid. The reaction was carried out with 2 cm^3 of the filtrate, 2 cm^3 acetic acid, and 2 cm^3 acidic ninhydrine reagent. After incubation in a boiling water bath for 1 h, the reaction product was extracted with 2 cm^3 toluene and the absorbance was read at 518 nm. Lipid peroxide content was determined according to the method of Cakmak and Horst (1991) based on the interaction of malonyldialdehyde (product of membrane lipid breakdown) with thiobarbituric acid. Fresh material (0.3 g) was homogenized with 3 cm^3 0.1 % solution (m/v) of trichloroacetic acid. The reaction was carried out with 0.5 cm^3 of the supernatant and 1.5 cm^3 20 % solution (m/v) of trichloroacetic acid containing 0.5 % (m/v) thiobarbituric acid. After incubation in a boiling water bath for 30 min, the extinction of the solution was measured at 532 nm. The extinction at 600 nm was subtracted from this value as a correction factor. For calculation of the content of lipid peroxides [mmol (malonyldialdehyde) kg^{-1}(d.m.)], molar extinction coefficient of 155 000 was used. For all absorbance measurements, as well as for the kinetics of PO reaction *Shimadzu* (Tokyo, Japan) *UV-1601* spectrophotometer was used

Experiments were repeated three times, with four replicates per experiment, using leaves of about 15 plants per replicate. Significance of differences was given by the Student's test at $P \leq 1$ %* or 5 %.

An important accumulation of anthocyanins was registered in the stage of light symptoms of reddening. The development of severe symptoms resulted in a dramatic (662 %) increase in anthocyanin content (Table 1). In contrast to anthocyanins, the content of total Chl (*a*+*b*) continuously declined to 63 % of control in the stage of light symptoms and to 36 % in the stage of severe reddening (Table 1). The content of proline revealed a tendency to decrease under mild stress (slight reddening), but at severe reddening an important accumulation of proline was observed (255 % of controls) (Table 1). In a separate experiment with leaves of ultra-severe symptoms (deep reddening occupying more than 3/4 of leaf surface) a 10 fold increase of proline was scored. In reddening leaves the lipid peroxide content followed a trend to decrease from the stage of light symptoms to the stage of severe symptoms. The activity of PO was strongly

Table 1. Contents of total anthocyanins and chlorophylls, proline, and lipid peroxides [mmol kg^{-1}(d.m.)] and activity of peroxidase [IEU] in cotton leaves with light and severe symptoms of reddening. Data are also presented as percent of control (leaves without symptoms of reddening). Means of three experiments, each with four replicates. Standard deviations are less than 10 % of the means. Values in the same vertical column followed by different letters are significantly different at $P \leq 1$ % (*) and 5 %. Data for Menemen location are presented.

Variants	Total anthocyanins	[%]	Total Chl (*a* + *b*)	[%]	Proline	[%]	Lipid peroxides	[%]	Peroxidase	[%]
Controls	1.00 a	100	3.20 a	100	10.70 a	100	0.28 a	100	20.04 a	100
Light symptoms of reddening	2.58 b*	258	2.03 b*	63	7.93 b	74	0.19 b	68	45.57 b*	227
Severe symptoms of reddening	6.62 c*	662	1.16 c*	36	27.28 c*	255	0.16 b	57	77.53 c*	387

enhanced (227 % of controls) already at the stage of light symptoms and the activity was even higher at severe reddening.

Thus the red colouring of cotton leaves was due to an important anthocyanin accumulation accompanied by a decrease in Chl content. Similar responses were observed in other plants following cold, high irradiance, and UV-radiation stresses (Dixon and Paiva 1995), mineral deficiencies (Hodges and Nozzolillo 1996), and increased osmotic pressure (Cormier and Do 1990, Suzuki 1995). Being mainly vacuolarly localised water-soluble molecules, anthocyanins may act as compatible solutes and contribute to the regulation of osmotic homeostasis under osmotic stress. Moreover, antioxidant and active oxygen scavenging properties of anthocyanins were established (Darmon *et al.* 1990, Okuda 1993, Yamasaki *et al.* 1996); thus anthocyanins interfere with oxidative stress induced by improper temperature, irradiance, and osmotic and high salinity constraints (Bohnert *et al.* 1999, Verma 1999).

The accumulation of proline in leaves with severe symptoms of reddening may serve similar roles. Abundant information about increased synthesis and reduced degradation of proline in different stress situations, particularly in those related to osmotic disbalance (drought, heat, cold, high salinity) is available (Hare *et al.* 1998, Verma 1999, Nanjo *et al.* 1999, Hernandez *et al.* 2000, Ramanjulu and Sudhakar 2000). Proline is a ubiquitous compatible solute acting as an osmoregulator. Recently, other functions of proline were reported: stabilisation of membranes and proteins, built-up of cell wall proteins, balancing the ratio of $NAD(P)^+/NAD(P)H$ (redox signalling), C and N storage, as well as active oxygen scavenging (Hare *et al.* 1998, Verma 1999, Hasegawa *et al.* 2000). These functions may be more important than osmoregulation if assuming that osmotic constraint induces a state of oxidative stress (Verma 1999). The assumption of protective action of both anthocyanins and proline in cotton is substantiated by the fact that in red leaves no accumulation of lipid peroxides indicative of membrane damage and fragmentation is observed. Moreover, even in the stage of severe reddening Chls are not completely lost. The fact that in the stage of light symptoms of reddening proline content was not increased was explained by Hare *et al.* (1998): cycling between proline and its precursors with no net accumulation of proline may be an important homeostatic mechanism to forestall redox imbalance associated with mild stress.

The enhancement of PO activity in reddening leaves may also have a protective function. PO, acting as a H_2O_2 scavenger, is involved in the regulation of the overall active oxygen pool, and *via* H_2O_2 in cell signalling. The PO-H_2O_2 system is also important for regulation of cell wall plasticity; it catalyzes the cross-linking of cell wall by formation of diferulate bonds between polysaccharides in cell wall and by built-up of isodityrosine bridges between the tyrosine residues of cell wall proteins (Gaspar et al. 1986, Cosgrove 1998). Marshall *et al.* (1999) showed that insolubilisation of cell wall proteins and improved cell wall elasticity and turgor following osmotic stress are consistent with a role of PO in these phenomena. PO is important in plant responses to abiotic stresses (Castillo 1992), including high salinity (Mittal and Dubey 1991, Lutts and Guerrier 1995).

Our results suggest that a multicomponent system encompassing anthocyanins, proline, and PO may act coordinately to overcome the stress caused by high soil salinity or other environmental factors in cotton plants.

References

Bates, L.S., Waldren, R.P., Teare, I.D.: Rapid determination of free proline for water-stress studies. - Plant Soil **39**: 205-207, 1973.

Bohnert, H.J., Su, H., Shen, B.: Molecular mechanisms of salinity tolerance. - In: Shinozaki, K., Yamaguchi-Shinozaki, K. (ed.): Molecular Responses to Cold, Drought, Heat and Salt Stress in Higher Plants. Pp. 29-60. R.G. Landes Co., Austin 1999.

Cakmak, I., Horst, W.J.: Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root of soybean. - Physiol. Plant. **83**: 463-468, 1991.

Castillo, F.J.: Peroxidases and stress. - In: Penel, C., Gaspar, T., Greppin, H. (ed.): Plant Peroxidases 1980-1990. Pp. 187-203. University of Geneva, Geneva 1992.

Cormier, F., Do, C.B.: Anthocyanin composition of grape (*Vitis vinifera*) cell suspension grown under conditions of osmotic stress. - Bull. Groupe Polyphénols **15**: 91-94, 1990.

Cosgrove, D.J.: Relaxation in a high-stress environment: the molecular bases of extensible cell wall and cell enlargement. - Plant Cell **9**: 1031-1041, 1998.

Darmon, N., Fernandez, Y., Canal, M.T., Mitjavila, S.: Activité antiradicalaire des flavonoïdes vis-à-vis de l'anion superoxyde et du radical hydroxyle. - Bull. Groupe Polyphénols **15**: 158-162, 1990.

Dastur, R.H., Asana, R.D., Sawhney, K., Sikka, S.M., Vasudeva, R.S., Khan, Q., Rao, V.P., Setti, B.L.: Cotton in India. A Monograph. - Indian Central Cotton Committee, Bombay 1960.

Dixon, R.A., Paiva, N.L.: Stress-induced phenylpropanoid metabolism. - Plant Cell **7**: 1085-1097, 1995.

Gaspar, T., Penel, C., Greppin, H.: Peroxidases: structures and catalytic reactions, biosynthesis, transport and location, physiological roles. - Bull. Groupe Polyphénols **13**: 159-176, 1986.

Hare, P.D., Cress, W.A., Van Staden, J.: Dissecting the roles of

osmolyte accumulation during stress. - Plant Cell Environ. **21**: 535-553, 1998.

Hasegawa, P.M., Bressan, R.A., Zhu, J.-K., Bohnert, H.J.: Plant cellular and molecular responses to high salinity. - Annu. Rev. Plant Physiol. Plant mol. Biol. **51**: 463-499, 2000.

Hernandez, S., Deleu, C., Larher, F.: Accumulation de proline dans les tissus foliaires de tomate en réponse de la salinité. - Compt. rend. Acad. Sci. Paris Sér. D **323**: 551-557, 2000.

Herzog, V., Fahimi, H.D.: A new sensitive colorimetric assay for peroxidase using 3,3'- benzidine as hydrogen donor. - Anal. Biochem. **55**: 554-562, 1973.

Hodges, D.M., Nozzolillo, C.: Anthocyanin and anthocyanoplast content of cruciferous seedlings subjected to mineral nutrient deficiencies. - J. Plant Physiol. **147**: 749-754, 1996.

Ishag, H.M., Ayoub, A.T., Said, M.B.: Cotton leaf reddening in the irrigated Gezira. - Exp. Agr. **23**: 207-217, 1987.

Low, P.S., Merida, J.R.: The oxidative burst in plant defense: Function and signal transduction. - Physiol. Plant. **96**: 533-542, 1996.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. - J. biol. Chem. **193**: 265-275, 1951.

Lutts, S., Guerrier, G.: Peroxidase activities of two rice cultivars differing in salinity tolerance as affected by proline and NaCl. - Biol. Plant. **37**: 577-586, 1995.

Marshall, J.G., Dumbroff, E.B., Thatcher, B.J., Martin, B., Rutledge, R.G., Blumwald, E.: Synthesis and oxidative insolubilization of cell-wall proteins during osmotic stress. - Planta **208**: 401-408, 1999.

Mittal, R., Dubey, R.S.: Behaviour of peroxidases in rice: changes in enzyme activity and isoforms in relation to salt tolerance. - Plant Physiol. Biochem. **29**: 31-40, 1991.

Nanjo, T., Kobayashi, M., Yoshiba, Y., Sanada, Y., Wada, K., Tsukaya, H., Kakubari, Y., Yamaguchi-Shinozaki, K., Shinozaki, K.: Biological functions of proline in morphogenesis and osmotolerance revealed in antisense transgenic *Arabidopsis thaliana*. - Plant J. **18**: 185-193, 1999.

Okuda, T.: Natural polyphenols as antioxidants and their potential use in cancer prevention. - In: Scalbert, A. (ed.): Polyphenolic Phenomena. Pp. 221-235. INRA, Paris 1993.

Ramanjulu, S., Sudhakar, C.: Proline metabolism during dehydration of two mulberry genotypes with contrasting drought tolerance. - J. Plant Physiol. **157**: 81-85, 2000.

Suzuki, M.: Enhancement of anthocyanin accumulation by high osmotic stress and low pH in grape cells (*Vitis* hybrids). - J. Plant Physiol. **147**: 152-155, 1995.

Verma, D.P.S.: Osmotic stress tolerance in plants: role of proline and sulfur metabolism. - In: Shinozaki, K., Yamaguchi-Shinozaki, K. (ed.): Molecular Responses to Cold, Drought, Heat and Salt Stress in Higher Plants. Pp. 153-168. R.G. Landes Co., Austin 1999.

Yamasaki, H., Uefuji, H., Sakihama, Y.: Bleaching of the red anthocyanin induced by superoxide radical. - Arch. Biochem. Biophys. **332**: 183-186, 1996.