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Photosynthetic changes that occur during aging of cypress (*Cupressus sempervirens* L.) needles

N. LA PORTA[*], M. BERTAMINI[*], N. NEDUNCHEZHIAN[**], and K. MUTHUCHELIAN[*,**,***]

Istituto Agrario di San Michele all' Adige, 38010 San Michele all' Adige, Italy[*]
Center for Biodiversity and Forest Studies, School of Energy, Environment and Natural Resources, Madurai Kamaraj University, Madurai 625 021, India[**]

Abstract

2-years-old cypress needles (A2) were physiologically most active with regard to net photosynthetic (P_N) and electron transport rates. Variable to maximum fluorescence (F_v/F_m) ratios of dark-adapted needles were higher in A2 needles than in current year (A1) or senescent (A4) needles. Lower F_v/F_m values in these stages seemed to be caused not by photoinhibition but by a low photochemical capacity as suggested from the chlorophyll (Chl) *a*/*b* ratios. In isolated thylakoids, lower rates of whole chain and photosystem 2 (PS2) activities were observed in A4 needles, while higher rates were observed in A2 needles. A similar trend was noticed for contents of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC) and total soluble proteins. The artificial exogenous electron donor Mn^{2+} failed to restore the loss of PS2 activity in 3-year-old (A3) and A4 needles, while diphenylcarbazide and NH_2OH significantly restored the loss of PS2 activity. The marked loss of PS2 activity in A4 needles was primarily the result of the loss of 33, 28–25, 23, and 17 kDa polypeptides. A marked loss of RuBPC activity in A4 needles is mainly due to the loss of 15 (SSU) and 55 (LSU) kDa polypeptides.

Additional key words: chlorophyll fluorescence; diphenylcarbazide; donor side; electron transport; photosystems 1 and 2; polyacrylamide gel electrophoresis; polypeptides; ribulose-1,5-bisphosphate carboxylase/oxygenase.

Introduction

Needle characteristics of temperate tree species vary along macro-environmental gradients. In temperate forest ecosystems, needle characteristics vary with latitude, altitude, soil fertility, and amount and distribution of rainfall over the year. On a local scale, needle characteristics vary with spatial and temporal distribution of constituent species, height of the forest, irradiance, and needle age (Larcher 2000). Ontogenetic changes of leaves with respect to photosynthetic properties of forest trees have often been studied (Šesták 1985, Kutík *et al*. 1993, 1996, Mazari and Camm 1993, Sobrado 1996, Kutík 1998). Net photosynthetic rates (P_N) typically increase as leaf expansion occurs and maximum P_N is achieved prior to the time of full expansion with rates often declining when leaves become senescent (Constable and Rawson 1980, Šesták 1985, Roper and Kennedy 1986, Sobrado 1996, Kutík 1998). P_N changes with individual leaf age (Davis and McCree 1978, Kennedy and Johnson 1981, Sobrado 1996) as well as on a whole canopy basis during the growing season (Christy and Porter 1982, Wells 1988). As leaves age further, photosynthetic capacity, stomatal conductance (Šesták 1985), leaf dry mass per area, nitrogen (Feller and Fisher 1994) and protein (Muthuchelian 1987) contents, activities of nitrate reductase (Muthuchelian 1987) and photosynthetic enzymes including RuBPC (Šesták 1985, Kutík 1998) decrease. The aging process of needles leads to disturbed biosynthesis and accumulation of chlorophyll (Chl) and carotenoid (Car) pigments as well as the formation of redox carriers and specific proteins, which is parallel to

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[***]Author for correspondence; fax: 91-452-2459181, e-mail: drchelian1960@yahoo.co.in
Abbreviations: Car – carotenoids; Chl – chlorophyll; DCBQ – 2,6-dichloro-p-benzoquinone; DCPIP – 2,6-dichlorophenol indophenol; DPC – diphenyl carbazide; DTT – dithiothreitol; EDTA – ethylene diaminotetraacetic acid; F_0 – minimal fluorescence; F_m – maximum fluorescence; LSU – large subunit; MV – methyl viologen; NRA – nitrate reductase activity; PAR – photosynthetically active radiation; PMSF – phenylmethylsulfonyl fluoride; PPFD – photosynthetic photon flux density; PS – photosystem; PAGE – polyacrylamide gel electrophoresis; RuBPC – ribulose-1,5-bisphosphate carboxylase; SDS – sodium dodecylsulphate; SiMo – silicomolybdate; SSU – small subunit; Tris – tris-hydroxyl methyl amino-methane.
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disorganization of thylakoids and gradual decline of photosynthetic activity in *Taxus* and *Pinus* trees (Endler *et al*. 1990, Miroslavov and Alekseeva 1990, Kutík 1998).

During ontogeny of photosynthetically active leaves, *i.e*. from their unfolding to yellowing, the ultrastructure of chloroplasts in the mesophyll cells changes substantially (Šesták 1985, Hudák 1997, Kutík 1998). The main features of this development are increase of chloroplast size in maturing leaves and decline of their number during leaf senescence, accumulation of starch in the chloroplasts of just mature leaves, accumulation of plastoglobulin (material) during leaf senescence, and changes in quantity of the thylakoid system and in the thylakoid stacking degree during whole leaf ontogeny.

Studies of leaf development of several woody perennials show that high P_N is typical for mature leaves and then declines (Kennedy and Johnson 1981, Roper and Kennedy 1986). However, patterns of leaf P_N as a function of leaf age vary among fruit tree species. In apple, mature well-exposed leaves showed little variation in P_N for about four months (Kennedy and Fujii 1986). In sour cherry, leaf P_N increased 4–5 fold during the period of rapid lamina expansion, it was stable for 4 weeks, and then decreased gradually (Sams and Flore 1982). We report here aging-induced changes of pigment and soluble protein contents, electron transport activities, Chl fluorescence, and RuBPC and nitrate reductase activities in needles of cypress (*Cupressus sempervirens* L.).

Materials and methods

Plants and experiment design: Cypress needles (*Cupressus sempervirens* L.) were collected from selected 10-year-old trees grown under field conditions in the Istituto Agrario di San Michele all' Adige, Italy (46°12'N, 11°08'E). The average time of sunlight per day to needle sample was 8 h (SD 2 h 50 min). The average maximum air temperature for this period was 25.6 °C (SD 3.7 °C), the highest air temperature was 32.5 °C. The average minimum temperature was 12.0 °C (SD 2.9 °C), the lowest air temperature was 4.2 °C. The maximal leaf temperature was 33 °C. The needle age classes were: expanding current year (A1), two-years-old (A2), three-years-old (A3), and more than three years old (A4).

Photosynthetic pigments were determined in crude acetone extracts. Needles were killed in liquid nitrogen, lyophilized, pulverized with a mill, and extracted with 80 % (v/v) acetone. Chl and Car were measured spectrophotometrically and their concentrations calculated using the extinction coefficients of Lichtenthaler (1987).

Gas exchange was measured using a portable gas analyzer system (model *LCA-3*, *ADC*, UK). These measurements were taken on 15–20 needles at PAR of >1 000 μmol m^{-2} s^{-1}, about 33 °C needle temperature, and about 34 Pa ambient partial pressure of CO_2.

Modulated Chl fluorescence was measured on intact needles using a *PAM 2000* fluorometer (*Walz*, Effeltrich, Germany). Before the measurements, the needles were dark adapted for 15 min. F_0 was measured by switching on the modulated radiation of 0.6 kHz; PPFD was less than 30 μmol m^{-2} s^{-1} at the leaf surface. F_m was measured at 20 kHz with a 1-s pulse of 6 000 μmol m^{-2} s^{-1} of "white light".

Thylakoid membranes were isolated as described by Nedunchezhian *et al*. (1997). Needles were homogenized in ice-cold buffer containing 330 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.8), and chloroplasts pelleted by centrifuging for 5 min at 8 000 rpm. After osmotic shock in the previous buffer, but lacking sucrose, thylakoid membranes were pelleted and re-suspended in 100 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, and 50 mM Tris (pH 7.8).

Activities of electron transport: Whole chain electron transport ($H_2O \rightarrow MV$) and partial reactions of photosynthetic electron transport mediated by PS2 ($H_2O \rightarrow DCBQ$, $H_2O \rightarrow SiMo$) and PS1 ($DCPIPH_2 \rightarrow MV$) were measured as described by Nedunchezhian *et al*. (1997). Thylakoids were suspended at 10 g(Chl) m^{-3} in the assay medium containing 20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM NH_4Cl, and 100 mM sucrose supplemented with 0.5 mM DCBQ and 0.2 mM SiMo.

DCPIP photoreduction: The rate of DCPIP photoreduction was determined as the decrease in absorbance at 590 nm using a *Hitachi 557* spectrophotometer (*Hitachi*, Tokyo, Japan). The reaction mixture contained 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM NaCl, 100 mM sucrose, 100 μm DCPIP, and thylakoid membranes equivalent to 20 g(Chl) m^{-3}. Where mentioned, the concentrations of $MnCl_2$, DPC, and NH_2OH were 5.0, 0.5, and 5.0 mM, respectively.

SDS-PAGE separation: Thylakoid membrane proteins were separated using the polyacrylamide gel system of Laemmli (1970), with the following modifications. Gels consisted of a 12–18 % gradient of polyacrylamide containing 4 M urea. Samples were solubilised at 20 °C for 5 min in 2 % (m/v) SDS, 60 mM DTT, and 8 % saccharose using SDS : Chl ratio of 20 : 1. Electrophoresis was performed at 20 °C with constant current of 5 mA. Gels were stained in methanol : acetic acid : water (4 : 1 : 5, v/v/v) containing 0.1 % (m/v) Coomassie brilliant blue R, and de-stained in methanol : acetic acid : water (4 : 1 : 5, v/v/v). Content of thylakoid membrane protein was estimated according to Lowry *et al*. (1951).

Content of total soluble proteins: The proteins were extracted by grinding needles [0.3–0.5 g(f.m.)] in a mortar with 6 cm^3 of 100 mM Tris-HCl, pH 7.8 containing 15 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 10 mM PMSF in the presence of liquid nitrogen. Homogenates were filtered through nylon cloth. After centrifugation at 11 000×*g* for 10 min, the concentration of soluble proteins was determined in the supernatant after Bradford (1976).

Extracts and assay of RuBPC activity: Needles were cut into small pieces and homogenized in a grinding medium of 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 5 mM DTT, and 0.25 mM EDTA. The extract was clarified by centrifugation at 10 000×*g* for 10 min. The clear supernatant was decanted slowly and used as the RuBPC. The assay of RuBPC activity was measured as described by Nedunchezhian and Kulandaivelu (1991).

Nitrate reductase activity: Needles of 100 mg were suspended in a glass vial containing 5 cm^3 of the assay medium consisting of 100 mM KH_2PO_4-KOH, pH 7.0, 100 mM KNO_3, and 1 % (v/v) *n*-propanol. The vial was sealed and incubated in the dark at 27 °C for 60 min. Suitable aliquots of the assay medium were removed for nitrate analysis (Jaworski 1971).

Statistical analysis (ANOVA) indicated that there were no differences in most measurements between the 3 repetitions with 5 replications of each experiment ($n = 3$ with 5 replications). Therefore, data presented here are the averages of the three repetitions of the experiment. Student *t*-tests were performed to determine significant difference ($p \leq 0.05$) between treatment means.

Results and discussion

The contents of Chl and Car on a fresh mass basis, and the Chl *a*/*b* ratio increased with needle development and then declined (Table 1). Similar changes were observed in cotyledons whose area and total Chl contents increased during the 15–40 d of their metabolic activity, the result being an increase and decline in the Chl amount per cotyledon (Millerd *et al*. 1971, Hong and Schopfer 1981). The low content of Chl *a* in A3 and A4 needles was manifested by low Chl *a*/*b* ratios. Our observations are in agreement with earlier reports (Šesták 1985, Kutík *et al* 1988, Kutík 1998). The reduction of Chl content in A4 needles was probably related to an enhanced activity of chlorophyllase (Šesták 1985, Reddy and Vora 1986). At the early developmental stages, the higher Chl content in A2 needles confirms the findings of other investigators (Šesták 1985, Kutík *et al.* 1988, Kutík 1998, Petrie *et al*. 2000).

The Chl *a*/*b* ratio was markedly higher in A2 than A4 needles (Table 1). The decrease in Chl *a*/*b* ratio in A4

Table 1. Chlorophyll (Chl) and carotenoid (Car) contents [g kg^{-1}(f.m.)] and their ratios, values of ground (F_0) and variable fluorescence (F_v), ratio of F_v and maximum fluorescence (F_v/F_m), net photosynthetic rate (P_N) [μmol(CO_2) m^{-2} s^{-1}], activities of total electron transport [whole chain ($H_2O \rightarrow MV$)] and photosystems PS2 ($H_2O \rightarrow DCBQ$; $H_2O \rightarrow DCPIP$) and PS1 ($DCPIPH_2 \rightarrow MV$) [mmol(O_2) kg^{-1}(Chl) s^{-1}], content of total soluble proteins [g kg^{-1}(f.m.)], activities of RuBPC [mmol(CO_2) kg^{-1}(protein) s^{-1}] and nitrate reductase (NRA) [$mmol^{-1}$(NO_2) kg^{-1}(f.m.) s^{-1}] as a function of needle age. Needles were: expanding current year (A1), two-years-old (A2), three-years-old (A3), and senescent needles more than three years old (A4). Statistical significance ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$) of difference between the needles A2 and A4 were tested by means of Student's *t*-test.

Parameter	Needle age A1	A2	A3	A4
Chl *a*	1.090±0.500	1.371±0.060**	1.223±0.070	0.929±0.030***
Chl *b*	0.326±0.020	0.369±0.010*	0.328±0.020	0.275±0.020**
Chl *a*+*b*	1.416±0.800	1.740±0.070*	1.550±0.500	1.204±0.400**
Chl *a*/*b*	3.30±0.10	3.70±0.15*	3.70±0.16	3.40±0.12***
Car	0.467±0.020	0.597±0.030**	0.549±0.020	0.420±0.020*
Car/Chl	0.330±0.010	0.340±0.010**	0.350±0.020	0.350±0.010**
F_0	0.56±0.02	0.82±0.03*	0.62±0.03	0.48±0.02***
F_v	1.10±0.04	1.80±0.09*	1.60±0.07	1.20±0.06**
F_v/F_m	0.70±0.03	0.70±0.02*	0.80±0.04	0.70±0.03***
P_N	14.2±1.0	28.9±1.4*	18.7±0.9	8.7±0.5*
Whole chain [$H_2O \rightarrow MV$]	115.8±5.9	130.2±6.3**	97.6±4.6	71.5±3.1**
PS2 [$H_2O \rightarrow DCBQ$]	125.5±6.1	142.3±7.1**	89.6±5.2	58.3±2.8***
PS2 [$H_2O \rightarrow SiMO$]	75.3±3.6	102.5±4.9*	67.6±3.2	47.2±2.8**
PS1 [$DCPIPH_2 \rightarrow MV$]	272.4±14.1	292.1±14.2**	102.2±6.2	43.80±2.5**
Total soluble proteins	19.84±0.93	47.48±1.90*	34.40±1.90	12.60±0.62*
RuBPCO	18.63±0.91	28.80±1.40***	22.70±1.40	8.27±0.52**
NRA	0.285±0.010	0.460±0.020*	0.385±0.020	0.112±0.010**

needles is mainly due to a decrease in Chl *a* during needle aging (Šesták 1985). Since Chl *a* is an exact characteristic of photosynthetic activity (Šesták 1966), the tendency towards a higher content might partially explain the higher P_N found in A2 needles. Chl/Car ratios varied from 0.33 in A1 to 0.35 in A4 needles (Table 1). The Car breakdown between A2 and A4 was different to Chl (Table 1). The Chl/Car ratio decrease in A4 needles reflected the relatively high retention of Cars. The changes of photosynthetic pigments during needle development and A4 in cypress were similar to those found in other species (Šesták 1985, Šiffel *et al*. 1993, Sobrado 1996).

Initial fluorescence (F_0) reflecting the size of antenna Chl of PS2 (Krause and Weiss 1984) did not show consistent changes with the needle age (Table 1). By contrast, variable fluorescence (F_v) and variable to maximum fluorescence ratios (F_v/F_m) of dark-adapted needles reached a peak in A3 needles, while lower values were obtained in A4 needles (Table 1). Hence, photons absorbed by photosynthetic apparatus were used more efficiently by A3 than A1 needles. High F_v/F_m values obtained at A3 were typical of non-photoinhibited mature tissue (Šiffel *et al.* 1985, Demmig and Björkman 1987, Somersalo and Aro 1987). High F_v/F_m is a result of a high photochemical capacity of PS2 reaction centres and is independent from Chl content. Lower F_v/F_m values in A1 and A4 needles in comparison with mature ones are probably not due to photoinhibition but to a low photochemical capacity as suggested by Chl *a*/*b*. During needle ontogeny, a rapid increase in the capacity of PS2 photochemistry (increasing F_v/F_m) to A3 needle and a decline with A4 have been reported (Lichtenthaler 1987, Somersalo and Aro 1987, Šiffel *et al*. 1993).

However, when photosynthetic electron transport was studied using isolated thylakoids from needles of different ages, all photosynthetic electron transport activities increased with needle development and then declined (Table 1). The PS2 mediated electron transport of $H_2O\rightarrow DCBQ$ and $H_2O\rightarrow DCPIP$ increased from A1 to A3 needles and then declined (Table 1). A similar trend was also noticed for the whole chain electron transport ($H_2O\rightarrow MV$) activity (Table 1). The high PS2 rate in A2 needles found in our experiments agrees with earlier reports (Strnadová and Šesták 1974, Šesták *et al.* 1977, 1978, Šesták 1985, Sobrado 1996, Kutík *et al.* 1999).

DCPIP collects electrons after plastoquinone (Lien and Bannister 1971, Ouitrakul and Izawa 1973) but benzoquinone at the reducing side of plastoquinone (Lien and Bannister 1971) in PS2. In the presence of the above PS2 electron acceptors, the loss of PS2 activity in A4 needles was approximately the same. Thus, A4-induced changes must be prior to plastoquinone in the electron transport. Among the artificial electron donors tested, DPC and NH_2OH donate electrons directly to the PS2 reaction centre (Wydrzyński and Govindjee 1975). In A4 needles, the PS2 activity was reduced to about 60 % when water or $MnCl_2$ served as electron donor (Table 2). In contrast, a significant restoration of PS2-mediated DCPIP reduction was observed when NH_2OH and DPC were used as electron donors (Table 2). Thus the inhibition of PS2 may be ascribed to an alteration of the water splitting system, since the addition of DPC and NH_2OH restored significantly its activity. This is in good agreement with findings that the water-oxidizing system is sensitive to ageing (Biswal and Biswal 1988, Nedunchezhian *et al*. 1995).

Table 2. Effect of various exogenous electron donors on photosystem 2 (PS2) activity ($H_2O\rightarrow DCPIP$) in thylakoids isolated from different age needles. Statistical significance ($^{*}p\leq0.05$ and $^{***}p\leq0.001$) of difference between the needle ages A2 and A4 was tested by means of Student's *t*-test.

Exogenous donors	[mmol(DCPIP red.) kg^{-1}(Chl) s^{-1}]	
	A2	A4
$H_2O\rightarrow DCPIP$	142.5±8.1	57.5±2.6*
$DPC\rightarrow DCPIP$	143.6±7.6	122.0±6.6***
$NH_2OH\rightarrow DCPIP$	143.1±7.1	121.8±6.1***
$MnCl_2\rightarrow DCPIP$	142.9±7.4	59.7±3.3*

The inactivation of PS2 electron transport activity in A4 needles is supported by the fact that the related protein(s) is(are) exposed at the thylakoid surface (Seidler 1994). A comparison of thylakoids from A4 needles with those of A2 needles showed specific losses of 33, 28–25, 23, and 17 kDa polypeptides (Fig. 1). The three extrinsic proteins of 33, 23, and 17 kDa associated with the lumen surface of the thylakoid membranes are required for optimal functioning of the oxygen evolving complex system (Murata *et al*. 1984, Enami *et al*. 1994). We found that the significant losses of 33, 23, and 17 kDa extrinsic

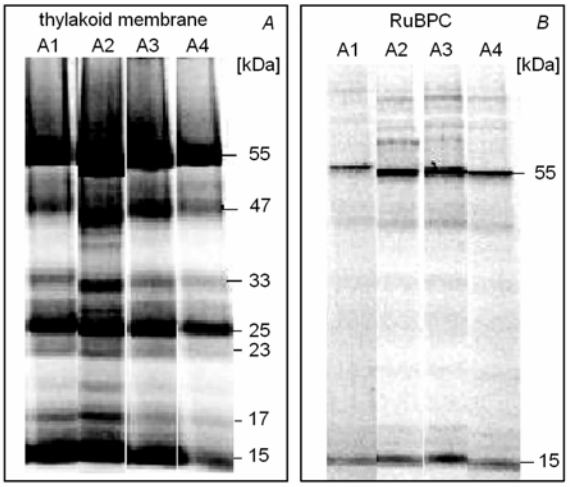

Fig. 1. Coomassie brilliant blue stained polypeptide profiles of thylakoid membranes (*A*) and crude needle extracts (*B*) isolated from needles at different ages: current year (A1), two-years-old (A2), three-years-old (A3), and senescent more than three years old (A4). Gel lanes were loaded with equal amount of protein (100 μg) for RuBPC and Chl (70 μg) for thylakoid membranes.

polypeptides and 28–25 kDa LHCP2 polypeptides could be the reason for marked losses of O_2 evolution in A4 needles (Fig. 2). Similar observations were made in dark-adapted *Vigna* seedlings during senescence (Nedunchezhian *et al*. 1995).

The amount of total soluble proteins gradually increased during needle development and then declined. The soluble protein content was lower (74 %) in A4 needles than in A2 needles (Table 1). This relatively low content of soluble proteins in A4 needles might have been the result of a decrease of the synthesis of RuBPC, the major soluble protein in needles. The reduction in the overall photosynthetic rates correlates well with the decrease of RuBPC activity in A4 needles. If the RuBPC activity was expressed on a protein basis, a low activity in young needles was followed by an increase to the maximum and then a final decrease. Our observations are concurrent with earlier reports (Daley *et al*. 1978, Zima and Šesták 1979, Zima *et al*. 1981, Šesták 1985). A higher RuBPC activity was observed in A2 needles, while a significant reduction was observed in A4 needles. A reduction of 57 % was noticed when compared to A2 needles (Table 1). The loss of RuBPC activity is also supported by SDS-PAGE analysis of crude needle extracts, a marked loss of LSU (nuclear encoded protein – 55 kDa) and marginal losses of SSU (chloroplast encoded protein – 15 kDa) polypeptides were observed in A4 needles (Fig. 1). The loss of LSU and SSU is one of the reasons for marked loss of RuBPC activity in A4 needles. Similar results were also found in dark-adapted *Vigna* seedlings during senescence (Nedunchezhian *et al*. 1995).

In vivo, a marked reduction of nitrate reductase activity (NRA) was noticed in A4 needles. This may be reflecting a balance between the synthesis of the active nitrate reductase enzyme or its activation on the one hand and degradation or inactivation on the other one. The decreased NRA might reflect the reduction in nitrate uptake by the roots. This reduced uptake might be due to the feedback inhibition of amino acids formed in needle blades and transported from there to the shoot (Clarkson 1986, Muthuchelian 1987).

We conclude that A2 (2-year-old) needles are photosynthetically more active than the cypress needles of the other ages. When needles become more than three years old, all photosynthetic activities/performance go down due to the senescence.

References

Biswal, U.C., Biswal, B.: Ultrastructural modifications and biochemical changes during senescence of chloroplasts. – Int. Rev. Cytol. **113**: 271-321, 1988.

Bradford, M.M.: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. – Anal. Biochem. **72**: 248-254, 1976.

Christy, A.L., Porter, C.A.: Canopy photosynthesis and yield in soybean. – In: Govindjee (ed.): Photosynthesis. Vol. II. Pp. 499-511. Academic Press, New York – London – Paris – San Diego – San Francisco – São Paulo – Sydney – Tokyo – Toronto 1982.

Clarkson, D.T.: Regulation of absorption and release of nitrate by plant cells. A review of current ideas and methodology. – In: Lambers, H., Neeteson, J.J., Stulen, I. (ed.): Fundamental, Ecological and Agricultural Aspects of Nitrogen Metabolism in Higher Plants. Pp. 3-27. Martinus Nijihoff Publ., Dordrecht 1986.

Constable, G.A., Rawson, H.W.: Effect of leaf position, expansion and age on photosynthesis, transpiration and water use efficiency in cotton. – Aust. J. Plant Physiol. **7**: 89-100, 1980.

Daley, L.S., Dailey, F., Criddle, R.S.: Light activation of ribulose bisphosphate carboxylase. Purification and properties of the enzyme in tobacco. – Plant Physiol. **62**: 718-722, 1978.

Davis, S.D., McCree, K.J.: Photosynthetic rate and diffusion conductance as a function of age in leaves of bean plants. – Crop Sci. **18**: 280-282, 1978.

Demmig, B., Björkman, O.: Comparison of the effect of excessive light on chlorophyll fluorescence (77 K) and photon yield of O_2 evolution in leaves of higher plants. – Planta **171**: 171-184, 1987.

Enami, I., Kitamura, M., Tomo, T., Isokawa, Y., Ohta, H., Katoh, S.: Is the primary cause of thermal inactivation of oxygen evolution in spinach PS II membranes release of the extrinsic 33 kDa protein or of Mn? – Biochim. biophys. Acta **1186**: 52-58, 1994.

Endler, W., Barsig, M., Weese, G., Hafner, L.: Cytomorphologische Untersuchungen an Nadeln von Kiefern (*Pinus silvestris* L.) eines Ballungsgebietes. I. Die Feinstruktur von Chloroplasten im Mesophyll in Abhängigkeit von Jahreszeit, Alter und Schadstoffbelastung. – Angew. Bot. **64**: 289-302, 1990.

Feller, U., Fisher, A.: Nitrogen metabolism in senescing leaves. – Crit. Rev. Plant Sci. **13**: 241-273, 1994.

Hong, Y.N., Schopfer, P.: Control by phytochrome of urate oxidase and allantoinase activities during peroxisome development in the cotyledons of mustard (*Sinapis alba* L.) seedlings. – Planta **152**: 325-335, 1981.

Hudák, J.: Photosynthetic apparatus. – In: Pessarakli, M. (ed.): Handbook of Photosynthesis. Pp. 27-48. Marcel Dekker, New York – Basel – Hong Kong 1997.

Jaworski, E.G.: Nitrate reductase assay in intact plant tissues. – Biochem. biophys. Res. Commun. **43**: 1274-1279, 1971.

Kennedy, R.A., Fujii, J.A.: Seasonal and developmental changes in apple photosynthesis: Enhancement effects due to flowering and fruit maturation. – In: Lakso, A.N., Lenz, F. (ed.): Regulation of Photosynthesis in Fruit Trees. Pp. 27-29. Publ. New York State Agr. Exp. Sta., Geneva 1986.

Kennedy, R.A., Johnson, D.: Changes in photosynthetic characteristics during leaf development in apple. – Photosynth. Res. **2**: 213-223, 1981.

Krause, G.H., Weiss, E.: Chlorophyll fluorescence as a tool in plant physiology. II. Interpretation of fluorescence signals. – Photosynth. Res. **5**: 139-157, 1984.

Kutík, J.: The development of chloroplast structure during leaf ontogeny. – Photosynthetica **35**: 481-505, 1998.

Kutík, J., Činčerová, A., Dvořák, M.: Chloroplast ultrastructural development during the ontogeny of the second leaf of wheat under nitrogen deficiency. – Photosynthetica **28**: 447-453,

1993.
Kutík, J., Kočová, M.: The development of chloroplast ultrastructure and photochemical activity during leaf ontogeny in maize (*Zea mays* L.). – Biológia (Bratislava) **51**: 59, 1996.
Kutík, J., Kočová, M., Holá, D., Kőrnerová, M.: The development of chloroplast ultrastructure and Hill reaction activity during leaf ontogeny in different maize (*Zea mays* L.) genotypes. – Photosynthetica **36**: 497-507, 1999.
Kutík, J., Zima, J., Šesták, Z., Volfová, A.: Ontogenetic changes in the internal limitations to bean-leaf photosynthesis. 10. Chloroplast ultrastructure in primary and first trifoliate leaves. – Photosynthetica **22**: 511-515, 1988.
Larcher, W.: Temperature stress and survival ability in Mediterranean sclerophyllous plants. – Plant Biosyst. **134**: 279-295, 2000.
Laemmli, U.K.: Clevage of structural proteins during the assembly of the head of bacteriophage T4. – Nature **227**: 680-685, 1970.
Lichtenthaler, H.K.: Chlorophyll fluorescence signatures of leaves during the autumnal chlorophyll breakdown. – J. Plant Physiol. **131**: 101-110, 1987.
Lien, S., Bannister, T.T.: Multiple sites on DCIP reduction by sonicated oat chloroplasts: Role of plastocyanin. – Biochim. biophys. Acta **245**: 465-481, 1971.
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.
Mazari, A., Camm, E.L.: Effect of cytokinins on plastid development and photosynthetic polypeptides during organogenesis of *Pinus ponderosa* Dougl. cotyledons cultured *in vitro*. – Plant Cell Tissue Organ Cult. **33**: 81-89, 1993.
Millerd, A., Simon, M., Stern, H.: Legumin synthesis in developing cotyledons of *Vicia faba* L. – Plant Physiol. **48**: 419-425, 1971.
Miroslavov, E.A., Alekseeva, O.A.: [Longevity of plastids in *Taxus* leaf chlorenchyma.] – Dokl. Akad. Nauk SSSR **313**: 1274-1276, 1990. [In Russ.]
Murata, N., Miyao, M., Omata, T., Matsunami, H., Kuwabara, T.: Stoichiometry of components in the photosynthetic oxygen evolution system of Photosystem II particles prepared with Triton X-100 from spinach chloroplasts. – Biochim. biophys. Acta **765**: 363-369, 1984.
Muthuchelian, K.: Bioproductivity Studies of Some Leguminous and Non-Leguminous Plants in Semi-Arid Ecosystem. – Ph.D. Thesis. Madurai Kamaraj University, Madurai 1987.
Nedunchezhian, N., Kulandaivelu, G.: Effect of UV-B enhanced radiation on ribulose-1,5-bisphosphate carboxylase in leaves of *Vigna sinensis* L. – Photosynthetica **25**: 431-435, 1991.
Nedunchezhian, N., Morales, F., Abadia, A., Abadia, J.: Decline in photosynthetic electron transport activity and changes in thylakoid protein pattern in field grown iron deficient peach (*Prunus persica* L.). – Plant Sci. **129**: 29-38, 1997.
Nedunchezhian, N., Ravindran, K.C., Kulandaivelu, G.: Changes in photosynthetic apparatus during dark incubation of detached leaves from control and ultraviolet-B treated *Vigna* seedlings. – Biol. Plant. **37**: 341-348, 1995.
Ouitrakul, R., Izawa, S.: Electron transport and photophosphorylation in chloroplasts as a function of the electron acceptor II. Acceptor-specific inhibition by KCN. – Biochim. biophys. Acta **305**: 105-118, 1973.
Petrie, P.E., Trought, M.C.T., Howell, G.S.: Influence of leaf ageing, leaf area and crop load on photosynthesis, stomatal conductance and senescence of grapevine (*Vitis vinifera* L. cv. Pinot noir) leaves. – Vitis **39**: 31-36, 2000.
Reddy, M.P., Vora, A.B.: Changes in pigment composition, Hill reaction activity and saccharide metabolism in bajra (*Pennisetum typhoides* S & H) leaves under NaCl salinity. – Photosynthetica **20**: 50-55, 1986.
Roper, T.R., Kennedy, R.A.: Photosynthetic characteristics during leaf development in 'Bing' sweet cherry. – J. amer. Soc. hort. Sci. **111**: 938-941, 1986.
Sams, C.E., Flore, J.A.: The influence of age, position and environmental variables on net photosynthetic rate of sour cherry leaves. – J. amer. Soc. hort. Sci. **107**: 339-344, 1982.
Seidler, A.: Expression of the 23 kDa protein from the oxygen-evolving complex of higher plants in *Escherichia coli*. – Biochim. biophys. Acta **1187**: 73-79, 1994.
Šesták, Z.: Limitations for finding a linear relationship between chlorophyll content and photosynthetic activity. – Biol. Plant. **8**: 336-346, 1966.
Šesták, Z. (ed.): Photosynthesis during Leaf Development. – Academia, Praha; Dr W. Junk Publ., Dordrecht – Boston – Lancaster 1985.
Šesták, Z., Zima, J., Strnadová, H.: Ontogenetic changes in the internal limitations to bean-leaf photosynthesis. 2. Activities of photosystems 1 and 2 and non-cyclic photophosphorylation and their dependence on photon flux density. – Photosynthetica **11**: 282-290, 1977.
Šesták, Z., Zima, J., Wilhelmová, N.: Ontogenetic changes in the internal limitations to bean-leaf photosynthesis. 4. Effect of pH of the isolation and/or reaction medium on the activities of photosystems 1 and 2. – Photosynthetica **12**: 1-6, 1978.
Šiffel, P., Lebedev, N.N., Šesták, Z.: Changes in chloroplast absorption and fluorescence spectra during ontogeny of primary bean leaves. – Photosynthetica **19**: 127-137, 1985.
Šiffel, P., Šantrůček, J., Lang, M., Braunová, Z., Šimková, M., Synková, H., Lichtenthaler, H.K.: Age dependence of photosynthetic activity, chlorophyll fluorescence parameters and chloroplast ultrastructure in aurea and green forms of *Nicotiana tabacum Su/su* mutant. – Photosynthetica **29**: 81-94, 1993.
Sobrado, M.A.: Leaf photosynthesis and water loss as influenced by leaf age and seasonal drought in an evergreen tree. – Photosynthetica **32**: 563-568, 1996.
Somersalo, S., Aro, E.-M.: Fluorescence induction in pea leaves of different ages. – Photosynthetica **21**: 29-35, 1987.
Strnadová, H., Šesták, Z.: Reliability of methods used for determining ontogenetic changes in Hill reaction rate. – Photosynthetica **8**: 130-133, 1974.
Wells, R.: Response of leaf ontogeny and photosynthetic activity to reproductive growth in cotton. – Plant Physiol. **87**: 274-279, 1988.
Wydrzyński, T., Govindjee: A new site of bicarbonate effect in photosystem II of photosynthesis: Evidence from chlorophyll fluorescence transients in spinach chloroplasts. – Biochim. biophys. Acta **387**: 403-408, 1975.
Zima, J., Šesták, Z.: Photosyntetic characteristics during ontogenesis of leaves. 4. Carbon fixation pathways, their enzymes and products. – Photosyntetica **13**: 83-106, 1979.
Zima, J., Šesták, Z., Čatský, J., Tichá, I.: Ontogenetic changes in leaf CO_2 uptake as controlled by photosystem and carboxylation activities and CO_2 transfer. – In: Akoyunoglou, G. (ed.): Photosynthesis. Vol. VI. Pp. 23-31. Balaban Int. Sci. Serv., Philadelphia 1981.