SENESCENCE AND REJUVENATION IN INTACT COTYLEDONS OF *CUCURBITA PEPO* L. (ZUCCHINI)

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Received: August, 03, 2005

Summary. Growth parameters and total pigment content (chlorophyll *a*+*b* and carotenoids) were investigated through postgermination, greening, natural senescence and subsequent rejuvenation of intact cotyledons of *Cucurbita pepo* L. (zucchini). Dark-induced senescence and recovery of the cotyledons after 2-day darkening of a whole plant were also studied. Results showed significant increase in the fresh weight of cotyledons accompanied by a reciprocal decrease in dry matter during the first two weeks of their postgerminative growth. During the period of greening cotyledons rapidly accumulated chlorophylls and carotenoids in a synchronous manner. Maximum pigment contents were reached by days 7-8 after the onset of germination, followed by a steady state by day 14-15, and a gradual decline thereafter during the period of natural senescence. Cotyledons intensively yellowed, lost their turgor and died about 25 days after seed germination. Epicotyl decapitation at the stage of visible yellowing resulted in a recovery of both chlorophyll and carotenoids amounts during the next 15 days of rejuvenation period. When 8-day-old seedlings were transferred to the dark for 2 days, a progressive reduction in fresh and dry weight as well as in the chlorophyll and carotenoids content and net photosynthetic rate was registered. The pigment losses and CO_2 assimilation as well as the growth reduction of dark-treated cotyledons were completely reversed after the return of the plants to normal light regime. Eleven days after ceasing the dark treatment chlorophyll content of the recovering plants was even increased. In contrast, five-day dark treatment of entire plants could not be overcome by returning to the light.

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Key words: chlorophyll and carotenoids content, decapitation, intact cotyledons of *Cucurbita pepo* L. (zucchini), natural and dark-induced senescence, rejuvenation

Abbreviations*:* DW – dry wight, FW – fresh weight, ABA – abscisic acid, MeJA – methyl ester of jasmonic acid, SAG – senescence associated genes

INTRODUCTION

Senescence is the final step of leaf development comprising the period from mature fully expanded state to cell death. Senescence is not a chaotic process of passive degeneration but a highly regulated loss of cell functions under the stringent control of the nucleus. In general, leaf senescence is characterized by the breakdown of macromolecules and membranes mostly involved in photosynthesis, and the subsequent mobilization of nutrient components to other parts of the plant, such as developing seeds or storage organs (Smart, 1994; Gan, 2004). Like other developmental processes, selective activation of specific genes (senescence-associated genes – SAGs), accompanied by a decrease of certain RNAs and/or proteins is likely to initiate and regulate this process (Buchanan-Wollaston, 1997; Hajouj et al., 2000; Hinderhofer and Zentgraf, 2001; He et al., 2003; Zentgraf et al., 2004). In contrast to other developmental processes such as seed germination, natural leaf senescence in a plant population does not proceed in a synchronous way beginning at a defined starting point. This is one of the practical problems when dealing with differentiated leaves, which are often circumvented by inducing leaf senescence artificially. The latter is commonly achieved by incubating detached leaves in the dark (Thimann, 1980). Much more convenient for studying senescence are cotyledons which represent specific reserve organs whose major physiological function is to ensure the development of the growing seedling until differentiation of photosynthetically efficient leaves. The pair of epigeal cotyledons, which emerge from the soil, besides having reserve-mobilizing functions, can also act as primary photosynthesizing organs (Lovel and Moore, 1971). After achieving oxygen evolution rates comparable to those of expanded leaves, the photosynthetic capacity of these cotyledons decreases and they senesce (La Rocca et al., 1996). So, cotyledons are short-lived organs which senesce with the progression of seedling development and die shortly after the appearance of differentiated leaves (Marshall and Kozlovski, 1976). Although the senescence of cotyledons is not fundamentally different from leaf senescence, organ specific differences between cotyledons and differentiated leaves in respect to their photosynthetic activity during natural senescence have been reported (La Rocca et al., 1996). Therefore, in cotyledons, senescence is regulated differently from that in true leaves.

Many internal and external factors may trigger the initiation of leaf senescence. Nowadays, the variations of the light regime as a fundamental physical factor for leaf

senescence are intensively studied. In spite of the similarity between natural and dark-induced senescence, some significant differences have been found with regard to gene expression (Weaver et al., 1998) and the activity of antioxidative enzymes (Kanazawa et al., 2000). Recently, some of the notions concerning darkness as a powerful inducer of leaf senescence have been modified based on accumulation of new experimental evidences using *Arabidopsis* differentiated leaves. The transfer of whole plants to the dark for two days did not induce transcription of SAG-genes as well as massive hydrolytic processes in the differentiated leaves (Weaver and Amasino, 2001). The leaves of the dark stressed plants even senesced later than those of the controls. Therefore, a question arises whether there is one senescence process, or whether several different senescence processes occur under normal and dark-induced conditions (Buchanan-Wollaston, 1997).

Decapitation of the shoot above certain leaves and /or of the entire epicotyl above the cotyledons leading to subsequent rejuvenation is an useful approach for studying the reversibility of senescence (van Staden and Carmi, 1982; Iordanov and Manolov, 1986). Despite of the similarities between the physiological processes in young and rejuvenated leaf tissues many differences were found out (Marek and Stewart, 1992).

Insufficient results were obtained using intact cotyledons as a model system for studying senescence (Biswal and Biswal, 1984; Weaver and Amasino, 2001). The main goal of the present work was to study the mechanisms of natural senescence of intact zucchini cotyledons as well as the effect of short-term darkening of whole plants on the morphophysiological (fresh weigh and dry weight) and physiological status (chlorophyll content and rate of photosynthesis) of cotyledons since changes in photosynthesis-related indices in chloroplasts and the yellowing of plant tissues are considered as symptoms of senescence (Gan, 2004). Furthermore, the capability of cotyledons to rejuvenate after entire epicotyl decapitation was also examined.

MATERIALS AND METHODS

Growth conditions and treatments

Seeds of *Cucurbita pepo* L. (zucchini) were germinated on moistened filter paper for 96 h at 28°C in darkness. The 4-day-old etiolated seedlings were grown further on a nutrient solution (Yamagishi and Yamamoto, 1994) in a growth chamber at a photon flux density of 100 $\mu mol.m^{-2}.s^{-1}$, 28±2°C, relative humidity of 60 % and a 12 h/12 h day/night cycle. Under these conditions cotyledons began to senesce very rapidly and 25 days after the onset of germination they became fully yellow, lost their turgor and died. The recovery from natural senescence was studied in rejuvenated cotyledons after decapitation of the epicotyl, with the differentiated leaves and apical bud,

above the cotyledonary node, done by day 25 after seed germination. After decapitation the arising sprouts from the lateral buds were removed periodically as they appeared. Similar procedure has been widely used by Yordanov and co-workers decapitating bean plants above the primary leaves (Iordanov and Merakchiiska-Nikolova, 1984; Yordanov and Weis, 1986). During the period of rejuvenation (15 days) cotyledons visibly regreened and recovered their turgor. Samples were collected 5, 10 and 15 days after decapitation.

In order to study the dark-induced senescence 8-day-old plants grown in the light for 4 days were darkened for 2 or 5 days at the same temperature and humidity conditions. The 8-day-old seedlings had only the cotyledon pair and the first primary leaf had just emerged. Following dark treatment the seedlings were transferred again to normal light regime. Samples were collected at different days after the seedlings had been returned to the light.

Chlorophyll and carotenoid content determination

Chlorophyll was extracted in 80 % v/v buffered acetone as described by Arnon (1949). Chlorophyll content was determined spectrophotometrically using Specol 11 spectrophotometer (Germany) at 3 wavelengths – 663 nm (for chlorophyll *a*), 645 nm (for chlorophyll *b*) and 460 nm (for carotenoids). Calculations were done using the extinction coefficients of Mackinney (1941). Results represent the means from 4 different experiments with three replicates each.

Net photosynthesis

Net photosynthetic rate was measured with a portable photosynthetic apparatus (Li Cor 6000, USA) at a quantum density of 800 μmol m^{-2} s^{-1} PAR)

The dynamics in the fresh weight per cotyledon as well as per cotyledon disc (d= 0.8 cm; S = 0,005 dm^2) were determined. The dry weight of a cotyledon disc was measured after drying at 105°C until reaching constant values. Results were obtained from triplicate measurements.

RESULTS AND DISCUSSION

Cotyledons of *Cucurbita pepo* L. (zucchini) are of epigeal type with an average life of about 25 days and maximal leaf surface of approximately 7 cm^2. Seed germination in *Cucurbitaceae* is accompanied by a massive degradation of reserve compounds accumulated in cotyledons during seed development. The increased amount of osmotically active substances after degradation leads to an intensive water uptake. Fig. 1, A presents the increase in cotyledon fresh weight (3-fold) during the first five days after transfer of seedlings to the light. In contrast, the dry weight per cotyledon disc

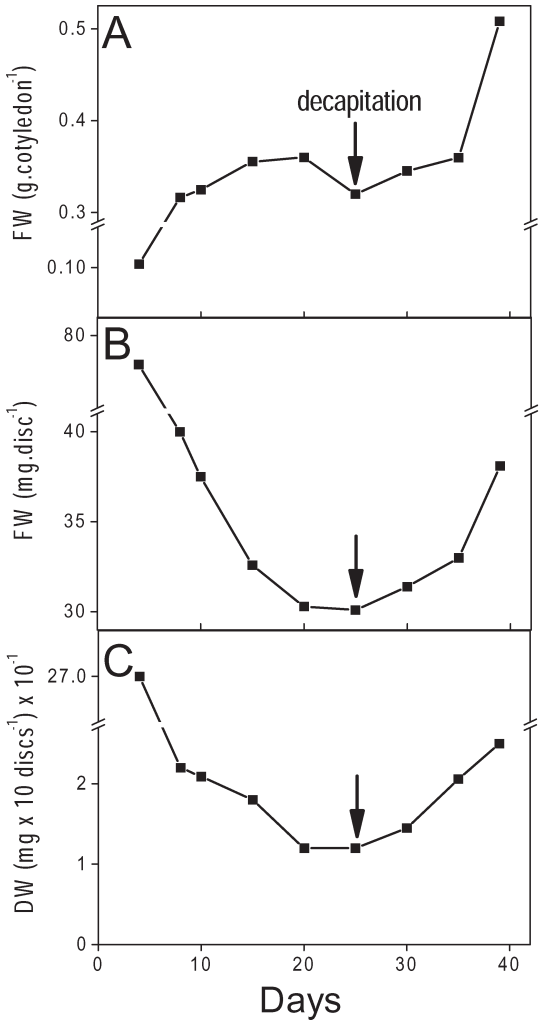

Fig. 1 Time course of cotyledon growth and development during postgerminative growth, greening, natural senescence and rejuvenation of intact cotyledons of *Cucurbita pepo* L. (zucchini) (see Materials and Methods). **A.** Cotyledon fresh weight; **B.** Fresh weight per cotyledon disc; **C.** Dry weight per cotyledon disc. Each data point represents the mean value of three experiments. The SE values averaged 6% and did not exceed 9% of the means.

decreased drastically (13.5-fold) for the same period of time (Fig. 1, C) due to degradation and utilization of cotyledon reserves by the growing young seedling. The same tendency was observed when the fresh weight was measured per cotyledon disc (Fig. 1, B). The differences between the fresh weight curves presented in Fig. 1, A and Fig. 1, B could be explained by the fact that growing cotyledons increased their size not only in fresh weight but also in surface (data not shown here).

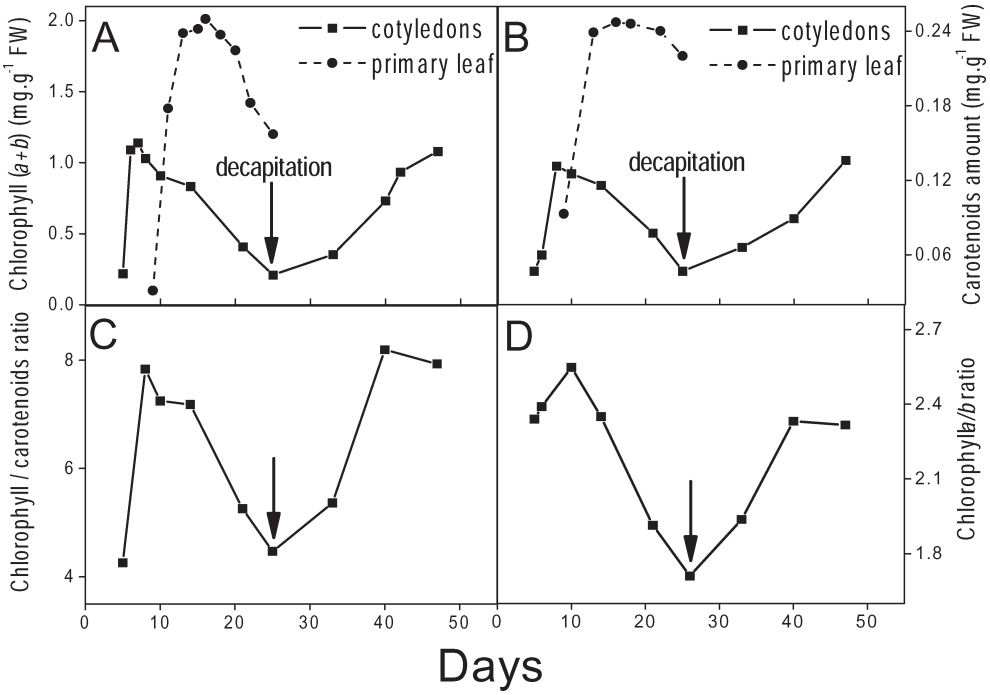

Fig. 2 Time course of total chlorophyll and carotenoid content in intact cotyledons and the primary leaf of *Cucurbita pepo* L. (zucchini). Seedlings were grown in a 12h/12h dark/light cycle and chlorophyll and carotenoid content were determined in the cotyledons and the first differentiated leaf from the day of transfer of the seedlings to light (4th day) till the period of natural senescence (25-day-old seedlings), followed by 22-day rejuvenation period. **A.** Total chlorophyll content (chlorophyll *a*+*b*) **B.** Total carotenoids amount **C.** Chlorophyll/carotenoids ratio **D.** Chlorophyll (*a*)/chlorophyll (*b*) ratio. Each data point represents the mean value of four experiments. The SE values averaged 5% and did not exceed 7% of the means.

The transfer of 4-day-old seedlings grown in the dark to the light led to a very rapid chlorophyll accumulation in the cotyledons (Fig. 2, A). After reaching a maximum by days 7-8, chlorophyll content slowly decreased by day 14 (steady state), followed by a gradual decline during natural senescence till the day 25 after the start of germination. The time course of chlorophyll accumulation in the primary leaf was similar to that of the cotyledons. A burst of chlorophyll accumulation was registered just after the appearance of the leaf by day 8, reaching a maximum by days 15-16 after the onset of germination. After that chlorophyll content sharply declined with progression of yellowing and soon after cotyledons death the primary leaves also died. So, the life span of zucchini primary leaf is a bit shorter in comparison to cotyledons. The time course of total carotenoid amount in cotyledons and the primary leaf was very similar to that of their chlorophyll contents (Fig. 2, B). Nowadays the photoprotective role of carotenoids as components of the xanthophyll cycle is widely

accepted (Stefanov and Yordanov, 1993). Carotenoids rapidly quench triplet excited states of chlorophylls before they can react with oxygen to form the highly reactive damaging excited singlet state of oxygen (Frank and Young, 2000). The maximum in both the chlorophyll/carotenoids and chlorophyll (*a*)/chlorophyll (*b*) ratios corresponded to the maxima of chlorophyll and carotenoid contents in cotyledons (Fig. 2, C, D) by day 7-8 after the start of germination. Therefore, the greatest part of chlorophyll amount in cotyledons at this life stage is represented mainly by chlorophyll (*a*) whereas the content of chlorophyll (*b*) is relatively low. It is worth noting that there is almost a complete identity in the curves of chlorophyll (*a*+*b*) content and chlorophyll *a*/chlorophyll *b* ratio, thus suggesting the highly synchronous way of chlorophyll (*a*) degradation and the respective increase in the relative amount of chlorophyll (*b*) during the period of cotyledon natural senescence (see Fig. 2, A and Fig. 2, D). These results confirmed previous findings that cotyledon and leaf senescence was accompanied by a decline in the amount of chlorophylls, proteins and RNA (Klyachko and Kulaeva, 1975; Yordanov and Weis, 1984). A highly reproducible correlation between the loss of chlorophyll and the decrease in protein and RNA levels was also reported (Lohman et al., 1994). Therefore, leaf yellowing is one of the most definitive signs of senescence and generally senescence is estimated in terms of the chlorophyll loss (Thomson and Platt-Aloia, 1987; Nooden et al., 1997). Cotyledon yellowing is due to an increased rate of chlorophyll degradation controlled by specific set of SAG genes and mainly by the genes coding for chlorophyllase (Smart, 1994).

Epicotyl decapitation at the stage of natural cotyledon senescence by day 25 resulted in a recovery of chlorophyll and carotenoids amounts during the subsequent period of rejuvenation. Eight days after decapitation when the cotyledons of the undecapitated control plants had already died, chlorophyll amount increased by 70 % compared to day 25 (Fig. 2, A). With progression of rejuvenation total chlorophyll and carotenoid contents increased very rapidly and by day 22 after decapitation (the 47th day after the start of germination) it was 4-5- fold higher than that in the beginning of the rejuvenation (Fig. 2, A, B). The same tendency was observed when measuring the chlorophyll/carotenoid ratio and chlorophyll *a/b* ratio which were 1.8-fold and 1.35-fold higher, respectively by day 22 after decapitation (Fig. 2, C, D). These results are in accordance with the results obtained with soybean cotyledons (Marek and Stewart, 1992) showing an increase of cotyledon surface, chlorophyll amount and the quantity of ribulose-1,5-bisphosphate carboxylase (Rubisco) following decapitation. Concomitantly to the renewed formation of pigments, rejuvenating cotyledons restored rapidly their growth, accumulating additional dry matter (Fig. 1, C) and reaching the levels characteristic of the young 1 week-old cotyledons. The increase in cotyledon fresh weight during rejuvenation even exceeded the growth parameters of cotyledons at their most expanded state before senescence (Fig. 1, A). The molecular mechanisms of rejuvenation are not revealed completely yet but the essential role of the hormonal balance in decapitated plants has been showed (van

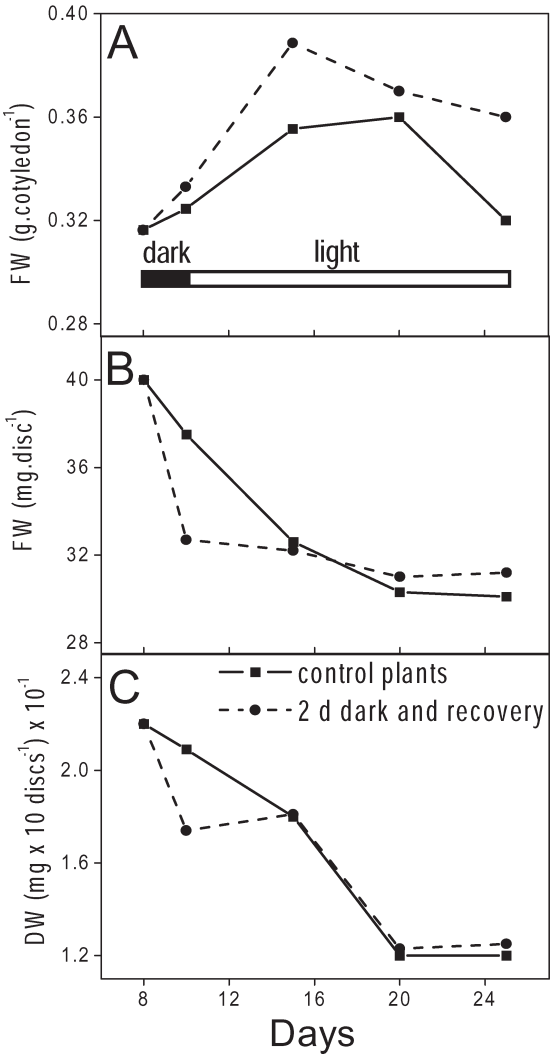

Fig. 3. Effect of 2-day darkening of entire seedlings on the growth parameters of intact cotyledons of *Cucurbita pepo* L. (zucchini). 8-day-old seedlings grown in a 12h/12h dark/light cycle were transferred to darkness for 2 days and after that returned to light regime (see Materials and Methods). Samples were collected 5, 10 and 15 days after ceasing of the dark treatment and return of the seedling to the light. **A.** Cotyledon fresh weight **B.** Fresh weight per cotyledon disc **C.** Dry weight per cotyledon disc. Each data point represents the mean value of three experiments. The SE values averaged 6% and did not exceed 9% of the means.

Staden and Carmi, 1982; Ananieva et al., 2004a). Recently, the recovery of the functional activity of the photosynthetic apparatus in greening cotyledons after epicotyl decapitation was explained as being due to the increase in the levels of physiologi-

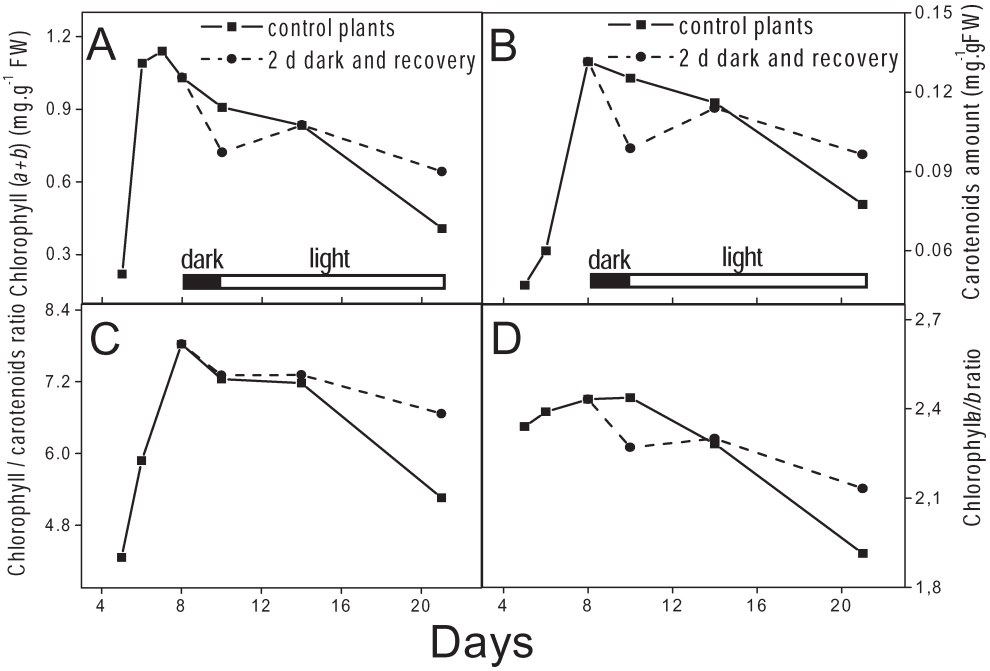

Fig. 4. Effect of 2-day darkening of entire seedlings on the chlorophyll and carotenoids content in intact cotyledons of *Cucurbita pepo* L. (zucchini). 8-day-old seedlings grown in a 12h/12h dark/light cycle were transferred to darkness for 2 days and after that returned to light regime (see Materials and Methods). Samples were collected 4 and 11 days after the seedlings had been returned to the light. **A.** Total chlorophyll content - chlorophyll (*a*+*b*) **B.** Total carotenoids amount **C.** Chlorophyll/carotenoids ratio **D.** Chlorophyl (*a*)/chlorophyll (*b*) ratio. Each data point represents the mean value of four experiments. The SE values averaged 5% and did not exceed 7% of the means.

cally active cytokinins and the decrease in the amount of storage and physiologically inactive cytokinins (Ananieva et al., 2004a).

Further on, we studied the effect of short-term darkening of entire plants on the physiological status of the cotyledons as a possible inducer of senescence. We used 8 day-old seedlings for a start of darkening when cotyledons demonstrated a maximum of CO_2 assimilation (results not shown). At that early seedling age no visible signs of natural senescence were observed. Using young seedlings allowed us to eliminate the impact of different endogenous factors, such as the senescence-related phytohormones ABA, ethylene and especially MeJA (Ananieva et al., 2004b). So, by this assay system we were able to study primarily the effect of darkness itself as a senescence-promoting factor. Two-day-dark treatment of the plants caused an inhibition of cotyledon fresh and dry weights as estimated per cotyledon disc (13 % and 17 %, respectively) (Fig. 3, B, C). In contrast, darkness provoked a slight increase in the fresh weight of the entire cotyledon (Fig. 3, A). This effect was preserved after the return of the plants to the light till the stage of natural senescence (Fig. 3, A). Transfer of the

seedlings to darkness resulted also in a decline both in total chlorophyll and carotenoids content by 21 % in comparison to the control (Fig. 4, A, B). Results indicated that together with chlorophyll content, fresh and dry weights of zucchini cotyledons could also serve as important parameters for quantifying senescence. The ability of cotyledons to recover after dark treatment was tested after return of the seedlings to the light. The return to normal light regime led to a full recovery of cotyledons as judged by the investigated parameters. Four days after returning to the light total chlorophyll and carotenoid contents reached the control values and even exceeded them by day 21 from the onset of germination (11 days after ceasing the dark treatment). At that age total chlorophyll amount in cotyledons exceeded that of the control by 58 %. In addition, similar stimulation in the amount of carotenoids (25 %) was also observed (Fig. 4, A, B). A higher chlorophyll/carotenoids ratio (27% stimulation compared to the control) as well as chlorophyll *a*/*b* ratio (12 %) in the recovering cotyledons were also registered (Fig. 4, C, D). The increase in the chlorophyll a/b ratio during delayed cotyledon senescence was similar to that observed after decapitation (Fig. 2) and deserves further analyses.

Decline in chlorophyll content during 2 days of dark treatment correlated with a drop in the rate of net photosynthesis measured by CO_2 assimilation (Fig. 5). While

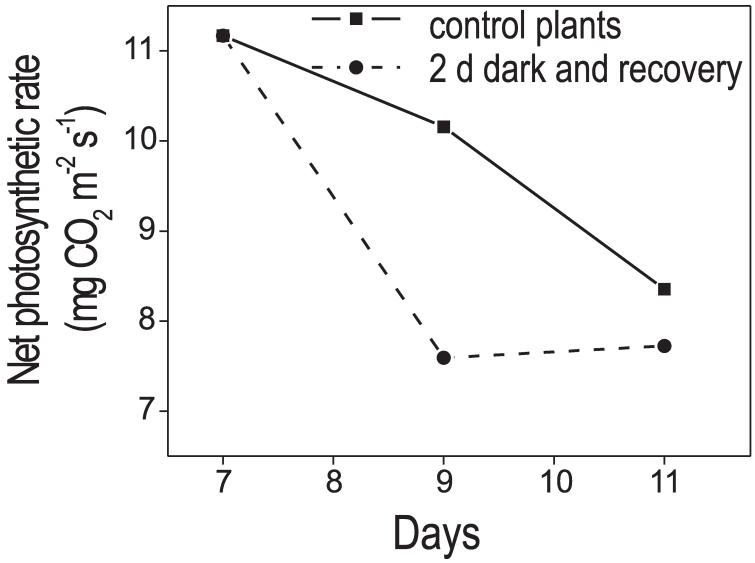

Fig. 5. Effect of 2-day darkening on the net photosynthesis rate in intact cotyledons of *Cucurbita pepo* L. (zucchini). 8-day-old seedlings grown in a 12h/12h dark/light cycle were transferred to darkness for 2 days and after that returned to light regime. Samples were collected 2 days after the seedlings had been returned to the light. Net photosynthesis rate was measured with a portable photosynthesis system (Li Cor 6000, USA) (see Materials and Methods). Each data point represents the mean value of three experiments. The SE values averaged 7% and did not exceed 11% of the means.

continuing to decrease in control variant, the photosynthetic rate in cotyledons after return of whole plants to light preserved the same values with a tendency of increase (Fig. 5).

Summing up, our results suggested that placing whole plants to darkness for 2 days did not induce irreversible symptoms of senescence in intact zucchini cotyledons. On the contrary, the process of senescence was even inhibited to some extent and its progress was delayed. Similar results were obtained with differentiated *Arabidopsis* leaves (Weaver and Amasino, 2001). In contrast to *C. pepo*, delayed senescence was not observed when intact *Arabidopsis* cotyledons were used, and their progressive yellowing was not reversed by the return of plants to the light. A slight increase in chlorophyll content of zucchini cotyledons after the return of 2-day-darkened plants to the light has recently been reported (Ananieva et al., 2004a). However, the measured values did not reach the control upon reillumination of the dark stressed plants. Recovery can be registered when entire plants are darkened but not in the case of individually darkened intact leaves (Weaver and Amasino, 2001). Apparently, the changes observed after whole plants' darkening are qualitatively different from those typical for natural senescence. At the same time the alterations in individually darkened leaves appear similar to those induced by age.

The transfer of entire plants to the dark for 5 days resulted in irreversible changes, which led to a significant delay in the growth and development of the plants (data not shown). Obviously, 5-day dark treatment of the cotyledons could be considered as an acute stress that could not be overcome. Similar results were previously reported for dark-induced senescence in wheat leaves (Lu, Zhang, 1998).

Our results showed that epicotyl decapitation at the age of natural cotyledon senescence led to recovery of pigment content, turgor and photosynthetic capacity of zucchini cotyledons. Therefore, rejuvenation of cotyledons in *Cucurbitaceae* can be used as an useful tool to study the mechanisms of senescence and its reversibility. In addition, this assay system is very convenient to investigate the differences in the dark-induced senescence between cotyledons and true leaves.

Acknowledgements: This work was funded by National Science Grant K-1403 2004.

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