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Limitations on photosynthesis under environment-simulating culture *in vitro*

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Abstract

Limitations on photosynthesis, characterized by leaf CO_2 exchange, chlorophyll fluorescence, and thylakoid structure, were studied under environmental conditions simulating culture *in vitro*. These were simulated by growing *Phaseolus vulgaris* plants in nutrient solution under high relative humidity of air (> 90 %), and CO_2 concentrations (c_a) that decreased with the development of photosynthetic activities during plant ontogeny (1200 to 300 mg m^{-3}). The ontogeny of such model plants was more rapid, primary leaves reached photosynthetic maturity 2 to 3 d earlier and their life span was 7 to 14 d shorter than in control plants. Their photosynthetic activity *in situ* was limited, after reaching "photosynthetic maturity", similarly to plants grown *in vitro*. When measured under optimal conditions, however, 50 to 70 % higher net photosynthetic rates (P_N) were found in leaves of different ages as compared with plants grown under c_a of 700 mg m^{-3} and a lower air humidity (30 - 35 %). This increase in P_N was associated with a high conductance for CO_2 transfer by adaxial and abaxial epidermes. In model plants, the dark respiration rate (R_D) was almost twice that in the control, while the photorespiration rates were similar to controls; CO_2 compensation concentration was about 50 % of that in controls. The ratios P_N/R_D were similar in control and in model plants. Chlorophyll *a+b* content in leaves of the model plants was lower than that in the control plants. Grana extent increased with plant age in the model plants while it decreased in the control ones. In both the stromal and granal membranes of the chloroplasts in model plants, a marked accumulation of carotenoids occurred independent of age. The ratio of variable to

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Abbreviations: c_a - ambient CO_2 concentration; Car - carotenoids; Chl - chlorophyll; d.m. - dry mass; E - transpiration rate; F_v - variable fluorescence; f.m. - fresh mass; g_{ab}, g_{ad} - stomatal conductance of abaxial and adaxial epidermes; P_N - net photosynthetic rate, PS - photosystem; q_{NP} - nonphotochemical quenching, q_P - photochemical quenching; Rfd - vitality index; R_D - dark respiration rate; R_L - photorespiration rate; ψ_w - water potential; Γ - CO_2 compensation concentration.

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maximal fluorescence, F_v/F_m, did not differ in the model and the control plants. In the control plants, photochemical quenching (q_P) slightly increased with plant age and was not affected by CO_2 concentration present during measurement. In the model plants, q_P increased with elevated CO_2 concentration in young plants and decreased in saturating CO_2 concentrations in older plants. Nonphotochemical quenching (q_{NP}) was lower in the model plants and increased under CO_2 saturating conditions. Vitality index, Rfd, was markedly lower in the model plants than in the control ones and a decline was found in saturating CO_2 concentration.

Key words: carotenoids, chlorophyll, conductances of adaxial and abaxial epidermes, dark respiration rate, dry mass, fluorescence, net photosynthetic rate, photorespiration rate, transpiration rate, water potential

Introduction

Photosynthetic activity of plantlets grown autotrophically or mixotrophically on agar substrate *in vitro* is frequently limited by low carbon dioxide concentration and low irradiance during the light period, and affected by high air humidity, increased ethylene concentration, *etc.* (Fujiwara *et al.* 1987, Kozai *et al.* 1986, 1987, 1990, 1991, Solárová 1989, Kozai 1991, Šantrůček *et al.* 1991, Pospíšilová *et al.* 1992). CO_2 levels in cultivation vessels often approaches CO_2 compensation concentration (Γ) for about three quarters of the light period, and P_N is near to zero. The considerable limitation on P_N results in a similar limitation on growth rate measured as the rate of dry matter accumulation (Solárová 1989). The daily carbon gain (24 h) of autotrophic plantlets cultivated *in vitro* using a standard procedure decreases - depending on the saccharose concentration in the medium - to 25 - 10 % of that in control plantlets cultivated under atmospheric CO_2 concentration. In spite of very low P_N in cultures *in vitro* (measured *in situ*) the photosynthetic apparatus is fully developed (Čatský and Solárová 1992). The limitation on P_N through CO_2 deficiency has been confirmed also by a 200 % increase in dry matter accumulation after increasing CO_2 concentration in cultivation vessels (Solárová *et al.* 1989, 1994) and by marked increases in dry matter accumulation after transplanting plantlets to the open air (Pospíšilová *et al.* 1989). The difference in *in vitro* dry matter accumulation between plantlets grown under atmospheric and increased CO_2 concentration is more pronounced on a medium without saccharides.

The experiments presented in this paper were aimed to characterize photosynthetic activities and limitations to them in model plants cultivated on mineral medium under atmospheric conditions simulating the conditions typical for developing autotrophic culture *in vitro,* namely high air humidity and decreasing CO_2 concentration during the light period.

Materials and methods

Plants: Seeds of French bean (*Phaseolus vulgaris* L. cv. Jantar) were germinated in Petri dishes on filter paper soaked with distilled water. After 4 d, thirty seedlings

were placed into holes in porcelain holders in 4500-cm^3 glass vessels, each containing 1000 cm^3 of IBP nutrient solution (Hewitt 1966). The plants were grown in a growth cabinet at an irradiance of 450 μmol m^{-2} s^{-1} inside the cultivation vessels. Nutrient solutions were changed every third day.

The model plants were grown in closed vessels under high relative humidity of air (92 - 94 %), temperatures of 27 - 29 °C, and CO_2 concentration decreasing with the development of photosynthetic activities during plant ontogeny (from 1200 to 300 mg m^{-3}). Further diurnal changes in CO_2 concentration in the model environmental conditions followed those in the environment of cultivation vessels during *in vitro* culture of tobacco (Fig. 1, Solárová 1989). Control plants were grown in similar vessels under CO_2 concentrations of *ca.* 700 mg m^{-3}, relative humidity of air 30 - 35 % and temperature 25 - 26 °C.

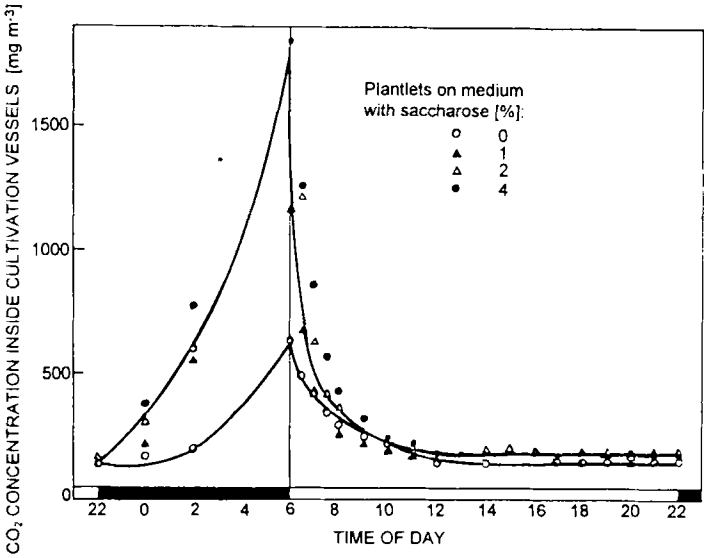

Fig.1. Diurnal variation in CO_2 concentration in cultivation vessels with tobacco plantlets. Solid half-strength Murashige-Skoog medium, day/night temperature 30/35 °C, relative humidity 90 ± 5 %, irradiance 40 ± 5 μmol m^{-2} s^{-1}. Modified from Solárová (1989).

Treatments started on the 4th day after sowing and measurements of all the studied photosynthetic parameters started between the 7th and 10th day from sowing, *i.e.* before reaching half the final area of primary leaves. Three to five times during plant ontogeny, the following parameters were measured:

Chlorophyll fluorescence induction kinetic was measured after a 15-min dark period with the *PAM Chlorophyll Fluorometer* (*Walz,* Effeltrich, Germany) on detached leaves at room temperature, and at CO_2 saturation using potassium bicarbonate or at atmospheric CO_2 concentration. Measuring beam intensity was 0.5 W m^{-2}, actinic irradiance 150 W m^{-2}, 700-ms saturated flashes of 1 400 W m^{-2} were fired every 10 s. Data sampling, control and calculation was served by the *DA 100 Data Acquisition System* (*Walz,* Effeltrich, Germany).

Chlorophyll and carotenoid contents were determined in 80 % acetone extracts of leaves with a spectrophotometer *PU-8740* (*Philips Scientific*, Cambridge, U.K.) by the methods of Arnon (1949) and Lichtenthaler (1987), respectively.

CO_2 exchange rates were measured on leaves reaching at least 3 cm^2 leaf area. The rates of net photosynthesis (P_N) and respiration (R_D) were determined as CO_2 flux in a closed gas exchange system with an infrared gas analyser *Infralyt IV* (*Junkalor*, Dessau, Germany) in a CO_2 concentration range from 20 to 1200 mg m^{-3}, leaf temperature 22 °C and a near-saturating irradiance (400-700 nm) of 860 μmol m^{-2} s^{-1} (Čatský and Tichá 1975, Kaše and Čatský 1983). Photorespiration rate (R_L) and CO_2 compensation concentration were calculated from CO_2 dependence of P_N. Daily net carbon gain was calculated for primary leaves from 24-h CO_2 exchange rates (Čatský *et al.* 1987).

Stomatal conductances of abaxial and adaxial epidermes were measured by a diffusion porometer *Delta-T* (type *Mk3*, *Delta-T Devices*, Cambridge, UK) at a temperature of 25 °C, irradiance of 860 μmol m^{-2} s^{-1}, and air humidity 50 %. Total conductance for CO_2 was calculated from the initial slope of the curve relating P_N to CO_2 concentration.

Transpiration rates (E) were determined from water loss curves measured gravimetrically on leaves originally fully turgid. Irradiance was 860 μmol m^{-2} s^{-1}, temperature 25 °C, and air humidity 35 %. The transpiration rate was calculated from the slope of the water loss curve (as the first derivative) in the first minutes after cutting, as it is supposed that in this time the rate of water loss corresponds to the E *in situ* under the same conditions. These measurements were done for comparison of transpiration rates in plants growing in conditions simulating *in vitro* culture with those measured previously in plantlets *in vitro* (leaves of which were not suitable for porometric determination of stomatal conductance).

Leaf water potential was measured by a *Dew Point Hygrometer HR-33T* (*Wescor*, Logan, USA) connected with a leaf chamber *L-51* which enables water potential to be measured on leaves *in situ*.

Membrane stacking estimation: Chloroplasts were isolated from leaves of 8 and 15 d old plants by the method of Melis and Anderson (1983). About 5 g of cut leaves were ground with a *Ultra-Turrax* homogenizer (*Janke & Kunkel*, Stauffen, Germany) in 50 cm^{-3} of isolation medium (50 mM Tricine, 10 mM NaCl, 5 mM $MgCl_2$, 0.33 M sorbitol with addition of 1 % polyvinylpyrrolidone, pH 7.8). After filtration through a gauze, the homogenate was centrifuged at 2 000 *g* for 10 min. The pellet was washed and resuspended in incubation medium (50 mM Tricine, 150 mM KCl, pH 7.3). Isolated membranes were treated with 0.5 % digitonin (Dg), Dg:Chl = 3, for 30 min at 4 °C in the dark. Further centrifugation at 10 000 *g* for 30 min separated the grana (pellet) and intergranal (supernatant) membranes. The relative amount of both types of membranes was estimated by determining chlorophyll content.

Irradiance and **air humidity** were measured with *Li-Cor LI 185B* radiometer with quantum sensor (*Li-Cor*, Lincoln, NE, USA) and with *JUMO Humitherm TDAc-70* (*M.K. Juchheim*, Fulda, Germany), respectively.

Dry mass was determined in samples oven-dried at 90 °C to constant mass.

Results

Growth: The ontogenetic changes in growth of experimental plants and their primary leaves were similar to those found in French bean earlier (Čatský *et al.* 1976, Čatský and Tichá 1980). The ontogenetic changes in area and dry mass of primary leaves followed a usual S-shaped curve. Leaf areas of control plants increased rapidly after

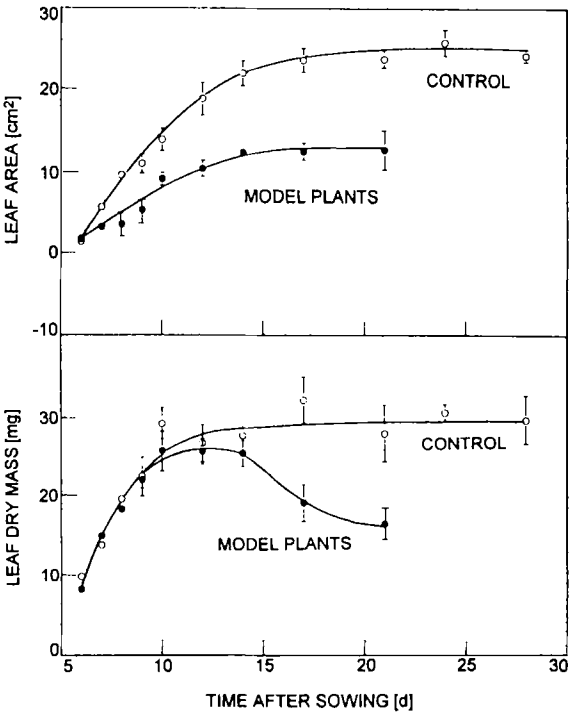

Fig. 2. Leaf area and dry mass during ontogeny of primary leaves of the model and the control French bean plants.

leaf unfolding 6 d after sowing to a maximum size on about the 15th day, while leaf dry mass was continuing to increase slowly (Fig. 2). The leaf life span was between 25 and 35 d. However, the ontogeny of the model plants grown in conditions simulating *in vitro* culture was more rapid, primary leaves reached photosynthetic maturity 2 to 3 d earlier and their life span was 7 to 14 d shorter than in the control plants.

Chlorophyll *a+b* (Chl) content in leaves calculated per unit leaf area or fresh leaf matter decreased somewhat with plant age. 7-d-old model plants exhibited lower Chl content than control plants, while in 14-d-old plants no significant difference was observed between them. The lower content of Chl *b* and excess of carotenoids (Car) in the model plants were manifested in slightly higher ratios of Chl *a/b* (Fig. 3) and significantly lower ratio Chl *a+b*/Car (xanthophylls+carotenes) than in the control plants. A statistically significant increase in Chl *a/b* ratio was observed in both types of plants within one week of treatment.

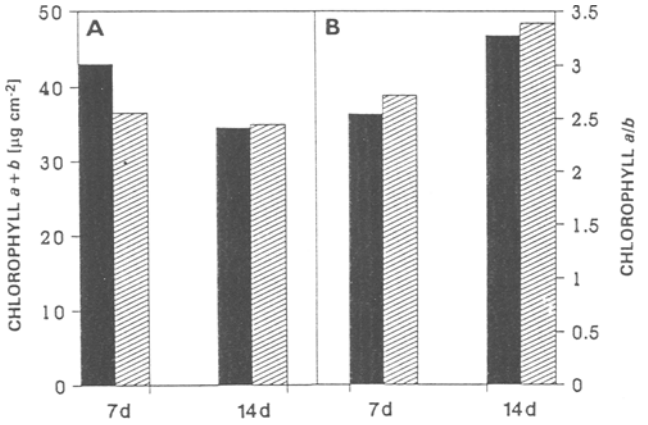

Fig. 3. Chlorophyll *a+b* content (*A*) and Chl *a/b* ratios (*B*) in primary leaves of 7- and 14-d-old model (*hatched columns*) and control (*full columns*) bean plants.

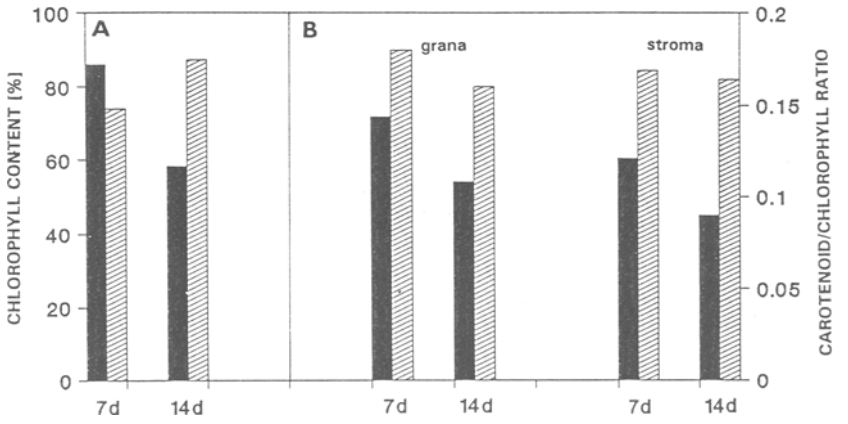

Fig. 4. Comparison of grana extent expressed as relative Chl content in 10 000 x *g* pellet after digitonin treatment (*A*) and of relative Car content in grana and stroma thylakoid membranes (*B*) in primary leaves of 7- and 14-d-old model (*hatched columns*) and control (*full columns*) plants.

Chloroplast membrane structure: As estimated by chlorophyll content, chloroplasts of both control and model plants contained a rather high proportion of grana membranes (Fig. 4A). An opposite trend in thylakoid stacking during plant ageing

was found in the model and control plants: the relative amount of grana membranes increased with age of the model plants, while it decreased in the control ones. Though senescence in model plants occurred earlier, grana degradation was not found, probably due to an effect of low CO_2 concentration in cultivation vessels.

Significantly higher relative content of carotenoids was observed in the model plants than in the control plants in both the granal fraction and even to higher extent in the stromal fraction. The influence of CO_2 was more pronounced in fractions from the 14-d-old plants (Fig. 4B).

Chlorophyll fluorescence kinetics was measured at two distinct CO_2 concentrations to demonstrate the adaptability of plants to a changed concentration of CO_2. There was no significant difference in the ratio of F_v/F_m between the control and the model plants (Fig. 5A). Photochemical quenching (q_P) slightly increased with plant age and was not affected by CO_2 concentration during measurement in the control plants, while in the model plants the effect of elevated level of CO_2 was age dependent. In

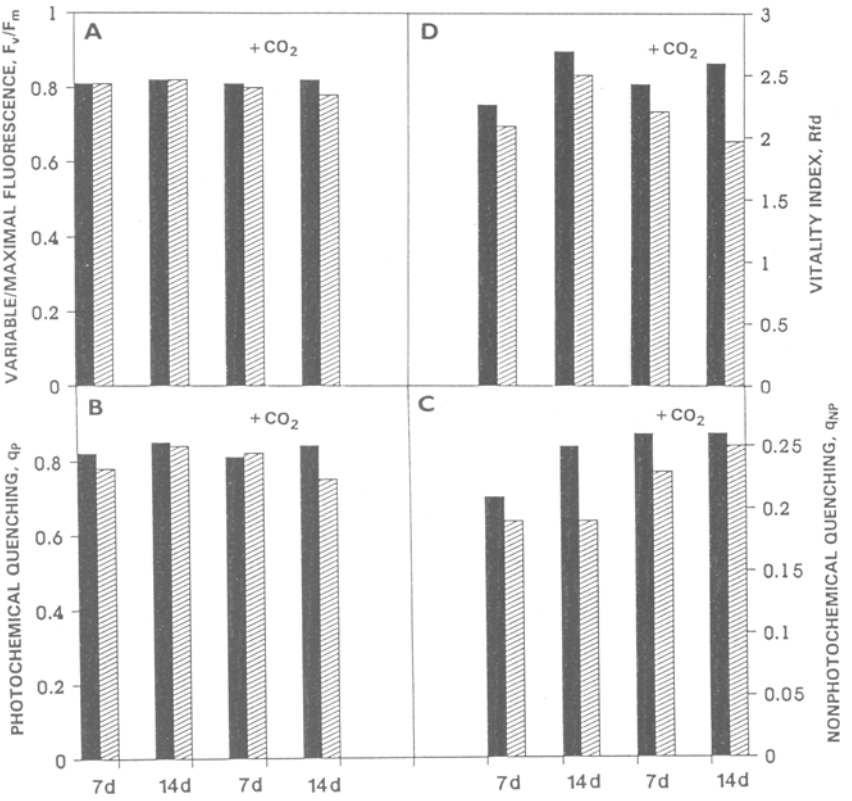

Fig. 5. *In vivo* Chl fluorescence parameters in primary leaves of 7- and 14-d-old model (*hatched columns*) and control (*full columns*) plants, measured in ambient or saturated (indicated as $+CO_2$) CO_2 concentrations: photochemical efficiency as ratio of F_v/F_m (*A*); photochemical quenching, q_P (*B*); nonphotochemical quenching, q_{NP} (*C*); relative fluorescence decrease, indicated as vitality index, Rfd (*D*).

7-d-old model plants q_P increased with increased CO_2 concentration, while in 14-d-old plants a decrease of about 12 % in q_P was found under saturating CO_2 concentration (Fig. 5B). The level of non-photochemical quenching (q_{NP}) was about 20 % higher under elevated CO_2 concentration in both control and model plants (Fig. 5C). The fluorescence decrease ratio from the maximum to the steady-state, *i.e.* Rfd (Lichtenthaler and Rinderle 1988), was markedly lower in the model plants than in the control plants. A decline of Rfd under saturating CO_2 was observed in the model plants, as with q_P (Fig. 5D).

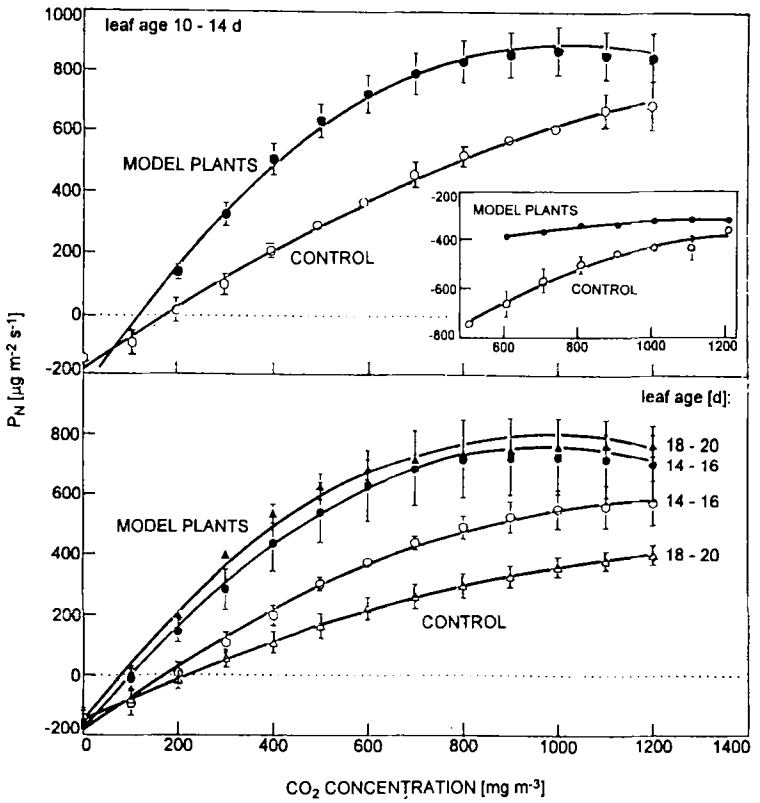

Fig. 6. Net photosynthetic rates in dependence on ambient CO_2 concentration during ontogeny of control and model primary leaves of French bean (*inset:* cotyledons).

CO_2 exchange: After reaching "photosynthetic maturity" (Šesták and Čatský 1962, Tichá *et al.* 1985), the net photosynthetic rate of the model plants was limited in cultivation vessels, similarly to plants grown *in vitro* (Solárová 1989). When measured under optimal conditions *ex vitro,* however, 50 to 70 % higher P_N was found in leaves of different ages of model plants as compared with control plants (Fig. 6). In model plants, dark respiration rate was almost twice that in the control, while photorespiration rate was similar to controls; CO_2 compensation concentration was about 50 % of that in the controls. The ratios P_N/R_D *ex vitro* were similar in

control and in model plants. In fully developed leaves, the daily net carbon gain (24 h) calculated from CO_2 exchange rates *ex vitro* was slightly higher in model plants; in cultivation vessels, however, the daily net carbon gain in model plants was around zero due to markedly lower CO_2 concentration.

Leaf conductances: Adaxial and abaxial epidermal conductances decreased during aging of plants in both variants. Both conductances were always higher in the model plants than in the control ones (Fig. 8). The ratio between abaxial and adaxial epidermal conductance decreased with aging of plants and was always higher in the model plants.

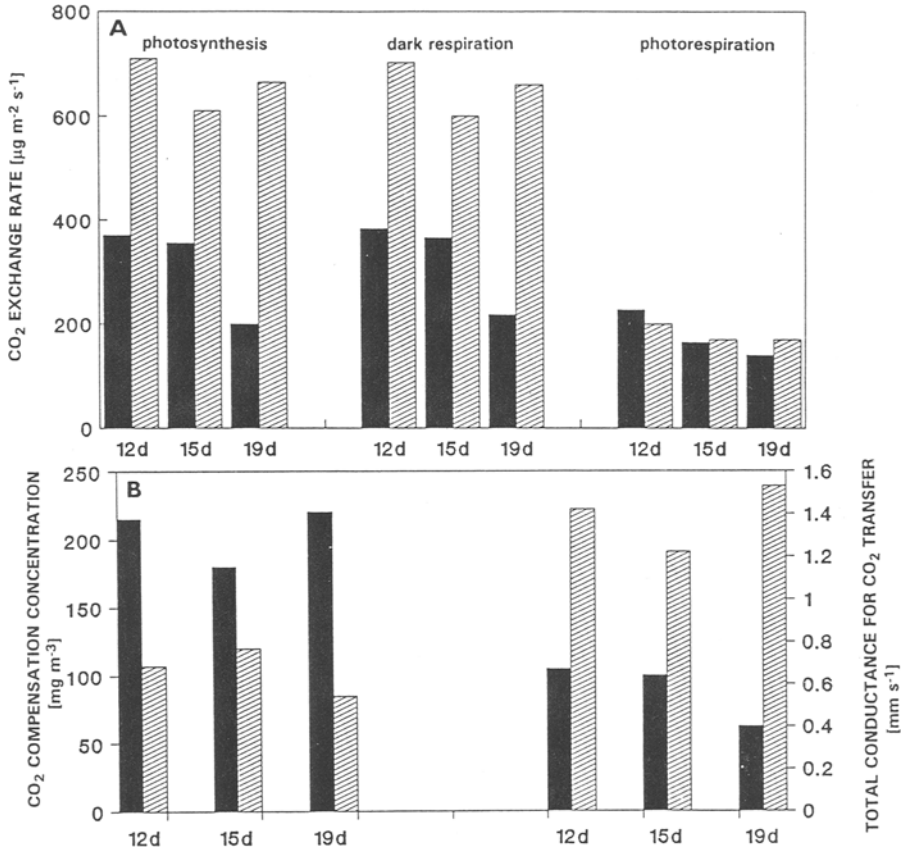

Fig. 7. Net photosynthetic rate, dark respiration rate, photorespiration rate, CO_2 compensation concentration and total conductance for CO_2 transfer in primary leaves of 12-, 15-, and 19-d-old model (*hatched columns*) and control (*full columns*) bean plants.

The total conductance for CO_2 transfer was also higher in the model plants than in the control ones during the whole leaf life span. It decreased with plant age in the control plants but not in the model plants (Fig. 8).

The transpiration rate (E) was also higher in the model plants than in the control ones. While in the control plants E decreased during their ontogeny (Fig. 9), the high E in the model plants remained for the whole life span and even increased.

Leaf water potential of model plants was very high during their whole life span (*ca.* -0.3 MPa). Leaf water potential in control plants was also about -0.3 MPa in the beginning of their ontogeny but decreased a little during their growth (to -0.5 MPa).

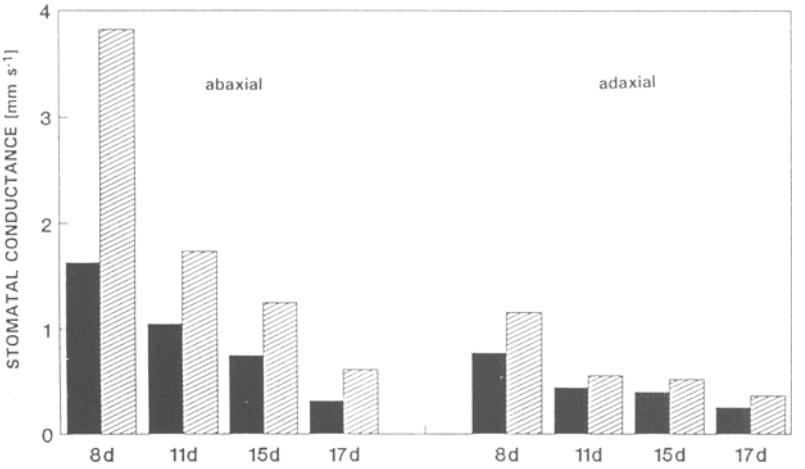

Fig. 8. Abaxial and adaxial stomatal conductances for water vapour transfer in primary leaves of model (*hatched columns*) and control (*full columns*). Measurements were done on 8-, 11-, 15-, and 17-d-old plants at temperature 25 °C, irradiance of 860 μmol m^{-2} s^{-1} and air humidity 50 %.

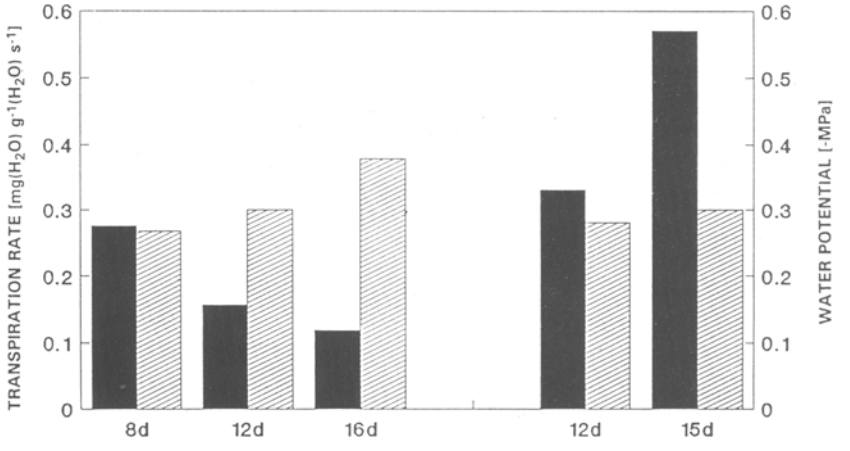

Fig. 9. Transpiration rate (measured at irradiance 860 μmol m^{-2} s^{-1}, temperature 25 °C, air humidity 35 %) and leaf water potential in primary leaves of model (*hatched columns*) and control (*full columns*) bean plants.

Discussion

Our earlier studies on photosynthesis of plantlets grown *in vitro* indicated several peculiarities in the biological limitations on leaf photosynthesis (Pospíšilová *et al.* 1987, 1988, 1989, 1990, 1992, Solárová *et al.* 1989). It was therefore the aim of this project to study photosynthetic processes on larger seedlings grown under environment simulating culture *in vitro*, because the rather small plantlets from *in vitro* culture do not allow a more detailed analysis of photosynthetic activities, and, moreover, there exist no really comparable control plants *in vitro*.

The main feature of this approach is that the model plants in very early stages of ontogeny were grown at a rather high CO_2 concentration. This was chosen according to the changes in CO_2 concentration in cultivation vessels during development of autotrophy in cultures *in vitro* (Čatský, unpublished). The high CO_2 concentration may markedly affect the development of photosynthetic apparatus before the photosynthetic activities are measured. Thus the differences between model and control plants presented here may reflect the action of higher CO_2 concentrations at this very early stage of development, even when the later CO_2 concentrations in the cultivation vessel are much lower.

CO_2 exchange rates and other photosynthetic parameters in both types of plants followed the ontogenetic changes generally known (Čatský *et al.* 1985, Čatský and Tichá 1985, Šesták *et al.* 1985).

CO_2 exchange rates were generally higher in model plants when measured under near-optimum conditions *ex vitro*. In cultivation vessels, however, the rather low CO_2 concentration markedly limited net photosynthetic rate as may be inferred from Fig. 6. The daily net carbon gain of model plants was therefore zero or even negative. This was reflected in a decrease in leaf dry mass during senescence of the model plants.

The increase in P_N in model plants was associated with very high adaxial and abaxial epidermal conductances. Even if leaf water potential in control plants decreased a little during their growth, this value was high enough that it was not reasonable to suppose that lower epidermal conductance in control plants was due to their wilting. As found in previous experiments, stomata in bean plants were closed at water potentials lower than -1.0 MPa (*e.g.* Pospíšilová and Solárová 1980). In connection with the high epidermal conductance, the transpiration rate was also higher in model plants than in the control ones and so detached leaves from model plants wilted very quickly. While in control plants the rate of water loss decreased during their ontogeny due to the development of cuticular waxes and of the ability of stomata to regulate water loss by their closing, the high rate of water loss in model plants remained for the whole life span and even increased. This suggests that in these plants the development of cuticular waxes and the ability of stomata to regulate water loss were retarded. All the above-mentioned properties of model plants were in close agreement with the properties of plantlets grown *in vitro* which were found in previous experiments (Pospíšilová *et al.* 1987, 1988, 1989, Solárová *et al.* 1989). These properties are probably connected with growing plants under high relative

humidity, as Sage and Reid (1992) did not find any changes in stomatal conductance in bean plants induced by growing under decreased CO_2 concentration.

The decrease in q_P, which measures the relative concentration of open PS 2 traps, and in F_v/F_m observed in 14-d-old model plants in comparison to the control ones could indicate damage to PS2 centres. However, the dependence of q_P on actinic radiation fluence rate must also be compared to verify whether the observed effect might have been induced by different cultivation conditions. The increase of non-photochemical quenching (q_{NP}) observed under elevated CO_2 concentration in both types of plants was similar to that found by Sharkey *et al.* (1988). As Rfd is a factor that integrates photochemical processes and CO_2 fixation (Lichtenthaler and Rinderle 1988), it seems useful in giving a true picture of plant acclimation. The course which Rfd followed in model plants supported the hypothesis that these plants are more adaptable to changes in CO_2 concentration during the initial phase of growth, while after 10 d they were adapted to lower CO_2 concentration and were not able quickly to adjust their photosynthetic apparatus to a surplus of CO_2, as exhibited by the decrease of q_P, F_v/F_m and Rfd. It will be necessary to supplement the present preliminary results with more detailed measurements of the initial phase of acclimation and of irradiance and CO_2 concentration dependences.

The increased ratio of Chl *a/b* observed in leaves of model 14-d-old plants, that under these stress conditions were in the phase preceding senescence, was probably due to priority degradation of Chl *b* (*cf.* Walmsley and Adamson 1989, Dean *et al.* 1993). The high retention of carotenoids as compared to total Chl could have a physiological significance as they protect the remaining Chl molecules from damage by free oxygen radicals (Young and Britton 1990), formed preferentially during stress and senescence.

In chloroplasts, we found a rather high relative content of granal membranes in the 14-d-old model plants that could be due to the shade character of these chloroplasts induced by the glass cover shielding. This phenomenon could not be explained simply by increase in content of light-harvesting chlorophyll-protein complexes, since in thylakoids the Chl *a/b* ratio increased. The differences in grana thylakoid stacking could be influenced by changes in membrane composition. In *Chlamydomonas*, decreased CO_2 concentration modulates thylakoid membrane lipid composition (Sato 1989) and induces synthesis of several membrane-associated polypeptides (Spalding and Jeffrey 1989).

The present results explain only some aspects of photosynthesis in plants cultivated *in vitro*. However, high initial ambient CO_2 concentration must also affect the carbon fixation reactions. This area remains unclear, and will be followed in a further study.

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