

Isolation of chloroplasts in the *Karwinskia* species and determination of their photochemical activity under *in vitro* conditions

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ABSTRACT

Reaction conditions for evaluation of the photochemical activity of isolated chloroplasts in the Hill reaction of *Karwinskia humboldtiana* (Roem & Schut) Zucc. and *Karwinskia parvifolia* Rose species were determined. Hill's reaction activity was measured spectrophotometrically at 630 nm as the amount of DCPIP reduction by the chloroplast suspension at an irradiance of 400 $\mu\text{mol}/\text{m}^2/\text{s}$ PAR. A significant difference was observed between the activity of chloroplasts isolated at 2°C and 25°C amounting to 27% in *Karwinskia humboldtiana* and 18.5% in *K. parvifolia*. After 24 hours of storage of chloroplasts at a suspension of 2°C, a significant decrease of chloroplasts activity was noted in both species, e.g. 38% in *Karwinskia humboldtiana* and 45% in *K. parvifolia*. The photochemical activity of chloroplasts increased also with the length of irradiation of the reaction mixture and the content of chlorophyll (*a* + *b*) in chloroplast suspension. The activity of chloroplasts was found to be significantly higher in the species *K. humboldtiana* than in *K. parvifolia* and it proved higher in both when these were grown under field conditions rather than in a greenhouse.

Keywords: *Karwinskia humboldtiana*; *Karwinskia parvifolia*; isolation of chloroplasts; Hill reaction activity

Species of the genus *Karwinskia* (family Rhamnaceae) are known for their high content of secondary metabolites on the basis of dimeric anthracenones (Dreyer et al. 1975) and (Hanáčková 1999) in vegetative and generative organs (Argalášová-Šútovská et al. 2000) with a selective antitumour effect (Piñeyro-López et al. 1994).

According to Masarovičová et al. (2000) a direct correlation was established between photosynthesis as a primary metabolism and production of peroxisomicine A₁ as the product of secondary metabolism of the *Karwinskia* species. The study of photochemical activity of isolated chloroplasts in the Hill reaction (as photosystem PS 2) under *in vitro* conditions is one of the important characteristics of photosynthetic activity in many species of plants (May 1975, Plesničar and Stanković 1979, Zunzunegui et al. 1999, Zhu et al. 2001).

The different rates of the Hill reaction observed during plant growth and ontogenesis of primary leaves of different species suggested that they might result from the method used for chloroplasts isolation or determination of reaction conditions (Moreland and Hill 1962, Fry 1970, Trebst 1972, Strnadová and Šesták 1974, Šesták 1985, Atal et al. 1991, Kutík et al. 1999, Subhan and Murthy 2000). Species of the genus *Karwinskia* growing in subtropical and tropical areas of Mexico (Fernández 1992),

but unfortunately no study of the photosystem PS 2 in these species was made.

The aim of the present study was to elaborate a method for isolating active chloroplasts and determining the reaction conditions and then to compare the photochemical activity in the Hill reaction in two species of *Karwinskia* with the highest content of the toxin peroxisomicine A₁.

MATERIAL AND METHODS

Plant material

The plants of *Karwinskia humboldtiana* (Roem & Schut) Zucc. and *K. parvifolia* Rose (Figure 1) of four and seven years old respectively, were grown in the greenhouse at the Department of Plant Physiology, Faculty of Natural Sciences, Comenius University in Bratislava. Plants of the same species grown in the experimental pots during the growing season were cultivated under field conditions from May 1 to October 30, 1999. The plants were grown from seeds originating from the locality Villa de García in the State of Nuevo León (*K. humboldtiana*) and from the state Sinaloa, Mexico (*K. parvifolia*). The plants were cultivated in a substrate containing 11.8% humus at pH 7.2 and

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Figure 1. Shape and size basal and apical leaves, four years old plants of *Karwinskia humboldtiana* (A) and *Karwinskia parvifolia* (B) grown under greenhouse conditions

phosphorus (P) 28, potassium (K) 90, magnesium (Mg) 680 and calcium (Ca) 4900 mg per 1 kg of substrate. The analysis of the soil was done at the Soil Science and Conservation Research Institute, Bratislava, Slovakia.

Preparation of chloroplasts

Chloroplasts for the Hill reaction assay were prepared as follows: *Karwinskia* leaves (10 g) from seven-years old plants, previously washed with deionized water, were homogenized for 60 s in a 100 cm³ medium containing 0.15M phosphate buffer at pH 7.4 with 0.5M sucrose. The phosphate buffer consisted of 0.15M Na₂HPO₄·12 H₂O and 0.15M KH₂PO₄ (8:2). The homogenate chloroplasts were filtered through four layers of gauze, centrifuged at 6700 × g for 15 min at 2°C and 25°C and

re-suspended in different volume of the phosphate buffer (pH 6.5) containing 10% glycerine (RM I) or in 0.05M Tris-buffer containing 0.35M sucrose (pH 6.5) (RM II) with a final concentration from 83.2 to 35.2 mg/l (*K. humboldtiana*) and from 84.9 to 35.1 mg/l (*K. parvifolia*) of chlorophyll (*a + b*). Comparison of the photochemical activity of isolated chloroplasts in four-years old plants was done in July 1999. The mean daily air temperature in the greenhouse during this month was 28 ± 1°C and under field conditions 31 ± 1°C, the relative air humidity being 80 ± 5% and 40 ± 5%, respectively. The suspension of the chloroplasts in this case (four-year old plants) was re-suspended only in RM II with the resulting concentration of chlorophylls (*a + b*) from 92 to 95 ± 9.08 mg/l. The content of chlorophyll in chloroplast suspensions was determined spectrophotometrically by measurements of A₆₄₉ and A₆₆₅ on 80% (v/v) acetone extracts (Vernon 1960).

Preparation of the redox-system

As a redox-system we used sodium salt of 2,6-dichlorophenol-indophenol (DCPIP), prepared from 58 mg in 100 cm³ phosphate buffer (pH 6.5) The DCPIP solution was diluted so that its concentration before irradiation was from 77.8 to 88.9 mmol/l.

Determination of the Hill reaction activity

The reaction mixture contained 9 cm³ of DCPIP solution and 1 cm³ of chloroplasts suspension diluted as stated above. The reaction mixture was exposed for 1 to 5 min at irradiance of 400 $\mu\text{mol m}^2/\text{s}$ PAR through 10 cm of an aqueous filter at a temperature $20 \pm 1^\circ\text{C}$. Hill reaction activity was measured spectrophotometrically at 630 nm as the amount of DCPIP reduction by the chloroplast suspensions during irradiation using the spectrophotometer Jenway 6400 (England) in glass cells of 1 cm light-pass. The activity of chloroplasts was evaluated at a rate of DCPIP photoreduction in % and as in Hill reaction activity (HRA) in mmol (DCPIP)/kg

(Chl)/s. The measurements were made in triplicate and the data are presented as arithmetic means of the various experiments. The results were statistically evaluated using Student's *t*-test.

RESULTS AND DISCUSSION

To study the photochemical activity of chloroplasts in the species *Karwinskia* it was necessary to find not only a suitable way of isolating chloroplasts, but likewise to determine the optimum conditions for measuring the speed of Hill's reaction *in vitro*. The methodical procedures for isolating chloroplasts described so far and utilized in various plant species differ by the composition of isolation and re-suspension media, number and length of centrifugations, use of various electron acceptors, the composition of reaction media and the procedure proper for measuring chloroplast activity in Hill's reaction. The most frequently utilized isolating chloroplast media and also the most accessible are sucrose-phosphate buffers, which we have also used in our experiments. It was shown that there is an imperative need to ensure that the entire isola-

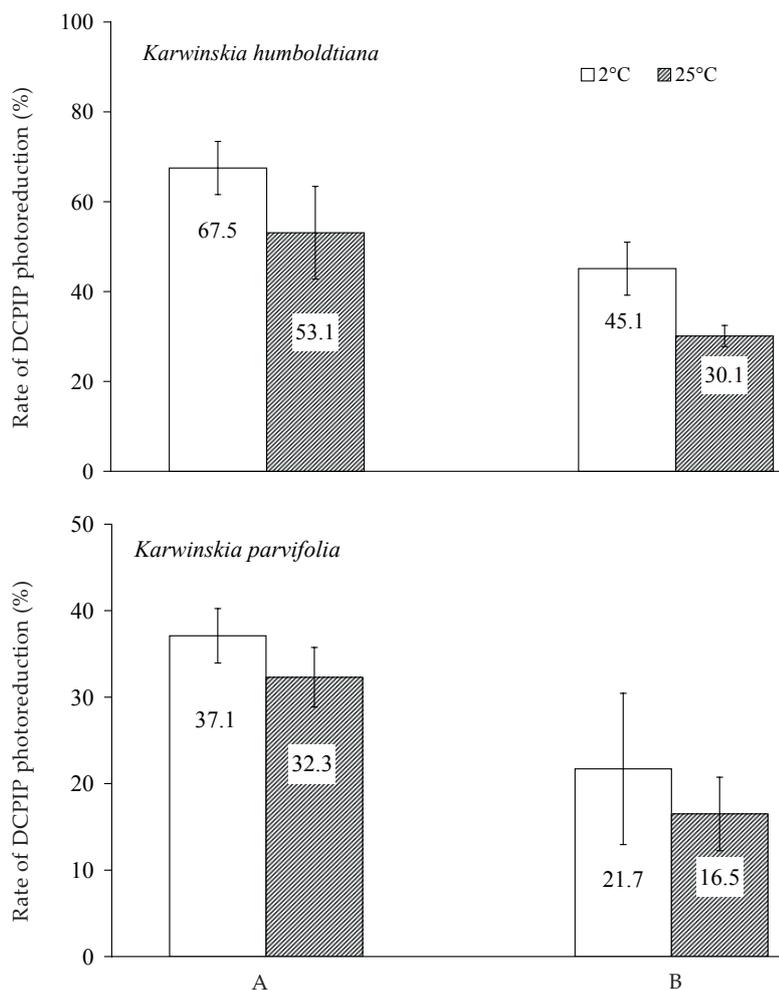


Figure 2. Rate of photoreduction of DCPIP (in %) by isolated chloroplasts prepared at 2°C and 25°C in time t_0 (A) and after 24 hours storage at 2°C (B) in *Karwinskia* species; plants were seven years old grown under greenhouse conditions; dates represent means \pm SE, $n = 3$

Values between *K. humboldtiana* and *K. parvifolia* are significantly different at $P = 0.01$; values of activity chloroplasts isolated in time t_0 and after 24 hours storage are significantly different at $P = 0.001$ in both species

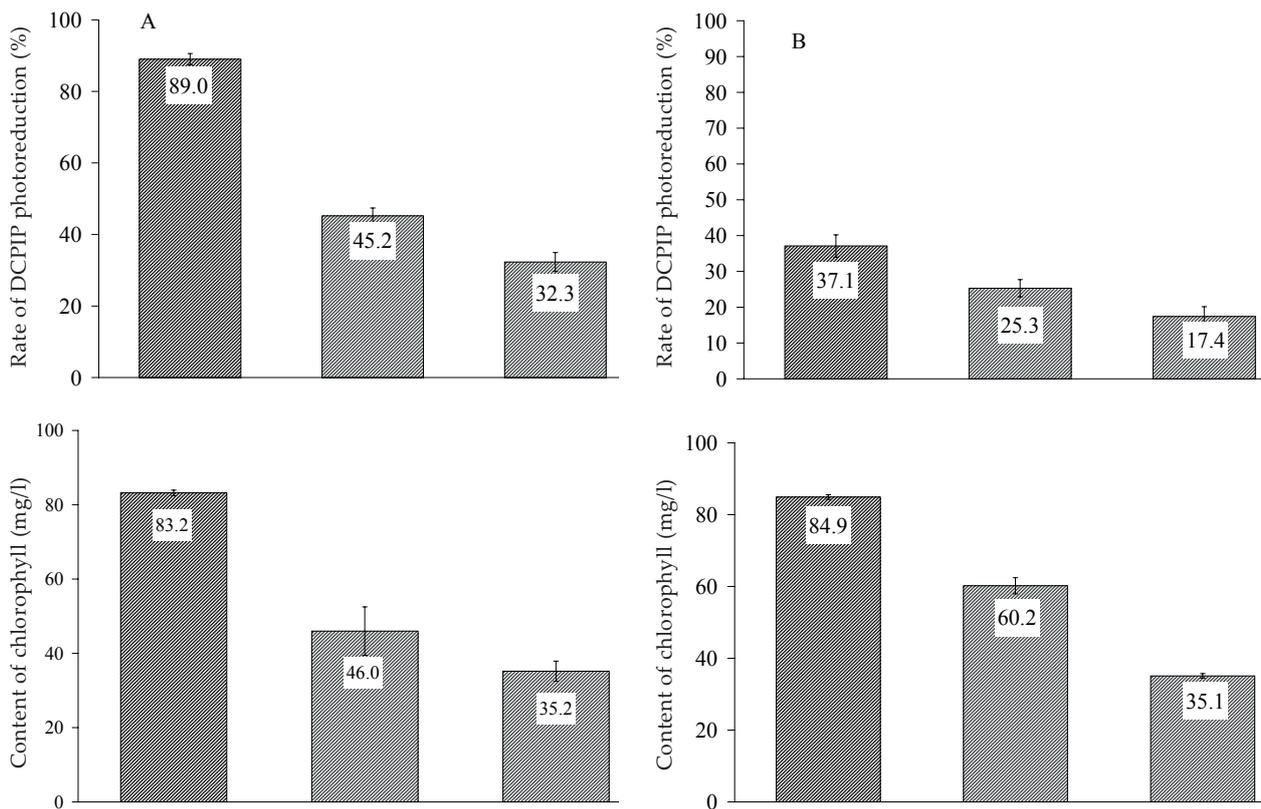


Figure 3. Rate of photoreduction of DCPIP (in %) depending on the concentration of the chlorophyll content ($a + b$) in 1 cm^3 of chloroplast suspension in the reaction mixture; the plants of *Karwinskia humboldtiana* (A) and *Karwinskia parvifolia* (B) were seven years old grown under greenhouse conditions; dates represent means \pm SE, $n = 3$

tion procedure take place under conditions of cold, with sufficiently cooled solutions. This finding is also supported by the results of the varying activity of chloroplasts isolated at 2°C which proved to be decidedly higher in the species *Karwinskia humboldtiana* by 27.3% and in that of *K. parvifolia* by 18.5% than the activity of chloroplasts isolated at 25°C (Figure 2) thus giving support to Wasserman's and Fleisher's view (1968) to the effect that chloroplast activity is generally maintained and prolonged by cooling. The enhanced sensitivity of chloroplasts of the *Karwinskia species* to the procedure as such and to their storage conditions is also attested to by the evident decline in chloroplast activity noted after their maintenance at $2 \pm 1^\circ\text{C}$. The decline in chloroplast activity at 2°C after 24 hours of storage was lower by 32.2% in *K. humboldtiana* and by 41.5% in *K. parvifolia* than on the day of their preparation, while in the case of chloroplasts isolated at 25°C this decline proved even more striking and amounted to 43.3% in *K. humboldtiana* and 48.9% in *K. parvifolia* (Figure 2). The above results also correspond to those obtained by Öquist et al. (1974) who found the most striking decline in chloroplast activity of the pine during the first days of storage. One of the possibilities of prolonging chloroplast activity in the

Karwinskia species might be other types of media, e.g. on the basis of Tricine and HEPES-buffers which, however, are more costly but, according to Synková and Šesták (1991) ensure a high pH stability.

The use of two different media failed to reveal evident differences in chloroplast activity of the *Karwinskia species*. A resuspension medium based on phosphate buffer containing 10% of glycerine (RM I) yielded an activity of $42.3 \pm 1.51\%$, and with the use of TRIS-buffer containing sucrose (RM II) the activity amounted to $43.3 \pm 0.81\%$. However, evident difference in chloroplast activity came out when it was compared in RM II ($29.3 \pm 0.71\%$) with that in RM I (25.1 ± 1.51) in the species *Karwinskia parvifolia*. This finding clearly points to a higher chloroplast sensitivity of the latter species to the composition of the media in their isolation and storage. An altered composition of the media used in the isolation of chloroplasts from leaves of legumes (Strnadová and Šesták 1974), pine and fir (Öquist et al. 1974) and *K. humboldtiana* followed here, had no effect on the change and course of Hill's reaction.

The photochemical activity of chloroplasts and reliability of its assay also depend on the chlorophyll concentration in the chloroplast suspension.

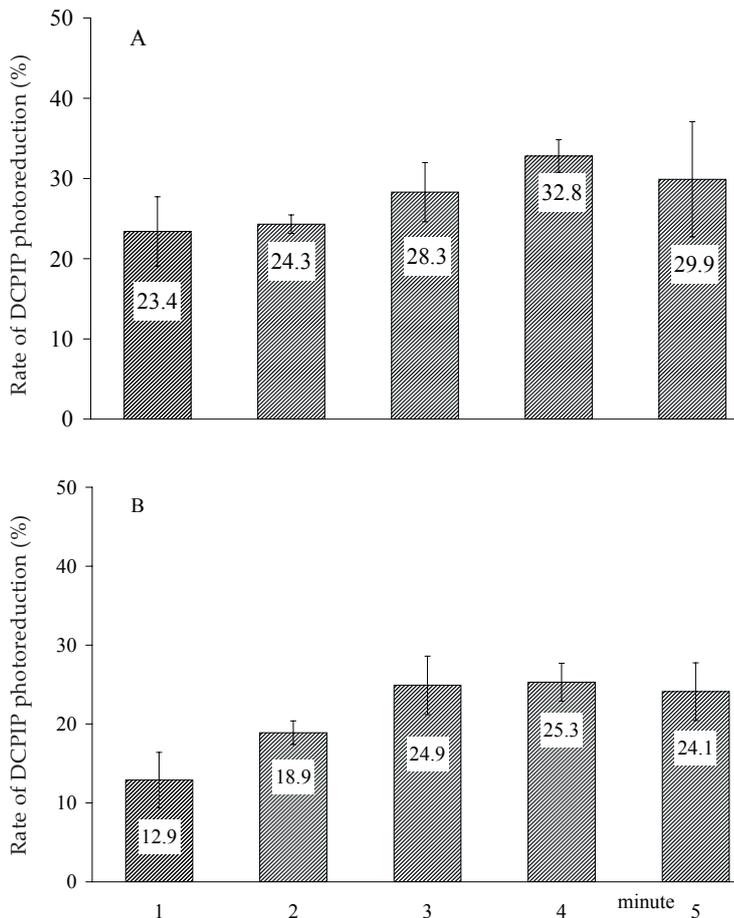


Figure 4. Rate of photoreduction of DCPIP (in %) by isolated chloroplasts of *Karwinskia humboldtiana* (A) and *Karwinskia parvifolia* (B) seven years old plants grown under greenhouse conditions depending on length of irradiation of reaction mixture; dates represent means \pm SE, $n = 3$

An increase in the chlorophyll content from 35.2 ± 0.09 to 83.2 ± 0.17 mg/l in the species *Karwinskia humboldtiana* was accompanied with a significant increase in chloroplast activity, from 32.3 ± 1.13 to $89.0 \pm 1.01\%$. A similar trend was also noted in the species *Karwinskia parvifolia* in which chlorophyll increase from 35.1 ± 0.75 to 84.9 ± 0.65 mg/l was likewise accompanied with an increase of chloroplast activity from 17.4 ± 1.29 up to $37.1 \pm 1.50\%$. The results also confirmed that with the same chlorophyll content in the suspension, the chloroplast activity in the species *K. parvifolia* was significantly lower than in the species *K. humboldtiana* (Figure 3). Thus comparable chlorophyll content in chloroplast suspensions in the *Karwinskia* species does not simultaneously assure their equal photochemical activity.

Chloroplast activity and the speed of Hill's reaction also depend on the length of irradiation of the reactive mixture. Determination of the optimum irradiation time, however, required verification of the stability of the various reactive components, i.e. both of the chloroplast suspensions as such and also of the redox-system DCPIP. It was found that no change takes place in the concentration of the chloroplast suspension until the fourth minute of exposure. However, after five minutes of exposure of the chloroplast suspension we found

a non-significant increase in absorbance of 1.2% in the species *K. humboldtiana* and a significant one of 5.6% in the species *K. parvifolia*, as against that prior to the exposure. The occurrence of a moderate increase of chloroplast concentration in both the species of the genus *Karwinskia* led, as is also evident from Figure 3, to a decline of chloroplast activity determined 5 minutes after exposure of the reaction mixture. We therefore assume that this decline in chloroplast activity might be due to an incipient heat stress. Yet, neither can we exclude a possibly destructive action of a transient rise in temperature on some of the thermolabile components of the electrontransporting chain of photosystem II, as pointed out by Mukohata et al. (1971), or an incipient degradation of the pigment-protein complex of chloroplasts detected in spinach legumes determined by Petková et al. (1973). Concentration of the redox-system DCPIP as such did not change up to the 5th minute of exposure. Hence, in virtue of the results of research into the stability of reaction components, a 4-minute interval has been chosen as the optimum irradiance time interval for determining and comparing the photochemical activity of chloroplasts in species of the genus *Karwinskia*. It was further noted that the activity of chloroplasts rises with the length of

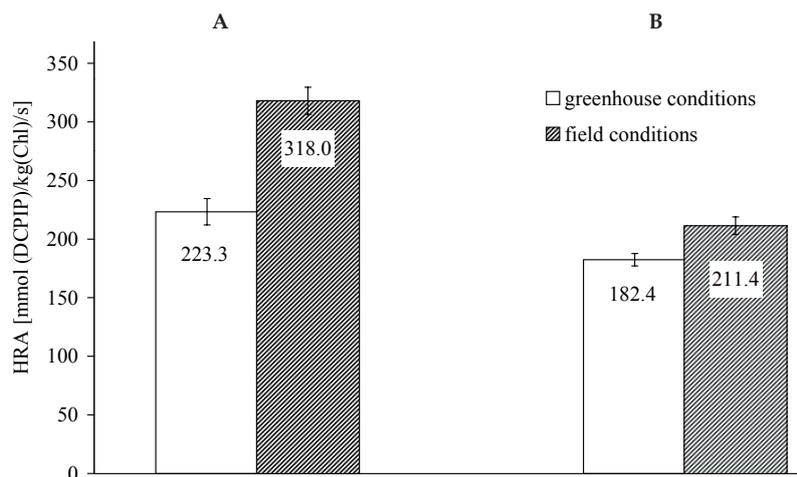


Figure 5. Comparison of Hill reaction activity (HRA) of isolated chloroplasts of *Karwinskia humboldtiana* (A) and *Karwinskia parvifolia* (B) of four years old plants grown in greenhouse and field conditions; plants were cultivated under field conditions from May 1 to October 30, and experiments were done in July 1999; dates represent means \pm SE, $n = 3$

Values between *K. humboldtiana* and *K. parvifolia* grown under greenhouse conditions are significantly different at $P = 0.05$ and values between plants grown under field conditions are significantly different at $P = 0.01$

exposure of the reaction mixture, with the species *K. humboldtiana* achieving a maximum of $32.8 \pm 0.93\%$ and those of *K. parvifolia* a maximum of $25.2 \pm 0.23\%$ after 4 minutes (Figure 4). The speed of Hill's reaction is most striking during the first minute of exposure and thus in both the species of the genus *Karwinskia* (Figure 4). Even though according to May (1975) and Souza Machado et al. (1977) a 1-minute exposure of the reaction mixture need not always be a guarantee of a high and sufficient chloroplast activity. In our experiments with a 4-minute exposure we already noted the highest reduction in the redox system after the first minute, i.e. 23.4% in the species *K. humboldtiana* and 12.9% in that of *K. parvifolia* (Figure 4).

The methodical procedure for isolating chloroplasts described here and determination of conditions for measuring their activity in Hill's reaction enabled us to compare the characteristics of the photosystem PS II in two species of the *Karwinskia* genus – the most significant as regards peroxisomicine A₁ content. As evident from Figure 5, in plants of the genus *Karwinskia* we found not only significant interspecies differences in chloroplast activity but also likewise differences in the activity of plants cultivated under field and greenhouse conditions. The species *K. humboldtiana* stood out by its significantly higher activity from 223.3 up to 318.0 mmol (DCPIP)/kg (Chl)/s than the species *K. parvifolia* from 182.4 up to 211.4 mmol (DCPIP)/kg (Chl)/s, hence we may state that the photochemical activity of chloroplasts in four-year old plants is inversely proportional to the peroxisomicine A₁ content in the species under study. The higher chlo-

roplast activity determined in four-year old plants cultivated under field conditions was probably due to a higher mean daily temperature and a greater light intensity. In addition, the varying climatic conditions also affected the differing production of new leaf biomass which proved higher in the species *K. humboldtiana* than in those of *K. parvifolia* and both the species produced more leaves under field conditions rather than greenhouse conditions. Therefore, the higher chloroplast activity in the species *K. humboldtiana* may possibly be derived also from a higher production of younger leaves than in the case of the species *K. parvifolia* for, according to Synková and Šesták (1991). Chloroplasts isolated from young, sufficiently developed leaves are by 8 to 59 percent more active than those from old, senescent leaves. This is also supported by the results reported by Kutík et al. (1999) who found that the values of activity in Hill's reaction in various maize genotypes continuously rise from young to mature leaves and decline during their senescence. The lower photochemical chloroplast activity is simultaneously related to a lower photosynthetic CO₂ assimilation which, according to Grover (1993), may also be caused by physiological leaf senescence. Chloroplasts isolated from 7-year old plants of the genus *Karwinskia* was essentially less active than those from 4-year old plants, while interspecific differences in their activity grew with their age which is in agreement with Šesták's findings (1985). According to Morris and Hall (1982), the activity of isolated chloroplasts may likewise vary among species during the vegetation period, as was also confirmed in our experiments following

the all-the-year dynamics of changers in chloroplast activity of the genus *Karwinskia* (Henselová et al. unpublished). Interspecific differences, and this not solely in photosynthetic activity but also in further growth parameters in the genus *Karwinskia*, were ascertained by Masarovičová et al. (2000). It is our opinion that chloroplast activity in the genus *Karwinskia* may be conditioned genetically but also by the environmental conditions of their natural incidence. While the species *K. humboldtiana* occurs under very disparate conditions of tropical and subtropical altitudes from 0 up to 2200 m a.s.l., the species *K. parvifolia* grows only under semi-arid conditions at altitudes from 20 to 150 m a.s.l. (Standley 1923 and Fernández 1992).

The diversity in chloroplast activity in different populations of the species *Deschampsia caespitosa* L. growing under varying environmental conditions was determined by Tieszen and Helgager (1968). Williams (1971) and Williams et al. (1975) registered a lower chloroplast activity in the more northerly populations of the species *Liquidambar styraciflua* L. (here, temperature had but a minimum effect on the values of Hill's activity; a much larger impact was that of the photoperiod). In the species *Verbascum thapsus* these authors found high values of chloroplast activity in the population with the longest growth period and this at a temperature of 35°C.

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Abbreviations

DCPIP = 2,6-dichlorophenol-indophenol
 Tris = Tris-(hydroxymethyl)-aminomethane
 Tricine = N-[tris(hydroxymethyl)methyl]glycine
 Hepes = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
 HRA = Hill reaction activity
 PAR = photosynthetically active radiation
 Chl = chlorophyll content

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ABSTRAKT

Izolace chloroplastů u druhů rodu *Karwinskia* a stanovení jejich fotochemické aktivity v podmínkách *in vitro*

Byl vypracován postup izolace chloroplastů a stanoveny podmínky měření rychlosti Hillovy reakce v podmínkách *in vitro* u druhů *Karwinskia humboldtiana* (Roem & Schut) Zucc a *Karwinskia parvifolia* Rose. Hillova reakce byla měřena spektrofotometricky při 630 nm jako intenzita redukce DCPIP izolovanými chloroplasty po 4min expozici při 400 μmol/m²/s PAR. Zjištěný rozdíl ve fotochemické aktivitě chloroplastů izolovaných při 2 °C a 25 °C byl u druhu *K. humboldtiana* 27.3 % a u druhu *K. parvifolia* 18.5 %. Průkazný pokles aktivity chloroplastů u druhu *K. humboldtiana* o 38 % a u druhu *K. parvifolia* o 45 % byl zaznamenán též po jejich 24h skladování při teplotě 2 °C. Prodloužení expozice reakční směsi a zvyšování obsahu chlorofylu (*a + b*) v suspenzi chloroplastů mělo za následek zvýšenou aktivitu chloroplastů. Druh *K. humboldtiana* se vyznačoval vyšší fotochemickou aktivitou chloroplastů nežli druh *K. parvifolia*. U obou sledovaných druhů byla stanovena vyšší aktivita chloroplastů rostlin pěstovaných v polních než ve skleníkových podmínkách.

Klíčová slova: *Karwinskia humboldtiana*; *Karwinskia parvifolia*; izolace chloroplastů; Hillova reakce

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