

## Photosynthetic characteristics of the cotyledon and first true leaf of castor (*Ricinus communis* L.)

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### Abstract

The photosynthetic characteristics of the cotyledon and the first true leaf were studied at various developmental stages of the castor seedlings. The cotyledons expanded at a much faster rate than the first true leaf. The area of the cotyledons expanded very rapidly during the first 4 days, then slowed down, reaching its maximum size 7 days after emergence. In contrast, the first true leaf growth was slower for the first 4 days, then expanded linearly from day 4 to day 18. The chlorophyll and carotenoid content accumulation is associated with the expansion rate of cotyledons and the first true leaf. The daily net photosynthetic rate ( $P_N$ ) for the cotyledon and the first true leaf increased gradually in the morning, reached peak at 01:00 PM, and then decreased rapidly until sunset. The  $P_N$  of the first true leaf was significantly greater than that of cotyledon 18 days after seedling emergence when the first true leaf was 13 days old. Our results showed that although the cotyledon did not keep its photosynthesis rate for the whole period of seedling development, it was sufficient to balance respiratory losses that occur during the early stages of seedling establishment. The photosynthesis by cotyledons provided enough carbohydrate and energy for the first true leaf to appear, and maintained growth of the seedling until the first true leaf had expanded.

**Keywords:** cotyledon; leaf age; photosynthesis; primary leaf; seedling.

### Abbreviations:

Car	carotenoids	$g_s$	stomatal conductance
Chl	chlorophyll	LS	stomatal limitation
Ci/Ca	intercellular CO <sub>2</sub> /ambient CO <sub>2</sub>	$P_N$	net photosynthetic rate
$E$	transpiration rate	WUE	water use efficiency

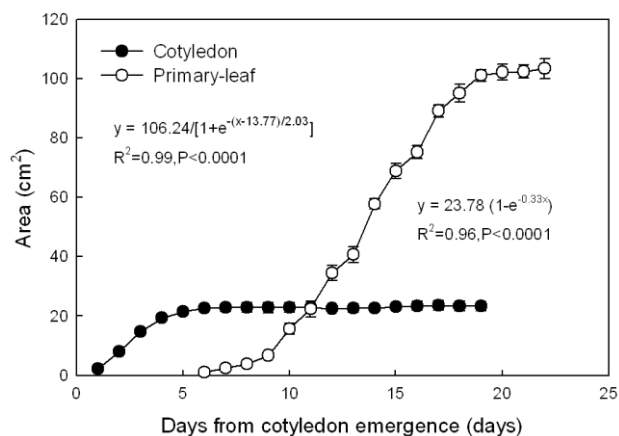
### Introduction

Cotyledons are the first leaves that appear on a plant, playing an important role in seedling development, especially in the early stages (Kitajima, 2003; Hanley and May, 2006; Zhang et al., 2008). They are pushed above ground by the hypocotyl during epigeal germination, and possess stomata, develop chlorophyll and expand to form leaf-like structures soon after emergence (Ampofo et al., 1976). Shortly after castor seedling emergence, rapid cotyledon expansion occurs and soon this leaf forms a high proportion of the total photosynthetic surface. It is the cotyledon that provides a major proportion of assimilates needed for seedling growth until the first true leaf becomes a significant exporter of photosynthate. The importance of cotyledon photosynthetic activity for early seedling growth has been stressed by several authors, particularly for woody plant species (Ampofo et al., 1976; Marshall and Kozlowski, 1976) and legumes (Yoshida and Kajimoto, 1978; Harris et al., 1986a) having seeds with epigeal germination. Zhang et al. (2008) showed that the contribution of cotyledons to seedling growth had significant effect on normal seedling development during late developmental stages. Experiments with <sup>14</sup>CO<sub>2</sub> by Webb and Gorham (1964) using straight-necked squash, and by Lovell and Moore (1970) using a number of species, have

demonstrated the photosynthetic capabilities and contributions made by cotyledons to the development of herbaceous seedlings. However, these methods have not quantified the total photosynthetic assimilation by cotyledons during their lifespan, nor do they provide clear information on the balance between the photosynthetic contribution and storage contribution of cotyledons during early seedling development. Lasley and Garber (1978) reported that cotyledonary leaves, with only 50% of the total photosynthetic area, were responsible for nearly 80% of the total net CO<sub>2</sub> exchange of cucumber seedlings. Lovell and Moore (1970) suggested that the extent and nature of cotyledon contribution to early seedling development, in a sense, defines development strategy. Both cotyledons and true leaves are photosynthetic organs, providing the photosynthate used in seedling growth, and in this respect are similar. Ampofo et al. (1976) determined for the *Acer* that the extent of this similarity with respect to comparative rates of CO<sub>2</sub> fixation, export of products and distribution of the photosynthate within the seedling is not known. Several studies showed that plant growth is affected by the response of leaf photosynthesis (Hogan, 1988; Makino et al., 1997). Generally, several studies were centralized on the relationships

**Table 1.** The changes in stomatal conductance ( $g_s$ ), transpiration rate ( $E$ ) and ratio of stomatal and sub-stomatal  $\text{CO}_2$  concentration ( $C_i/C_a$ ) of cotyledons at various development stages. Means  $\pm$  SE (n = 3). Values with the same letter are not statistically significant at  $P < 0.05$ .

		$g_s$ (mol $\text{H}_2\text{O m}^{-2} \text{s}^{-1}$ )	$E$ (mmol $\text{H}_2\text{O m}^{-2} \text{s}^{-1}$ )	$C_i/C_a$
7:00 AM	4d-age	0.41 $\pm$ 0.05c	6.59 $\pm$ 0.31c	0.93 $\pm$ 0.01ab
	7d-age	0.44 $\pm$ 0.08c	7.02 $\pm$ 0.17ab	0.91 $\pm$ 0.005b
	10d-age	0.65 $\pm$ 0.04a	6.88 $\pm$ 0.08bc	0.96 $\pm$ 0.022a
	13d-age	0.54 $\pm$ 0.05b	7.23 $\pm$ 0.12a	0.92 $\pm$ 0.004b
	17d-age	0.53 $\pm$ 0.04b	6.02 $\pm$ 0.09d	0.96 $\pm$ 0.003a
10:00 AM	4d-age	0.74 $\pm$ 0.06a	8.10 $\pm$ 0.19b	0.88 $\pm$ 0.001a
	7d-age	0.79 $\pm$ 0.10a	10.63 $\pm$ 0.59a	0.85 $\pm$ 0.009ab
	10d-age	0.74 $\pm$ 0.08a	10.98 $\pm$ 0.25a	0.83 $\pm$ 0.013b
	13d-age	0.65 $\pm$ 0.02b	11.29 $\pm$ 0.23a	0.80 $\pm$ 0.009b
	17d-age	0.46 $\pm$ 0.01c	8.79 $\pm$ 0.16b	0.82 $\pm$ 0.003b
01:00 PM	4d-age	0.64 $\pm$ 0.06a	10.04 $\pm$ 0.09c	0.72 $\pm$ 0.008a
	7d-age	0.60 $\pm$ 0.05a	13.34 $\pm$ 0.73b	0.66 $\pm$ 0.011b
	10d-age	0.47 $\pm$ 0.02b	12.82 $\pm$ 0.14bc	0.65 $\pm$ 0.007b
	13d-age	0.46 $\pm$ 0.06b	15.35 $\pm$ 0.69a	0.66 $\pm$ 0.007b
	17d-age	0.39 $\pm$ 0.01b	10.31 $\pm$ 0.31c	0.69 $\pm$ 0.006ab
04:00 PM	4d-age	0.49 $\pm$ 0.01a	11.67 $\pm$ 0.18a	0.81 $\pm$ 0.020a
	7d-age	0.47 $\pm$ 0.02a	11.89 $\pm$ 0.32a	0.80 $\pm$ 0.020a
	10d-age	0.37 $\pm$ 0.05b	11.81 $\pm$ 0.11a	0.77 $\pm$ 0.020a
	13d-age	0.36 $\pm$ 0.02b	11.23 $\pm$ 0.39a	0.78 $\pm$ 0.012a
	17d-age	0.27 $\pm$ 0.02c	10.30 $\pm$ 0.30b	0.68 $\pm$ 0.012b
06:00 PM	4d-age	0.36 $\pm$ 0.02a	4.80 $\pm$ 0.12d	0.93 $\pm$ 0.006a
	7d-age	0.35 $\pm$ 0.03a	6.34 $\pm$ 0.36b	0.84 $\pm$ 0.005bc
	10d-age	0.32 $\pm$ 0.03a	8.21 $\pm$ 0.19a	0.89 $\pm$ 0.014ab
	13d-age	0.24 $\pm$ 0.01b	5.28 $\pm$ 0.18c	0.84 $\pm$ 0.005bc
	17d-age	0.22 $\pm$ 0.03b	5.25 $\pm$ 0.29c	0.80 $\pm$ 0.010c



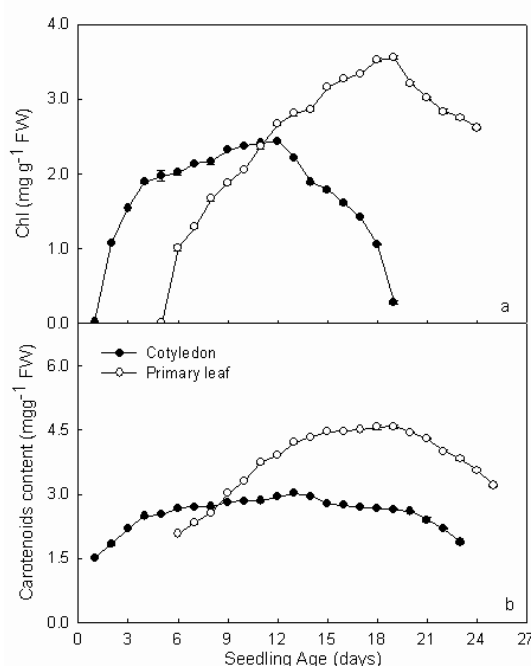
**Fig 1.** Expansion of cotyledon and primary leaf area with seedling age. Vertical bars represent  $\pm$  SE.

between photosynthetic responses with low or high elevation (Körner and Diemer, 1987), with nitrogen (Evans, 1983, 1989), leaf loss (Kitajima, 2002), with soil quality (Efthimiadou et al., 2010), and with salt-, alkali- and drought stress (Sultana et al., 1999; Loreto et al., 2003; Yang et al., 2008a; Yang et al., 2008b; Yang et al., 2009; Mafakheri et al., 2010). Seedling development is a very sensitive stage in the plant life cycle (Harper, 1977; Westoby et al., 1992; Armstrong and Westoby, 1993; Leishman and Westoby, 1994; Bonfil, 1998) and the initial growth and survival can be strongly influenced by the photosynthetic responses of cotyledons and true leaves in some higher plants (Penny et al., 1976). Although there is some information on the photosynthetic responses in leaf-like

cotyledons and true leaves, little is known about the difference of photosynthetic capabilities between cotyledons and the first true leaf during seedling growth. Therefore, it is necessary to understand the contributions of the cotyledons and the first true leaf to the initial growth and establishment of seedlings in order to evaluate how cotyledons and leaves can affect plant development in later stages. Castor (*Ricinus communis* L.), which belongs to the Euphorbiaceae family, is an annual, epigeal, dicotyledonous, herbaceous plant. It is an important oilseed crop that produces an oil rich in ricinoleic acid (Gharibzadeh et al., 2011). The seeds contains 46.0 to 51.8% oil, 17.1 to 24.4% protein, 18.2 to 26.5% crude fiber and 2.1 to 3.4% ash (Yuldashaeva et al., 2002), and also can be used as a

**Table 2.** The changes in stomatal limitation (LS), water use efficiency (WUE) and transpiration rate ( $E$ ) of cotyledon and primary leaf at the same age. Means  $\pm$  SE ( $n = 3$ ). Values with the same letter are not statistically significant at  $P < 0.05$ .

		LS	WUE ( $P_N / E$ )	$E$ (mmol $H_2O\ m^{-2}\ s^{-1}$ )
4d-old-age	Cotyledon	0.17 $\pm$ 0.04a	1.43 $\pm$ 0.17a	10.49 $\pm$ 3.80a
	Leaf	0.19 $\pm$ 0.05a	0.95 $\pm$ 0.05b	10.19 $\pm$ 3.15a
7d-old-age	Cotyledon	0.18 $\pm$ 0.04a	1.38 $\pm$ 0.44a	10.07 $\pm$ 3.90a
	Leaf	0.17 $\pm$ 0.02a	1.18 $\pm$ 0.30b	10.26 $\pm$ 3.14a
11d-old-age	Cotyledon	0.22 $\pm$ 0.05a	1.07 $\pm$ 0.14a	11.55 $\pm$ 3.20a
	Leaf	0.19 $\pm$ 0.03a	1.16 $\pm$ 0.38a	9.07 $\pm$ 3.00b
14d-old-age	Cotyledon	0.21 $\pm$ 0.04a	1.31 $\pm$ 0.47a	9.55 $\pm$ 2.18b
	Leaf	0.17 $\pm$ 0.02a	1.21 $\pm$ 0.30a	12.99 $\pm$ 3.51a
17d-old-age	Cotyledon	0.19 $\pm$ 0.02a	1.18 $\pm$ 0.34a	8.07 $\pm$ 2.07b
	Leaf	0.18 $\pm$ 0.03a	1.09 $\pm$ 0.21a	14.87 $\pm$ 3.84a



**Fig 2.** Production of chlorophyll (a) and carotenoids (b) content of cotyledon and primary leaf with seedling age. Vertical bars represent  $\pm$  SE.

Chinese herbal medicine. In this paper, we compared the difference between photosynthetic capabilities of cotyledons and the first true leaf during early seedling growth and establishment.

## Materials and methods

### Study site and material

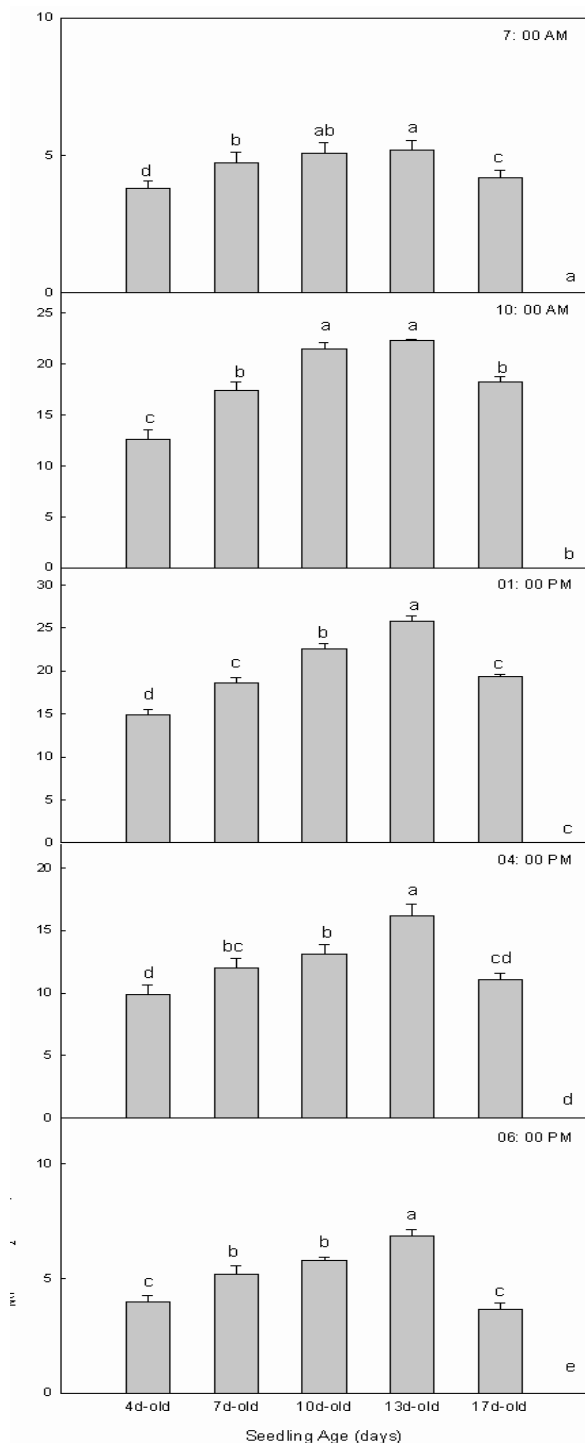
A pot experiment was performed in the summer (July) of 2006 in the Grassland Ecosystem Experimental Station of Northeast Normal University (44°30'–44°45'N, 123°31'–123°56'E). This area is of typical mesothermal monsoon climate with plain topography in the south of the Songnen plain (altitude: 137.8–144.8 m). The mean annual temperature is 4.6–6.4°C. The annual accumulated temperature is 2545–3 374°C, with a frost-free period of 136 $\pm$ 16 d. The mean annual rainfall is about 400–500 mm, which is mainly concentrated from June to August, and accounts for more than 60% of the rainfall of the whole year. The annual evaporation capacity is 2–3 times more than the rainfall. The seeds of castor were collected during autumn 2005 from the Songnen plain in the Western Jilin province in Northeast China, and stored in cloth bags at room temperature (20  $\pm$  2°C).

### Experimental design

Pots (20 cm diameter and 25 cm height) were filled with a soil-sand mixture (1:1 by volume). There were 10 replicate pots for each day, for 15 continuous days, thus 150 pots in total. Two seeds were sown in each pot, then covered with the mixture to the required depth of 2 cm and cultivated in the open air. Irrigation was provided when needed. Seedlings were thinned to one per pot after cotyledon emergence, and growth continued under natural light. The time of cotyledon and leaf emergence was recorded. The experiment was carried out when the seedlings of the 15<sup>th</sup> sowing day emerged.

### Measurements

Net photosynthesis rate ( $P_N$ ), transpiration rate ( $E$ ), and stomatal conductance ( $g_s$ ) of cotyledons were recorded for seedlings which were 4d, 7d, 10d, 13d and 17d-old after emergence, and those of primary leaves were measured using the first leaf present on the seedlings at days of 4, 7, 11, 14 and 17d-old. The instrument used was a Li-6400 portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA). The results were expressed as  $\mu\text{mol}(\text{CO}_2)\ \text{m}^{-2}\text{s}^{-1}$ ,



**Fig 3.** The changes in net photosynthetic rate ( $P_N$ ) of cotyledons of 4d, 7d, 10d, 13d and 17d-old seedling at 7:00 AM (a), 10:00 AM (b), 01:00 PM (c), 04:00 PM (d) and 06:00 PM (e). Vertical bars represent  $\pm$  SE. Values with the same letter are not statistically significant at  $P < 0.05$

$\mu\text{mol (H}_2\text{O) m}^{-2}\text{s}^{-1}$  and  $\text{mol (H}_2\text{O) m}^{-2}\text{s}^{-1}$  respectively. At each age studied, the result given is the mean of five plants, and cotyledons and the primary leaf of five plants were removed to measure length, width and area with SigmaScan Pro (Systat Software Inc., Richmond, California, USA), and three pairs of them were extracted in 80% acetone. Carotenoids (Car) and chlorophyll (Chl) a and b were extracted with acetone, the

spectrophotometric determination at wavelengths of 440, 645 and 663 nm of each sample was performed three times (*SpectrUV-754*, Shanghai Accurate Scientific Instrument Co.) and the concentrations, calculated according to the Arnon (1949) procedure, were expressed as  $\text{mg g}^{-1}$  FW.

We calculated stomatal limitation (LS) and water use efficiency (WUE) using the following equations:

$$\text{LS} = (1 - C_i/C_a) \times 100\% \quad (1)$$

$$\text{WUE} = \frac{P_N}{E} \quad (2)$$

Where  $C_i/C_a$  is the intercellular  $\text{CO}_2$  / ambient  $\text{CO}_2$ ,  $P_N$  is the photosynthetic rate and  $E$  is the transpiration rate.

### Statistical data analysis

Data were analyzed by one-way analysis of variance (ANOVA) using the statistical software *SPSS 13.0* (SPSS Inc., Chicago, IL, USA). The treatment mean values were compared by post hoc least significant difference (LSD) test, with the statistical significance set at  $P \leq 0.05$ .

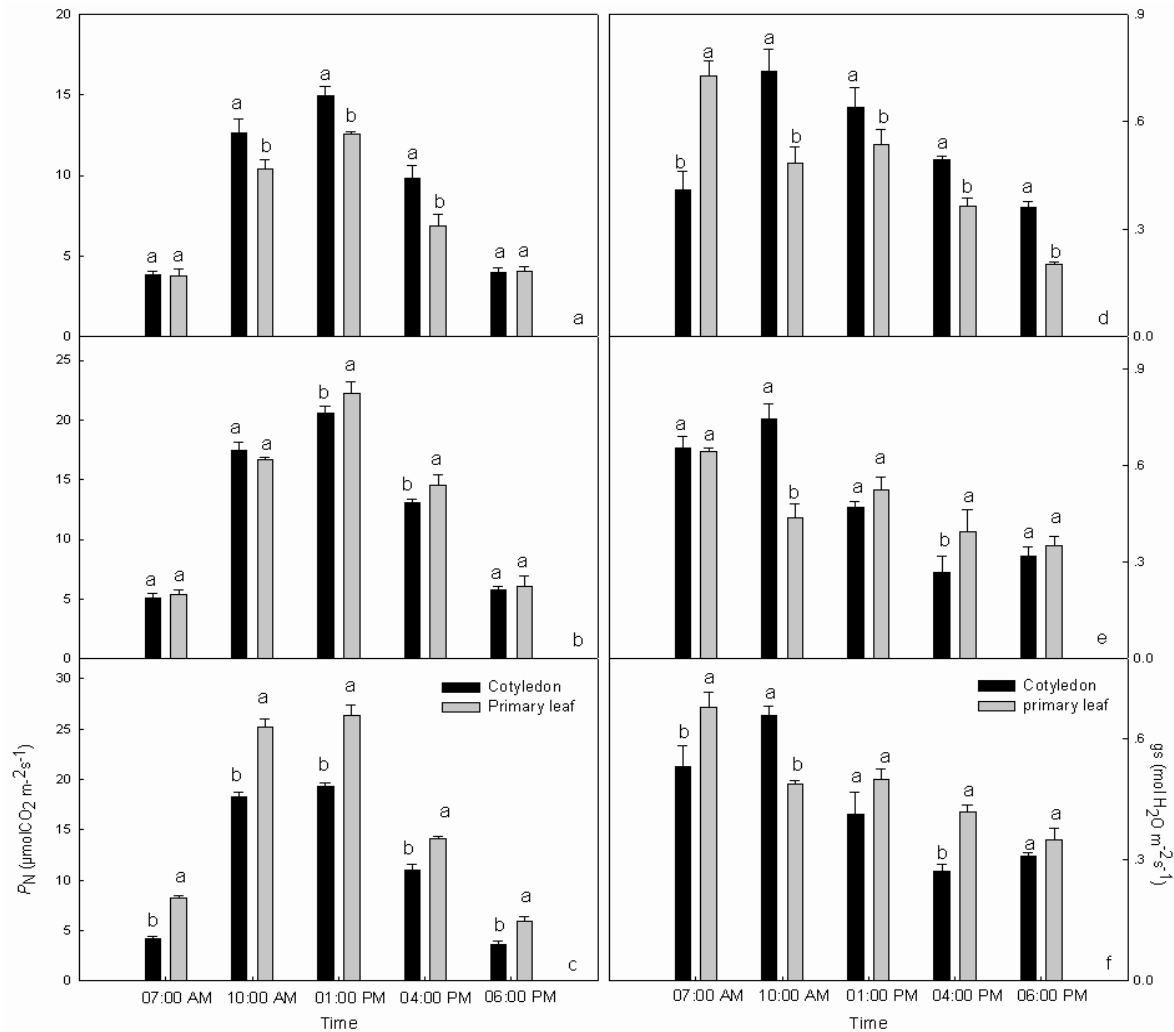
## Results

### Development of photosynthetic area and pigment contents

The area of the cotyledon was in accord with the exponential growth mode  $y = a*(1-e^{-bx})$ . The cotyledons expanded very rapidly as soon as emerging from soil and reach a plateau at day 5 after emergence and maintained its photosynthetic area until day 19 (Fig. 1). The first true leaf growth was in accord with the logistic growth mode  $y = a/[1+e^{-(x-x_0)/b}]$ . The first true leaf appeared at day 5 after seedling emergence and grew slowly for the first 4 days, then expanded linearly from day 4 to day 18 (Fig. 1). The leaf area of the true leaf attained the equivalent size of the cotyledonary area at day 11. By day 19, the true leaf was fully expanded and the area was 4.5 times greater than that of the cotyledon (Fig. 1). Cotyledonary chlorophyll content rose rapidly during the first 4 days and maintain a high level until day 13, and then decreased rapidly. The chlorophyll content of the first true leaf rose slowly until day 18, and then decreased rapidly (Fig. 2a). Cotyledonary carotenoids content rose rapidly during the first 4 days and remained at a constant level until day 20, and then decreased slowly, but the carotenoids of the first true leaf rose slowly until day 20, and then decreased slowly (Fig. 2b). The Chlorophyll and Carotenoids content of the first true leaf were already greater than those of cotyledon by days 11 and 8 after seedling emergence, respectively (Fig. 2).

### Changes in photosynthetic parameters

The  $P_N$  of cotyledons at various seedling ages showed a consistent trend correlating to the time of day (Fig. 3), which initially increased with increasing cotyledon age. The maximum  $P_N$  of the cotyledon was at 13d-old as compared with that of all other time points measured, and then the  $P_N$  decreased with increasing cotyledon age. The maximum daily  $P_N$  of all cotyledons ages was at 01:00 PM. The  $g_s$  displayed significant differences at each time point. It decreased with cotyledon age except at 07:00 AM, when it initially increased with cotyledon age and then decreased. The maximum  $g_s$  was at 10d-old-age. From 07:00 AM to 06:00 PM, the daily changes of  $g_s$  showed a consistent trend, which increased significantly before 01:00 PM, and then decreased until 06:00



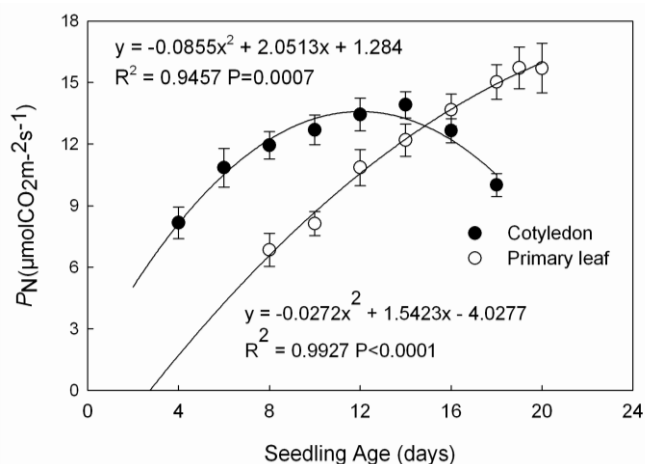
**Fig 4.** Daily changes in net photosynthetic rate ( $P_N$ ) and stomatal conductance ( $g_s$ ) of cotyledons and primary leaves at different developmental stages. (a, d: 4d-old-age; b, e: 11d-old-age; c, f: 17d-old-age). Vertical bars represent  $\pm$  SE. Values with the same letter are not statistically significant at  $P < 0.05$ .

PM. The changes of  $E$  also differed greatly in different developmental stages at each time point and through the course of the day. The daily change of Ci/Ca differed greatly in each developmental stage, but differed slightly for the different developmental stages at each time point (Table 1). The daily  $P_N$  of the cotyledon and the first true leaf had similar trends during three periods at 4d, 11d and 17d-old-age, respectively (Fig. 4a, b, c). Their  $P_N$  rose and achieved the peak at the 01:00 PM. The relative  $P_N$  of the cotyledon and the first true leaf was compared at the three day-age periods, and that of the cotyledon was greater than that of the first true leaf at the same day-age development (Fig. 4a). The  $P_N$  of the first true leaf was not statistically different from the cotyledon before 01:00 PM, and then the  $P_N$  of the first true leaf was greater than that of cotyledon, but there was no significant difference at the final time point on day 11 (Fig. 4b). The  $P_N$  of the first true leaf was significantly greater than that of the cotyledon at the end of cotyledon lifespan (Fig. 4c). A clear change of  $g_s$  was detected at various developmental stages (Fig. 4d, e, f). The  $g_s$  of the first true leaf was significantly greater than that of the cotyledon at 07:00 AM at day 4, but the  $g_s$  of the cotyledon was significantly greater than that of the first true leaf at all other times. But when comparing the  $g_s$  of the cotyledon with the first true leaf at day 17, it showed that the  $g_s$  of the first true leaf was significantly greater than

that of the cotyledon at all times except at 10:00 AM. The  $P_N$  of cotyledon and leaf was in accord with the binomial growth modes  $y = -0.0855x^2 + 2.0513x + 1.284$  and  $y = -0.0272x^2 + 1.5423x - 4.0277$ , respectively (Fig. 5). We calculated that cotyledons took 13 days to reach the maximum net photosynthetic rate. The  $P_N$  of the first true leaf (primary leaf 8d-age) was lower than that of the cotyledons, and then, the  $P_N$  of cotyledons began to decline and that of the first true leaf increased steadily. The  $P_N$  of the first true leaf was significantly greater than that of the cotyledon on 18d-age (primary leaf 13d-age) after seedling emergence.

#### **Changes in stomatal limitation, water use efficiency and transpiration rate**

To analyze further the basis for the capacity for photosynthesis of castor, measurements were made of LS, WUE and  $E$ . The comparison of LS, WUE and  $E$  between the cotyledon and the first true leaf of all day-age stages is presented in Table 2. There is no significant change in LS or WUE at any stage of cotyledon growth. However, the WUE of the first true leaf was significantly greater than that of the cotyledon in initial stages of the cotyledon lifespan. In contrast, the  $E$  of the first true leaf was significantly greater than that of the cotyledon at the end of the cotyledon lifespan.



**Fig 5.** The changes in net photosynthetic rate ( $P_N$ ) of cotyledons and primary leaves at development stages. Vertical bars represent  $\pm$  SE.

## Discussion

The cotyledon of castor, like some other epigeal cotyledons, bears stomata on both surfaces, undergoes a period of expansion, is dark green and persistent. Initially, the growth of the seedling depends upon the mobilization and transfer of stored material from the cotyledon (Kitajima, 1996; Moles and Westoby, 2004; Hanley and Fegan, 2007). Our results supported this conclusion, prior to day 4, the seedlings use stored reserves. Concurrently, there is an increase of Chl and Car from days 1 to 4 as a preparation for photosynthesis (Fig. 2). Apart from major reserves, the cotyledon may also provide growth substances (Wheeler, 1966) and minerals (Okamoto, 1962; Vaňousová, 1968). After emergence, cotyledon photosynthesis becomes important, especially from days 4 to 13 (Fig. 5), but the growth of the cotyledon and the first true leaf follow different patterns (Fig. 1), which results in different photosynthetic capacity as the seedling matures. Cotyledons expand very rapidly and reach the maximum area soon after emergence; therefore, they play a predominant role in photosynthesis that is essential to early seedling survival (Lane and Hesketh, 1977). The first true leaf appeared later and reached the maximum area more slowly. All of these observations make it highly probable that photosynthesis by the first true leaf provides material and energy later than that by the cotyledon during the seedling development stage (Fig. 5). The results presented here show that by day 18, 13 days after expansion of the first true leaf has begun, the photosynthetic rate of the first true leaf has become greater than that of the cotyledons, from day 13, the first true leaf takes over and the role of cotyledon is finished. Therefore, the early photosynthesis by the cotyledon was found to be essential to seedling growth and survival in this investigation. With respect to the timing of the onset of cotyledon photosynthesis, it was different with various number and function of cotyledon (Harris et al., 1986a; Harris et al., 1986b; Milberg and Lamont, 1997; Kennedy et al., 2004). In our investigation, cotyledon photosynthesis was first measurable on day 4 and increased markedly with cotyledon growth (Fig. 5). It is possible that the function of cotyledon photosynthesis is to supply the carbohydrates necessary for respiration during this brief period of carbohydrate and lipid reserve depletion, and then, at the onset of senescence on day 17, photosynthesis declines sharply. Another factor that may contribute to the decrease in photosynthesis is the shading of older leaves by

younger leaves. Moreover, experimental evidence indicates that photosynthesis by cotyledons provides enough material and energy for the first true leaf to appear, while simultaneously, it was enough to maintain growth of the seedling until the unfolding of the first true leaf. The water application in our experiment was similar to what would be experienced by castor seedlings growing in the field, so this should not affect the main conclusion that the photosynthetic capacity is different between the cotyledon and the first true leaf at early seedling stage. In summary, the cotyledon of castor has a high photosynthetic capacity at the initial period of seedling development, comparable to that of the first true leaf. This high photosynthetic capacity by cotyledons is not sustained during all seedling development stages; the photosynthetic capacity of the first true leaf is greater than that of the cotyledon as the seedling matures, which is caused by the area expansion of the primary leaf and the concomitant increase of chlorophyll. The first true leaf can then provide all material and energy necessary for plant growth. Therefore, although photosynthetic contribution of the cotyledon and the first true leaf is different during early seedling growth and establishment, each is important to seedling development and survival at various stages. Castor has semi-photosynthetic reserve cotyledons with a prolonged dual function: storage and photosynthesis. We still cannot explain how the castor cotyledon diverts vital resources into the synthesis of the photosynthetic system, contributing to the overall seedling growth and development. However, our results show that although cotyledon photosynthesis is not sustained for seedling development, it is sufficient to balance respiratory losses that occur during the early stages of seedling establishment, at least until the first true leaf has expanded. Therefore, further research on species with leaf-like photosynthetic cotyledons that will start to depend on light availability earlier should be carried out, including seedling growth and development under different environmental conditions.

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