

BRIEF COMMUNICATION

Specific leaf area, leaf nitrogen content, and photosynthetic acclimation of *Trifolium repens* L. seedlings grown at different irradiances and nitrogen concentrations

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Abstract

Clover seedlings were grown at different nitrogen concentrations (5, 10, 15, 20, 25 mM NO_3^- , i.e. N_5 to N_{25}) and two irradiances, I (200 and 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of photon flux density, i.e. I_{200} and I_{400}). Net photosynthetic rate (P_N), photosynthetic nitrogen use efficiency (PNUE), leaf chlorophyll (Chl) content, maximum photochemical efficiency (F_v/F_m), and actual photochemical efficiency of photosystem 2 (PS2) (Φ_{PS2}) increased from N_5 to N_{15} and decreased with N_{15} to N_{25} . P_N , PNUE, and Φ_{PS2} were higher at I_{400} than at I_{200} , but F_v/F_m and leaf Chl contents at I_{400} were lower than at I_{200} . The effects of the N and I on specific leaf area (SLA) and N contents per unit dry mass (N_m) were similar, the SLA and N_m increased from N_5 to N_{25} and they were higher at I_{200} than at I_{400} . The nitrogen contents per unit area (N_a) increased from N_5 to N_{20} , but decreased from N_{20} to N_{25} . The N_a was higher at I_{200} than at I_{400} when *Trifolium repens* grew at N_5 and N_{10} , but it was higher at I_{400} than at I_{200} at N_{15} to N_{25} .

Additional key words: actual photochemical efficiency of photosystem 2; chlorophyll content and fluorescence; clover; photosynthetic acclimation; irradiance; photosynthetic nitrogen use efficiency; specific leaf area.

As a leguminous species, *Trifolium repens* L. is ecologically the most important herbaceous species in the temperate zone (Jiang 2004) and prefers shady places. Plant photosynthesis, leaf anatomic plasticity and morphology, and growth at different N supply or irradiance (I) are different (Schwank *et al.* 1986, Malinowski *et al.* 1998, Shangguan *et al.* 2000a,b, Pandey and Kushwaha 2005, Shi and Cai 2006, Gregoriou *et al.* 2007). According to Moskvina *et al.* (1998) the chlorophyll (Chl) fluorescence of clover leaves differs when growing at varying N supply and I . We studied the responses of *T. repens* in specific leaf area (SLA), leaf N and Chl contents, leaf Chl fluorescence, and net photosynthetic rate (P_N) to different I and N concentrations in a climate chamber. The objective of the study was to better understand the physio-ecological adaptability of plant photosynthesis by combining Chl fluorescence determination and leaf morphology.

T. repens was grown from seeds in a growth chamber (ZPW-280, Heilongjiang Dongtuo Instrument Manufacture Co., China) with the temperature set at 28 °C; the relative humidity was above 75 %. The germinated seeds were transplanted into pots (15 cm deep and 18 cm in diameter) and grew under the 13-h day at photosynthetic photon flux density (PPFD) of $200 \pm 20 \mu\text{mol m}^{-2} \text{s}^{-1}$ (I_{200}) or $400 \pm 20 \mu\text{mol m}^{-2} \text{s}^{-1}$ (I_{400}). Uninterrupted I was provided with tungsten-halogen lamp, temperature was 25 ± 2 °C, and the relative humidity was about 75 %. At night the temperature was 18 ± 2 °C. The seedlings grew on the substrate composed of vermiculite and turf (1 : 1, v/v), and were irrigated with 150 cm³ of modified Hoagland solution [5 mM $\text{Ca}(\text{NO}_3)_2 \times 4 \text{H}_2\text{O}$, 5 mM KNO_3 , 2.5 mM $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, 2.0 mM KH_2PO_4 , 5.0 mM KCl , 0.3 μM $\text{CuSO}_4 \times 5 \text{H}_2\text{O}$, 0.7 μM $\text{ZnSO}_4 \times 7 \text{H}_2\text{O}$, 50 μM H_3BO_3 , 7 μM $\text{MnCl}_2 \times 4 \text{H}_2\text{O}$, 0.5 μM $\text{H}_2\text{MoO}_4 \times \text{H}_2\text{O}$, and 20 μM Fe-EDTA] per pot once a week. The seedlings

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were irrigated with different N solutions, 5 to 25 mM (N_5 to N_{25}), and N_{15} was used as control. pH was adjusted to 5.8–6.2 using KOH and HCl. Each N concentration and growth I was replicated three times. The plants were measured 90 d after transplanting germinated seeds into pots. All measurements were done between 09:00 and 11:30.

P_N of fully expanded leaves was measured with a portable open gas exchange system (LI-6400, Li-Cor, USA). Irradiation was provided by LED model 6400-02B (Li-Cor). The gas entry was connected to a gas pole 3 m above ground. The open pathway was turned on to adjust the airflow rate to $0.5 \text{ cm}^3 \text{ min}^{-1}$, the CO_2 partial pressure was set at $360 \text{ } \mu\text{mol mol}^{-1}$, and the cuvette temperature was approximately that of air. Six to eight fully expanded leaves were placed inside the cuvette, and their P_N were recorded 2 min after photosynthesis became stable.

Maximum photochemical efficiency (F_v/F_m) and the actual photochemical efficiency of photosystem 2 (Φ_{PS2}) of the fully expanded leaves were measured with an FMS 2.02 pulse modulation fluorescence meter (Hansatech, King's Lynn, UK). The initial fluorescence (F_0) of the leaves was measured after the 30-min dark-adaptation, and maximal fluorescence (F_m) after the leaves received a strong flash ($6000 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$, 0.7 s pulse duration). After the steady-state fluorescence of the leaves had been measured under natural irradiance (F_s), their maximum fluorescence at adaptation to irradiance (F_m') was measured after exposure to a strong flash. The efficiencies were finally calculated as $F_v/F_m = (F_m - F_0)/F_m$; $\Phi_{PS2} = (F_m' - F_s)/F_m'$ (Schreiber *et al.* 1986).

Leaf chlorophyll (Chl) contents were checked in five leaflets previously used for P_N measurements using a portable Chl meter (SPAD-502, Minolta Camera Co., Osaka, Japan). The measurement of SPAD-502 was based on the comparison of leaf transmittance at wavelengths of 650 and 940 nm (Manetas *et al.* 1998).

10–12 fully expanded leaves were used to measure specific leaf area (SLA). The projected leaf areas were measured with a digital scanner and image analysis application (Motic Images Advanced 3.0, Micro-Optic Industrial Group Co., China). After measurement they were oven dried at 70°C for 48 h and then the leaves were weighed with a precision balance. The SLA was calculated as the ratio of leaf area to dry mass. Afterwards, the dried leaf samples were ground into a homogenous fine powder with a plant-sample mill (1093 Sample Mill, Foss, Sweden) and sieved with a 2-mm mesh screen before the sub-samples were weighed for N determination. 200 mg of each sub-sample was taken to determine the leaf N content by the modified Kjeldahl procedure (Bremner and Mulvaney 1982), using an auto-analyser (Kjeltec 2300, Foss, Sweden). The leaf N content was expressed per mass (N_m) or area (N_a) (Hikosaka 2004). The photosynthetic nitrogen use efficiency (PNUE) was calculated as the ratio of P_N to N_m

(Poorter and Evans 1998).

P_N of *T. repens* was higher at I_{400} than at I_{200} (Fig. 1A), it increased from N_5 to N_{15} and decreased from N_{15} to N_{25} ; P_N separately decreased at I_{200} by 21 and 30 % and at I_{400} by 35 and 39 %. The effects on SLA were different, it increased from N_5 to N_{25} at both I_{400} and I_{200} (Fig. 1B) but was always higher at I_{200} than at I_{400} (Fig. 1B). The plants grown at high I generally have thick leaves with a low SLA.

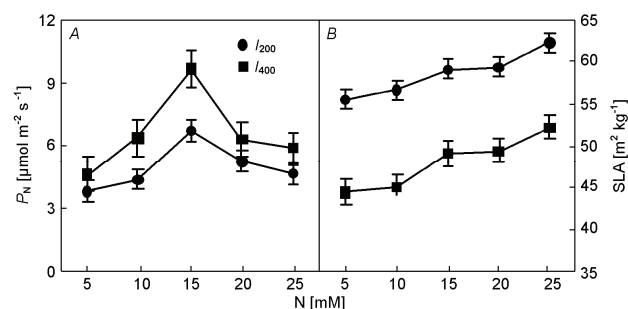


Fig. 1. Net photosynthetic rate, P_N (A) and specific leaf area, SLA (B) of *Trifolium repens* at different nitrogen concentrations (N) and irradiances (I).

N_m increased at I_{200} and I_{400} from N_5 to N_{25} . N_a increased from N_5 to N_{20} and decreased to N_{25} (Table 1). When clover grew at N_5 and N_{10} , N_a was higher at I_{200} than at I_{400} ($p < 0.05$), but at N_{15} to N_{25} it was higher at I_{400} than at I_{200} . The N_m was higher at I_{200} than at I_{400} ($p < 0.05$). The PNUE increased from N_5 to N_{15} and decreased from N_{15} to N_{25} ; it markedly ($p < 0.01$) differed between I_{200} and I_{400} (Table 1).

The leaf Chl content increased from N_5 to N_{15} and decreased from N_{15} to N_{25} (Table 1). The Chl contents at I_{200} were higher than at I_{400} . F_v/F_m of a dark-adapted leaf reflects the potential quantum efficiency of PS2 and is used as a sensitive indicator of plant photosynthetic performance. The F_v/F_m and Φ_{PS2} increased from N_5 to N_{15} and then decreased to N_{25} . F_v/F_m was also higher at I_{200} than at I_{400} , but Φ_{PS2} was lower at I_{200} than at I_{400} .

Plants acclimate to different I by several approaches regulating leaf morphology and photosynthetic capacity (Le Roux *et al.* 2001, Warren and Adams 2001, Walcroft *et al.* 2002). Zhang and Feng (2004) suggest that plants acclimate to different I by regulating SLA and leaf N content, and by changing the relative investment of N among the different pools of photosynthetic apparatus. A change in SLA alters the amount of photons that can be intercepted per unit leaf dry mass (Evans and Poorter 2001). This is advantageous under low I . Blackman *et al.* (1955) reported that SLA is negatively linked with I . SLA is lower for the leaves growing under sun than for those growing in the shade (Kitao *et al.* 2000, Le Roux *et al.* 2001, Wang and Feng 2005), and species with lower SLA accumulate more photon-absorbing compounds in their leaves or radiation-reflecting compounds on the adaxial

Table 1. Nitrogen contents per unit dry mass (N_m) [g kg^{-1}] or unit area (N_a) [g m^{-2}], photosynthetic nitrogen use efficiency (PNUE) [$\text{mmol m}^{-2} \text{s}^{-1}$], leaf chlorophyll (Chl) content [relative], maximum photochemical efficiency (F_v/F_m), and actual photochemical efficiencies of photosystem 2 (Φ_{PS_2}) of *Trifolium repens* grown at different nitrogen (N) concentrations and irradiances (I). Different uppercase letters in the same column indicate significant difference at different growth I , and different lower case letters indicate significant difference at the same growth I at $p < 0.05$.

		N_m	N_a	PNUE	Chl	F_v/F_m	Φ_{PS_2}
I_{200}	N_5	36.55±1.05 Ab	0.67±0.02 Ab	0.104±0.009 Bb	45.14±2.57 Ac	0.842±0.005 Ab	0.808±0.013 Bb
	N_{10}	38.56±1.22 Ab	0.68±0.06 Aab	0.113±0.015 Bb	45.50±2.07 Aabc	0.846±0.003 Aab	0.810±0.007 Bab
	N_{15}	43.47±6.17 Aa	0.73±0.10 Aa	0.159±0.042 Ba	49.40±3.86 Aa	0.852±0.002 Aa	0.819±0.003 Ba
	N_{20}	45.54±1.74 Aa	0.77±0.11 Aa	0.115±0.013Bbc	48.97±2.15 Aab	0.849±0.009 Aab	0.808±0.017 Bab
	N_{25}	46.02±0.67 Aa	0.74±0.07 Aa	0.102±0.012 Bc	44.99±4.34 Abc	0.839±0.012 Ab	0.798±0.009 Bab
I_{400}	N_5	20.58±3.45 Bb	0.46±0.05 Ab	0.232±0.054 Ab	40.14±1.15 Ac	0.830±0.013 Bb	0.810±0.007 Ab
	N_{10}	27.09±4.20 Bb	0.61±0.12 Aab	0.240±0.040 Ab	44.96±3.02 Aabc	0.840±0.003 Bab	0.818±0.002Aab
	N_{15}	36.68±3.45 Ba	0.75±0.07 Ab	0.266±0.038 Aa	48.97±1.29 Aa	0.850±0.001 Ba	0.832±0.001 Aa
	N_{20}	38.19±1.65 Ba	0.78±0.09 Ab	0.165±0.006 Abc	48.83±1.22 Aab	0.841±0.005 Bab	0.818±0.003Aab
	N_{25}	40.78±4.00 Ba	0.78±0.04 Ab	0.145±0.016 Ac	44.33±1.24 Abc	0.836±0.008 Bb	0.817±0.018 Aab

surface (Hikosaka 2004). Wang *et al.* (2003) found that irradiance-demanding species had higher SLA, lower thermal dissipation, and higher quantum yield of PS2 non-cyclic electron transport when they survived and kept growing under extremely low I . SLA of *T. repens* was higher at low I than at high I , which is consistent with information in previous studies (Kitao *et al.* 2000, Le Roux *et al.* 2001, Wang *et al.* 2003, 2005, Zhang and Feng 2004, Wang and Feng 2005). Witkowski and Lamont (1991) found that plants of two Australian tree species growing in more nutrient-poor soils had a lower SLA and thicker, denser leaves, and Van Arendonk *et al.* (1997) found that SLA decreased with decreasing N supply. The SLA of *T. repens* increased with increasing N supply, which is consistent with what Van Arendonk *et al.* and Witkowski *et al.* reported.

Generally, the leaves of plants growing at low I exhibit higher N contents than those growing at high I (Evans and Poorter 2001, Zhang and Feng 2004), because shade leaves contain less mechanical tissue (consisting mainly of cellulose and lignin) per unit area than sun leaves (Niinemets and Kull 1998), and a greater proportion of N is partitioned into light-harvesting thylakoid components when plants grow at lower I (Evans 1989b, Frak *et al.* 2002). The changes in N partitioning occur in response to varying N contents or growth I . The total N content per dry mass is higher for *T. repens* growing at low I which is in agreement with reports of Evans and Poorter (2001), and Zhang and Feng (2004). The ability to proliferate thylakoid membranes when grown in the shade is a feature of many species and occurs at the expense of both soluble protein and the other fraction. The acclimation to shade at thylakoid level is consistent with the reduction in the N cost of photon capture (Evans 1989b). In shade-tolerant species whose premium is efficient photon capture, there is a greater investment of leaf N in light-harvesting thylakoid components (Evans 1989b, Niinemets 1997). The major

proportion of N directly related to photosynthesis is in pigment-protein complexes, photosystem reaction centres, components of the electron transport chain (primarily the cytochrome *b/f* and ferredoxin-NADP reductase complexes), and the coupling factor (ATP synthase). The majority of thylakoid N (60–85 %) is in pigment-protein/reaction centre complexes (Evans 1989b). The N cost is influenced by I at what the leaves grow. The electron transport capacity per unit of Chl at high I is greater than that at low I . This is mainly due to a relative increase in the amounts of cytochrome *b/f* complex and coupling factor (Davies *et al.* 1987). Some studies showed smaller N allocation to photosynthetic apparatus in species with lower PNUE (Warren and Adams 2000, Ripullone *et al.* 2003, Takashima *et al.* 2004). Therefore, N allocation to photosynthetic apparatus may be a major factor for inter-specific variation in PNUE. The PNUE was higher at I_{400} than at I_{200} , which is in agreement with what Zhang and Feng (2004) reported.

Leaf photosynthetic characteristics exhibit remarkable adaptability to I (Evans 1989a). Because more than 50–60 % of total leaf N is allocated to photosynthetic proteins (Hikosaka *et al.* 1998), photosynthetic acclimation to I has been described largely in terms of changes in total leaf N and/or in the partition of total leaf N among the different pools of the photosynthetic apparatus (Hikosaka and Terashima 1995). The fractional N investments in ribulose-1,5-bisphosphate carboxylase/oxygenase (P_R) and photosynthetic electron transport (P_B) increase with increasing irradiance (Evans 1989a, Niinemets and Tenhunen 1997). Generally, leaves growing at high I exhibit higher photosynthetic capacity per unit leaf area compared with shade leaves (Niinemets *et al.* 1998). In our experiment, P_N of *T. repens* at I_{400} was higher than at I_{200} . Photosynthetic rate per unit leaf area of plants growing at high I was three times higher than for plants growing at low I (Evans and Poorter 2001). Plants grown in high I generally have increased number

of chloroplasts and amount of photosynthetic enzymes thereby enhancing the photosynthetic capacity per unit leaf area. Zhang and Cao (2002) found that the leaves of *Pometia tomentosa* and *Horsfieldia tetratelpala* growing at low *I* possessed similar F_v/F_m , higher than that of the

leaves growing at high *I*. The changes in Chl content of *T. repens* are consistent with earlier reports (Niinemets 1997, Niinemets and Tenhunen 1997). Shade tolerant plants usually have low photosynthetic capacities and low plasticity in photosynthetic acclimation to *I*.

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