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Ecosystem carbon fluxes of a ryegrass and clover fodder crop in a Mediterranean environment

L. VITALE[*,+], P. DI TOMMASI[*], C. ARENA[**], M. OLIVA[*], T. BERTOLINI[*], S. RANUCCI[*], A. VIRZO DE SANTO[**], and V. MAGLIULO[*]

Istituto per i Sistemi Agricoli e Forestali del Mediterraneo - Consiglio Nazionale delle Ricerche, Via C. Patacca, 85, 80056 Ercolano (Na), Italy[*]
Dipartimento di Biologia Strutturale e Funzionale,Università degli Studi di Napoli Federico II, Via Cinthia, 80126 Napoli, Italy[**]

Abstract

A mixture of ryegrass (*Lolium italicum* A. Braun) and clover (*Trifolium alexandrinum* L.) was sown in Eboli (Salerno, Southern Italy) in September 2007. Crop growth, leaf and canopy gas exchange and ecophysiological traits were monitored throughout the growth cycle. The gross primary production (GPP) was not affected by air temperature (T_{air}); on the contrary the ecosystem respiration (R_{eco}) decreased as T_{air} decreased while net ecosystem CO_2 exchange (NEE) increased. When was normalized with leaf area index (LAI), GPP decreased with T_{air}, a likely response to cold that down-regulated canopy photosynthesis in order to optimize the light use at low winter temperatures. Net photosynthetic rates (P_N), the effective quantum yield of PSII (Φ_{PSII}) and photosynthetic pigment content were higher in clover than ryegrass, in relation to the higher leaf N content. The lower Φ_{PSII} in ryegrass was linked to lower photochemical quenching coefficient (q_P) values, due to a reduced number of reaction centres, in agreement with the lowest Chl *a* content. This behaviour can be considered as an adaptive strategy to cold to avoid photooxidative damage at low temperature rather than an impairment of PSII complexes.

Additional key words: carbon fluxes; carbon sequestration; *Lolium italicum* A. Barum; photochemistry; productivity; *Trifolium alexandrinum* L.

Introduction

Croplands represent about 12% of the earth's surface and agroecosystems have received considerable scientific attention in the last few years for their potential carbon sequestration role in the soil (Lal *et al.* 1998, Vleeshouwers and Verhagen 2002, Suyker *et al.* 2004, Verma e*t al.* 2005, Vitale *et al.* 2007, 2009). Although several papers have been recently published about C fluxes at the biome and ecosystem scales (Mahecha *et al.* 2010, Yi *et al.* 2010), it is often difficult to predict the C sequestration strength of arable lands due to the intensive agricultural management practices, which impact on both the amount of carbon fixed by plants and on carbon released by organic matter degradation.

CO_2 fixation by crops as well as CO_2 release by soil and plant respiration determine the net ecosystem production and are in turn affected by biotic and abiotic factors, including canopy structure, soil and air temperature. Leaf area development modulates light interception and thereby determines the amount of radiant energy absorbed by the crop, thus affecting photosynthetic rates. Temperature is another key factor in influencing both ecosystem photosynthesis and respiration.

Grass-legume mixtures are used as forage crops because of the legumes nitrogen fixation capability and the production of a highly digestible herbage with good nutritional value. In the southern Mediterranean zone

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[+]Corresponding author; tel: (+39) 0817717325, fax: (+39) 0817718045, e-mail: luca.vitale@isafom-cnr.it

Abbreviations: Car – carotenoids; C_i – intercellular CO_2 concentration; Chl *a* – chlorophyll *a*; Chl *b* – chlorophyll *b*; DAS – days after sowing; F_v'/F_m' – the intrinsic quantum yield of PSII; g_s – stomatal conductance; GPP – gross primary production; GPP_T – gross primary production normalized for LAI; LAI – leaf area index; NEE – net ecosystem CO_2 exchange; P_N – net photosynthetic rate; q_P – photochemical quenching coefficient; PSII – photosystem II; R_{eco} – ecosystem respiration; T_{air} – air temperature; Φ_{PSII} – effective quantum yield of PSII.

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annual legumes, such as berseem clover (*T. alexandrinum*) and *Vicia* spp., play an important role in pastoral livestock systems (Porqueddu and Gonzalez 2006). Legumes show a variable relative plant density in mixed swards due to both the seasonal and annual variability of growth and to competition for resources with other species in the mix (Parson *et al.* 1991, Elgersma and Schlepers 1997ab, Schils *et al.* 2000); for this reason, the relative growth of each species is a function of their competitive abilities. When cultivated in mixed swards, clover tends to be positioned in the lower layers of the canopy (Ross *et al.* 2005). This limits its ability to compete for light, thus determining lower photosynthetic rates that leads to a reduced relative proportion of clover plants in the sward compared to that of the grass (Kendall and Stringer 1985, Buxton and Mertens 1995). This competition might become more critical at lower temperatures, because optimal temperature for clover is higher than those for grass (Woledge *et al.* 1989). However, competition with grass should have a positive effect on N fixation derived from symbiosis as a consequence of increased competition for nitrate (Nesheim and Boller 1991). The grass is in fact more efficient in soil N uptake. This reduces soil N availability so that the legume is forced to fix N by means of the symbiotic pathway. In grass-legume swards the proportion of nitrogen legume derived from symbiosis is therefore higher than in pure legume swards (West and Wedin 1985).

Italian ryegrass (*Lolium italicum* A. Braun) and berseem clover (*Trifolium alexandrinum* L.) are commonly cultivated as mixed fodder crops in southern Italy and used either as fresh forage or hay. The two species show a differential sensitivity to low temperatures. Both species are cold tolerant, but berseem growth is reduced by cold and frost temperatures in a more pronounced way, thus reducing its competitive ability with regard to ryegrass in a mixed-species sward.

Plants from cold environments or adapted to low temperatures present an optimum of temperatures for growth and photosynthesis lower than plants from warmer environments; suboptimal air temperatures may limit leaf and canopy photosynthesis by impairing the activity of enzymes involved in carbon metabolism (Sage and Sharkey 1987, Sassenrath *et al.* 1990, D'Ambrosio *et al.* 2006). A relationship between CO_2 fixation and air temperature is well documented. Exposure to chilling temperature has a direct impact on the photosynthetic apparatus by limiting stomatal conductance and CO_2 supply to mesophyll, disturbing water balance (Vernieri *et al.* 1991, Pardossi *et al.* 1992) and by disrupting specific components of the photosynthetic apparatus, including the thylakoid electron transport and the carbon reduction cycle (Baker *et al.* 1994, Allen and Ort 2001). Chilling may also reduce the demand for reductans of the Calvin cycle, determining an increase in photosynthetic electron flux to O_2 and a consequent production of reactive oxygen species that have a harmful effect on the photosynthetic apparatus (Cadenas 1989, Foyer *et al.* 1994). Some species are able to tolerate low temperatures by adaptive mechanisms known as hardening (Hanslin and Höglind 2009) and by diverting the photosynthetic electron flux to alternative pathways other than CO_2 fixation (Flexas *et al.* 1999, Hendrickson *et al.* 2003, Hendrickson *et al.* 2004, D'Ambrosio *et al.* 2006).

The aim of this work was to examine the dependence of carbon fluxes on abiotic and biotic (leaf area) factors and to investigate the regulation of photosynthesis and photochemistry between *L. italicum* and *T. alexandrinum* in response to wintertime cold temperatures.

Materials and methods

Study site and crop management: The study was carried out at "Borgo Cioffi" site (IT-Bci, Eboli, Italy, latitude: 40°31'25.5'', longitude: 14°57'26.8'', mean altitude: 15 m a.s.l.) an experimental station part of the CarboEuropeIP and NitroEuropeIP European networks. The experimental field is the northern half (18 ha) of a circular field served by a center pivot irrigation system. Cropping systems in rotation such as fennel/maize and ryegrass-clover/maize are typical of the area. According to agrometeorological data collected from 1975 to 2005 by the nearby micrometeorological station in Torre Lama (Bellizzi, Sa, Italy; mean altitude: 15 m a.s.l.), the mean temperature of the warmest month (August) is 23°C while the mean temperature of the coldest month (January) is 9.0°C.

Berseem clover (*Trifolium alexandrinum* L.) and Italian ryegrass (*Lolium italicum* A. Braun) were sown on 27 September 2007 at a seed density of 15 kg ha^{-1} and 30 kg ha^{-1}, respectively after the main spring-summer crop (*i.e.* maize). No irrigation water nor fertilizer or pest control sprays were applied. Starting at the end of January and through February 2008 part of the field was harvested. Several cuttings were made at different dates, harvesting areas variable in size, located in the NE section of the field. Final overall sward harvest took place on April 30th 2008.

Environmental variables: The sensors for measuring micrometeorological variables were placed above the canopy at 3.8 m height above ground. The following parameters were measured: incident and reflected global radiation (*Eppley black & white, Eppley Laboratory Inc.*, Newport, RI, USA), net radiation (*Rebs Q7.1, Campbell Scientific, Ltd.*, Shepshed, UK), incident and reflected photosynthetically active radiation (*LI-190SA, LI-COR Inc.*, Lincoln, NE, USA), canopy radiative temperature (*IR4000-GL, Fullerton*, CA, USA), precipitation (*Rain Collector II, Davis Instruments*, CA, USA), air temperature and relative humidity (*Rotronic MP100, Campbell Scientific, Ltd.*, Shepshed, UK), wind speed and direction (*R3 sonic anemometer, Gill Instruments Ltd.*, Lymington, UK). Soil temperature at 0.03 m depth was also monitored by means of thermocouple probes (*TCAV, Campbell Scientific, Ltd.*, Shepshed, UK) .

Biometric determinations: Crop height, LAI, and aboveground biomass were monitored during the entire crop cycle. LAI and crop biomass were assessed on a monthly basis by destructively sampling an area of 0.25 m^2, with four replicates along a NE–SW transect within the footprint of the flux tower. Starting on 124 days after sowing (DAS), the sward was partly harvested in the footprint zone, as described above. Destructive samplings were continued along the section of the NE–SW transect of the field where the grass was not harvested. LAI of harvested plants was determined by detaching leaves and determining green leaf surface by means of an area-meter (*LI-3100, LI-COR Inc.,* Lincoln, NE, USA). Standing biomass and its partition among leaves and stems was determined gravimetrically after oven drying samples at 65°C to constant mass.

Turbulent fluxes: A 3-dimesional sonic anemometer (*Gill Solent R3, Gill Instruments, Ltd.*, Lymington, UK) and an open path nondispersive CO_2-H_2O infrared gas analyser (*LI-7500, LI-COR Inc.*, Lincoln, NE, USA) provided high frequency (50 Hz) data streams for the calculation of turbulent mass and energy exchange (Baldocchi *et al.* 1988).

The Eddymeas and Eddysoft packages (Kolle and Rebmann 2007) were used for collecting and post-processing data, for the calculation of net ecosystem CO_2 exchange (NEE), which can be defined as NEE = GPP – R_{eco}. The partitioning of NEE in its two components, ecosystem respiration R_{eco} and gross primary production GPP, was attained by downloading level 4 data from the CarboEuropeIP database, which routinely estimates these variables according to Reichstein *et al.* (2005). Negative and positive NEE values represent the net carbon uptake and the release from the ecosystem, respectively. For filling gaps in NEE time series, we used an online tool available on the CarboEuropeIP web site, based on procedures set-up by Reichstein *et al.* (2005). Further information can be found at http://www.carboeurope.org. An average of 35% of half-hourly data was rejected by EDDYFLUX package according to several criteria based on the presence of spikes, acceptable ranges for concentration and fluxes, integral turbulence test, flux divergence test, and stationary test (Aubinet *et al.* 2000).

A footprint analysis was also performed routinely by the EDDYFLUX software. An average 80% of observations had sufficient upwind fetch. Records with insufficient fetch were flagged and excluded by the computations. The mast was located in the centre of the field, so as to maximize footprint from all possible wind directions. The field featured a westward slope of 3 to 5%. The wind regime was fairly constant year-round, due to a local breeze circulation and to the orientation on Sele river valley, surrounded by hills on all directions except to the west, where it is bordered by the sea. Under these circumstances, over 95% of the semihourly observations featured a SW–NE wind direction. Wind regime is characterized by a NE land breeze up until noon, followed by a calm wind transition and the by sea breeze thereafter, lasting until dusk. Only occasionally, synoptic circulation determined winds from the second and fourth quadrants.

Gas-exchange and Chl fluorescence measurements: Leaf gas-exchange measurements were performed simultaneously to fluorescence determinations on fully expanded apical leaves by a portable gas-exchange system (*Portable Photosynthesis LI-6400, LI-COR Inc.*, Lincoln, NE, USA) combined with a fluorometer (*LI-6400-40, LI-COR Inc.*, Lincoln, NE, USA). Measurements were performed twice along the crop season, on 62 DAS (average T_{air} = 11.8°C) and 140 DAS (average T_{air} = 7.2°C) between 11:00 and 15:00 h. A leaf temperature of 20°C and a relative humidity of 50%, as well as a CO_2 concentration of 380 μmol mol^{-1} and a saturating irradiance of 1,200 μmol(photon) m^{-2} s^{-1} were setting in the cuvette of the gas-exchange system. The leaves were kept in the cuvette for 5 min, a time sufficient to obtain the steady-state conditions.

The net photosynthetic rate (P_N), stomatal conductance to water (g_s), transpiration rate (E), and intercellular CO_2 concentration (C_i) were calculated according to von Caemmerer and Farquhar (1981).

Under illumination, the steady-state fluorescence (F_s) was measured by setting measuring light at a frequency of 20 kHz. The maximum fluorescence under light exposure (F_m’) was obtained by 0.8-s saturating flash of 10,000 μmol(photon) m^{-2} s^{-1}. The effective quantum yield of PSII (Φ_{PSII}) was expressed according to Genty *et al.* (1989) as Φ_{PSII} = (F_m’ – F_s)/F_m’. The photochemical quenching coefficient (q_P) and the intrinsic quantum yield of PSII (F_v’/F_m’) were calculated as q_P = (F_m’ – F_s)/(F_m’ – F_0’) and F_v’/F_m’ = (F_m’ – F_0’)/F_m’, respectively. The minimum fluorescence emission of light-adapted leaves (F_0’) was determined by rapidly darkening the leaf in the presence of far-red light.

Leaf N, C, Chl, and total carotenoid content: Representative leaf samples of the two species were collected on 69 and 116 DAS and C and N content were determined by using a CSN analyzer (*Flash EA 1112 Series CHN Report.*, *Thermo Finnigan*). Photosynthetic pigments (chlorophylls and carotenoids) were extracted by smashing leaves in a mortar in 100% ice-cold acetone and then quantified by means of a spectrophotometer according to Lichtenthaler (1987).

Statistical analysis was performed using *SigmaStat* statistical package (*version 3.1, Aspire Software International*, Ashburn, VA, USA). A regression analysis was applied to assess the relationship between the various measured parameters and temperature, for the growth period before partial harvesting (*i.e.* 0–123 DAS). Student *t*-test was performed to assay the differences between measurement dates and between species, based on a significance level of 0.05. For leaf gas-exchange measurements were used in each sampling data 15 different plants.

Results

Weather conditions: Precipitation and air temperature regime of the entire study period was typical of the Mediterranean region. Chilling soil and air temperatures between 0 and 5°C were recorded during periods corresponding to 78–80 DAS and 143–144 DAS (Fig. 1).

Biometric determinations and canopy turbulent fluxes: Green LAI of the mixed crop increased during the season, reached the highest values (about 6.7 m^2 m^{-2}) close to 114 DAS (Fig. 2*A*) and decreased afterwards. Total and green aboveground biomass increased with time (Fig. 2*A*) according to a power relationship. Canopy height of ryegrass and that of berseem were similar for most of the sampling dates. Differences in canopy height were only detected in spring when higher air temperature favoured ryegrass relative growth (data not shown). Daily gross primary production (GPP) increased during the growth season reaching the higher values in spring (Fig. 2*B*). On the contrary, the ecosystem respiration (R_{eco}) did not show definite trends during the great part of the crop cycle and only increased during spring (Fig. 2*C*). NEE showed a similar trend to that of GPP (Fig. 2*D*).

GPP, R_{eco} and NEE were plotted against interpolated LAI values (Fig. 3). Absolute values of GPP and NEE increased with LAI, reaching a plateau in correspondence of LAI values of about 4.0 m^2 m^{-2} (Fig. 3*A*,*C*); for higher LAI values, both GPP and NEE showed a tendency to

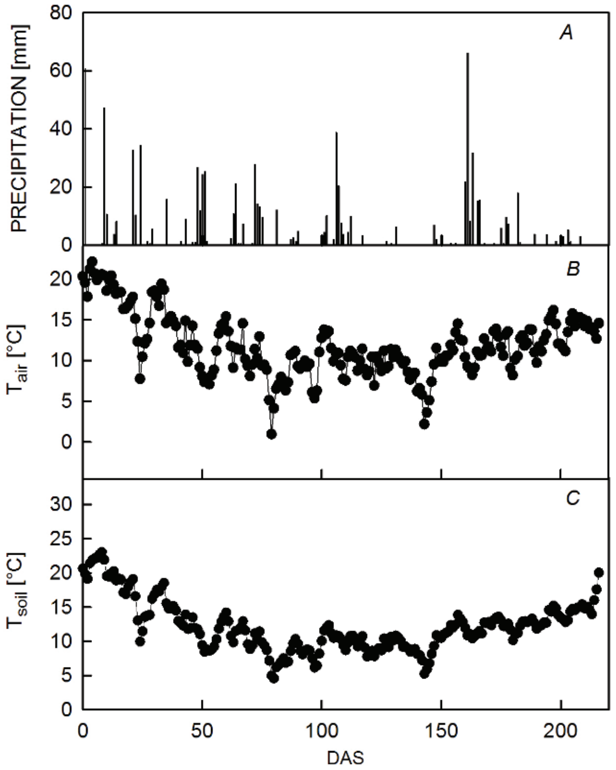

Fig. 1. *A*: Monthly rainfall, *B*: air temperatures, and *C*: soil temperatures throughout crop growth cycle (27 September 2007 – 30 April 2008).

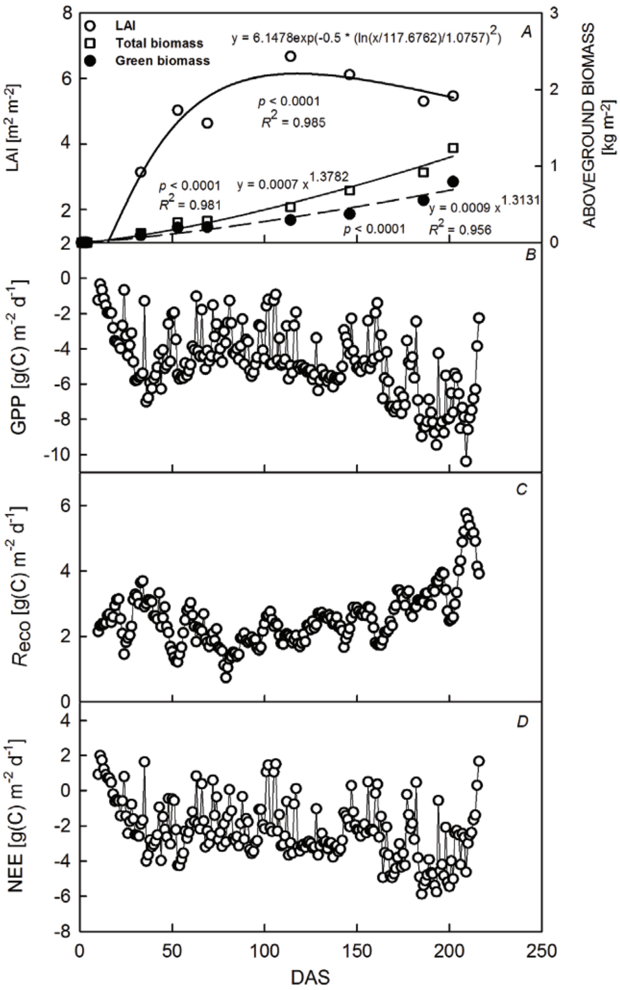

Fig. 2. Seasonal trends of *A*: leaf area index (LAI), total and green aboveground biomass, *B*: daily gross primary production (GPP), *C*: daily ecosystem respiration (R_{eco}), and *D*: daily net ecosystem exchange (NEE). Panel *A*: values are mean (n = 4).

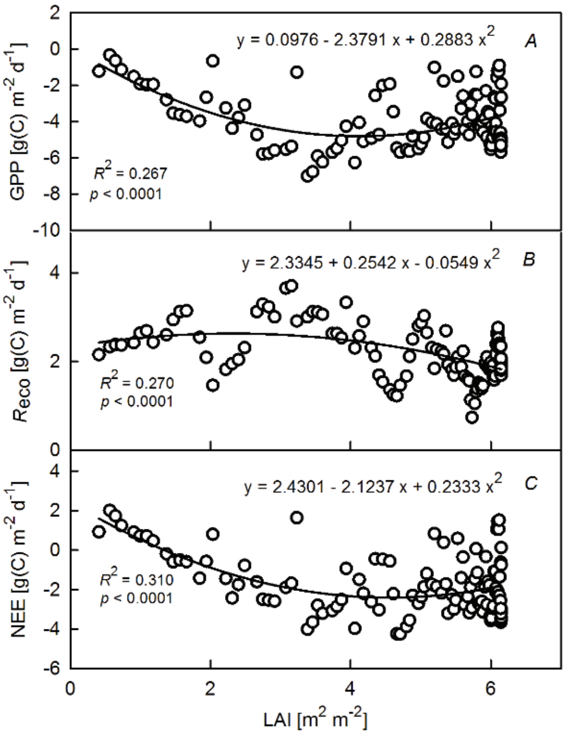

Fig. 3. *A*: Daily gross primary production (GPP), *B*: daily ecosystem respiration (R_{eco}), and *C*: daily net ecosystem exchange (NEE) as a function of leaf area index (LAI) throughout the period 0–123 DAS.

decrease. R_{eco} was only little affected by LAI when leaf area expansion influenced ecosystem photosynthesis positively; a reduction in R_{eco} was evident for LAI values higher than 4.0 m^2 m^{-2} (Fig. 3*B*). Although a significant quadratic relationship could be found, the regression does not capture the dynamic of R_{eco}, which featured several peaks and falls.

GPP was apparently not influenced by air temperature, as it remained almost constant throughout the study period (Fig. 4*A*); on the contrary, R_{eco} decreased with air temperature while NEE increased (Fig. 4*B*,*C*). In order to distinguish the relative effects of temperature and LAI on ecosystem photosynthesis, GPP values recorded before cutting were normalized by LAI to derive the GPP$_T$. Absolute values of GPP$_T$ steadily decreased with decreasing T_{air} (Fig. 4*A*).

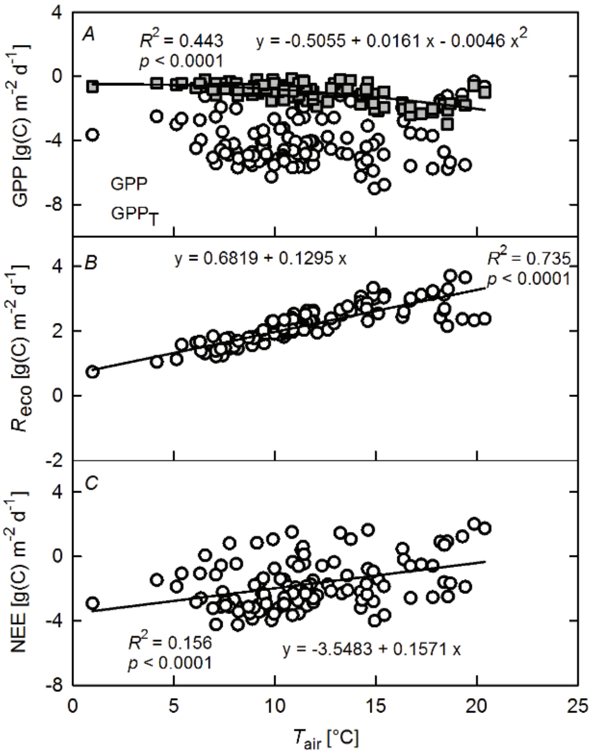

Fig. 4. *A*: Daily gross primary production (GPP), *B*: daily ecosystem respiration (R_{eco}), and *C*: daily net ecosystem exchange (NEE) throughout the period 0–123 DAS (*open circles*); GPP normalized for LAI (*gray squares*) as a function of daily average air temperature (T_{air}).

Leaf gas exchange, Chl fluorescence, pigment composition and leaf N and C content: No difference in P_N was found between 62 DAS and 140 DAS for both species; on the contrary, g_s was lower at 140 DAS as compared to 62 DAS in both species. *T. alexandrinum* always showed higher values for the above-mentioned parameters, as compared to *L. italicum* (p<0.01) (Fig. 5*A*,*B*).

The effective quantum yield of PSII, Φ_{PSII}, the intrinsic quantum yield of PSII, F_v'/F_m' and the photochemical quenching, q_P were also higher in *T. alexandrinum* than in *L. italicum* (Fig. 5*C*,*D*,*E*) although these differences were only significant at 140 DAS (p=0.01). No difference was found between the two measurement dates.

Chl *a*, total Chl and carotenoids (Car) content, and Car/Chl ratio measured at 116 DAS were higher in clover compared to ryegrass (p<0.05) (Table 1); total leaf nitrogen determined at 69 DAS and 116 DAS was higher in berseem while no difference in Chl *b* or leaf carbon content was found (Table 1).

Discussion

Ecosystem carbon fluxes: Winter temperature represents an environmental factor controlling the growth and NEE of natural and cropped ecosystems. The fodder crop grown at "Borgo Cioffi " site was able to maintain high CO_2 fixation rates throughout the fall-winter growing season, as showed by seasonal trends of GPP.

It is well known that temperature as well as the structural characteristics of vegetation canopies regulate ecosystem photosynthesis of crop and grass ecosystems (Dugas *et al.* 1999, Suyker *et al.* 2004, Li *et al.* 2005, Zhang *et al.* 2007). Leaf area and light extinction coefficient determine the amount of light absorbed along the crop profile and thus canopy photosynthetic rates.

NEE increased with increasing leaf expansion and decreasing air temperature suggesting that the steady increase in CO_2 uptake observed during the growth season could be mainly explained in terms of LAI development. Daily GPP increased with LAI expansion but it appeared to be unaffected by air temperature throughout the growing season. On the other hand, since GPP remained fairly constant, it should be pointed out that the sharp response of R_{eco} to T_{air}, which decreased more than three-fold when temperature decreased from 20°C to 5°C, also featured to diminish NEE throughout the season. The crop growth effect was then capable of compensating the temperature dependence of GPP. In order to analyze the relative effects of temperature and LAI on the ecosystem photosynthesis during the growing season, GPP was normalized by LAI. The variable thus calculated, referred to as GPP_T, is better suitable to describe the influence of temperature alone on ecosystem photosynthesis. The differences between GPP and GPP_T, is an indication of the relative contribution of LAI to the dynamics of GPP. As the season progressed, leaf area development was the determinant of crop photosynthesis. When this dominant effect was partly removed by normalizing for LAI, it was observed that the progressive reduction in T_{air} lead to lower GPP_T due to the dependence of canopy gross photosynthesis on temperature. As a result, it is evident that canopy photosynthesis was affected by both leaf area and air temperature. The negative effect of decreasing air temperature on GPP was compensated by the concurrent LAI increase that, in turn, determined a raise in ecosystem CO_2 uptake (NEE) during fall-winter growth season. As previously described, the reduction in R_{eco} as T_{air} decreased, also contributed to the increase in ecosystem CO_2 uptake, due to the effect of low temperature on biological processes. Partitioning the R_{eco} in the plant and soil components could help in further understanding the respective plant and soil responses to air temperatures and their contribution to NEE.

The steady reduction in GPP_T as T_{air} decreased could be regarded as a response to cold that down-regulates canopy photosynthesis in order to optimize the light use at low winter temperatures and avoid photoinhibitory and photoxidative damages. The fodder crop grown at "Borgo Cioffi" site was, in fact, dominated by ryegrass, which requires very low cardinal temperatures for growth and photosynthesis, and is capable of winter-hardening and frost tolerance (Hanslin and Höglind 2009). The recover of GPP during the following spring, when higher air temperatures enhanced crop photosynthesis, supports our view about the down-regulation of crop photosynthesis during winter.

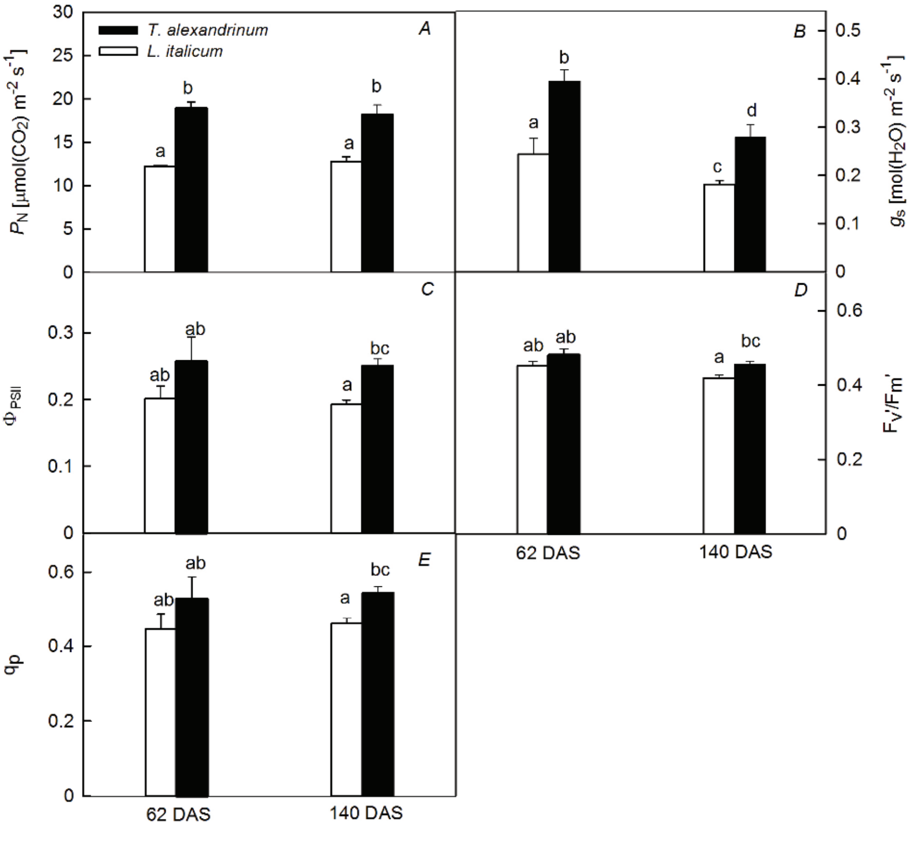

Fig. 5. *A*: The net photosynthetic rate (P_N), *B*: stomatal conductance (g_s), *C*: effective quantum yield of PSII (Φ_{PSII}), *D*: intrinsic quantum yield of PSII (F_v'/F_m'), *E*: coefficient for photochemical quenching of fluorescence (q_P) at 62 and 140 DAS. *Different letters* denote significant differences between species and days. Data represent means ± SE (n = 15).

Table 1. Leaf nitrogen (N) and carbon (C) content, chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophylls [Chl (*a*+*b*)], total carotenoids [Car (*x*+*c*)] content, and carotenoids to total Chl ratio (Car/Chl) in ryegrass (*L. italicum*) and clover (*T. alexandrinum*). nd – not determined. *Different letters* denote significant differences between species. Data represent means ± SE ($n = 3$).

Parameter	69 DAS Ryegrass	Clover	116 DAS Ryegrass	Clover
Leaf N [%]	3.6 ± 0.4^a	5.2 ± 0.1^b	4.2 ± 0.3^a	5.1 ± 0.3^b
Leaf C [%]	42.6 ± 2.7^a	45.4 ± 1.5^a	45.0 ± 1.2^a	47.4 ± 3.2^a
Chl *a* [μg cm^{-2}]	nd	nd	17.8 ± 2.5^a	30.7 ± 2.8^b
Chl *b* [μg cm^{-2}]	nd	nd	6.9 ± 1.1^a	8.2 ± 1.0^a
Chl (*a*+*b*) [μg cm^{-2}]	nd	nd	24.7 ± 3.5^a	38.9 ± 3.9^b
Car (*x*+*c*) [μg cm^{-2}]	nd	nd	4.8 ± 0.5^a	9.6 ± 0.9^b
Car/Chl	nd	nd	0.20 ± 0.01^a	0.25 ± 0.00^b

In conclusion, ecosystem CO_2 exchange was affected either by leaf area and air temperature. The negative effect of decreasing air temperature on GPP was compensated by the concurrent increase in LAI, that appeared to be the main determinant of ecosystem CO_2 exchange. As a consequence, the crop steadily increased its aboveground biomass and the ecosystem was an active sink for CO_2 during wintertime.

Physiological response of. *L. italicum* and *T. alexandrinum*: When considering photosynthetic response of individual species, it should be noted that *T. alexandrinum* showed higher P_N values than *L. italicum* due likely to its higher leaf N content. As previously reported, photosynthesis increases with increasing leaf nitrogen (Llorens *et al.* 2003, Takashima *et al.* 2004). Ross *et al.* (2005) found in mixed clover/oat swards (sowing density between 135 and 17 kg ha^{-1} for oat and 15 kg ha^{-1} for berseem) that the taller oat plants determined considerable shading of berseem clover plants, limiting their growth and biomass production when compared to a sole berseem crop, as a consequence of lower photosynthetic rates (Kendall and Stringer 1985, Buxton and Mertens 1995). In our study, during the fall-winter period when gas-exchange measurements were performed, the mean plant height of the two species was comparable (data not shown) likely due to the mild air temperature that did not limit berseem growth; the upper leaves of *T. alexandrinum* were therefore able to intercept a significant amount of incident radiation thus sustaining canopy growth, which in turn allowed to sustain the competition for light with *L. italicum*.

The higher leaf N content in *T. alexandrinum* than *L. italicum* is the result of N_2 fixation activity by nodules. It is known that in grass-legume swards, the proportion of legume N derived by symbiosis is higher than in pure legume swards (Wets and Wedin 1985) because the competition with grass reduces soil N availability to clover (Nesheim and Boller 1991).

From a physiological viewpoint, the lowest P_N rates in *L. italicum* compared to *T. alexandrinum* occurred together with lower Φ_{PSII}. The latter is due to the lower reduced q_P rather than to a reduced F_v'/F_m', which was only slightly lower in *L. italicum* compared to *T. alexandrinum*. Low q_P, which represents the approximate fraction of open PSII reaction centres, is associated with an increased risk of photoinhibition. Thermal energy dissipation in the antennae has been proposed as an effective way to maintain the fraction of open PSII high and to prevent photoinhibition (Demmig-Adams and Adams 1992, Demmig-Adams *et al.* 1996). In the present study, however, thermal energy dissipation, indicated by lowest F_v'/F_m' values, was slightly higher in *L. italicum* compared to *T. alexandrinum*, in spite of lowest q_P. This suggests that thermal energy dissipation might not be sufficient to prevent a decline in q_P at low temperatures. In our view, the lowest q_P in *L. italicum* should be linked to a reduced number of reaction centres, in agreement with the lowest Chl *a* content, and it is to be interpreted as an adaptive strategy to cold rather than an impairment to PSII complexes, in order to avoid photooxidative damage at low temperature. A partial loss of PSII reaction centres, reduced needle Chl per unit area, and reduced daily carbon gain was observed in cold-acclimated *Pinus contorta* L. plants (Savitch *et al.* 2002). The acclimation strategy in *L. italicum* proved to be different from that of *T. alexandrinum*. This could be linked to the lower Car content and Car/Chl ratio found in ryegrass. It is accepted that carotenoids act as scavengers of triplet Chl and singlet oxygen, and mediate the harmless dissipation of excess excitation energy as heat protecting photosynthetic machinery from photoinhibitory and photooxidative damages (Schreiber *et al.* 1994, Demmig-Adams and Adams 1996, Kyparissis *et al.* 2000, Demmig-Adams and Adams 2006, Logan *et al.* 2007).

This study shows that at the individual plant level, both *T. alexandrinum* and *L. italicum* were able to maintain high photosynthetic activity during winter. The higher net photosynthetic rate in clover was likely due to its elevated leaf N content depending on symbiotic N fixation, a process which is known to take place also at moderately low temperature, typical of the Mediterranean climate. The lowest Φ_{PSII} in *L. italicum* could be interpreted as a strategy to tolerate cold in order to avoid photooxidative damage at low temperature.

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