RESEARCH ARTICLE

Analysis of mesophyll conductance in five understory herbaceous species

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Abstract Mesophyll conductance (g_m) has received over time much less attention than stomatal conductance (g_s), although it affects leaf photosynthesis to about the same extent as stomatal conductance does. The objective of this study was to analyze the g_m trend in five understory herbaceous species growing in a close-canopy forest in the north-west of Italy. In particular, three of analyzed species were monocots: *Carex brizoides* Lam., *Carex pilosa* Scop., and *Oplismenus undulatifolius* P. Beauv and the others dicots species: *Circaea lutetiana* L., and *Pulmonaria officinalis* Ced. The results showed, on one hand, the absence of correlation between g_m and the considered environmental variables in the forest understory (i.e. air temperature, photosynthetic photon flux density and carbon dioxide concentration). Moreover, we carried out a principal component analysis considering all the analyzed morphological and physiological variables for the five species. The following correlation between the first component, related to the leaf mass per unit of leaf area and the leaf tissue density, and g_m seem to suggest a key role of the leaf structural features in determining g_m variations across the five species.

Keywords Mesophyll conductance · Herbaceous species · Understory species · Functional group

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Introduction

Net photosynthetic rate is affect by several parameters, and among all, stomatal conductance is certainly the best characterized receiving the greatest attention over time (Kumar et al. 1999; Park and Furukawa 1999; Massacci et al. 2008). However, another important factor having a key role as photosynthetic parameter, but just more recently recognized, is the mesophyll conductance (Nascimento and Marenco 2013). Mesophyll conductance (g_m) is the ease with which carbon dioxide (CO_2) diffuses from intercellular airspace within a leaf to the sites of carboxylation within chloroplasts, and it constrain photosynthesis to about the same extent as stomatal conductance does (von Caemmerer and Evans 1991; Flexas et al. 2012).

Based on the currently knowledges (review by Flexas et al. 2008) the observed variations of g_m among different plant groups (i.e. evergreen gymnosperm, evergreen angiosperms, semi-deciduous angiosperms, deciduous angiosperms, herbaceous annual, perennial herbs, CAM plants) are mainly associated to the leaf forms and plant functional group rather than reflecting evolutionary trends. Taking as example the herbaceous group, in this case, the greater differentiation is found between the functional groups of annuals/biannuals versus perennials herbs, while no differences were found along the evolutionary differentiation of dicots versus monocots. Anyway, further the general trend based on the average data per groups, studies show significant variability within a single group, genus and species. In fact, in annual and biannual herbaceous species, the g_m range can go from 0.08 mol CO_2 m^{-2} s^{-1} bar^{-1} to g_m values over 1 mol CO_2 m^{-2} s^{-1} bar^{-1}. This last aspect, about the greater variability within group, genus and species, suggest that g_m is a parameter that can adapt very quickly and is therefore involved in the

differences in photosynthetic efficiency found between species. Besides this large variability among species, g_m shows a long-term response to different environmental factors (Flexas et al. 2008). Concerning the abiotic factors, as underlined by previous works, g_m is affect by water stress, low nitrogen availability and salinity (Jones 1973; Evans and Terashima 1988; Bongi and Loreto 1989). Moreover, g_m responses to change in light conditions. In fact, shade leaves show a lower g_m values compared to sun leaves (Hanba et al. 2002; Piel et al. 2002; Laisk et al. 2005; Warren et al. 2007; Catoni et al. 2015a), whilst a complex response in the acclimation to different growth temperature has been suggested (Yamori et al. 2006; Diaz-Espejo et al. 2007). Moreover, referring to internal factors, such as leaf development and ageing, they seem to strongly affect g_m (Flexas et al. 2008). On one hand, during leaf development (i.e. from unfolding to maturation) g_m increases in parallel with leaf photosynthesis (Miyazawa and Terashima 2001), while on the other hand, leaf ageing results in a g_m decreases (Bernacchi et al. 2005; Grassi and Magnani 2005; Catoni et al. 2015a). Finally, a relationship between g_m and leaf structural traits such as leaf mass per area (LMA) has been reported (De Lucia et al. 2003; Warren et al. 2003).

Based on these observations, g_m seems to have a long-term response to several environmental factors, and in turn, these changes are important in affecting photosynthesis response to environmental conditions. Nevertheless, others studies reported a stable g_m in response to changes in environmental variables such as CO_2 concentration, irradiance and temperature (von Caemmerer and Evans 1991; Tazoe et al. 2009; Nascimento and Marenco 2013).

The objective of this study was to analyze, on one hand the g_m long-term response to the variations of environmental conditions in five herbaceous understory species; and on the other hand, how g_m varies among these species belonging to different evolutionary groups (dicots and monocots) and functional groups (annual and perennial).

Materials and methods

Plant materials and study area

The study was carried out in the period June-October 2018 on five understory herbaceous species. In particular, three monocots: *Carex brizoides* Lam., *Carex pilosa* Scop., and *Oplismenus undulatifolius* P. Beauv and two dicots species: *Circaea lutetiana* L., and *Pulmonaria officinalis* Ced. were considered. All these represent the most important and representative species constituting the herbaceous layer of a typical alluvial lowland forest inside a Strict Natural Reserve "Bosco Siro Negri" (45°12′39″N; 09°03′26″E,

74 m a. s. l) in Italy. During the study period all the considered species were well representative and without any sign of senescence. Specifically, in the herbaceous layer of the considered forest *C. lutetiana* and *O. undulatifolius* are generally found from the middle of May to beginning of November (i.e. annual species), *C. pilosa* and *P. officinalis* are present throughout the year (i.e. perennial species), while *C. brizoides* seems to have a borderline behavior between annual and perennial herbaceous species, showing up later during the year compared to the two perennial species. For each species five plots (1 m^2 each) were randomly select and inside each plot three representative individual were identify. The structure of the forest is that of a typical closed canopy forest, with a forest tree density of 237 ± 100 stems ha^{-1}, a total basal area of 74.5 ± 24.6 m^2 ha^{-1} and a Leaf Area Index (LAI) of 4.5 ± 0.3 (Catoni et al. 2015a, b). Moreover, the high tree density determines a high light extinction at soil level, with a value of the relative intercept irradiance by the canopy at soil level inside the forest of $0.77 \pm 0.12\%$ and a value of the ratio between irradiance in the red and far red wavelengths (R/FR) of 0.5 ± 0.1 (data from Granata et al. 2016).

The soil is characterized by a pH of 5.46 ± 0.11, a nitrogen concentration (N) of 1.61 ± 0.12 mg g^{-1}, a carbon to nitrogen ratio (C/N) of 17 ± 1 and a soil organic matter (SOM) concentration of 44.47 ± 0.04 mg g^{-1} (Catoni et al. 2015a).

The climate of the area is characterized by a total annual rainfall of 627 mm most of it falling in autumn and winter. The mean minimum air temperature (T_{min}) of the coldest month (January) is 0.1 ± 1.5 °C, the mean maximum air temperature (T_{max}) of the hottest month (July) 30.2 ± 1.2 °C and the mean annual temperature (T_m) 13.8 ± 8.2 °C (Lombardia Regional Agency for Environmental Protection, Meteorological Station of Pavia, Ponte Ticino SS35, data for the period 2002–2017). Floods occurred sporadically every 5–10 years during the last 40 years, with water levels up to 1.50 m height in the forest during exceptional events (Castagneri et al. 2013; Motta et al. 2009). On average, groundwater level is around -4.50 m in winter reaching -3.50 m in summer due to irrigation in the surrounding area.

During the study period total rainfall was 256 mm, T_m 22.2 ± 3.9 °C and T_{max} (July and August) 30.7 ± 0.1 °C (Lombardia Regional Agency for Environmental Protection, Meteorological Station of Pavia, Ponte Ticino SS35, data for the period June–October 2018).

Leaf morphology and ambient conditions

Fully expanded leaves (for each species n = 2 leaves per 3 plant per plot) were collected in June. Leaf samples were

sealed in plastic bags and transported immediately to the laboratory. Measurements included leaf surface area (LA, cm^2), obtained by the image analysis system (Delta-T Devices, UK) and leaf dry mass (DM, mg), determined by drying leaves at 80 °C to constant mass.

Leaf mass per unit of leaf area (LMA, mg cm^{-2}) was calculated by the ratio of DM and LA and leaf tissue density (LTD, mg cm^{-3}) was calculated by the ratio of LMA and leaf thickness (LT, μm). This last was determined using digital calipers (precision of 0.01 mm) on the middle portion of the leaf blade and at two different points per leaf avoiding major veins.

During the study period, photosynthetic photon flux density (PPFD), air relative humidity (RH), air temperature (T_{air}) and atmospheric CO_2 concentration (CO_2, ppm) in the forest understory were recorded using specific sensors connected to data loggers.

Gas exchange

Gas exchange measurements were carried out in the period June–October 2018. Leaves (n = 5 leaves per each species) were retained in their natural position during measurements. Net photosynthetic rate [P_N, μmol(CO_2) m^{-2} s^{-1}], stomatal conductance [g_s, mol(H_2O) m^{-2} s^{-1}], leaf transpiration [E, mmol(H_2O) m^{-2} s^{-1}], photosynthetic photon flux density [PPFD, μmol(photons) m^{-2} s^{-1}] leaf temperature (T_l, °C), and substomatal CO_2 concentration (C_i, ppm) were measured by an infrared gas analyzer (LC-$Pro +$, ADC, UK) equipped with a leaf chamber (PLC, Parkinson Leaf Chamber, UK). On each sampling occasion, leaf respiration [R_D, μmol(CO_2) m^{-2} s^{-1}] was measured after P_N measurements (on the same leaves) as CO_2 efflux by darkening the leaf chamber with a black paper, according to Cai et al. (2005) for 30 min prior to each measurement, to avoid the release of CO_2 transient post-irradiation bursts (Atkin et al. 1998).

Chl fluorescence and mesophyll conductance

Chl fluorescence measurements were carried out by a portable modulated fluorometer (OS5p, Opti-Sciences, USA). Maximum PSII photochemical efficiency (F_v/F_m) was estimated by darkening leaves (n = 10 leaves per each species) for 20 min, then a saturating pulse was applied to measure initial (F_0) and maximum (F_m) fluorescence. F_v/F_m was estimated as:

$$(F_m - F_0)/F_m$$

Additional gas-exchange and Chl fluorescence measurements were made on leaves (n = 10 leaves per each species) comparable to those previously used in order to estimate mesophyll conductance [g_m, mol(CO_2) m^{-2} s^{-1} bar^{-1}].

The g_m was calculated according to Harley et al. (1992) by a single point method which combines gas-exchange and Chl a fluorescence measurements, as:

$$g_m = P_N/\{Ci - [\Gamma* \times [ETR + 8 \times (P_N + R_D)]]/[ETR - 4 \times (P_N + R_D)]\}$$

where $\Gamma*$ was the CO_2 compensation point under non respiratory conditions. The temperature dependency for $\Gamma*$ was calculated according to Bernacchi et al. (2002).

P_N, Ci, and R_D were obtained from gas-exchange measurements as described in the above section. ETR was the electron transport rate calculated from Chl fluorescence measurements, according to Krall and Edwards (1992) as:

$$ETR = \Phi_{PSII} \times PPFD \times 0.5 \times 0.84$$

where Φ_{PSII} was the actual PSII photochemical efficiency of light-adapted leaves calculated according to Genty et al. (1989) as:

$$(Fm' - Fs)/Fm'$$

Fm' was the maximum fluorescence obtained with a light-saturating pulse ($\sim$ 8000 μmol m^{-2} s^{-1} PPFD) and Fs was the steady-state fluorescence of illuminated leaves (1500 μmol m^{-2} s^{-1} PPFD). Gas-exchange and Chl fluorescence measurements were taken concurrently by fitting the portable infrared CO_2 gas analyzer with a fluorometer adapter chamber (F.LCI-FL, ADC, UK).

Data analysis

The obtained data [expressed as mean ± standard error (± SE))] were analyzed by the analysis of variance (ANOVA). As the same leaves were measure during the course of the study period, data were analyze using a repeated measures analysis of variance. Tukey's post hoc test ($p < 0.05$) was use to assess the significant differences among the species and between months. Linear regression analysis was carried out between g_m and the considered environmental variables (i.e. CO_2 concentration, PPFD and T_{air}). A principal component analysis (PCA) was carried out in order to summarize the considered morphological (LMA, LTD and LT) and physiological (P_N, R_D, g_m, g_s, F_v/F_M, Φ_{PSII}, ETR) leaf traits into major components which explained their variation between the five considered species. Then, a linear regression analysis between the axis explaining the largest proportion of the variance (i.e. PC1) and g_m was carried out.

Results

Leaf morphology and ambient conditions

At morphological level significant differences were found among the considered species (Table 1).

In particular LMA ranged from 1.55 ± 0.15 mg cm^{-2} (*O. undulatifolius*) to 5.85 ± 0.48 mg cm^{-2} (*C. brizoides*) while LTD ranged between 319.7 ± 13.8 mg cm^{-3} (*P. officinalis*) to 1024.1 ± 65.1 mg cm^{-3} (*C. brizoides*). *P. officinalis* showed the highest LT (110.0 ± 8.9 μm) and *O. undulatifolius* and *C. brizoides* the lowest (50.65 ± 0.01 μm). During the study period in the forest understory, mean monthly PPFD ranged between 51 ± 5 μmol m^{-2} s^{-1} in September to 122 ± 48 μmol m^{-2} s^{-1} in June and monthly understory T_{air} varied between 20.9 ± 0.5 °C in October to 28.4 ± 0.5 °C in July. The average value of CO_2 concentration during the study period was 449 ± 29 ppm.

Gas exchange

During the study period the highest P_N was measured in June for all the considered species, with *C. pilosa* having the highest value [4.33 ± 0.3 μmol(CO_2) m^{-2} s^{-1}] and *O. undulatifolius* the lowest [1.31 ± 0.06 μmol(CO_2) m^{-2} s^{-1}] (Fig. 1a). A steady trends of R_D has been observed (Fig. 1b) with higher rates in July [0.56 ± 0.05 μmol(CO_2) m^{-2} s^{-1}, mean value of the species] decreasing by 11% (mean value) in September–October. Stomatal conductance showed the same P_N trend, with the highest values in June for all the species [0.171 ± 0.063 mol(H_2O) m^{-2} s^{-1}, mean value] (Fig. 2).

Chl fluorescence and mesophyll conductance

Values of Φ_{PSII} and F_V/F_M are show in Fig. 3a and b. Quite constant trends were monitored during the study period for both the parameters and in all the considered species, with *C. pilosa* having the lowest Φ_{PSII} during the study period

(0.691 ± 0.023, mean value of the considered months) and *P. officinalis* the highest (0.742 ± 0.003, mean value). This last showed also the highest F_V/F_M (0.793 ± 0.003, mean value of the considered months) and *C. lutetiana* the lowest (0.765 ± 0.002, mean value).

During the study period (Fig. 4) the highest g_m value was monitored in July [0.015 ± 0.011 mol(CO_2) m^{-2} s^{-1} bar^{-1}, mean value], with *P. officinalis* having the highest value. The results of the linear regression showed that none of the considered environmental variables seems to affect g_m variations during the study period (Table 2).

Principal component analysis

The PCA highlighted that the first two components accounted for 99.9% of the total variance, with the first component explained the 99.4% of the total variance and it was positively related to LMA ($r = 0.805$) and LTD ($r = 0.999$). The second component explained 0.62% of the total variance was only related to LT ($r = 0.891$). Three different groups can be observed (Fig. 5): one group was represented by *C. pilosa* and *P. officinalis*, and the second group by *O. undulatifolius* and *C. lutetiana*, while *C. brizoides* was placed far from the other two groups. Along the first component *C. brizoides* showed the highest value and the *P. officinalis*, *C. lutetiana* and *O. undulatifolius* the lowest one. There was a significant linear regression between PC1 and g_m ($R^2 = 0.24$, $p < 0.01$) (Fig. 6).

Discussion

On the whole, during the analyzed months, in which all the species are well evident and representative and without any sign of senescence, the lowest g_m rates are observed in June [0.012 ± 0.007 mol(CO_2) m^{-2} s^{-1} bar^{-1}, mean value of the considered species] and the highest ones in July [0.015 ± 0.011 mol(CO_2) m^{-2} s^{-1} bar^{-1}, mean value]. Overall, the average g_m value for the five species over the entire study period [0.014 ± 0.008 mol(CO_2) m^{-2} s^{-1}

Table 1 Morphological and anatomical leaf traits of the considered species

	LMA (mg cm^{-2})	LTD mg (cm^{-3})	L (μm)
Carex brizoides	5.85 ± 0.48a	1024.1 ± 65.1a	50.6 ± 3.0a
Carex pilosa	4.84 ± 0.38a	406.3 ± 16.0b	94.4 ± 4.4b
Circaea lutetiana	2.01 ± 0.06b	327.0 ± 29.5b	63.3 ± 3.9a
Oplismenus undulatifolis	1.55 ± 0.15b	324.1 ± 16.9b	50.7 ± 4.8a
Pulmonaria officinalis	3.18 ± 0.06c	319.7 ± 13.8b	110.0 ± 8.9b

LMA leaf mass per unit of leaf area, *LTD* leaf tissue density, *L* leaf thickness

Mean values ($\pm$ SE) are shown (n = 35 leaves). Different letters indicate significant differences among the species (Tukey-test, $p < 0.05$)

Fig. 1 Trend of **a** net photosynthetic rates (P_N) and **b** dark respiration rates (R_D) during the study period in the five considered species. Mean values ($\pm$ SE) are shown (n = 5)
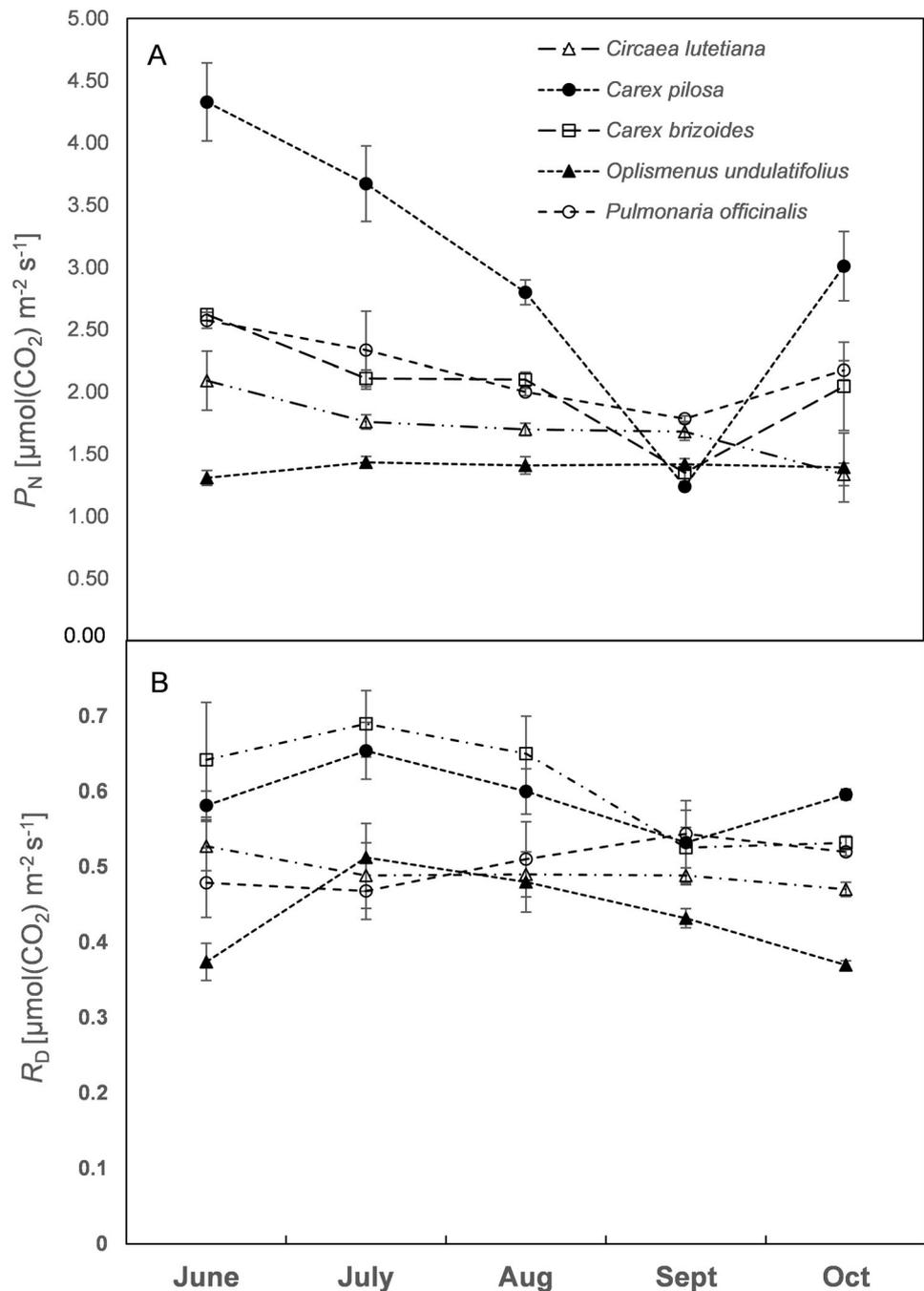
bar^{-1}] results lower compared to an expected value for herbaceous species (see Flexas et al. 2008). Nevertheless, taking into account the typical shade environment of the forest understory in which they grow, this value may not be surprising considering that normally shade leaves have lower g_m values than leaves in high-light environments. In fact, understory species might behave similarly to shade-leaves, which appear to have stronger limitations from g_m (Tosens et al. 2012; Cano et al. 2013). As suggested by Laisk et al. (2005) the higher mesophyll diffusion resistance (i.e. lower g_m) can reflects the smaller air-exposed mesophyll area in the thinner shade leaves. Moreover, according to the results of Taylor and Pearcy (1976) for understory species, the observed g_m rates are substantially lower compare to g_s. Another typical behavior observed in the understory environment is the photosynthetic response that generally parallels the change in the light environment induced by overstory canopy closure. In fact, the highest P_N rates are found, in all these species, in June prior to the complete canopy closure, with a further

Fig. 2 Trend of stomatal conductance (g_s) during the study period in the five considered species. Mean values ($\pm$ SE) are shown (n = 5)
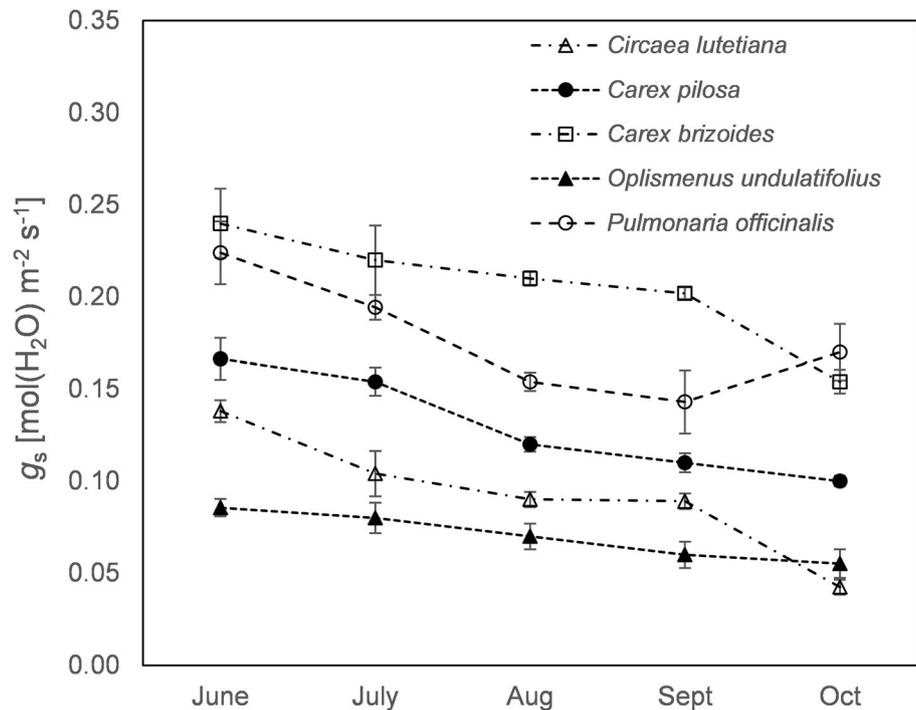

increase observed in October in *C. pilosa, P. officinalis* and *C. brizoides* when approaching overstorey canopy senesces, while the annual species (*C. lutetiana* and *O. undulatifolius*) show a more steady P_N trend during the study period, without the further increase in autumn. Therefore, the analyzed understory herbaceous species differ in their photosynthetic characteristics in way that are consistent with differences in their above-ground phenologies according to the results of Rothstein and Zak (2001).

Concerning the first question analyzed in this study and related to the g_m response to environmental conditions, we did not find any significant relationship between g_m and the considered environmental variables (i.e. atmospheric CO_2 concentration, T_{air} and PPFD). In our study, the absence of these correlations can be related to the fact that the environmental changes occurring in the forest understory are not large enough to determine g_m variations according to the results of Nascimento and Marenco (2013). This is because the forest understory experiences damped environmental fluctuations, due to shelter by the overstory tree canopy (Fotelli et al. 2003), that, in this case is characterized by a tree layer constituted by *Quercus robur* L., *Robinia pseudoacacia* L., *Populus nigra* L. and *Populus alba* L., and by an underlying layers with the presence of the species of the dominant tree layer and by *Corylus avellana* L., *Acer campestre* L., *Prunus padus* L. and *Crataegus monogyna* Jacq. (Granata et al. 2019). Moreover, it is worth noting that, the g_s values in

the forest tree layer (i.e. high-light level), during the more favorable environmental condition, ranges from 0.263 ± 0.017 mol(H_2O) m^{-2} s^{-1} in *P. alba* and *P. nigra* (mean value), followed by *R. pseudoacacia* [0.221 ± 0.005 mol(H_2O) m^{-2} s^{-1}] and by *Q. robur* [0.196 ± 0.009 mol(H_2O) m^{-2} s^{-1}]. In the underlying layers (i.e. lower light level), the g_s value varies from 0.195 ± 0.006 mol(H_2O) m^{-2} s^{-1} in *R. pseudoacacia*, followed by *A. campestre* [0.170 ± 0.005 mol(H_2O) m^{-2} s^{-1}], *C. avellana* [0.160 ± 0.003 mol(H_2O) m^{-2} s^{-1}], *C. monogyna* [0.154 ± 0.006 mol(H_2O) m^{-2} s^{-1}], *P. padus* [0.117 ± 0.006 mol(H_2O) m^{-2} s^{-1}], and by *Q. robur*, *P. alba* and *P. nigra* [0.101 ± 0.006 mol(H_2O) m^{-2} s^{-1}, mean value] (data not published). These results allow us to highlight a decrease in g_s rates between the same species present in different light conditions (tree layer vs underlying tree layer).

To understand the similarity between the species, based on the analyzed morphological and physiological variables, we carried out a Principal Component Analysis, which identifies three groups for the five considered species: one group is characterized by *C. lutetiana* and *O. undulatifolius,* the other by *C. pilosa* and *P. officinalis*, while *C. brizoidez* is positioning far from the others species. In particular, the first component (i.e. PC1) which explains the major variation among the species is significantly correlate to the leaf morphological traits (i.e. LMA and LTD). Thus, we can conclude that the similarity and then the association between these species is mainly drive

Fig. 3 Trend of **a** the actual PSII photochemical efficiency (Φ_{PSII}) and **b** the maximal quantum efficiency of PSII (F_v/F_m) during the study period in the five considered species. Mean values ($\pm$ SE) are shown (n = 10)
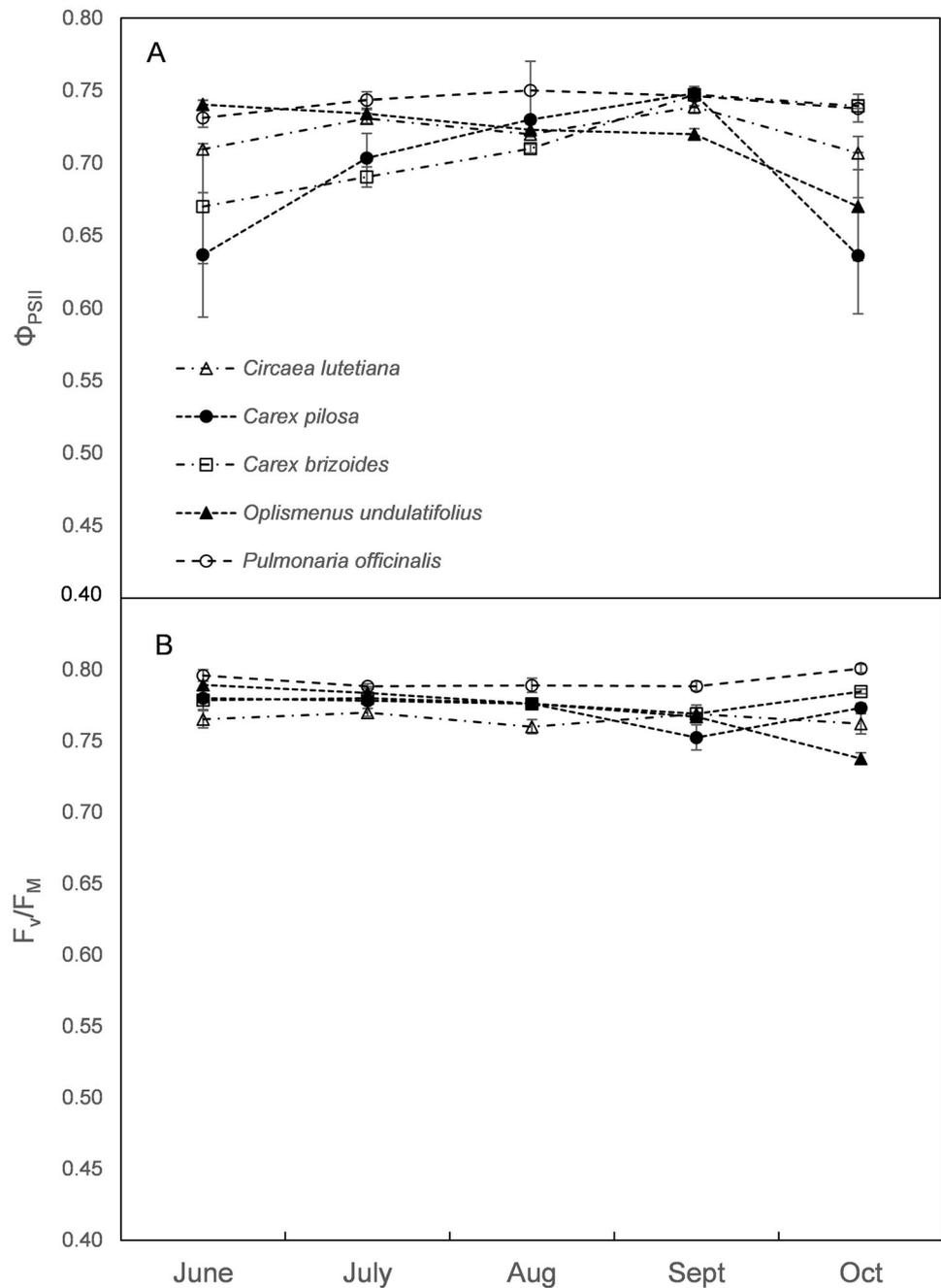

by the morphological leaf traits. In particular, among the considered species, *C. brizoides* is characterize by the highest values of both LMA and LTD (5.85 ± 0.48 mg cm^{-2} and 1024.1 ± 65.1 mg cm^{-3}, respectively), while *C. lutetiana* and *O. undulatifolius* have the lowest LMA (1.78 ± 0.32 mg cm^{-2}, mean value). The others two species have a 31% lower LMA compared to *C. brizoides,* but due to a highest LT, they have a 65% lower LTD (406.3 ± 16.0 mg cm^{-3} in *C. pilosa* and 319.7 ± 13.8 mg cm^{-3} in *P. officinalis*).

Furthermore, the significant relationship between the PC1 and g_m highlights that these morphological leaf traits are good predictors for the g_m variations across the considered species. In fact, leaf structural features are believe to play a central role in determining g_m (Hassiotou et al. 2009).

Thus, if we can suppose that these associations are driven by leaf structural traits and we found an association between one dicot and one monocot (i.e. *C. lutetiana* and *O. undulatifolius* in one group and *C. pilosa* and *P. officinalis* in the other group) and in turn these

Fig. 4 Trend of mesophyll conductance (g_m) during the study period in the five considered species. Mean values ($\pm$ SE) are shown (n = 5)
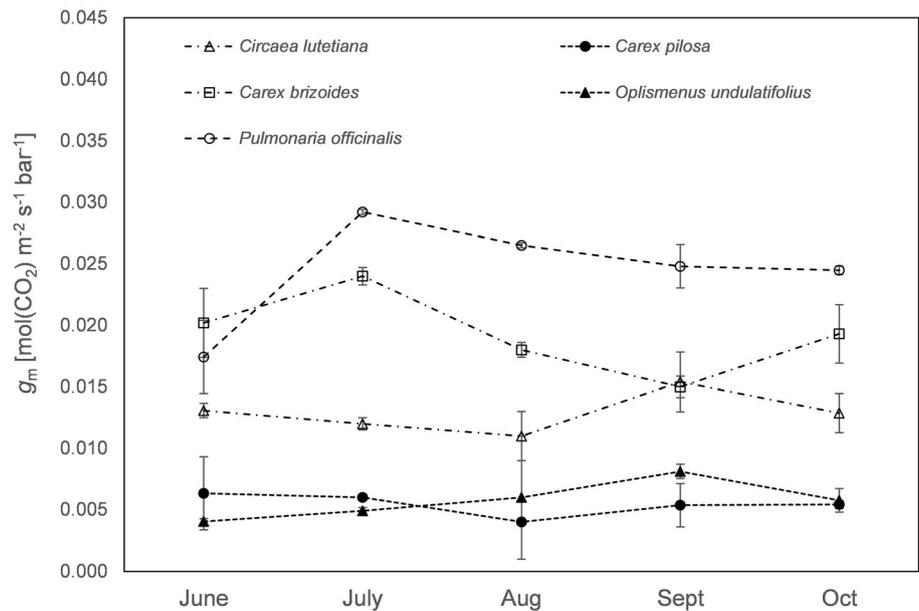

Table 2 Regression analysis between mesophyll conductance (g_m) and air temperature (T_{air}), between g_m and photosynthetic photon flux density (PPFD) and between g_m and atmospheric CO_2 concentration [CO_2]. Regression analysis equation determination coefficient (R^2) are shown

	Regression equation	R^2
g_m versus T_{air}	y = 0.004x + 0.0037	0.00208
g_m versus PPFD	y = $- 1 e^{-05x}$ + 0.0141	0.0148
g_m versus [CO_2]	y = $- 9 e^{-06x}$ + 0.0171	0.0011

morphological leaf traits are the best predictors of g_m variation in these species, we can conclude that the g_m variations are more linked to the functional group rather than reflecting an evolutionary trend. In fact, at the level of the functional groups, *C. lutetiana* and *O. undulatifolius* can be considered as annual species, while *C. pilosa* and *P. officinalis* are perennial species and *C. brizoides* seem to have an intermediate behavior between the two groups.

Fig. 5 Principal component analysis (PCA) carried out using morphological (LMA, LTD and LT) and physiological (P_N, R_D, g_m, g_s, F_v/F_M, Φ_{PSII}, ETR) leaf traits for the five considered species. The 1st component, accounting for 99.4% of the total variance, was positively related to LMA and LTD. The 2nd component explained 0.62% of the total variance and it was correlated to LT
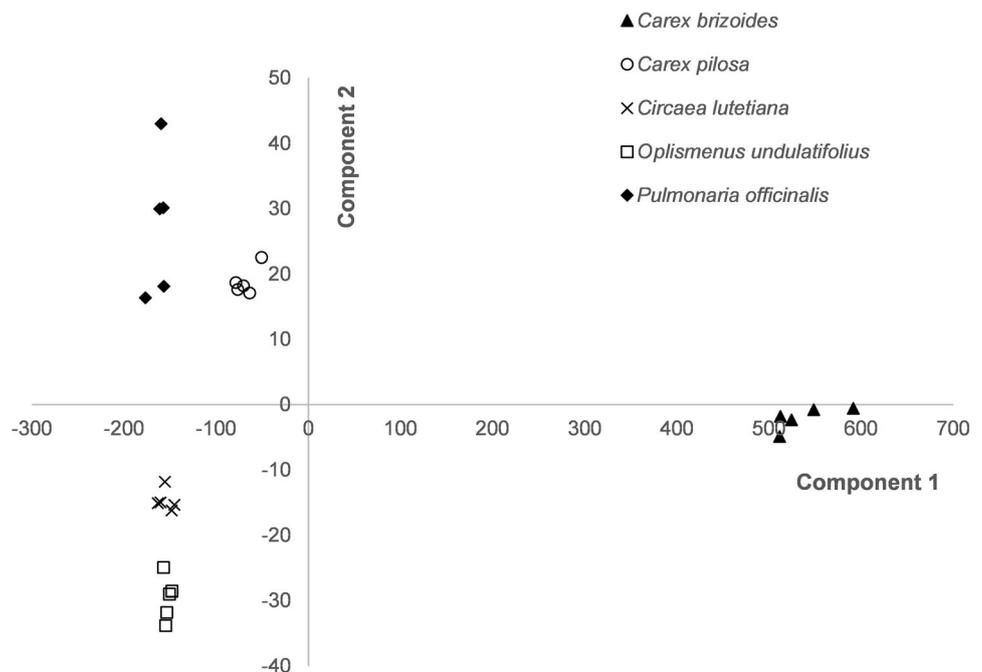
Fig. 6 Linear relationship between the first principal component combining LMA and LTD (PC1) and the mesophyll conductance [g_m, mol(CO_2) m^{-2} s^{-1} bar^{-1}] across the five considered species. The equations as well as their R^2 are shown. (**$p \leq 0.01$)
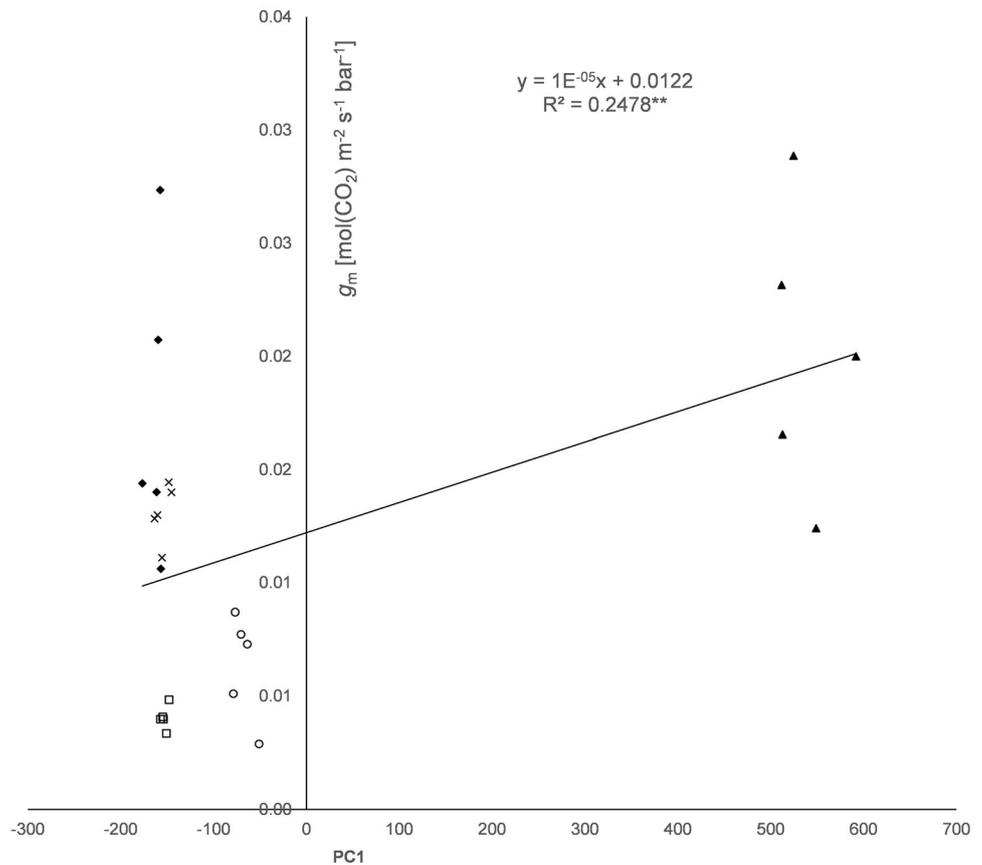
$$y = 1E^{-05}x + 0.0122$$
$$R^2 = 0.2478**$$

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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