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CHLOROPHYLL *a* FLUORESCENCE ANALYSIS IN FORESTS

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ABSTRACT – A European-wide assessment of chlorophyll *a* fluorescence (ChlF, prompt fluorescence on dark-adapted samples) parameters in forest ecosystems was carried out in the years 2012-2013, within the 7FP FunDivEUROPE project. A total of 1596 trees growing in 209 stands distributed in six countries, from Mediterranean to boreal sites, were sampled. This paper shows the applicability of the ChlF in forest ecology surveys, the protocols adopted for leaf sampling and ChlF measurements, the variability of the ChlF parameters within and between trees, their dependence to environmental factors and the relationships with other functional leaf traits. The most relevant findings were as follows: (i) the least variable ChlF parameter within and between the trees was the maximum quantum yield of primary photochemistry (F_V/F_M), whereas the performance indices (PI_{ABS} and PI_{TOT}) showed the highest variability; (ii) for a given tree, the ChlF parameters measured at two heights of the crown (top and bottom leaves) were correlated among the and, in coniferous species, the ChlF parameters were correlated between different needle age classes (the current year and previous year); (iii) the ChlF parameters showed a geographical pattern, and the photochemical performance of the forest trees was higher in central Europe than in the edge sites (northernmost and southernmost); and (iv) ChlF parameters showed different sensitivity to specific environmental factors: F_V/F_M increased with the increase of the leaf area index of stands and soil fertility; ΔV_{IP} was enhanced under high temperature and drought. The photochemical responses of forest tree species, analyzed with ChlF parameters, were influenced by the ecology of the trees (i.e. their functional groups, continental distribution, successional status, etc.), and by the tree species' richness and composition of the stands. Our results support the applicability and usefulness of the ChlF in forest monitoring investigations on a large spatial scale and its possibility of being integrated with remote sensing surveys.

KEYWORDS: CHLOROPHYLL FLUORESCENCE, ECOLOGICAL FACTORS, FORESTS, FOREST MONITORING, TREE CROWN, FUNCTIONAL LEAF TRAITS

INTRODUCTION

Chlorophyll *a* fluorescence (ChlF) techniques and parameters are extensively used in plant physiology researches, both to study the mechanism of light harvesting and electron transport between and beyond the two photosystems (photosystem II, PSII and photosystem I, PSI) and in applied studies to assess the mechanisms and effects of biotic and abiotic stresses on the photosyntetic efficiency and performances of plants (Papageorgiou and Govindjee, 2004). Furthermore, ChlF is a useful tool for experimental research in agriculture, forestry and arboriculture (for reviews, see Ball et al., 1994; Maxwell and Johnson, 2000; Mohammed et al., 2003; Bussotti et al., 2010; Kalaji et al., 2014). The application of ChlF in forest monitoring to assess the ecological conditions and productivity of forests is more problematic than application in agriculture, and it mainly concerns the use of remote sensing techniques, based on the passive fluorescence (Grace et al., 2007; Meroni et al., 2009; Joiner et al., 2011, 2014; Garbulsky et al., 2014; Porcar-Castell et al., 2014; Zhang et al., 2014).

The photosynthetic properties of "tall" crowns, assessed by

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means of ChlF fluorescence and analyzed using the JIP-test, were revealed to be sensitive to the growth conditions of the trees, such as stand density and leaf area index (Pollastrini et al., 2016), above-ground competition between trees due to different growth rates (Pollastrini et al., 2014) and tree species diversity (both species' richness and composition, Bussotti and Pollastrini, 2015a; Pollastrini et al., 2015; Pollastrini et al., *submitted*). ChlF parameters, moreover, can describe the health status of trees and their physiological reactions to defoliation, although with species-specific behaviors (Pollastrini et al., 2016; Gottardini et al., 2016), as well as the more general processes of acclimation to varying environmental conditions (Pollastrini et al., *submitted*; Gottardini et al., 2016). The simultaneous analysis of the ChlF parameters and other functional leaf traits on the same foliar sample is a tool for a comprehensive assessment of the overall conditions of the crowns (Bussotti and Pollastrini, 2015b).

In field experiments and surveys in forests, the measurement of ChlF parameters on leaves is not easy, due to the difficulty of reaching and collecting the leaves at the canopy height (e.g. in mature forests, trees can reach a height of 20 m or beyond). Available data concerning the ChlF characteristics of tall trees come mostly from research carried out in experimental areas equipped for long-term assessment (Gielen et al., 2007; Mänd et al., 2012; Hallik et al., 2012) or as validation of remote sensing surveys (Rossini et al., 2006; Pieruschka et al., 2014). Specific researches on tall trees in forests were carried out by Bussotti (2004) (diurnal and seasonal variations of photosynthetic efficiency in *Quercus ilex* L.), Koprowski et al. (2015) (fertilization experiments on tall *Pinus sylvestris* L. trees), Fusaro et al. (2015) different ecophysiological behavior of *Quercus ilex* L. in urban and periurban forests and Gottardini et al. (2016) (assessment of the responses of *Picea abies* (L.) Karst. along an altitudinal gradient).

Besides the difficulty of sampling leaves, the assessment of ChlF in forest trees proposes specific challenges, such as (i) the heterogeneity of the photosynthetic properties in leaves living in different positions in the crown and, consequently, exposed to different light regimes (Niinemets, 2007; Yoshimura, 2010; Desotgiu et al., 2012a), namely the distinction between sun and shade leaves; (ii) the influence of the hour of the day when the leaves are collected, before the ChlF measurement, (Desotgiu et al., 2012b, 2013), that determines the state of photoinhibition, or the lack of it, of the leaves (Werner et al., 2002); (iii) the comparability of data coming from different instruments (Bussotti et al., 2011); and (iv) the variability of ChlF parameters within and among trees.

This paper reports the experience carried out within the FunDivEurope project (7FP), where a comprehensive foliar sampling and analysis was carried out in six European forests, from Mediterranean to boreal. Our purpose was to provide an overview of the applicability, usefulness and difficulties encountered in a terrestrial assessment of the ChlF properties of tree species, suggesting solutions to enhance the effectiveness of ChlF surveys in forests and their comparability. Moreover, we want to address the variability of the ChlF parameters within and between trees, their dependence on environmental factors and their relationships with other functional leaf traits.

Materials and Methods

The prompt fluorescence (PF) and the JIP-test

The prompt fluorescence (PF) refers to the fluorescence induction curve from the minimum fluorescence intensity (F_0) to the maximum fluorescence (F_M) in dark-adapted samples. This curve, called "fluorescence transient" (Strasser et al., 2000, 2004), represents the "fast induction kinetics" of the fluorescence emission. Plotted on a logarithmic time scale, the fluorescence transients show a polyphasic shape. In a dark-adapted sample, the fluorescence began to be measured at the time of 20 μs (or 50 μs, depending on the temporal resolution of the fluorimeter). This time step is the first level of the ChlF emission and is indicated as O. Then, three intermediate levels of fluorescence emission, indicated, respectively, as K (at 300 μs), J (~2 to 3 ms) and I (~30 ms), and then the last level of fluorescence emission, at 500-800 ms - 1s, indicated as P (peak level of fluorescence), are achieved. The latter indicates the maximum fluorescence intensity (F_M) when saturating light is procured on the leaf. The fluorescence O-J-I-P transient is analyzed using the JIP-test (Strasser et al., 2000, 2004; Tsimilli-Michael and Strasser, 2008), a methodology to study the structure and functions of the photosystem II, through the translation of the shape changes of the O-J-I-P-induced transient to quantitative changes of several parameters. The JIP-test parameters link the different steps and phases of the PF transient with the redox states of the PSII, describing the efficiency of the electron transfer in the intersystem chain to the end-electron acceptors at the PSI acceptor side. The JIP-test defines the maximal (subscript "0") energy fluxes in the energy cascade for the events absorption (ABS), trapping (TR_0), electron transport (ET_0), dissipation (DI_0), reduction of end acceptors of PSI (RE_0) in the PSII.

The most common parameter used in the prompt fluorescence is the maximum quantum yield of primary photochemistry ($\varphi_{Po} = TR_0/ABS = [F_M-F_0]/F_M = F_V/F_M$, Paillottin, 1976). This parameter is defined as the ratio of the total energy flux trapped by the reaction centers of PSII.

It can be expressed also by means of a de-excitation constants ratio [$F_V/F_M = k_P/(/k_P+k_N)$] (Strasser et al., 2000). Other parameters applied in this study for the ChlF transient analysis are as follows: the number of the active reaction centres per total chlorophyll content in the antennae of the PSII (RC/ABS); the probability of an electron to reduce the primary quinone acceptor and to move into the electron transport chain beyond the PSII ($\Psi_{Eo} = ET_0/TR_0$); the amplitude of the relative variable fluorescence of the I-to-P rise (ΔV_{IP} = relative contribution of the I-to-P- phase to the OJIP transient), which is a semi-quantitative indicator of the abundance of PSI with respect to PSII and is related to the electron transport chains beyond PSI (Ceppi et al., 2012). Finally, the performance indices (PIs) measure the potential energy conservation of photons in the intersystem between PSII and PSI (PI_{ABS}) and the potential energy conservation from photons absorbed by PSII to the reduction flux of PSI end-acceptors (PI_{TOT}). The explication and the formulae of the ChlF transients and JIP-test applied in this research are shown in Table 1.

Table 1. Chlorophyll *a* fluorescence parameters used in the fluorescence transient analysis.

Technical fluorescence parameters	
F_t	Fluorescence emission from a dark-adapted leaf at the time t
F_0	Minimal fluorescence from a dark-adapted leaf
F_M	Maximum fluorescence from a dark-adapted leaf
F_J	Fluorescence intensity at the J-step (at 2 ms)
F_I	Fluorescence intensity at the I-step (at 30 ms)
F_V	Maximum variable fluorescence from a dark-adapted leaf. $F_V = F_M - F_0$
V_J	Relative variable fluorescence at 2 ms. $V_J = (F_{2ms} - F_0) / (F_M - F_0)$
V_I	Relative variable fluorescence at 30 ms. $V_I = (F_{30ms} - F_0) / (F_M - F_0)$
M_0	Slope of the curve at the origin of the fluorescence rise. It is a measure of the rate of the primary photochemistry. $M_0 = 4(F_{300\mu s} - F_0) / (F_M - F_0)$
Derived parameters	
$\varphi_{Po} = TR_0/ABS = [F_M - F_0] /F_M = F_V/F_M$	Trapping probability, or maximum quantum yield of primary photochemistry of a dark-adapted leaf. It is the probability that an absorbed photon will be trapped by the PSII reaction centre.
$\Psi_{Eo} = ET_0/TR_0$	Probability that a photon trapped by the PSII reaction centre enters in the electron transport chain. $\Psi_{Eo} = 1 - V_J$
$\delta_{Ro} = RE_0/ET_0$	Probability that an electron is transported from the reduced plastochinone (PQ) to the electron acceptor side of PSI. $\delta_{Ro} = (1 - V_I) / (1 - V_J) = (F_M - F_I) / (F_M - F_J)$
ΔV_{IP} = IP-phase	The amplitude of the relative variable fluorescence of the I-to-P-rise (= relative contribution of the I-to-P phase to the OJIP-transient). $\Delta V_{IP} = 1 - V_I$ = IP phase
RC/ABS	Number of active RCs for chlorophyll molecule constituting the antenna. $RC/ABS = \varphi_{Po} (V_J/M_0)$
PI_{ABS}	Performance Index (potential) for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors. $PI_{ABS} = (RC/ABS) [\varphi_{Po} / (1 - \varphi_{Po})] [\Psi_{Eo} /(1 - \Psi_{Eo})]$
PI_{TOT}	Performance index (potential) for energy conservation from the photons absorbed by PSII to the reduction of PSI end-electron acceptors. $PI_{TOT} = PI_{ABS} [\delta_{Ro} / (1 - \delta_{Ro})]$

Study sites and sampling procedures

This survey was carried out within the exploratory platform of the FunDivEUROPE project, whose general description was reported by Baeten et al. (2013) and Jucker et al. (2014). The project investigated six of the most important forest types in Europe, from boreal to Mediterranean regions: North Karelia (Finland); Białowieża (Poland); Hainich (Germany); Râşca (Romania); Colline Metallifere (Italy); and Alto Tajo (Spain). The general ecological features of the forests and the tree species compositions of the stands are reported in Table 2. Moreover, we used in this study some data coming from the

Table 2. Main features of the six study sites belonging to the explorative platform of the FunDivEUROPE project (Baeten et al., 2013, modified). MAT = Mean Annual Temperature; MAP = Mean Annual Precipitation; Martonne Aridity Index: (annual precipitation / (mean annual temperature + 10)); GSR = Global Solar Radiation, daily averaged for the period 2009-2013 (April – September), data from CGMS database of interpolated meteorological data (AGRI4CAST, http://mars.jrc.ec.europa.eu/mars). (*) data of Leaf Area Index and mean basal area refer only to monocultures (Co= coniferous species; TB= temperate broadleaf species; MO= Mediterranean oaks).

Forest name **Country**	**North Karelia** **Finland**	**Bialowieza** **Poland**	**Hainich** **Germany**	**Râsca** **Romania**	**Colline Metallifere** **Italy**	**Alto Tajo** **Spain**
Latitude-Longitude	62.60° - 29.83°	52.72° - 23.95°	51.10° - 10.51°	47.32° - 26.03°	43.27° - 11.26°	40.77° - 1.95°
Altitude (m asl)	100-150	35-185	500-600	600-1000	250-550	960-1400
Main forest type	Boreal	Hemiboreal	Beech	Mountainous beech	Thermophilous deciduous	Continental - Mediterranean
Focal Tree Species	*Picea abies* *Pinus sylvestris* *Betula pendula*	*Picea abies* *Pinus sylvestris* *Betula pendula* *Carpinus betulus* *Quercus robur*	*Picea abies* *Fagus sylvatica* *Quercus* sp. *Fraxinus excelsior* *A. pseudoplatanus*	*Picea abies* *Abies alba* *Fagus sylvatica* *A. pseudoplatanus*	*Castanea sativa* *Ostrya carpinifolia* *Quercus cerris* *Quercus ilex* *Quercus petraea*	*Pinus sylvestris* *Pinus nigra* *Quercus ilex* *Quercus faginea*
Leaf Area Index (*) (m^2 m^{-2})	2.775 (Co) 2.338 (TB)	6.400 (Co) 4.440 (TB)	4.840 (Co) 6.813 (TB)	5.950 (Co) 5.703 (TB)	3.380 (TB) 4.535 (MO)	2.102 (Co) 1.142 (MO)
Mean Basal Area (*) (m^2 ha^{-1})	24.74 (Co) 15.64 (TB)	43.46 (Co) 31.12 (TB)	34.97 (Co) 34.74 (TB)	59.49 (Co) 36.47 (TB)	28.48 (TB) 27.50 (MO)	35.89 (Co) 16.37 (MO)
GSR Apr.-Sept. (kJ m^{-2} d^{-1})	14,907	16,634	16,018	17,917	21,022	23,428
MAT (°C)	2.1	6.9	6.8	6.8	13.5	10.2
MAP (mm)	629	627	775	800	850	499
Martonne Aridity Index (mm $°C^{-1}$)	62.5	52.7	51	47.2	43.2	40.77
Topography	Flat	Flat	Flat	Medium to steep slopes	Medium to steep slopes	Flat-medium slopes
Soil N concentration (mg g^{-1})	14.48 (Co) 6.85 (TB)	14.44 (Co) 13.39 (TB)	12.53 (Co) 13.75 (TB)	12.96 (Co) 13.63 (TB)	10.50 (TB) 10.19 MO)	9.00 (Co) 10.28 (MO)
Soil C/N	29.29 (Co) 22.71 (TB)	26.07 (Co) 25.61 (TB)	28.51 (Co) 27.53 (TB)	30.21 (Co) 30.08 (TB)	25.61 (TB) 35.27 (Mo)	49.41 (Co) 34.15 (Mo)
Soil depth (cm)	80	80	50	80	60	40

experimental platform of the FunDivEurope project (the experimental site at Satakunta in Finland, Pollastrini et al., 2014). In each country, 28 to 42 forest stands (30x30 m wide) with different levels of tree species richness were selected (from monospecific to five tree species). In each forest stand, between six and 15 dominant trees for each species were selected. Six trees were selected in monospecific stands, and three trees per species were selected in the plots with the other species richness levels. The trees were randomly selected among those with the largest diameter at breast height. In total, 1596 trees growing in 209 stands were sampled.

The leaf sampling was carried out by means of tree climbers, extension loppers and gun shooters according to the height of the trees, the stand structure and the operational conditions in each country. Branches 40-50 cm long with attached leaves were sampled in the highest southern exposed part of the crown, in the upper portion (top leaves, fully exposed to sun) and at a lower part of the crown (bottom leaves). The cut branches were immediately placed in hermetic plastic bags and humidified to avoid dehydration. The bags with the samples were then kept at a constant temperature in an insulated box.

The form of sampling from the ground was applied in Spain

and Finland. This method was the easiest, fastest and cheapest one. With extension loppers (telescopic poles equipped with scissors), it was possible to reach a height of 4-5 (8) m up to 14-18 m. Tree-climbing was applied in stands with tall trees, as in Italy, Germany and Poland (mean height of trees of 20-22 m). This sampling was expensive and time-consuming. A tree climber can climb and sample from 8 to 12 plants per day, depending on the size and shape of the stem and crown architecture and the site characteristics (e.g. soil slope, the presence of shrubs and understory vegetation that can make it difficult to reach the trees). Finally, the branches were collected by shooting, with a shotgun, in Romania. This method to collect leaves was rapid and relatively easy, but it can be dangerous for human activities in forests.

In 2012, we sampled the forest stands in Italy, Germany and Finland; in 2013, the leaves were collected in Spain, Romania and Poland. The samplig period was between mid June and mid August each year. Only mature, well-developed leaves were sampled and measured. In the presence of mature leaves and before the onset of the leaf senescence, we can assume that the photosynthetic properties of the leaves were relatively constant for the same tree species (excluding specific conditions such as severe summer drought or attacks of pathogen, Holland et al., 2014). As an example, in Fig. 1, we showed the ChlF transients of mature and immature leaves of *Quercus faginea* Lam. sampled in Spain in June 2013, to check the leaf development and to avoid including ChlF measurements from immature leaves in this study.

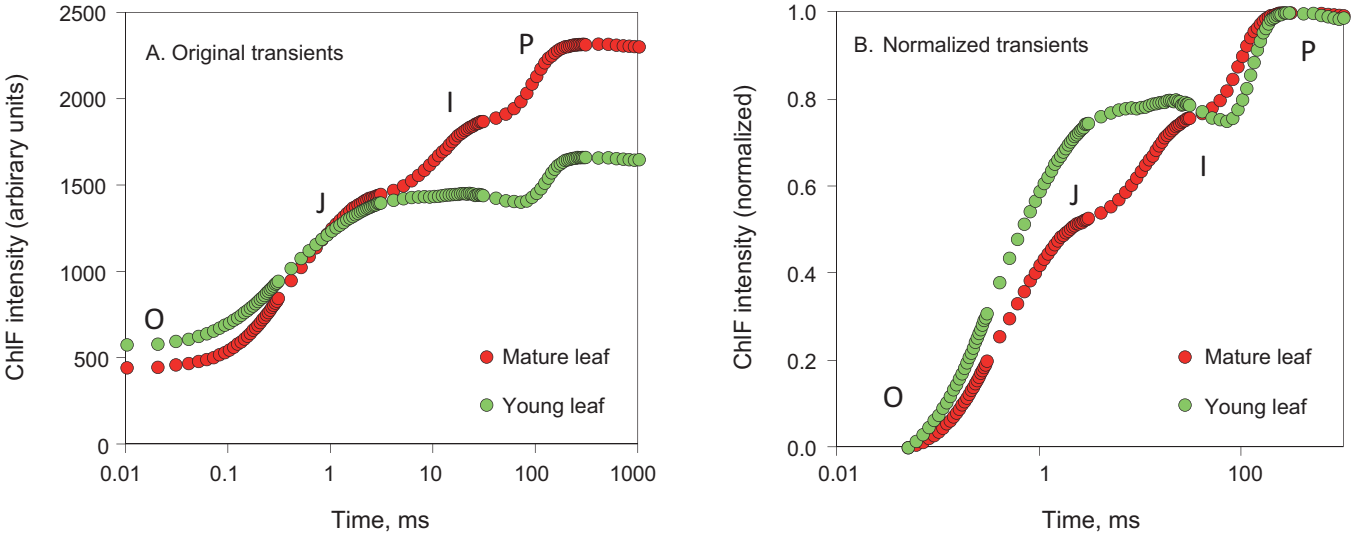

Fig. 1. Example of chlorophyll *a* fluorescence transients of mature and immature leaves of *Quercus faginea* collected in Spain.

Chlorophyll a fluorescence measurements

Among ChlF techniques, the prompt fluorescence and the analysis of the fluorescence transient by means of the JIP-test are the most efficient for screening purposes, since PF combines speed of execution with manoeuvrability of the instrument in field conditions. Measurements were done using a HandyPEA fluorimeter (Hansatech Instruments Ltd., Petney, Norfolk, UK). The fluorescence rise O-J-I-P curves was induced by 1-s pulses of red light (wavelength of 650 nm, intensity of 3500 μmol m^{-2} s^{-1}).

When it is necessary to use more than one fluorimeter, systematic errors can be introduced. The intensity of the light flash is the most important source of differences between instruments. Beside the adoption of common settings, it is recommended to calibrate the instruments by means of crossed exercises, consisting of the measurement of common samples. In this way the instrumental differences can be traced, and it is possible to apply correction factors to the dataset (Bussotti et al., 2011). Our experience suggests that F_V/F_M is very robust and comparable among different instruments and illumination conditions, as along with the parameters calculated on the normalized ChlF transients (V_I and V_J). The main problems of comparability, between two or more fluorimeters, concern the absolute values of the parameters (namely the extreme points of the transient F_0 and F_M) and the initial slope of the ChlF transient (M_0). In particular, this latter parameter affects the calculation of the antenna size (ABS/RC) and its reciprocal RC/ABS (that is, the number of the reaction centres of PSII for the total

amount of chlorophyll), as well as the performance indices. Special care must be taken when these parameters are used for the comparison of results from different instruments.

The time of the day when the leaf sampling is performed is an important variable to take into account when comparing ChlF data between different studies. In field conditions, the values of many fluorescence parameters vary according to the time of the day, due to the duration of the sunlight exposure of leaves (Desotgiu et al., 2012b). The exposure to strong light intensity can trigger the photoinhibition of the photosynthetic apparatus, which reduces its capacity to convert the solar energy into electron transport (Takahashi and Murata, 2008). The dark-adaptation of leaves prior to ChlF measurements is performed using leaf clips, for a time period of 20–30 minutes, which eliminates the dynamic photoinhibition of leaves, but not their potential chronic photoinhibition (Werner et al., 2002). Dark-adaptation of the samples for longer periods (at least four-five hours) is necessary to effectively reduce most components of the photoinhibition of the leaves, thus reducing the margin of error in the comparison between the ChlF properties of leaves. Only under extreme winter conditions (e.g. winter-acclimated boreal conifers or frost-acclimated Mediterranean evergreen broadleaf trees/shrubs), photoinhibitory processes may continue even after such a long darkening period.

Results and Discussion

Variability of ChlF parameters

The frequency of the distribution of the values of the selected ChlF parameters, for all species and countries together, is shown in Fig. 2. Many of the ChlF parameters show a non-normal distribution of the values. F_V/F_M, Ψ_{Eo} and ΔV_{IP} revealed a narrow range of distribution.

Factors of variation of the ChlF parameters within a tree canopy, analyzed in this paper, include the age of the needles in the coniferous species. The comparison between current year (c) and previous year (c+1) needles of *Picea abies* and *Pinus sylvestris* was carried out at the experimental site of Satakunta, in Finland, in 2011. Differences and correlations between c and c+1 needles are shown in Table 3. Previous-year needles showed higher performance indices (PI_{ABS} and PI_{TOT}) than current-year needles. The values of all the considered parameters were correlated between the two age classes with $p<0.01$.

The influence of the position of the leaves in the canopy (top or bottom leaves) has also been analyzed. The relationships between the ChlF parameters measured in top and bottom leaves were explored in all tree species grouped per coniferous species and broadleaf species (Table 4). Significant differences in the photosynthetic properties of

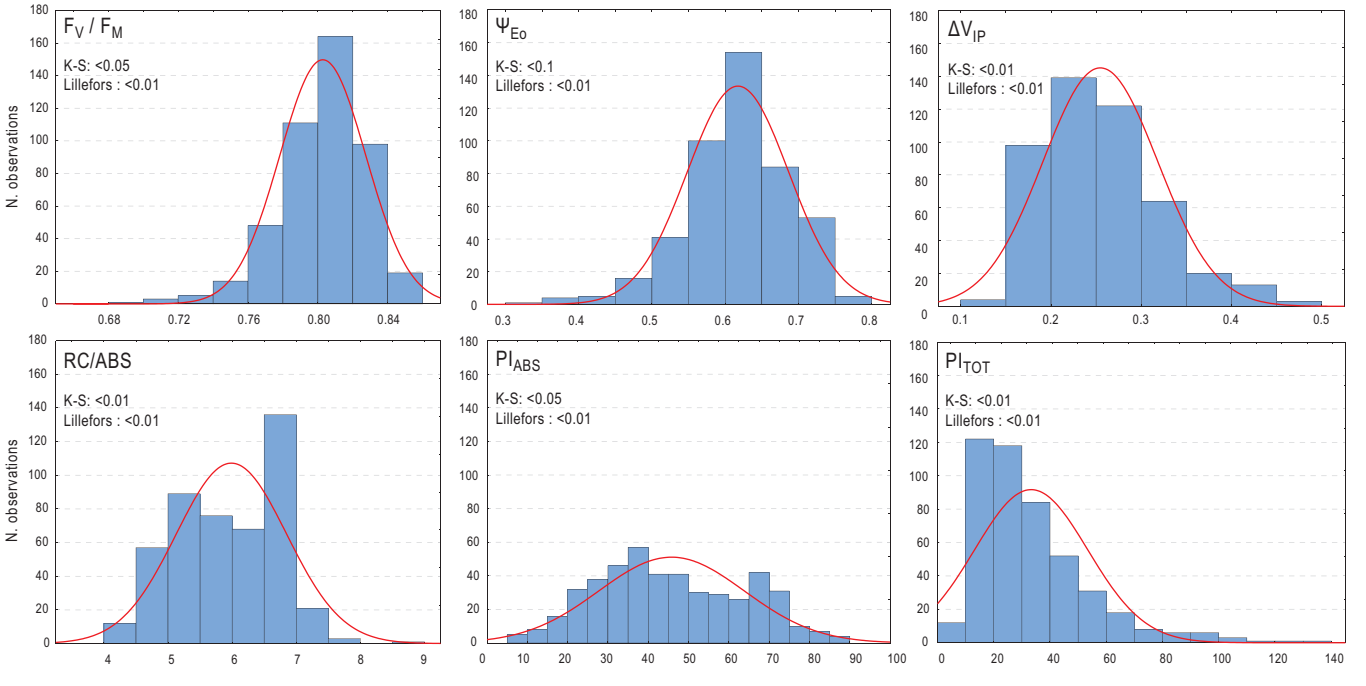

Fig. 2. Distribution of the frequency of the values of the chlorophyll *a* fluorescence parameters in the European forests (all tree species in all countries). The significance of the difference with respect to the normal distribution is indicated (per $p<0.05$ with Kruskas-Wallis (K-S) test and per $p<0.01$ with Lillefors test).

Table 3. Descriptive statistics (mean ± standard deviation) of chlorophyll fluorescence parameters in current (c) and previous year (c+1) needles of *Picea abies* and *Pinus sylvestris* sampled at the experimental site of Satakunta (Finland). The significance of the differences tested using Kolmogorov-Smirnov test and correlations (Spearman Test, r) between the two needle ages are indicate per $p<0.05$. For ChlF parameters meaning see Table 1.

	Needles age				
	c M ± sd	c + 1 M ± sd	Difference p	Correlation r	 p
Picea abies					
F_V/F_M	0.82 ± 0.02	0.83 ± 0.02	0.027	0.8	<0.01
RC/ABS	5.73 ± 0.59	6.39 ± 0.64	<0.001	0.34	<0.05
Ψ_{Eo}	0.62 ± 0.04	0.64 ± 0.04	ns	0.55	<0.01
ΔV_{IP}	0.23 ± 0.04	0.24 ± 0.04	ns	0.76	<0.01
PI_{ABS}	45.99 ± 14.98	59.94 ± 18.10	<0.001	0.47	<0.01
PI_{TOT}	27.04 ± 11.61	36.74 ± 15.10	0.004	0.61	<0.01
Pinus sylvestris					
F_V/F_M	0.83 ± 0.01	0.84 ± 0.01	ns	0.34	<0.05
RC/ABS	5.37 ± 0.42	6.28 ± 0.49	<0.001	0.42	<0.01
Ψ_{Eo}	0.67 ± 0.03	0.68 ± 0.03	ns	0.53	<0.01
ΔV_{IP}	0.27 ± 0.03	0.27 ± 0.03	ns	0.37	<0.01
PI_{ABS}	57.67 ± 11.76	77.31 ± 17.48	<0.001	0.4	<0.01
PI_{TOT}	40.51 ± 11.74	51.38 ± 16.89	0.001	0.58	<0.01

Table 4. Descriptive statistics (mean ± standard deviation) of chlorophyll fluorescence parameters in top and bottom leaves per functional groups of tree species (Coniferours and broadleaved species) sampled in the exploratory sites of the FunDivEurope project. The significance of the differences tested using Kolmogorov-Smirnov test and the correlations (Spearman Test) between the two leaf/needle positions in the canopy are indicated per $p<0.05$. For ChlF parameters meaning see Table 1.

	Leaf position				
	Top M ± sd	Bottom M ± sd	Difference p	Correlation r	 p
Coniferous species					
F_V/F_M	0.82 ± 0.02	0.82 ± 0.01	ns	0.86	<0.01
RC/ABS	6.27 ± 0.80	6.11 ± 0.78	<0.001	0.83	<0.01
Ψ_{Eo}	0.63 ± 0.07	0.64 ± 0.05	ns	0.91	<0.01
ΔV_{IP}	0.28 ± 0.07	0.26 ± 0.08	<0.001	0.87	<0.01
PI_{ABS}	57.95 ± 19.79	54.21 ± 14.09	<0.001	0.87	<0.01
PI_{TOT}	49.21 ± 29.03	39.23 ± 21.42	<0.001	0.84	<0.01
Broadleaved species					
F_V/F_M	0.79 ± 0.03	0.80 ± 0.02	<0.001	0.61	<0.01
RC/ABS	5.97 ± 0.84	5.80 ± 1.01	<0.001	0.86	<0.01
Ψ_{Eo}	0.60 ± 0.08	0.61 ± 0.07	<0.001	0.85	<0.01
ΔV_{IP}	0.26 ± 0.06	0.23 ± 0.06	<0.001	0.85	<0.01
PI_{ABS}	41.76 ± 19.30	39.64 ± 15.58	<0.001	0.88	<0.01
PI_{TOT}	31.51 ± 17.16	24.36 ± 13.91	<0.001	0.8	<0.01

leaves were found for most of the parameters studied, with RC/ABS, ΔV_{IP} and PI_{TOT} being higher in the top than in bottom leaves, and F_V/F_M, Ψ_{Eo} and PI_{ABS} being higher in bottom than in top leaves. The values of ChlF parameters at the two levels of the crown were significantly correlated between them, with $p<0.001$, in all tree species and in all countries.

The standard deviations and the standard errors of the ChlF parameters were calculated per species at different levels of aggregation: (i) within the trees (on the 16 measurements taken for each sampled tree, including both top and bottom leaves); (ii) among the trees standing in the same plot (six trees in monocultures and three trees in mixed plots); and (iii) among the plots in the same country. The results are reported for Poland (Table 5). The variability of ChlF data observed at tree species and country levels, expressed as a coefficient of variation (percent of the standard deviation with respect to the mean), reflects the findings already described by Pollastrini et al. (2014), with F_V/F_M being the most robust parameter and PIs the most variable.

Differences among functional groups of tree species and countries

The differences between the ChlF parameters measured in the functional groups of tree species (coniferous species, temperate broadleaf species, Mediterranean oak species, Table 6), were analyzed in the whole sample (all tree species in all countries). The coniferous species showed the highest values of F_V/F_M and PI_{ABS} and PI_{TOT}, whereas Mediterranean oaks had the lowest F_V/F_M values. The tree species with the highest PI_{ABS} values and related parameters (namely F_V/F_M and Ψ_{Eo}) were in the central Europe countries (namely Poland and Germany). In the opposite, the species with lower PI_{ABS} values occurred in the southernmost (Italy and Spain) and northernmost (Finland) regions. No significant differences between the ChlF parameters were found among Mediterranean oaks in Italy and Spain.

Table 5. Variability of the ChlF parameters per tree species in Poland. Standard deviation (sd) and standard error (es) are expressed as percent with respect to the mean. sd% represents the coefficient of variation (CV). CV measures: (A) the variability among the leaves in the same crown (16 leaves per tree); (B) the variability among trees of the same species in monospecific plots (six trees per plot); (C) the variability among the trees of the same species in mixed plots (three trees per plot); (D) the variability among the trees of the same species in both monospecific and mixed plots (about 23 plots per species). For ChlF parameters meaning see Table 1.

	A. Leaves within the crown n=16		**B. Trees within the plot n=6**		**C. Trees within the plot n=3**		**D. Among the plots n=23**	
	sd (%)	**es (%)**	**sd (%)**	**es (%)**	**sd (%)**	**es (%)**	**sd (%)**	**es (%)**
Betula pendula								
F_V/F_M	1.42	0.44	1.46	0.60	1.06	0.64	0.95	0.20
RC/ABS	10.15	3.18	10.86	4.43	9.24	5.44	7.73	1.65
Ψ_{Eo}	4.02	1.24	2.76	1.13	4.57	2.69	5.75	1.23
ΔV_{IP}	13.48	4.09	13.43	5.48	12.01	7.17	15.21	3.24
PI_{ABS}	20.21	6.31	18.30	7.47	18.91	11.21	16.85	3.59
PI_{TOT}	28.61	8.76	27.67	11.30	25.63	15.12	30.57	6.52
Carpinus betulus								
F_V/F_M	1.90	0.52	2.02	0.83	1.37	0.80	1.68	0.35
RC/ABS	11.47	3.11	6.63	2.71	5.57	3.28	5.39	1.12
Ψ_{Eo}	5.26	1.42	7.94	3.24	4.28	2.45	6.08	1.27
ΔV_{IP}	18.22	4.88	14.10	5.76	14.40	8.46	10.99	2.29
PI_{ABS}	22.03	6.08	29.81	12.17	15.04	8.80	21.66	4.52
PI_{TOT}	35.72	9.71	33.79	13.79	24.82	14.36	25.10	5.23
Picea abies								
F_V/F_M	1.40	0.38	1.16	0.47	1.02	0.59	1.15	0.24
RC/ABS	16.11	4.41	7.60	3.10	6.14	3.54	6.32	1.32
Ψ_{Eo}	5.65	1.55	2.56	1.04	3.43	1.98	2.79	0.58
ΔV_{IP}	15.99	4.37	6.92	2.82	11.00	6.35	11.95	2.49
PI_{ABS}	33.52	9.18	17.94	7.32	17.43	10.06	14.97	3.12
PI_{TOT}	43.89	12.02	15.71	6.41	26.53	15.32	22.47	4.68
Pinus sylvestris								
F_V/F_M	1.52	0.45	1.46	0.59	1.19	0.69	1.14	0.24
RC/ABS	16.66	4.90	11.33	4.62	10.18	5.88	10.64	2.22
Ψ_{Eo}	3.70	1.07	3.58	1.46	3.38	1.95	2.77	0.58
ΔV_{IP}	14.01	4.00	8.15	3.33	11.83	6.83	12.33	2.57
PI_{ABS}	29.13	8.53	24.81	10.13	18.48	10.67	15.72	3.28
PI_{TOT}	39.11	11.30	26.31	10.74	26.07	15.05	23.73	4.95
Quercus robur								
F_V/F_M	1.60	0.46	1.77	0.72	1.14	0.66	1.71	0.36
RC/ABS	12.60	3.60	5.04	2.06	8.34	4.82	8.04	1.68
Ψ_{Eo}	4.63	1.33	4.33	1.77	4.21	2.43	6.33	1.32
ΔV_{IP}	14.22	4.03	9.45	3.86	10.86	6.27	11.00	2.29
PI_{ABS}	27.64	7.90	22.77	9.30	21.98	12.69	26.55	5.54
PI_{TOT}	38.03	10.76	29.29	11.96	32.38	18.69	33.24	6.93

Table 6. ChlF parameters (mean and standard deviation) per functional groups of tree species (C: coniferous species, TB: temperate broadleaved species; MO: Mediterranean oak species) in the whole sample (all species in all countries) and per each country. The significance of the differences between the functional groups and country were analyzed by means of two-samples Kolmogorov-Smirnov test. The significances between countries (on the column) are reported for $p<0.05$. Different letters indicate the significant differences. For ChlF parameters meaning see Table 1.

Funct. group	Country	RC/ABS			F_V/F_M			Ψ_{Eo}			ΔV_{IP}			PI_{ABS}			PI_{TOT}		
		M	sd		M	sd		M	sd		M	sd		M	sd		M	sd	
C	*All sites*	6.22	±0.63	a	0.83	±0.01	a	0.62	±0.05	a	0.26	±0.06	a	53.75	±14.28	a	41.01	±22.82	a
TB	*All sites*	5.73	±0.80	a	0.79	±0.02	b	0.59	±0.07	a	0.24	±0.06	a	35.86	±14.33	b	25.72	±14.43	b
MO	*All sites*	5.48	±1.49	a	0.76	±0.03	c	0.53	±0.07	b	0.29	±0.03	a	26.76	±19.47	b	29.27	±12.51	b
C	Finland	6.25	±0.55	ab	0.82	±0.02	a	0.60	±0.05	b	0.27	±0.07	a	47.92	±12.70	b	43.17	±22.43	a
	Poland	6.29	±0.90	ab	0.83	±0.01	a	0.69	±0.04	a	0.27	±0.06	a	72.15	±12.92	a	69.24	±35.58	a
	Germany	6.98	±0.00	a	0.84	±0.01	a	0.70	±0.04	a	0.22	±0.02	a	66.09	±9.09	ab	25.94	±6.00	a
	Romania	6.68	±0.53	ab	0.83	±0.01	a	0.63	±0.03	a	0.20	±0.02	a	57.60	±13.55	ab	28.06	±7.50	a
	Spain	5.60	±0.61	b	0.82	±0.02	a	0.59	±0.05	b	0.26	±0.03	a	43.15	±16.96	b	32.78	±13.48	a
TB	Finland	5.24	±0.77	b	0.77	±0.04	b	0.50	±0.07	b	0.18	±0.04	a	22.29	±12.75	b	13.19	±8.85	a
	Poland	5.58	±0.85	b	0.80	±0.01	a	0.62	±0.04	a	0.26	±0.07	a	39.02	±10.65	ab	32.80	±17.53	a
	Germany	6.97	±0.00	a	0.80	±0.01	a	0.66	±0.07	a	0.23	±0.06	a	57.52	±16.06	a	26.23	±17.09	a
	Romania	5.75	±0.47	ab	0.79	±0.01	b	0.62	±0.02	a	0.21	±0.02	a	37.71	±7.07	ab	20.48	±4.92	a
	Italy	5.47	±0.67	b	0.78	±0.02	b	0.57	±0.05	ab	0.27	±0.07	a	28.83	±10.61	b	29.32	±16.42	a
MO	Italy	6.59	±2.14	a	0.78	±0.03	a	0.58	±0.06	a	0.28	±0.04	a	42.13	±27.73	a	34.08	±16.19	a
	Spain	5.12	±0.84	a	0.76	±0.04	a	0.51	±0.07	a	0.29	±0.04	a	21.95	±12.43	a	28.95	±14.68	a

Relationships between ChlF parameters, leaf traits and environmental factors

The multivariate relationships between ChlF properties of tree species and their leaf functional traits and some ecological features of the forest stands were assessed on monocultures, to avoid the possible confounding factors due to the ecological interactions between tree species. The ecological and structural parameters of the stands were selected from among those listed in Table 2 (latitude, global solar radiation, Martonne aridity index, leaf area index, basal area, C/N ratio in the soil and depth of the soil). The functional leaf traits analyzed were the specific leaf area (SLA), i.e. the projected leaf area per unit of leaf dry mass; nitrogen concentration on a mass basis in leaves (N); the C/N ratio in leaves; and the carbon isotope composition of leaves ($\delta^{13}C$; it is a key parameter for exploring carbon sequestration and strategies for efficient water use of trees under water stress condition, Farquhar et al., 1982; Gessler et al., 2001). All the foliar data were measured within the FunDivEUROPE project from different research groups (noted in Acknowledgements). The data of SLA were partially from literature (Gratani and Foti, 1998; Bussotti et al., 2000; Bréda, 2003; Bruschi et al., 2003; Legner et al., 2014).

As far as the ChlF parameters are considered, a preliminary selection was carried out by exploring their relative relationships. Fig. 3 shows the results of the principal component analysis (PCA) applied on all the considered ChlF parameters (all tree species, in all the countries). Two clusters were identified: the first was on the principal component 1; it explains 63% of the variability of the data and includes F_V/F_M, Ψ_{Eo}, RC/ABS and PI_{ABS}. The second cluster was on the principal component 2; it explains 24.7%

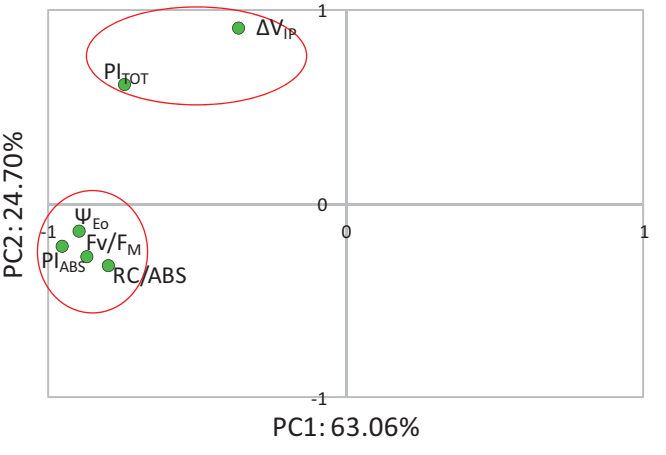

Fig. 3. Principal Component Analysis plot of the distribution of the chlorophyll *a* fluorescence parameters in according to the principal component 1 (PC1) and 2 (PC2). The clusters of the parameters are highlighted. The scale on the axes (-1 to 1) indicates the loading values of the individual parameter to the principal component.

of the variability of the data and includes ΔV_{IP} and PI_{TOT}. On the basis of these results, we decided to select F_V/F_M and ΔV_{IP} as representatives of the two main underlying physiological processes reflected in the ChlF signature.

The results of the PCAs of the data sets for these ChlF parameters together with leaf traits and environmental factors, separately per broadleaf species and coniferous species, are shown in Fig 4. In broadleaf species, three clusters were identified. The first, on the principal component 1 (PC1), includes the parameters ΔV_{IP}, GSR, $\delta^{13}C$ and soil C/N. At the opposite position on the PC1, we found the latitude, the index of aridity, soil depth and SLA. F_V/F_M is included in a cluster on the principal component 2, with basal area and leaf area index of the stands. The leaf properties and the stand parameters of the coniferous species showed a similar behavior of broadleaf species, although the distribution of the parameters was more scattered.

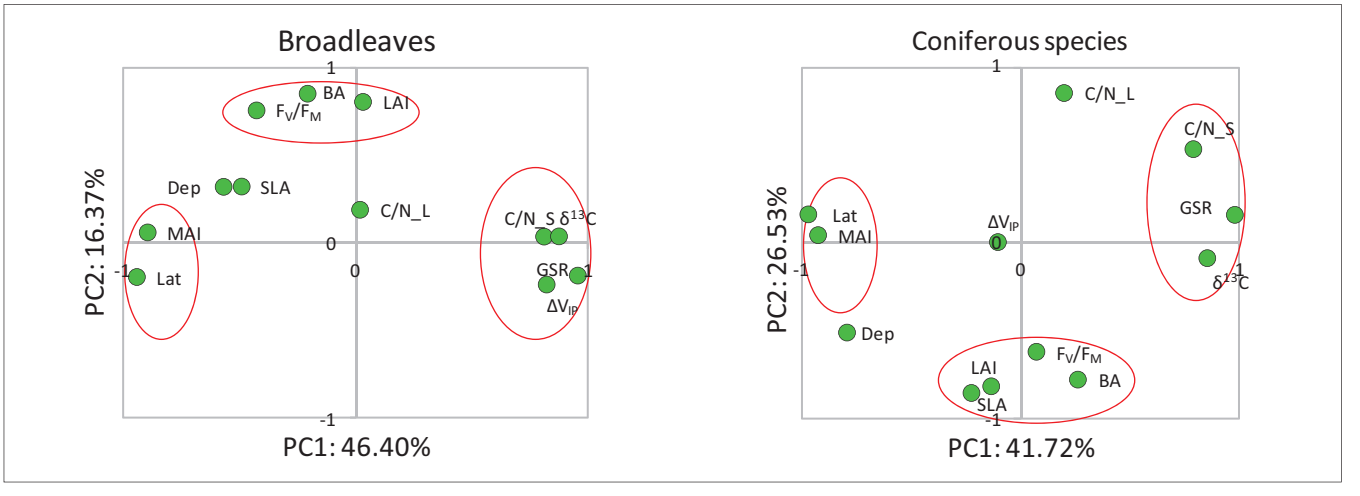

Fig. 4. Principal Component Analysis plot of the distribution of the chlorophyll *a* fluorescence parameters and stand parameters according to the principal component 1 (PC1) and 2 (PC2). Broadleaves include both temperate broadleaf species and Mediterranean oaks. F_v/F_M and ΔV_{IP} = see Table 1; Lat = Latitude; GSR = Global Solar Radiation; MAI = Martonne Aridity Index; BA = stand basal area; LAI = Leaf Area Index of the stand; Dep = soil depth; C/N_S = carbon/nitrogen ratio in the soil; C/N_L = carbon/nitrogen ratio (mass basis) in the leaves; SLA = Specific Leaf Area; $\delta^{13}C$ = carbon isotope composition of leaves. The scale on the axes (-1 to 1) indicates the loading values of the individual parameter to the principal component.

The univariate correlations between the stand parameters and the leaf parameters of trees, shown in Table 7 and Figs. 5-6, show evidence of opposite patterns in F_V/F_M and ΔV_{IP} in relation to GSR (Fig. 5) and LAI (Fig. 6). F_V/F_M was enhanced in conditions of lower solar radiation intensity and higher canopy closure, whereas ΔV_{IP} showed higher values in open canopies, with leaves exposed to high radiation intensity.

These results suggest that ΔV_{IP} was related to the factors that indicate the "Mediterranean conditions", i.e. high solar radiation intensity and shallow soils, with a limited water availability. This finding was coherent with the experimental results obtained by Cascio et al. (2010) and Desotgiu et al. (2012a, b) in *Fagus sylvatica* L. and *Populus*, where ΔV_{IP} values were enhanced by high light intensity and drought treatment, suggesting the onset of the photochemical de-excitation processes in the leaves to manage the excess of the absorbed solar radiation. F_V/F_M decreased with increasing of the solar radiation, from central Europe to Mediterranean sites. The decrease of F_V/F_M with increasing GSR indicates a global strategy of plants for acclimation to light (Adams and Demmig-Adams, 2004) and the dissipation of the excess of the absorbed solar radiation. High sunlight intensity is, indeed, the most powerful factor that influences the ChlF parameters in tree species, inducing the photoinhibition processes (Gilmore, 2004). Moreover, the significant relation between F_V/F_M with the density of the forest stand (high LAI and stand basal area) suggests that this parameter may be indicative of the overall forest fertility and productivity.

CONCLUSIONS

The terrestrial assessment of the ChlF properties and of other foliar features in high forest canopies has indisputable difficulties, mainly due to the difficulty of reaching the canopies and, consequently, the cost for the leaf sampling.

Table 7. The correlation matrix (coefficient of correlation of Spearman, r) between the stand parameters and leaf parameters. The results are reported for $p<0.05$. Lat = Latitude; GSR = Global Solar Radiation; C/N_S = Carbon/ Nitrogen ratio in the soil; Dep_S = soil depth; MAI = Martonne Aridity Index; BA = basal area of the stand; LAI = Leaf Area Index of the stand; C/N_L = Carbon/Nitrogen ratio (mass basis) of leaves; $\delta^{13}C$ = carbon isotope composition of leaves; F_V/F_M and ΔV_{IP} = see symbols in Table 1.

Lat	1.00										
GSR	**-0.95**	1.00									
C/N_S	**-0.65**	**0.65**	1.00								
Dep_S	**0.61**	**-0.51**	**-0.43**	1.00							
MAI	**0.83**	**-0.88**	**-0.55**	**0.54**	1.00						
BA	ns	ns	ns	ns	ns	1.00					
LAI	**0.23**	**-0.27**	**-0.55**	0.21	**0.25**	**0.61**	1.00				
C/N_L	ns	ns	**0.36**	ns	ns	ns	ns	1.00			
$\delta^{13}C$	**-0.70**	**0.73**	**0.49**	**-0.54**	**-0.73**	0.13	-0.18	ns	1.00		
F_V/F_M	**0.23**	**-0.25**	-0.06	**0.24**	**0.24**	**0.44**	**0.25**	**0.40**	-0.07	1.00	
ΔV_{IP}	ns	**0.23**	**0.49**	ns	**-0.35**	ns	**-0.25**	ns	**0.36**	ns	1.00
	Lat	GSR	CN_S	Dep_S	MAI	BA	LAI	CN_L	$\delta^{13}C$	F_V/F_M	ΔV_{IP}

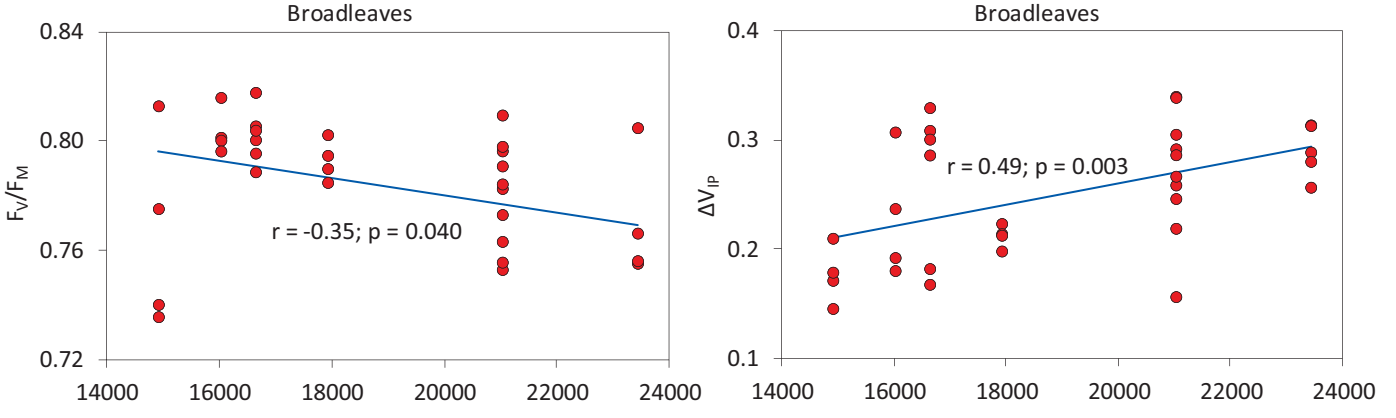

Fig. 5. Univariate correlation (Spearman correlation coefficient, r) between the selected ChlF parameters and Global Solar Radiation in broadleaved species (including both temperate broadleaf species and Mediterranean oaks) across European forests. The significant value of the correlation is indicated (p).

However, there are some potential applications. The measurement of ChlF parameters at the canopy level can support large-scale surveys as a tool to validate the remote sensing data. ChlF is assessed with remote sensing on light-adapted canopies (passive fluorescence, Meroni et al., 2009) and cannot be directly comparable with the JIP-test parameters, measured from the ground, on dark-adapted samples. Among the parameters measured on dark-adapted leaves, F_V/F_M correlates with the Photosynthetic Reflectance Index (Peñuelas et al., 1995), which is used in remote sensing surveys. To enhance the comparability between the terrestrial and remote sensing surveys, it is therefore necessary to promote further studies aimed at combining the JIP-test findings with remotely assessed parameters (Serbin et al., 2012).

Another potential application of ChlF concerns the large-scale and long-term surveys of forest condition (i.e. the ICP-Forests program, Meining and Fischer, 2011). The foliar analysis that is currently applied to assess the nutritional status of trees (Jonard et al., 2015) can gain positive inputs from the ChlF analysis and from a more complete assessment of the functional leaf traits.

Finally, the ChlF analysis can be used to assess the responses of trees to silvicultural interventions, thus making it possible to define the best forestry practices on a physiological basis.

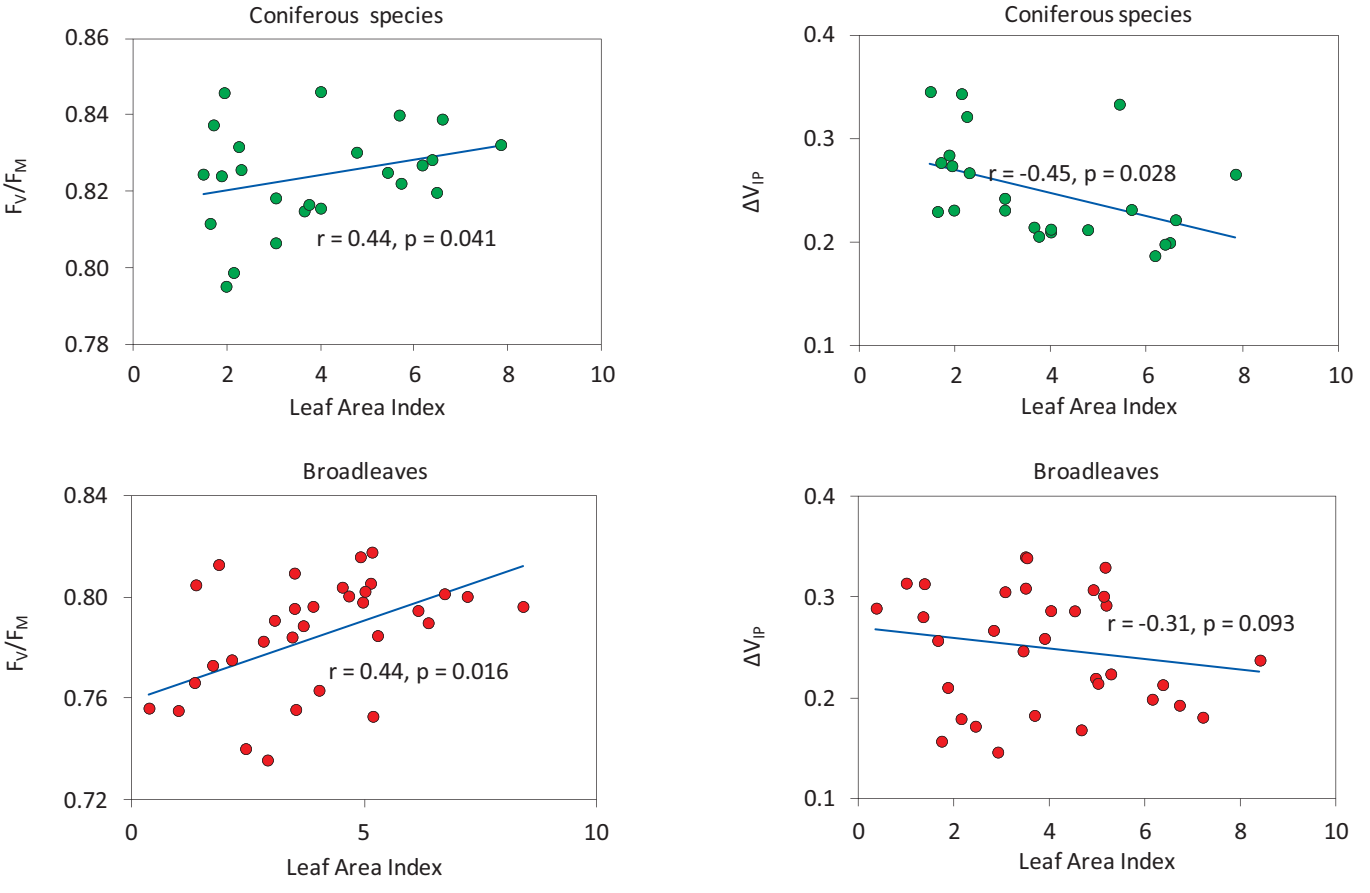

Fig. 6. Univariate correlation (Spearman correlation coefficient, r) between the selected ChlF parameters and Leaf Area Index in broadleaf species and coniferous species across European forests. Broadleaves include both temperate broadleaved species and Mediterranean oaks. The significant value of the correlation is indicated (p).

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