Physiological significance of forest tree defoliation: Results from a survey in a mixed forest in Tuscany (central Italy)

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Abstract: A survey of tree crown defoliation and leaf physiological traits (chlorophyll a fluorescence, nitrogen content, and stable carbon isotope composition) was carried out in the thermophilous deciduous forests in Tuscany (central Italy). In contrast to large scale surveys, where variation in defoliation can be associated with the change in environmental conditions, in a limited homogenous area the defoliation of co-existing tree species may have different significance and depends on the interaction between the characteristics of each individual species with biotic stress and environmental conditions. The survey included measurements of structural and vegetational characteristics of the forest stands, such as Leaf Area Index (LAI), basal area and tree diversity, which is expressed as the Shannon diversity index. The five tree species studied (Castanea sativa, Quercus cerris, Quercus ilex, Quercus petraea and Ostrya carpinifolia) showed species-specific crown conditions and physiological features relative to stand structure and diversity. The shape of the crowns and their area (LAI) affected forest defoliation. Tree diversity reduced defoliation in C. sativa, which was the tree species most affected by defoliation, and likewise for Q.ilex. Chlorophyll a fluorescence parameters showed lower photosynthetic efficiency in defoliated C. sativa, O. carpinifolia and Q. petraea trees. Similarly, foliar nitrogen content decreased in defoliated C. sativa and O. carpinifolia trees, whereas $\delta 13C$ was higher in defoliated C. sativa. These responses may be related to the health status of C. sativa, since it was subjected to pathogen damages and insect attacks. In contrast, the mast year in O. carpinifolia may have diverted the nutrient resources from leaves to fruits, and consequently explaining the physiological effects on the tree crown. These results suggest that the combined analysis of defoliation with foliar features and stand characteristics can provide insights into tree health and vitality.

Physiological significance of forest tree defoliation: results from a survey in a mixed forest in Tuscany (central Italy)

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Highlights

- Crown condition and leaf physiological traits were assessed in mixed forests
- Tree species and Leaf Area Index were the most important predictors of defoliation
- Tree diversity reduced the defoliation in chestnut and in holm oak
- Defoliation has different physiological significance in forest tree species
- Crown assessment is more informative when defoliation and leaf traits are combined

Summary

A survey of tree crown defoliation and leaf physiological traits (chlorophyll *a* fluorescence, nitrogen content, and stable carbon isotope composition) was carried out in the thermophilous deciduous forests in Tuscany (central Italy). In contrast to large scale surveys, where variation in defoliation can be associated with the change in environmental conditions, in a limited homogenous area the defoliation of co-existing tree species may have different significance and depends on the interaction between the characteristics of each individual species with biotic stress and environmental conditions. The survey included measurements of structural and vegetational characteristics of the forest stands, such as Leaf Area Index (LAI), basal area and tree diversity, which is expressed as the Shannon diversity index. The five tree species studied (*Castanea sativa, Quercus cerris, Quercus ilex*, *Quercus petraea* and *Ostrya carpinifolia*) showed species-specific crown conditions and physiological features relative to stand structure and diversity. The shape of the crowns and their area (LAI) affected forest defoliation. Tree diversity reduced defoliation in *C. sativa*, which was the tree species most affected by defoliation, and likewise for *Q.ilex*. Chlorophyll *a* fluorescence parameters showed lower photosynthetic efficiency in defoliated *C. sativa*, *O. carpinifolia* and *Q. petraea* trees. Similarly, foliar nitrogen content decreased in defoliated *C. sativa* and *O. carpinifolia* trees, whereas δ^{13}C was higher in defoliated *C. sativa*. These responses may be related to the health status of *C. sativa*, since it was subjected to pathogen damages and insect attacks. In contrast, the mast year in *O. carpinifolia* may have diverted the nutrient resources from leaves to fruits, and consequently explaining the physiological effects on the tree crown. These results suggest that the combined analysis of defoliation with foliar features and stand characteristics can provide insights into tree health and vitality.

Keywords: crown condition, defoliation, foliar analysis, FunDivEUROPE, mixed forests, tree diversity

Abbreviations List:

BA = Basal Area (m^2 ha^{-1});

CC = crown compression;

ChlF = Chlorophyll *a* fluorescence;

C/N = Carbon/ Nitrogen ratio;

F_0: minimum (basal) fluorescence in dark adapted samples;

F_M: maximal fluorescence in dark adapted samples;

F_V: total variable fluorescence (F_M-F_0);

$F_V/F_M = \varphi_{P0} = TR_0/ABS = [F_M-F_0]/F_M$: maximum quantum yield of PSII photochemistry of a dark adapted sample;

LAI = Leaf Area Index (m^2 m^{-2});

LI = Light Interception Index;

M_0: initial normalized slope of the fluorescence transient;

OJIP: labels of the different time-steps of the fluorescence transient;

PI_{ABS}: Performance Index on absorption basis. Index for energy conservation of photons absorbed by PSII, through the electron transport chain to the reduction of the electron acceptors in the intersystem between PSII and PSI;

PI_{TOT}: Performance Index total. Potential capacity for energy conservation until the reduction of the final acceptors beyond the PSI;

PSI: photosystem I;

PSII: photosystem II;

V_I: relative variable fluorescence at I step (30 ms);

V_J: relative variable fluorescence at the J step (2 ms);

$\delta^{13}C$: carbon isotope composition (‰);

$\Delta V_{I\text{-}P} = 1 - V_I$ = I-P phase: relative contribution of the I-P phase to the fluorescence transient OJIP (it is regarded as a measure for the efficiency of the electron flux through PSI to reduce the final acceptors of the electron transport chain);

$\Psi_{Eo} = 1 - V_J$ = Jstep = ET_0/TR_0: probability of an electron to move from reduced Q_A, the secondary PSII electron acceptor, into the electron transport chain.

1. Introduction

Tree crown defoliation is the main parameter adopted in surveys (International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests, ICP Forests programme, www.icp-forests.net) to assess the health of European forests (Eichhorn et al., 2010). Defoliation is a raw visual indicator of the relative amount of foliage on the tree crown compared to a reference standard tree, and is assessed visually by trained field teams (Ferretti et al., 1999). Defoliation is an unspecific parameter integrating intrinsic tree genetic variability, site effects (soil fertility, climatic features, structure and composition of a forest stand), and external factors such as abiotic and biotic stresses. Consequently, defoliation (as assessed according to the ICP Forests criteria) is not necessarily equivalent to damage and can be considered indicative of the plastic equilibrium of a tree in a given environment.

Crown defoliation has been assessed extensively in many European countries since the 1980s, and the trends recorded are assumed to correlate with the effects of environmental stress, such as air pollution and climate change (Van Leeuwen et al., 2000; Meining and Fischer, 2011; Bussotti et al., 2014, 2015). Recent papers examining these historical trends found relationships between the increasing defoliation levels and the change of climatic conditions, with special reference to drought and heat waves (Seidling, 2007; Carnicer et al., 2011; De la Cruz et al., 2014). To increase the effectiveness of the surveys, and to evaluate the overall conditions of the trees, visual assessment of defoliation and crown status could be combined with the analysis of the functional traits most likely linked to the responses of trees to environmental stress (Bussotti and Pollastrini, 2015).

The physiological consequences of defoliation have not been thoroughly explored. Defoliation implies reduction of the leaf area, light absorbing area and the whole tree photosynthetic apparatus, and is commonly assumed that defoliated trees have reduced growth. This assumption, though supported by observational evidence (Augustatis and Bytnerowicz, 2008), does not take into account the so-called *compensatory photosynthesis*, i.e. the capacity to compensate the loss of leaves with higher photosynthetic rates in the remaining foliage (Nowak and Caldwell, 1984; Desotgiu et al., 2012a). Eyles et al. (2011) observed compensatory photosynthesis in aphid-defoliated *Pinus radiata* D.Don, and attributed this effect to the enhanced exploitation of sunlight by leaves in the inner layers of the crown. It is likely that there is a threshold of defoliation whereby the remaining foliage is no longer able to restore the full photosynthetic activity. The altered light regime within a thinned crown affects photosynthetic function (Lavigne et al., 2001; Turnbull et al., 2007) and leaf nitrogen content that is directly related to the protein composition of the photosynthetic apparatus (Ellsworth and Reich, 1995; Wright et al., 2004). Moreover, foliar transpiration can be either enhanced or suppressed by the altered microclimate inside the canopy (Quentin et al., 2011). It is reasonable to assume that the altered physiological functions in defoliated trees may be reflected by an array of leaf features detectable with foliar analysis (Bussotti and Pollastrini, 2015).

Among stand features, tree species composition and diversity are thought to be important. Mixed forests are assumed to be more productive (Jucker et al., 2014) and more resilient to environmental stress (Grossiord et al., 2014a, b) than monospecific ones that result from positive interactions among tree species and the ability to exploit resources more efficiently (Bengtsson et al., 2000; Balvanera et al., 2006; Knoke et al., 2008). Eichhorn et al. (2005) identified tree diversity as a relevant factor that positively influences the crown conditions (i.e. reduced defoliation) at the stand level in mixed oak – beech forests in Germany.

The present research was part of a project on the functional significance of forest biodiversity in Europe (FunDivEUROPE, Baeten et al., 2013), and was carried out in mixed broadleaved forests in central Italy (Tuscany). Defoliation and crown conditions, assessed according to the guidelines of the ICP Forests manual (Eichhorn et al., 2010), were studied in the context of stand characteristics and foliar features. Stand characteristics provide information on the possible detrimental (or beneficial) effects of forest structure and composition (basal area, leaf area index, tree species mixture) on crown condition. Foliar features are relevant to investigation of the cause and/or consequences of defoliation on tree health and relative physiological functions. In contrast to large scale surveys, where variation in defoliation can be associated with the change in environmental conditions, for example drought or elevation gradients (Michel and Seidling, 2014) in a local homogenous area, with uniform climatic and soil conditions, defoliation may have contrasting significance to different tree species and depends on the interaction between the characteristics of each individual species with biotic stress and environmental conditions.

Within the hypothesis that defoliation may have different ecological and physiological meanings in tree species sharing the same environment, the present survey is aimed at exploring the effectiveness of comprehensive foliar analysis, combined with the structure and composition of the forest stands, to analyze species-specific responses connected to defoliation.

2. Materials and Methods

2.1.Site description

This study was carried out in the Italian forests (Tuscany, Colline Metallifere) of the exploratory platform of the FunDivEUROPE project (www.fundiveurope.eu, Baeten et al., 2013). The study design of the survey in Tuscany has been described by Bussotti et al. (2012). The sites were located at 43.27° N, 11.26° E, mainly at 350-450 m asl (for detailed characteristics of the plots see Table S1). The mean annual precipitation in the plots is 733 ± 42 mm and the mean annual temperature is 13.35 ± 0.38 °C (data from WorldClim-Global Climate Data, www.worldclim.org, with spatial resolution of 1x1 km). The bedrock is predominantly siliceous (sandstones and various conglomerates) and the soil is Cambisol (FAO classification), with a mean soil depth of 68 cm. Almost all of the plots have northern exposure and mean slope <50%. Thirty-six plots (30x30 m) with five focal tree species (*Quercus ilex* L.; *Quercus cerris* L.; *Quercus petraea* (Matt.) Liebl.; *Ostrya carpinifolia* Scop.; and *Castanea sativa* Mill.) were selected. Forest stands are around 50 -70 years old and originate from old coppices (the cutting of the stumps was suspended after the World War II). The trees of *C. sativa* are 60 year-old-stumps sprouted from the oldest trees cultivated in the past for fruits and then abandoned. At present, the forests considered in this study are public and managed as natural reserves.

In this survey thirty-two plots were used (four plots were discarded because data were biased by uncontrolled conditions). The plots had different levels of tree species diversity, ranging from monocultures to a maximum of four species. The level of tree diversity was calculated as the Shannon diversity index (Staddon et al., 1997; Spellerberg and Fedor, 2003), taking into account tree basal area, for each plot.

2.2.Leaf Area Index and Light Interception Index

Canopy closure of the forest stands was assessed by means of Leaf Area Index (total one-side area of leaf tissue per unit ground surface area, m^2m^{-2}, Watson, 1947). Five measurements of Leaf Area Index (LAI) in each plot were carried out at two time points, either early in the morning (shortly before sunrise) or late in the evening (shortly after sunset) in order to work in the presence of diffuse solar radiation and thus reduce the effect of scattered blue light in the canopy. LAI measurements were carried out in early September 2012, before the beginning of leaf shedding, using a Plant Canopy Analyzer LAI-2000 (LI-Cor Inc., Nebraska). With the LAI-2000, the incident light above the canopy and the light transmission below the canopy were measured using one sensor with five fisheye light sensors (lenses), with central zenith angle of 7°, 23°, 38°, 53° and 68° (LAI-2000 manual, Li-Cor 1991). The protocol used in each plot consisted of five measurements within the plots (light transmission below the canopy), and five measurements outside the forest (as proxy of the light incidence above the canopy), in an open space that was in close proximity of the sampled plots. LAI data were processed using Li-Cor's FV2200 software (LI-COR Biogeosciences, Inc. 2010). The light transmittance measurements of the fifth ring were removed to minimize the boundary effects on LAI. The LAI value per plot was the mean value of the five measurements for each plot.

Furthermore, the capacity of trees to intercept light was determined. The light interception index (LI, King et al., 2005) was calculated for each tree, according to Jucker et al. (2015):

$$LI = CPA \times CI^2$$

where CPA is the crown projected area of each tree (in m^2, calculated using the crown radius measurements taken in the field), and CI is the crown illumination index, which scores each tree on a scale of 1 to 5 based on exposure to direct sunlight (Clark and Clark, 1992).

2.3.Crown condition assessment

In each plot, between six and 12 dominant trees were selected. Six trees were selected in monocultures, and three trees per focal species in mixture plots. The trees were randomly selected among the trees with the largest diameter breast height. Defoliation and damage symptoms on leaves and branches were assessed for each selected tree, on the visible portion of the crown, in June 2012 following the guidelines of the ICP Forests (Eichhorn et al., 2010). Defoliation was evaluated according to a proportion scale in 5% intervals (0= not defoliated tree; 5; 10; 15 ... 100%= dead tree), by comparing the sampled tree with a photographic standard ("photoguide" method, Müller and Stierlin, 1990; Ferretti, 1994). Defoliation was defined as leaf loss (fallen and undeveloped leaves, dieback of parts of the crown, as well the loss of foliar surface as a consequence, e.g. of herbivores and/or by hail) compared to a reference tree, regardless of the cause of foliage loss. Damage on leaves and branches due to biotic and abiotic factors were evaluated from the ground, with binoculars, in the visible part of the crown, according to a scale with 5% intervals from 0 to 100%. Causes of the damage symptoms (meteorological, mechanical, leaf senescence, biotic attacks, etc.) were determined when visually recognizable. The interaction with the neighbouring trees ("crown compression") was also assessed on an ordinal scale (0= crown completely free in all four sides; 1= one side of the crown compressed by neighbouring trees; 2= two sides of the crown compressed; 3= three sides of the crown compressed; and 4= all four sides of the crown compressed).

2.4.Leaf sampling

From each selected tree, branches with leaves attached were sampled in two parts of the crown, the highest, southern exposed part, and the lower third of the same side. The sampling was done in June 2012, with the assistance of tree climbers and extension loppers. Fully developed current-year leaves were collected both from deciduous and evergreen species.

2.5.Chlorophyll a fluorescence analysis

Measurements of chlorophyll *a* fluorescence (ChlF) were done, after four to five hours of dark adaptation of the sample, on 16 leaves for each tree. The dark adaptation of leaves was carried out in hermetic black plastic bags immediately after sampling, and stored in a dark room at ambient temperature. Leaves were humidified to avoid de-hydration. A long dark adaptation period was necessary to reduce both dynamic and chronic leaf photoinhibition (Werner et al., 2002; Desotgiu et al., 2012b; 2013) and allow leaves to return to standard conditions. Measurements were done with a HandyPEA fluorimeter (Hansatech Instruments Ltd., Petney, Norfolk, UK). Plotted on a logarithmic time scale, the fluorescence induction transient shows polyphasic behaviour. The different time-steps of this polyphasic transient are labelled as: O (20-50 µs), J (2 ms), I (30 ms) and P (peak). The latter indicates the highest fluorescence intensity (F_M), when saturating light is used. Generally, F_M is reached around 0.8 s. For reviews of the theoretical background of ChlF parameters obtained from the ChlF induction curve (fast kinetics) of dark-adapted leaves, see Strasser et al. (2000, 2004, 2010) and Kalaji et al. (2014). ChlF parameters used in this study, calculated on the basis of JIP-test (Strasser et al., 2004), were: F_V/F_M, Ψ_{Eo}, $\Delta V_{I\text{-}P}$ and Performance Indices (PI_{ABS} and PI_{TOT}) (see Abbreviation list). PI_{ABS} combines three parameters related to the photosynthetic activity: (1) the density of reaction centres; (2) the quantum yield of primary photochemistry; (3) and the ability to feed electrons into the electron chain between PSII and PSI. PI_{TOT} considers also the efficiency by which an electron can move from the reduced intersystem electron acceptors to the PSI end electron acceptors.

2.6.Chemical analyses: total foliar nitrogen and carbon content; carbon isotope composition

Twenty fully expanded leaves were sampled from each sampled tree for the analysis of carbon isotope composition ($\delta^{13}C$, ‰), total carbon (C, %) and nitrogen (N, %) contents. Foliar samples were dried at 60°C for 48 h, after which the foliar samples from the same species in the same plot were pooled together and finely grounded.

For the analysis of $\delta^{13}C$ about 1.0 mg of the dried powdered material from each sample was placed into tin capsules. The analyses were performed by the Technical Platform of Functional Ecology at the INRA Forest Ecology and Ecophysiology Unit (Champenaux, France), with an isotope ratio mass spectrometer (Delta S, Finnigan MAT, Bremen, Germany). The isotopic composition of the sample was reported in delta notation ($\delta^{13}C$) relative to Vienna Pee Dee Belemnite standard.

For the determination of the N and C content, 2.5 mg of the dried sample was used. All samples were analysed by Near Infra Red Spectroscopy (NIRS), as described by Niederberger et al. (2015). For this purpose, a subset of the samples was analysed for N and C with a flash CHN Elemental Analyser (Flash EA1112 Series, ThermoFinnigan, Milan, Italy) to calibrate the NIRS spectra. The spectra were averaged from five replicates taken with a Fourier Transform Mid- and Near Infrared combination instrument (Tensor 37, Brukeroptics, Ettlingen, Germany). Each single spectrum was a mean of 32 individual scans over the range of 12000 to 4000 cm^{-1} wave numbers with a resolution of 8 cm^{-1}. The choice of samples for calibration was done for each component separately and carried out with an automatic function in the OPUS spectroscopy software (version 6.5, Brukeroptics Ettlingen, Germany) after a first prediction with an existing model. A second subset of the samples was analysed with the same equipment to validate the calibration. Calibration was performed with cross validation with one leave-out sample. The statistical parameters r^2 (coefficient of determination), RMSECV (root mean square error of cross validation) for calibration or RMSEP (root mean square error of prediction) for validation and RPD (ratio of standard error of prediction to standard deviation) were used for the evaluation of prediction quality. Leaf N and C content at the species level for each plot was calculated as average value of three or six trees per species sampled in the plot.

2.7.*Symptoms of parasitic attacks on leaves*

A number of fresh leaves were selected to assess the presence of fungi (25-60 leaves per branch; 50-100 leaves per tree) and insects symptoms (30 leaves per branch). Damage symptoms on leaves that were not visible during crown evaluations were assessed. Fungal symptoms were classified into two categories: powdery mildew and leaf spots. The percentage of leaves with the presence of either type of symptom was counted. After fungal assessment, the leaves were frozen at -18°C until the assessment of the endophagous insects. Four insect guilds were considered for all tree species: miners, gallers, rollers and tiers. As the sap-feeder species *Trioza remota* Foerster was the only one easily detectable on deciduous oak leaves (nymphs stay on the underside of the leaves), there was a fifth insect guild for *Q. cerris* and *Q. petraea*. A mite guild was also assessed for *Q. ilex*. The number of leaves with at least one of the six guilds was counted. Insect damage at the tree level was then aggregated by calculating the percentage of leaves with at least one damage present. Ectophagous insects were not included in the analysis since the loss of foliar surface was already accounted in the assessment of defoliation, according to the definition of Eichhorn et al. (2010). Data of fungi and insect damage are reported as percent of damaged leaves (separately for fungi and insects) relative to the total number of assessed leaves (including both damaged and healthy leaves).

2.8.*Data analysis*

To test the univariate correlations of defoliation with the foliar and stand structure parameters on the whole sample (i.e. all tree species considered together) and for each species individually, the Spearman rank correlation test was applied. Significant differences between tree species for each parameter were assessed with a two-sample Kolmogorov-Smirnov test (for independent samples). Linear mixed models were used to determine the importance of stand structural parameters, diversity and crown properties of trees as predictors of tree defoliation. A number of alternative mixed effects models of defoliation were fitted and compared using Aikake Information Criteria (AIC). Models included different combinations of predictors: plot basal area (BA), Leaf Area Index (LAI), Shannon diversity index, crown compression and light interception index were treated as fixed effects, and tree and plot were treated as random effects in the model. We performed two sets of models: (1) linear mixed models for the whole dataset (i.e. all tree species together). In this case the variable ‘species’ was included in the models as a fixed-effect variable, and (2) linear mixed models for each tree species separately to examine species-specific responses to defoliation. Before running the models, the correlation between the predictor variables was checked to avoid autocorrelation between them. The assumptions of normality and homogeneity of variance of defoliation were checked. All analyses were implemented in R (3.1.2; R Core Team 2014). For the linear mixed models, the package lmerTest (Kuznetsova et al., 2014) was used.

3. Results

The results of the survey carried out in Tuscany considered 244 trees in 32 plots (Table 1). The variability of each foliar and crown trait assessed, both between trees in the same plot and between the plots, is provided in Table 2. The traits estimated by visual assessment (crown defoliation, damage to leaves and branches, insect and pathogen attacks) showed high variability among the trees and the plots. Photosynthetic performance indices also had high coefficient of variability (CV). The lowest CV values were in $Fv/F_{M,}$ C content and $\delta^{13}C$.

The *C. sativa* monocultures had the highest BA and the lowest LAI compared to the other species, and *Q. ilex* monocultures had the highest LAI (Table 1). Crown condition features for each species, as visually assessed from the ground, are reported in Table 3. *C. sativa* showed the highest levels of defoliation and damage to leaves and branches, followed by *O. carpinifolia*. Oak species had the lowest defoliation levels and crown damage. Defoliation was significantly correlated, in many species, to the presence of dead or dying branches (*C. sativa*: $r^2 = 0.53$; *O. carpinifolia*: $r^2 =$

0.22; *Q. cerris*: $r^2 = 0.21$; all species: $r^2 = 0.53$). On *C. sativa*, crown dieback was related to past attacks by the fungus *Cryphonectria parasitica* (Murr.) Barr. and oomycete *Phytophthora cambivora* (Petri) Buisman. More recently, the new agent causing defoliation and damage to leaves was attributed to the Asian wasp *Dryocosmus kuriphilus* Yasumatsu that produces galls on buds (and subsequent desiccation of branches) and leaves. The causes of branch dieback in oaks were not easily identifiable in the field, but were most likely caused by insects, e.g. *Coroebus florentinus* (Herbst), and by the opportunistic fungus *Biscogniauxia mediterranea* (De Not.) Kunze that acts synergistically with drought. Climatic agents (drought and high summer temperatures) probably induced early senescence and loss of leaves in *O. carpinifolia*.

The effects of the stand structure and tree composition as predictors of tree defoliation were examined using of univariate correlations (Table 4) and linear mixed models (Tables 5 and 6). Species and LAI were the most important predictors for defoliation in the whole sample (Table 5), whereas specific factors were important in each individual tree species. LAI was negatively correlated with defoliation in *C. sativa*, *O. carpinifolia* and *Q. cerris*. Since LAI was positively correlated with plot BA ($r^2 = 0.21$), we expected to observe an inverse correlation between BA and defoliation. That was indeed observed for *O. carpinifolia*, *Q. cerris and Q.ilex*, whereas for *C. sativa*, the opposite trend was noted. Tree diversity (Shannon diversity index) showed significant negative correlation with defoliation in *C. sativa* and *Q. ilex*. Light interception index (LI) and crown compression (CC) exerted significant effects on defoliation in the whole sample and in some species, but in opposite directions: LI was negatively correlated with defoliation, and CC was positively correlated with tree defoliation (Table 4).

Physiological features were measured on detached leaves. The highest foliar concentration of N was detected in *C. sativa* and the lowest in the sclerophyllous evergreen species *Q. ilex* (Table 3). The highest $\delta^{13}C$ values were observed in *Q. petraea* and the lowest in *O. carpinifolia* (Table 3). For ChlF parameters (Table 3), Fv/F_M and Ψ_{Eo} were not significantly different among species, whereas the lowest values of ΔV_{I-P} and PI_{TOT} were observed both in *O. carpinifolia* and *Q. ilex*. The univariate correlations between defoliation and leaf features are shown in Table 4. C/N ratio was positively correlated to defoliation in many species, and negatively in the whole sample. ChlF parameters were negatively correlated with defoliation in *C. sativa* (Fv/F_M and PI_{ABS}), *O. carpinifolia* and *Q. petraea* (Ψ_{Eo}, ΔV_{I-P}, PI_{ABS} and PI_{TOT}), but positively in *Q. ilex*. $\delta^{13}C$ was positively correlated with defoliation in the whole sample and in *C. sativa*. Insect and pathogen damage (assessed on detached leaves) were negatively correlated with defoliation in *Q. cerris* and *O. carpinifolia,* respectively. Pathogen damage was negatively correlated with defoliation in the whole sample.

4. Discussion

In the present survey, the main patterns of defoliation were associated with parasitic attacks (*C. sativa*) or abiotic agents (*O. carpinifolia*). Stand factors could enhance or suppress tree crown defoliation. The closure of the canopies, expressed with high LAI, was the main stand factor associated with reduced defoliation. The positive correlation between crown compression and defoliation in some species seemed to suggest an opposite trend, i.e. enhanced defoliation. This apparent contradiction can be explained by a negative effect of the aboveground competition between crowns, resulting in mechanical abrasions (Hajek et al., 2015). In *C. sativa*, the positive correlation between basal area and defoliation was explained by the high defoliation rates of old large chestnut trees under parasitic attacks.

Tree diversity reduced tree defoliation in *C. sativa* and *Q. ilex*. In the studied forests, *C. sativa* was severely affected by the Asian gall wasp *D. kuriphilus*, which constitutes a tremendous threat for tree health and fruit production (Quacchia et al., 2008; Panzavolta et al., 2012; Battisti et al., 2014). Tree diversity is expected to reduce the intensity of insect herbivore attacks (Jactel and Brockerhoff, 2007). Several hypotheses have been proposed to explain this associational resistance effect of diverse plant communities (Tahvanainen and Root, 1972). According to the “resource concentration hypothesis” (Root, 1973), the probability of a host plant to be located by insects decreases in plurispecific systems. Non-host plants could disrupt chemical or physical cues used by herbivores to locate a suitable host (Huber and Borden, 2001; Castagneyrol et al., 2013). In addition, the “natural enemy hypothesis” (Root 1973; Russell 1989) suggests that richer plant assemblages provide natural enemies with more complementary resources and habitats, thus promoting top-down regulation of herbivores. Tree species richness *per se*, however, has no effect on the probability of attack by pests. The infestation rate is also strongly dependent on plot composition (Castagneyrol et al., 2014) and species-specific interactions. Guyot et al. (2015) found the decrease of *D. kuriphilus* attacks on chestnut tree crowns in more diverse forests. The authors suggested that it is connected with the presence of oak species, housing cynipid galls and associated parasitoids (Aebi et al., 2006, 2007, Panzavolta et al., 2013, Quacchia et al., 2013). At opposite, in *Q. ilex*, the negative correlation between defoliation and the Shannon diversity index was probably due to the smaller size of this species in comparison with the tallest deciduous oaks, and as a result, *Q. ilex,* a sciaphilous species, benefits from the shading effect of dominant canopies.

Foliar damage assessed from the ground (ICP Forests protocol) and on detached leaves gave contrasting information. Ground assessment provides a general overview and allows the identification of the most relevant foliar attacks affecting the status of the whole crown. Fungal and insect damages assessed directly from leaves on hand may, however, be indicative of a demography in equilibrium with the crown status (Leather, 2005), although these parameters showed high variability (Table 2).

Among the physiological traits assessed, the C/N ratio was related to defoliation in many species. For each species considered individually, the C/N ratio was positively associated with defoliation (reduction of N with increasing defoliation), the opposite was observed in the whole sample. This apparent contradiction can be explained by the result of the combination of species-specific behaviors, with the most N-rich species (*C. sativa*) being also the most defoliated. In *O. carpinifolia*, we found increasing C/N ratio associated with decreasing levels of photosynthetic efficiency in defoliated trees. Nikiforou and Manetas (2011) found that the decrease of ΔV_{I-P} may be indicative of low nitrogen foliar concentrations. In *O. carpinifolia*, defoliation was probably related to intense fructification and seed production (masting) that occurred during the sampling period. Masting is a well know phenomenon that has a relevant role in forest ecology (Kelly and Sork, 2002; Packham and Hilton, 2002), whereby the recurrence of mast year may be indicative of altered environmental conditions (Jonard, 2009). In *O. carpinifolia*, this behavior was already observed in previous surveys in Tuscany (Bussotti, data not published). Inverse relationships between fructification and defoliation was found on *Fagus sylvatica* L. (beech) in central Europe by Eichhorn et al. (2005) as a consequence of nutrient allocation strategies (carbon and nitrogen were diverted from leaves to increase its availability for fructification, Jonard et al., 2009). Thomas et al. (2002) demonstrated that unbalanced nutritional status (in this case decreased K/N and P/N ratios, and decreased production of allochemicals caused by high nitrogen deposition) was responsible of poor crown conditions in European species of deciduous oak (*Quercus robur* L. and *Q. petraea*), and pathogenic attacks were favoured.

Defoliation in *C. sativa* was correlated to the capacity to trap the solar energy (Fv/F_M and light interception index). A reduction of the photosynthetic efficiency, measured with the JIP-test, was also observed by Ugolini et al. (2014) in *C. sativa* leaves attacked by *D. kuriphilus*. According to the authors, the quantum yield efficiency (Fv/F_M) was not negatively affected by the presence of galls, but Ψ_{Eo} was affected. Deciduous oaks had low levels of defoliation, almost always falling below the value of 25%, which is the threshold that defines “healthy” trees (Eichhorn et al., 2010). This narrow range of defoliation impeded the effective analysis of the relationships with ChlF parameters, although contrasting patterns were detected on *Q. petraea* and *Q. ilex*. The positive correlation between defoliation and ChlF parameters in *Q. ilex* can be attributed to better exploitation of sunlight in lighter crowns, as explained by the enhanced electron transport (Bussotti, 2004).

Higher values of $\delta^{13}C$ (less negative values) in defoliated trees revealed a potential relation between defoliation and drought stress. High $\delta^{13}C$ content in plant tissues indicates decreased leaf internal CO_2 concentration that can be caused by a decrease in stomatal conductance induced, for example, by drought (Francey and Farquhar, 1982). In the present research, we found a positive correlation between $\delta^{13}C$ and defoliation in the whole sample, and among individual tree of *C. sativa*. The loss of foliage mass may cause higher irradiation levels and a drier microclimate in the crown, which may induce stomatal closure. As far as *C. sativa* is concerned, the relatively high values of $\delta^{13}C$ may be a result of parasitic attacks on the functionality of roots (*P. cambivora*) and xylem (*C. parasitica*), and by the leaf damage (*D. kuriphilus*).

There are many papers about the relationship between crown defoliation and drought, where water shortage is a causal factor, both in space (increasing defoliation in drier sites, Zierl, 2002) and in time (increasing defoliation after drier years, Solberg, 2004; Ferretti et al., 2014). In previous research carried out in Italian beech forests, Bussotti et al. (2005) found that the most defoliated trees, growing in the southernmost drought-prone sites, had smaller and thicker leaves, and higher C/N ratio, than trees growing in mesic sites. Drought is expected to play an increasing role in Mediterranean forests in light of climate change (Galiano et al. 2012; Bussotti et al., 2014, 2015). The present paper supports the idea that the use of physiological indicators in forest surveys may be relevant to validate the responses observed in controlled conditions and field studies realized “ad hoc” over a larger scale (Iovi et al., 2009; Galiano et al., 2012; Moreno-Gutiérrez et al., 2012; Granda et al., 2014).

5. Conclusions

The results presented here suggest that defoliation is not an unequivocal phenomenon, but may have different causes and physiological significance in different tree species. Defoliation in *C. sativa* was associated with insect and pathogen attacks, and subsequently resulted in increased exposure to drought. In *O. carpinifolia*, the effect of fruiting with the diversion of N from leaves to fruit was found. In these two species, defoliation was accompanied by the decrease of photosynthetic efficiency, as observed by JIP-test parameters. The opposite pattern observed in *Q. ilex* (higher photosynthetic efficiency in more defoliated trees) maybe related to specific physiological strategies to exploit sunlight.

This "leaf trait analysis" approach can be applied both at local and large scale for purposes of forest management and policy. Locally it can be used to individuate the most appropriate structural characteristics of the forests to enhance the physiological efficiency of the different tree species. Moreover, through surveys repeated over time, it would be possible to assess the responses of trees to sylvicultural practices. At a large scale, this analysis can contribute to strengthening the informative potential of the ongoing routine monitoring activities (e.g., ICP Forests) providing insights about the ecological equilibrium of different tree species in a changing environment. In this perspective the leaf traits analysis can provide basic knowledge to support the elaboration of adaptive strategies.

The main limitation of this study relies in the difficult of representing, with a consistent number of replicates (within and among forest stands), all the possible combination of tree species mixture whilst avoiding bias deriving from the variability of environmental factors (e.g., bedrock and exposure). Such limitations are due to the characteristics of landscape fragmentation and the difficulty of sampling leave in high forests. A large scale survey with a consistent number of forest stands may provide more robust data evidencing general tendencies and avoiding interference from local factors. The analysis of the variability of the foliar features assessed in this study (Table 2) can help to select the more robust and reliable parameters to design an effective cost-benefit surveys according to the criteria of ecological monitoring (Elzinga et al., 2001).

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Table 1 Number of plots and trees sampled in the study. For Basal Area (BA, m^2 ha^{-1}) and Leaf Area Index (LAI, m^2 m^{-2}), mean and standard deviation for each species and mixture level are indicated.

Species	Mixture level	No. Plots	No. Trees	BA		LAI	
				M	±sd	M	±sd
Castanea sativa	1-sp	2	11	29.33	±0.49	2.95	±0.13
	2-sp	3	9	29.07	±3.54	4.13	±0.12
	3-sp	5	15	26.01	±1.66	3.24	±0.70
	4-sp	4	12	27.95	±5.15	4.24	±0.54
Ostrya carpinifolia	1-sp	2	11	23.72	±2.51	3.49	±1.69
	2-sp	3	8	22.80	±1.84	4.09	±0.61
	3-sp	4	12	26.45	±4.67	4.16	±0.65
	4-sp	5	13	28.10	±3.84	3.77	±0.54
Quercus cerris	1-sp	1	12	28.30	±0.29	3.48	±0.04
	2-sp	3	9	30.24	±6.42	5.25	±1.15
	3-sp	5	14	26.83	±4.06	3.72	±1.00
	4-sp	6	18	27.65	±5.24	4.13	±0.58
Quercus ilex	1-sp	2	12	28.48	±5.60	4.54	±0.67
	2-sp	4	12	28.46	±7.74	4.77	±1.32
	3-sp	5	13	27.84	±3.62	4.06	±0.65
	4-sp	7	19	27.89	±5.10	4.05	±0.60
Quercus petraea	1-sp	2	12	28.85	±0.97	3.76	±0.28
	2-sp	1	3	21.85	±0.00	4.91	±0.00
	3-sp	5	15	25.72	±2.27	3.47	±0.84
	4-sp	5	14	26.03	±2.92	3.82	±0.49
All species	1-sp	10	58	27.77	±3.38	3.65	±0.93
	2-sp	7	41	27.39	±6.09	4.61	±1.01
	3-sp	8	69	26.53	±3.34	3.70	±0.83
	4-sp	7	76	27.53	±4.55	4.00	±0.57

Table 2 Variability (coefficient of variability, CV) of the tree and foliar parameters among trees in the same plot and between plots. For foliar nitrogen content (N), carbon content (C) and carbon isotope composition (Leaf $\delta^{13}C$), the variability is indicated between plots because these parameters were measured at the tree species level in each plot.

	Castanea sativa		*Ostrya carpinifolia*		*Quercus cerris*		*Quercus ilex*		*Quercus petraea*	
	CV (%)		CV (%)		CV (%)		CV (%)		CV (%)	
	tree	plot	tree	plot	tree	plot	tree	plot	tree	plot
Tree characteristics										
Defoliation	29.36	46.06	29.47	36.65	38.33	53.63	76.56	111.13	42.02	54.49
Damage to leaves	57.00	64.36	43.89	63.82	93.38	81.33	141.29	165.92	74.47	59.20
Damage to branches	38.21	65.60	52.72	59.12	66.91	63.28	121.45	111.95	77.31	55.85
Foliar characteristics										
Insect damage	20.68	19.53	75.13	55.62	35.08	27.84	31.98	22.59	47.54	32.95
Pathogen damage	61.81	224.32	71.69	249.72	83.75	134.85	44.32	43.63	115.30	96.29
Nitrogen (N %)		8.23		10.26		6.71		8.31		6.49
Carbon (C %)		1.52		1.59		1.42		1.19		1.79
Leaf $\delta^{13}C$ (‰)		-1.49		-2.37		-1.51		-2.32		-2.37
C/N		9.37		9.06		5.92		9.02		5.54
Fv/F_M	1.30	1.19	0.99	1.61	1.84	1.78	1.82	1.83	1.19	1.97
Ψ_{Eo}	5.07	4.70	3.23	8.33	6.47	9.11	5.59	7.75	4.07	8.48
ΔV_{I-P}	8.53	10.49	9.52	21.41	8.65	15.27	11.17	15.80	8.12	12.67
PI_{ABS}	17.84	14.54	11.34	24.39	22.31	25.07	24.27	41.40	15.02	26.22
PI_{TOT}	19.92	25.10	19.83	43.99	22.90	34.03	25.47	45.00	19.83	33.53

Table 3 Mean (± standard deviation) of the crown and leaf parameters assessed for each tree species in the whole sample (i.e. all tree species together). The data are pooled for all plots (monospecific and mixed plots). Letters indicate the significant differences of the parameters among the species (Kolmogorov-Smirnov test for independent samples). Descriptions of each parameter can be found in the Materials and Methods. See Abbreviation list.

	Castanea sativa			*Ostrya carpinifolia*			*Quercus cerris*			*Quercus ilex*			*Quercus petraea*		
	Mean	±sd		Mean	±sd		Mean	±sd		Mean	±sd		Mean	±sd	
Crown parameters:															
Defoliation (%)	36.90	±16.99	a	17.44	±6.39	b	12.16	±6.51	b	6.51	±7.23	c	12.76	±6.95	b
Damage to leaves (%)	23.02	±14.81	a	10.77	±6.87	b	2.97	±2.41	b	0.93	±1.53	c	4.29	±2.54	b
Damage to branches (%)	32.57	±21.36	a	13.77	±8.14	b	7.08	±4.48	b	1.94	±2.17	c	7.24	±4.04	b
Foliar parameters:															
Insect damage (%)	45.26	±14.23	a	3.25	±2.71	c	33.10	±13.97	b	38.72	±15.44	b	36.46	±20.10	b
Pathogen damage (%)	17.73	±0.19	a,b	24.68	±0.26	a,c	4.96	±0.06	c	41.57	±0.14	b	8.39	±0.07	c
Nitrogen (N %)	2.62	±0.21	a	2.36	±0.26	b	2.26	±0.14	c	1.28	±0.12	d	2.27	±0.16	c
Carbon (C %)	48.93	±0.81	b	49.22	±0.90	c	50.22	±0.66	a,b	50.37	±0.56	a	49.70	±0.73	b,c
Leaf $\delta^{13}C$ (‰)	-27.06	±0.38	c	-28.26	±0.64	c	-27.54	±0.39	a	-27.79	±0.67	b	-27.04	±0.64	b
C/N	18.83	±1.74	d	21.06	±2.08	c	22.35	±1.25	b	39.58	±3.86	a	22.04	±1.62	b
Fv/F_M	0.79	±0.01	a	0.79	±0.02	a	0.78	±0.02	a	0.78	±0.02	a	0.79	±0.02	a
Ψ_{Eo}	0.60	±0.04	a	0.58	±0.05	a	0.57	±0.06	a	0.58	±0.06	a	0.59	±0.05	a
ΔV_{I-P}	0.28	±0.04	a	0.20	±0.04	c	0.31	±0.06	a	0.26	±0.05	b	0.29	±0.04	a,b
PI_{ABS}	36.19	±7.96	a	25.93	±7.00	b	31.38	±10.39	a	34.24	±17.44	a	34.30	±10.99	a
PI_{TOT}	33.75	±10.77	a	13.78	±6.25	c	39.83	±15.83	a	27.67	±14.35	b	35.44	±14.91	a

Table 4 Correlations (Spearmann rank correlation) between defoliation and stand parameters and leaf traits in the whole sample (i.e. all tree species together) and in each tree species. Significant correlation coefficients ($p < 0.05$) are in bold.

	All species	*C. sativa*	*O. carpinifolia*	*Q. cerris*	*Q. ilex*	*Q. petraea*
Stand and tree characteristics						
Tree Diversity (Shannon Index)	-0.107	**-0.325**	0.083	0.074	**-0.350**	-0.203
Plot basal Area (BA)	-0.010	**0.427**	-0.178	**-0.436**	-0.119	0.058
Leaf Area Index (LAI)	-0.333	**-0.401**	**-0.586**	**-0.397**	0.037	-0.257
Crown compression (CC)	0.051	**0.313**	**0.381**	0.114	-0.01	-0.165
Light Interception (LI)	**-0.365**	-0.187	-0.119	-0.075	-0.198	-0.110
Foliar characteristics						
Leaf δ^{13}C	**0.175**	**0.426**	0.074	-0.072	0.168	0.157
C/N	**-0.572**	**0.517**	**0.330**	0.206	**0.534**	0.224
Fv/F_M	**0.155**	**-0.332**	-0.244	0.036	0.224	-0.274
Ψ_{Eo}	0.088	-0.172	**-0.328**	0.005	**0.428**	**-0.492**
ΔV_{I-P}	0.044	-0.057	**-0.378**	-0.007	**0.349**	**-0.401**
PI_{ABS}	0.123	**-0.402**	**-0.403**	-0.048	**0.409**	**-0.413**
PI_{TOT}	0.085	-0.208	**-0.452**	-0.081	**0.493**	**-0.437**
Insect damage	0.005	0.056	0.084	**-0.315**	-0.035	-0.278
Pathogen damage	**-0.240**	-0.109	**-0.395**	0.057	0.160	-0.185

Table 5 Degrees of freedom (df), F and p-value from the linear mixed models used to test the fixed effects of stand parameters (plot basal area, Shannon diversity index and LAI), tree species, crown characteristics of trees (light interception index, crown compression) and the effects of fungal attacks on tree defoliation in the whole sample (all tree species together). The significant effect ($p<0.05$) of the predictor on the variability of defoliation is noted in bold.

Predictor	df	F	p-value
Plot basal area	1	0.080	0.780
Shannon diversity index	1	0.205	0.655
LAI	1	5.047	**0.034**
Light interception index	1	0.000	0.993
Crown compression	1	1.210	0.273
Species	4	33.741	**0.000**
Pathogen damage	1	3.750	0.054

Table 6 Degree of freedom (df), F and p-values from the linear mixed models used to test the fixed effects of stand parameters (plot basal area, Shannon diversity index, LAI), and crown characteristics of trees (light interception index, crown compression) on defoliation in each tree species. The models did not include the correlated predictors (plot basal area and Leaf Area Index in the model for *Q. petraea* and *Q. ilex*).

	Castanea sativa			*Ostrya carpinifolia*			*Quercus cerris*			*Quercus petraea*			*Quercus ilex*		
Predictor	df	F	p value	df	F	p value	df	F	p value	df	F	p value	df	F	p value
Plot basal area	1	7.338	**0.01**	1	0.038	0.846	1	1.513	0.24				1	0.023	0.88
Shannon diversity index	1	1.716	0.197	1	0.033	0.857	1	0.056	0.816	1	0.159	0.7	1	1.736	0.207
Light interception index	1	3.975	0.053	1	1.669	0.204	1	2.614	0.112	1	0.306	0.583	1	1.316	0.257
Crown compression	1	2.93	0.095	1	0.879	0.355	1	10.846	**0.002**	1	0.06	0.807	1	0.645	0.426
Leaf Area Index							1	1.504	0.241	1	4.354	0.063			

Highlights

- Crown condition and leaf physiological traits were assessed in mixed forests
- Tree species and Leaf Area Index were the most important predictors of defoliation
- Tree diversity reduced the defoliation in chestnut and in holm oak
- Defoliation has different physiological significance in forest tree species
- Crown assessment is more informative when defoliation and leaf traits are combined

FORECO15463R2

Reviewers' Comments
Authors responses

Reviewer #1:
The authors followed the main recommendations of the first review. From my view, the value of the paper increased significantly. I recommend accepting the paper for printing after some minor changes. One point for further considerations and improvement: Some phrases and statements of the paper are not precise enough. Some examples:
Introduction gives the scientific environment of the study and questions that might be still open. However, it should be stated even more clearly what is the task of the study and what can be achieved by the scientific concept. It seems important to specify what can be achieved and what is out of reach by methodological limitations and difficulties of the study.
The limitation are discussed in Conclusions. In Introduction some statements are changed:
"The present research was part of a project on the functional significance of forest biodiversity in Europe (FunDivEUROPE, Baeten et al., 2013), and was carried out in mixed broadleaved forests in central Italy (Tuscany). Defoliation and crown conditions, assessed according to the guidelines of the ICP Forests manual (Eichhorn et al., 2010), were studied in the context of stand characteristics and foliar features. Stand characteristics provide information on the possible detrimental (or beneficial) effects of forest structure and composition (basal area, leaf area index, tree species mixture) on crown condition. Foliar features are relevant to investigation of the cause and/or consequences of defoliation on tree health and relative physiological functions. In contrast to large scale surveys, where variation in defoliation can be associated with the change in environmental conditions, for example drought or elevation gradients (Michel and Seidling, 2014) in a local homogenous area, with uniform climatic and soil conditions, defoliation may have contrasting significance to different tree species and depends on the interaction between the characteristics of each individual species with biotic stress and environmental conditions.
Within the hypothesis that defoliation may have different ecological and physiological meanings in tree species sharing the same environment, the present survey is aimed at exploring the effectiveness of comprehensive foliar analysis, combined with the structure and composition of the forest stands, to analyze species-specific responses connected to defoliation." [line 139-155]

I appreciate to combine aspects of site, stand, LAI, defoliation, C/N, Carbon isotopes and ChlF as well as insects and fungi in the given study. However, my concern is: The main questions raised are quite general. Is the paper able to give answers to general questions such as (Summary 41) "to detect the main causes of tree defoliation in forest tree species"…. E. g. water use and drought are not at all included in the concept.
The main question is the different responses of tree species sharing the same environment. In Summary the statement was changed:
"In contrast to large scale surveys, where variation in defoliation can be associated with the change in environmental conditions, in a limited homogenous area the defoliation of co-existing tree species may have different significance and depends on the interaction between the characteristics of each individual species with biotic stress and environmental conditions". [line 40-44]

Another example: the authors relate the results to forest management. However, is there a real good argumentation how to use the results for practical forestry? Under which conditions show the results a general applicability in practical forestry?
Forest management issues are not included now in Summary and Introduction. In Conclusions the statements were changed:

“This “leaf trait analysis” approach can be applied both at local and large scale for purposes of forest management and policy. Locally it can be used to individuate the most appropriate structural characteristics of the forests to enhance the physiological efficiency of the different tree species. Moreover, through surveys repeated over time, it would be possible to assess the responses of trees to sylvicultural practices. At a large scale, this analysis can contribute to strengthening the informative potential of the ongoing routine monitoring activities (e.g., ICP Forests) providing insights about the ecological equilibrium of different tree species in a changing environment. In this perspective the leaf traits analysis can provide basic knowledge to support the elaboration of adaptive strategies.” [line 459-467]

Is the investigation and inclusion ChlF sufficient to state: "the present paper demonstrated the feasibility of the use of physiological indicators in forest surveys?
This statement is deleted. It is now added a discussion about the limits and difficult of the survey. [line 468-476]

Discussion tries to explain major results quite completely. However, it is not adequately stated, which contradictions might be given by restrictions of the data base or by the complexity of the concept. Questions such as: What might be a needed next step to clarify complex results. Which consequences can be derived from the experimental set up (samples, repetitions). Which limitations are given by statistical methods?
In conclusions the statements were changed:
“The main limitation of this study relies in the difficult of representing, with a consistent number of replicates (within and among forest stands), all the possible combination of tree species mixture whilst avoiding bias deriving from the variability of environmental factors (e.g., bedrock and exposure). Such limitations are due to the characteristics of landscape fragmentation and the difficulty of sampling leave in high forests. A large scale survey with a consistent number of forest stands may provide more robust data evidencing general tendencies and avoiding interference from local factors. The analysis of the variability of the foliar features assessed in this study (Table 2) can help to select the more robust and reliable parameters to design an effective cost-benefit surveys according to the criteria of ecological monitoring (Elzinga et al., 2001).” [line 468-476]

Therefore, I would prefer to be more precise what the authors really intend to explain. This is mainly valid in the chapter introduction (questions) and discussion/summary. In my opinion, this would be a further step of improvement of the overall value of the paper.

***Cover Letter**

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E DELL'AMBIENTE

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Subject:FORECO15463R2: **Submission revised manuscript**

Pollastrini M., Feducci M., Bonal D., Fotelli M., Gessler A., Grossiord C., Guyot V., Jactel H., Nguyen D., Radoglou K., Bussotti F., '**Physiological significance of forest tree defoliation: results from a survey in a mixed forest in Tuscany (central Italy)'**

Dear Editor,

We herewith enclose a new revised version of the manuscript and a detailed list of responses to each point raised by Reviewers.

We thank the two anonymous Referees for their constructive comments and suggestions, which has helped us to improve the paper.

Waiting for your kind consideration,

Yours Sincerely,

Martina Pollastrini