

## Effect of fungal infection on leaf gas-exchange and chlorophyll fluorescence in *Quercus ilex*

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**Abstract** – Experiments were conducted to study the susceptibility to infection by two fungal pathogens, *Cryphonectria parasitica* or *Phomopsis* spp. of undisturbed holm oaks (*Quercus ilex*) and the resprout from the stump of trees after excision of the shoot. Leaf gas-exchange and chlorophyll *a* fluorescence were recorded on plants growing in natural conditions for two years, as markers of disease progress at the first stages of infection. In infected plants, pathogen-induced stomatal closure limited photosynthesis and increased contribution of energy dissipating processes protecting PSII integrity, as shown by higher non-photochemical quenching (NPQ). Excision treatment reduced susceptibility to infection and favoured water availability in resprouts, which showed higher gas-exchange rates.

*Quercus ilex* / *Cryphonectria parasitica* / *Phomopsis* spp. / gas-exchange / chlorophyll fluorescence

**Résumé** – Effet de l'infection fongique sur les échanges gazeux et la fluorescence de la chlorophylle chez *Quercus ilex*. Le but de ce travail est l'étude de la susceptibilité des plants élagués (cas du chêne vert), à l'infection par *Cryphonectria parasitica* et *Phomopsis* spp. Pendant deux années, les échanges gazeux et la fluorescence de la chlorophylle d'un groupe de plants croissants dans des conditions environnementales naturelles ont été étudiés. Ces paramètres ont été des marqueurs convenables de l'évolution de la maladie durant les premières étapes de l'infection. Chez les plants infectés, le progrès de la maladie a été mis en évidence par la réduction de l'assimilation de CO<sub>2</sub> et l'augmentation de la participation des processus de dissipation thermique de l'énergie révélée par un NPQ élevé (Non-photochemical quenching). L'élagage induit une diminution de la susceptibilité à l'infection et permet une haute disponibilité hydrique chez les rejets assurant ainsi des taux élevés d'échanges gazeux.

*Quercus ilex* / *Cryphonectria parasitica* / *Phomopsis* spp. / échanges gazeux / fluorescence de la chlorophylle

### Abbreviations

A, net photosynthesis; g, stomatal conductance; F, fluorescence intensity at any point; F<sub>o</sub>, F'<sub>o</sub> minimum fluorescence yield in dark-adapted and light-adapted state; F<sub>m</sub>, F'<sub>m</sub> maximum fluorescence yield in dark-and light-adapted state; F<sub>v</sub>/F<sub>m</sub>, quantum yield of PS<sub>II</sub> photochem-

istry in dark-adapted state; ΔF/F'<sub>m</sub>, quantum yield of PS<sub>II</sub> photochemistry in light-adapted state); NPQ, non-photochemical quenching; q<sub>p</sub>, photochemical quenching; F'<sub>v</sub>/F'<sub>m</sub> intrinsic efficiency of open PS<sub>II</sub> centers during illumination; PS<sub>II</sub>, photosystem II; PAR, photosynthetic active radiation.

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## 1. INTRODUCTION

Holm oaks (*Quercus ilex* L.) are often subject to environmental constraints (drought, high and low temperatures and fire), typical of the Mediterranean forests. Resprouting from underground organs after perturbations is common [40]. Increased rates of gas-exchange and growth have been observed in resprouts after fire or clear-cut [14, 15, 26, 36], due to increased water [13, 17, 30] and/or nitrogen [16, 20] availability for smaller crowns. *Quercus* species are also often affected by fungal pathogens such as *Cryphonectria parasitica* (Murrill) Barr. and *Phomopsis* spp. [31, 32, 33]. In Mediterranean forests, *C. parasitica* is common in chestnut and rare in holm oak, but this fungus could become a serious threat to the latter because several species of *Quercus* (*Q. ilex*, *Q. pubescens* and *Q. petraea*) are susceptible to infection [37, 39, 40]. *Phomopsis* spp. infects holm oaks weakened by drought or other factors. *C. parasitica* causes yellowing and wilting of the foliage and localised necrosis of the bark and cambium on stems, branches or twigs. The fungus grows in the inner bark and cambium, producing small brownish mycelial fans. Yellow tendrils of conidia may be present when cankers encircle the stem, killing the vascular cambium and leading to the death of the tree.

Lesions of *Phomopsis* canker on holm oak branches are slightly depressed, purplish or greyish in colour, darkening later and spreading to encircle the stem. Young twigs in the diseased area become stunted and leaves turn brown and dry [32]. Wilting in plants affected by vascular pathogens has been attributed to reduction of water transfer in plants due to destruction of cortical tissue or increased resistance to water flow through xylem elements [1, 2, 9, 23, 27].

We studied the susceptibility of holm oak to infections by *C. parasitica* or *Phomopsis* spp. and we have determined leaf gas-exchange and chlorophyll fluorescence for two years to assess whether these non-destructive techniques are suitable tools for recording disease progress in plants inoculated with fungal pathogens in natural conditions. The second aim was to elucidate whether plants subjected to excision of the shoot showed different susceptibility to *C. parasitica* or *Phomopsis* spp. infection than undisturbed plants, and if so, whether this would be reflected in gas-exchange and chlorophyll fluorescence measurements.

During the summer, when soil and atmospheric water deficits are high, species of the Mediterranean forests undergo a midday depression of photosynthesis and leaf conductance [38] due to stomatal closure, which can be accentuated by vascular pathogens. Moreover, restricted CO<sub>2</sub> fixation enhances susceptibility to photoinhibition

[10, 11], since the light absorbed can greatly exceed that required for carbon assimilation. Due to the fact that resprouts from the stump of trees after excision exhibit better photosynthetic performance under stressing conditions due to greater water availability [15], we measured gas-exchange and chlorophyll fluorescence at midday in order to identify possible differences in the responses to environmental constraints in infected plants submitted to excision or undisturbed.

## 2. MATERIALS AND METHODS

### 2.1. Plant and fungus material

Studies were carried out on sixty 3-year-old *Q. ilex* plants (ranging from 8 to 12 mm in diameter) growing in 6.5 L pots with loam in natural conditions in the Experimental Fields of the Faculty of Biology at the University of Barcelona (Spain). The climate at the site is typically Mediterranean with cold winters, cool wet springs and autumns and hot dry summers, with a mean annual temperature of 13–14 °C and an annual precipitation of 500–700 mm. Plants were irrigated daily with 1.33 L water during autumn and winter and with twice this quantity in spring and summer in order to avoid superimposed soil water stress.

Pathogenic cultures of *C. parasitica* isolated from chestnut (*Castanea sativa* Mill.) and *Phomopsis* spp. isolated from twigs of holm oak (*Q. ilex*) were maintained on 3.9% Difco potato dextrose agar (PDA) in Petri dishes.

### 2.2. Plant inoculation and experimental design

For the *Cryphonectria parasitica* study, 30 plants were divided into two groups; in 15 plants (E-plants), shoots were subjected to excision (28.4.1997) below the lowest branch and removed; the remaining 15 were left undisturbed (U-plants). One week later (5.5.1997), in each group, 10 plants were inoculated in the trunk with *C. parasitica* and 5 plants were given only an agar plug and used as controls. Inoculation was performed after disinfecting the bark surface in 95% ethanol for 10 s. Thereafter, a 15 mm long superficial wound was made with a scalpel on the bark tissues approximately 30 cm above the ground level. A 5 mm diameter plug was removed from the margin of culture that had been grown on PDA for 6 days at 25 °C and placed mycelium-side-down on the wound. The inoculated area was sealed with parafilm.

In the *Phomopsis* spp. study, 30 plants were divided into two groups; in 15 plants (E-plants), shoots were

subjected to excision (28.4.1997) above the lowest branch. This remaining branch was left for inoculation. The other 15 plants were left undisturbed (U-plants). One week later (5.5.1997), in each group, 10 plants were inoculated with *Phomopsis* spp. on the lowest branch and 5 plants were given only an agar plug and used as controls. Inoculation was performed in the same way as for *C. parasitica*. *Phomopsis* spp. was inoculated on a thin branch since its effect on the trunk or thicker branches is only slight.

Two months after inoculation, *Q. ilex* plants were inspected for the presence of cankers and their length was recorded. The presence of mycelia, pycnidia and conidia was recorded and canker size was measured every two months throughout the study. Vascular cambium colonization was determined at the end of the study.

Gas-exchange and fluorescence measurements were carried out over two years on four leaves at similar ontogenic stage (young and fully expanded) of four randomly selected plants for each treatment combination (i.e. Inoculated U-plants, Inoculated E-plants, Control U-plants, Control E-plants). Measurements were always conducted on the same group of leaves, which were marked at the beginning of the study and showed no chlorosis or senescence symptoms. In the *C. parasitica* experiment, leaves were selected from the first branch up from the wound. In the *Phomopsis* spp. experiment, leaves of the wounded branch were selected.

### 2.3. Measurements

Gas-exchange measurements were carried out with a portable LI-6200 (Li-Cor, Inc. Lincoln, NE, USA) system. In one measurement day, net photosynthesis ( $A$ ), stomatal conductance ( $g$ ), transpiration ( $E$ ) and intercellular  $\text{CO}_2$  concentration ( $C_i$ ) on attached leaves were determined for the different groups of plant. Each replicate was carried out in 20–40 s. Leaf area was estimated from leaf images obtained with an Epson GT5000 scanner. Images were then processed using image analyser software supplied by Servei Científic-Tècnic (Universitat de Barcelona).

Immediately after gas-exchange measurements, components of chlorophyll fluorescence were quantified on the same leaves with a portable modulated fluorometer (Mini-Pam Photosynthesis Yield Analyzer, Walz, Effeltrich, Germany). The instrument was equipped with a leaf-clip holder (2030-B, Walz) including a micro-quantum sensor to monitor PAR and a thermocouple to measure temperature at the lower leaf surface. After clamping the leaf-clip holder onto the leaf, the actual fluorescence  $F$ , was monitored to ascertain that it was sta-

ble. The maximum fluorescence yield was measured by exposing the leaf to a 0.8 s saturating flash at  $6000 \mu\text{mol m}^{-2} \text{s}^{-1}$  during exposure to natural illumination and the effective PS<sub>II</sub> quantum yield,  $\Delta F / F'_m$  (equivalent to  $(F'_m - F) / F'_m$ ) [18] was recorded. After these measurements, leaves were wrapped in aluminium foil to measure dark-adapted fluorescence:  $F_o$ ,  $F_m$  and  $F_v / F_m$  (potential quantum yield of PS II equivalent to  $(F_m - F_o) / F_m$ ). The adaptation time was at least 20 minutes, after which values of  $F_v / F_m$  reach about 95% of pre-dawn ones in *Q. ilex* [17]. Data were corrected for changes in measuring light intensity induced by temperature changes in the Mini Pam. Correction was calculated by monitoring the fluorescence signal of a standard provided with the instrument. Non-photochemical quenching coefficient (NPQ, equivalent to  $(F'_m - F'_m) / F'_m$ ) was calculated and photochemical quenching ( $q_p$ , equivalent to  $(F'_m - F) / (F'_m - F'_o)$ ) and intrinsic efficiency of open PS<sub>II</sub> centers ( $F'_v / F'_m$ , equivalent to  $(F'_m - F'_o) / F'_m$ ) were estimated [29].

Gas-exchange and chlorophyll fluorescence measurements were conducted around midday (12:00-14:00).

### 2.4. Statistical design and analyses

Statistical analyses were conducted by repeated measures ANOVA, using SPSS for Windows (versions 6.31 and 8.0.1, SPSS Inc.). A complete, repeated measures design was used, with two fixed main factors, their interaction, and time as the factor for repetition. This results in a quite complex model in which differences accepted or rejected after the statistical tests are not always obvious when directly looking at the data in graphs and tables. Main effects and interactions were tested against appropriate error terms, for perturbation (excised vs undisturbed), infection (inoculated vs control plants), and day of measurement (a random factor). For parameters sensitive to variations in light, PAR was used as a covariate. Number of replicates is indicated in figure legends.

## 3. RESULTS

### 3.1. Disease progress

#### *Cryphonectria parasitica* treatment:

Inoculation by *C. parasitica* was effective in all holm-oak plants (undisturbed or subjected to excision). The extent of the lesion was 53% lower in E-plants. During the first year after infection, the vegetative growth and sporulation of *C. parasitica* was abundant,

and the growth of the canker was 19% lower in E-plants (*figure 1*). Visual symptoms of *Q. ilex* infected by *C. parasitica* during the first year included localised necrosis of the bark on inoculated stems. The bark on the canker was split, and irregularly swollen with sunken areas. Lesions were purplish with an irregular outline around the canker. Pycnidia were produced in orange brown erumpent stromata and yellow tendrils of conidia were present. During the second year, the length of the lesion and canker dimensions were respectively 75% and 25% lower in plants subjected to excision (*figure 1*). The canker development was mainly in length. Many of the infected trees had stems completely affected by the canker, but plants subjected to excision developed healthy resprouts below the canker. At the end of the study, 47% of the vascular cambium was affected in undisturbed plants and 20% in E-plants.

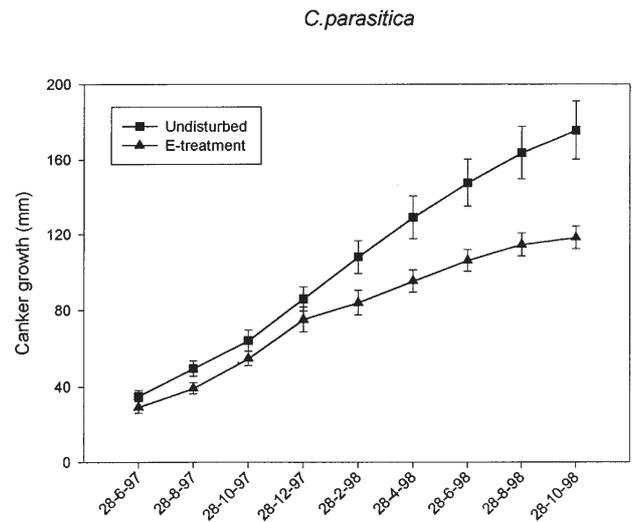
### *Phomopsis* spp. treatment:

Inoculation by *Phomopsis* spp. was effective in all kinds of holm-oak plants (U-plants or E-plants). Vegetative growth and sporulation of *Phomopsis* spp. was low and plants showed canker extension only in the inoculated branches. Canker length increased during the first 6 months after inoculation, decreasing thereafter in association with the beginning of callus tissue formation on the edges of the canker. Plants subjected to excision showed 16% lower growth of the canker (*figure 2*) and a faster healing than U-plants. The second year after inoculation cankers were completely healed in all treatments.

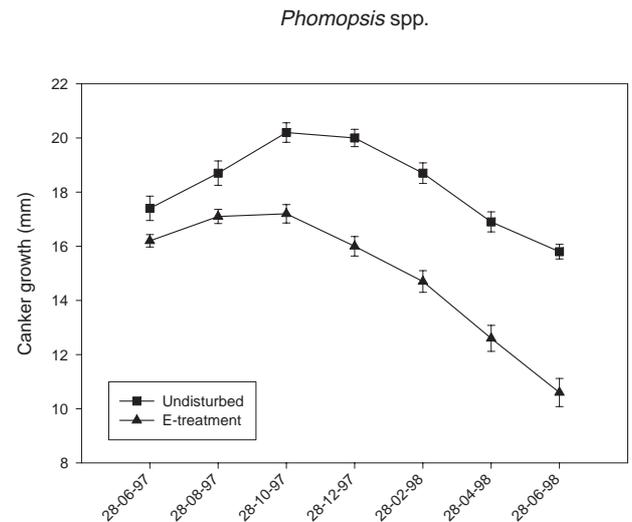
Leaves of plants selected for gas-exchange and chlorophyll fluorescence measurements did not show chlorosis or senescence symptoms during the first year of study in either infection treatment. At the end of the second year after inoculation (April 1999), leaves of the infected plants by *C. parasitica* were chlorotic, whereas leaves from *Phomopsis* spp. inoculated plants were still asymptomatic.

### 3.2. Gas exchange

A significant reduction in net photosynthesis (*A*) and stomatal conductance (*g*) in undisturbed or E-plants inoculated with *Cryphonectria parasitica* was observed during the following summer, autumn and especially in the winter (1997–1998) (*figures 3a,b,c,d*). A significant effect of excision on gas exchange rates was only observed during the first summer (1997): E-plants of control and infected plants showed higher rates than undisturbed plants. From spring 1998 and during the second year, the effect of infection on gas-exchange was not detected in spite of the progress of the disease in inocu-

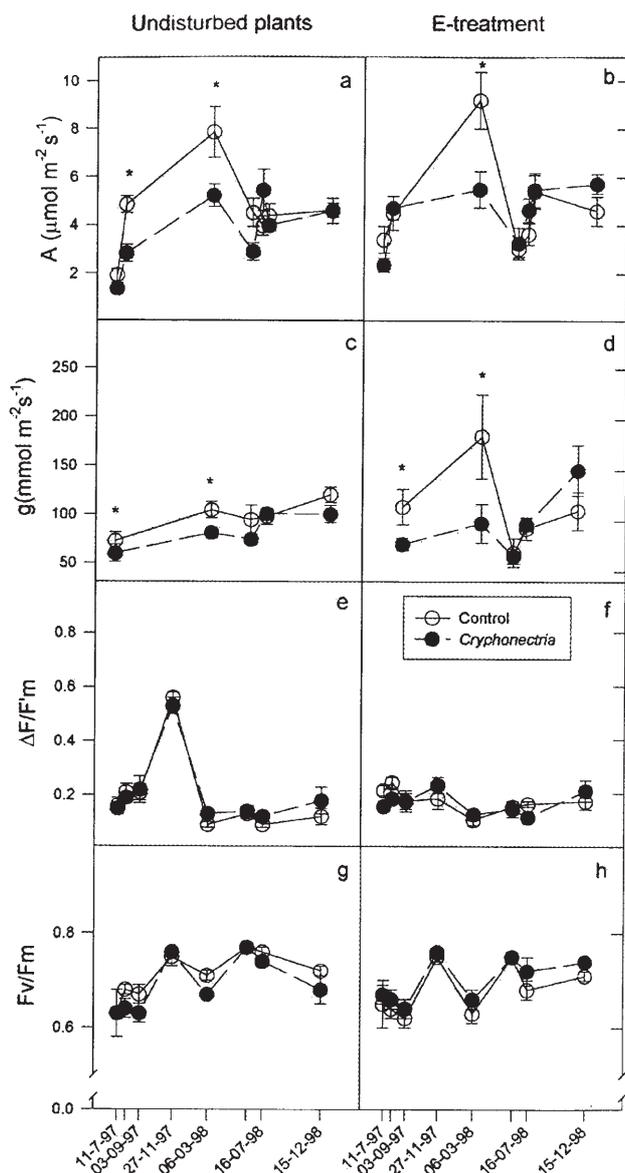


**Figure 1.** Canker length progression in undisturbed plants and plants subjected to excision infected by *Cryphonectria parasitica*.



**Figure 2.** Canker length progression in undisturbed plants and plants subjected to excision infected by *Phomopsis* spp.

lated plants (*figure 1*), that lead to the mortality of 10% of the plants in spring 1999. The surviving plants had brownish leaves and their photosynthesis rates were 25% lower than controls.



**Figure 3.** Gas exchange and fluorescence parameters of *Q. ilex* leaves of undisturbed plants (U) or plants subjected to excision (E), during two years after the inoculation with *Cryphonectria parasitica* in comparison to control plants. a, b: Net photosynthesis (A); c, d: stomatal conductance (g); e, f: effective PS<sub>II</sub> quantum yield ( $\Delta F/F_m$ ); g, h: PSII quantum yield in the dark adapted state ( $F_v/F_m$ ); i, j: non-photochemical quenching (NPQ). Values per day of measurement are means  $\pm$  S.E. for 4 leaves per 4 plants per each treatment combination. Asterisks mark significant differences between data ( $P < 0.05$ ) according to the statistical model.

Inoculation with *Phomopsis* spp. in undisturbed plants reduced A and g during the following year, espe-

cially during winter 1997-1998 (figures 4a,c), whereas E-plants showed no significant effect of infection (figures 4b,d). No differences in gas-exchange between inoculated and control plants from winter 1997-1998 until the end of the study (spring 1999) were observed. The effect of excision was only observed during the first summer, with higher gas-exchange rates in E-plants.

### 3.3. Chlorophyll fluorescence parameters

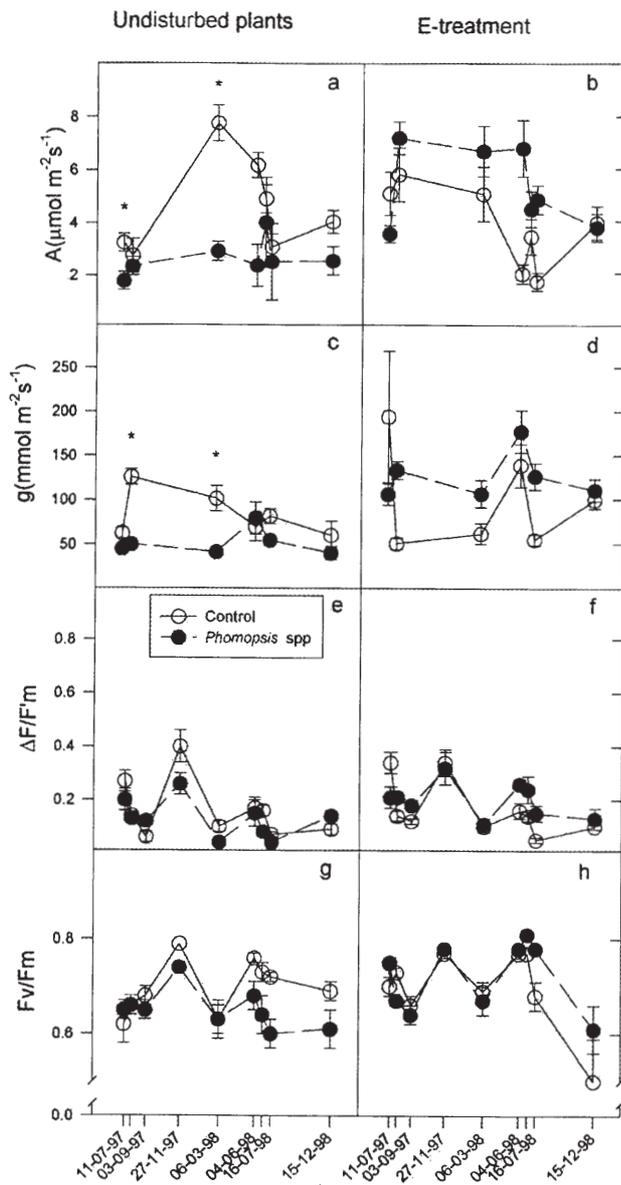
Inoculation with *C. parasitica* or *Phomopsis* spp. or excision treatment did not affect the effective PS<sub>II</sub> quantum yield ( $\Delta F/F_m$ ) and midday potential quantum yield of PS<sub>II</sub> ( $F_v/F_m$ ) throughout the study (figures 3e,f,g,h, figures 4e,f,g,h).  $q_p$ , (which represents fraction of open PS II centers) were not affected by inoculation with *C. parasitica* or *Phomopsis* spp. but  $F'_v/F'_m$  (which represents the efficiency of open centers) was higher in plants infected by *C. parasitica* during the first summer. (figures 5c,d, e,f and figures 6c,d,e,f). Excision effect was not detected.

During the first summer, non-photochemical quenching (NPQ) was higher in plants infected with *C. parasitica* or *Phomopsis* spp (figures 5a,b and figures 6a,b), but excision treatment had no significant effect.

## 4. DISCUSSION

*Q. ilex* plants infected by *C. parasitica* showed a disease progression that led to the death of 10% in two years (figure 1), whereas plants infected by *Phomopsis* spp. showed infection proliferation only the first six months after inoculation, healing thereafter (figure 2). Plants reacted to *Phomopsis* spp. infection with structural and chemical defence mechanisms, periderm formation and activated lignification that reduced colonization. Moreover, *Phomopsis* spp. can be aggressive on young holm oaks already weakened by overcrowding or drought, which was not our case, since inoculated trees were watered at regular intervals. Consequently, they closed the wound and inhibited fungus colonization.

The pathology described was only reflected on leaf gas-exchange and chlorophyll *a* recording at the first stages of infection by *C. parasitica* or *Phomopsis* spp. During the first nine months following inoculation, (April 1997-January 1998), photosynthetic rates (A) and stomatal conductance (g) decreased due to infection both in undisturbed plants and plants subjected to excision (figures 3a,b and figures 4a,b). Differences between infected plants and controls were especially marked during the first winter after infection, probably because



**Figure 4.** Gas exchange and fluorescence parameters of *Q. ilex* leaves of undisturbed plants (U) or plants subjected to excision (E), during two years after the inoculation with *Phomopsis* spp. in comparison to control plants. a, b: Net photosynthesis ( $A$ ); c, d: stomatal conductance ( $g$ ); e, f: effective  $PS_{II}$  quantum yield ( $\Delta F/F_m$ ); g, h: potential  $PS_{II}$  quantum yield ( $F_v/F_m$ ); i, j: non-photochemical quenching (NPQ). Values per day of measurement are means  $\pm$  S.E. for 4 leaves per 4 plants for each treatment combination. Asterisks mark significant differences between data ( $P < 0.05$ ) according to the statistical model.

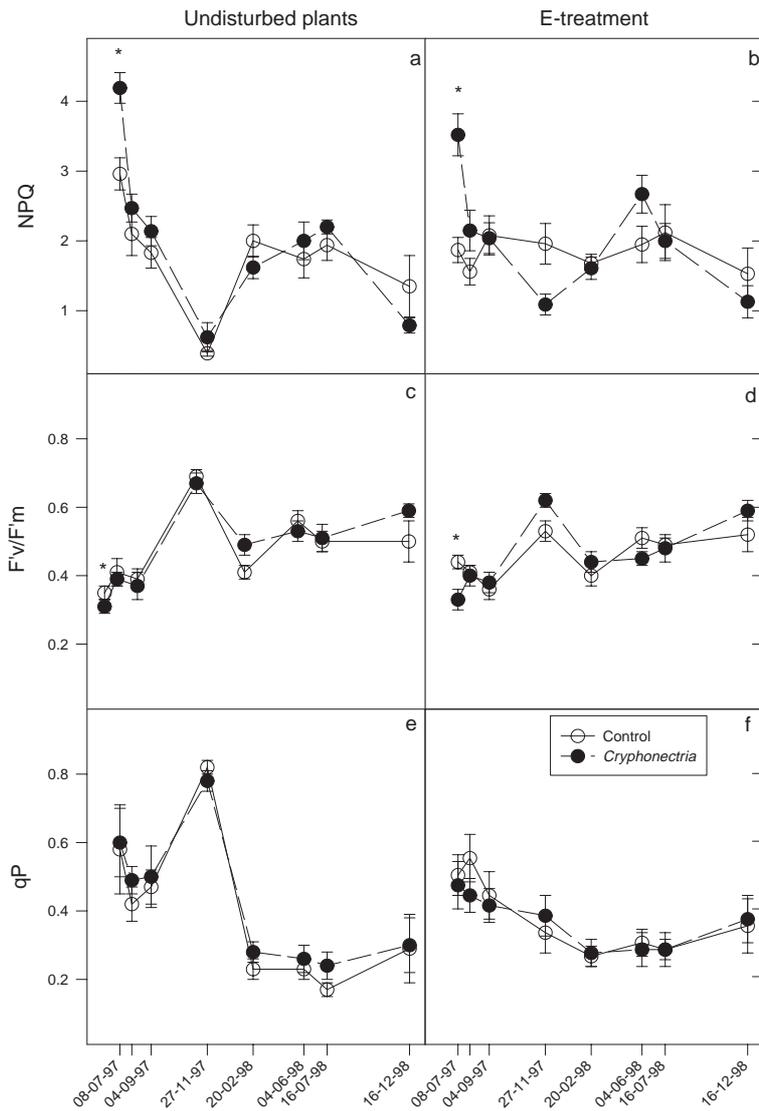
pathogen-related effects might be more restricted in other seasons, especially during summer stress, when drier atmospheric conditions would limit  $g$  and  $A$  in con-

trol plants. The parallelism between  $A$  and  $g$  variations and the constant concentrations of intercellular  $CO_2$  ( $C_i$ , data not shown) suggests a direct effect of infection on the biochemistry of photosynthesis. Nevertheless, some authors suggest that a close coupling between  $A$  and  $g$  might rely on a mechanism other than  $C_i$ ; as a consequence, intercellular  $CO_2$  would remain constant [25]. Then, in a long-term experiment like ours in which acclimation is likely to occur, it would be difficult to determine which parameter changed first. Both fungal infections provoke bark and vascular cambium alterations that may affect water relations (e.g. reduction of flux to the leaves) as observed in cork oak plants inoculated with vascular pathogens such as *Botryosphaeria stevensii* or *Hypoxylon mediterraneum* [24]. In our work, the decrease in stomatal conductance in infected plants might be indicative of water stress and consequent photosynthetic reduction. No measurements of leaf water potential were undertaken in order to avoid an excessive defoliation that would alter sink-source relationships, but depression in photosynthetic activity due mainly to drought in plants inoculated with wilt fungi has been described by several authors [4, 5, 6, 22, 34].

Although our calculations of  $C_i$  might be affected by stomatal patchiness, as has been described in Mediterranean species under drought, we do not believe this is the case. Cornic and Masacci [8] concluded that patchiness probably occurs only when dehydration is very rapid and thus might not occur in the field. This is especially true for our case, as plants were watered daily.

The effect of infection was detected on non-photochemical quenching (NPQ) (figures 5a,b and figures 6a,b), during the first summer 1997. Plants infected with *C. parasitica* or *Phomopsis* spp. showed higher NPQ, indicative of the participation of thermal energy dissipation by the xanthophyll cycle [3, 19]. Photosynthesis limitation by pathogen induced stomatal closure favoured dissipation of excess energy as heat [12, 28] in infected plants, preventing damage to the  $PS_{II}$  reaction centers.

The effect of infection with *C. parasitica* or *Phomopsis* spp. on the effective  $PS_{II}$  quantum yield ( $\Delta F/F_m$ ) was not statistically significant, although a decreasing trend was observed in infected trees (figures 3e,f and figures 4e,f). In spite of this, we did observe that one of the components of  $\Delta F/F_m$ ,  $F'_v/F'_m$  (which represents efficiency of open  $PS_{II}$  centers) was lower in infected plants in the *Cryphonectria* experiment in summer 1997 (figures 5c,d). The other component,  $q_p$ , (which represents fraction of open  $PS_{II}$  centers) was not affected by infection (figures 5e,f). These results indicate that differences in fluorescence parameters due to



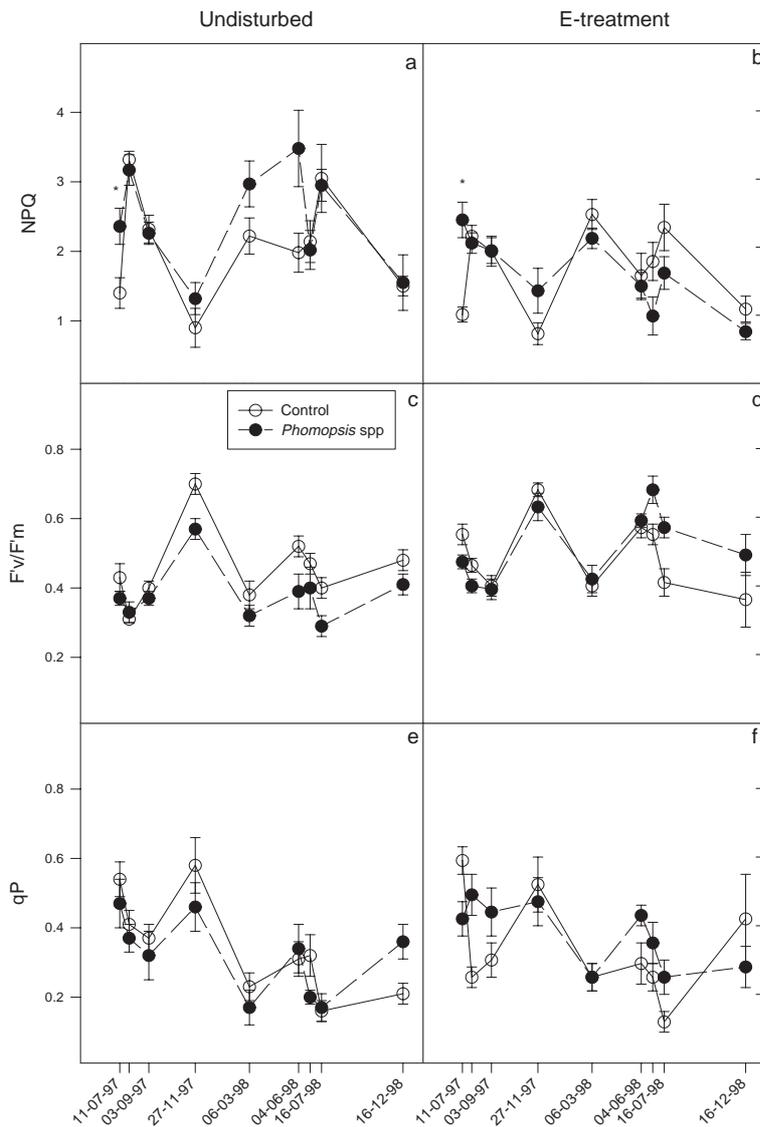
**Figure 5.** Photochemical quenching ( $q_p$ ), intrinsic efficiency of open PS<sub>II</sub> centers during illumination ( $F_v/F_m$ ) and non-photochemical quenching (NPQ) of *Q. ilex* leaves of undisturbed plants (U) or plants subjected to excision (E), for two years after the inoculation with *Cryphonectria parasitica*. Values per day of measurement are means  $\pm$  S.E. for 4 leaves per 4 plants per 4 per each treatment combination. Asterisks mark significant differences between data ( $P < 0.05$ ) according to the statistical model.

infection during the first summer were in some cases not significant due to the complexity of the statistical design.

Mean values of midday potential quantum yield of PS<sub>II</sub>, ( $F_v/F_m$ ) were also similar for all kinds of treatment ( $0.7 \pm 0.01$ ) (figures 3g,h and figures 4g,h) and only slightly lower than reported pre-dawn values for this species (0.78) [17]. The lack of differences in  $F_v/F_m$  between inoculated and control plants denoted that light processing structures in PS<sub>II</sub> were not affected by infection.

Plants subjected to excision were less susceptible to infection by *C. parasitica* or *Phomopsis* spp., with lower lesion extensions and canker growth (figures 1 and 2), probably due to the lack of upper branches which act as nutrient source for the growth of mycelial fans [7].

This lower susceptibility to infection was reflected in higher leaf gas-exchange rates in E-plants during the first summer with respect to undisturbed plants. Under stressing conditions at summer midday, with high temperatures, PAR and vapour pressure deficit, water availability by sprouts is greater than in undisturbed plants due



**Figure 6.** Photochemical quenching ( $q_p$ ), intrinsic efficiency of open PS<sub>II</sub> centers during illumination ( $F'_v/F'_m$ ) and non-photochemical quenching (NPQ) of *Q. ilex* leaves of undisturbed plants (U) or plants subjected to excision (E) plants, for two years after the inoculation with *Phomopsis* spp. Values per day of measurement are means  $\pm$  S.E. for 4 leaves per 4 plants per each treatment combination. Asterisks mark significant differences between data ( $P < 0.05$ ) according to the statistical model.

to a reduced shoot-to-root ratio [13, 35]. This fact enables resprouts to maintain higher stomatal conductance and thereby increase net photosynthesis and growth during the first year [21]. Moreover, during the first summer photosynthetic activity was higher in resprouts of plants infected by *C. parasitica* than in infected plants not submitted to excision.

The results indicate that leaf gas-exchange and chlorophyll a fluorescence measurements can be used to detect early alterations in asymptomatic leaves of plants infected by fungal pathogens that provoke colonization into the vascular cambium. Nevertheless, in long-term

studies of infections, these methods are not conclusive, since disease progression can be stimulated or depressed by changes in the environment. Moreover, in natural conditions, the effect of fungal infections can be masked by the interactions of different stresses (high or low temperatures, high light and drought). Excision treatment reduced the susceptibility to infection and improved gas-exchange of resprouting infected plants under stressing atmospheric conditions.

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