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Ecophysiological responses and vulnerability to other pathologies in European chestnut coppices, heavily infested by the Asian chestnut gall wasp

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ABSTRACT

Dryocosmus kuriphilus Yasumatsu, the Asian chestnut gall wasp (ACGW), is an invasive alien species, which is causing alarm in the chestnut stands of Italy and Europe. It has extensively colonised the *Castanea sativa* Miller range throughout the country and highly conspicuous, alarming symptoms have appeared on plants. In addition, chestnut trees growing in Mediterranean climates are more frequently subjected to stressful environmental conditions, such as hot-dry periods or extreme weather events, which compromise both yield and productivity. It will be useful, therefore, to gain further insights into the biotic and abiotic disturbances affecting chestnut and their interactions in order to develop management strategies to counteract the loss of productivity.

This study was aimed at investigating the effects of ACGW on European chestnut ecophysiology during warm, dry summer conditions, typical of Mediterranean environments. We studied the functional and structural responses of young chestnut sprouts in a coppice stand in the Apennines (Tuscany, Italy), focussing in particular on photosynthetic capacity, leaf morphology, shoot growth, and hydraulic architecture. We also assessed the vulnerability of sprouts to chestnut blight *Cryphonectria parasitica* (Murrill) Barr. ACGW is a gall-making insect with strong dispersal potential. It spends its larval stages inside the chestnut buds and grows to adult state inside galls, which are located mainly on the main leaf axis or petiole. In this study, we found a reduction of about 30% in CO_2 assimilation and stomatal conductance in the blades of galled leaves. PSII efficiency (Φ_{PO}) was not negatively affected by the presence of galls, although lower electron transport to PSI acceptors (ΔV_{IP}) was found in galled leaves, which could have negative consequences for $NADP^+$ production and carboxylation.

ACGW strongly affected the photosynthesizing leaf area, which was reduced by about 40% compared with a non-galled leaf. Carbohydrate concentration was higher in leaf blades while galls were richer in starch.

Shoot vigour was affected by a massive presence of ACGW, resulting in a smaller leaf area and biomass, and very low capacity for water transport through the wood xylem compared with vigorous shoots. In fact, compared with vigorous shoots, non-vigorous shoots had a higher percentage of impaired xylem conductive area (5.7% vs. 1.3%) and a higher number of obstructed vessels per mm^2 (31.2 vs. 7.4).

Assessment of shoot vulnerability to chestnut blight revealed a prevalence of hypovirulence in blight infections, probably not directly due to ACGW, but instead related to loss of vigour in the shoot. However, ACGW could play a role in the appearance and spread of *Gnomoniopsis* sp., which was the most common endophyte on vigorous and non-vigorous shoots and the main coloniser of old galls.

ACGW is opening up unpredictable scenarios in European chestnut forests, which are also susceptible to environmental stress factors. Therefore, the simultaneous study of disturbance dynamics and chestnut response, and advanced monitoring of insect spread and climate change would allow timely and effective implementation of adaptive forest management strategies.

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1. Introduction

Recently, increasing concern has been raised about the appearance and spread of new alien (or exotic) invasive pests and pathogens (Bale and Hayward, 2010). The number of immigrant species reported worldwide is increasing dramatically and almost 10% of them (Williamson, 1996) have become invasive, with implications at socio-economic (yield losses, increased production costs, changes in the landscape, endanger of human health), environmental and ecological levels (reduction of native biodiversity and genetic variability, impacts on ecosystem services) (Wheeler and Hoebeke, 2009).

Among invasive species, insect pests and diseases can have direct effects on their host plants. For example, feeding insects directly reduce leaf biomass by redirecting photosynthates from plant organs for their own benefit (Shorthouse et al., 2005), but they also induce physiological responses in the host plant, which affect the remaining tissue (Zangerl et al., 2002), or activate strategy for carbon-based defence mechanisms in plants (King and Caylor, 2010) with an increase of carbohydrate production.

Gall-inducing insects cause in their host plants tumour-like structures that provide a warmer microenvironment for the pest insect. These galls may also have the ability to photosynthesize (Fernandes et al., 2010), thus serving as either a shelter or a source of food for the larval instars of the insect. There is controversy over the effects of leaf gall-inducing insects on the physiological behaviour of leaves. Galls have been found to have either negative (Gailite et al., 2005; Aldea et al., 2006; Fernandes et al., 2010) or positive effects on photosynthesis (Fay et al., 1993; Bogatto et al., 1996), including mechanical damage and/or arthropod-derived chemical signals, which affect endogenous plant signals and, consequently, the physiology of the host plant (Raman, 2007). The feeding strategies of gall-inducing insects may alter several parameters associated with leaf energy balance, such as stomatal conductance and absorbance of solar radiation (Pincebourde et al., 2005). Gall infestation might, therefore, produce effects at the macroscopic level; for example, an induced change in leaf coloration could alter the leaf microclimate and amplify the effects of environmental variations on the transpiration rate (Pincebourde and Woods, 2012); more generally, it might affect canopy processes and ecosystem functions.

One of the most prolific invasive gall-inducing insects is *Dryocosmus kuriphilus* Yasumatsu, the Asian chestnut gall wasp (ACGW) (Hymenoptera: Cynipidae). Native to China, this pest has rapidly spread out across chestnut woods and orchards of Europe, USA and Asia (Fig. 1).

Fig. 1. ACGW galls on new leaves of European chestnut trees.

The gall wasp was first recorded in northern Italy in the early years of this century (Brussino et al., 2002) and since then it has established extensively throughout the country. ACGW has now colonised almost the whole European chestnut (*Castanea sativa* Miller) range in Italy, which covers a conspicuous and contiguous surface area of 788,408 ha (INFC, 2008) and is present in the whole peninsula and in Sardinia and Sicily islands.

Aside from the absence of natural limiting factors, such as parasitoids and predators, this formidable colonisation rate can be ascribed to the high reproductive potential of this hymenoptera. ACGW is a parthenogenetic species and its populations are entirely constituted by female individuals, which are able to lay 10–150 eggs in chestnut buds (EPPO, 2005). Moreover, the absence of evident symptoms in scions and seedlings between July and April, owing to the long asymptomatic growth phase from egg to the second larval stage, has resulted in the diffusion of infected commercial material, that has triggered the insurgence of new foci. Finally, the ability of ACGW to move with prevailing winds has probably been underestimated and has concurred in its massive invasion (Graziosi and Rieske, 2012). These factors have frustrated eradication attempts and prompted an immediate and extensive programme of biological control, which involved the release of the specific parasitoid *Torymus sinensis* Kamijo in the areas of chestnut cultivation (Quacchia et al., 2008).

Until now, only scattered evidence has been gathered on the impact of ACGW on fruit and wood production and on chestnut vitality. Bosio et al. (2010, 2013) reported a reduction in the fruit harvest in the north-western Italy, while Battisti et al. (in press) found a clear correlation between the number of galls and the decrease in fruit production in the northeast. Maltoni et al. (2012a, 2012b) analysed and classified different kinds of damage on leaves, shoots and buds in chestnut sprouts, confirming the observations carried out in Japan by Kato and Hijii (1997) on *C. crenata* Siebold and Zucc.

Moreover, there have been warnings of a possible negative interaction between ACGW and *Cryphonectria parasitica* (Murrill) Barr, the fungal agent of chestnut blight. Hypovirulent strains of *C. parasitica* are well established in Italian chestnut woodlands and the disease can be considered as endemic but not hazardous (Turchetti et al., 2008). Recently, new virulent chestnut blight infections following ACGW colonisation were reported by Turchetti et al. (2010a) in Italy and by Prospero and Forster (2011) in Switzerland. The latter authors suggested that abandoned galls might facilitate penetration and infection by *C. parasitica* in twigs.

However, plants are not subjected only to biological diseases. Environmental conditions and changes in climate conditions are additional factors exacerbating the host functionality and consequently insect outbreaks, even though no clear trends have been found yet (e.g., Raffa et al., 2008; Büntgen et al., 2009). In plants, changes in climatic variables can affect leaf gas exchange, productivity and phenology, and potentially also the frequency and degree of insect damage. Given that extreme climate events (such as summer drought) are increasing in frequency and/or intensity (Brunetti et al., 2004; Nanni et al., 2007), they should not be mistreated and further knowledge of how these diverse factors interact may be useful in order to develop strategic proactive management plans to counteract adverse effects.

Therefore, the aim of this study was to investigate the effects of ACGW on European chestnut physiology during warm, dry summer conditions, typical of Mediterranean environments. To this purpose, we studied the functional and structural responses of young chestnut sprouts in a coppice stand in the Tuscan Apennines (Italy) colonised by ACGW. The study focused on physiological responses to environmental stresses, by measuring gas exchange, chlorophyll fluorescence, leaf morphology, stomatal density, stem

growth and hydraulic architecture. Eventually, the vulnerability of sprouts to chestnut blight was also assessed.

2. Materials and methods

2.1. Study area and meteorological data

Physiological responses of European chestnut to ACGW infestation were studied at three sites located within an extensive chestnut coppice area on the gently sloping hills of the Pistoia Apennines (Tuscany, Italy), no more than two km from each other. Rock substrate and exposure are similar at the three sites, but they are at different elevations: San Mommè (Lat. 44.0213; Long. 10.9198) is at 700 m a.s.l., Collina A (Lat. 44.0209; Long. 10.9285) 915 m a.s.l., and Collina B (Lat. 44.0148; Long. 10.9322) 850 m a.s.l.

The study was carried out in 2012 when the coppice stands in San Mommé and Collina B were 3 years old and Collina A 4 years old.

ACGW infestation had occurred in the area a few years earlier (ARSIA, 2008), and hence the wasp was already present during our study.

These mountains have a continental Mediterranean climate with a mean annual rainfall of about 2000 mm, falling mainly from October to March, and abundant snow at higher elevations. Summertime is characterised by a high daily temperature range: the average difference between minimum and maximum temperatures is 10–12 °C.

The meteorological station closest to the sites is Acquerino, seven km from the study area, at 920 m a.s.l. It is part of the LaMMa (Laboratorio per la Meteorologia e la Modellistica Ambientale) network and has been recording data almost continuously since 1961. Meteorological data used in this work are from the year 2012 and the long-term series 1961–1988.

2.2. Sampling and analysis of European chestnut responses to the Asian chestnut gall wasp

San Mommé was deemed the reference study site because it is located at the *optimum* elevation for *C. sativa*. Investigations at this site focussed on the physiological responses of European chestnut to ACGW infestation at the leaf level (gas exchanges and chlorophyll *a* fluorescence, leaf area and stomatal density, sugar compounds) and shoot level (shoot vigour, xylem conductivity, xylem anatomy, leaf and wood biomass).

Finally, a cluster of stumps representative of the site was selected to evaluate sprout vulnerability to chestnut blight and to assess the general damage caused by the galls.

Collina A and Collina B served as replicates for leaf physiology and traits.

Table 1 summarizes the investigated parameters.

2.2.1. Leaf analyses

2.2.1.1. Leaf gas exchanges. Leaf gas exchange was monitored at San Mommé in 2012 (19th and 25th May; 16th and 29th June; 14th July; 4th and 21st August) using a gas analyser CIRAS-1 (PP Systems, Hitchin, UK). Measurements of stomatal conductance (*gs*), leaf transpiration (*Tr*), net photosynthesis (*Pn*) and internal CO_2 (*Ci*) were taken on sunny days at 10–11 a.m. with PAR > 1000 μmol photons m^{-2} s^{-1}.

A total of 20 sunlit leaves (10 non-galled and 10 galled) were selected from ten stumps. One non-galled leaf and one galled leaf were chosen from a sunlit position on each stump and only one measurement was taken on each leaf.

2.2.1.2. Chlorophyll a fluorescence. Direct chlorophyll *a* fluorescence (Chl *a* fluorescence) was measured at San Mommé, Collina A and Collina B using a Handy-PEA fluorimeter (Hansatech, Instruments Ltd., King's Lynn, UK). Measurements were taken at 10–11 am on May 28th, June 16th and 29th, July 14th, and August 4th and 21st.

A total of 40 leaves (20 non-galled and 20 galled) were selected from two sprouts on each of the same stumps as above. One non-galled and one galled leaf, very close to each other and under the same exposure conditions (sunlit/partially sunlit), were selected from each sprout.

Leaves were dark-adapted for 25 min with leaf clips, and direct fluorescence was then detected during 5 s of exposure to actinic light. Data were downloaded using the Winpea 32 software (v.1.00, Hansatech, Instruments Ltd., King's Lynn, UK) and processed in a Biolyzer 3.0 (JIP-Test Analysis Program v.3.0, Laboratory of Bioenergetics, University of Geneva). The Time Marks for calculating the parameters for the JIP-test were set at 0.05; 0.1; 0.3; 2; 30 ms. Finally, the parameters were exported into Office Excel for statistical analysis. Analysis of the fluorescence transient was performed using the JIP-test (Strasser et al., 2000, 2004), which translates the original data into the biophysical parameters – all referring to time zero (onset of fluorescence induction) – that quantify PSII behaviour.

The analysed parameters are reported in Table 2.

2.2.1.3. Leaf morphological traits and stomatal density. At the end of June, a total of 120 leaves were collected from all sites (San Mommé, Collina A, Collina B) for the analysis of leaf morphological traits.

At each site, 20 non-galled leaves and 20 galled leaves were collected from 2 sprouts of each of 10 stumps. The non-galled and galled leaves chosen from each sprout were very close to each other and under the same exposure conditions (sunlit/partially sunlit).

The samples were sealed in separate plastic bags and stored in a refrigerator bag surrounded by ice packs to keep them cool during transportation to the laboratory.

Table 1
Summary of the analysis.

Plant organ	Parameter	Month	Sites
Leaf	Leaf gas exchanges	May–August	Reference: San Mommé;
	Chl a fluorescence	May–August	Replicates: Collina A and Collina B
	Leaf morphological traits and stomatal density	June	
	Sugars/starch in leaf blades and galls	July	
Shoot	Qualitative analysis: vigour	July	Reference: San Mommé
	Quantitative analysis:		
	Hydraulic conductivity	September/october	
	Xylem anatomy		
	Fungal isolation		
Sprout/stump	Chestnut blight presence	February	Reference: San Mommé
	Gall damage evaluation		

Table 2
Chlorophyll *a* fluorescence parameters.

Symbol	Parameter
F_0	Initial fluorescence from a dark adapted leaf
Fm	Maximum fluorescence from a dark adapted leaf
Φ_{P0}	(Fv/Fm) or TR_0/ABS. Maximum quantum yield of primary photochemistry of a dark-adapted leaf. It expresses the probability that an absorbed photon will be trapped by the PSII reaction centre
Ψ_0	$(1 - Vj)$ or ET_0/TR_0. Probability that a photon trapped by the PSII reaction centre enters the electron transport chain
ΔV_{IP}	(1-Vi) Efficiency/probability with which a PSII trapped electron is transferred until PSI acceptors
PI_{ABS}	Performance Index (potential) for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors

The following parameters were measured on each leaf: leaf area and leaf blade in galled leaves (*LA*) (in this case the blade was re-cut around the gall) using a portable Li-3000 leaf-area meter (Licor, Lincoln, Nebraska, USA); fresh weight (*FW*) and dry weight (*DW*) of non-galled leaves and blades of galled leaves after desiccation for 36 h at 70 °C. Leaf mass per area (*LMA*) was then calculated as *DW*/*LA* of leaf blades.

In addition, stomatal density (*SD*) was measured at San Mommé, to investigate the different physiological behaviours with respect to leaf gas exchanges in galled and non-galled leaves.

Since most of galled leaves were located in partial shade, three sample types were compared: 20 partially shaded galled leaves, 20 partially shaded non-galled leaves and 20 non-galled sunlit leaves. Two leaves of each sample type were taken from each stump (10 stumps) and put into separate plastic bags, which were stored in a refrigerator bag to keep them cool during transportation to the laboratory.

An epidermal impression of the lower surface of each leaf was taken by coating the leaf surface with nail varnish, then peeling the dried layer of nail varnish off using transparent tape and sticking it to a slide. The epidermal impressions obtained were examined with an Eclipse E400 optical microscope (Nikon) and pictures were taken using a Digital Net Camera–Camera Control Unit (Nikon) at 40×. Images were imported to Image-J analysis software (freeware, available at http://rsbweb.nih.gov). Stomata were counted and the number of stomata on the image area of the examined leaf portion was recorded.

2.2.1.4. Analysis of soluble sugars and starch in leaves. The dried samples used to analyse leaf morphological traits were also used to analyse soluble sugars and starch. Three kinds of samples were analysed: the galls, the leaf blades of galled and shaded non-galled leaves.

Dried samples were ground to a fine powder using an MM 400 Mixer Mill (Retsch, Haan, Germany). Soluble sugars were determined using the anthrone-sulphuric acid method (McCready et al., 1950; Fales, 1951; Yemm and Willis, 1954) after extraction in 80% ethanol. Starch was extracted from the sugar-free residue with chloridric acid and determined using the anthrone-sulphuric acid method. In both cases D-(+)-glucose was used to prepare the calibration curve.

2.2.2. Shoot analysis

Twelve sprouts, one per stump, were randomly selected and collected at San Mommé. Two kinds of analysis were carried out on the samples: a qualitative analysis of the effects of ACGW on shoot vigour and a quantitative analysis of biomass and hydraulic properties altered by the presence of galls. The total height of each sprout and the diameter at breast height were also measured.

2.2.2.1. Qualitative analysis of shoot vigour. Evaluation of shoot vigour was carried out on the basis of the classification proposed by Maltoni et al. (2012b); single shoots (primary, secondary and tertiary) and the stem internodes of the 12 sprouts were assessed (Table 3). The vigour class was assigned to shoots with a prevalence of S0 while non vigour shoots were mainly in dominate positions with a prevalence of Sa and S2 and low number of developed leaves.

In addition, two primary shoots (two years old), one non-vigorous and one vigorous, depending on the prevalent class of minor twigs, were selected from each sprout for quantitative analysis of biomass and wood hydraulic properties (description below).

2.2.2.2. Quantitative analysis: biomass and hydraulic properties. The effects of ACGW infestation on two-year-old primary shoots, which are vital for plant productivity, were quantified using the 12 sprouts described above. One non-vigorous and one vigorous shoot were taken from each sprout to analyse: (i) shoot biomass, (ii) xylem hydraulic conductivity, and (iii) xylem conductivity area.

(i) The following parameters were measured on each shoot: length of growth (*SL*) for the years 2011 and 2012; number of leaves (*NL*); leaf area or leaf blade in galled leaves (*LA*), and fresh and dry weights of leaves after drying for 36 h at 70 °C to determine leaf biomass (*DWl*).
(ii) Xylem hydraulic conductivity was measured on a larger sample. Vigorous and non-vigorous shoots ($N = 30$) were cut from the sprouts, put in wet paper and sealed in plastic bags. The bags were stored in refrigerator bags surrounded by ice packs to keep the temperature inside at around 10 °C and the relative humidity at 100% during transportation to the laboratory. On arrival at the laboratory, all material was put in water; the lowest few centimetres were cut away from each stem to

Table 3
Damage classification and vigour indication. The classification was carried out on shoots and stem internodes of each sprout selected (adapted by Maltoni et al., 2012b).

Class	Damages on the twigs	Twigs vigour class vigour (V)/ non-vigour (NV)
S0	The galls (one or more) are located along the axis or on adjacent leaves and the axis development is nearly normal. The galls on the axis do not cause any abrupt reduction in shoot diameter	V
S1	The galls (one or more) are located along the axis or on adjacent leaves; deviation of the axis direction and reduction of axis growth and diameter are evident. Usually this damage causes a reduction in photosynthetic activity area during the current growing season	V
S2	The galls (usually more than 1) cause a definitively abnormal shoot development. The agglomerate can be peduncled	NV
Sa	The gall causes a deformed bud, and the leaf development is aborted	NV

avoid further embolism and the remainder kept in a cold room at 4 °C until analysis. Hydraulic conductivity was measured using the Smith and Ennos (2003) method.

From each shoot of the 2011 growth, one specimen with a length 15–20 times the diameter (10–20 cm in length) was cut in water at mid length. Length (L) and median diameter (Ø) of the specimens were noted. Specific hydraulic conductivity by length ($kh = FL/\Delta P$) was calculated on the basis of the time needed for distilled water pushed by a pressure head to pass through the whole specimen. Hydraulic conductivity per segment section was then calculated as $ka = 4kh/\pi d2$.

Wood density (D) was then calculated by measuring fresh volume and dry weight of sections ~5 cm in length. Fresh volume was measured using the water displacement method (Hacke et al., 2000). Samples were then dried for 72 h at 80 °C and dry mass recorded.

(iii) Two sub-samples of the same non-vigorous and vigorous shoots ($N = 7$) used to assess hydraulic conductivity were used to analyse wood xylem anatomy. Specimens of about 5 cm in length were cut from the shoots and stored in glycerol until analysis. Cross-sections about 20 μm thick were cut perpendicular to the grain using a microtome (Leica Microsystems Ltd., Milton Keynes, UK). The sections were stained with Safranin in 70% alcohol and washed with distilled water. Each section was captured at ×20 magnification using an Eclipse E400 optical microscope (Nikon) and photographed with a Digital Net Camera-Camera Control Unit (Nikon). The images were corrected in Adobe Photoshop CS2 (v. 9.0.2) to facilitate automatic classification of vessels by pixel value and to distinguish the lumen of vessels and the occlusions inside the lumen (tyloses, other deposits, and wall collapses).

The areas were measured using ImageJ software. For each portion of area we measured and derived: (iii.i) potential conductive area, defined as the lumen vessel area without occlusions; (iii.ii) vessel area occluded by tyloses or wall collapses; (iii.iii) functional conductive area, defined as the difference between the potential conductive area and the occlusion area; (iii.iv) number of vessels and (iii.v) size distribution of total and obstructed vessels.

2.2.2.3. Fungal isolation from shoots. The same non-vigorous and vigorous shoots ($N = 30$) used to determine hydraulic conductivity were also used to assess fungal isolations. Shoot specimens of 8–10 cm (of both the 2011 and 2012 growth seasons) were cut from them, their surfaces disinfected for 10 s in 95% ethanol followed by 4 min in 2% NaOCl solution with two drops of Tween 80 per L (Stanosz et al., 2001), then rinsed with sterile distilled water. Ten fragments of bark tissue and ten of wood tissue were cut from various positions on each specimen and placed on Petri dishes filled with potato dextrose agar (PDA, Oxoid), five in each.

Isolations were incubated at 25 ± 1 °C for 7 days in the dark, after which the growing fungi were identified by their cultural characteristics.

2.2.3. Evaluation of sprout vulnerability to chestnut blight

The damage caused by ACGW galls to European chestnut trees and the presence of chestnut blight were evaluated at San Mommé in February 2013. Infections were defined in accordance with the classification proposed by Turchetti et al. (2008).

All the sprouts on twelve representative stumps higher than 1 m and ⩾1 cm in diameter were examined in order to acquire the following data: (i) sprout diameter at breast-height; (ii) sprout height; (iii) number of healing cankers; (iv) number of intermediate cankers; (v) number of virulent cankers; (vi) number of initial infections; (vii) number of blight infections from galls; (viii) number of dead primary shoots; (ix) number of healthy primary shoots; (x) number of S2 shoots; (xi) number of Sa shoots and (xii) total number of galls still on the sprouts.

In addition, all the galls still attached were observed to identify the presence of *C. parasitica* or *Gnomoniopsis* sp.; a sample of 36 galls collected randomly from the shoots was taken for more accurate examination in the laboratory.

2.3. Statistical analysis

The statistical analysis was carried out using Statistica software (Release 4.5, StatSoft Inc., 1993).

A *t*-test for independent samples was used to compare data on leaf gas exchanges (photosynthesis, stomatal conductance, leaf transpiration, internal CO_2 concentration) in non-galled and galled leaves at San Mommé.

The same test was also used to investigate differences in the number of leaves, leaf area, leaf fresh weight, leaf dry weight, 2012 shoot length, 2011 shoot length and hydraulic conductivity between vigorous and non-vigorous shoots, and for the xylem anatomy analysis.

When the three sites samples (San Mommé, Collina A and Collina B) or when more than two samples had to be compared, an ANOVA followed by a Tukey's test for post hoc comparison of means was performed. For instance, this approach was used to compare soluble sugars and starch in three sample types (galls, leaf blades of galled leaves, and non-galled leaves), morphological leaf traits and Chl *a* fluorescence parameters at the three sites, and stomatal density in shaded galled leaves, shaded non-galled leaves and sunlit non-galled leaves.

For each type of fungal isolation from bark and wood tissues, vigorous and non-vigorous shoots were compared using a Chi-squared ($\chi 2$) test for 2-way tables. $\chi 2$ values were compared with critical $\chi 2$ value at significance level $\alpha = 0.05$ and 1 degree of freedom.

Tables and charts in the text report mean values, standard deviations and significance with *P*-values.

3. Results

3.1. Meteorological data

Abundant precipitation was recorded in April and May 2012, 174 and 84 mm respectively above the monthly averages. The summer months, however, were drier, with only about 50–60 mm of rain recorded in June and August, and very little in July. Drought conditions were exacerbated by high air temperatures. Mean temperatures in March, June, July and August were 4.9, 3.4, 2.3 and 4.8 °C, respectively, higher than the monthly averages (Table 4).

3.2. Analysis of European chestnut responses to the Asian chestnut gall wasp

3.2.1. Leaf analysis

3.2.1.1. Leaf gas exchange. Stomatal conductance (*gs*), transpiration (*Tr*), and net photosynthesis (*Pn*) measures were significantly higher in non-galled than in galled leaves during the whole vegetative period (Fig. 2), the only exception being *gs* and *Tr* on the first date of the survey. Moreover, the values of the parameters increased from May, reaching their maximum at the end of June. From May to mid-June *Pn* was ~30% higher in non-galled leaves than galled leaves and the difference increased to 77% on the last measurement date, though both show a decreasing trend.

Table 4
Meteorological data of the year 2012 and climatic series (1961–1988) from Acquerino meteorological station. Monthly minimum, mean and maximum temperatures and cumulated precipitation are reported.

Month	Meteorological series from 1961–1988				Year 2012				Differences between year 2012 and historical series			
	Temperatures			Cum. precip.	Temperatures			Cum. precip.	Temperatures			Cum. precip.
	Min (°C)	Mean (°C)	Max (°C)	(mm)	Min (°C)	Mean (°C)	Max (°C)	(mm)	Min (°C)	Mean (°C)	Max (°C)	(mm)
1	−1.6	1.3	4.2	224	0.3	2.5	5.6	–	1.9	1.2	1.4	n.a.
2	−1.1	1.8	4.8	188	−4.1	−1.9	1.3	43	−3.0	−3.7	−3.5	−145
3	0.8	4.0	7.3	206	5.5	9.0	13.3	62	4.7	4.9	6.0	−144
4	3.4	7.3	11.2	167	5.2	7.9	11.1	340	1.7	0.6	−0.1	173
5	6.8	11.2	15.6	130	8.6	12.3	16.4	214	1.7	1.1	0.8	84
6	10.2	14.9	19.6	102	15.0	18.2	21.5	39	4.8	3.4	2.0	-63
7	12.5	17.8	23.1	56	16.1	20.1	24.4	5	3.6	2.3	1.3	-52
8	12.3	17.6	22.9	91	18.0	22.4	27.4	40	5.7	4.8	4.5	-51
9	10.3	14.8	19.2	137	12.4	15.4	19.3	130	2.1	0.6	0.1	-7
10	7.0	10.7	14.4	213	9.1	11.7	15.2	319	2.1	1.0	0.8	106
11	2.7	5.8	8.9	256	5.8	7.8	10.5	429	3.1	2.0	1.7	172
12	−0.4	2.5	5.5	218	0.9	3.3	6.3	283	1.3	0.8	0.8	65
				683				768				140

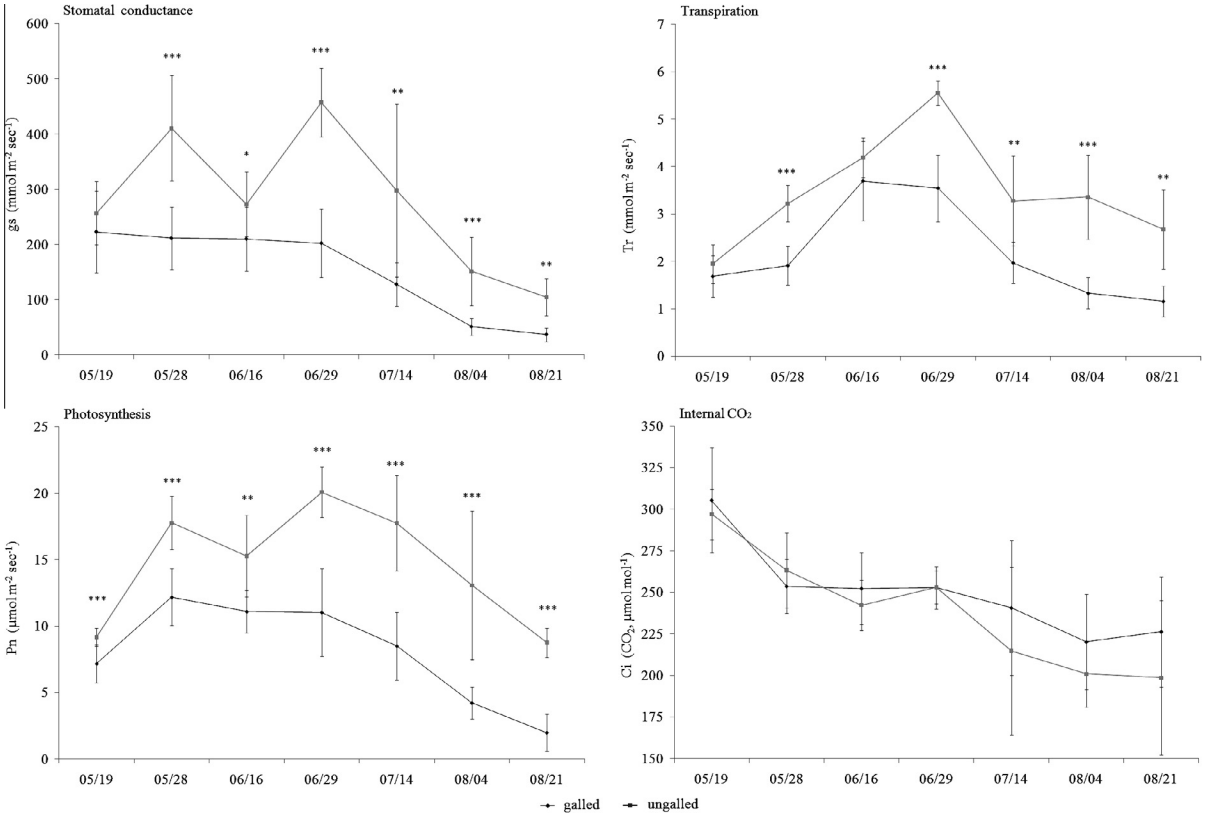

Fig. 2. Leaf gas exchanges parameters: stomatal conductance (*gs*); leaf transpiration (*Tr*); net CO_2 assimilation (*Pn*); internal CO_2 (*Ci*) measured in San Mommè in sunny days at 10 a.m. and PAR>1000 μmol photons m^{-2} s^{-1}. Mean values ± standard deviations are reported for each date. Significant differences between the two samples were obtained by the *T*-test for independent samples. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Internal concentrations of CO_2 (*Ci*) were not, however, significantly different in non-galled and galled leaves, the trend generally decreasing from May to August.

3.2.1.2. Chlorophyll a fluorescence. F_0 values for non-galled and galled leaves at San Mommè (Fig. 3) were similar throughout the whole period, with a decreasing trend from May to August.

No significant differences between the two samples were observed for Fm, with the exception of August when it was higher in galled leaves. Like F_0, Fm decreased during the vegetative period.

Maximum efficiency of photosynthesis (Φ_{PO}) was similar in non-galled and galled leaves (except on the first date, when values were higher in galled leaves), with an increasing trend in both samples.

Electron transport beyond the PSII, indicated by Ψ_0, was similar in the two samples, except on 29th June when Ψ_0 was higher in galled leaves than non-galled leaves. In addition, electron transport capacity increased in summertime (from May to mid-July) followed by a decrease. The same trend was observed for PI_{ABS}.

Both non-galled and galled leaves showed a slight decreasing trend in electron transport around PSI (ΔV_{IP}); galled leaves had significantly lower values than non-galled leaves.

At the replicate sites, Collina A and Collina B, Fm, Φ_{PO}, Ψ_0, and PI_{ABS} were significantly higher in galled leaves than in non-galled

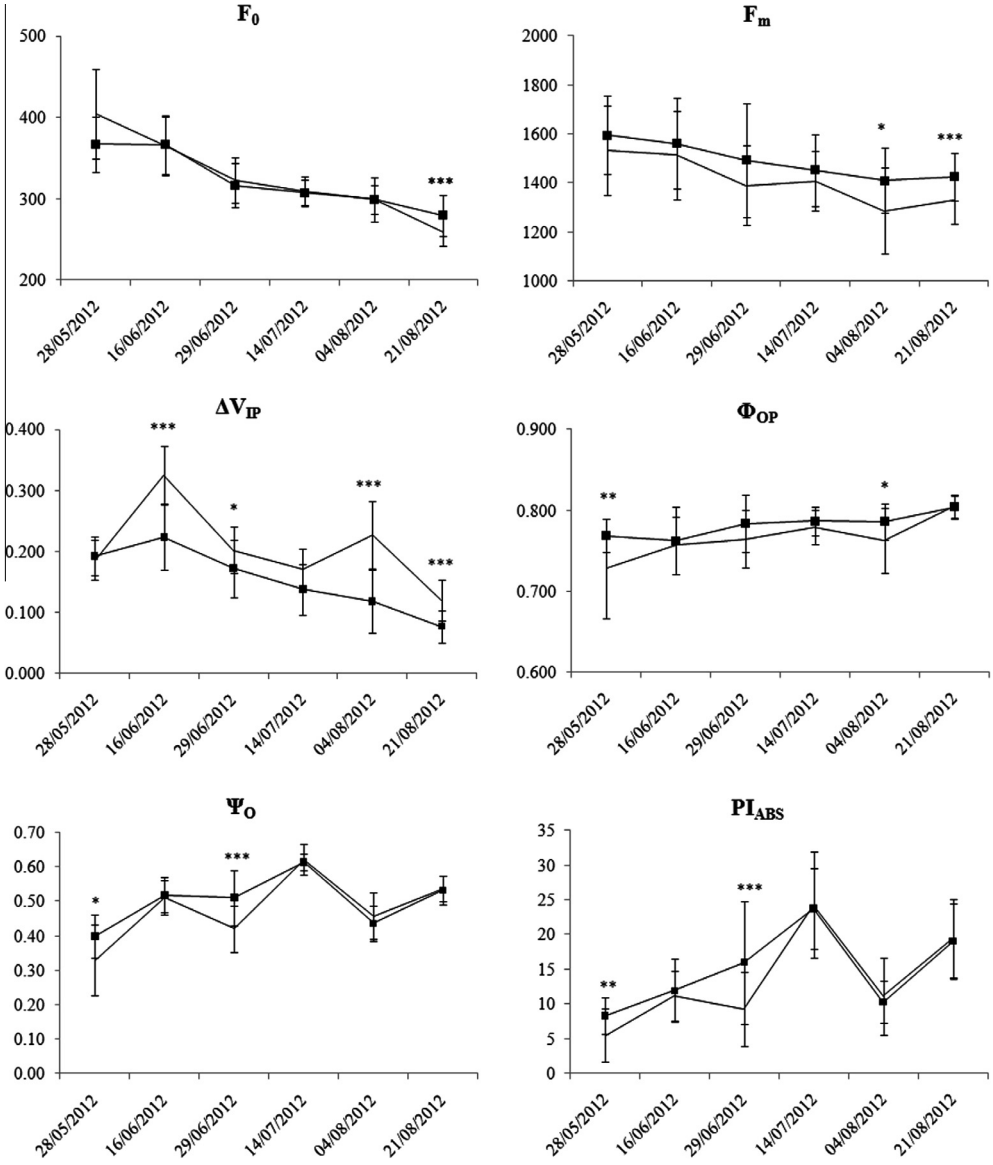

Fig. 3. Parameters of direct fluorescence analysis: F_0 (Initial fluorescence); Φ_{P0} (Maximum quantum yield of primary photochemistry of a dark-adapted leaf; Ψ_0 (Probability that a photon trapped by the PSII reaction centre enters the electron transport chain); ΔV_{IP} (Probability with which a PSII trapped electron is transferred until PSI acceptors); Fm (Maximum fluorescence); PI_{ABS} (Performance Index for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors). Comparison between healthy and gall-infected leaves. Mean values and standard deviations are shown. Significant differences resulted from the *T* test for independent samples are also shown: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

leaves, more frequently than at San Mommé, while ΔV_{IP} was significantly higher in non-galled leaves, as at San Mommé, except on 16th June at Collina A, and on 28th May, 16th June and 14th July, when no significant differences were observed (data not shown).

3.2.1.3. Leaf morphological traits and stomatal density. The surface of galled leaves is strictly dependent on gall position, which affects blade development (Table 5). The reduction in leaf size induced by gall development was around 40% at Collina A and Collina B, and 48% at San Mommé compared with non-galled leaves, though no differences were observed between sites. However, at San Mommé non-galled leaves were smaller and LMA was higher, in both non-galled and galled leaves.

Stomatal density (*SD*) measured at San Mommé did not change with the presence of galls. Partially shaded non-galled leaves (*SD*: 324 ± 22) and partially shaded galled leaves (*SD*: 334 ± 28) had similar *SD* values, about 50% lower than in sunlit non-galled leaves (*SD*: 698 ± 24) ($p < 0.05$). Insufficient numbers of galled leaves were found in fully sunlit position to enable comparison with other galled leaves.

3.2.1.4. Analysis of soluble sugars and starch in leaves. In all three sites, analyses of carbohydrates and starch (Table 6) revealed higher concentrations of carbohydrates in the leaf blade (in both non-galled and galled leaves) than in the galls. Conversely, galls were richer in starch.

3.2.2. Analysis of shoots

3.2.2.1. Qualitative analysis of shoot vigour. The 12 selected sprouts had an average height of 2.7 m and an average diameter of 16.8 mm. All classes of shoot damage were found in the sprouts, as shown in Table 3. Table 7 reports the number of shoots per class of damage for each sprout examined: S0 shoots ranged from 9 to 38, S1 from 5 to 13, S2 from 24 to 79, while Sa shoots were found

Table 5
Leaf characteristics of non-galled and galled leaves (fresh weight (*FW*); dry weight (*DW*); leaf area (*LA*); leaf mass per area (*LMA*). Mean values and standard deviations are reported. Letters mark significant differences at $P < 0.05$ among the three sites through Anova followed by Tuckey test. *Marks significant differences between healthy and gall-infested leaves in each site through the T-test for independent samples.

	FW (g)	DW (g)	LA (cm^2)	LMA (g cm^{-2})
Collina A non-galled	1.25 ± 0.17*	0.37 ± 0.05b*	70 ± 9.2ab*	0.0053 ± 0.0005b
Collina B non-galled	1.52 ± 0.31*	0.52 ± 0.1a*	82.6 ± 14.1a*	0.0065 ± 0.0007a
San Mommé non-galled	1.27 ± 0.34*	0.42 ± 0.13ab*	65.4 ± 12.9b*	0.0065 ± 0.0011a
Collina A galled	0.5 ± 0.17	0.16 ± 0.06	26.3 ± 8.5	0.006 ± 0.007B
Collina B galled	0.61 ± 0.23	0.2 ± 0.07	33.4 ± 11.3	0.0061 ± 0.006B
San Mommé galled	0.66 ± 0.2	0.24 ± 0.03	31.4 ± 7.4	0.0077 ± 0.004A

Table 6
Concentration of carbohydrates and starch in non-galled leaves, leaf blades around the gall and in the galls. Mean value and standard deviation and standard error are of the three sites are reported. Anova test was followed by Tuckey test for post hoc comparison of means. Marked differences are significant at $P < 0.05$.

Soluble sugars and starch					
			Mean	s.d.	s.e.
Sugars g/kg DW	Healthy leaf	49.1	9.4	1.7	a
	Leaf around the gall	50.5	13.5	2.3	a
	Gall	25.2	10.1	1.9	b
Starch g/kg DW	Healthy leaf	59.0	5.5	0.9	b
	Leaf around the gall	57.4	5.5	0.9	b
	Gall	75.1	9.9	1.9	a
Starch/sugars	Healthy leaf	1.2	0.3	0.04	b
	Leaf around the gall	1.2	0.4	0.07	b
	Gall	3.6	1.9	0.36	a

only on three sprouts (although only one had a very high count of 27).

3.2.2.2. Quantitative analysis: biomass and hydraulic properties.

(i) Shoot leaf biomass. Vigorous shoots had a higher number of leaves, greater leaf biomass and more extensive leaf area, about ten times more than non-vigorous shoots (Table 8). Shoot length was more pronounced in vigorous than in non-vigorous shoots of the 2012 growth, but did not differ significantly between vigorous and non-vigorous shoots of the 2011 growth.

(ii) Xylem hydraulic conductivity was measured in specimens of vigorous shoots, but it was possible to measure this only in two of the non-vigorous shoots; of course, vigorous shoots were found to have a more efficient hydraulic system, but they also had a thicker diameter and higher wood density than non-vigorous shoots (Table 9). Non-vigorous shoots had a higher percentage of obstructed area (T-value = 3.01, $P < 0.05$) and a greater number of obstructed vessels (T-value = 3.36, $P < 0.01$) (Table 9).

(iii) The chemistry of obstructed elements (occlusions) with respect to the xylem conductive area was not determined and no mycelia were observed inside the vessel or in the wood tissues. More than 90% of conductive vessels had a lumen area smaller than 0.0001 mm^2 in both samples (Table 10). Obstructed elements were observed in conductive vessels with a lumen area larger than 0.0001 mm^2 and smaller than 0.01 mm^2 in both samples. Larger vessels (lumen area > 0.01 mm^2) had a higher proportion of obstructed vessels, especially in the cross-sections of non-vigorous shoots (Table 10).

Table 7
Number of shoots for each class of damage in twelve sprouts selected randomly, one per stump.

Sprout	Height (m)	$\varnothing_{1.3}$ (mm)	Shoot damage classes				
			S0	S1	S2	Sa	(S2 + Sa)/Tot (%)
1	3.5	20	21	10	40	27	68
2	3	20	19	5	28	0	54
3	3	15	26	6	38	0	54
4	2	10	9	9	25	3	61
5	2	10	20	7	30	0	53
6	3.5	24	18	13	79	0	72
7	1.8	15	11	7	52	3	75
8	4	22	15	8	62	0	73
9	2	12	15	7	48	0	69
10	3.5	16	22	10	65	0	67
11	2.5	24	38	9	57	0	55
12	2	13	13	5	24	0	57
Mean	2.7	16.8	18.9	8.0	45.7	2.8	63
s.d.	0.8	5.1	7.7	2.3	17.8	7.7	8

3.2.2.3. Fungal isolation from branches. Several positive isolations were obtained from both vigorous and non-vigorous shoot subsample fragments, among which *Gnomoniopsis* sp. and *C. parasitica* prevailed. The percentages of isolations from 300 fragments taken from 30 vigorous and 30 non-vigorous shoots (wood and bark tissues) are reported in Table 11.

Of the bark fragments, *Gnomoniopsis* sp. colonies were found in 70% of vigorous shoot fragments and in 62% of non-vigorous shoot fragments. Very few fragments had colonies of *C. parasitica*: 4% in vigorous shoots and 7% in non-vigorous shoots.

Regarding wood tissue fragments, *Gnomoniopsis* sp. was found more frequently in non-vigorous than in vigorous shoot fragments (13% vs. 2%) ($\chi^2 = 8.72 > 3.84$, d.f. = 1 at $\alpha = 0.05$), while *C. parasitica* was not observed at all in wood tissues.

3.3. Evaluation of sprout vulnerability to chestnut blight

In February 2013 a total of 104 sprouts from 12 stumps were examined for the presence of blight. The mean number of sprouts on the stumps was 8.7 ± 3.4, mean height was 2.6 ± 0.3 m, and

Table 8
Leaf biomass parameters for the two classes of vigour of the selected shoots (number of leaves (*NL*); leaf area of whole shoot (*LA*); leaf fresh weight (FWl); leaf dry weight (*DWl*); 2012 shoot length (SL_{2012}); 2011 shoot length (SL_{2011}). *Marks significant differences between vigorous and non vigorous through the *T*-test for independent samples.

	NL	LA (cm^2)	FWl (g)	DWl (g)	SL_{2012}	SL_{2011}
V	64 ± 31***	3135.3 ± 1535.3***	64.6 ± 33.2	27.5 ± 14.4***	47.7 ± 8.2***	50.9 ± 23.2
NV	8 ± 6	212.9 ± 172.3	3.7 ± 3.2	1.5 ± 1.4	17.9 ± 12.5	42.4 ± 14.7

Table 9
Specimen characteristics in terms of size, xylem hydraulic conductivity and xylem vessels traits taken from specimens of vigorous and non-vigorous shoots. Parameters shown in the table: mid diameter (∅); wood density (WD); hydraulic conductivity specific for length (k_h); hydraulic conductivity specific for area (k_a), vessels density in wood sections (VD), Potential conductive area (*pot. cond. A*), occlusions area (*occ. A*), obstructed vessels density (*obstr.* VD). *Marks significant differences between vigorous and non-vigorous through the T-test for independent samples.

	Ø (mm)	WD (g cm^{-3})	k_h (m^4 Pa^{-1} s^{-1})	k_a (m^2 Pa^{-1} s^{-1})	VD (N mm^{-2})	pot. cond. A (%)	occl. A (%)	obstr.VD (N mm^{-2})
V	8.1 ± 2.1***	0.47 ± 0.04**	$2.1^{-14} \pm 4.2^{-14}$	$3.3^{-10} \pm 4.7^{-10}$	7513 ± 2565	25.9 ± 3.0	1.8 ± 1.3	7.4 ± 6.5
NV	6.0 ± 1.1	0.43 ± 0.04	$3.7^{-16} \pm 1.4^{-16(n)}$	$1.5^{-11} \pm 8.0^{-12(n)}$	6337 ± 940	30.5 ± 5.5	5.7 ± 3.2*	31.2 ± 17.5**

(n)In non-vigorous sample, k_h and k_a could be measured only in two specimens.

Table 10
Percentage of vessels and obstructed vessels per portion area and percentage of obstructed vessels in the cross section of specimens, per class of vessel area, for vigorous and non-vigorous shoots.

Classes of vessel area (mm^2)	Vigorous			Not vigorous		
	Vessels per portion (%)	Obstructed vessels per portion (%)	Obstructed vessels (%)	Vessels per portion (%)	Obstructed vessels per portion (%)	Obstructed vessels (%)
0–0.000025	33.6	2.4	0.0	39.5	1.1	0.0
0.000025–0.00005	36.2	3.5	0.0	33.9	0.6	0.0
0.00005–0.000075	15.8	1.2	0.0	15.8	0.6	0.0
0.000075–0.0001	6.7	0.0	0.0	5.4	0.0	0.0
0.0001–0.0005	6.1	11.8	0.3	3.8	12.4	2.3
0.0005–0.001	0.5	22.4	6.0	0.5	20.8	27.0
0.001–0.01	1.0	51.8	7.6	1.2	62.4	37.1
0.01–0.1	0.0	7.1	30.0	0.0	2.2	57.1

Table 11
Mean percentage of fungal isolations from bark and wood tissue of vigorous and non-vigorous shoots (two-years old). Bold numbers are significantly different. The Chi-squared test for two-way table was performed (fungi species in vigorous vs. non-vigorous shoots). Critical χ^2 value at $\alpha = 0.05$ and 1 d.f.

	V Bark (%)	NV Bark (%)	V Wood (%)	NV Wood (%)
Gnomoniopsis sp.	70	62	2	13
Cryphonectria parasitica	4	7	0	0
Other fungi	5	13	0	13
Sterile fragments	21	18	98	74

mean diameter 14.3 ± 3.2 mm (ranging between 7 and 35 mm). Infections were found in 29 sprouts (Table 12): 9 sprouts presented healing cankers with completely girdled stems, 12 sprouts had virulent cankers (chestnut blight) and dead wood tissue in the infected part of the stem or branch, 2 sprouts had intermediate cankers and 10 sprouts showed initial infections, as yet undifferentiated. The correlation between type of infection and sprout diameter was not significant, even though during the field survey it was observed that virulent infections were present mainly on branches, shoots or the upper part of the main stem, while healing cankers were mainly observed on the lower part of the main stem.

Only one fungal infection (blight) had clearly started from one gall, while none of the other galls was colonised by *C. parasitica*. Instead, black pycnidia of *Gnomoniopsis* sp. were observed on the galls, as confirmed by accurate examination of the gathered galls. A total of 4334 galls were counted on the assayed sprouts (42 ± 29 per sprout) and pycnidia and conidia of *Gnomoniopsis* sp. were observed on all of them.

In addition, mean counts of 11 ± 8 dead or S2 shoots, and 9 ± 4 S0 shoots were found on the sprouts. Moreover, they presented several lateral (secondary and tertiary) compromised shoots, although without signs of blight: 530 dead shoots out of 2067 (26% of total) located mostly in the lower, inner part of the crown, and 627 S2 shoots (30% of total).

Some of the dead shoots revealed the presence of *Gnomoniopsis* sp. as well as fructifications of *Alternaria* spp, although no fructifications or mycelia of *C. parasitica* were present.

4. Discussion

Insect galls are generally expected to produce mechanical and chemical responses in the host plant and consequently to affect its physiology (Raman, 2007). In some hardwood species, gall-inducing insects reduce photosynthesis and alter the water balance

Table 12
Examination of 104 sprouts from 12 stumps. Number of sprouts per stump (*Ns*); mean diameter (∅); mean height (*H*) are shown. Total observations are given.

	Sprout		Number of sprouts with					Number of		
Ns	Ø (mm)	H (m)	Initial infection	Healing canker	Intermediate canker	Virulent canker	Infection from galls	Healthy shoots	S2 shoots	Dead shoots
8.7 ± 3.4	14.3 ± 3.2	2.6 ± 0.3	10 (29%)	9 (26%)	2 (6%)	12 (35%)	1 (3%)	991 (44%)	627 (30%)	530 (26%)

of the leaf blade (Nabity et al., 2009); gall formation in red maple and black oak was found to reduce the quantum yield for electron transport, implying down-regulation of the PSII reaction centres in the area around galls (Aldea et al., 2006). The results of the present study are consistent with these findings and demonstrate that also ACGW induces physiological responses in European chestnut sprouts. Physiological leaf parameters generally show a similar behaviour in galled and non-galled leaves, even though some values, such as lower carbon assimilation and stomatal conductance, in galled leaves are different.

Although down-regulation of the photosynthetic apparatus protects it from oxidative damage, a decreased photosynthetic activity may also free up resources, especially nitrogen-rich compounds (Schwachtje and Baldwin, 2008), making these available for use in secondary defence pathways. Decreased photosynthetic rates may be part of a global inhibition of protein synthesis, possibly in anticipation of the need to redirect resources to defensive functions (Schwachtje and Baldwin, 2008).

Nevertheless, despite the reduction in net photosynthesis, an increased capacity for energy transfer from the Light Harvesting Complex of Photosystem II to the reaction centres (Φ_{PO}) was observed during leaf development, apparently uninfluenced by gall development. In addition, a positive effect on maximum fluorescence (Fm), more evident at Collina A than at the other sites, indicated a reduction in all electron acceptors.

However, both galled and non-galled leaves displayed decreased electron transport around Photosystem I (ΔV_{IP}) during summertime, and lower values were found in galled leaves. This may have negative consequences for $NADP^+$ production and carboxylation, probably associated with down-regulation mechanisms, which might explain the lower rate of CO_2 assimilation in galled leaves.

In addition, a reduction in electron transport without a decrease in the efficiency of PSII (Φ_{PO}) regulates acclimation and stress responses, as reported by Samsone et al. (2012). In this study, Φ_{PO} was not negatively affected by the presence of galls, as found by Aldea et al. (2006), at a certain distance from the gall; similar findings have been reported for *Ulmus* and *Salix* (Samsone et al., 2007).

Aside from down-regulation mechanisms, an alternative hypothesis to explain the negative insect-derived effects on photosynthesis might be that it is costly for the resource-rich photosynthetic apparatus to provide the metabolic precursors and energy needed for proper deployment of the supply.

In addition, a combination of biotic and abiotic factors plus stressful environmental conditions might be the cause of unbalanced excitation of PSII and PSI, and consequently of suboptimal quantum efficiency in photosynthetic electron transport (Oukarroum et al., 2007). It is worthwhile pointing out that PI_{ABS} (Performance Index for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors) was higher in galled leaves than in non-galled leaves, likely associated with the efficiency of PSII photochemistry.

The lower rate of CO_2 assimilation and greater efficiency in PSII photochemistry might depend upon a combination of biotic and abiotic factors. Leaf physiology parameters vary with micro-environmental conditions, such as leaf exposure or position, and with macro-environmental conditions, such as water stress and/or high temperature (Lu and Zhang, 1998; Oukarroum et al., 2009). Desotgiu et al. (2012) found greater efficiency of PSII, better electron transport beyond the excitation of PSII reaction centres, and higher PI_{ABS} in shaded than in sunlit leaves of beech trees. This would explain the higher values of these parameters in galled leaves, which were prevalently in shade during sprout foliation. Again, alterations in the external light environment could have a marked influence on stomatal conductance and CO_2 assimilation (Ashton and Berlyn, 1994; Sellin et al., 2011), in addition to the insect effect. This would be confirmed by the lower stomatal density found in galled leaves and in partially shaded non-galled leaves. On the other hand, sunlit leaves, characterised by higher stomatal density, could greatly increase the potential for behavioural control over gas exchanges and regulation of leaf heating.

Leaf gas exchanges also depend on the efficiency of water transport. In this study, shoot hydraulic conductivity could not be measured in non-vigorous shoots (mostly S2 and Sa), mainly due to the lower xylem area and higher xylem conductive area obstructed by tyloses (Clérivet et al., 2000) or wall collapses. Tyloses are generally considered to be a primary defence mechanism against endophytes (Beckman and Talboys, 1981), but they may be also associated with embolism phenomena (Cochard and Tyree, 1990). Embolism formation could explain the reduced conductivity and the complete winter desiccation of non-vigorous shoots observed during the field survey on sprout vulnerability to chestnut blight. Furthermore, an alteration of water transport in the shoot xylem may disrupt water transport to leaves, thus altering stomatal aperture.

It was not clear whether stomata in attacked leaves conserved the same functionality as stomata in intact leaves. Nevertheless, stomatal conductance was reduced in gall-infested leaves. In addition, the gall-induced change in leaf coloration, which takes place more quickly than in non-galled leaves, would likely vary the leaf microclimate, amplifying the effects of environmental variation on transpiration rate (Pincebourde and Woods, 2012).

It is widely believed that insects redirect photosynthesis products from plant organs for their own benefit (Shorthouse et al., 2005), although galls are also able to photosynthesize (Fernandes et al., 2010). In this study, leaf blades were generally characterised by higher concentrations of carbohydrates, likely caused by the depletion of starch linked to the activity of alpha-amylase and other enzymes (Garg and Mandhar, 1975; Shekhawat, 1980). Conversely, galls, which are often described as physiological sinks, were richer in starch. This could alter the carbohydrate source-sink relationship in galled leaves, with a reduction in the mobilisation of photosynthesis products.

Regarding morphological leaf traits, no differences in LMA were found between galled and non-galled leaf blades; the comparison of the three sites showed LMA to be significantly higher at San Mommé. This index varies considerably with light, temperature, and nutrient and water stress (Poorter et al., 2009), with high values that likely indicate a higher investment in organic compounds resulting in higher leaf density (Castro-Díez et al., 2000). Although the three sites were located on the same hill with the same exposure and slope, the differences found at San Mommé could be explained by soil characteristics, a factor that was not, however, analysed.

The data on shoot biomass confirmed a direct impact of ACGW. Bud development was highly constrained, as indicated by the large number of Sa, S2 and dead shoots on all sprouts. Comparable results were reported by Maltoni et al. (2012b) for similar chestnut coppices, while Kato and Hijii (1997) observed a delay in leaf development and a reduced biomass production during the following year in *C. crenata*. Indeed, there was also a decrease in leaf area due to the presence of galled leaves, which were smaller and reached quickly senescence and desiccation, which, together with a reduced hydraulic conductivity, may aggravate the desiccation of less vigorous shoots during the winter. The final effect was an increase in crown transparency in the shoots of sprouts and trees, and consequently a sizeable reduction in photosynthetic surface. The reduction in leaf biomass might then have an influence on leaf litter, soil organic matter and element cycles, with plausible cumulative effects on the vigour of chestnut sprouts.

The investigation of fungal isolations showed that *Gnomoniopsis* sp. was highly influenced by ACGW. This fungus is the causal agent

of brown rot in chestnut nuts, which is emerging in several chestnut production areas (Maresi et al., 2013). Although the appearance of brown rot on chestnut nuts is not clearly related to the spread of the wasp, *Gnomoniopsis* sp. has been reported to be a coloniser of new and old galls (Magro et al., 2010; Maresi et al., 2013). In this work, we found a colonisation of all overwintering galls by *Gnomoniopsis* sp. and an abundant pycnidia and conidia production, which could be explained by the high amount of starch in gall tissues, as in chestnut nuts. Moreover, *Gnomoniopsis* sp. was found also in isolations from shoot bark, confirming its endophytic status (Maresi et al., 2013). Nevertheless, it is probable that the fungus in bark tissue was not influenced by shoot vigour; it was also difficult to ascribe the desiccation of non-vigorous shoots to *Gnomoniopsis* sp., since no signs of mycelia were observed in vessels of the shoot cross-sections. As a latent pathogen and in its response to the presence of ACGW and environmental stresses, *Gnomoniopsis* sp. warrants further study. It is interesting to note that no symptoms of the fungus were observed on fresh galls at the three sites. This might weaken its effectiveness as a biological control agent against *D. kuriphilus*.

The behaviour of chestnut blight (*C. parasitica*) at San Mommé was similar to that observed by Davini et al. (1998) and Turchetti et al. (2008) in young chestnut coppices: a rather high level of damage and several initial, still undifferentiated, infections. In general, a clear predominance of healing and healed infections may occur during the growth and differentiation of chestnut sprouts. Hypovirulent infections were already present at San Mommè suggesting a possible evolution toward hypovirulence, as generally observed in most of the chestnut orchards and coppices in Italy (Turchetti et al., 2008). Death of shoots was mostly attributable to low vigour, uninfluenced by *C. parasitica*, while the blight infections on galls observed by Prospero and Forster (2011) were rare (1 out of 4334 examined galls). The rare presence of *C. parasitica* on galls could be due to the massive presence of *Gnomoniopsis* sp. as a competitor.

In any case, recurrent drought events and ACGW attacks could in the future depress hypovirulence, since it is known to be affected by stress factors (Turchetti et al., 2010b). Moreover, it is possible that the progressive decrease in plant biomass production could trigger a decline in chestnut tree performance, thus increasing lethal infections.

Sprout vigour at the study site was closely associated with the residual top branches, where leaves were healthy and well developed. The high precipitation in spring 2012 favoured plant growth. Similarly, excellent shoot growth was observed in cultivated chestnut orchards manured at the beginning of the vegetative season (Turchetti et al., 2012), whereas in forest stands (even those close to the study site), where neither manuring nor irrigation is possible, the cumulative effects of ACGW spread and drought could severely compromise the vitality of sprouts and stumps.

As suggested by Maltoni et al. (2012a), innovative silvicultural practices are needed to reduce the risk of chestnut decline, while coppices and abandoned stands must be continuously monitored in order to understand the possible evolution and apply proactive management strategies. In this context, the positive results obtained with biological control using the parasitoid *T. sinensis* (Bosio et al., 2013) are encouraging, although several more years are needed before clear effects in the whole chestnut range will be seen.

In conclusion, this study has provided evidence that:

(1) ACGW affected the photosynthetic apparatus in galled leaves, with a reduction in CO_2 assimilation and stomatal conductance, although further investigation is needed to assess the effects of environmental variables, including shaded environment and extreme drought.
(2) ACGW strongly affected the photosynthesizing leaf surface in galled leaves.
(3) ACGW influenced shoot growth in terms of leaf area and biomass. Massive attack may either constrain bud development or reduce shoot vigour.
(4) ACGW effects on chestnut blight were indirect and mainly related to the loss of shoot vigour.
(5) ACGW may play a role in the emergence and spread of *Gnomoniopsis* sp.

As an alien species in European chestnut forests, ACGW is opening up unpredictable scenarios. A combination of this biotic element and other disturbances is likely to diminish European chestnut vitality. Good ACGW management requires in-depth knowledge of how different factors, in particular the environmental conditions that stress the plant, can interact in the development of infestation. Again, proper monitoring of insect spread and climate change would be useful in order to apply timely and effective adaptive forest management strategies.

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