



## FIELD SURVEYS OF OZONE SYMPTOMS IN EUROPE. PROBLEMS, RELIABILITY AND SIGNIFICANCE FOR ECOSYSTEMS

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**ABSTRACT** – The ICP-Forest program for the monitoring of forest conditions includes the assessment of ozone symptoms in the European forests. This contribute to discussion points out the problems related to the recognition of such symptoms, with a special focus on the difficulties to extend the results obtained in experimental conditions to woody plant species growing in the field. Non specific symptoms (such as reddening, yellowing, early senescence and leaf loss), and the concurrent action of modifying factors (high light, drought, nutrient deficiency, pest attack and fungi) make the recognition elusive. In these cases, the action of ozone cannot be proven or excluded with “ad hoc” experiments. Apparently “good” bioindicators (*Rubus* sp. *Cornus* sp. pl., *Prunus* sp. pl., *Viburnum* sp. pl. etc.) are not suitable to assess the impact of ozone on vegetation. Symptoms are not necessarily related to the ozone dose taken up by stomata, and don’t are reliable indicator for biomass and productivity losses. Symptoms can be considered an epiphenomenon of more complex ecosystem processes.

**KEYWORDS:** CONTROLLED EXPERIMENTS; FIELD SURVEYS; ICP-FORESTS; MICROSCOPIC VALIDATION; MODIFYING FACTORS; OXIDATIVE PRESSURE

### INTRODUCTION

“A theory which is not refutable by any conceivable event is nonscientific. Irrefutability is not a virtue of a theory (as people often think) but a vice” (Karl Popper, 1957).

There are no doubts that the current concentrations of tropospheric ozone [O<sub>3</sub>] are sufficient to induce foliar symptoms in many sensitive European woody plant species (trees and shrubs), at least in experiments carried out in controlled and semi-controlled conditions (Skelly et al., 1999; VanderHeyden et al., 2001; Novak et al., 2003, 2005; Gravano et al., 2003, 2004; Bussotti et al., 2007; Gerosa et al., 2008, 2009; Marzuoli et al., 2009; Calatayud et al., 2007, 2010, 2011). Experiments allowed the researchers to identify the most sensitive species and their morphological, anatomical and ultrastructural responses. According to Innes et al. (2001) and Schaub et al. (2002) typical symptoms in broadleaved trees consist in interveinal stipples (reddish or

brownish) visible on the upper (adaxial) leaf surface (Fig. 1). At microscopical level, stipples corresponds to the collapse and death of groups of cells in the upper parenchyma, i.e. the palisade tissue (Vollenweider et al., 2003; Gravano et al., 2003, 2004; Bussotti et al., 2005). The symptomatic leaves are located in the basal part of the branches (the first leaves emitted in the growing season are mostly affected), and in the lowest part of the crown. In conifers, ozone symptoms consist in chlorotic mottles on the oldest needles, with a correspondent degeneration of mesophyll cells (Soda et al., 2000; Kivimäenpää et al., 2010). Visible manifestations with a lower degree of specificity, including leaf reddening, bronzing, bleaching, yellowing, early senescence and premature leaf loss, have also been described by Innes et al. (2001).

Based on these experimental evidences, field surveys to

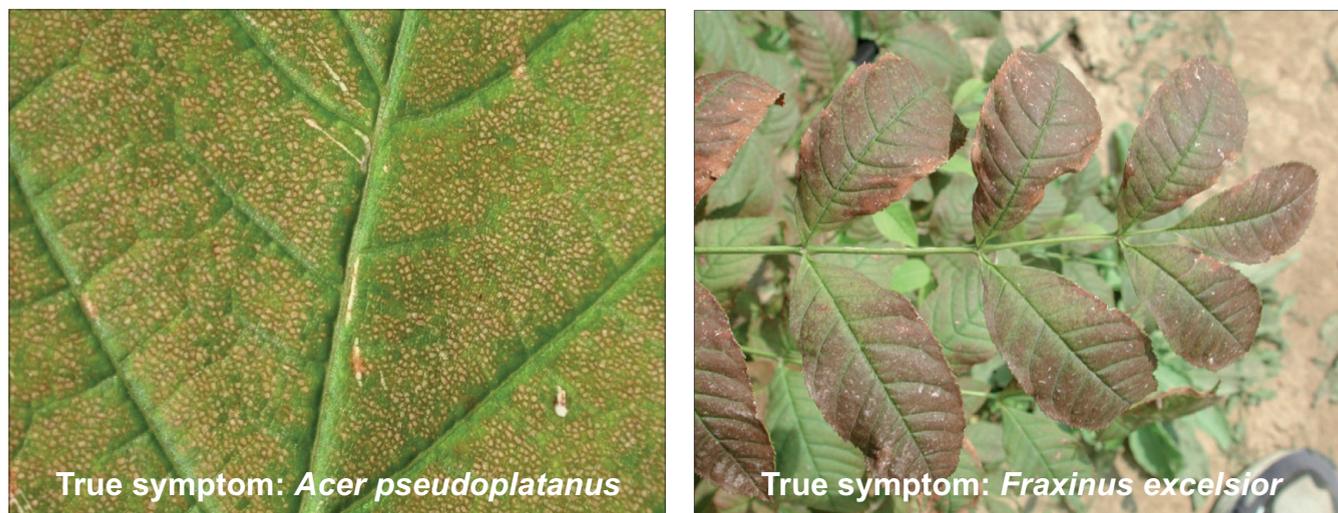


Fig. 1. Examples of typical ozone symptoms on *Acer pseudoplatanus* and *Fraxinus excelsior*, with interveinal stipples on the upper leaf blade. Stipples affect the leaves in basal position on the branches.

assess the spread and intensity of ozone symptoms on the natural vegetation have been carried out. In previous experiences in North America, surveyors take in account only few selected species displaying unambiguous symptoms, and that were well tested in experimental conditions (Coulston et al., 2003; Smith et al., 2003). In Europe the field surveys are currently carried out at the Level II plots belonging to the ICP-Forests program, and consider all the woody species falling within a specific sampling design (Schaub et al., 2010). Since symptoms were not reproduced experimentally for all species, a criterion of “ozone-like” symptoms was adopted. This criterion was based on the similarity between the symptoms observed in a variety of field grown species and those experimentally reproduced on a limited number of species.

The notation “ozone-like” was extensively used in the first decade of the present century to remark the possibility, but not the certainty, that a given manifestation may be caused by ozone (Manning et al., 2002; Manning and Godzik, 2004; Bussotti et al., 2003a; 2006a; Ferretti et al., 2007; Bussotti and Ferretti, 2009), although the first results suggest no (or very weak) correlations between ozone levels and the diffusion of foliar injuries. Field experiences have been recently reviewed by Mills et al. (2011), but a serious reflection on the limits of this first generation of foliar symptoms assessment surveys is still lacking. In the first period the concept “ozone-like” was useful to promote field surveys and experiments, but it is now necessary to exit this ambiguity and decide what it is really attributable to ozone and what must be discharged, reconsidering the current strategies of field assessment.

#### UNCERTAINTIES AND ERRORS IN RECOGNIZING OZONE SYMPTOMS

Among the factors that reduce the reliability of the field surveys, the first issue concerns the accordance among different surveyors in evaluating the symptoms in the same leaf sample (Bussotti et al., 2003b, 2006b). In general, experienced surveyors use more prudential criteria than non experienced ones. In many cases the source of errors relies in the uncertainties to assess non specific responses like early senescence, yellowing and reddening (see Figs. 2-3). When these symptoms appear in experimental conditions it is easy to relate them to the action of ozone, but the same manifestations in the field can be induced by a variety of uncontrolled factors. In some *taxa* leaves become red as early response to many environmental stress, with a pattern similar to that induced by ozone (for ex., species belonging to the genus *Rubus*, *Cornus*, *Viburnum*, *Prunus* etc.). These species are apparently good bioindicators, but may induce errors in evaluating the results of a survey. In recent years a number of local surveys on field assessment of foliar symptoms were reported for Europe and Asian far East countries (Hunova et al., 2011; Calderon Guerrero et al., 2013; Wan et al., 2013, 2014). There are several reasons because in most cases the symptoms described in literature are not suitable indicators of ozone stress in field surveys. In particular:

- The symptoms observed in the field can be explicated with causes different from ozone (high sun radiations, lack of nutrients, senescence etc.), or are different from those reproduced experimentally on the same species;

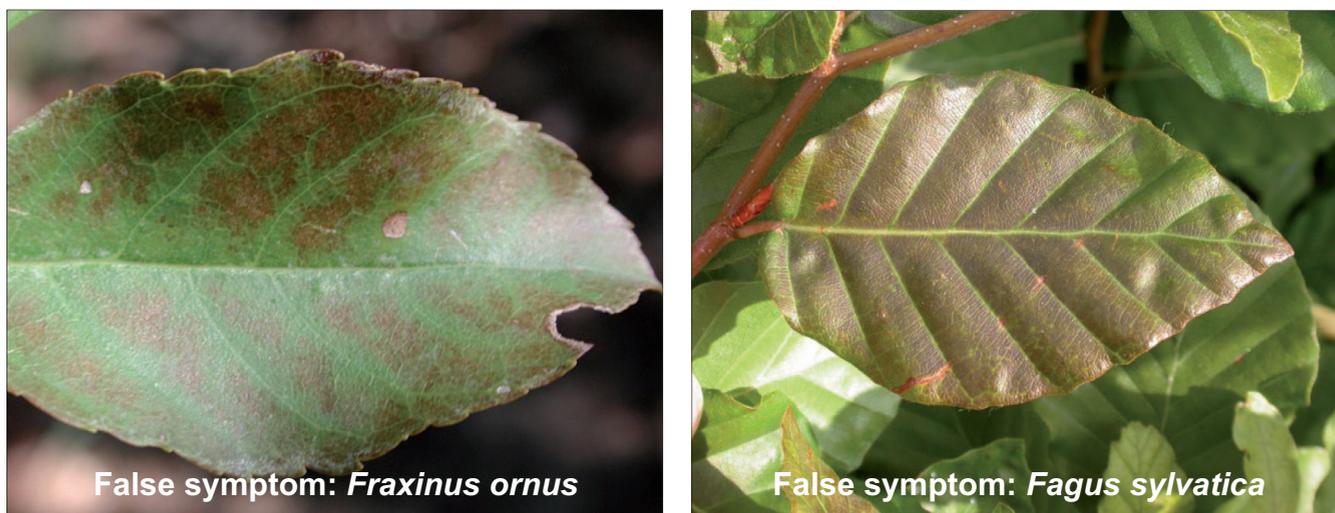


Fig. 2. False or ambiguous symptoms. Leaf bronzing on *Fraxinus ornus* and *Fagus sylvatica*. The pattern of the symptoms interests the outer leaves, in the apical position on the branches and directly exposed to light. This behavior is opposite to the typical ozone symptoms and can be attributed directly to high light. Leaf bronzing on *F. ornus* was not reproduced with ozone treatment in experimental conditions (Paoletti et al., 2009). Ozone can enhance bronzing on *F. sylvatica*, but this symptoms was observed also at low ozone concentration (Cascio et al., 2010).

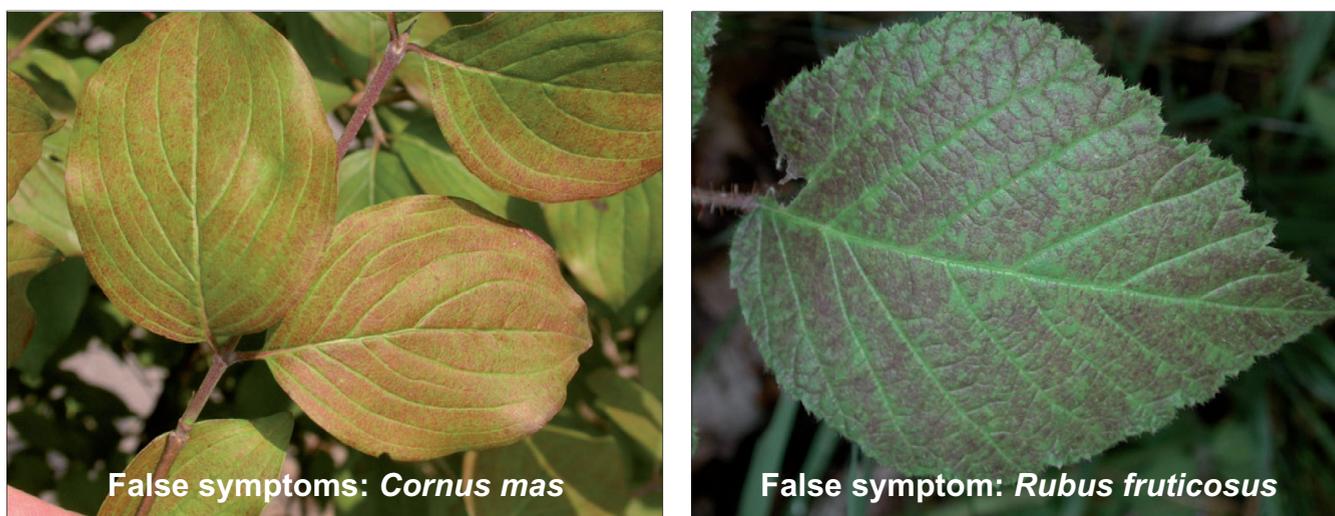


Fig. 3. False or ambiguous symptoms. Leaf reddening in leaves of *Cornus mas* and *Rubus fruticosus*. These species are very common in the forest understory of Mediterranean and Central Europe, and have been considered as bioindicator. Leaf reddening, however, can be induced by a variety of environmental stress factors including high light, drought, senescence. Their evaluation is therefore problematic.

- Symptoms occur on species considered ozone resistant, and were not reproduced in experimental conditions.

In the European program an important role is attributed to the so-called “microscopic validation”, i.e. the confirmation of the actual role of ozone as causal agent by means of the observation of the microscopic alteration in the mesophyll (Vollenweider et al., 2003a). In our opinion, because of the low degree of specificity of the responses, the microscopic analysis is more reliable in excluding rather than in confirming the action of ozone.

We underline that a supposed ozone symptom can be considered as such only when it has been reproduced unequivocally in experimental condition, without the possibility of any confounding factor. The possibility that the “ozone like” manifestations recognized in the field can be attributable or not to ozone only by visual and/or microscopic observations, without an effective experimental validation, is not testable (always there is the possibility that an environmental factor not considered may have, or not have, the ability to induce the same symptom, with or without a possible synergistic effect with ozone), and consequently not

refutable. According to the general laws of epistemology (Popper, 1957) a theory with these characteristics must be rejected.

### **OCCURRENCE OF SYMPTOMS AND ABSORBED OZONE**

There is a general (tacit) agreement that the appearance and spread of symptoms depend from the absorbed dose of ozone (Marzuoli et al., 2009), but comparing data from different open-top chamber experiments (see literature cited in Introduction) such assumption appears weak. In semi-controlled and field conditions the response of the plant depends by the interaction of different factors that can promote or depress the onset of symptoms, and not always ozone plays a primary role. High light is perhaps the major driver for ozone and ozone like symptoms (Davison et al., 2003). Light intensity, especially in Southern Europe conditions, is always exceeding the saturation requirements of photosynthesis. High light produces an over-excitation usually disposed of by photochemical and non-photochemical de-excitation processes, but a fraction of radiations exceeds the de-excitation capacity and is involved in ROS formation and accumulation. This process may have more deleterious consequence when the Calvin cycle is suppressed, as consequence of the inactivation of Rubisco by the action of high light itself (Jagtap et al., 1998), drought (Flexas and Medrano, 2002) or ozone (Fontaine et al., 2003), so compromising the photochemical de-excitation pathways. Such situation provokes the accumulation of reactive oxygen species (ROS), inducing metabolic and ultrastructural changes having the same nature of those usually attributed to ozone. In a long period the photosynthetic apparatus can be damaged from the action of the ROS, and senescence processes, including the breakdown of chlorophyll, are triggered.

Drought stress is believed to delay or avoid the appearance of symptoms, but drought and ozone are not always coincident and we can find a series of interactive effects not fully explored. Summer drought doesn't avoid the ozone uptake in the first part of the season (when there is not still water deficit in the soil), so producing an overlap of oxidative stress factors (Pollastrini et al., 2014). During the summer the concomitant action of ozone and drought stress in an environment characterized by high solar radiations may further increase the oxidative pressure since the Rubisco is inactivated (mesophyll limitation, Fontaine et al., 2003; Flexas and Medrano, 2002), or because of the lack of intercellular carbon due to the stomatal closure (stomatal limitation, Flexas and Medrano, 2002; Kitao et al., 2012). In these cases the pressure of electron flow in the ETC and

consequently the production of ROS is enhanced because of the lack of sinks for photochemical de-excitation. This situation can be further exacerbated by rain pulses during the summer season. In leaves subjected to strong oxidative pressure, the uptake of a moderate dose of ozone can trigger a cascade of consequences including widespread foliar symptoms. In this case, foliar symptoms are not produced by the "critical" dose absorbed by leaves, but by the "marginal" dose that allow to overcome the oxidative pressure tolerated by leaves.

### **DOES FOLIAR SYMPTOMS AFFECT ECOSYSTEM FUNCTIONS?**

Ozone impacts negatively ecosystem functions and services such as carbon sequestration and timber production (Wittig et al., 2009), but the reduction of tree growth doesn't seem to be related to foliar symptoms. In some cases field surveys (Vollenweider et al., 2003b) and open-top chamber experiments (Novak et al., 2007) allowed to find appreciable stem growth reduction connected to widespread symptoms in sensitive species (*Prunus serotina* Ehrh. and sensitive poplar clones). In other studies, the reduction of photosynthetic surface was compensated by the emission of new leaves and/or the increased efficiency of the remaining leaves (*compensatory photosynthesis*) without loss of woody biomass (Desotgiu et al., 2012, Pollastrini et al., 2013). Growth reduction may be consequence of many different physiological processes that compromise the photosynthesis and that don't imply the onset of foliar symptoms. In the Kranzberg experimental forest (Kitao et al., 2012, Matyssek et al., 2015) the reduction of photosynthetic activity and loss of growth was not connected to high ozone fluxes and the damage to photosynthetic apparatus but to the stomatal closure (*avoidance* mechanism). From an ecological point of view, we hypothesize that maintaining living leaves (not symptomatic) in a stressful environment determines metabolic costs for the tree (diversion of resources for defense and repair; increasing respiration rates) leading to the reduction of growth. In this perspective foliar injuries (HR – Hypersensitive Response) can be interpreted as a mechanism to protect the whole tree against metabolic losses, since the death of the damaged cells and the leaf loss allow to redirect the resources to the healthy cells/ leaves so maintaining high levels of photosynthesis.

## CONCLUSIONS

The experiences developed during many years in controlled and semi-controlled experiments, as well in field surveys, convinced us that the ozone foliar symptoms is an epiphenomenon in the more general context of the impact of oxidative stress on forests. Ozone symptoms don't represent *per se* a threat for forest functioning and health, but may be the "signal" of ongoing impacts on the ecosystem. This signal, however, is often very confused and its interpretation is extremely difficult; moreover the links between visible manifestation and ecosystem functions and services are ambiguous. Developing a conceptual framework to link visible manifestations of oxidative stress and the reduction of forest growth and efficiency is a challenge for future researches.

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