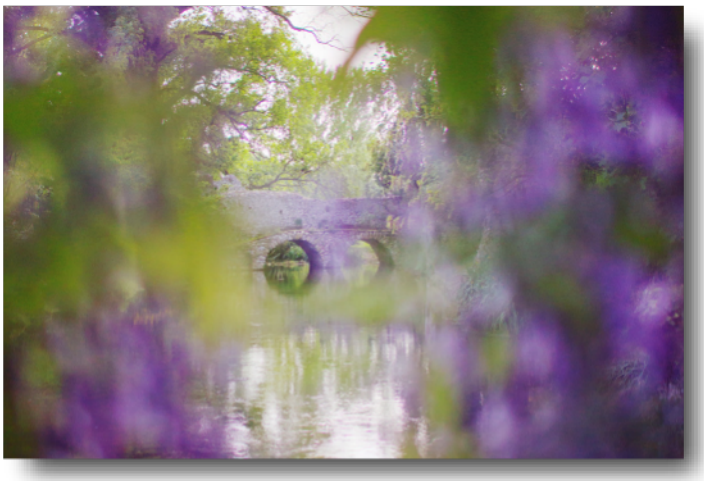
Effect-based activities on air pollution:

What is the state of the natural and anthropogenic Italian ecosystems?

Edited by Ilaria D'Elia, Alessandra De Marco, Giovanni Vialetto



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Effects of air pollution on crops and semi-natural vegetation

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CHAPTER 5 - EFFECTS OF AIR POLLUTION ON CROPS AND SEMI-NATURAL VEGETATION

5.1 Introduction

The International Cooperative Programme on Effects of Air Pollution on Natural Vegetation and Crops (ICP Vegetation, formerly ICP Crops) was established in 1987 as a subsidiary body of the Working Group on effects of the UNECE Convention on long-range transboundary air pollution (LTRAP). ICP Vegetation is an international research programme investigating the impacts of air pollutants (particularly tropospheric ozone (O_3), heavy metals and nitrogen) on crops and semi-natural vegetation.

Participants meet each year at the Task Force Meeting (TFM) to discuss recent results and the future development of the programme. Recently, Italy hosted the 25th and the 28th of the TFM, respectively organized by the Math and Physics Department of the Catholic University of Brescia in 2012, and by the National Focal Centre, Department of Environmental Biology, Sapienza University of Rome, in 2014. Some of the works that were presented during the Rome meeting are published in Annali di Botanica, Vol. 5, 2015.

5.2 Pressures

Tropospheric ozone (O_3),nitrogen deposition and heavy metals are currently identified by the ICP Vegetation as the main sources of pressure for crops and (semi)-natural vegetation.

5.2.1 Ozone

O_3 is considered as the main oxidizing agents in the near-surface atmosphere of the Mediterranean region (Cristofanelli and Bonasoni, 2009), also acting as a major greenhouse gas (Cooper et al., 2014). Despite the increasing efforts to regulate the precursor emissions this secondary air pollutant (Directive 2008/50/EC, EC, 2008), background O_3 levels are continuously increasing, particularly in Southern Europe (Coll et al., 2009; Cooper et al., 2014). Although the reduction of emissions has decreased the magnitude of O_3 peaks over the last decade, high daily summer O_3 concentrations still occur in the Mediterranean area, favored by high values of irradiance and temperature (Fernández-Fernández et al., 2011; Sicard et al., 2013). Furthermore, nocturnal O_3 pollution episodes are also frequent in the Mediterranean area, particularly along coastal sites located downwind of large urban conurbations, thus posing a concrete risk to vegetation (Fares et al., 2009; Mereu et al., 2009).

Fig. 5.1 describes the temporal trend of the target value for the protection of vegetation, indicated by the EU Directive (EC, 2008) as the AOT40 calculated over a given period using only the one-hour values measured between 08:00 to 20:00 CET in selected monitoring stations with different characteristics. The 5-y average of 9 ppm h (target value for vegetation protection) is systematically exceeded in most of them (see fig. 5.1).

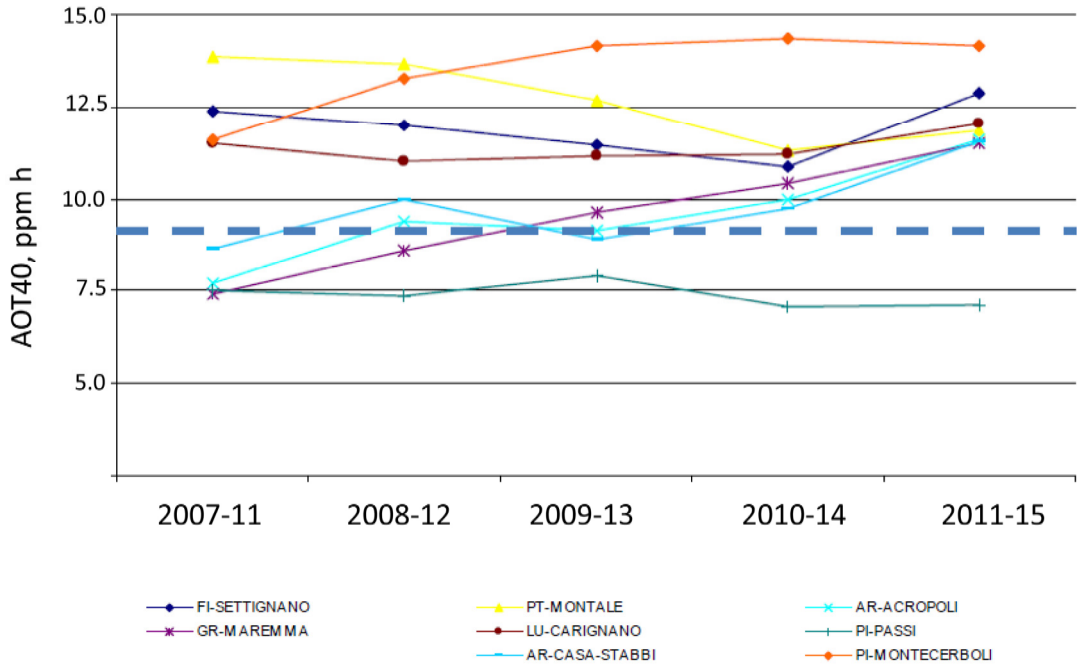

Figure 5.1 – AOT40 (expressed in ppm h) 5-y running averages as observed in 8 Tuscan monitoring stations (Data source: Tuscan Regional Environmental Agency, ARPAT, Florence). The dashed horizontal line shows the target value for the protection of vegetation. GR-Maremma is a rural site; AR-Casa Stabbi is rural/background; AR-Acropoli, FI-Settignano, PT-Montale, LU-Carignano, PI-Passi, PI-Montecerboli are suburban.

It is also interesting to estimate the biological additive effects of O_3 pollution and summer heat waves. This because the meteoclimatic conditions typical of the heat waves (i.e. high temperature, intense solar radiation, dryness) are also responsible of the formation and build-up of photochemically generated ozone (Lorenzini et al., 2014) (Fig. 5.2).

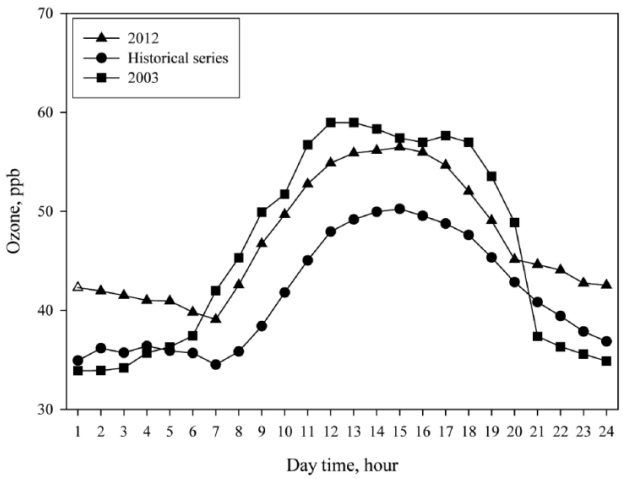

Figure 5.2 – Diurnal variation of ozone hourly concentration in the rural/background monitoring station of Casa Stabbi (Arezzo Province) (1 July-15August) in 2003 and 2012 (two years with dramatic summer heat waves), in comparison with an historical series (1999-2002 + 2004-2011) (After Lorenzini et al., 2014).

5.2.2 Nitrogen

Among the main concerns for the health of Mediterranean vegetation, increasing attention is given to anthropogenic perturbation of the nitrogen (N) cycle (Phoenix et al., 2006; Ochoa-Hueso et al., 2011). Nitrogen is often a limiting factor in many terrestrial ecosystems, but its cycle at global scale has been altered by post-industrial human activities, mainly because of combustion of fossil fuels and fertilization practices (Haeber Bosh reaction) aimed at increasing agricultural production, and resulting in increased N deposition (mainly nitrates and ammonium) (Galloway et al., 2008). NO_x and NH_3 emitted to the air are the main nitrogen pollutants contributing to the amount of reactive nitrogen rising levels of atmospheric deposition on soils, vegetation surfaces and waters (The European Nitrogen Assessment, 2011).

5.3 Impacts

5.3.1 Ozone

O_3 is known to affect plant photosynthetic function in different ways, and the effects of the O_3 stress can be observed at biochemical, microscopic and macroscopic level. The first interaction between O_3 and plants occurs at the level of the guard cells of stomata, in which O_3 triggers the production of Reactive Oxygen Species (ROS), thus activating a signalling cascade inducing an overall efflux of anions and K^+ (Vahisalu et al., 2010; Vainonen and Kangasjärvi, 2015). The consequent loss of guard cells turgor, leading to stomatal closure, reduces the gas uptake through stomata; this mechanism restricts the O_3 flux into the leaves ("avoidance mechanism", Castagna and Ranieri, 2009) but, at the same time, also limits the photosynthetic CO_2 assimilation (Astorino et al., 1995). The O_3 molecules that enter the sub-stomatal chamber can directly peroxidate the membrane lipids of the mesophyll cells (Vitale et al., 2008) or, in the apoplastic space, can generate toxic ROS (like OH^-, O_2^-, H_2O_2), which can then move inside the cells (Vainonen and Kangasjärvi, 2015). Furthermore, O_3 triggers the ROS production from different endogenous, enzymatic sources, a process known as "oxidative burst" (Manes et al., 1990a; Scalet et al., 1995). Despite acting as signalling molecules, that leads to the activation of several plant defence responses (Vainonen and Kangasjärvi, 2015), ROS are also responsible of direct oxidative damages to different molecules involved in the photosynthetic process, such as chlorophylls a and b, and Rubisco, whose activity and content have been reported to decline under O_3 stress (Goumenaki et al., 2010). Moreover, detrimental effects of O_3 on the photosynthetic electron transport chain, and in particular on Photosystem II (PSII) function, have been demonstrated (Guidi et al., 2002; Pellegrini, 2014). A reversible, photoprotective down regulation of PSII photochemistry is, however, the most commonly observed PSII response to O_3, being a consequence of the reduced demand of NADPH and ATP from the Calvin cycle; the latter can be caused by both stomatal closure and biochemical limitations (Bussotti et al., 2011; Mereu et al., 2011; Salvatori et al., 2013).

Also, the role of Photosystem I (PSI) in plant photosynthetic response to O_3 has received increasing attention in the last years. In fact, the measurement of prompt chlorophyll "a" fluorescence (PF) and the JIP test analysis (Strasser et al., 2004; 2010) have shown that the I-P part of the PF transient, which correlates to PSI content and activity (Ceppi et al., 2012; Schansker et al., 2005), is particularly sensitive to O_3 and other oxidative stress factors (Oukkarroum et al., 2009; Bussotti et al., 2011; Pollastrini et al., 2014; Bernardini et al., 2016).

In particular, the amplitude of the I-P phase of the PF (ΔV_{I-P}) was reduced by O_3 stress in many studies, indicating a negative effect of this pollutant on the efficiency of electron transport through PSI, to reduce the end acceptors beyond PSI (Bussotti et al., 2011; Mereu et al., 2011; Pollastrini et al., 2014). Recent studies carried out with the innovative technique of multi signal fluorescence measurement (Fusaro et al., 2016a; Salvatori et al., 2015) have highlighted that, in both crop and natural species, it is possible to distinguish an early O_3 response of the photochemical apparatus, involving PSII only, and a late response, occurring when O_3 cumulative stress becomes more severe, in which PSI activity and content are also modulated.

Ultrastructural alterations and visible leaf injury are reported in sensitive species after ozone stress. The appearance and diffusion of visible leaf injuries due to ozone exposure were reported in several experiments on bean (Gerosa et al., 2009) and durum wheat (Gerosa et al., 2014; Monga et al., 2015). Results showed that microscopical leaf symptoms, assessed as cell death and H_2O_2 accumulation, preceded by three-four days the appearance of visible symptoms.

Iriti et al. (2006) obtained similar results on currant tomato, suggesting the potential use of this species for ozone biomonitoring protocols, since there was a linear relationship between the intensity and diffusion of visible leaf injuries and the hourly ozone concentration mean.

Basile et al. (2010) have applied Transmission Electron Microscope (TEM) analysis on O_3-resistant (NC-R) and O_3-sensitive (NC-S) clones of *Trifolium repens* L. cv. Regal, exposed to ambient O_3 conditions in the Botanical Garden of the Sapienza University of Rome, Italy, from June to October 2005, following the ICP Vegetation Experimental Protocol. This work has highlighted that ultrastructural injuries appeared to be widespread in NC-S leaves, showing dead cells and heavily damaged living cells, while the NC-R clones showed much less damage: the chloroplast maintained an almost intact organization of thylakoids (granal and intergranal regions), but the chloroplast side facing the apoplastic space appeared with no thylakoids. Interestingly, Hsp70 levels, an important stress marker, were only slightly increased in the O_3-sensitive clone with respect to the O_3-resistant clone; in contrast, a strong decrease (–60%) in phosphoenolpyruvate carboxylase (PEPCase) protein levels was measured in the ozone-resistant clone.

Pellegrini et al. (2015a) conducted a comparative study on functional leaf treats and the diurnal dynamics of photosynthetic processes on plants of two grape (*Vitis vinifera*) varieties (Aleatico, ALE, and Trebbiano giallo, TRE), exposed under controlled conditions to realistic concentrations of O_3 (80 ppb for 5 h day^{-1}, 8:00-13:00 h, + 40 ppb for 5 h day^{-1}, 13:00-18:00 h). At the constitutive level, morphological functional traits of TRE improved leaf resistance to gas exchange, suggesting that TRE is characterized by a potential high degree of tolerance to ozone. At the end of the treatment, both varieties showed typical visible injuries on fully expanded leaves and a marked alteration in the diurnal pattern of photosynthetic activity. This was mainly due to a decrease in stomatal conductance (-27 and -29% in ALE and TRE, respectively, in terms of daily values in comparison to controls) and mesophyllic functioning (+33 and +16% of the intercellular carbon dioxide concentration). Although the genotypic variability of grape regulates the response to oxidative stress, similar detoxification processes were activated, such as an increased content of total carotenoids (+64 and +30%, in ALE and TRE), enhanced efficiency of thermal energy dissipation within photosystem II (+32 and +20%) closely correlated with the increased de-epoxidation index (+26 and +22%) and variations in content of some osmolytes.

In summary, it can be concluded that: the daily photosynthetic performance of grapevine leaves was affected by a realistic exposure to ozone. In addition, the gas exchange and chlorophyll *a* fluorescence measurements revealed a different quali-quantitative response in the two varieties. The genotypic variability of *V. vinifera* and the functional leaf traits seem to regulate the acclimation response to oxidative stress and the degree of tolerance to ozone. Similar photoprotective mechanisms were activated in the two varieties, though to a different extent.

Valletta et al. (2016) have investigated the physiological and metabolic effects of O_3 on two wine cultivars of *V. vinifera*, a red grape (San Giuseppe) and a white grape (Maturano) (Fig. 5.3), both autochthonous cultivars of the Latium region and having an economic importance at local scale. The results showed that the white grape cultivar appeared more sensitive to O_3 stress. Moreover, differently from what expected O_3 did not activate stilbene production but, in both cultivars, influenced the content of chlorogenic acid (CGA), an important molecule in the biosynthetic pathway of polyphenols and an antioxidant itself, which decreased in the fumigated samples and recovered after 8 days. The decrease in CGA not necessarily indicates a decrease in its biosynthesis, since it is highly probable that CGA is consumed or as antioxidant, or as precursor to other phenolic compounds.

Figure 5.3 – "Walk-in" Chamber facility of the Department of Environmental Biology, Sapienza University of Rome, with V. vinifera plants fumigated with O_3.

As a result of the above described invisible and visible alterations, and of the increase in metabolic cost for detoxification and repair, a reduction of total biomass, as well as a decreased carbon allocation to heterotrophic tissues (i.e. roots and fruits) (Andersen, 2003), is frequently reported for different species (Salvatori et al., 2013). In crops, such effects translate in a reduction of yield, as well as of food quality, with consequent economic losses to the agricultural sector that are mostly reported for Italy and Spain. In particular, Nali et al. (2002), on the basis of O_3 concentrations recorded by ten monitoring stations in Tuscany, have estimated yield losses due to ozone, which varied in Florence from 8% for corn and alfalfa to 27% for soybean, in Pisa from 5% for corn to 24% for soybean, in Lucca from 3% for corn to 17% for soybean. A preliminary economic estimate for corn, wheat, barley, soybean, tomato and alfalfa, calculated annual damage to be 4.6 MEuro in Florence, 0.5 MEuro in Lucca and MEuro in Pisa.

Figure 5.4 – Open-Top chambers facility of C.R.IN.ES. at Curno (Bergamo).

Several experiments on horticultural yield losses due to ozone were carried out in the Po plain climatic conditions at the CRINES research site (Centro di Ricerca Inquinamento atmosferico ed Ecosistemi, Curno, Bergamo) by the Environmental Physics and Ecophysiology research group of the Catholic University of Brescia (Fig. 5.4).

Results showed mean yield reductions of 40% on bean (cv. "borlotto nano lingua di fuoco" Gerosa et al., 2009), 36% on tomato (cv. "Oxheart", Gerosa et al., 2008), 18% on lettuce cv. "Romana" and 14% on lettuce cv. "Canasta" (Marzuoli et al., 2016a). In the same experimental site Monga et al. (2015) performed an experiment on durum wheat, highlighting yield losses up to 16% (in cv. "Sculptur") in plants that were exposed to O_3 enriched air (+50% of the ambient ozone).

Dry biomass reductions up to 25% due to O_3 were observed on alfalfa in the Po plain, and significant differences of forage quality parameters were also found for the same species. However, in all of the above mentioned experiments, a strong intraspecific variability of the response to ozone was observed. For example, ozone caused a slight increase of fruit yield in the tomato cv "San Marzano" (Gerosa et al., 2008), Monga et al. (2015) in a varietal screening experiment found that only 2 of the 5 studied cultivars of durum wheat were ozone sensitive.

The dataset of the experiment on tomato contributed to the definition of a dose-response relationship and critical levels based on ozone flux and fruit yield loss (Gonzalez-Fernandez et al., 2014) that was included in current version of the "Manual on Methodologies and Criteria for Modelling and Mapping Critical Loads and Levels and Air Pollution Effects, Risks and Trends" (CLRTAP, 2010). Other experimental datasets have been required for the definition of analogous dose-response relationships for leafy crops species (lettuce, Marzuoli et al., 2016a), bean (Gerosa et al., 2009) and durum wheat (Monga, 2015; Gerosa et al., *in preparation*) in order to define their specific critical levels.

In addition to controlled conditions experiments (with Open-Top Chambers), some open field experiments were conducted in order to characterize the ozone uptake at agricultural ecosystem level, and to quantify the partition of the total ozone flux between the stomatal and non-stomatal components of the deposition patway. These studies are strictly necessary to properly calculate the

ozone dose absorbed by vegetation for the regional level risk assessment, and for the application of the critical levels.

In this context, it is important to mention the field experiments performed on winter wheat (Gerosa et al., 2003) and soybean (Gerosa, 2002) that highlighted the presence of a significant non-stomatal component of the ozone total flux that could cause serious overestimation of the effects in case it would not be taken into account (Bassin et al., 2004; Tuovinen et al., 2007). These results contributed in the fine calibration of the EMEP model (which contain the DO_3SE module embedded) for the estimation of the non-stomatal component (Tuovinen et al., 2004).

Finally, a dataset of measurements on onion fields (Gerosa et al., 2007) was used to further improve the ozone flux partition process, identifying the agrometeorological parameters that mostly can influence the quality of the flux estimations.

The ecophysiological and biochemical effects of realistic O_3 exposure under controlled conditions upon medicinal species have been also investigated.

In particular, *Salvia* (Pellegrini et al., 2015b) and the perennial herbaceous lamiacea *Melissa officinalis* (known as lemon balsam) have been considered. Lemon balsam has been selected as a test species to elucidate the variations on biochemical composition of an officinal plant under short- and long-term exposure to O_3. Whole plants and cell cultures were investigated and biotechnological practical implications envisaged. A consistent fraction of this project (the one focused on rosmarinic acid biosynthesis at the molecular level - Döring et al., 2014a, b) has been performed jointly with a German team. Signaling molecules, membrane integrity, secondary metabolism, programmed cell death, photosynthetic performances are amongst the other key issues taken into account (Pellegrini et al., 2011, 2013b; Tonelli et al., 2015; D'Angiolillo et al., 2015).

The lichen response to O_3 exposure has been evaluated (Pellegrini et al., 2014a), thorough description of the biochemical and physiological mechanisms that are at the basis of the O_3-tolerance of lichens. Chlorophyll *a* fluorescence emission, histochemical ROS localization in the lichen thallus, and biochemical markers [enzymes and antioxidants involved in the ascorbate/glutathione(AsA/GSH) cycle; H_2O_2 and O_2 were used to characterize the response of the epiphytic lichen *Flavoparmelia caperata* exposed to O_3 (250 ppb, 5 h d^{-1}, 2 weeks) at different watering regimes and air relative humidity in a fumigation chamber. After a two-week exposure Chl*a*F was affected by the watering regime but not by O_3. The watering regime influenced also the superoxide dismutase activity and the production of ROS. By contrast O_3 strongly influenced the AsA/GSH biochemical pathway, decreasing the AsA content and increasing the enzymatic activity of ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase independently from the watering regime and the relative humidity applied.

5.3.2 Nitrogen

Increasing in N deposition is a growing threat to semi-natural grasslands that are listed as a priority habitat for biodiversity conservation in European Union Habitats Directive (92/43/CEE). A study carried out on grassland in Central Italy highlighted that N deposition markedly increases aboveground biomass, maintaining low species diversity (Bonanomi et al., 2006). The strong growth response the authors found out using a relatively low level of nitrogen enrichment, clearly indicated a nitrogen limited community.

Nitrogen eutrophication could exacerbate the deleterious effects of land abandonment on grassland biodiversity (Bonanomi et al., 2006) through enhanced above-ground competition and litter accumulation.

Moreover, since stressors like O_3 or drought can have an impact on roots biomass (Fig. 5.5), puzzling interaction effects might occur. In order to understand the effects of ongoing global environmental change on Mediterranean ecosystems, it is necessary take into account how the multiple stresses affect vegetation since a comprehensive picture is far from being given (Tattini and Loreto, 2014). A recent work by Fusaro et al. (2016b) has investigated how functional and structural traits of two Mediterranean species with different leaf habits (*Fraxinus ornus* and *Quercus ilex*) shift because of nitrogen (N) addition (30 kg ha^{-1} y^{-1}), also exploring the effect that nitrogen has on the water stress response. Their results have shown that the early successional, deciduous *F. ornus* tends to invest more nitrogen at leaf level enhancing photosynthetic machinery, whereas late successional evergreen *Q. ilex* invests resources on non-photosynthetic biomass, keeping constant N content at leaf level. These differences between species can modify their competitive relations, through influence temperate forest community structure and dynamics. This study also highlights that N can have role in water stress response in both species, but in different way. In *F. ornus* N has ameliorative effect on water stress, determining high gas exchange rates and photosystems functionality. On the contrary, in *Q. ilex* the N addition seems to increase the susceptibility to water stress, possibly because of changes in biomass partitioning due to N. Moreover, studies relative interaction between N deposition and O_3, highlighted an adverse synergistic effect, since exposure to ambient O_3 concentrations was shown to reduce the Nitrogen Use Efficiency. On the other hand, the growth of plants in response to N (i.e, root development), mitigates impact on biomass and physiology due to O_3 (Marzuoli et al., 2016b). Mechanism of action of N on tree species can contribute to provide a more reliable risk assessment, and accordingly it should be implemented for natural and semi-natural vegetation.

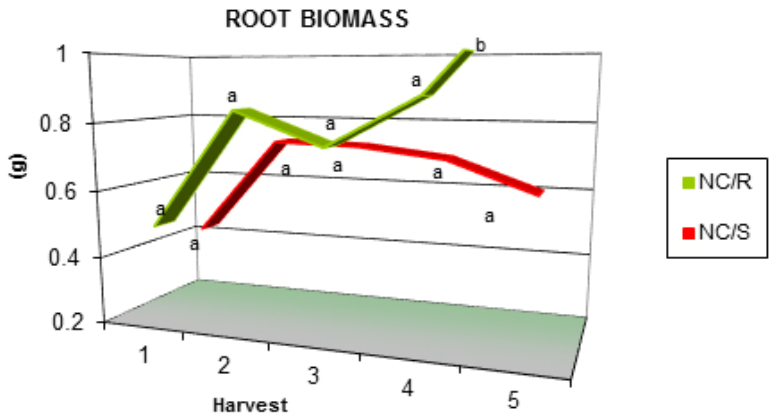

Figure 5.5 – Root biomass of NC/R and NC/S clover clones, harvested after 1, 2, 3, 4 and 5 months of exposure to ambient O_3 in the Botanical Garden of Sapienza University of Rome.

5.4 Risk assessment

The methodologies developed within UN/ECE to assess the impacts of air pollution on crops are described in the Chapter 3 of the "Manual on Methodologies and Criteria for Modelling and Mapping Critical Loads and Levels and Air Pollution Effects, Risks and Trends." (UN/ECE, 2010).

5.4.1 Ozone

Chapter 3 of the mapping manual considers two main approaches to set critical levels to protect vegetation from tropospheric ozone: the concentration-based critical level (O_3 accumulated over a threshold of x ppb, AOTx) and the uptake-based critical level (accumulated O_3 dose above a threshold of Y or phytotoxic ozone dose, PODY). The critical levels are defined as the dose that causes a reduction by 5% of the yield at 95% confidence level (UN/ECE, 2010). The two approaches differ by their data requirements (UN/ECE, 2015a), because PODY is linked to stomatal uptake that is limited by environmental condition such as temperature, light, soil moisture, vapor pressure deficit, phenology and wind (Mills et al., 2011). Since their first establishment in 1996, the O_3 critical levels have been intensively debated among scientists. In particular, many studies carried out in Italy have shown that, despite O_3 exposure at Italian background sites exceeds the AOT40-based critical level, the occurrence of adverse effects of O_3 on forests and crops appears controversial (Manes et al., 2005; Ferretti et al., 2007). At this regard, the use of the stomatal uptake concept (PODY) is highly recommended since, besides pollutant concentration, it takes into account vegetation characteristics and environmental constraints that affect the dose of O_3 effectively absorbed by the plant, an aspect that is of particularly importance under Mediterranean climatic conditions (Ferretti et al., 2007; Manes et al., 2005; Anav et al., 2016; De Marco et al., 2015).

The most common methodology to define the risk assessment is biomonitoring. Biological monitoring can be defined as the measurement of the response of living organisms to changes in the air quality of their environment (Nali et al., 2006), and may provide integrated information on air quality impacts. As plants are more sensitive in terms of physiological reaction to the most prevalent air pollutants than humans and animals (Nali et al., 2006), they are more suitable to be used for biomonitoring. Biomonitoring can be performed through the analysis on the vegetation already present in a given study area (so-called passive biomonitoring) or carried out with selected test plants introduced at the study site (active biomonitoring) (Nali and Lorenzini, 2007). The selection of plants with different sensitivity/resistance to O_3 has been a challenge since 1950's (Karlsson et al., 2003), with the purpose of biomonitoring phytotoxic effects of this pollutant under conditions of ambient exposure. From 1987, these efforts have been coordinated under the ICP Vegetation (Harmens et al., 2015a). Many plant species have been tested as active O_3 biomonitors on pan-European scale, such as radish (*Raphanus sativus* L. cv. Cherry Belle, Manes et al., 1990b; Allegrini et al., 1994), *Phaseolus vulgaris* cv Lit (Astorino et al., 1995), and *Trifolium subterraneum* cv Geraldton. From 1996 to 2004, the ICP Vegetation international biomonitoring programme has involved exposure of ozone sensitive (NC-S) and ozone resistant (NC-R) biotypes of white clover (*T. repens* L. cv. Regal) (Heagle et al., 1994) (Figg. 5.6, 5.7). The clover system has proven to be useful to detect detrimental effects of ambient O_3 (Manes et al., 2003; Nali et al., 2009), however visible injury and biomass reductions proved to be more related to stomatal O_3 uptake than to air O_3 concentration, expressed as AOT40 (Mills et al., 2011).

Figure 5.6 – "Biomonitoring station" with NC-S and NC-R clover clones, in the Botanical Garden of Sapienza University of Rome.

(a) (b)

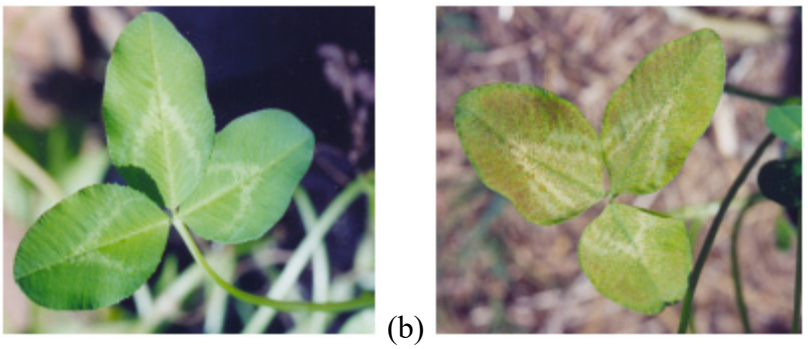

Figure 5.7 – Healthy (a) and O_3-injuried (b) leaves of white clover, NC-S clone.

Moreover, studies carried out in Mediterranean climatic conditions, have shown the importance to consider the effect of environmental variables, such as high air temperatures and Vapour Pressure Deficit (VPD), which can act as confounding factors on the O_3 dose-response relationship of the clover system, even if plants are grown following standard protocols under non limiting water availability (Ferretti et al., 2007; Manes et al., 2005).

Ozone-hypersensitive tobacco (*Nicotiana tabacum* L.) Bel-W3 has been also used worldwide as a reliable and sensitive bioindicator of ozone, showing a characteristic and specific foliar response. The typical foliar lesions induced by ozone under realistic exposure to ambient air are bi-facial greyish necrotic spots, scattered over the lamina (Manes et al., 1990b; Lorenzini et al., 2000). The tobacco system has been extensively investigated, also from the quantitative point of view. Biomonitoring campaigns have been successfully performed all over the world. Ozone-resistant tobacco plants (cv. Bel-B) are routinely inserted in the plots; their sensitivity threshold, in terms of visible injury, for 2 h exposures is 220 ppb vs. 100 ppb of Bel-W3.

Therefore, the appearance of injury on Bel-W3 but not on Bel-B provides further confirmation that such injury is due to ozone. Conventional biomonitoring is performed with adult tobacco plants (about 2 months old, 60 cm in height). However, some years ago, an innovative miniaturized kit for ozone biomonitoring was developed and patented at the University of Pisa, employing plates with tobacco germlings (typically (Lorenzini, 1994; Lorenzini et al., 1995 – Fig. 5.8). These plates are exposed to ambient air for 7 days in shaded conditions, and the intensity of the injury of cotyledons and the first leaf is visually assessed and related to the dose of ozone to which the seedlings have been exposed (Nali et al., 2004; 2007). More precisely, at the University of Pisa two are the applicative fields based on the tobacco mini-kit, i.e. (i) educational projects of environmental education with young pupils (and their teachers and families) (e.g. Nali and Lorenzini, 2007; Pellegrini et al., 2014b), and (ii) regional wide season-long campaigns to fulfill commitments of local/national environmental authorities to allow permitting of industrial plants such as thermal power stations. Under the guidance of their teachers, the pupils had several opportunities to practice with many basic and applied study areas and disciplines and were initiated into the scientific method in a simple and absorbing manner. Though primarily an educational exercise, the survey provided sound research elements and the picture of pollution that emerged has increased the knowledge of air quality in the investigated area.

Figure 5.8 – The miniaturized kit for biomonitoring of O_3 with tobacco Bel-W3 germlings. The external dimensions are 12.5x8.5 cm. Please note necrotic lesions induced by the exposure to ambient air in the presence of O_3.

Recently, ozone sensitive (S156) and ozone resistant (R123) genotypes of snap bean (*Phaseolus vulgaris* L.), selected through genetic crosses, have been proposed as a new biomonitoring system (Burkey et al., 2005) (Fig. 5.9). Several studies have tested the new system under semi-controlled environmental conditions, showing that pod yield was similar in S156 and R123 under O_3 concentrations lower than 30 ppb (S156/R123 pod yield ratio equal to 1), and consistently reduced by increased O_3 levels in the sensitive genotype only (S156/R123 pod yield ratio < 1) (Burkey et al., 2005, 2012; Flowers et al., 2007). Given these promising results, the ICP Vegetation O_3 programme has conducted field trials using the snap bean system, following a standardised experimental protocol at pan-European scale since 2008 (Fig. 5.10), the advantage being also the cost-effectiveness of the seed-propagated bean plants, in respect to the vegetative-propagated clover clones (Burkey et al., 2005).

The results of the field trials, however, have shown that the extent of leaf injury on S156 variety, as well as the S156/R123 pod yield ratio, were often not directly related to the AOT40 at the experimental site. This was particularly true for Mediterranean sites, such as the Castelporziano Estate (Rome, Italy), in which the response of the snap bean system during three consecutive summer periods (years 2008-2010), characterized by different meteorological conditions and O_3 levels, was not related to seasonal ozone concentration (Fusaro et al., 2015). Recently, Salvatori et al. (2013) pointed out that the O_3 response of S156 and R123 bean lines varied within plant growth stage, with the different O_3 sensitivity of the two genotypes being more apparent during vegetative growth and flowering. Such differences can be due to different stomatal conductance of the sensitive and resistant plants, and to different effect of O_3 on the I-P phase of the fluorescence transient (Salvatori et al., 2015). A large-scale application of the snap bean biomonitoring system, under different climatic conditions and O_3 levels found at pan-European scale, appears therefore unsuitable.

Figure 5.9 – Ozone sensitive (S156) and ozone resistant (R123) genotypes of snap bean (Phaseolus vulgaris L.), during a fumigation experiment carried out in the "walk-in" chambers of the Department of Plant Biology, Sapienza University of Rome.

Figure 5.10 – O_3-resistant (R123) and O_3-sensitive (S156) snap bean plants, after three months of ambient air exposure in the Castelporziano Presidential Estate, following the ICP Vegetation biomonitoring protocol (Fusaro et al., 2015).

5.4.2 Nitrogen

The risk assessment of nitrogen pollution in European Mediterranean natural and semi-natural ecosystems is difficult since only few experiments were carried out in the whole region. In Chapter 5 of the Mapping Manual, a list of critical loads (kg N ha^{-1} y^{-1}) is reported for natural and semi-natural ecosystems classified according to the European Nature Information System (EUNIS). For example, over a range of 15 and 25 kg N ha^{-1} y^{-1} Mediterranean xeric grasslands are expected to increase production and dominance of graminoids. Between 20 and 30 kg N ha^{-1} y^{-1}, Mediterranean shrub ecosystem changes species richness and composition; however, critical loads have not yet been set.