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"Protoplasts as a novel source for Comet assay in forest tree species exposed to elevated troposheric ozone"

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ABSTRACT

The aim of the present thesis is to enlighten the effect that elevated tropospheric ozone concentration has in the DNA integrity of two plant species of different tolerance to ozone. Ozone in the troposphere is a major pollutant that causes oxidative stress to humans and plants. The concern of it is increasing because of its elevated concentration especially in the Mediterranean region due to high temperatures and solar radiation which favor its formation. The present study focuses in the set-up of a novel technique to detect the level of DNA damage by using Comet assay in sensitive and tolerant species which had undergone ozone fumigation. The experiments were conducted in free air exposure of *Arbutus unedo* and *Populus maximoviczii x berolinensis* in the facilities of the Institute of Sustainable Plant Protection of the National Research Council of Italy in Florence. Comet assay was used in protoplasts of plants that were exposed to ambient concentration of ozone, elevated per 1.5 and double concentration of ozone regarding to the ambient one. The isolation of protoplast was feasible only when young leaves were used, which means that the analysis was conducted in material that was exposed to different concentrations of ozone for an estimated period of one month. Comet assay detected an increase of the DNA damage because of the oxidative stress due to ozone exposure in leaf tissues that were exposed to increased concentration of tropospheric ozone in leaves without visible symptoms in both species. Macroscopic damage was observed in leaves exposed to ozone for a longer period than one month only in the sensitive hybrid poplar genotype. Indeed, this clone appeared more affected also by using some physiological parameters like the annual growth and the photosynthesis. Protoplasts isolated from young leaves of the poplar genotype and *A. unedo* exposed to different concentration of ozone were suitable as a source material for Comet assay. From the absence of macroscopic damage in leaf tissues of *A. unedo* plants it can be assumed that *A. unedo* is a species with higher tolerance probably due to a better DNA repair capability.

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INTRODUCTION

Current situation of tropospheric ozone and effects that it has to the plants

Ozone in the stratosphere is fundamental for life as it is present in the ozone layer which absorbs dangerous UV radiations, but in the troposphere it is considered as a major air pollutant especially in the Mediterranean basin because of its climate. 20-40% of the ozone in the troposphere comes from the stratosphere, while the remaining is product of photochemical processes taking place in the troposphere (Ribas & Peñuelas, 2004). Its production is enhanced by high solar radiation and temperatures, which make South Europe having greater concentrations compared to the Northern countries. Ozone precursors are nitrogen oxides and volatile organic compounds, usually originated by human activities. Volatile organic compounds, also known as VOCs, may also originate from plants emissions (Calfapietra et al., 2013). More specifically, as it results from Calfapietra's papers, VOCs can be divided in Biogenic Volatile Organic Compounds (BVOCs) and Anthropogenic Volatile Organic Compounds (AVOCs). The latter are mainly produced by transport, solvent use and production, storage and combustion processes. Regarding BVOCs, they are mainly isoprenoids or terpenes, but it has been proved that tree species that emit VOCs, also show greater ozone uptake, so the conclusion if trees are responsible for more ozone production or they act like sinks for ozone, is not so simple (Fares, 2008).

Generally, half of the percentage of the *in situ* produced ozone has anthropogenic origin (Ribas & Peñuelas, 2004). To make clear how ozone precursors like nitrogen oxides originated by human activity lead to the creation of tropospheric ozone, the following reactions (Fares, 2008) are given:

O_2 + UV radiation → O + O

NO_2 + UV radiation → NO + O

O + O_2 + M → O_3 + M (molecule that allows the stabilisation of ozone)

NO + O_3 → NO_2 + O_2

Ozone is an important gas pollutant because in high doses can provoke necrosis to plants like pathogens and wounds do, causing visible injuries to the leaves. Apart

from that, it makes faster leaf senescence and it decreases photosynthesis resulting to overall decreasing of the plants growth (Tai et al., 2010). It is the major regional air stressor in Europe and it affects over 30% of the forests of the planet (Karnosky et al., 2005). In addition, it alters carbon partitioning and it is proved to cause stress-induced gene expression (Tai et al., 2010). The way it works is by entering inside the plant tissues mainly from the stomata and enhancing the creation of reactive oxygen species (ROS) which produce oxidative stress. A small amount of ozone is also entering from the cuticle, but it is almost negligible compared to the stomatal uptake (Calfapietra et al., 2013). This does not mean that ozone does not provoke damage to the cuticle too, as chronic exposure harms it, changing its protective action (Fares et al., 2008). As it comes to ROS, they may serve as indicators to show that there is a problem to the plant and their excessive production can destroy cellular structures and molecules (Tai et al., 2010).

Before the industrial revolution the concentration of the ozone near the ground was no more than 10 ppb (parts per billion), but during the last years the average annual concentration is about 35-50 ppb (Paoletti et al., 2017), as you may see in Figure 1.

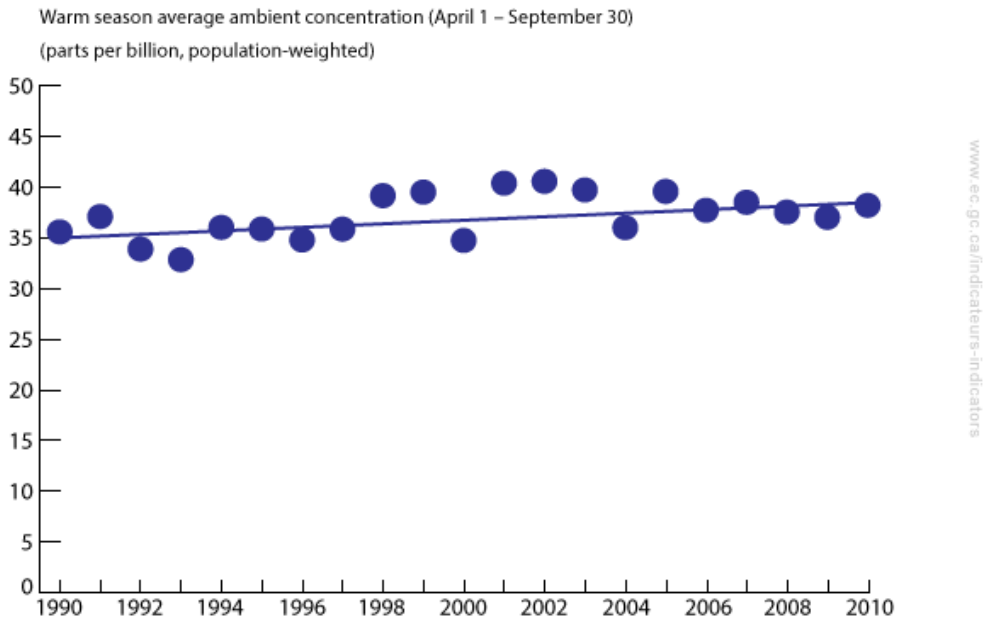


Fig. 1: Tropospheric ozone concentrations during last decades

Especially after 1975 ozone concentration has increased 0.5-2% per year and in some places in Asia have been measured peaks of 165-316 ppb (Paoletti et al., 2017). In the

same paper is mentioned that predictions for the future say that the expected values of annual average are around 80 ppb, while the peaks are expected to be more than 200 ppb in 2100. European legislation has achieved to decrease the peaks of ozone concentrations by regulating the emissions of its precursors, but in the next years climate change may increase again the ozone levels as heat and sunlight favor photochemical reactions that lead to the production of ozone such the ones described above.

Given the fact that heat and solar radiation enhance ozone production, there is no problem with high ozone concentrations during the night as well as lower concentrations are observed during winter. This is the reason why in some researches only warm season concentrations are taken into account and this is why in the present study the experiments took place during the summer. Of course, this does not mean that during the whole season or even day the concentrations are the same. As ozone production is a dynamic process that happens every moment according the availability of ozone precursors and the meteorological conditions, the levels of ozone are constantly changing.

As it comes to legislation, the primary standard in USA is 75 ppb, while in Canada the target standard is 65 ppb (Tai et al., 2010). As primary standard is defined the one that has been calculated according to the human health, while there is also the term of secondary standard which is welfare-based. In Europe, the legislation is following the instructions of the World Health Organization (WHO). The suggested level in the "WHO Air Quality Guidelines" before 2005 was 120 μg m^{-3} for 8-h mean concentration but then for human health the critical level was reduced to 100 μg m^{-3} for 8-h mean concentration (WHO, 2017). 8-h mean concentration is referring to the level of ozone concentration during a period of 8 hours that usually is the workday and it should not exceed the previously mentioned value. To convert the values given in ppb (parts per billion) to μg m^{-3} the following equation as given in: http://www2.dmu.dk/atmosphericenvironment/expost/database/docs/ppm_conversion.pdf can be used: μg m^{-3}= (ppb)*(12.187)*(M) / (273.15 + °C).

Concerning plant protection, the official threshold value is 65 μg m^{-3} for 10-h mean concentration, but it is more used the AOT40 of 3000 ppb during day for three consecutive months (Ribas & Peñuelas, 2004). AOT40 is a very used way of measuring

ozone concentrations and it measures the accumulated over threshold 40 ppb value. Can be calculated from the equation:

$$AOT40 = \sum_{\substack{[O_3]>40 \\ Rad.>50 Wm^{-2}}} [[O_3] - 40]\Delta t$$

The integral is taken only for daytime as ozone concentrations during the night are considered negligible. In reality, this index expresses "the sum of average hourly ozone concentrations exceeding 40 ppb during the day hours in which the global radiation is equal or above than 50 W m^{-2}" (Fares, 2008).

As it is already mentioned, ozone concentrations in the Northern hemisphere are higher in South countries than in Northern ones because of more intense summers and generally higher temperature and sunlight. A research that was made in five European countries (Spain, Italy, Luxemburg, France and Switzerland) confirmed that higher values were observed in Italy, Southern Switzerland and Spain. The reason that Southern Switzerland appeared more contaminated although it is not in the South, is that it is near to the zone of Milan, where industries are located. This fact, together with high traffic emissions, increases the ozone precursors, which can explain more elevated values of ozone concentration in Milan and nearby regions (Sanz et al., 2007). The effect of wind is affecting places that are near industrial regions since fluxes of VOCs and NOx are determined by the multiplication of the vertical wind velocity by the gas concentration at the set height (Fares, 2008). Another important factor that affects ozone concentrations it seems to be the altitude, with concentrations to increase from lower places to higher even during the night. A possible explanation is that mountain areas probably remain inside reservoir layers which means air masses rich in ozone (Sanz et al., 2007).

As it results from all the above, it is obvious the significance of further study of the impacts of ozone to the plants in South Europe where ozone concentrations are higher. It is of high importance to know the distribution of ozone in different places and during different seasons of the year, as well as the values of AOT40. In addition, it would be useful to study the stomatal flux of ozone, in order to combine the previous knowledge with the responses of the plants. This goal is difficult to be achieved as macroscopic symptoms like defoliation or decrease of tree growth may happen due to

other stressors too. Moreover, visible symptoms that are clearly impacts of ozone pollution are not always connected to growth reduction. Consequently, this connection between the two has also to be investigated (Ferretti et al., 2007).

It is an indisputable fact that a factor that also plays important role for the health and growth of the plants is the pests that are attacking them. In a study that was conducted in USA, it is observed that ozone may also play a role to forest pests' occurrence. In some of the species that were used for this research it was shown that forest tent caterpillars were increased in higher ozone concentrations, same as the population of aphids. Additionally, the effects of *Melampsora medusae* were worsen with ozone, maybe because for some species even the structure and the chemical composition of the leaves was changed making them more susceptible to infections. What is more, ozone enhanced leaf abscission and had negative effects on Leaf Area Index (LAI). In some cases, it also reduced the volume of xylem, with the effects to be earlier noticeable for pioneer species while slower growing, late successional species took more time to show symptoms (Karnosky et al., 2005).

In corresponding studies for Europe that were investigating the response of European forest species in ozone, was found that for deciduous species like birch and beech that are considered sensitive, the decrease in growth was 5% for an AOT40 value of 5 ppm h. Generally, it was observed that mesophylic species are more sensitive than xerotolerant ones. Schlerophylls are more tolerant to high ozone levels as they have low gas exchange rates and better stomatal control. However, even species with high gas exchange rates like *Phillyrea latifolia* may show tolerance to ozone. Role may play also the number of stomata in each leaf, as higher stomatal density means less ozone uptake per each stoma. Another important factor is the season of the year, as in different months plants have different stomatal conduction (Paoletti, 2006).

Furthermore, apart from the month, it seems that ozone has diverse effects to plants, especially at cellular level, also during different time of the day. More damage in the DNA is observed in the morning, maybe because of the higher levels of oxidative damage at the end of dark period. This means that the plants are suffering a lot during the morning, but after that, they have the capacity to repair more or less the damage that occurred, although this repair activity is not the same for every plant (Tai et al.,

2010). The fact of DNA damage associates ozone with genotoxicity and high levels of lipid peroxidation. Lipid peroxidation is provoked by ROS, but the latter can be controlled by production of antioxidants (Tai et al., 2010).

Another study conducted by Nali and collaborators held in Europe in species of Mediterranean Macchia, like strawberry tree that was also used for the present study, showed that high ozone concentrations had visible symptoms to the plants. The treatment was applied to the plants for 5 hours per day during three months and the maximum level of ozone concentration was 110 ppb. Conditions of that high ozone concentration for so long time are not easy to be observed in the field. Some of the visible symptoms appeared were the appearance of foliar chlorotic mottles in the leaves of laurel and phillyrea and reddish interveinal stipple-like necrotic lesions in the leaves of strawberry tree, but in any case the damaged area did not exceed the 8% of the leaf (Nali et al., 2004). In Figure 2, there are some visible symptoms due to ozone exposure in the sensitive hybrid poplar which was employed in the present study.

Fig. 2: Ozone visible foliar injury in poplar

Additionally, in the same research, when they measured the relative water content (RWC) in laurel and strawberry tree, they found out that ozone reduced membranes retention capacity. Apart from the concentration of ozone, another important factor is the time. For membrane retention capacity it was confirmed that even 45 days were enough to change it. The differences of effects between the species may be explained because each species has also different stomata functioning. Moreover, we have to

take into consideration that these studies are about evergreen broadleaves that are supposed to be more resistant than pines for example, due to their schlerophylic adaptations. Generally speaking, species that are acclimated to drought and have high levels of antioxidants can protect themselves better from the oxidative damage that ozone provokes (Nali et al., 2004).

In any case, in Mediterranean climates species develop mechanisms to protect themselves from both air pollutants and drought. More specifically, two groups of different mechanisms can be observed. The drought avoiding species and the drought tolerant species. The first group avoids drought by closing their stomata during water-stressed periods, while the second one maintain relatively high stomatal conductance during drought periods. Pines can be considered as drought avoiding species, while hardwoods like Holm oak can be considered to belong to the second group. Of course, the stomatal conductance affects also the ozone uptake, with higher stomatal ozone flux to be associated with higher maximum stomatal conductance (Hoshika et al., 2017a).

The Single Cell Gel Electrophoresis as a tool to detect DNA damage

The Single Cell Gel Electrophoresis (SCGE) or Comet assay has its origins 40 years ago, back in the 1970s when two scientists named Rydberg and Johanson used it to quantify DNA damage in nuclei (Lanier et al., 2015). Its realization would not be possible without the investigation of nuclear structure by Cook and collaborators (1976) who discovered a way to "wash" the cell with a special detergent removing membranes, cytoplasm and nucleoplasm leaving just the nucleoids (Collins, 2004). According to his model of nuclear structure, the DNA is supercoiled around the histones and even if the histones are not there after washing, the loops of DNA remain constrained because of their matrix attachment. However, if there is presence of strand breaks, the supercoiling is relaxed and the broken loops are more free to migrate (Collins, 2014).

Ostling & Johanson who developed a microelectrophoretic technique to observe directly the DNA damage in individual cells of mammals improved the Comet assay in 1984. Back then, they did not call it Comet assay, although the image they obtained was the typical one with the broken DNA to form tails (Collins, 2004). Only in 1990, Olive and collaborators named it Comet assay, as nucleoids take this form because the high-molecular-weight DNA is located in the head, while the tail contains the ends of migrating fragments (Singh et al., 2014). The Comet assay, is a promising method for the detection of DNA damage because it is economic, fast and simple to apply and very sensitive (Pourrut et al., 2014). However, until the 1990's it was applied only in mammalian and fish cells (Lanier et al., 2015). The reason why the Single Cell Gel Electrophoresis took almost twenty years to be applied in plant cells is due to the presence of cell wall which provokes some technical problems (Santos et al., 2015).

The main idea of Single Cell Gel Electrophoresis is that the cells are embedded in slides with agarose and after lysis an electrical field is applied under neutral or alkaline conditions (Ventura et al., 2013). The DNA is negatively charged (Collins, 2004) which makes it moving towards the anode of the electrophoretic field. As the chromatin is organized in the form of "loops", for the healthy cells the loops are well supercoiled and the DNA does not migrate. On the contrary, when there is damage the

DNA is fragmentized and under the electric field it gets uncoiled forming a “halo” around the nucleus (Ventura et al., 2013). The more breaks there are in the DNA the more it migrates creating a big tail, while when it is entire and undamaged there is only the head as we can see in figure 3.

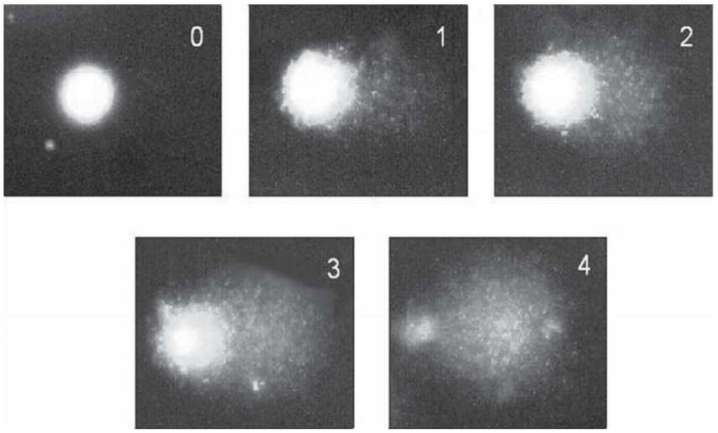


Fig. 3: Images of "comets" ranging from undamaged DNA without migration (0) to totally fragmented DNA (4)

Briefly, the steps of Comet assay are the ones described below. First, most of the researchers agree that the lysis of the membranes has to be achieved for the nucleoids to be released, although Pourrut and collaborators in 2014 claimed that this step in unnecessary. In the next step, the nucleoids are suggested to be exposed to alkaline conditions to let the DNA be uncoiled and denaturated and at the end the electrophoresis has to be conducted in preferably alkaline conditions again (Tice et al., 2000). There are some disagreements about the pH that has to be applied, as some researchers declare that both denaturation and electrophoresis can be carried out also in neutral conditions, but that means that unwinding and denaturation are prevented and consequently only double strand breaks can be observed (Ventura et al., 2013). Another option is to use alkaline solution for the electrophoresis and a neutral one to conduct the electrophoresis (Lanier et al., 2015). Generally, alkaline conditions are suggested, but there is not a minimal standard because of the variability in Single Cell Gel Electrophoresis (Tice et al., 2000). In any case, most of the researchers that deal with the Comet assay in plants use the alkaline conditions while only a small minority prefers the neutral protocol (Lanier et al., 2015).

Although the sensitivity, simplicity and versatility of the Comet assay are an indisputable fact, the above disagreements set a question about its reliability. As each laboratory follows its own protocol, the comparison of the results between different laboratories becomes impossible (Azqueta et al., 2011). In some occasions investigators decide to conduct the experiments two times, one under neutral and one under alkaline conditions, in order to compare then the differences between the two different protocols, since Single Strand Breaks (SSB) can be detected with the use of alkaline buffer, while Double Strand Breaks (DBS) are detected with the use of neutral one (Abas et al., 2007). The pH of the unwinding and electrophoresis solution is not the only problem as there are also differences in the voltage gradient applied, which varies from 0.76 V/cm to 1.6 V/cm; in the unwinding or electrophoresis time that usually vary from 20 minutes to 40 minutes and from 20 minutes to half an hour respectively; and also, in the current applied, with values that range from 260 mA to 300 mA (Azqueta et al., 2011). In addition, there are different approaches also in the steps before electrophoresis which imply the isolation of the nuclei and diverse methods are suggested, such as chopping or slicing (Pourrut et al., 2014). Finally, the findings of the comparison of different parameters showed that the more time unwinding and electrophoresis are implied, the more damaged the nuclei appear, with unwinding time to be more important than electrophoresis time (Azqueta et al., 2011). Generally, more damage was evident when alkaline conditions were used. In addition, the step of nuclei isolation plays an important role (Abas et al., 2007). Furthermore, regarding the other parameters mentioned above, higher voltage gradient shows bigger damage too (Azqueta et al., 2011).

As it is mentioned before, apart from the unwinding and electrophoresis, another significant factor that has to be taken under consideration is the isolation of nuclei (Pourrut et al., 2014). As everyone agrees that cell wall is an inhibitory factor for Comet assay, researchers chop or slice the leaf in order to relief the nuclei into the buffer that they use (Tai, 2010). In this step, light and high temperature can induce damage to the nuclei, but the extent of this damage is also depending on the part of the plant from which the nuclei are obtained, as nuclei taken from roots are more sensitive to light compared to those obtained from leaves (Pourrut et al., 2014). In addition, usually a lysis solution is used in order to remove the membranes and the histones from DNA and obtain nucleoids (Collins, 2014). However, in the research of Pourrut and colla-

borators in 2014, they claimed that not only lysis is unnecessary, but also filtration that is normally used to clean the final solution. Actually, on the one hand, they declare that during lysis DNA repair may occur inducing a bias to the results of comet assay and on the other hand they said that filtration provokes a little damage to the nuclei.

Instead of the use of nuclei as a source material, for the extraction of nucleoids also protoplasts can be used. With this method, researchers also agree that light causes damage to the protoplasts although there are some mechanisms like photorepair (Abas et al., 2007). In any case, finally the protocol that each investigator follows is upon to the needs of the experiment with the source material to depend on the operator. On the other hand, the Comet assay parameters depend on the plant or organ because factors like the growing stage or the leaf position may also alter the results of the experiment (Pourrut et al., 2014).

After the completion of the electrophoresis, the next steps that have to be followed are the neutralization of the slide that was under alkaline conditions, the staining of the DNA and in the end the visualization and scoring of the comets (Tice et al., 2000). The most commonly used DNA-binding dye to visualize comets is the ethidium bromide which binds better to double-stranded DNA and the next most used dye is DAPI whose official name is 4,6-diamidino-2-phenylindone (Collins, 2004). Other dyes like propidium iodide, SYBR Green and YOYO-1 have also been reported (Tice et al., 2000). An interesting case that has to be mentioned is this of acridine orange (AO) which stains with red colour the single-stranded DNA and with yellow-green the double-stranded DNA giving images of comets with yellow-green heads and red tails (Collins, 2004).

The last part of the Comet assay, which is the scoring of comets, is usually made using specific software, although visual interpretation is said to be valid too and that it gives results similar to the image analysis (Collins et al., 2004). The parameters that are scored have to do with the head of the comet, the tail or the entire comet. In the first group there are the head diameter and the percentage of DNA in the head, in the second one the tail length, the tail area, the percentage of DNA in the tail, the tail length/head diameter ratio, the tail intensity and the tail moment, while parameters

related to the entire comet are the comet length, comet area, comet intensity and percentage of nuclei with tails (Lanier et al., 2015). The most used of them is the tail moment (TM) which is defined as the product of the tail length and the fraction of total DNA in the tail. Tail moment incorporated a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed / broken pieces (represented by the intensity of DNA in the tail) (Olive et al., 1990). However, there are researchers that do not appreciate it, as they claim that it is about a term that is not clearly depended on the dose and consequently suggest instead other parameters like the use of percentage of total fluorescence in the tail as more reliable and easy to use and to interpret (Collins, 2014).

For the scoring different number of comets is used by each researcher, with the most popular number to be fifty comets per slide, although there are researchers who claim that a minimum of twenty comets per slide still gives statistical reliability (Lanier et al., 2015). In any case, independently of the number of comets that each one prefers to score, the total number of nuclei in the slide should not be too high as if there are too many comets with migrating DNA the tails may overlap making the scoring difficult if not impossible (Tice et al., 2000). However, enough nuclei have to be present in each slide, as if there are a lot of nuclei, the border effect can be avoided and the scoring of the slide becomes easier and faster (Pourrut et al., 2014). Additionally, it has to be mentioned that apart from the number of the scored comets, for a robust statistical analysis is recommended the scoring of two slides per condition, although most of the authors prefer to score three slides (Lanier et al., 2015).

Despite the fact that comet assay indicates the damaged DNA it is not sure that can identify apoptotic cells (Santos et al., 2015). This has two explanations. The first one is that the cells visualized in the Comet assay sometimes may have repaired their damage and return to their initial state, while apoptosis is irreversible. The second one is that apoptotic cells have too fragmented DNA that it would disappear during procedures like lysis or electrophoresis (Collins et al., 2004).

As it was mentioned before, the cells can in some cases repair their damage, so the Single Cell Gel Electrophoresis technique can be also used to measure the repair of DNA (Collins, 2014; Ventura et al., 2013). In addition, it has a lot of applications in

environmental pollutant bio-monitoring for *in vivo* and *in vitro*, like for instance in the study of Restivo et al., 2002, where the effects of ozone were measured in the leaves of two tobacco clones. Another study that uses the Comet assay to measure ozone damage, this time in a poplar species is the one of Tai et al., 2010. It has also been used in a study for the detection of DNA damage to plants that were located near the road and others that were away from them and consequently were subjected to less pollution because of the vehicle emissions (Sriussadaporn et al., 2003). Generally, it can be considered suitable for genotoxic assessments in both field and indoor atmospheres (Restivo et al., 2002).

Protoplasts: Isolation & Use

One of the differences between plant cells and animal cells is the presence of cell wall (Figure 4) which is made mainly by cellulose and it is useful for structural support of the plants (Wu et al., 2009). As it was also mentioned before, Single Cell Gel Electrophoresis (SCGE) or Comet assay is mainly implemented to animal cells because of the difficulties the cell wall provokes to the extraction of the nuclei. Protoplasts are plant cells without cell wall, rounded just by the cell membrane. The isolation of protoplasts can be made with two ways. The first one is the mechanical disruption, which was the first method that historically was applied for isolation of protoplasts, and the other one is enzymatic digestion of the cell wall (Davey et al., 2010).

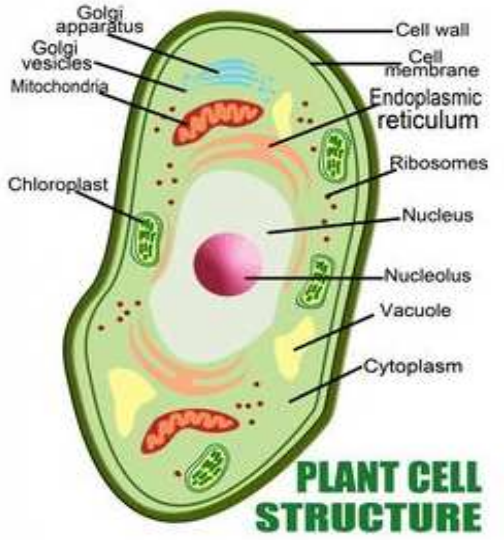


Fig. 4: Plant cell

In mechanical disruption the plant tissues had to be sliced, but as with this method only a few protoplasts could be obtained, it is not used anymore nowadays (Davey et al., 2010). For the method that involves enzymatic degradation of the cell wall is used an enzymatic solution that usually contains cellulase and macerozyme (Wu et al., 2009). Macerozyme, a mixture of pectinase, cellulose and hemicellulose, serves for the degradation of middle lamella between neighboring cells, leaving the cells separated and exposed to cellulase that destroys the cell wall. Different amounts of these enzymes have been tried to different enzymatic cocktails according to the protocol of each species, as well as different types of enzymes, but always containing pectinases and cellulases (Tan et al., 2013). Conclusions may differ from study to study, as while according to Conde & Santos (2006) the best results are acquired with the use of Cel-

lulase Onozuka RS instead of Cellulase Onozuka R10 and with the use of Macerozyme Onozuka R10 rather than pectinase alone. On the other hand, in the study of Tan and collaborators in 2013, the best enzymatic cocktails were containing Cellulase C2605 and Pectinase P2611. The enzymatic solution also contains an osmoticum, whose concentration is important for the isolation of the protoplasts. Mannitol or sucrose are usually used as an osmoticum and if the concentration is too high, the protoplasts instead of being spherical as they have to be, they shrink. On the other hand, if the concentration is too low there is the danger of lysis because of the water that will enter into the naked cell. The same concentration of osmoticum must be also used for the Cell and Protoplast Washing Solution, known as CPW (Davey et al., 2010).

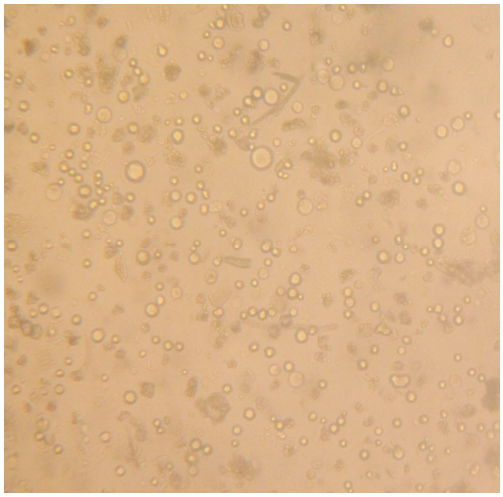


Fig. 5: Isolated protoplasts from *Arbutus unedo* before the purification process

For the cell wall to be digested different incubation periods have been suggested, traditionally ranged from three to sixteen hours, although alternative methods have been investigated (Lin et al., 2014). After the digestion of the cell wall with the use of the enzymatic solution, the next steps that have to be followed are the filtration and a series of centrifugations (Giles, 1983). In the Figure 5, there is a solution of protoplasts isolated in the laboratories of Tuscia University during the classes of Forest Biotechnology, before filtering. The blurred places of the image exist because not all of the protoplasts and debris are on the same level and consequently it is impossible an ideal focus that would include every part of the field to be achieved. Protoplasts are present, but also cell debris and undigested plant tissues. For the filtering little metal or plastic sieves are used with size that may differ according to the size of the protoplasts of each species. The goal is to remove undigested plant tissues and cell debris and to fi-

nally obtain a cleaner suspension. To complete this procedure is suggested the washing of the suspension in Cell and Protoplast Washing solution (CPW) with the same concentration of the osmoticum as the enzymatic solution as it was mentioned before, and the subsequent centrifugation. The centrifugation serves for the removal of finer cell debris that has different weight than the protoplasts, so it is supposed to stay in the supernatant while the protoplasts are forming a pellet. The washing and centrifugation steps may have to be repeated in order to take an as much as possible purer suspension of protoplasts (Davey et al., 2010).

Apart from the composition of the enzymatic solution and the subsequent purification of the solution, another important factor for the success of the isolation of protoplasts is the physiological status of the tissues that are used (Kuzminsky et al., 2016). Protoplasts can be isolated from different parts of the plant. Usually leaves and embryogenic or dedifferentiate cell suspension are used as source material, but petals and seedling organs like hypocotyls, cotyledons and roots can also be used (Davey et al., 2005). When the isolation is made from the leaves, the age seems to play an important role, as younger leaves tend to give higher yield of vital protoplasts (Davey et al., 2010; Kuzminsky et al., 2016). Indeed, according to a study that Conde and Santos conducted in 2006 in *Ulmus minor*, when leaves from greenhouse plants were used, protoplasts were released only from very young leaves, while better results were obtained from in vitro material. In addition, it is observed that protoplasts that derive from leaves and consequently are full of chloroplasts are easier to be lysed in comparison to those coming from cell suspensions (Davey et al., 2010). In addition, there are some restrictions, as only plants actively growing and in healthy conditions can give protoplasts (Lin et al., 2014).

Generally, the successful isolation of protoplasts is also depending on the species, as many species are recalcitrant making the isolation impossible, with more difficulties appearing in woody species that show a resistance to cell wall degradation (Jones et al., 2015). In woody plants, one of the problems is hydroxycinnamic acids that were created through phenylpropanoid pathway. What happens is that phenolic compounds like ferulic and coumaric acids are binding to the cell wall making it stronger and inhibiting enzymatic degradation (Jones et al., 2015). According to Jones and collaborators (2015), a possible solution could be to stop phenylalanine ammonia lyase by add-

ing to the culture of source tissues an inhibitor called 2-aminoindane-2-phosphonic acid.

The isolation of protoplasts is not an easy procedure as it is time-consuming, in some occasions it has to be done in strict, sterile conditions and in the case of leaves as source material the slicing in little pieces of 0.5-1mm has to be manually and with lot of attention (Wu et al., 2009). Attempts to overcome these disadvantages have been made in the past, with researchers to suggests the isolation of protoplasts from xylem (Lin et al., 2014) or the removal of the lower epidermis from the leaves with the use of tape instead of manually in order to be easier the release of protoplasts (Wu et al., 2009). The technical difficulties are not the only disadvantages, but there are also disadvantages for the cell, as the isolation by itself is a stress-inducing procedure for the cells that creates Reactive Oxygen Species known as ROS and provoke oxidative stress to the plants (Papadakis & Roubelakis-Angelakis, 2002).

However, the isolation of protoplasts deserves to be further studied as it may have many useful applications. Till now, the most extended use is for creating new plants because of the totipotency of protoplasts that permits them to re-enter to the cell cycle, create a new cell-wall, elongate and divide. The generation of new plants may happen after the fusion of protoplasts that allows the intake of new and different DNA into the new plants (Davey et al., 2005). Consequently, protoplasts are very important for the biotechnology of transformation, but also just for the study of cell division as well as for the understanding of injection and fusion procedures (Bergounioux et al., 1988). Furthermore, apart from its importance for genetics and cell ultrastructure, the study of protoplasts is a useful tool for studies of physiology and plant-pathogen interaction (Davey et al., 2005). Protoplasts are a novel source for obtaining nuclei from plants and make easier the implementation of SCGE in plants (Kuzminsky et al., 2016) as also happened in the experiments conducted for the completion of this thesis.

Already till now a lot of studies have been conducted showing the importance of the protoplasts for both applications of DNA transfection (Lin et al., 2014) and studies of gene functions but also for the understanding of physiological perceptions and responses to environmental factors (Tan et al., 2013). An interesting discovery for this sector is that protoplasts can be isolated not only from the mesophyll but also from the

epidermis as it was shown in a study where Petunia's petals were used for isolation (Faraco et al., 2013) and that even guard cells can give protoplasts, usually smaller in size than mesophyll protoplasts (Pandley et al., 2002). For the isolation of the guard cell protoplasts the same procedure as for the mesophyll protoplasts is used, with the difference that the concentration of the osmoticum is lower (Zhu et al., 2008). In the first case, the difference of the provenience was obvious because of anthocyanins that were present in the epidermal cells giving the protoplasts a pink colour (Faraco et al., 2013). In the second case, the isolation of guard cells protoplast is considered very important as it can illustrate the "electrophysiological, biochemical and molecular genetic pathways of guard cell function" as it is mentioned in the text (Pandley et al., 2002). Other studies about physiological responses using protoplasts have been conducted, like for instance the one of D'Angeli and collaborators in 2003 that studied the response of protoplasts to fast temperature decreases. The response was measured according to the increase of free cytosolic calcium, which is involved in cold acclimation, so the use of protoplasts was preferable in order to avoid the interference of apoplastic Ca^{2+} which is bound to the cell wall.

Plant species:

Arbutus unedo

Arbutus unedo is an evergreen shrub of Ericacea family with common name strawberry tree (Fiorentino et al., 2007). It is distributed in most of the countries of the Mediterranean basin, namely Turkey, Greece, Italy, France, Spain, Morocco, Algeria, Tunisia, Libya, as well as Portugal. There are isolated populations also in Lebanon, Israel, Syria and Ireland. Its height can reach 5-10 m, although very often, the shrub does not exceed the 2-3 metres, and the trunk diameter can reach 80 cm. The leaves have a dark green colour and they are glossy with a serrated margin. Their length is 5-10 cm and the width 2-3 cm. The flowers are hermaphrodite of white colour and bell shape with 4-6 cm diameter. During the autumn, they are organized in panicles of 10-30 together, which have a mild sweet scent. Bees carry out the pollination. At the same time of flowering in the autumn, the tree still has the fruits of the previous pollination, which are red berries, of 1-2 cm diameter with a rough surface. They need 12 months to mature and they are edible, sweet and soft when reddish. Frugivorous birds can disperse seeds. The strawberry tree is easy in its cultivation as it is adaptable to a wide range of climates. It is shade tolerant, salt tolerant, drought resistant and frost resistant, so it can easily survive in both cases of cold and hot weather (Celikel et al, 2008).

It is an important species from an economic point of view too, as it is used for ornamental purposes, and also for pharmaceutical and chemical industrial applications. In addition, liquors can be produced from its fruit and consequently, this tree is a subject of study because of its diverse uses (Ribeiro et al., 2017). It use for pharmaceutical purposes can be justified as it is known the antioxidant activity of its acetone and ethanolic extracts (Andrade et al., 2009). However, the strawberry tree is underutilized and some organizations like for instance FAO, does some efforts to give a boost to the further use of this species (Ribeiro et al., 2017). There are also studies that analyze the effects that ozone has to this species, since it is considered generally a species acclimatized to the reality of the Mediterranean region and, therefore, to its elevated ozone concentrations too (Paoletti, 2005).

Arbutus unedo, same as other Mediterranean woody plants, is considered to be tolerant to tropospheric ozone thanks to its morpho-functional adaptations to distinct oxidative stress factors typically occurring in the Mediterranean region. In fact, Nali and collaborators (2004) described the presence of visible foliar symptoms (in any case less than 8% of the total leaf surface) only for plants that were exposed to high level of ozone (which means 110 ppb per 5 h every day) under an accumulated exposure over a threshold of 40 ppb (AOT40) of 31.5 ppm h during the three months of the experiment.

Fig. 6: One of the *Arbutus unedo* plants used for the experiments

***Populus maximowiczii* Henry *x berolinensis* Dippel**

The Oxford genotype of poplar is a hybrid with parent plants *Populus maximowiczii* (seed parent) and *Populus berolinensis* (pollen parent). During the summer its characteristics described by Schreiner and Stout in 1934 are the following. The stems are round and somehow sparsely pubescent, with colour that is brownish red toward tip and olive green toward base. The lenticels are white, linear toward tip and pinkish brown, and oval toward base. Their length ranges from 0.5-4.0 mm. The secondary shoots are somewhat less red. The shape of the leaves is broadly ovate to oval with apex short acute and base obtuse. Their color is dull dark green above and glaucous below and the margins are medium finely crenate. The midrib and the basal portion of large veins is red and puberulose above, while in the below surface they are green and glabrous. The shape of the petioles is round to broadly oval and their color is red and puberulose above and green and sparsely puberulose below. Their length is 30-50 cm. The stipules are rather small, almost triangular and green. The leaves on secondary shoots are broadly elliptical, in the apex short acute and in the base broadly acute to obtuse with margin more finely crenate. Finally, the buds are lanceolate with length of 6-10 mm, somehow glossy, with red to reddish brown colour, viscid, aromatic and appressed (Schreiner and Stout, 1934).

Fig. 7: One of the Oxford poplar plants used for the experiments

About the winter characteristics, Schreiner and Stout (1934) refer that the stems are round, of reddish brown colour toward tip and olive grey or greenish toward base. They also have pinkish lenticels, which are linear toward tip and elliptical to oval or even circular toward base. The pith is five-sided, of light brown colour and homogeneous. The leaf scars are broadly triangular, decurrent and winged. The stipule scars are linear to narrow V-shaped. As it comes to buds, the terminal buds are ovate, while the axillary buds are broadly lanceolate with narrow acute tips. Their dimensions are 10-14 *x* 3-5 mm of reddish brown colour, resinous, viscid, aromatic and closely appressed.

This species, contrarily to strawberry tree, is considered very sensitive to ozone (Desotgiu et al., 2010). Indeed, in the work of Desotgiu and collaborators (2010), this poplar showed macroscopic symptoms of visible foliar injury (such as interveinal browning) because of ozone exposure very early (less than two months from the beginning of the experiment). But apart from the macroscopic damage, microscopic analysis also revealed extended collapsed areas in the palisade mesophyll of the leaves and in the inner cells of spongy tissue.

MATERIALS AND METHODS

Experimental site

The experimental site for plant ozone fumigation was located in Sesto Fiorentino, nearby Florence, Italy (43° 48’ 59” N, 11° 12’ 01” E, 55 m a.s.l.) at the CNR (National Research Council) campus. The climate there is classified as hot-summer Mediterranean according to the Koppen-Geiger classification. For the months May to September, the mean daily temperature was 24.0^{o}C in 2015, while in 2016 it was 22.9^{o}C. For the same months, total precipitation was 536.2 mm in 2015 and 226.6 in 2016. As the solar radiation and the temperature in summer are very high in this place, it is ideal for the study of the effect of high level of tropospheric ozone, since the previously mentioned factors favor ozone production.

FO_3X (Free-air Ozone eXposure) facility

From June 2015 a fumigation device able to increase the concentration of tropospheric ozone (FO_3X) was installed in Sesto Fiorentino, under the scientific responsibility of Dr. Elena Paoletti (Institute for Sustainable Plant Protection IPSP – CNR). To our knowledge, FO_3X is the only ozone FACE currently available in Mediterranean climate.

FO_3X is considered as a new generation 3D ozone FACE experiment where there were 9 different plots, three per each different ozone treatment. Each plot was 5×5 meters squared with distance 17 meters between different ozone treatments. Every month, the plant position within each plot was change in order to avoid positional effects (Dr Hoshika, personal communication). The different treatments were one with ambient ozone concentration, one with intermediate ozone concentration (1.5 x ambient) and one with doubled concentration than the ambient one. The ozone was being produced by an ozone generator and the diffusion was made through micro-holes in the tubes that were around of the plants. Further details on FO_3X facility are available in the following link:
http://www.ipsp.cnr.it/products-2/facilities/fo3x/?lang=en

The tubes were fixed in a metal scaffolding of 2 meters height. They were made of PVC-C and their diameter was 32 mm. From there, smaller Teflon tubes of 10 mm diameter, 25 per plot, were hanging (Figure 8). They were fixed in the ground in order to avoid moving because of the wind or other reasons. Every 20 cm from where the air-ozone mix was releasing there were micro-holes of 0.2 mm diameter. The flow was tested to be more or less the same from every hole (Paoletti et al., 2017).

Fig. 8: View of a plot with the scaffolds and the tubes providing ozone

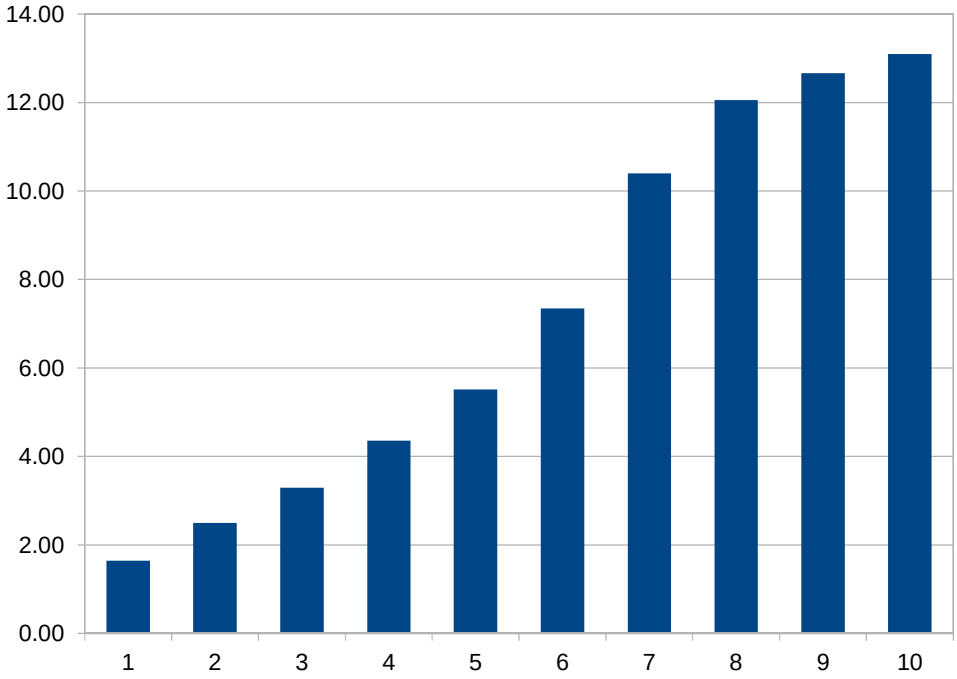
The ozone distributed by this construction was produced by TOGC13X Mk2 compact ozone generators generating 13 g ozone h^{-1} each. The generators were installed in an air-conditioned power-supplied shed with ambient temperature no more than 30^{o}C. The mix of air that was exiting the tubes was ambient air flowed by an oil-free vane compressor mixed with ozone provided by a Venturi tube. The concentration of ozone in each plot was being monitored every 10 s (Paoletti et al., 2017). The fumigation of the plants began the 12^{th} of June 2017 and ended the 15^{th} of October 2017.

Plant material

Young plants of the evergreen species *Arbutus unedo* and of a hybrid poplar clone *Populus maximowiczii x berolinensis* (Stout and Schreiner), were used in the Free-air Ozone exposure experiment. Healthy seedlings of strawberry tree (*Arbutus unedo*) were obtained from a nursery the 4th of May 2017 and their age was 2-3 years. Subsequently, they were moved to the experimental site. Ten plants were used immediately for the measurement of biomass, while the rest of them were transplanted in pots of 30 L with a mixture of sand:peat:soil = 1:1:1 (v:v:v) the 17th of May 2017. In total 27 plants of strawberry tree were divided into the three different plots (9 per treatment). Attempts have been made in order for each plot to have plants from all of the sizes. Contemporarily strawberry tree plants were pruned at 1 m height in order to facilitate the recovery after transplanting and stimulate the sprouting of new branches.

The plants of the hybrid poplar, from now on referred as Oxford genotype of poplar, were already in Sesto Fiorentino since the previous year, derived from rooted cuttings propagated in December 2015 by technicians of IPSP-CNR. For the Oxford poplar clone, 2017 was the second year of ozone fumigation. They were planted in plastic pots of 10 L filled with the same mixture of soil as the Strawberry trees on April 2016 and then, on April 2017 they were transferred in 20 L pots in order to avoid limitation of root growth. Every two or three days all of the plants were irrigated to avoid water stress. Same as for the strawberry tree, for this species also 9 plants were used in each plot of different ozone treatment, which sums up to a number of 27 plants.

For the improvement of the protocol of protoplasts isolation from the Oxford genotype of poplar, two plants were kept in Viterbo where they were closely monitored. Actually, in the beginning only one plant was kept, but as it stopped growing because of the extraordinarily cold April of this year, a second one was taken from Sesto Fiorentino the 4th of May. For almost one month measurements of the leaves were taken every 3 days approximately in order to find out in which age the leaves can be considered fully developed. As it is shown in graphic 1, the growth rate reduces when leaves reach the eighth from apex.



Graph 1: Average leaf length of the oxford clone of poplar in cm

For the sampling, young, fully expanded leaves were used, with ages ranged from 6th to 8th from the apex. As it was mentioned before, the ideal age would be 8th order from the apex, but if they were too hard the protoplasts could not be isolated. Consequently, the softer ones were chosen (as you may see in Figure 9) and cut the same day of the experiment in the arch of the morning. The samplings took place on 21st of July and 30th-31st of August. After sampling, the leaves were kept in water and transferred in the laboratory where they were processed within 1 hour (Choury et al., 2017). For the procedure of the protoplasts isolation leaf tissue of ~0.045 g were used, taken from the middle part of the leaf avoiding the main rib.

Fig. 9: Examples of leaves used for protoplasts isolation

Protoplast isolation

Sampling for the protoplasts isolation was made on July and August. For the protoplasts isolation the little squared pieces of the leaves were put in a petri dish contained Cell and Protoplast Washing solution (CPW) and PVP-40 powder. The CPW was prepared beforehand according to Kuzminsky and collaborators (2016) by adding in distilled water 0.2 mM KH_2PO_4, 1 mM KNO_3, 10.1 mM $CaCl_2.2H_2O$, 1 mM $MgSO_4.7H_2O$ and 0.96 μM KI. Few days before the experiment 11% of Mannitol and 0.1% of BSA were being added. The pieces of leaves were placed with the abaxial surface facing up and subsequently the epidermis layer was removed by scratching gently with a fresh sharp razor blade. Then, they were sliced into little pieces with the same razor blade and forceps (Figure 10) and left inside CPW for 10 minutes (Figure 11). After that, they were transferred into the enzyme solution with the abaxial surface facing down and the Petri dishes were put under Vacuum conditions for half an hour to favor penetration of the enzyme solution inside the leaf tissue (Figure 12). The enzymatic solution was made upon a stock solution of KH_2PO_4 (1 mM), NH_4NO_3 (5 mM), sodium citrate (5 mM) and Mannitol (0.6 M) diluted in distilled water at pH 5.0 (Kuzminsky et al., 2016). The stock solution was being kept in the fridge at 4^oC and just before the isolation of protoplasts 1% of Macerozyme R-10 and 3% of Cellulase RS were added, as well as 2 mM of DTT. After the preparation, the enzymatic solution was being warmed at 55^oC for 10 minutes in order to improve enzyme solubility and inactivate DNAse and proteases. After that, the solution was cooled under cold tap water and filtered with a plastic filter (0.45 μM). Then, 2.5 ml of the solution was placed in a 35 mm Petri dish and antibiotic (Carbenicillin and cefotaxime, 0.5% v/v) as well as 1% w/v of PVP-40 powder were added (Choury et al., 2017). Subsequently, they were left at 25^oC to shake gently in the dark for 4 hours (Figure 13). In the end of 4 hours the presence or absence of protoplasts was checked with an inverted microscope.

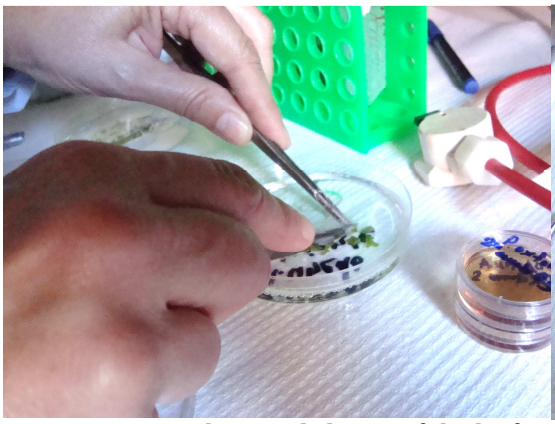


Fig. 10: Scratching and slicing of the leaf tissue

Fig. 11: Washing of the tissue for 10 mins in CPW

Then, 2 ml of the solution of each Petri dish were filtered and put into a 2 ml Eppendorf tube. For the strawberry tree, filters of 50 μm were used, while for the oxford genotype of poplar, 30 μm filters were used, as the poplar's protoplasts were supposed to be smaller. Then, the first centrifugation was conducted in 2000 rpm for 5 minutes in order to maintain only the protoplasts in the pellet and the pellet was kept while the supernatant was removed. The 0.5 ml that was remained of the solution in the Petri dish was transferred to the Eppendorf tube with the pellet and CPW was added till the Eppendorf tube was filled. A second centrifugation was conducted under the same conditions. The supernatant was removed again and the pellet was kept. Now, for the washing of the protoplasts 2 ml of CPW were added to the Eppendorf tubes with the pellet. The third centrifugation was done as before and only the pellet was kept again, this time as drier as possible.

Fig. 12: Vacuum for 30 minutes

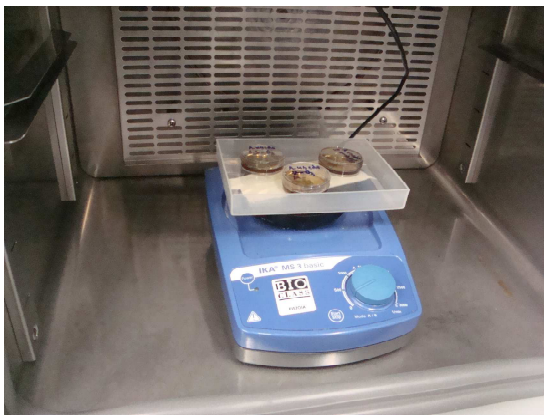


Fig. 13: Orbital shaking for 4 hours

As the protocol of protoplast isolation for this clone of poplar was not well established, another protocol was also tried in order to save time. An eighth leaf was taken from the plant that was kept in Viterbo and cut in two pieces. Then, a strip of Time tape was affixed to the adaxial surface of the leaf, according Wu and collaborators 2009 and the leaf was submerged into CPW containing also PVP-40 powder. Subsequently, a strip of scotch was put to the other side of the leaf. After ensure that it was well stuck, the scotch was carefully removed in order to peel the abaxial epidermis cell layer. The leaf was cut in big pieces just to fit in the 35 mm Petri dish with the enzymatic solution, the antibiotic and the PVP-40 powder. Then, it was left for one hour in gentle orbital shaking at 25^{o}C in the dark. A second observation was done after 3 hours, another one after 4 and a last one after 24 hours.

Nuclei isolation

In order to avoid the time-consuming protoplast isolation some attempts have been with purpose the direct isolation of nuclei according the Tai and collaborators protocol, 2010. Briefly, a segment of both a frozen and a fresh leaf of the Oxford clone of poplar with dimensions 1 cm x 2 cm was cut and placed into 300 μl of ice-cold PBS. The frozen leaf was flash frozen in liquid nitrogen some days before and then stored at -80^{o}C. 50 mM EDTA was added in the PBS just before its use. The thawed segment was sliced in a 60 mm Petri dish with a fresh scalpel blade. Then it was gently squeezed with a pestle till the liquid obtained a green colour. After that, the liquid was put in an Eppendorf tube after filtration with a 30 μm filter and was centrifugated in 3500 rpm for 5 minutes. Then the supernatant was thrown away and the protocol continues as described below, same as the procedure followed with protoplasts' pellet, without the step of incubation in lysis.

An attempt was also made to try a different buffer according Loureiro and collaborators, 2007, so WPB was prepared. For its preparation 0.2 M Tris Hcl, 2 mM $MgCl_2.6H_20$, 86 mM NaCl, 10 mM $Na_2S_2O_5$ and 2 mM EDTA $Na_2.2H_20$ were used. Just before its use also 1% of Triton-X and 1% of PVP-40 were added. For this protocol, one piece of leaf of same dimensions as in the previously described protocol was chopped for 2 minutes with a blade in a Petri dish of 60 mm placed on ice which contained 2 ml of WPB. Then it was left for two more minutes in darkness and afterwards the liquid was collected and placed in an Eppendorf tube. Then the step of centrifugation in 3500 rpm for 5 minutes followed and the slides were prepared as before.

Single Cell Gel Electrophoresis

For each slide to be prepared 90 μl of Low Melting Point Agarose was added in the Eppendorf tube with 10 μl of protoplast suspension and then 100 μl of the mix was put in a microscope slide previously coated with a layer of 1% Normal Melting Point Agarose. The cover slip was put and the slides were left for 10 minutes at 4^{o}C in the fridge. Afterwards, the cover slips were removed and the slides were put in a Coupling jar with lysis solution for 1 night. The lysis solution was prepared before and was containing 2.5 M of NaCl, 100 mM of EDTA, 10 mM of Tris-Base and 0.2 M of NaOH diluted in distilled water. The pH was adjusted to 10 before storing the stock solution. Just before the use 1% of triton X-100 and 10% of DMSO were freshly added.

The next day the slides were horizontally placed into an electrophoresis unit and were cover with cold electrophoresis buffer (temperature $+4^{o}$C). The buffer was previously prepared by mixing 30 ml of solution A with 5 ml of solution B and fill with distilled water till the volume of 1 l. The solution A was containing 10 M of NaOH in distilled water and the solution B was containing 200 mM of EDTA in distilled water, with final pH 10. The final pH of the electrophoresis buffer was more than 13. The slides of *Arbutus unedo* were left in the buffer for fifteen minutes in order for the DNA to unwind and then the electrophoresis was conducted for twenty minutes at 25 V and 280 mA. As it comes to the slides of the oxford genotype of poplar were left for 5 minutes in unwinding and then 10 minutes in electrophoresis under the same conditions. After the completion of the electrophoresis the slides were gently washed three times with 500 μl of neutralization buffer containing 0.4 M of Tris-Base in distilled water, with final pH 7.5. The slides remained with the neutralization buffer for five minutes and a last wash only with distilled water for another five minutes was conducted. Subsequently, they were left to dry in the dark and when they were totally dry they were stained with 50 μl of ethidium bromide (20 μg / ml) and a cover slip was placed to each one of them. Then, they were examined with the Comet assay program in a fluorescent microscope and the Tail Moments of 50 or 100 nucleoids per each slide were scored.

Physiological measurements

Fully expanded sun leaves were used for leaf gas exchange measurements with a use of a portable infra-red gas analyzer (CIRAS-2 PP Systems, Herts, UK) at controlled values of CO_2 concentration (400 μmol mol^{-1}), leaf temperature (25 °C) and of leaf-to-air vapour pressure deficit (VPD, 1.0-1.8 kPa) under the light-saturated conditions (PPFD, Photosynthetic Photon Flux Density: 1500 μmol mol^{-1}). The measurements were made in the morning, between 8:00 and 12:00 and always on days with clear sky. For Strawberry tree the measurements were carried out on 1st of June, 17th of July, 6th and 29th of September. For the Oxford genotype of poplar, the measurements were carried out on 29th of May, 14th of July and 14th of September. From these measurements, the light-saturated net photosynthetic rate (A_{sat}), the Ci/Ca ratio and the stomatal conductance (Gs) were determined (Dr Hoshika, personal communication).

Biomass measurements

For the strawberry tree, in May 2017, 10 plants were harvested before the beginning of the experiment and separated into leaves, stem, branch, coarse and fine roots (as fine roots are determined those with diameter less than 2 mm). Roots were washed very carefully in order for soil particles to be removed. After the end of the experiment, all treated plants were harvested according to the previously described procedure. Most of the organs were dried at 103 ˚C, apart from leaves and fine roots that were dried at 70 ˚C, until a constant weight was reached. The dry mass of the organs was determined by a scale (Mod. Bp110, Sartoris weighing technology, Germany; 0.01 g accuracy) after oven-drying. The growth was calculated with the subtraction of the initial biomass from the final one. Same approach was followed for the poplars but this time only the final biomass was measured (Dr Hoshika, personal communication).

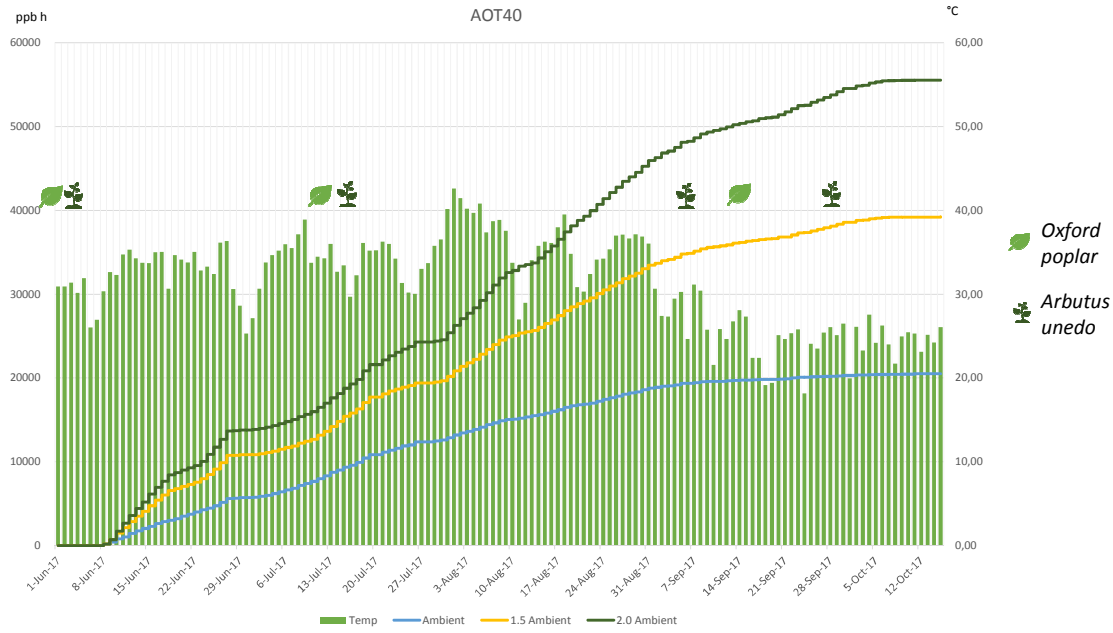
Statistical analysis

For the results that came out from the Comet assay, a Univariate two sample t-test was conducted comparing all of the given parameters (Head Length, Tail Length, Head Intensity, Tail Intensity and Tail Moment) between the different treatments of ozone into the same sampling and for the same species. Past 3 software was used. For the percentages of damaged, moderately damaged and undamaged cells according their Tail Moment for each ozone treatment a chi squared test was conducted per species and sampling. For biomass and physiological measurements ANOVA was used to give the significance for ozone level applied, time and their combination.

RESULTS

Meteorological conditions

Given that the period of fumigation lasted from the 12th of June till the 15th of October, in the diagram below the temperatures during the whole experiment are shown (secondary axis, measured in °C), as well as the AOT40 for the three different ozone treatments (main axis, measured in ppb h).



Graph 2: Temperature and AOT40 ozone concentration from 01/06 to 15/10 combined with the measurements of leaf gas exchange. The leaf sign and the sign of little tree indicate the physiological measurements for the Oxford clone and the strawberry, tree respectively

We can see that the accumulated ozone concentration over the threshold of 40 ppb (AOT40), measured only during the day, is increasing a lot during the experiment, with the values of the most elevated treatment to reach 60000 ppb h, which is more than the double of the value of around 20000 ppb h which is given for the ambient AOT40. In the beginning the values seem to be zero because the fumigation begun the 12th of June, while the graphic start from the 1st of June. During the summer of 2017 very high temperatures (more than 40°C) were observed in Sesto Fiorentino which after September started to decrease but without falling under 20°C apart from some sporadic times. Precipitation is not shown in the graphic as during those months it rained only occasionally.

Physiological measurements

For the ozone sensitive Oxford genotype of poplar, the physiological measurements before fumigation (29th of May), after 4 weeks of fumigation (14th of July) and after 13 weeks of fumigation (14th of September), showed that photosynthesis under ozone fumigation was decreasing with time for plots with 1.5 x ambient and 2 x ambient as we may see in table 1. For ANOVA conducted for the results of photosynthesis measurement, both the parameters of time and ozone level were statistically significant either alone and combined. More specifically, ozone treatment showed 1 level of significance with p value < 0.05, time showed three levels of significance with p value < 0.001 and the combination ozone treatment x time showed two levels of significance with p value < 0.01.

Table 1: Photosynthesis measurements and standard errors for the oxford genotype of poplar

	Photosynthesis measurements for poplar		
	Before fumigation	4 weeks of fumigation	13 weeks of fumigation
Ambient conc.	18.0 ± 1.1	16.2 ± 0.1	16.9 ± 0.8
1.5 x ambient	16.0 ± 0.9	15.1 ± 1.7	11.2 ± 0.6
2.0 x ambient	19.2 ± 0.9	13.8 ± 1.0	7.9 ± 1.6

For strawberry tree the photosynthetic rate was in any case lower than the poplar's one, but even here there is a pattern of decrease when the ozone treatment increases, as we can see in Table 2. An exception to this pattern can be observed in the late September measurement where the plot with the ambient concentration and the one with the 1.5 times elevated show more or less the same value. In this case, ANOVA indicated as statistically significant only the parameter of time with p value < 0.05, although the p value for the parameter of ozone treatment was small too (0.066). We also have to underline that the first measurement gave very similar values of photosynthesis because on 1st of June, when the measurement was taken, the fumigation had not begun yet so all of the three plots had photosynthetic values for ambient concentration of ozone. More specifically, the measurements for this species were made on 1st of June (before the beginning of fumigation), on 17th of July (5

weeks of fumigation), on 6th of September (12 weeks of fumigation) and on 29th of September (the end of the experiment).

Table 2: Photosynthesis measurements and standard errors for *Arbutus unedo*

	Photosynthesis measurements for strawberry tree			
	Before fumigation	5 weeks of fumigation	12 weeks of fumigation	End of the experiment
Ambient conc.	7.7 ± 0.7	7.2 ± 1.2	11.9 ± 0.8	9.8 ± 1.4
1.5 x ambient	7.2 ± 0.1	6.8 ± 1.7	8.6 ± 1.1	10.1 ± 1.6
2.0 x ambient	7.1 ± 1.1	6.3 ± 0.3	5.6 ± 0.8	7.9 ± 0.2

Apart for photosynthesis measurements, also measurements of stomatal conductance have been taken and they are shown in the tables 3 and 4.

Table 3: Stomatal conductance and standard errors for the oxford genotype of poplar

	Stomatal conductance for poplar		
	Before fumigation	4 weeks of fumigation	13 weeks of fumigation
Ambient conc.	0.40 ± 0.04	0.38 ± 0.02	0.44 ± 0.04
1.5 x ambient	0.37 ± 0.03	0.37 ± 0.05	0.42 ± 0.01
2.0 x ambient	0.32 ± 0.03	0.30 ± 0.05	0.38 ± 0.03

The results of stomatal conductance for the oxford genotype of poplar according ANOVA were not statistically significant for ozone treatment, time or the combination of the two parameters. However the p value for ozone treatment was near to the one that is required to show significance (0.092).

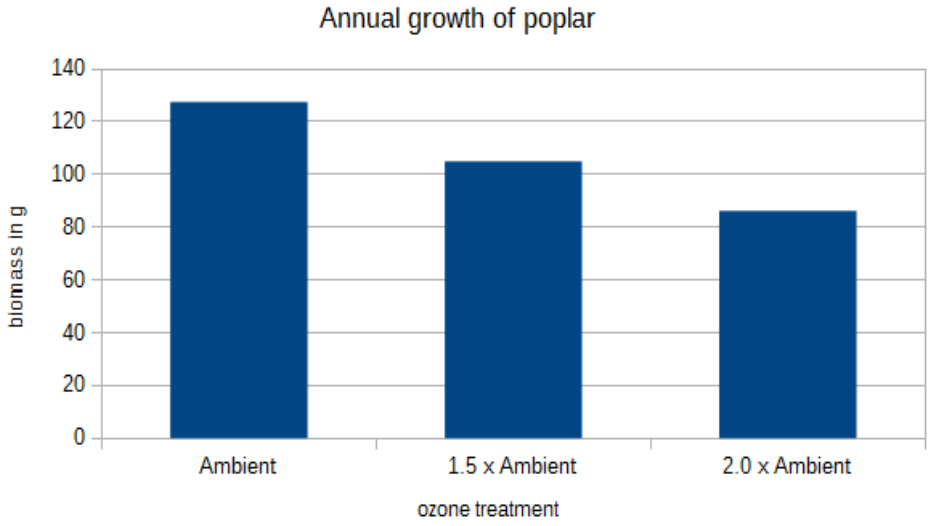
Table 4: Stomatal conductance and standard errors for *Arbutus unedo*

	Stomatal conductance for the strawberry tree			
	Before fumigation	5 weeks of fumigation	12 weeks of fumigation	End of the experiment
Ambient conc.	0.07 ± 0.01	0.07 ± 0.01	0.16 ± 0.03	0.10 ± 0.02
1.5 x ambient	0.06 ± 0.00	0.08 ± 0.03	0.09 ± 0.02	0.11 ± 0.02
2.0 x ambient	0.06 ± 0.01	0.06 ± 0.00	0.08 ± 0.01	0.07 ± 0.00

According to ANOVA, the stomatal conductance for the strawberry tree was affected only by the parameter of time with p value < 0.01.

Biomass measurements

The growth rate of the poplar was decreased as the ozone concentration per treatment was increasing as it is shown in the graph 3.



Graph 3: Annual growth of the oxford genotype of poplar for the years 2016-2017 in the three different ozone treatments

The decrease of annual growth of poplar with the increase of ozone level treatment was statistically significant according to ANOVA with p value < 0.05.

The growth of strawberry tree is not shown as it was not statistically significant. We can just refer that the growth from May to the end of the experiment was 106.45 g for the plot with ambient concentration of ozone, 123.48 g for the plot with 1.5 times elevated ozone concentration respect to the ambient one and 111.36 g for the plot with 2 times elevated ozone concentration.

Comet assay

The attempts for direct nuclei isolation did not give any results. We were able to process only the nucleoids derived from protoplast isolation. The different parameters of Comet assay are given for each treatment per species to the tables 5 and 6. The tables are divided in two parts, showing the results of the two different samplings we made, one the 21st of July and the next one 30th-31st of August.

Table 5: Mean values and standard errors of the different parameters of Comet assay for *Arbutus unedo* for the samplings of July and August

	Source material	Tail moment	Tail intensity	Tail length	Head intensity	Head Length
		Arbitrary unit	%	µm	%	µm
July	***AmbO₃***	0.15±0.04	1.56±0.37	11.68±0.40	98.44±0.37	27.05±1.17
	AmbO₃ x 1.5	0.84±0.09*	8.46±1.00*	20.99±1.75*	91.54±1.00*	22.05±0.91*
	AmbO₃ x 2.0	1.25±0.21*	12.20±1.95*	16.12±1.50*§	87.80±1.95*§	17.99±0.80*§
August	***AmbO₃***	0.45±0.05	7.35±0.65	8.82±0.26	92.65±0.65	16.71±0.39
	AmbO₃ x 1.5	0.86±0.09*	10.93±0.82*	10.51±0.37*	89.07±0.82*	15.91±0.38*
	AmbO₃ x 2.0	1.46±0.13*	17.09±1.24*§	13.05±0.62§	82.91±1.24*§	16.86±0.70*§

* student t-test statistically significant (p-value < 0,01) for Amb O_3x1.5 and Amb O_3x2 vs Amb O_3
§ student t-test statistically significant (p-value < 0,01) for Amb O_3x1.5 vs Amb O_3x2

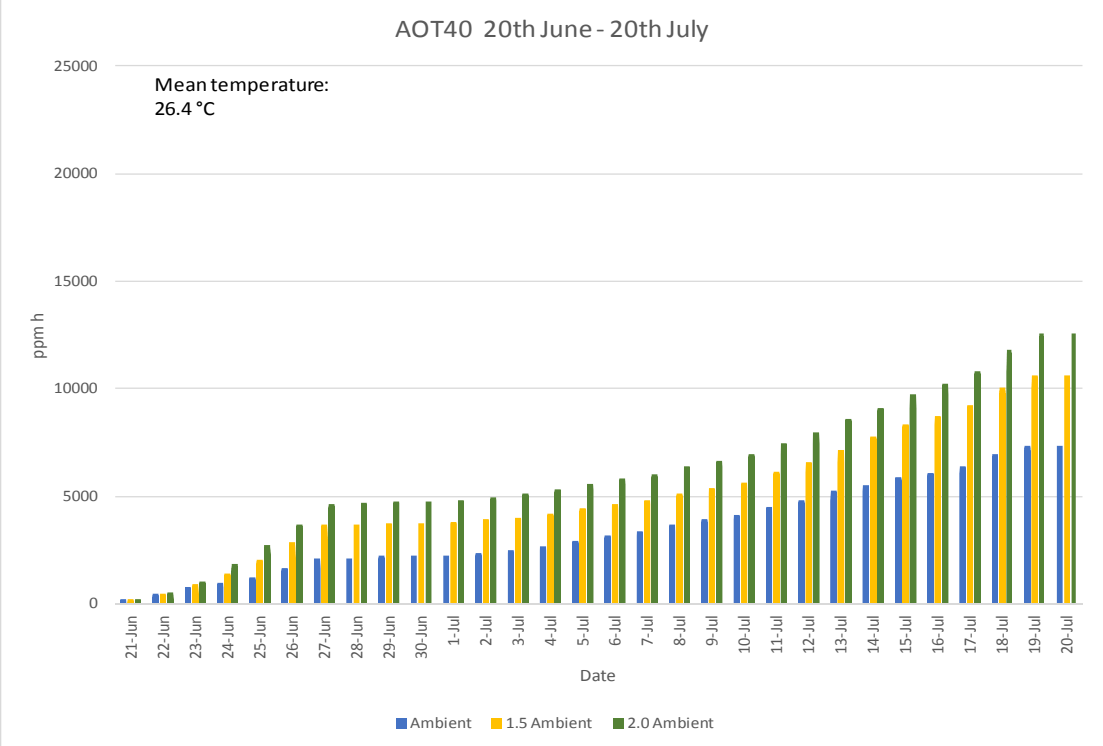
In the present study, we are mainly using the parameter of Tail Moment which is an arbitrary unit based on the tail intensity and tail length. In the cases shown here it behaves like the tail intensity most of the times, showing the same difference between the two treatments of ozone concentration versus the ambient one but not between them. The tail intensity has to do with the amount of DNA in the tail while the tail length shows the distribution of the DNA. Accordingly, the head intensity suggests how "bright" the head is, which is associated with the amount of DNA in the head, while the head length gives the size of the head.

Table 6: Mean values and standard errors of the different parameters of Comet assay for Oxford genotype of poplar for the samplings of July and August

	Source material	Tail moment Arbitrary unit	Tail intensity %	Tail length µm	Head intensity %	Head Length µm
July	***AmbO$_3$***	0.16±0.03	2.08±0.30	10.72±0.40	97.92±0.30	23.24±1.41
	AmbO$_3$ x 1.5	0.78±0.10*	9.15±1.13*	14.91±1.03*	90.85±1.13	17.47±0.72*
	AmbO$_3$ x 2.0	1.07±0.11*	13.35±1.45*	16.91±1.18	86.65±1.45*§	16.1±0.94*
August	***AmbO$_3$***	0.35±0.060	5.51±0.66	8.94±0.39	94.49±0.66	17.05±0.60
	AmbO$_3$ x 1.5	0.95±0.11*	14.01±1.20*	12.68±1.25	85.99±1.20*	16.46±0.70*
	AmbO$_3$ x 2.0	1.32±0.16*	16.41±1.59*	14.10±1.41	83.59±1.59*	13.72±0.59*

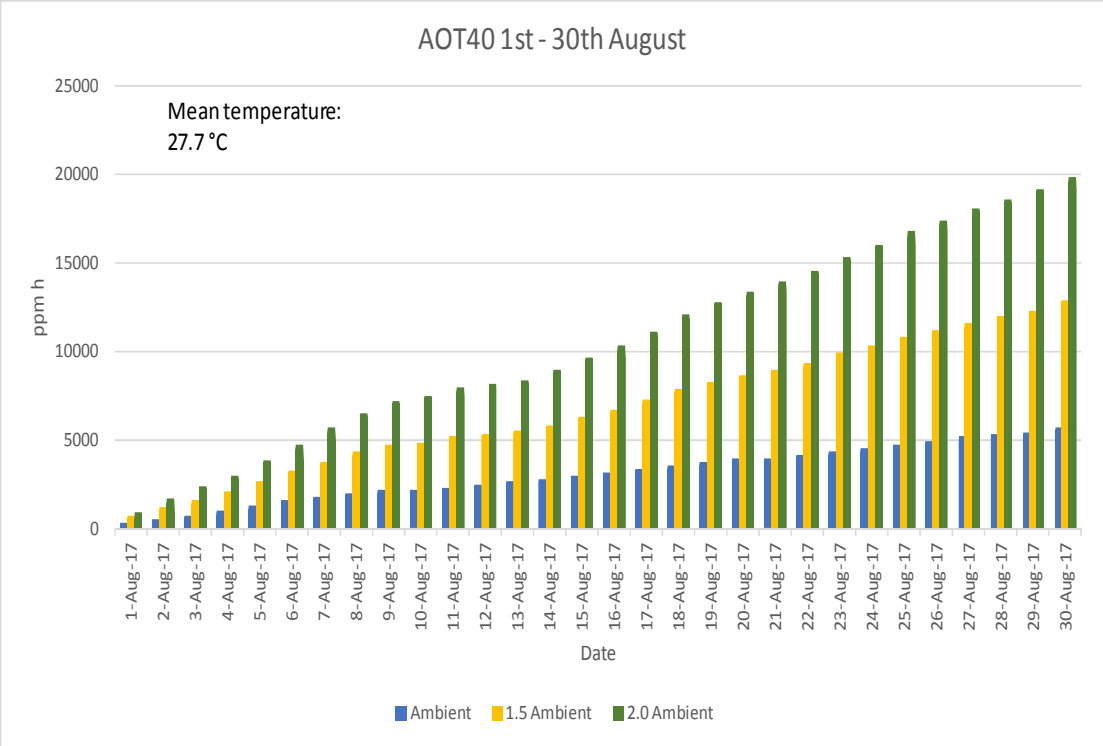
* student t-test statistically significant (p-value < 0,01) for Amb O_3x1.5 and Amb O_3x2 vs Amb O_3
§ student t-test statistically significant (p-value < 0,01) for Amb O_3x1.5 vs Amb O_3x2

As for Comet assay young leaves were used, for the better understanding of its results graphics 4 and 5 show the AOT40 for one month before the sampling that it is estimated to be the age of the used leaves.



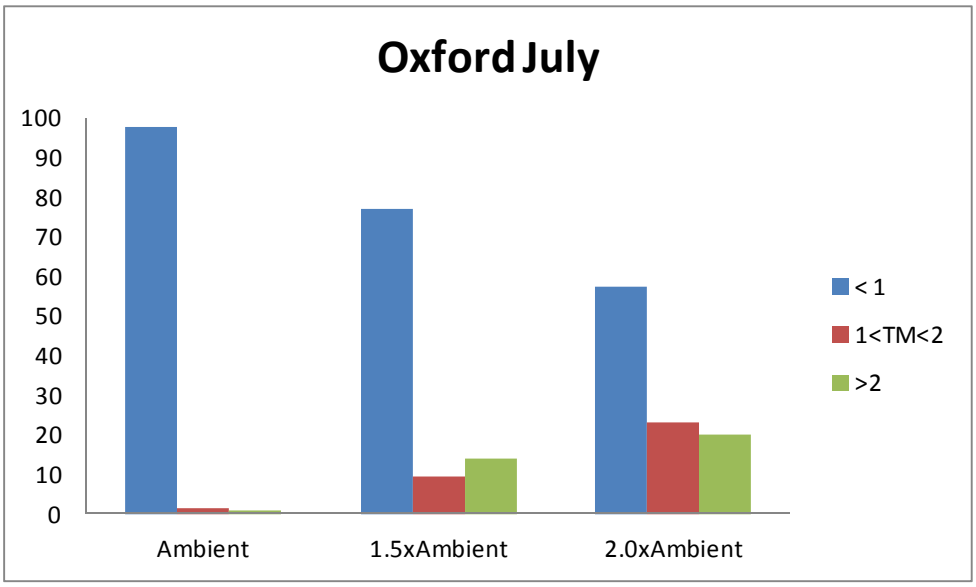
Graph 4: AOT40 for the month before the first sampling for the Comet assay

In graph 4, the plateau observed in the end of June – beginning of July is because of technical problems that appeared with the air generator in this period. For the drawing of this graph the concentrations anticipated the one month before the sampling were not taken into consideration and the values began from 0. After the 21st of June the concentrations over 40 ppb started to accumulate again till the day of the sampling in order to estimate the total ozone to which the leaves were exposed during their lifetime.



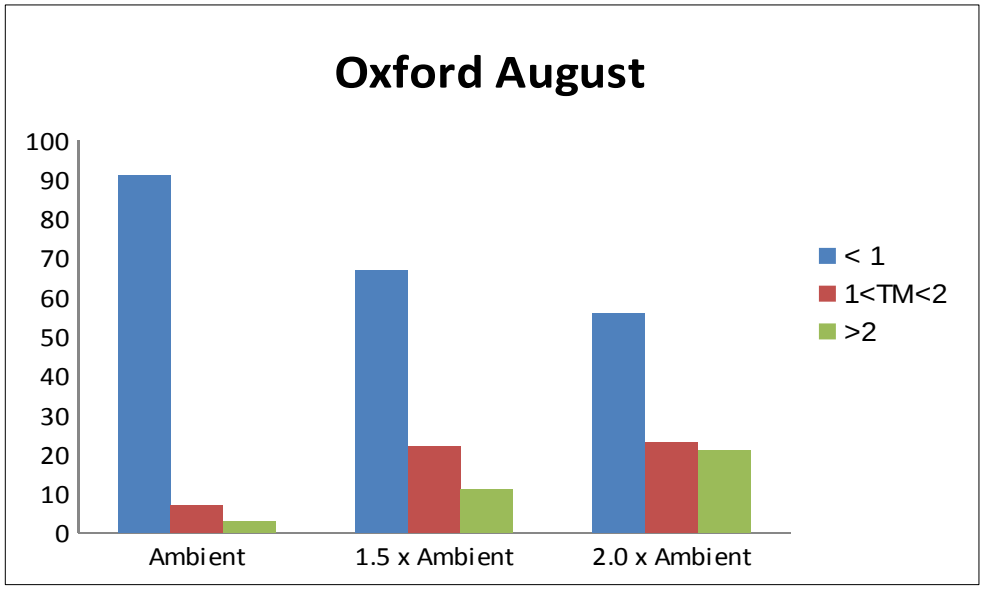
Graph 5: AOT40 for the month before the second sampling for the Comet assay

For the results of Comet assay the more relevant Tail Moments data obtained were put all together in an Excel file where they have been ordered from the lowest value to the highest one. Then the values of Tail Moment lower than one, from one to two and over two were counted and the percentages out of the total values per treatment, species and sampling were found. The results per species and sampling are shown in the graphs 6-9.



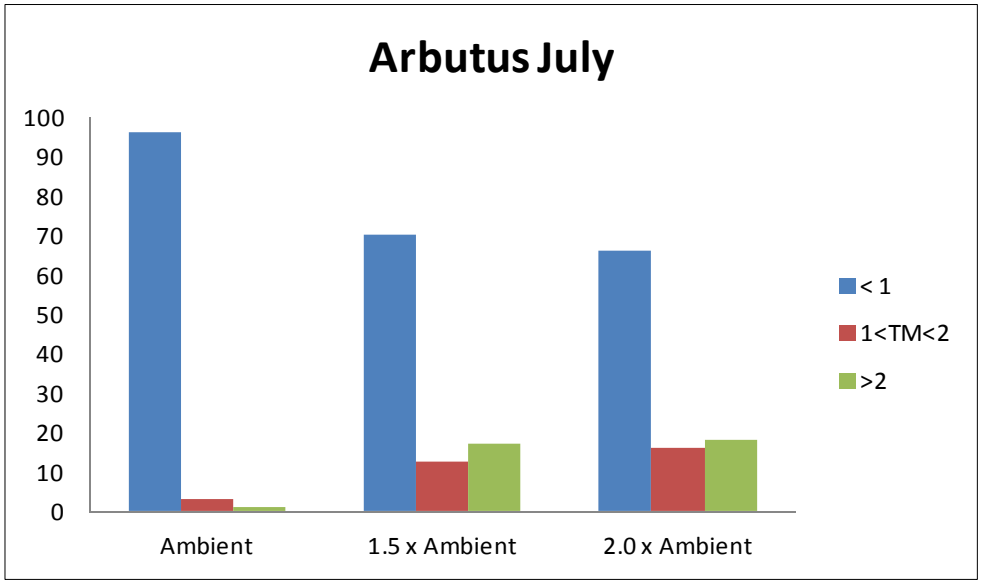
Graph 6: Percentages of cells with Tail Moment lower than 1, from 1 to 2 and higher than 2 per treatment for the sampling of the Oxford genotype of poplar in the end of July

For the Comet assay of poplar in July is observed that as the treatment with ozone level increases, the percentage of healthy nucleoids (damage lower than 1) decreases, while the percentage of nucleoids moderately damaged (1 < Tail Moment < 2) increases. The percentage of nucleoids with Tail Moment more than 2 (green columns in the graph) increases too.



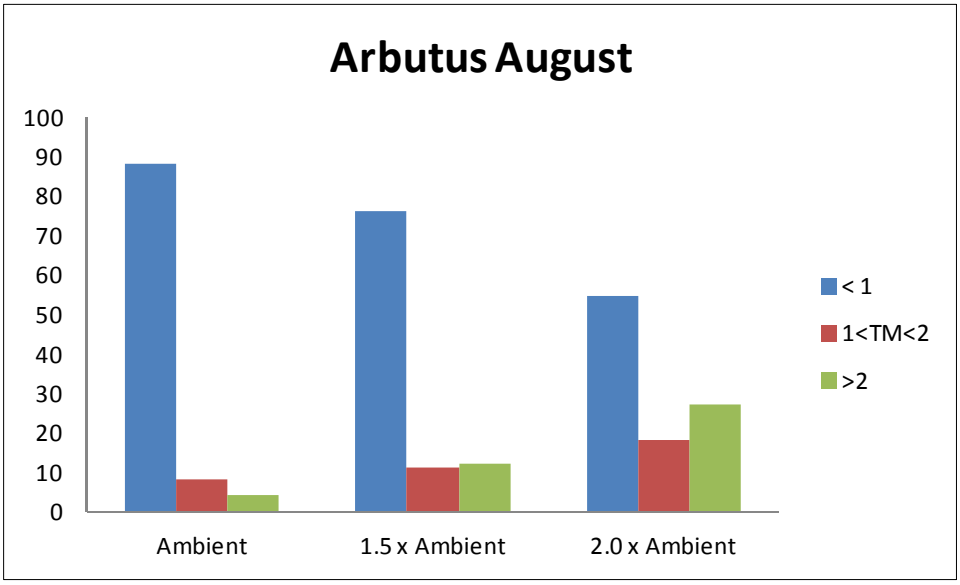
Graph 7: Percentages of cells with Tail Moment lower than 1, from 1 to 2 and higher than 2 per treatment for the sampling of the Oxford genotype of poplar in the end of August

In the sampling conducted in August, for the Oxford poplar the results also show that the percentage of healthy nucleoids is higher in the ambient concentration and as the concentration of ozone increases, this percentage (blue colour in the graphic) decreases. Also, the percentage of moderately damaged cells (red colour in the graphic) is increasing according to the treatment as well as the percentage of nucleoids with Tail Moment higher than 2.



Graph 8: Percentages of cells with Tail Moment lower than 1, from 1 to 2 and higher than two per treatment for the sampling of the strawberry tree in the end of July

For the sampling of the strawberry tree in July, the results also show that the higher the ozone concentration, the greater the percentages of damaged cells.



Graph 9: Percentages of cells with Tail Moment lower than 1, from 1 to 2 and higher than two per treatment for the sampling of the strawberry tree in the end of August

The sampling of August showed again the typical trend of increase of the damaged groups (moderate damage with values of Tail Moment from 1-2 and high damage with values of Tail Moment higher than 2) while the ozone concentration is increasing.

Table 7: Results of the chi squared test conducted to test the influence of ozone level to the damage of the cells

	Chi squared values	**Significance**
Oxford genotype of poplar, July	58.46	***
Oxford genotype of poplar, August	44.05	***
***Arbutus unedo*, July**	30.53	***
***Arbutus unedo*, August**	31.45	***

*** p value < 0.001

For the chi squared test conducted between the three different treatments of ozone and the percentages of Tail Moment lower than 1, between 1 and 2 and more than 2, the results showed that the null hypothesis which was that the level of ozone does not affect the level of damage, can be rejected. For both species and samplings the relationship between ozone level and damage of cells was statistically significant (Table 7), which means that DNA damage increased with increasing ozone concentrations. The chi squared test had four degrees of freedom.

DISCUSSION

As far as we are concerned, this is the first time that mesophyll protoplasts were employed for the implementation of Comet assay in order to be measured the DNA damage caused by tropospheric ozone in an ozone sensitive and an ozone tolerant plant species (the hybrid oxford poplar genotype and the strawberry tree respectively). The only other similar study in which Comet assay was used to measure DNA damage due to tropospheric ozone in clones of another poplar species (*Populus tremuloides*) was the one by Tai and collaborators (2010). However, in this case direct nuclei isolation was done by sliding frozen leaves with a razor blade. In the present study, preliminary trials of nuclei isolation showed that sliding was not a suitable technique to give a satisfactory yield of nuclei (200-500) for the implementation of the Comet assay. Other protocols that were also tried, employing mechanical methods such as chopping (Gichner and Plewa, 1998; Scintu, 2017) gave as a result an increased tail moment. On the contrary, the enzymatic method employed for the present study allowed the isolation of healthy protoplasts in a very short time (4h), ensuring a reduced oxidative stress, as shown by a very high percentage (>90%) of undamaged cells in ambient ozone treatment in the first sampling (21th of July).

The present work was conducted in the only facility for free air ozone fumigation in Mediterranean climate (Paoletti et al., 2017). This innovative system gave us the opportunity to better simulate the actual environmental condition without the effects of an open top chamber (OTC) system. The advantages of the free air ozone fumigation facility also allow the better prediction of a future elevated ozone levels atmosphere, giving more realistic condition of the effects that the tropospheric ozone will have to the plants. In addition, by increasing the ozone concentration according to the ambient ones, a more natural result is given, since the ozone concentration does not remain stable in any case in the environment.

During the experiment in Sesto Fiorentino in the summer 2017 (from 12th of June to 15th of October 2017) the meteorological conditions were particularly hot and dry with only five days of little rain events (28th-29th of June, 25th of July and 10th-12th of August) for the months June, July and August. The peak of temperature (more than

40°C) was from the end of July to the beginning of August, soon after the first sampling for the Comet assay (21th of July) and the physiological measurements for the Oxford clone of poplar (14th of July) and for the strawberry tree (17th of July). Indeed, while for the previous years the daily mean temperature for the summer was 24.0°C in 2015 and 22.9°C in 2016 (Hoshika et al., 2017b), this year the mean temperature for the months of the experiment was 24.9°C. Concerning precipitation, while the data for the previous years show total precipitation of 536.2 mm in 2015 and 226.6 in 2016 (Hoshika et al., 2017b), this year the total precipitation was 191 mm, including half of October, that it is already considered as autumn and the rainfall is supposed to be more elevated. Although the raining events were sporadic, the plants did not suffer stress because of drought as they were sufficiently irrigated every two to three days.

Along the experiment the ozone concentration of the ambient treatment was automatically registered in order to adapt the ozone fumigation level of the other two treatments (1.5 x Ambient and 2.0 x Ambient). AOT40 value, which is the sum of the hourly values over the threshold of 40 ppb, was measured in ppb h in each treatment to monitor the level of the ozone stress. It is worth noting that this parameter increases a lot and not proportionally between the different ozone treatments, because in each case all of the measurements that reveal ozone concentration higher than 40 ppb during the day are added to the previous value. That means that maybe for some measurements the ambient concentration does not exceed this threshold, but the elevated 1.5 times concentration it can be possible that it exceeds it. This is even more possible for the ozone treatment with double values respect to the ambient one. Therefore it could happen that at the end of the experiment the AOT40 of the 1.5 treatment was almost the double compared to the AOT40 of the ambient concentration while the AOT40 of the most elevated treatment (2.0) was almost three times greater than the ambient one. It is because the AOT40 does not increase proportionally to the ozone concentration but taking into consideration how much this concentration exceeds the threshold of 40 ppb and always accumulating this value to the previous one.

For a better comprehension of the Comet assay data we calculated the AOT40 of the 30 days before the sampling date of the leaves, because protoplast isolation needs young and non hardened material. In particular on the 21th of July we obtained values of 7.4, 10.6, and 12.5 ppm h for ambient ozone, 1.5 x ambient ozone and 2.0 x am-

bient ozone, respectively; while at the end of August the corresponding values were 5.9, 12.5, and 20.0 ppm h. All these values were less than the threshold of 31.5 ppm h reported by Nali and collaborators (2004) as necessary to reveal a macroscopic leaf damage in the tolerant *A. unedo* over a period of 3 month. Indeed, the strawberry tree leaves employed in the Comet assay did not show any macroscopic damage due to ozone exposure. Regarding Oxford genotype of poplar the onset of macroscopic damage depends on different conditions, such as the water status of the plant, for which in dry condition the damage could be postponed due to the stomatal closure (Pollastrini et al., 2014). The damaged leaves were normally located in the bottom part of the crown and in 2017 Hoshika and collaborators mentioned that visible foliar injury in Oxford clone was observed when AOT40 value was equal to 11.7 ppm h after a period of 2.5 months in the same FO_3X facility. In the present experiment the first visible foliar injury due to ozone were observed in the oxford clone of poplar 2.5 months after the beginning of fumigation, which is in agreement with the literature till now (Hoshika et al., 2017b). In any case the leaves collected for Comet assay did not show any symptom of leaf damage. Nevertheless, the results of Comet assay revealed an increasing DNA damage with the increase of ozone concentration according to the percentages of the Tail Moment that indicate healthy, moderately damaged and damaged cells, both in July than in August (Chi square $p< 0.001$). These data confirmed that Comet assay is a rapid and sensible technique to reveal early DNA damage in the isolated protoplast even in absence of macroscopic symptoms. The presence of different level of damage inside the cell population is a well-known phenomenon, as ozone exposure lead to localized macroscopic symptoms (Desotgiu et al., 2010).

Respect to the physiological measurements, for both of the species till September the photosynthetic rate is reducing during the time even in the plot without treatment which is quite reasonable given that the ozone concentration increases during summer because of high temperatures and solar radiation, as it was mentioned also in the introductory part. The oxford clone of poplar looks more affected by the ozone treatment than the strawberry tree, although a slight decrease is observed for the latter too. For the measurement of September, even the plot of ambient concentration of poplar showed a slight decrease compared to previous measurements, while the strawberry tree gave higher values after September even for the plot of 1.5 elevated ozone concentrations. In the oxford clone of poplar ozone seem to be more effective, as the pho-

tosynthetic rate in the treated plots decreases with time, without showing the recovering of *Arbutus unedo* during September.

Strawberry tree is normally characterized by a lower photosynthetic rate respect to the one of poplar. Our results showed that the ozone treatment did not significantly affect this parameter, even if we can observe different values along the experiments. An exception to the general decrease of photosynthetic rate while the ozone concentration increases, can be observed in the late September measurement where the plot with the ambient concentration and the one with the 1.5 times elevated show more or less the same value. This increase in the photosynthetic rate after the third measurement can be possibly explained if we take into consideration that both temperatures and solar radiation were decreased in September. Another possible explanation could be that the strawberry tree managed to adapt in the high ozone conditions and to increase its photosynthetic rate. Our results for the photosynthetic rate of strawberry tree agree with the literature (Fyllas, 2016), where the A_{sat} of schlerophyllous evergreen species is estimated to be around 10 μmol / m s.

Stomatal conductance, although it was not statistically different for most of the times, it was following more or less the pattern of photosynthesis, if being observed between the different ozone treatments but in the same date of measurement. This is quite reasonable since stomatal conductance expresses how open the stomata are and therefore is linked to the amount of CO_2 entering the leaf. A change to stomatal conductance was expected as ozone is known to produce stomatal sluggishness that leads to leaky stomata which is associated to more ozone uptake and water consume (Hoshika et al., 2015.

Regarding biomass measurements, the decrease of annual growth is obvious in poplar while ozone concentration increases. The non-significant pattern of change in the strawberry tree may be explained if we take into account that when the seedlings were transferred in the campus of Sesto Fiorentino, they were already too big to show great differences to their growth rate. On the contrary, the oxford seedling, derived from cuttings, were always there and could be closely observed and grow up in a controlled environment since the beginning.

CONCLUSION

Comet assay is a reliable technique to detect DNA damage in ozone treated material. In all of the experiments we were able to find damaged cells that were increased as the ozone concentration was increasing. In fact, Comet assay is a very sensitive technique, since it reveals DNA damage, even without macroscopic symptoms as dots in the leaves, leaf senescence or decrease in the growth. Furthermore, we should take into consideration that the leaves used for the conductance of Comet assay were young, recently expanded leaves, usually the 8^{th} order from the apex. Consequently, they were not exposed in ozone concentrations during the whole fumigation period which was four months; on the contrary they were exposed only for one month more or less, which was the estimated period for their full expansion. Beside the short period of exposure to ozone, Comet assay was able to detect the different levels of DNA damage according to the different levels of ozone concentration. This technique could be employed in an early detection of the ozone damage in order to sustain ecophysiological data.

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