UNIVERSITÀ
DEGLI STUDI
FIRENZE

DOTTORATO DI RICERCA IN SCIENZE AGRARIE ED AMBIENTALI

CICLO XXX

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Excess light and drought stress in the urban environment: response in cyanic and acyanic Norway Maple cultivars
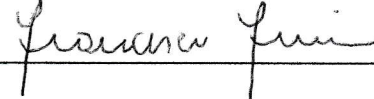
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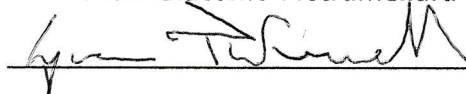
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Anni 2014/2017

La felicità di mio padre,
quando appoggiavo il naso freddo sul suo collo
e lo abbracciavo da dietro
e con le sue mani stringeva le mie…
e non sapeva che quella felice ero io

A te, per tutto quello che mi hai dato…

Riassunto

Gli alberi in città, importanti dal punto di vista economico, sociale ed ambientale, si trovano frequentemente a vegetare in condizioni micro- e macro-ambientali particolarmente severe, condizioni dovute alla modificazione delle caratteristiche chimico-fisiche del suolo e dell'aria, all'inquinamento atmosferico, alla ridotta disponibilità di spazio per apparati radicali e chiome nonché al peculiare bilancio idrico dei sistemi urbani. Esistono poi una moltitudine di stress abiotici, soprattutto durante la stagione estiva, in cui si ha una combinazione di elevate temperature, elevata radiazione solare e bassa disponibilità di acqua nel terreno, che complicano ancora maggiormente il corretto sviluppo dell'albero. In questo contesto si inseriscono delle specie arboree che hanno a disposizione una serie di caratteristiche morfo-anatomiche, biochimiche e fisiologiche che contribuiscono ad aumentare la loro tolleranza al 'drought' e allo stress luminoso. In particolare, le piante 'rosse' sono in grado di sintetizzare un'ampia gamma di metaboliti tra cui gli antociani. Questi assorbono in vivo le bande di luce verde e gialla, comunemente tra 500 e 600 nm. Essi proteggono le foglie dallo stress dei flussi fotoinibitori assorbendo i fotoni in eccesso che altrimenti sarebbero intercettati dalla clorofilla. Inoltre, fungono da utile filtro ottico, deviando l'eccesso di quantità di energia elevata lontano dalla catena di trasporto di elettroni fotosintetici già saturata. Gli antociani riducono il carico ossidativo in una foglia semplicemente filtrando la luce giallo-verde, poiché la maggior parte dell'ossigeno reattivo nelle cellule vegetali deriva dall'eccitazione della clorofilla. Questi composti sono inoltre eccellenti *scavenger* dei radicali liberi per cui l'induzione di antociani fogliari è stata implicata nell'acquisizione di tolleranza a molti diversi tipi di fattori di stress ambientali.

Proprio per questo motivo diventa fondamentale conoscere le risposte fisiologiche delle specie arboree, per poter valorizzarle e utilizzarle per una migliore progettazione del verde urbano.

L'obiettivo di questa tesi, quindi, è essenzialmente quello di valutare le strategie fisiologiche messe in atto da tre cultivars della specie *Acer platanoides* ('Summershade', 'Crimson King' and 'Deborah') a due diversi tipi di stress luminoso: il primo uno stress 'improvviso', il secondo invece uno stress cronico. Ciò che differenzia queste cultivars è il colore delle foglie: la prima cultivar è costitutivamente verde, la seconda è costitutivamente rossa e la terza è temporaneamente (nella fase giovanile) rossa.

A questo scopo, nel 2016 e nel 2017 sono stati condotti due esperimenti presso la Fondazione Minoprio in Vertemate con Minoprio (CO).

Nel primo (durata 3 mesi) lo stress luminoso improvviso veniva indotto da uno stress idrico, nel secondo (durata 16 mesi) lo stress luminoso cronico veniva realizzato con l'uso di pavimenti bianchi che riflettevano un eccesso di luce.

Sono state effettuate misure fisiologiche (scambi gassosi, relazioni idriche e fotochimica del fotosistema) e ottiche.

Complessivamente, i risultati ottenuti da questo progetto di dottorato sono stati i seguenti:

1. Caratterizzazione ottica fogliare delle tre cultivars in oggetto.

2. Le cultivar rosse sono adatte a siti urbani con un'irraggiamento molto elevato durante l'anno e sono meglio attrezzate per far fronte all'improvvisa e breve siccità.

3. In condizioni ottimali, le cultivar con foglie verdi hanno fornito maggiori benefici, in termini di stoccaggio di CO_2 e raffreddamento traspirante rispetto alle cultivar cianiche perché gli antociani sono "costosi" per le piante.

Abstract

The trees in the city, important from an economic, social and environmental point of view, are frequently found to vegetate under particularly severe micro- and macro-environmental conditions, due to the modification of the chemical-physical characteristics of the soil and the air atmospheric pollution, the reduced availability of space for root systems and foliage as well as the peculiar water balance of urban systems. There are also a multitude of abiotic stresses, especially during the summer season, in which there is a combination of high temperatures, high solar radiation and low availability of water in the soil, which further complicate the correct development of the trees. In this context there are some tree species that have at their disposal a series of morpho-anatomical, biochemical and physiological characteristics that contribute to increasing their tolerance to 'drought' and to light stress. In particular, 'red' plants are able to synthesize a wide range of metabolites including anthocyanins. These absorb *in vivo* the green and yellow light bands of light, commonly between 500 and 600 nm. They protect leaves from the stress of photo-inhibiting flows by absorbing excess photons that would otherwise be intercepted by chlorophyll. Furthermore, they serve as a useful optical filter, diverting the excess of high energy amount away from the already saturated photosynthetic electron transport chain. Anthocyanins reduce the oxidative load in a leaf by simply filtering the yellow-green light, since most of the reactive oxygen in plant cells comes from the excitation of chlorophyll. These compounds are also excellent scavengers of free radicals for which the induction of leaf anthocyanins has been implicated in the acquisition of tolerance to many different types of environmental stressors.

In this context it becomes fundamental to know the physiological responses of the arboreal species, in order to enhance them and use them for a better urban green design.

The objective of this thesis, therefore, is essentially to evaluate the ecophysiological strategies adopted by three cultivars of the species *Acer platanoides* ('Summershade', 'Crimson King' and 'Deborah') to two different types of light stress: the first a 'sudden' stress, the second a chronic one. What differentiates these cultivars is the colour of the leaves: the first cultivar is constitutively green, the second is constitutively red and the third is temporarily (in the juvenile phase) red.

To this end, in 2016 and 2017 two experiments were conducted at the Minoprio Foundation in Vertemate con Minoprio (CO).

During the first (duration 3 months) the sudden light stress was induced by a water stress, while during the second (duration 16 months) the chronic light stress was realized with the use of white concrete slabs that reflected an excess of light.

Physiological measurements (gas exchanges, water relations and photochemistry of the photosystem) and optics were carried out.

Overall, the results obtained from this doctoral project were as follows:

1. Leaf optical characterization of the three cultivars in question.

2. Cyanic cultivars are suitable for urban sites with very high irradiance through the year and are better equipped to cope with sudden, short drought spell.

3. Under optimal conditions the cultivars with green leaves provided higher benefits, in terms of CO_2 storage and transpirational cooling than cyanic cultivars because anthocyanins are "costly" to plants.

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1. GENERAL INTRODUCTION

1.1. Climate change

According to the Intergovernmental Panel on Climate Change 2018 (IPCC 2018; http://www.ipcc.ch/), human activities are estimated to have caused approximately 1.0°C of global warming above pre-industrial levels, with a likely range of 0.8°C to 1.2°C. Global warming is likely to reach 1.5°C between 2030 and 2052 if it continues to increase at the current rate.

Climate models project robust differences in regional climate characteristics between present-day and global warming of 1.5°C, and between 1.5°C and 2°C. These differences include increases in: mean temperature in most land and ocean regions, hot extremes in most inhabited regions, heavy precipitation in several regions, and the probability of drought and precipitation deficits in some regions (IPCC 2018).

This scenario will subject plants to a greater range and number of environmental stresses that could occur simultaneously with severe consequences (Mittler and Blumwald 2010). Abiotic stress conditions, including heat, cold, drought, salinity, oxidative stress and nutrient deficiency, are the primary cause of plant's stress worldwide (Carmo-Silva al. 2012). These environmental challenges that adversely affect plant growth and productivity, lead to a wide range of responses in plants, including morphological, physiological, biochemical and molecular changes.
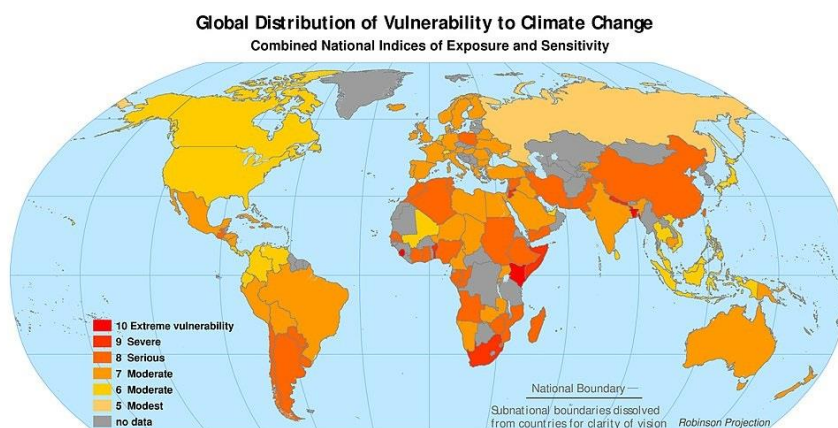

FIGURE 1. GLOBAL DISTRIBUTION OF VULNERABILITY TO CLIMATE CHANGE. PHOTO BY HTTP://SEDAC.CIESIN.COLUMBIA.EDU/

1.2. Urban environment and conditions

The current ecological condition of cities in respect to the surrounding countryside (higher temperatures and CO_2 levels, drought, photochemical pressure and particulate matter pollutants), may anticipate the effects of climate change at a broader scale.

In fact, the last century has been characterized by a rapid urbanization which took cities to interact with the surrounding environment. In this context, the definition of 'Urban Forest' was born and it is generally defined as 'the art, science and technology of managing trees and forest resources in and around urban community ecosystems for the physiological, sociological, economic, and aesthetic benefits trees provide society', definition provided by the Society of American Foresters (Helms, 1998). Many forests have begun to 'urbanize' both with the development and the economic, social, political growth and the related changes (Basnyat, 2008). So, scientists started to identify urban ecosystem as a dynamic ecosystem that has similar interactions and behaviours as natural one, but it is a hybrid of natural and man-made elements whose interactions are affected not only by the natural environment, but also culture, personal behaviour, politics, economics and social organisation (Srinivas, 2014).

In this context urban green spaces, urban forest and urban trees are typical natural-based solutions (or even just natural solutions) as they provide a range of ecosystem services that help cities meet important challenges, such as those related to climate change and public health and wellbeing. (Ferrini et al., 2017)

Urban ecosystem services are important for maintain the well-being of urban dwellers. Therefore, urban forests, as common (and often managed) natural components of urban areas, provide a variety of ecosystem services (Dobbs et al., 2011; Escobedo et al., 2011; Pataki et al., 2011). Urban tress can be found in a wide range of settings, including parks, streets, natural areas, housing areas, wetland areas, and along streams and ponds. Forests and trees in urban landscapes are among the main providers of services including climate amelioration, recreation and air pollution removal, capable of enhancing and maintaining human well-being (Dobbs et al.,2011; De Groot et al., 2010). However, the urban ecosystem present negative situations (Ferrini et al.,2014) for plants that are:

- digging, paving, asphalting;
- loss of soil fauna and flora;
- altered or interrupted organic matter cycle;
- modification of the vegetative cycles of plants depending on the urban climate;
- direct action of pollutants on the plants and on the parasite/plant relationships;

- planting in the wrong and harsh places;
- altered physiology and phenology.
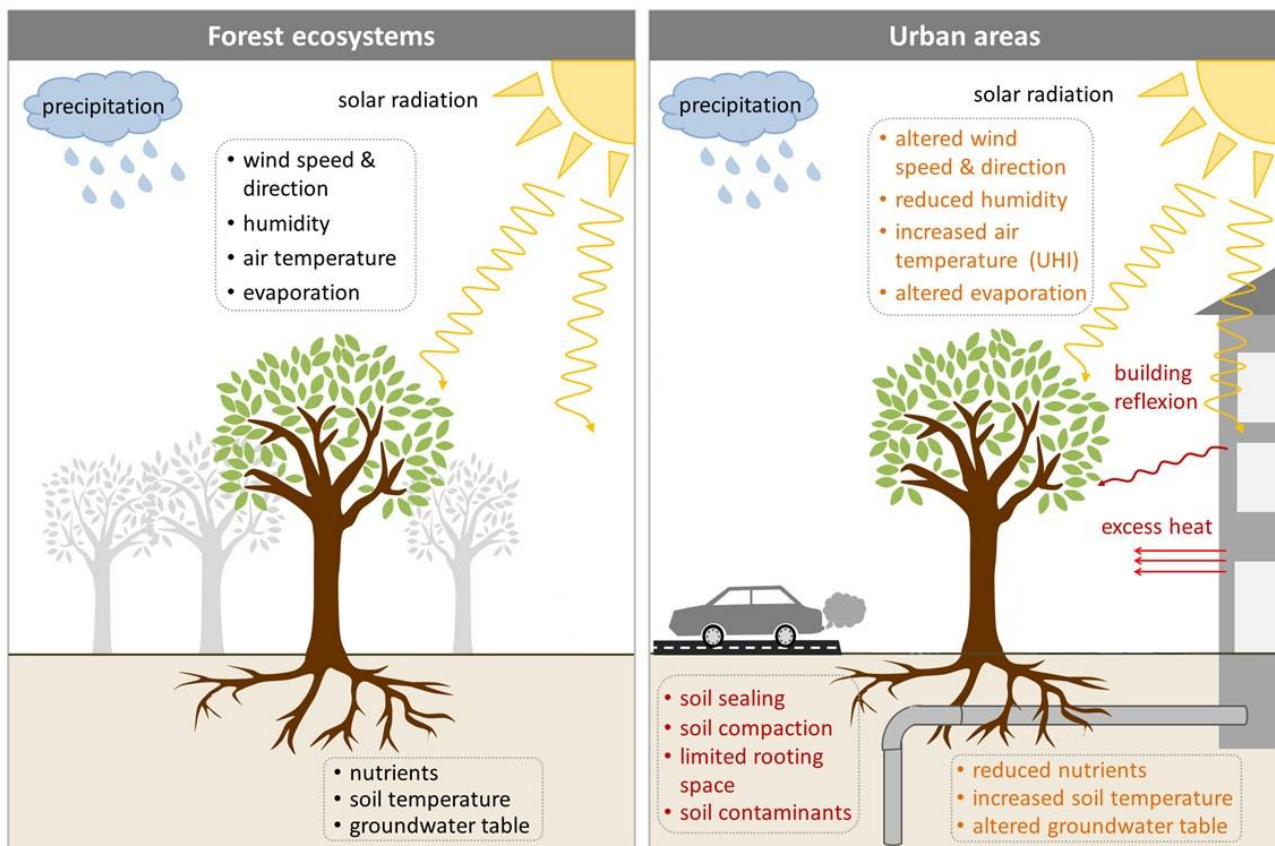
FIGURE 2. DIFFERENCES BETWEEN FOREST ECOSYSTEM AND URBAN AREA.

Therefore, the conditions of the urban system determine adverse conditions for tree growth and physiology and modify the relationships between trees and the urban environment. As well as population dynamics, sometimes the interactions can change in the city, even rapidly, resulting in very serious situations much more difficult to control compared to natural systems. Indeed, cities are responsible for more than 80% of global greenhouse gas emissions (Hoornweg et al., 2011), and given current demographic trends, this will likely increase over time.

For this reason, the effects of the climate change will exacerbate the stresses of urban environment which is characterized by specific microclimatic conditions, such as temperature 3-5°C higher than the surrounding countryside ('urban heat island') (McCarthy *et al.,* 2010), low-quality and impermeable soils (Fini and Ferrini, 2007), which impose several stresses on plants. There are multiple interactions between various stresses such as water stress, increased temperature (diurnal

and nocturnal) and increased atmospheric CO_2 that are particularly interesting and controversial. In fact, scientists observed that the reduction of stomatal conductance caused by CO_2 increasing allows a greater water use efficiency and, therefore, a lower incidence of water stress (Tognetti *et al.*, 1998). Moreover, it has been observed that the reduction of transpiration reduces the loss of heat by convection, and this leads to leaf lamina overheating (Nobel, 1999).

So, it is very important trying to understand how an urban tree species respond to climate change. In particular, the comparison of the mechanisms adopted by woody plants in urban areas to safeguard the integrity and maximize the functionality of the photosynthetic apparatus in a changing climate, is a prerequisite for the proper design of cities. There is a critical need to develop strategies to mitigate the impact of climate on long-term development and growth of plants (Ferrini *et al.* 2014).

1.3. Responses of trees to environmental stress factors

1.3.1 Excess light

Light can be considering an important parameter since it influence plant growth and survival driving the energy for photosynthesis (Canham et al. 1990, Valladares 2003).

In the urban context, two light environments are commonly found:

- *urban canyons*, defined as those environments where trees are heavily shaded by buildings for several hours per day and that may not be exposed to direct sunlight for entire weeks in late fall, winter or spring; (Figure 3b)

- *urban plazas* defined as those locations where no shade is provided by buildings or other structures and trees are exposed to continuous direct sunlight and to that reflected by pavements throughout the year (Ferrini *et al.*, 2014) (Figure 3a).
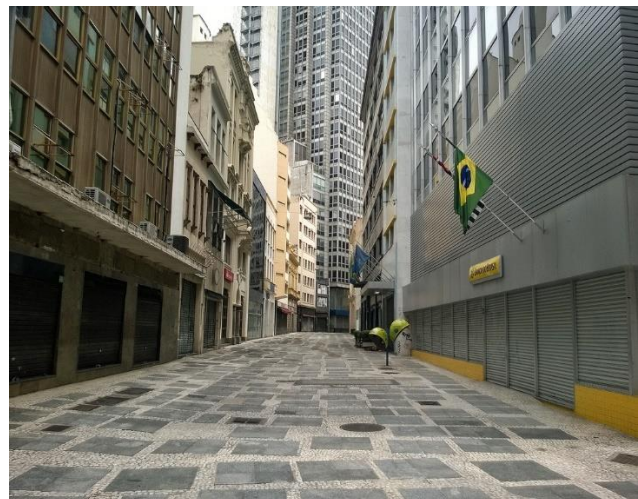
FIGURE 3A. URBAN PLAZA IN CENTER CITY OF PHILADELPHIA (USA). FIGURE 3B. URBAN CANYON (RUA DA QUINTADA, PORTUGAL)

In these environments light conditions are various, from very high intensities in full sunlight (approximately a PAR of 2000 µmol photons $m^{-2}s^{-1}$ in summer, at midday) to heavily shaded conditions with little available light. In normal light conditions, the rate of light absorption is well matched with the rate of photosynthesis, contributing to maximal photosynthetic efficiency (Bjökman and Demmig,1987). As light intensity increases, the rate of photosynthesis becomes saturated, but light in excess is still absorbed. Plants are usually able to cope with normal fluctuations in light levels and to adapt to seasonal changes over time. Because of these changes, plants can suffer both of sudden excess light and a chronic one, two types of excess light. The first

one occurs when there is short-term adjustment of stomatal aperture since guard cells usually contain chloroplasts and it is reasonable to suppose that photosynthesis in guard cells could affect stomatal aperture (Raschke et al., 1978). Meanwhile the second one is when plants are exposed for a long time period to a direct sunlight (Robinson, 2001).

Both excesses can lead to overexcitation and if plant cannot manage this, it can cause to the production of molecules that cause photo-oxidative damage to the two photosynthetic reaction centers, photosystem PSII and PSI, or to other proteins, lipids, and nucleic acids in the cell (Niyogi, 1999). These molecules are reactive oxygen species (ROS), which include radical molecules such as superoxide ($O_2^{\cdot}$), the hydroxyl radical ($OH^{\cdot}$), the non-radicals hydrogen peroxide (H_2O_2) and singlet oxygen ($1O_2^{-}$). ROS are products of over-reduction of the photosynthetic electron transport chain and can cause cellular damage, but they also function as important signals in development and disease (Suzuki et al., 2012). These are produced in the chloroplast because it contains a high concentration of chlorophyll (Chl), which can act as a photosensitizer of molecular oxygen. The excited state molecule $1O_2$ is generated predominantly at the reaction center of PSII and, to a lesser extent, in the light-harvesting complexes (LHCs) (Krieger-Liszkay et al., 2008), and it is the main ROS responsible for photo-oxidative damage in plants (Triantaphylides et al., 2008). In contrast, H_2O_2, (O_2^{-}) and $OH^{\cdot}$ are formed mainly on the acceptor side of PSI by direct electron transfer to O_2 by the PSI reaction center or by NADPH and ferredoxin (Tjus et al., 2001; Asada, 2006). Thus, this process of absorption excess light can result in photoinhibition, defined as a decrease in the maximum efficiency and/or rate of photosynthesis (Erikson et al., 2015; Kok, 1956; Long et al., 1994).

To face an excess light stress, plants evolved both short term and long strategies that consist in physiological adjustments to reduce the excess of excitation caused by light. Short-term acclimation involves a process called non-photochemical quenching (NPQ) that is the dissipation of the excitation energy as heat (Ballottari et al., 2007; Van Kooten & Snel, 1990; Niyogi, 1999). Another important mechanism involves the Calvin cycle in which ribulose-1,5-bisphospate carboxylase oxygenase (Rubisco) catalyses the carboxylation of ribulose- 1,5-bisphosphate (RuBP) and produces the Calvin cycle intermediate, glycerate-3-P. However, under CO_2-limiting conditions, Rubisco catalyses the oxygenation of RuBP and produces glycolate-2-P. This is subsequently metabolized in the photorespiratory carbon cycle to form the glycerate-3-P. During this photorespiratory carbon cycle, ammonia and CO_2 are produced by the mitochondrial Gly decarboxylase. Ammonia is subsequently refixed into Glu by plastidic isozymes of Gln synthetase and Fd-dependent Glu synthase in the photorespiratory nitrogen cycle. Therefore, it can be argued that the

photorespiratory pathway can aid in avoiding inhibition of the repair of photodamaged PSII by maintaining the energy utilization in the Calvin cycle, which is important for reducing the generation of ROS, under conditions where the supply of CO_2 is limited (Takahashi & Badger, 2011).

Long term adjustments involve several morphological and physiological changes in order to reduce the absorption of photons. First of all, light interception can be moderate by vertical orientation of the leaf. Another strategy is increased leaf reflectance due to surface waxes or hairs. These mechanisms also reduce heat load and water loss by plants and are therefore common features of xeric plants. The physiological defence mechanisms are more complex because plants activate other strategies of response, such as the modulation of the light harvesting apparatus and metabolic energy sinks. In fact, in highlight, the stoichiometry of electron carriers and enzymes of the Calvin cycle increases (Seeman et al., 1987; Walter & Horton, 1994), whereas the relative abundance of antenna proteins with respect to the reaction center complexes decreases (Anderson & Andersoon, 1988; Melis,1991). Excess light induces the reorganization of the photosynthetic apparatus to optimize light harvesting while avoiding photo-oxidative damage (Boardman, 1977; Anderson, 1986; Ballottari et al., 2007). In this way, long-term acclimatory responses include the modulation in the PSII/PSI ratio, the regulation of Rubisco and the ATPase content, as well as the cytochrome b6/f level. Chloroplasts also change their position in the cell to optimize the intensity of light. Under strong light conditions, chloroplasts gather at cell walls parallel to the direction of the light to avoid the absorption of excessive light and to maximize absorption of CO_2 from the intercellular air spaces. Linking to a light stress there is an oxidative stress and to avoid this, chloroplasts scavenge ROS effectively using multiple enzymes (i.e. superoxide dismutase, ascorbate peroxidase and peroxiredoxin) and antioxidants (the water-soluble ascorbate and membrane-bound a-tocopherol and carotenoids, such as zeaxanthin, neoxanthin and lutein) in chloroplasts (Robinson,2001).

1.3.2. Drought stress

Drought is identified as the most significant factor of death. In fact, limited water availability adversely affects plant productivity, growth, and survival (Boyer, 1982; Chaves et al., 2003; Flexas et al., 2006). It is clear that plant survival and plant performance under water deficit depend on different adaptations to drought stress conditions, with an array of physiological and biochemical interventions. Numerous studies have clearly shown that water stress primarily affects photosynthetic CO_2 assimilation and therefore sets a limit to plant productivity and growth (Quick et al., 1992; Flexas et al., 2002; Lawlor and Cornic, 2002; Monclus et al., 2006; Galle et al., 2007; Haldimann et al., 2008). Indeed, water stress has long been reported as imposing excess photosynthetic active radiation (PAR) to the photosynthetic apparatus, because the usage of PAR in the photosynthetic process decreases as a consequence of stomatal and biochemical limitations to net CO_2 assimilation (Bota et al., 2004; Flexas et al., 2004; Chaves et al., 2009). Then drought stress leads to a massive generation of reactive oxygen species (ROS; Munné-Bosch et al., 2001; Jubany-Marí et al., 2010) and hence promotes photooxidative stress (Hernandez et al., 2012). Plants respond to drastic changes in ROS generation by activating a net-work of 'antioxidant' defenses to keep ROS at a concentration efficient for signaling, while avoiding ROS-induced cellular damage (Pogson et al., 2008). These metabolic adjustments are common when plants acclimate to stress, and may confer stress tolerance (Suzuki et al., 2012). Antioxidant enzymes are believed to constitute a general primary defense against photooxidative stress (Apel & Hirt, 2004). Moreover, limitation to CO_2 assimilation was also due trough leaf internal resistances. These consist of the CO_2 pathway from the intercellular airspaces to the mesophyll cells, the chloroplasts, and the sites of carboxylation (Flexas et al., 2008) and it is known as mesophyll conductance (g_m) (Evans et al., 1986; Harley et al., 1992; Ethier and Livingston, 2004; Warren, 2006; Sharkey et al., 2007; Flexas et al., 2008; Warren, 2008). One of the mechanisms by which plants adapt to drought-stress is by osmoregulation. In this way plants can tolerate temporary or long-term periods of high soil water deficit (Hsiao et al., 1985). Osmotic adjustment has been reported for several woody plants (Grammatikopoulos, 1999; Huang et al., 2000; Lakso et al., 1984; Larcher et al., 1981; Patakas and Noitsakis, 1999; Rieger, 1995; Tyree and Jarvis, 1982). The solutes that accumulate in plants during water stress and contribute to active osmotic adjustment can be soluble carbohydrates, inorganic cations, organic acids or free amino acids (Huang et al., 2000)

The contribution of these tolerance processes to the adaptation to water stress can vary among species, as well as with the intensity and duration of imposed stress. In fact, the recovery phase after rewatering (i.e. rainfall or irrigation) becomes another important part of the overall plant physiological response to a water stress period. The capability for photosynthetic recovery from an extreme water stress event determines future growth and survival of plants in their habitat. (Ennahli and Earl, 2005; Miyashita et al., 2005; Flexas et al., 2006; Galle et al., 2007; Galmes et al., 2007).

So, trees display a wide range of mechanisms to survive to drought, including avoidance (ability to avoid large decreases in leaf pre-dawn water potential and relative water content during drought) and tolerance (ability to maintain physiological and metabolic processes during decreasing pre-dawn water potential) (Gucci et al., 1999; Valladares and Sánchez-Gómez, 2006; DeMicco and Aronne, 2012). Due to these properties, plants can be divided into two groups:

- Drought avoidant species means the maintenance of a sufficient plant water content. Drought avoiding species are in turn divided into 'water-spending' and 'water-saver'. 'Water-spending' plants can rapidly change their water potential (Ψw) and consequently they arable to extract water from the soil to compensate for water loss (Lo Gullo and Salleo, 1988). 'Water-saver' plants reduce transpiration either by stomatal closure (Nardini et al., 2014) or by decreasing evaporative surface (reducing leaf area or losing leaves) (Boyer, 1985). This second mechanism adopted by some water-saver plants is a conservative strategy, aimed to save soil water during vegetative stages in favour of reproductive stages. This conservative strategy fits most severe and long drought scenarios than the first mechanism (stomatal closure). Indeed, an early reduction in stomatal conductance allows the growth of vegetative and reproductive tissues under mild drought, but it is accompanied by an increase in leaf temperature and by the risk of a higher damage under severe stress (Tardieu et al., 2014).

- Drought tolerant species is an ambiguous concept, because these strategies are not mutually exclusive and we can realize that often plants adopt a mixture of stress avoidance and tolerance strategies that vary with genotype. Furthermore, this classification roughly corresponds to the actually widespread categorization of plants in two categories across a continuum of stomatal regulation of water status: 'isohydric' and 'anisohydric' species (Tardieu and Simmoneau, 1998). Isohydric plants reduce g_s (stomatal conductance) as soil water potential decreases and atmospheric conditions dry, thus maintaining are actively constant plant water status (water potential and/or water content regardless of drought conditions). On the other hand, anisohydric species allow a midday decline in water potential

(Ψw) following the decline in soil water potential (Nardini et al., 2014). Anysohydric species display wide fluctuations in water potential maintaining relatively high gas exchange rates even during arid season and are characterized by a more resistant xylem to negative water potentials (McDowell et al., 2008).

Thus, strategies of tolerance, adaption, and survival will be of major importance for plants growing under adverse environmental conditions.

1.3.3 Excess light and drought: how plant respond when they co occur

Although plant responses to drought and light are relatively well known, plant performances under simultaneous stresses (e.g. drought, excessive light and heat) and their response are usually not predictable from single factor studies (Harris and Bassuk, 1993; Schulze et al., 2002; Valladares and Pearcy, 2002; Valladares and Niinemets, 2008).

So many scientists studied plant responses under these concurrent stress conditions and it is well known, for example, that the excess light stress experienced by trees growing in *urban plazas* may be exacerbated by drought, which limits the use of radiation in the photosynthetic process because of both biochemical and non-stomatal limitations (Fini *et al.*, 2012). This is particularly true in Mediterranean-like climates, when high irradiance is coupled in the long period with drought during the Mediterranean dry summer, all conditions that strongly limit carbon assimilation and promote photoinhibition of the photosynthetic apparatus (Bussotti *et al.,* 2014). Another important defence mechanism against drought and light that plants use are flavonoids which are products of secondary metabolism. In fact they have, primarily, a role in antioxidant mechanisms in response sunlight irradiance. These data are corroborated by the preferential accumulation of flavonoids with an ortho-dihydroxy structure in the B ring with respect to flavonoids having a mono-hydroxy substitution, in response to high doses of both UV and sunlight radiation (Markham et al ., 1998; Wilson et al .,2001; Agati & Tattini, 2002; Hofmann et al ., 2003). Nevertheless, the accumulation of flavonols and dihydroxyflavones almost exclusively in epidermal cells of leaves exposed to light stress (Olsson et al ., 1998; Gould et al ., 2000; Kolb et al ., 2001),and their vacuolar distribution in most species (Hutzler   et al .,1998; Neill & Gould, 2003), still generate conflicts regarding their localization–functional relationships (Olsson et al .,1998).

Secondary, they has been shown also to be up regulated by drought-induced oxidative load, while photosynthetic processes are concomitantly downregulated (Smirnoff, 1993; Mackerness et al., 2001; Rizhsky et al ., 2002; Casati & Walbot, 2003). Nevertheless, conclusive data about how water stress affects leaf polyphenol concentration (Nogués et al., 1998; Pääkkönen et al ., 1998; Estiarte et al ., 1999), depending on both interspecific drought tolerance and stress intensity (Stephanou & Manetas, 1997; Nogués & Baker, 2000; Alexieva et al. , 2001),have not been reported. We note that excess light and drought stress markedly affect both secondary metabolism (Balakumar et al ., 1993; Knight & Knight, 2001) and the amount of carbon available for synthesis of secondary metabolites (Estiarte et al ., 1999; Jifon & Syvertsen, 2003; Xu & Baldocchi, 2003).Preliminary reports suggest that a contemporary analysis of light- and drought-induced changes in photosynthetic performance and polyphenol content should help in solving this complex matter (Agrell et al ., 2000; Hampton, 1992; Tattini et al., 2004).

In this context researchers have suggested a protective role played by anthocyanins, one of the most conspicuous classes of flavonoids together with proanthocyanidins and flavonols, which are important pigments responsible for the red, pink, purple, and blue colors in plants (Grotewold, 2006). Anthocyanins accumulate in the vacuoles of a wide range of cells and tissues in both vegetative and reproductive organs (Hatier and Gould, 2009) and localized in the upper epidermis of the leaf, in the lower and in the mesophyll, have shown efficacy in reducing the incident light on the photosystems. In cultivars of herbaceous species this has been translated into a greater ability of red-leaved individuals to live in environments characterized by high irradiance, than green leafy counterparts (Tattini et al., 2014). They have the potential to reduce both the incidence and the severity of photo-oxidative damage (Feild et al., 2001) by intercepting a portion of supernumerary photons that would otherwise strike the chloroplasts, thus increasing the ROS production and ROS-triggered damage (Nishio, 2000). Their glycosides show potent antioxidant activities in vitro at concentrations comparable to those that occur naturally in the cell vacuole (Ferreres et al., 2011; Agati et al., 2012)

Both adaxially- and abaxially-located anthocyanins apparently constitute an effective sunblock, as reported for a variety of species irrespective of whether the pigments reside in the leaf mesophyll (Hughes and Smith, 2007; Hughes et al., 2013) or epidermis (Hughes et al., 2007; Landi et al., 2014). As further evidence of photo-abatement by anthocyanins, it is noteworthy that anthocyanic leaves often display typical characteristics of leaves growing under shaded conditions, such as lower

chlorophyll a:b ratios than green leaves of the same species (Manetas et al., 2003) and higher total chlorophyll content (Hughes et al., 2008)

Anthocyanin biosynthesis may be of particular benefit to leaves for which the photosynthetic machinery is vulnerable to high sunlight irradiance, as in the cases of evergreen understory species in the temperate zone, which are suddenly exposed to full sunlight when the overstory senesces (Hughes et al., 2005, 2013), immature (Hughes et al., 2007; Manetas et al., 2002) and senescing leaves (Schaberg et al., 2003).

For all these reasons, it is very important to analyse physiology of trees in order to select species which face the challenges of the urban environment in view of climate change.

1.4. Maple in the urban environment

The *Acer* genus makes a significant contribution to the urban forest with its variety of aesthetic qualities and its potential to deliver ecosystem services such as the regulation of microclimate and mitigating the *urban heat island* effect (Sjöman et al., 2015).

1.4.1. The genus *Acer*

The genus *Acer* (Aceraceae), commonly known as maple, comprise 129 species with many infraspecific taxa. These species are mainly trees or shrubs, occupying a significant part of the northern hemisphere, especially in the temperate regions of East Asia, eastern North America, and Europe (van Gelderen et al., 1994). Due to the large number of microspecies and inconsequential varieties and forms, this genus is taxonomically difficult to classify (Delendick, 1990; Park et al., 1993; Bi et a., 2016).

Leaves of the genus <u>Acer</u> are highly various in form, single to compound or palmate; furthermore single-form leaves are undivided to lobed in shape. Leaf shape seems generally characteristic for respective species, and it is, of course, useful for identification in specific level. In the classification of the section Pax (1885) seems to use leaf shape in some case such as the sections of Palmata, Integrifoli a and Trifoliata. However, leaf shape or form are frequently variable even within one species as well known, and are not always reliable for a criterion of the section (Tanai, 1978; Hasebe et al. 1998)

The maple used in this project is Norway Maple (*Acer Platanoides*) and his three cultivars: 'Summershade', 'Crimson King' and 'Deborah'
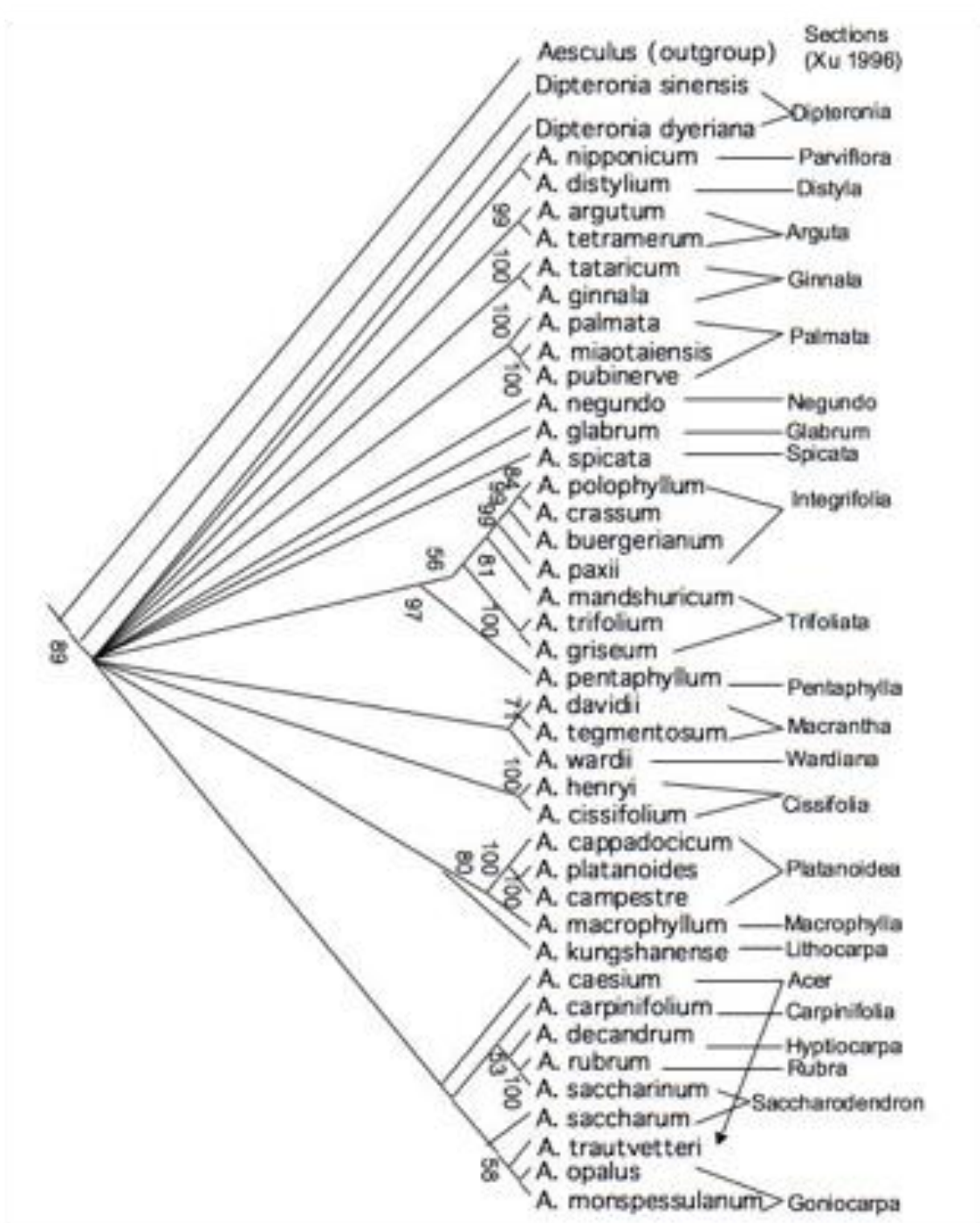
FIGURE 4 GENUS ACER: CLASSIFICATION AND PHYLOGENETIC RELATIONSHIP.

1.5. Botanical and ecological description of Norway Maple

The Norway maple (*Acer platanoides* L.) is a large and tall-domed tree, sometimes very broad, growing to 25-30 m tall and 60-80 cm in diameter, although exceptionally over 150 cm. The stem is straight, short with perpendicular shoots and the crown is dense with foliage. The leaves are opposite, simple, 10-15 cm long, very variable in dimension depending on the age and the vigour of the tree. They have five lobes with long and acuminate teeth and smooth margins. The colour is bright to shiny green turning yellow in autumn; the stalk is reddish, 10-20 cm long. The Norway maple is a monoecious hermaphrodite species with inflorescences grouped in panicles of 30-40 flowers, each 6-8 mm long with five yellow-green petals. In this species flowers appear from about 25-30 years and are insect pollinated. The fruit is a double samara, 3-5 cm each, greenish-yellow, dispersed by the wind. The two samaras are set at a wide angle approaching 180°. The bark of young trees is smooth and grey-brown; on older trees the bark becomes darker and shallowly furrowed with long narrow ridges in a network. The wood is dark reddish-brown; the grain is straight, with a fine, uniform texture. Many cultivars have been selected for their distinctive leaf shape and coloration and for crown shape (Mitchell, 1974; Jonhson & More, 2006; Goldstein, 1995; Kerr & Niles, 1998).

The Norway maple is a fast-growing tree species, able to grow well across a wide range of soil conditions, shade, drought and pollution. However, it thrives best in deep, fertile, moist soils, which are adequately drained and with a sub-acid pH. Exposure and strong calcareous soils are well tolerated4, 5. It is intolerant of low soil nitrogen conditions, high evapo-transpiration or prolonged drought and it is rare on acidic soils (pH near 4) (Nowak & Rowntree,1990).

It tends to be located at the base of hills where it receives a surface runoff and subsurface soil water flow. It also thrives at higher elevations with sufficient precipitation (Nowak & Rowntree,1990). It germinates and grows quickly in shade, even under close canopy. When mature, it becomes more light-demanding (Rhoads & Block, 2011). The height increment is about 1 m/year in the first 10 years. With its wide crown it tends to shade and suppress other slow-growing competitor species. Under optimal conditions Norway maple may live for more than 250 years. Over Europe it occurs in fresh and humid sites both in coniferous and deciduous forests. In natural stands Norway maple occurs as a secondary species with low frequency, thus not forming pure stands but generally found in small groups or as individual trees.

1.5.1. Norway Maple 'Summershade'

'Summershade' Norway Maple in cultivation has a height of 12 to 15 meters but can grow taller. The oval crown maintains a central leader and fills with greenish-yellow flowers in the spring. The tree is easily transplanted, grows more quickly than the species, is adapted to a wide variety of soils and has brilliant yellow fall colour. It can also tolerate coastal conditions and is adapted to street tree plantings. It is tolerant of urban conditions, including alkaline soil, drought and pollution. Norway Maple can naturalize if located near open areas and roots can heave sidewalks, so locate it at least four to two meters away. 'Summershade' is probably the most heat tolerant of the Norway Maples but it is the less drought tolerant (Gilman and Watson, 1993, Fini et al., 2009).

FIGURE 5 TREES AND LEAVES OF *ACER PLATANOIDES* 'SUMMERSHADE'

1.5.2. Norway Maple 'Crimson King'

Acer platanoides 'Crimson King' is a fast-growing, deciduous shade tree. Under ideal conditions it may live up to 250 years. Leaves have a wide, pale green color. Many variaties have very attractive leaf color such as bark purple, red or green-white variagation. In spring cluster of yellow flowers appear followed by a winged fruit. Very attractive specimen tree. Crimson Sentry is perfect for small places and garden. The leaves are dark red just like ' Crimson King' This species is very tolerant of a wide array of urban soils. It is considered drought tolerant (Urban Forest and Trees, Handbook)

FIGURE 6 TREES AND LEAVES OF *ACER PLATANOIDES* 'CRIMSON KING'

1.5.3. Norway Maple 'Deborah'

Acer platanoides 'Deborah' is a fast growing cultivar of Norway maple which has bright red new foliage.
This tree has discrete yellow flowers that appear in spring and contrast fantastically with the deep red foliage which gradually hardens to green throughout the summer period.

The red foliage emerges each time the tree has a growth spurt, giving a continuous contrast throughout the summer of red and green foliage. In the autumn the deeply lobed leaves, with a distinctive wavy margin, change to a rich orangey/yellow.

Acer platanoides 'Deborah' is a stalwart performer, tolerating most soil types, air pollution and resists the perils of drought. It is more drought tolerant than *Acer platanoides* 'Summershade' (Fini et al., 2009).

This stunning large tree is perfect as a statement tree for large gardens and also is a great choice for avenues as the contrasting foliage and autumn colour offers varying interest throughout the year

FIGURE 7 TREES AND LEAVES OF *ACER PLATANOIDES* 'DEBORAH'

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1.6. Objectives of the thesis

As mentioned in the preceding paragraphs plants are subjected to several environmental stresses that adversely affect growth, metabolism, and yield. In the urban environment, they are often exposed to an excess of light due to the action of the direct solar radiation and, in *urban plazas*, of reflection by constructed surfaces. Both reduce the amount of radiation used for photosynthesis, exposing the photosynthetic apparatus to an excess of light. Due to climate change the frequency of drought episodes will determine, in the coming decades, an increase in oxidative damage to the photosynthetic apparatus. It's very important to better understand the physiology of urban trees and the interaction between the urban environment and the physiological processes in trees as a key feature to increase the health of plants, their value and their life span (Liakopoulos et al. 2006; Manetas & Buschmann 2011; Hughes 2011; Landi et al. 2015; Logan et al. 2015).

So, in order to understand the ecophysiological processes that act during high light stresses, the aim of my thesis is to analyse the response to two different stresses (sudden and chronic) on cyanic (one constitutively red and one temporarily red) and acyanic cultivars of *Acer platanoides*.

The project studies the relative significance of the ecophysiology of three cultivars of *Acer platanoides*. which differ for the presence of anthocyanins in the red one and for their strategy to cope with drought and high light stress.

The aim of my thesis is that the use of cyanic cultivars can improve the health status and benefits provided by moderately shade tolerant species in areas characterized by excess light of various kinds. This could be a guide to planning, increasing the diversity of species that can be used in urban plazas

To study this, two experiments have been conducted in the experimental station of Fondazione Minoprio in Vertemate con Minoprio (CO).

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2. Dynamics of physiological traits in three cultivars of *Acer platanoides*: how a cyanic and acyanic cultivars cope with a sudden excess light

2.1 Abstract

Plants are subjected to several environmental stresses that adversely affect growth, metabolism, and yield. In the urban environment, they are often exposed to an excess of light due to the action of the direct solar radiation and, in *urban plazas*, of reflection by constructed surfaces, exposing the photosynthetic apparatus to an excess of light. Moreover, due to climate change, the frequency of drought episodes will determine, in the coming decades, an increase in oxidative damage to the photosynthetic apparatus that will be exacerbate by high light stress. In this context researchers have suggested a protective role played by anthocyanins against the adverse effects of excess light, although the adaptive significance of compounds that they primarily absorb in the green is still the subject of scientific debate. Anthocyanins localized in the upper epidermis of the leaf, in the lower and in the mesophyll, have shown efficacy in reducing the incident light on the photosystems and have a 'defence role' against ROS, generated by an oxidative damage.

So in order to understand the ecophysiological processes that act during drought and high light stresses, the aim of this experiment is to analyze the response to these stresses on cyanic and acyanic cultivars of *Acer platanoides* ('Summershade', 'Crimson King' and 'Deborah').

Our study permitted a better understanding of the mechanisms adopted by these cultivars to cope with a sudden excess light and drought stress. In particular, we noticed that under optimal water availability, green leaves are photosynthetically more efficient, but the more conservative use of light and water by red leaves allow better tolerance to severe drought stress compared to green leaves

2.2 INTRODUCTION

Future climate changes are likely to include further increases in mean temperature (about 1.5 °C globally between 2030 and 2052) with significant drying in some regions (Christensen et al., 2007; Seager et al., 2007), as well as increases in frequency and severity of extreme droughts, hot extremes, and heat waves (IPCC, 2018; Sterl et al., 2008; Allen et al., 2010).

In this scenario, plants will encounter new or more severe combinations of abiotic stresses such as heat, cold and drought (Rizhskyet al. 2004, Mittler 2006, Suzuki et al. 2014). Although past and current research on plants subjected to a single abiotic stress has provided important information, many of these results cannot be utilized to infer the effects of a combination of two or more different stresses on the physiology of plants (Mittler 2006, Suzuki et al. 2014). Indeed, plant physiological and biochemical responses to multiple stresses can be very complex, since their effects could be additive (Valladares et al.,2000).

Drought is one of the most devastating natural hazards and is a pervasive climate phenomenon across the world. Under water stress, plants are also exposed to an excess of photosynthetic active radiation (PAR) on the photosynthetic apparatus, because the usage of PAR in the photosynthetic process decreases as a consequence of stomatal and biochemical limitations to net CO_2 assimilation (Bota et al., 2004; Flexas et al., 2004; Chaves et al., 2009). This could become more severe under natural field conditions, because of the concomitant action of both high irradiance and high temperature (Flexas and Medrano, 2002; Chaves et al., 2009). Under these environmental constrains there is a massive generation of reactive oxygen species (ROS; Munné-Bosch et al., 2001; Jubany-Marí et al., 2010; Tattini et al.,2012).

Plants display a wide range of mechanisms to protect themselves from oxidative stress, such as antioxidant enzymes which constitutes the 'first line of defence' against ROS which are divided in two main classes consisted of non-radical species (H_2O_2) or free radical forms (O_2^-; $OH·$; $OH_2·$). Accumulation of high concentrations of ROS is potentially detrimental to plants cells causing damage to valuable biomolecules like DNA, proteins, lipids, chlorophyll, membrane etc (Blokhina et al. 2003; Smirnoff, 1998; Apel and Hirt, 2004). However, in plants concomitantly exposed to water stress and high sunlight irradiance, the activity of enzymes aimed at detoxifying H_2O_2 declines significantly (Peltzer and Polle, 2001; Peltzer et al., 2002; Sharma and Dubey, 2005; Guidi et al., 2008; Liu et al., 2011).

In addition, plants accumulate polyphenols, which are products of the secondary metabolism that have a main role as UV-B screening pigments (Agati & Tattini,2002). However, polyphenols have been shown to be up regulated by drought-induced oxidative stress, while photosynthetic processes are concomitantly downregulated (Smirnoff, 1993; Mackerness et al., 2001; Rizhsky et al., 2002; Casati & Walbot, 2003). In particular, when exposed to high doses of both UV and sunlight radiation, plants preferentially accumulate flavonoids with an ortho-dihydroxy structure in the B ring with respect to flavonoids having a mono-hydroxy substitution (Markham et al., 1998; Wilson et al.,2001; Agati & Tattini, 2002; Hofmann et al., 2003).

Nevertheless, the accumulation of flavonols and dihydroxyflavones almost exclusively in epidermal cells of leaves exposed to light stress (Olsson et al., 1999; Gould et al., 2000; Kolb et al., 2001), and their vacuolar distribution in most species (Hutzler et al.,1998; Neill & Gould, 2003), still generate conflicts regarding their localization–functional relationships (Olsson et al.,1999).

Secondary, they have been shown also to be up regulated by drought-induced oxidative stress, while photosynthetic processes are concomitantly downregulated (Smirnoff, 1993; Mackerness et al., 2001; Rizhsky et al., 2002; Casati & Walbot, 2003).

In this context, researchers have suggested a protective role played by anthocyanins, one of the most conspicuous classes of polyphenols that are responsible for the red, pink, purple, and blue colours in plants (Grotewold, 2006). Anthocyanins accumulate in the vacuoles of a wide range of cells and tissues in both vegetative and reproductive organs (Hatier and Gould, 2009). These compounds have shown to have efficacy in reducing the incident light on the photosystems (Agati & Tattini,2002). In herbaceous species, this function has been translated into a greater ability of red-leaved individuals to live in environments characterized by high irradiance, than their green counterparts (Tattini et al., 2014). Both adaxially- and abaxially-located anthocyanins apparently constitute an effective sunblock, as reported for a variety of species irrespective of whether the pigments reside in the leaf mesophyll (Hughes and Smith, 2007; Hughes et al., 2013) or epidermis (Hughes et al., 2007; Landi et al., 2014).

Preliminary reports have suggested that a contemporary analysis of light- and drought-induced changes in photosynthetic performance and polyphenol content should help to understand the role of these pigments on the physiology of plants under environmental constrains (Agrell et al., 2000; Hampton, 1992; Tattini et al., 2004).

For this reason, the present study was planned to be useful for understanding the role of anthocyanins. Indeed, our aim was to analyse if red leafed cultivars are better suited to cope with

both drought and excess light stress because of the screening and antioxidant roles of these polyphenols.

Moreover, we wanted to characterize *Acer platanoides* cultivars about how they behave under drought and excess light stress many abilities to adjust morphoanatomical leaf traits, and, hence, mesophyll conductance to CO_2 diffusion, to sustain photosynthesis. Indeed, Norway maple has been used as an ornamental, shade and street-side tree because of its attractiveness, colourful foliage and large, spreading crown, in combination with its tolerance of urban conditions. The analysis of functional traits related to photosynthesis and stress tolerance will be a guide for choosing the appropriate combinations of species to face these combined stresses.

2.3. MATERIAL AND METHODS

2.3.1. Plant material and growth conditions

In March 2016, seventy-two two-year-old grafted plants of *Acer platanoides* 'Summershade', *A. platanoides* 'Deborah' and *A. platanoides* 'Crimson King' were potted in 20 l pots with a peat/pumice substrate (3:1, v:v), and were grown outdoors in a screen house covered with a plastic film, Nowoflon (ET6235–Z; Nowofol, Siegsdorf, Germany) to avoid natural rainfall which reduce incoming irradiance by about 20% (i.e average irradiance at midday 1600 mmol m^{-2} s^{-1} under the tunnel). Trees were not fertilized at potting and were watered daily using a sprinker until August 22nd. Container capacity, wilting point, and water holding capacity (WHC) were determined on 10 pots using a gravimetric method, as described in detail in previous works (Fini et al., 2011).

The experimental area was located at the Fondazione Minoprio (Como, Italy, 45°44'N, 9°04'E). Weather conditions, over the experimental period, were recorded at the weather station near the experimental situ. The average maximum and minimum temperatures, during the experiment, was 24.5 °C and 13.7°C during the experiment. Year averages of mean daily global irradiance (W/m^2) during the days of measurements was 395.7 ± 26.4 W/m^2. After five months of acclimation to the growing conditions, the drought treatment was imposed.

Paired spectroradiometers (LightScout® 3670I, Spectrum Technologies, Inc.) were used to measure irradiance. Reflected irradiance was negligible.

The day before the onset of drought, all plants were watered by hand until leaching, and allowed to drain overnight to reach desiderated actual water content (AWC = 100%, T0). Then, water stress was applied according to the following treatments:

- WW: plants were daily hand-watered to container capacity throughout the experiment;
- WS: irrigation was suspended and the substrate was allowed to dry out until it reached pre-established Actual Water Contents (AWC), corresponding to moderate stress (AWC = 40-45%, T1) and stress (AWC = 25-30%, T2). The Actual Water Content (AWC, %) was calculated daily (at 17.00) during the stress period as: ((daily$_{pot\ weight}$ – (initial$_{pot\ weight}$ – substrate $_{weight}$))/ (initial$_{pot\ weight}$ – (initial$_{pot\ weight}$ – substrate$_{weight}$)))*100 where daily$_{pot\ weight}$ is the hydrated weight of the pot with plant at the moment of measurement, initial$_{pot\ weight}$ is the hydrated weight of pot with plant at the beginning of the experiment (T0) and substrate$_{weight}$ is the weight of substrate without plant at 100% AWC (Fini et al., 2011).

Cultivars with high transpiration (E) reached the desired AWC few days before those with lower E. To avoid biases, substrate moisture of fast transpiring cultivars was prevented to fall below the desired AWC threshold by adding daily the exact amount of water required to restore AWC to 45% (T1) or 30% (T2) until all cultivars reached the desired AWC. In this case, watering was done at 18.00, except for the days before pre-dawn water potential measurements. The day after all cultivars reached the desired AWC, physiological parameters were measured. When moisture of all cultivars felt below 25%, plants were re-watered daily to field capacity for two weeks, thereafter physiological and biochemical parameters were measured (T3).

Biomass and leaf mass area were measured at the beginning of the experiment (day 0) and at the end of the stress (day 45). Biomass was determined on 4 replicate plants per treatment. For biomass determination, plants were cut at the root flare and leaves, stem and roots were weighted separately. To determine dry weight, leaves, stem and roots were over dried at 70 °C until constant weight was reached (approximately 72 h). Leaf Mass per Area (LMA, g DW m^{-2}) was calculated as the ratio between leaf dry mass and leaf area. Root to shoot ratio were measured on 6 plants per cultvivar and treatment (24 plants). Relative Growth Rate (RGR) was calculated as RGR = (ln DW$_{final}$ – ln DW$_{initial}$)/n days. Wilting was assessed according to visual stages proposed by Engelbrecht and Kursar (2003).
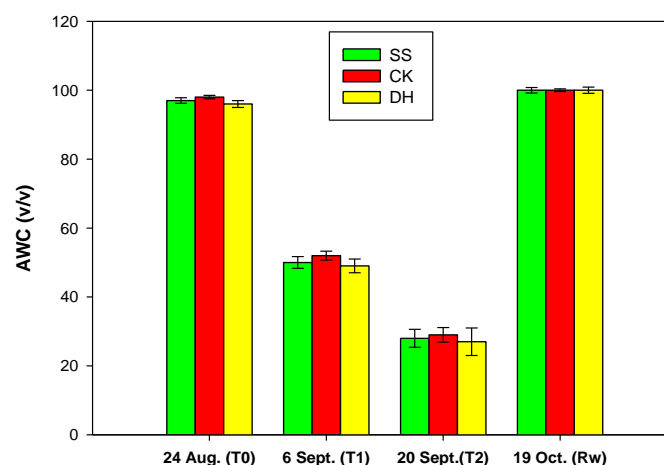

FIGURE 1. VOLUMETRIC SOIL MOISTURE. SS STANDS FOR *ACER PLATANOIDES* 'SUMMERSHADE', CK STANDS FOR 'CRIMSON KING' AND DH STANDS FOR 'DEBORAH'. BARS REPRESENT SD.

2.3.2. Leaf gas exchange and chlorophyll fluorescence measurements

Measurements between 9:00 and 17:00 of leaf gas exchange (net CO_2 assimilation rate (A or P_N), stomatal conductance (g_s) and transpiration (E)) were taken on the first fully expanded leaf with an infrared gas analyser (LI-COR Inc., Lincoln, NE, USA) on three leaves per treatment, species, and replicate. During all the three stages of stress, assimilation (A) to chloroplastic CO_2 concentration (C_c) curves were drawn with an infrared gas analyser (Ciras-2, PP-System, Hertfordshire, UK) by varying the ambiental CO_2 concentration (C_a) using an external cartridge, which allows to switch external CO_2 (C_a). For each cultivar at each stage of stress, a curve A/C_c was performed consisting of 7–8 steps, starting from about 370 and decreasing to 200, 100 and 40 μmol CO_2 mol^{-1}, before increasing to 370, 800, 1600 and 2000 μmol CO_2 mol^{-1}. The Quantitative Limitation Analysis (Grassi and Magnani, 2005) was used to interpret the curves and to identify stomatal (L_s), mesophyll (L_m) and biochemical limitations (B_L). Estimates of the apparent maximum rate of carboxylation by Rubisco (V_{cmax}) and the apparent maximum electron transport rate contributing to ribulose 1,5-BP regeneration (J_{max}) were made using the equations found by Sharkey et al. (2007).

During stress conditions, the A/PPFD curves were done with LI-COR suppling leaves with 1800 μmol PAR $m^{-2}s^{-1}$, then irradiance was decreased stepwise to darkness (Guidi et al., 2008). CO_2 evolution in light conditions, an indicator of the respiration rate in the light (R_{day}), was estimated from A/PPFD curves using the Kok method (Kok 1948). Light compensation (LCP) and light saturation (LSP) were estimated by fitting a non-rectangular hyperbola function to individual A/PPFD curves (Avola et al. 2008). The determination of the maximal quantum yield of PSII (F_v/F_m) was measured daily on the same leaf of leaf gas exchange by using a HandyPEA portable fluorescence spectrometer (Hansatech Instruments Ltd., King's Lynn, UK) and was obtained after adapting leaves to darkness for 40 min by attaching a light exclusion clips to the leaf surface and then using a saturating flash of actinic light at 3000 μmol m^{-2} s^{-1}.

2.3.3. Optics

Sections of leaves were examined in a Leica DM LB2 compound micro-scope (Leica, Wetzlar, Germany), equipped with a high-resolution TK 870E JVC (JVC, Tokyo, Japan) video camera. Hand-cut transverse sections from fresh leaves were examined in a light microscope to visualize anthocyanin location

2.3.4. Leaf water relations and plant conductivities

Leaf pre-dawn (Ψ_w), xylem (Ψ_x), and midday (Ψ_m) water potentials were measured during the stress period and at the end of relief with a pressure bomb (PMS Instruments, Albany, OR, USA; Scholander et al. 1964). Pre-dawn water potential was measured between 04.00 and 06.00 hours on two fully expanded leaves per specie, treatment and block. After Ψ_w measurements, leaves were frozen in liquid nitrogen and stored at −80 °C for the analysis of osmotic potential. Leaf osmotic potential (Ψ_π, MPa) was measured on expressed sap of frozen and thawed leaves with a freezing-point osmometer (Osmomat 030, Gonotech Gmbh, Berlin) equipped with a 20 µl measuring cell (Tattini et al., 2002). Leaf turgor pressure (Ψ_p, MPa) was calculated as the difference between Ψ_w and Ψ_π. Relative Water Content (RWC, %) was calculated on two leaves per plant, treatment and block as described by Tattini et al. (2006) as RWC= ((FM-DM)/(TM-DM))*100 where FM, TM and DM denote fresh, turgid and dry masses respectively. Then, osmotic potential at full turgor ($\Psi_{\pi FT}$, MPa) was calculated as in Tattini et al. (2006). For xylem water potential, the leaves opposite or next to that used for Ψ_w were enclosed at predawn in a plastic bag covered with aluminium foil for subsequent xylem water potential measurements (Ψ_x) which was performed between 13.00 and 14.30. At the same time, water potential was also measured on unwrapped leaves of the same shoot and midday water potential was determined. Soil-to-plant (K_{sp}, mm s^{-1} MPa^{-1}), soil-to-xylem conductivity (K_{sx}, mm s^{-1} MPa^{-1}), and leaf conductance (K_l, mm s^{-1} MPa^{-1}) were calculated from Ψ_w, Ψ_x and Ψ_m and E (measured between pre-dawn and midday) using *in vivo* method as previously described (Costa e Silva et al., 2004; Fini et al., 2011).

2.3.5. Statistical analysis

Plants were arranged following a randomized block design with 4 blocks, each consisting of 3 three plants per cultivar and per treatment.

Measurements were conducted in control, moderately (T1) (30% AWC), severely (T2) (50% AWC) and recovery drought-stressed plants.

Leaf gas exchange data were analysed using a two-way ANOVA, where treatments and hours were the between subject factors and sampling dates was the within subject factor using SPSS 24 (SPSS, Chicago, IL).

Leaf water relations and plant conductivities data were analyzed using Two-way ANOVA, where treatment was the between subject factor and sampling date was the within subject factor using SPSS 24.00 (SPSS, Chicago, IL). In both cases, significant differences among means were estimated at the 5% ($P<0.05$) level, using Tukey's test

2.4. RESULTS

2.4.1. Gas exchange

In all the three cultivars, photosynthesis gradually declined during the progression of drought stress (Figure 2). In fact, WW plants had higher A respect to WS plants throughout the experiment. In particular, at severe stress (T2), WS plants of *Acer platanoides* 'Deborah' showed lower values of P_n than the other stressed cultivars (respectively -24% to 'Summershade' and -62% to 'Crimson King'), indicating that A is affected by water regime (Figure 2). After rewatering, photosynthesis recovered in all the three species. In particular, 'Summershade' recovered of +121% compared to T2 WS plants, while 'Crimson King' recovered of +74% and 'Deborah' recovered of +266% (Figure 2).

Stomatal conductance showed the same trend of P_n during the experiment. In particular, in 'Deborah' WS plants g_s decreased from 0.20 mmol at T0 to 0.03 mmol at T2. After the recovery period, g_s were partially recovered in all the cultivars respect to stressed plants at severe stress (Figure 3).
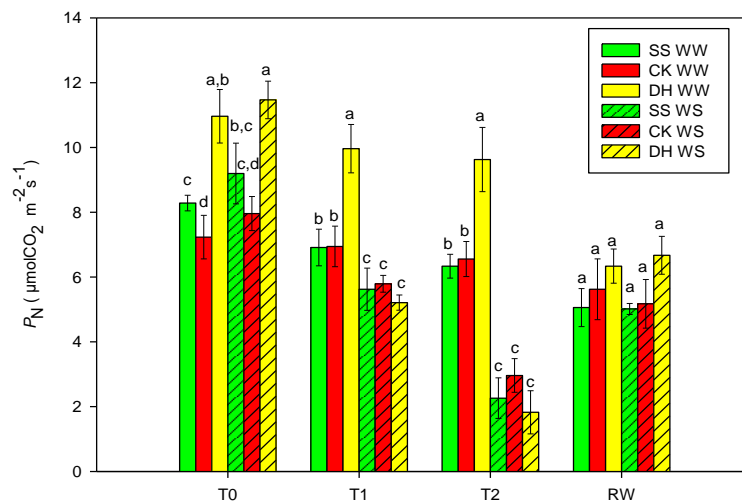

FIGURA 2. CO_2 ASSIMILATION (PN) IN LEAVES OF *ACER PLATANOIDES* 'SUMMERSHADE', 'CRIMSON KING' AND 'DEBORAH' DURING ALL OF THE THREE STAGES OF STRESS. MEAN VALUES WITH THE SAME LETTERS ARE NOT SIGNIFICANTLY DIFFERENT (P≥0.05).
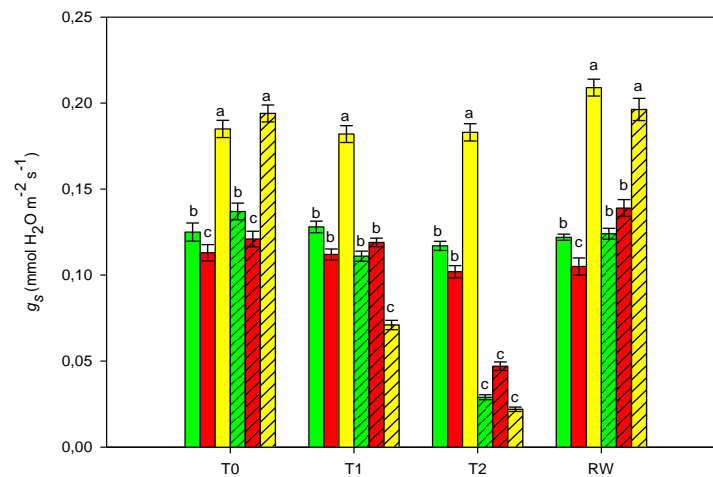
FIGURE 3. STOMATAL CONDUCTANCE TO CO_2 (G_S) IN LEAVES OF *ACER PLATANOIDES* 'SUMMERSHADE', 'CRIMSON KING' AND 'DEBORAH' DURING ALL OF THE THREE STAGES OF STRESS. MEAN VALUES WITH THE SAME LETTERS ARE NOT SIGNIFICANTLY DIFFERENT ($P \geq 0.05$).

At severe stress (T2), E values declined compared to the beginning of the experiment (Figure 4). In particular, in 'Summershade' and 'Deborah' E declined much more than in 'Crimson King' (-375% and -384%, respectively). E values of Acer platanoides 'Crimson King' was lower affected than the other cultivars by severe drought (-244% at T2 compared to T0). However, after rewatering, E recovered in all three cultivars.

FIGURE 4. E VALUES IN LEAVES OF *ACER PLATANOIDES* 'SUMMERSHADE', 'CRIMSON KING' AND 'DEBORAH' DURING ALL OF THE THREE STAGES OF STRESS. MEAN VALUES WITH THE SAME LETTERS ARE NOT SIGNIFICANTLY DIFFERENT ($P \geq 0.05$).
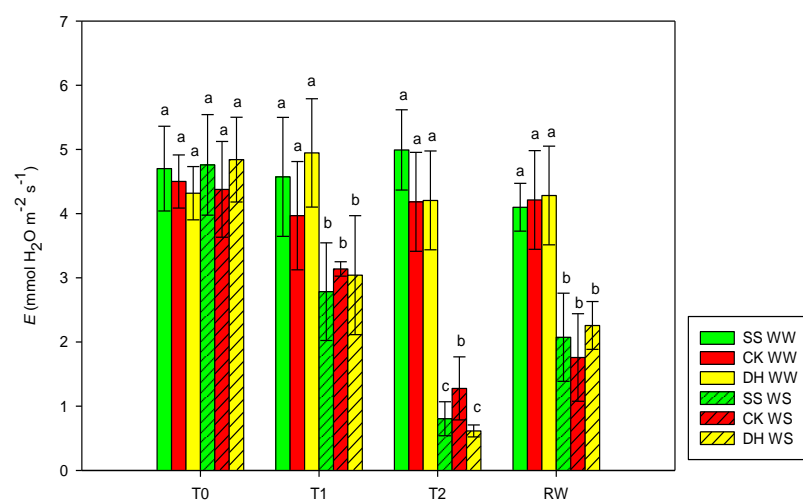
From the beginning of the experiment (T0) till the end of water stress (T2), Water Use Efficiency (WUE) is lower in cyanic leaves of WS plants than acyanic ones (Table 1). At rewatering WUE did not differ in the three cultivars.

WUE

		T0	T1	T2	RW
Acer Platanoides 'Summershade'	WW	2.56 a	2.41 a	2.45 a	2.36 a
	WS	2.44 a	2.02 a	2.31 a	2.41 a
Acer Platanoides 'Crimson King'	WW	2.10 b	2.12 a	2.44 a	2.99 a
	WS	2.06 b	2.04 b	1.81 b	2.37 a
Acer Platanoides 'Deborah'	WW	2.46 a	2.01 a	2.28 a	2.77 a
	WS	2.36 a	1.73 b	2.02 a	2.95 a

TABLE 1. DATA OF WATER USE EFFICIENCY OF THE THREE CULTIVARS OF *ACER PLATANOIDES* DURING STRESS PERIOD AND AT THE END OF THE RELIEF. MEAN VALUES WITH THE SAME LETTERS ARE NOT SIGNIFICANTLY DIFFERENT (P≥0.05) USING TUKEY'S TEST.

F_v/F_m was statistically unaffected by the drought treatment in all the three cultivars as shown in Figure 5.
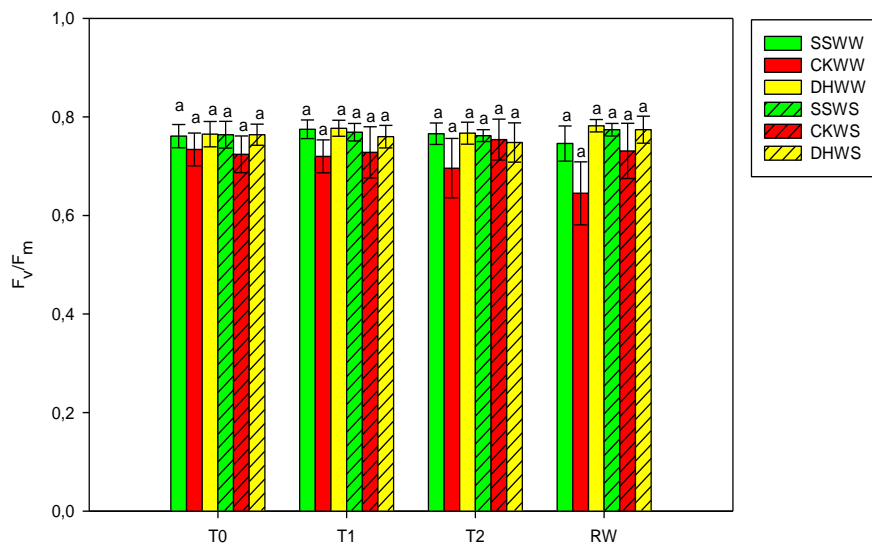
FIGURE 5. F_v/F_m'S VALUES IN LEAVES OF *ACER PLATANOIDES* 'SUMMERSHADE', 'CRIMSON KING' AND 'DEBORAH' DURING ALL OF THE THREE STAGES OF STRESS. MEAN VALUES WITH THE SAME LETTERS ARE NOT SIGNIFICANTLY DIFFERENT (P≥0.05).

For what concern limitations to photosynthesis, diffusive limitation (stomata + mesophyll) increased more in green (+70%) than in red (+45%) leaves from T0 to T2 (Table 2).

Moreover, cyanic leaves had a higher respiration day (R_{day}) than acyanic ones both at the beginning of the experiment and at T2 (Table 2).

	Stomatal Limitation (L_s,%)		Mesophyll Limitation (L_m,%)		Respiration day ($\mu mol\ m^{-2}\ s^{-1}$)	
	T0	T2	T0	T2	T0	T2
Acer Platanoides 'Summershade'	2	20	2	45	0.3 b	0.08 b
Acer Platanoides 'Crimson King'	5	15	12	45	0.9 a	0.1 a
Acer Platanoides 'Deborah'	3	22	14	40	0.8 a	0.06 b

TABLE 2. STOMATAL (L_S, %) AND MESOPHYLL (L_m, %) LIMITATIONS TO PHOTOSYNTHESIS IN THREE *ACER PLATANOIDES* CULTIVARS GROWN IN WATER STRESS CONDITIONS. RESPIRATION DAY ($\mu MOL\ M^{-2}\ S^{-1}$) CALCULATED FROM A/PPFD CURVES. MEAN VALUES WITH THE SAME LETTERS ARE NOT SIGNIFICANTLY DIFFERENT (P≥0.05).

For what concerns A/PPFD curves, as shown in Figure 6, drought induced an excess light stress in green leaves that was higher in 'Summershade' and 'Deborah' than in 'Crimson King'. In fact, from the beginning of the stress to severe stress, drought reduced usage of photons more than -50% for 'Summershade' and -80% for 'Deborah', besides for cyanic leaves drought reduced usage of photons by -34% (Figure 5). As shown in Table 3, light response curves indicated higher saturation point for acyanic leaves than cyanic leaves. However, 'Crimson King' showed a higher compensation point than acyanic leaves (Table 3).

	Light Compensation Point (LCP,$\mu mol\ m^{-2}\ s^{-1}$)		Light Saturation Point (LSP, $\mu mol\ m^{-2}\ s^{-1}$)	
	T0	T2	T0	T2
Acer Platanoides 'Summershade'	18	7	800	1000
Acer Platanoides 'Crimson King'	35	23	1000	1100
Acer Platanoides 'Deborah'	32	10	1100	1200

TABLE 3. DATA FROM A/PPFD CURVES. LIGHT COMPENSATION POINT (LCP)T IS THE VALUE OF PPFD WHEN $A_N = 0$ AND IT IS CALCULATED AS THE INTERCEPT ON THE X-AXIS, LIGHT SATURATION POINT (LSP) IS THE VALUE OF PPFD WHEN $A_N = 90\%\ A_{N\ MAX}$
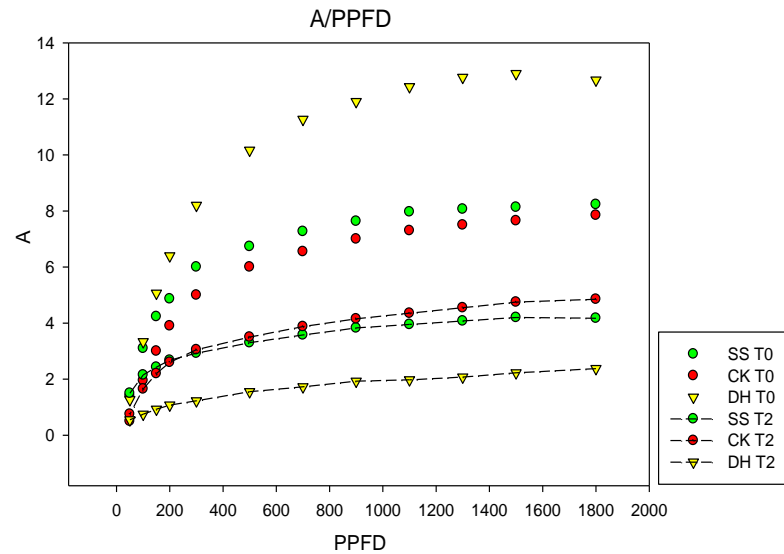
FIGURE 6. DATA FROM LIGHT RESPONSE CURVES. SS STANDS FOR *ACER PLATANOIDES* 'SUMMERSHADE' (GREEN SYMBOLS), CK FOR *ACER PLATANOIDES* 'CRIMSON KING' (RED SYMBOLS) AND DH FOR *ACER PLATANOIDES* 'DEBORAH' (YELLOW SIMBOLS), T0 FOR THE BEGINNING OF THE EXPERIMENT AND T2 FOR SEVERE STRESS.

2.4.2. Water relations

Fig. 7 showed the trends of predawn water potential (Ψ_w), osmotic potential (Ψ_π), leaf turgor potential (Ψ_P), midday potential (Ψ_w) and RWC (relative water content, %) in the study cultivars during drought.

RWC had a decline during T2, in particular in 'Deborah'. Indeed, during T2 RWC of WS plants of 'Deborah' was much lower than ones of T0 (-20%). At the end of the relief, WS plants of 'Crimson King' and 'Deborah' recovered similarly (+10% and +16%) respect to severe stress.

Predawn leaf water potential of WS plants of 'Deborah' significantly decreased from TO (-0.3 MPa) to T1 (-1.57 MPa) dropping below -3MPa at T2 and recovered to the level of WW plants at the end of relief in all the cultivars. Interestingly, in 'Deborah' Ψw declined much more than the other cultivars.

Ψ_π decreased in all species because of water stress, but in the green cultivars decreased more than in the red one. Indeed in 'Summershade' and in 'Deborah' Ψ_π declined of -56% and -64% from T0 to T2, while in 'Crimson King' the decline was of -53%. During rewatering, Ψ_π returned to level of T0 in all the cultivars. Ψ_p at T2 showed a higher decline in both WS plants of 'Summershade' and 'Deborah' than in 'Crimson King' compared to T0.

Ψ_m showed the same trend of leaf predawn potential. In particular WS plants of 'Deborah' significantly decreased from T0 (-1.39 MPa) to T1 (-2.71 MPa) dropping below -4MPa at T2 and recovered to the level of WW plants at the end of the relief.
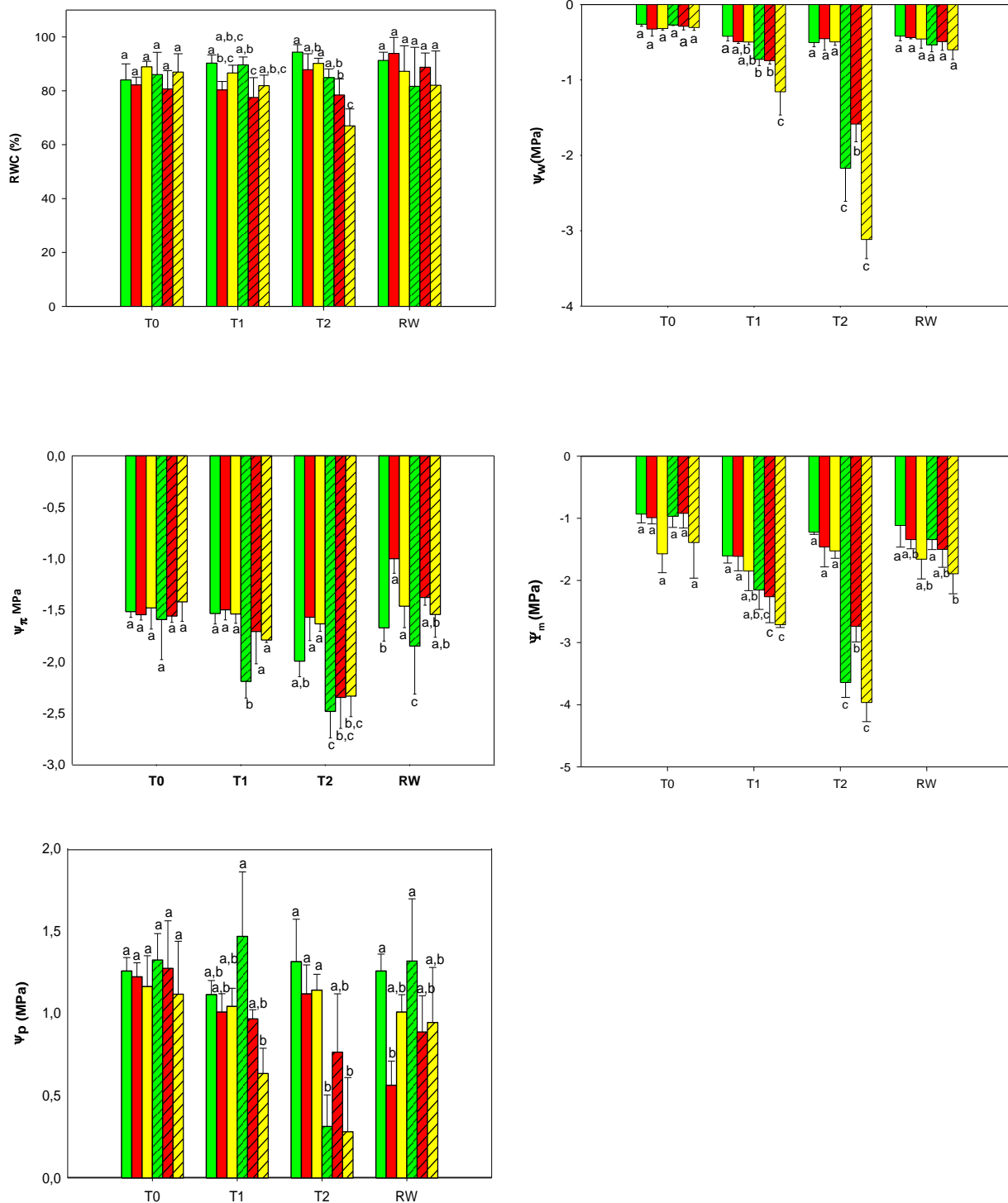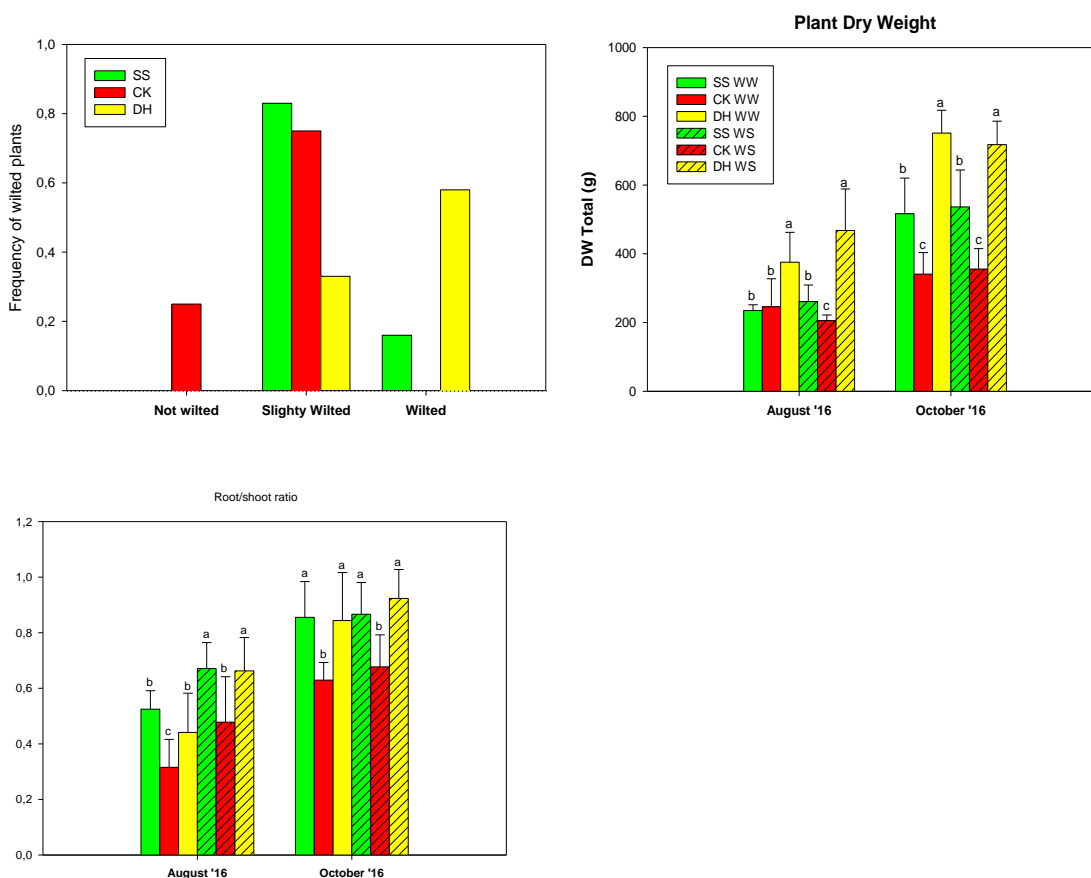

FIGURE 7. WATER RELATIONS OF THREE CULTIVARS OF *ACER PLATANOIDES* DURING ALL OF THE THREE STAGES OF STRESS. MEAN VALUES WITH THE SAME LETTERS ARE NOT SIGNIFICANTLY DIFFERENT (P≥0.05).

2.4.3. Wilting assessment and biomass determination

At the beginning of the experiment, 'Deborah' WS plants had a higher plant biomass than both 'Summershade' (+ 70%) and 'Crimson King' (+ 87%). At the end of the stress WS plants of 'Crimson King' had a minor plant-dry weight compared to ones of 'Deborah' (-101%) and 'Summershade' (-51%). Moreover, both at the beginning and at the end, in root to shoot ratio WS plants of 'Crimson King' displayed lower allocation to roots compared to ones of 'Summershade' (-42%) and 'Deborah' (-41%) (Figure 8).

At the end of the stress many plants showed selective leaf yellowing (indicated as slightly wilted, see Engelbrecht and Kursar, 2003). In particular 'Summershade' had the highest frequency. Moreover 'Deborah' showed the highest frequency of wilting because of the presence of necrotic leaves (see Engelbrecht and Kursar, 2003).

FIGURE 8. WILTING ASSESSMENT AND BIOMASS PARAMETERS OF THE THREE CULTIVARS OF *ACER PLATANOIDES* DURING ALL OF THE THREE STAGES OF STRESS. MEAN VALUES WITH THE SAME LETTERS ARE NOT SIGNIFICANTLY DIFFERENT (P≥0.05).

2.4.4. Optics

Acer Platanoides Crimson King

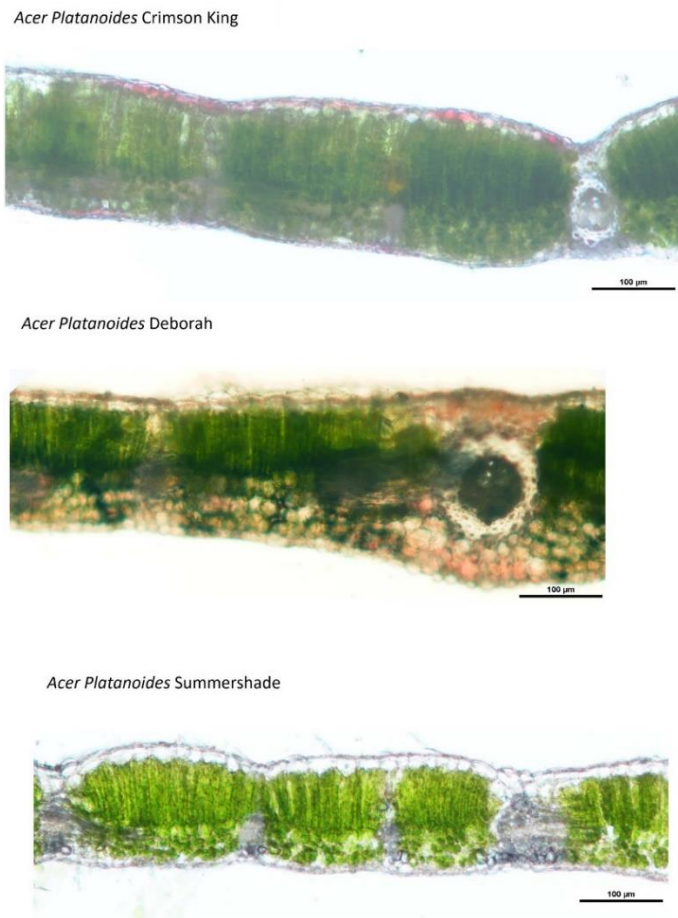Acer Platanoides Deborah

Acer Platanoides Summershade

FIGURE 9. FRESHLY CUT, CYANIC, RED LEAF OF *A. PLATANOIDES* 'CRIMSON KING', TEMPORARILY RED LEAF OF *A. PLATANOIDES* 'DEBORAH' AND GREEN LEAF OF *A. PLATANOIDES* 'SUMMERSAHDE' IN TRANSVERSE SECTION.

There were no obvious structural differences between the leaves of the three cultivars of *Acer platanoides*. The difference was in the localization of anthocyanins. In 'Crimson King', the anthocyanins were in the epidermis of leaf and chlorophyll in the mesophyll. In 'Deborah', these red compounds were localized in the upper (adaxial) and lower surface (abaxial). In 'Summershade' there was an upper palisade layer of vertically elongated cells, one to two cells thick, directly beneath the adaxial epidermis. Its cells contain many chloroplasts. The cells of the spongy layer are more branched and not so tightly packed, so that there are large intercellular air spaces between them for oxygen and carbon dioxide to diffuse in and out of during respiration and photosynthesis. These cells contain fewer chloroplasts than those of the palisade layer. The pores or stomata of the

epidermis open into substomatal chambers, which are connected to the intercellular air spaces between the spongy and palisade mesophyll cells.

2.5. Discussion

Acer platanoides L., commonly known as Norway maple, is a fast-growing tree species, able to grow well across a wide range of soil conditions, shade, drought and pollution. However, it thrives best in deep, fertile, moist soils, which are adequately drained and with a sub-acid pH. Exposure and strong calcareous soils are well tolerated. It is intolerant of low soil nitrogen conditions, high evapo-transpiration or prolonged drought and it is rare on acidic soils (pH near 4) (Nowak & Rowntree, 1990).

However, the strategy adopted from *Acer platanoides* to withstand dry period and excess light has not been investigated at research level.

So in this experiment we described the response of three woody cultivars of *Acer platanoides* to drought and high light conditions.

Gas exchange parameters measured between 07.00 and 18.00 h in leaves of *Acer platanoides* 'Summershade', 'Crimson King' and 'Deborah', at the beginning and at the end of the drought period showed that big differences existed among the three cultivars.

The data presented in this study clearly indicate that, under progressively increasing soil water deficit and high light stress, the red cultivar had a better tolerance than green ones because of the more conservative use of light and water, while under optimal water availability, green leaves are photosynthetically more efficient.

Indeed at T0, *Acer platanoides* 'Crimson King' had a lower P_n (Figure 2) and this could be due to a lower CO_2 availability in chloroplasts because anthocyanins have a general negative effect on the capacity of the mesophyll to conduct CO_2 from the substomatal chamber to the chloroplasts (Flexas et al., 2013). Transpiration was less affected than P_n by anthocyanins, thus WUE was lower in cyanic leaves (Table 2). Moreover, epidermal anthocyanins are costly to plants, in fact the red cultivar displayed a lower growth rate and a lower allocation to roots than the green ones (Figure 7). On the contrary, 'Deborah' and 'Summershade' at well-watered conditions has a higher photosynthetic gain (P_n) (Figure 2). However, these cultivars are more sensitive to water stress, and, in particular 'Deborah' showed the highest reduction in photosynthesis and stomatal conductance (Figure 2 and 3).

This could be due to diffusive limitations (stomata + mesophyll), which increased steeply in green leaves during stress (Table 2).

For what concern water relations, our data showed different types of strategies that plants use to cope with drought stress.

The relationship between stomatal conductance (g_s) and leaf water potential (Ψ_W) is key to the understanding of plant function under changing climate. The variability among tree species gave rise to selection towards either of two contrasting water management types: isohydric or anisohydric. The effect of stomatal regulation (isohydric/anisohydric) on the ability of trees to survive adverse conditions has been addressed in numerous studies (e.g. West et al. 2007; McDowell et al. 2008; Klein et al., 2011; Domec & Johnson, 2012; Klein, 2014)

'Crimson King' and Summmershade' showed a similar trend for predawan leaf water potential and midday potential, while in 'Deborah' data showed a greater decrease compared to the ones of the other cultivars (Figure 6). Only during T2 it decreased RWC (Figure 7).

However, the decrease in Ψ_π was mainly driven by leaf turgor pressure (Ψ_p), which progressively declined as drought progressed. So we could identified two main strategies when facing drought conditions namely isohydric and anisohydric mechanisms (Ryan 2011, McDowell et al. 2008). Isohydric species generally possess effective mechanisms of stomatal regulation of transpiration. They decrease leaf stomatal conductance if soil water potential declines, thereby reducing water loss and preventing a decrease of leaf water potential (McDowell et al. 2008). Furthermore, they often show extensive root systems, rather small leaves and a high leaf mass to area ratio (Aroca 2012). The downside of an isohydric response is the risk of carbon starvation as photosynthesis declines with the closing of the stomata but plant respiration still takes place (Allen et al. 2010). This mechanism is particular evident in 'Summershade' and 'Crimson king'. Anisohydric species only have a limited capacity of stomatal control of transpiration, maintain higher leaf stomatal conductance, and thus face higher water losses and a decline in leaf water potential, especially around midday. Additionally, they can react with a decrease of osmotic potential due to their capacity of osmotic adjustment (McDowell et al. 2008). The risk of carbon starvation is lower than for isohydric species, because stomata remain open. Anisohydric cope better with prolonged droughts but are more likely suffer from hydraulic failure under short but especially intense droughts (Gill et al. 2013) and this mechanism is particular evident in 'Deborah'.

Moreover, leaves of 'Crimson King' did not wilt (intended as strong changes in leaf insertion angle, and/or leaf rolling and folding, Engelbrecht and Kursar, 2003) in response to water shortage (Figure 8). In 'Deborah' extended leaf yellowing was observed and this trait conclusively indicated this cultivar as the most drought-sensitive (Figure 7). Root to shoot ratio underlined that 'Crimson King'

plants had a lower allocation to roots compared to the other cutivars (Figure 8) and this is probably due to anthocyanins that are costly to plants. The synthesis and vacuolar sequestration of anthocyanin molecules represent a considerable metabolic investment for plant cells. First, there are metabolic costs associated with enzyme production and activity; at least seven enzymes are involved in the biosynthesis of cyanidin from its precursors, 4-coumaroyl-CoA and malonyl-CoA (Shirley, 1996). Then there is a cost associated with the conjugation of each cyanidin molecule to a monosaccharide molecule. Finally, there are costs associated with the transport of cyanidin-3-O-glucoside into the cell vacuole via a tonoplast Mg-ATP-requiring glutathione carrier (Alfenito et al., 1998). This investment suggests that the accumulation of anthocyanins in leaf cells is unlikely to be an 'extravagancy without a vital function' (Gould,2004; Matile, 2000).

2.6. Conclusion

This study aimed to bring new knowledge about mechanism adopted by *Acer platanoides* cultivars to withstand drought and high light, which are still largely unknown. Results of this study indicated an extremely water-conservative behavior, achieved through a drought-avoidance (for 'Deborah') and a drought-tolerant mechanism (for 'Summershade' and 'Crimson King'). For these cultivars basics of this mechanism were early stomatal closure to reduce transpiration water losses and to avoid an excessive decline in leaf water potential and relative water content, and partial and selective leaf shedding, anticipated by nutrient re-absorption and leaf yellowing, and followed by an opportunistic leaf flushing behavior as water becomes never available. On the other hand, even a drought of moderate intensity was sufficient to induce heavy detrimental effects on morphological and physiological traits of 'Deborah. Indeed, plants of 'Deborah' were found to be less drought tolerant than those from the other two cultivars because this cultivar show the highest decreasing in water relations.

To cope with sudden drought and high light stress, cyanic cultivars are better equipped for the presence of anthocyanins, even if they are costly for plants. Even if, under well-watered conditions, cultivars with green leaves provided higher benefits, in terms of CO_2 storage and photosynthesis than cyanic cultivars.

Indeed, this study presents opportunities for research that will expand our knowledge of how trees acclimate and adapt to their environment.

The frequency and intensity of severe drought stress events are expected to increase dramatically in the near future, especially in urban areas, in correspondence to the increasing presence of urban heat island.

In conclusion, this study presents new insights on the physiology of three different cultivars of *Acer platanoides* under high light and drought stresses and should be helpful for choosing the right varieties in urban planning and maximise ecosystem services in the face of future climate change projections.

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3. Dynamics of physiological traits in three cultivars of *Acer platanoides*: how a cyanic and acyanic cultivars cope with a chronic excess light

3.1 Abstract

Plants are subjected to several environmental stresses that adversely affect growth, metabolism, and yield. In the urban environment, they are often exposed to an excess of light due to the action of the direct solar radiation and, in *urban plazas*, of reflection by constructed surfaces, exposing the photosynthetic apparatus to an excess of light.

 In this context researchers have suggested a photoprotective role played by anthocyanins against the adverse effects of excess light, although the adaptive significance of compounds that they primarily absorb in the green is still the subject of scientific debate. Anthocyanins localized in the upper epidermis of the leaf, in the lower and in the mesophyll, have shown efficacy in reducing the incident light on the photosystems and have a 'defence role' against ROS, generated by an oxidative damage.

So in order to understand the ecophysiological processes that act during high light stress, our study analyse the response to this stress on cyanic and acyanic cultivars of *Acer platanoides* ('Summershade', 'Crimson King' and 'Deborah').

Our aim was a better understanding of the mechanisms adopted by these cultivars to cope with a chronic excess light like in *urban plaza*. In particular, we noticed red cultivar appear an interesting opportunity when high light is a chronic issue because of anthocyanins.

3.2 Introduction

Although the plant growth is controlled by a multitude of physiological, biochemical, and molecular processes, photosynthesis is a key parameter, which contributes substantially to the plant growth and development (Asharaf & Harris, 2013).

Light is one of the main factors affecting photosynthesis and plant growth as it is the source of energy for carbon fixation (Walters & Horton, 1994). During the day, the quality and quantity of photosynthetically active radiation (PAR) changes frequently and plants try to keep a balance between the conversion of light energy and protection of the photosynthetic apparatus from photoinhibition or repair of eventual damage (Bailey et al., 2004, Kalaji et al., 2012). Indeed, sunlight damages photosynthetic machinery, primarily photosystem II (PSII), and causes photoinhibition that can limit plant photosynthetic activity, growth and productivity. Plants have therefore developed mechanisms that can quickly and effectively repair photodamaged PSII (Aro, E.M. et al., 2005); as a result, net photoinhibition only occurs when the rate of damage exceeds that of the repair (Takahashi & Badger, 2011).

Under this scenario, scientists showed that red individuals perform better than green ones when exposed to excessive solar radiation, even if after more than three decades of extensive research it is still a debate for many reasons (Hughes, 2011; Landi et al., 2015). First of all, the red colouring of leaves can be a transient or permanent condition. The leaves of some species are red during the juvenile phase (Liakopoulos et al., 2006; Zeliou et al., 2009) or become red during autumn– winter (i.e. 'winter reddening'; Kytridis et al., 2008; Hughes, 2011). On the other hand, plants display leaves that remain red throughout the entire life cycle (Kyparissis et al., 2007; Tattini et al., 2014; Logan et al., 2015). Secondly, anthocyanins can be located in different leaf tissues, they may occur in both the adaxial and abaxial epidermises (Hughes and Smith, 2007; Tattini et al., 2014), in just one of the two epidermises (Hughes et al., 2005; Hughes and Smith, 2007), or be distributed in the mesophyll (Gould and Quinn, 1999; Kytridis and Manetas, 2006). Thirdly, anthocyanins have different abilities to absorb the visible wavelengths of the solar spectrum (Merzlyak et al., 2008; Jordheim et al., 2016). Strikingly different experimental set-ups, which include both plant material and growth conditions, add further complexity in providing conclusive evidence on the photoprotective role of anthocyanins. However, many studies confirmed that the photosynthetic machinery is better protected in cyanic than in acyanic leaves under transient exposure to severe excess light (Feild et al., 2001; Hughes and Smith, 2007). Photosystem II (PSII) photochemistry also recovers faster in

red than in green leaves during relief from photoinhibitory light (Landi et al., 2014; Logan et al., 2015). More contradictory observations stem from long-term studies conducted in the field (Steyn et al., 2002; Gould, 2004). The ability of anthocyanins to absorb over the blue-green region of the solar spectrum (Feild et al., 2001) may profoundly alter cryptochrome-dependent photomorphogenesis in red morphs, and evoke shade avoidance responses (Keller et al., 2011; Keuskamp et al., 2011; Zhang and Folta, 2012; Pedmale et al., 2016). Red individuals show less plasticity than green individuals to changes in sunlight availability, sometimes referred as to the 'shade syndrome' displayed by red leaves (Manetas et al., 2003). In line with this, red cultivars display leaves that are thinner, with less compact mesophyll than the leaves of green morphs when fully exposed to solar radiation (Kyparissis et al., 2007; Lan et al., 2011; Tattini et al., 2014). The relative abilities of green and red individuals to modify leaf morpho-anatomical traits (e.g. leaf thickness and mesophyll compactness) depending on light availability (not only transmitting blue-green light at different rates in the leaf), may considerably affect their gas exchange performance (Terashima et al., 2011; Tattini et al., 2017).

In our study, analyses were conducted to detect physiological and morphological response mechanisms to a chronic high solar irradiance in three cultivars of *Acer Platanoides*: 'Summershade', 'Crimson King' and 'Deborah'. Our aim was to understand if red individuals cope better with a chronic light stress compared to green ones.

3.3 Material and methods

In March 2016, one hundred and eight grafted plants of *Acer platanoides* 'Summershade', *A. platanoides* 'Deborah' and *A. platanoides* 'Crimson King' were potted in 50 l pots with a peat/pumice substrate (3:1, v:v), and were grown outdoors at the Fondazione Minoprio (Como, Italy, 45°44'N, 9°04'E). Weather conditions, over the experimental period, were recorded at the weather station near the experimental situ. The average maximum and minimum temperatures were 21.5 °C and 10.8°C during 2016, while 21.1°C and 9.4°C during 2017. Average annual rainfall for 2016 was 109 mm, while for 2017 was 107 mm. During the experiment mean daily global irradiance (W/m^2) 172 W/m^2 in 2016, while in 2017 201 W/m^2.

Control plants (CTRL plants) were grown on a black polypropylene fabric with an albedo of 9%, while high light plants (HL plants) were grown on concrete slabs with an albedo of 30%. The experiment lasted 16 months, from May 2016 to September 2017. Plants were kept well-watered throughout the experiment.

Paired spectroradiometers (LightScout® 3670I, Spectrum Technologies, Inc.) were used to measure irradiance and albedo throughout the experiment.

Biomass and leaf mass area were measured at the beginning of the experiment (day 0) and at the end of the experiment. Biomass was determined on 4 replicate plants per treatment. For biomass determination, plants were cut at the root flare and leaves, stem and roots were weighted separately. To determine dry weight, leaves, stem and roots were over dried at 70 °C until constant weight was reached (approximately 72 h). Leaf Mass per Area (LMA, g DW m^{-2}) was calculated as the ratio between leaf dry mass and leaf area.

3.3.1. Leaf gas exchange and chlorophyll fluorescence measurements

Measurements of net CO_2 assimilation rate (A or P_N) were taken on the first fully expanded leaf with an infrared gas analyser (Ciras-2, PP-System, Hertfordshire, UK) on three leaves per treatment, species, and replicate on May and July 2017.

The determination of the maximal quantum yield of PSII (Fv/Fm) was measured on May and July 2017 on the same leaf of leaf gas exchange by using a HandyPEA portable fluorescence spectrometer (Hansatech Instruments Ltd., King's Lynn, UK) and was obtained after adapting leaves to darkness for 40 min by attaching a light exclusion clips to the leaf surface and then using a

saturating flash of actinic light at 3000 μmol m^{-2} s^{-1}. Chlorophyll (Chl) fluorescence kinetics is an informative tool for studying the effects of different environmental stresses on photosynthesis (Clijsters & Van Assche, 1985; Allakhverdiev & Murata, 2004). It is one of the main methods to investigate the function of PSII and its reactions to changes in the environment and growth conditions (Bukhov & Carpentier, 2004). However, only few reports illustrate the effects of exposure to low or high light intensity on photosynthetic activity (Slatyer,1973) expressed as Chl *a*, fluorescence parameters and do not deal with energy flux and its destination within PSII (Kalaji et al., 2012).

The Chlorophyll meter (SPAD) is a portable and non-destructive tool that measures the greenness from Chlorophyll content in plant leaf (Kariya et al. 1982). It estimates chlorophyll (Chl) content by emitting two wavelengths of 650 nm (red) and 940 nm (far red) through the leaf. A SPAD value is calculated instantly from an optical density differential between the red and infrared wavelength detected by a photodiode. Leaf greenness index was determined both in the first (2016) and second (2017) growing season with a Minolta SPAD meter (Spectrum Technologies, Plainfield, IL, USA) on one leaf per cultivar per block. For each leaf, the SPAD value was obtained averaging three different measurements made in different points of the leaf blade. This parameter is a good indicator of leaf chlorophyll, N and carotenoid content (Percival et al., 2005)

FIGURE 1A. FIELD EXPERIMENT IN VERTEMATE CON MINOPRIO (CO): PLANTS WERE ARRANGED FOLLOWING A RANDOMIZED BLOCK DESIGN WITH 6 BLOCKS, EACH CONSISTING OF 3 THREE PLANTS PER CULTIVAR AND PER TREATMENT. FIGURE 1B. PAIRED SPECTOMETERS (LIGHTSCOUT® 3670I, SPECTRUM TECHNOLOGIES, INC). USED TO MEASURE IRRADIANCE AND ALBEDO IN THE EXPERIMENT.

3.3.2 Statistical analysis

Plants were arranged following a randomized block design with 6 blocks, each consisting of 3 three plants per cultivar and per treatment.

Measurements were conducted in control condition (CTRL) with polypropylene fabric and high light (HL) one imposed with white concrete slabs.

Leaf gas exchange data, fluorescence parameters, SPAD data and biomass determination were analysed using a two-way ANOVA, using SPSS 24 (SPSS, Chicago, IL).

Significant differences among means were estimated at the 5% ($P<0.05$) level, using Tukey's test.

3.4. Results

3.4.1 Climatic conditions

The average precipitation and temperatures during the experimental period are shown in Fig.2. Concerning the weather conditions, the two study years differed in the seasonal distribution and the total amount of rainfall and temperature fluctuations.

In 2016, from May to December, the average minimum and maximum temperatures were 10.9 °C and 21.1 °C, while in 2017, from January to September, were 9.4 °C and 21.1 °C.

Paired spectrometers were used to measure the incoming and reflected visible radiation. Fig 2. showed the difference between plants on polypropylene fabric (CTRL plants) and plants on concrete slabs (HL plants) (34%).
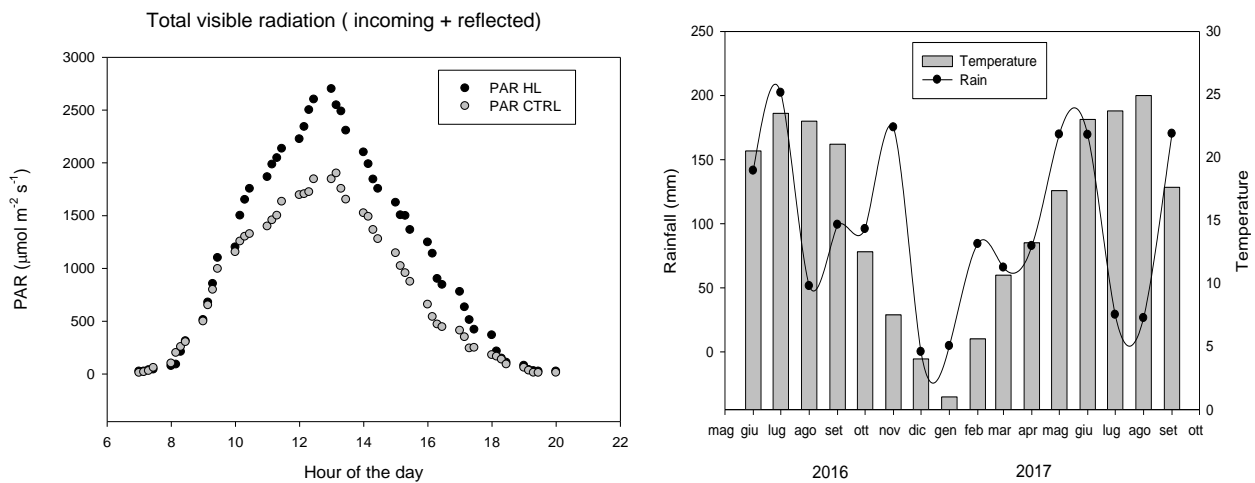
FIGURE 2A. TOTAL VISIBLE RADIATION MEASURED BY PAIRED SPECTROMETER DURING A DAY. PAR CTRL WAS MEASURED ON POLYPROPYLENE FABRIC WHERE CONTROL PLANTS WERE GROWN, WHILE PAR HL WAS MEASURED ON CONCRETE SLABS WHERE HIGH LIGHT PLANTS WERE GROWN. FIGURE 2B. MONTHLY TOTAL PRECIPITATION (MM) AND DAILY AVERAGE OF MAXIMUM AND MINIMUM AIR TEMPERATURE THROUGHOUT THE STUDY PERIOD (DATA OF METEOROLOGICAL STATION OF VERTEMATE CON MINOPRIO).

3.4.2. Gas exchange

From May to July 2017, in 'Crimson King' HL plants photosynthesis declined of -32% compared to ones of May. In 'Summershade' HL plants photosynthesis declined of -19% from May to July. During July, in 'Deborah' HL plants photosynthesis declined of -21 % compared to ones of May. In CTRL plants photosynthesis did not change significantly in all the three cultivars (Figure 3). However, both on May and on July, in 'Crimson King' HL plants photosynthesis had a higher value compared to 'Summershade' HL ones (+8% and +10%) (Figure 3).
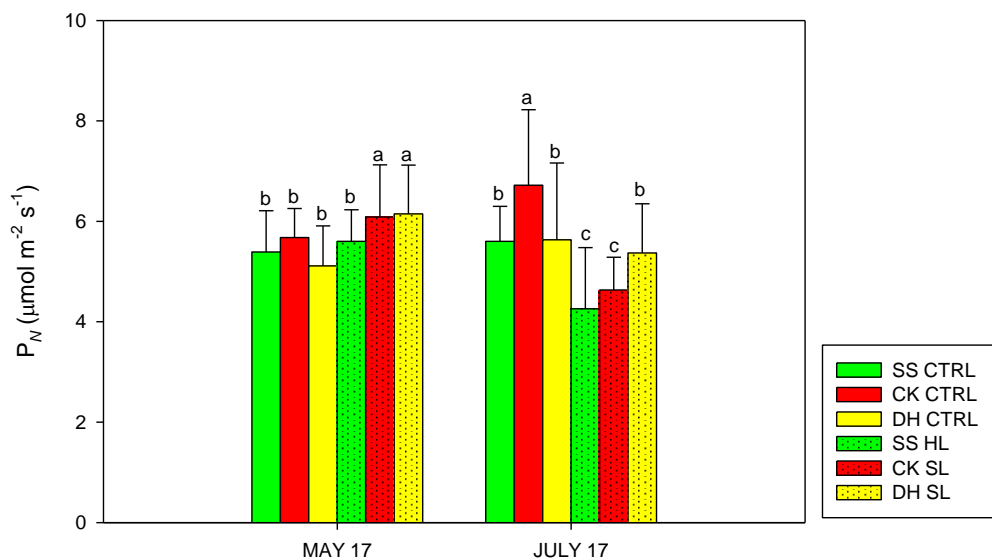

FIGURE 38. CO_2 ASSIMILATION (P_N) IN LEAVES OF *ACER PLATANOIDES* 'SUMMERSHADE', 'CRIMSON KING' AND 'DEBORAH' DURING THE EXPERIMENT. MEAN VALUES WITH THE SAME LETTERS ARE NOT SIGNIFICANTLY DIFFERENT (P≥0.05).

3.4.3. Chlrophyll fluorensce

From May to July 2017, F_v/F_m did not affect significantly in CTRL plants of all the three cultivars. During July, in 'Summershade' and 'Deborah' HL plants the values declined respectively of -5% and -2%. While, in 'Crimson King' HL plants, the value is significantly higher compared to one of May (+5%) (Figure 4).
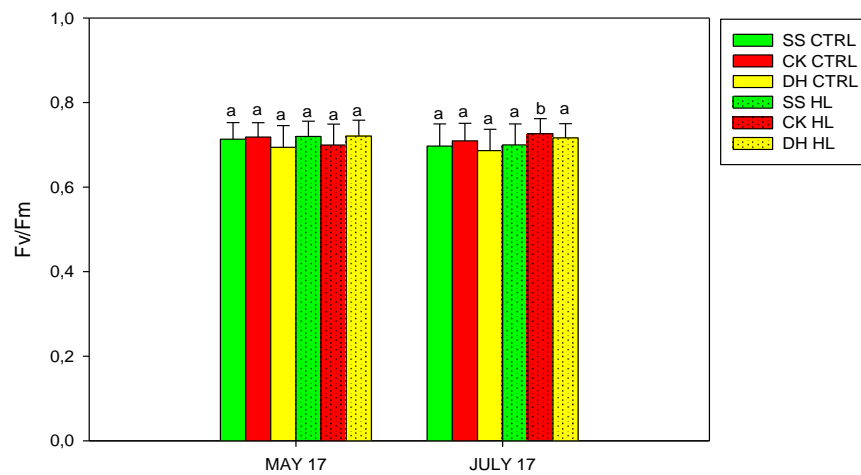

FIGURE 4. F_v/F_m IN LEAVES OF *ACER PLATANOIDES* 'SUMMERSHADE', 'CRIMSON KING' AND 'DEBORAH' DURING THE EXPERIMENT. MEAN VALUES WITH THE SAME LETTERS ARE NOT SIGNIFICANTLY DIFFERENT ($p≥0.05$).

3.4.4. SPAD

In 2016, 'Crimson King' both CTRL plants and HL ones had the highest values of SPAD compared to acyanic cultivars. In 2017, 'Crimson King' HL plants had a higher value (+29%) compared to 'Summershade' HL ones, while + 8% compared to 'Deborah' HL ones. The lowest data were found in 'Summershade' both CTRL and HL plants both in 2016 and in 2017.
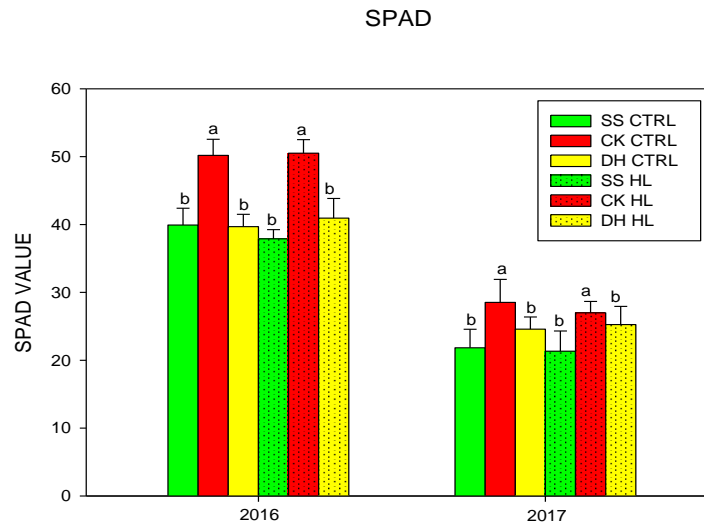

FIGURE 5. SPAD VALUES IN LEAVES OF *ACER PLATANOIDES* 'SUMMERSHADE', 'CRIMSON KING' AND 'DEBORAH' DURING THE EXPERIMENT. MEAN VALUES WITH THE SAME LETTERS ARE NOT SIGNIFICANTLY DIFFERENT (P≥0.05).

3.4.5. Biomass determination

At the beginning of the experiment in 2016, both 'Deborah' CTRL plants and HL ones had the highest plant biomass dry weight (Fig. 6A). In 2016, 'Deborah' CTRL plants had a higher value than both 'Summershade' and 'Crimson King' CTRL ones (+ 70% and +87%). 'Deborah' HL plants had a higher value than both 'Summershade' and 'Crimson King' HL ones (+80% and +128%). At the end of the experiment (2017), HL plants of 'Crimson King' had a minor plant-dry weight compared to HL ones of 'Deborah' (-26%) and 'Summershade' (-21%). However, in 2017, 'Crimson King' HL plants had a higher value compared to CTRL ones (+32%). Moreover, both at the beginning and at the end, in root to shoot ratio HL plants of 'Crimson King' displayed lower allocation to roots (Fig. 6B). Indeed, in 2016, HL plants of 'Crimson King' had a minor value compared to HL plants of 'Summershade' (-

43%) and 'Deborah' (-40%). Moreover, in 2017, 'Crimson King' HL plants showed a higher value compared to one of CTRL ones (+25%).
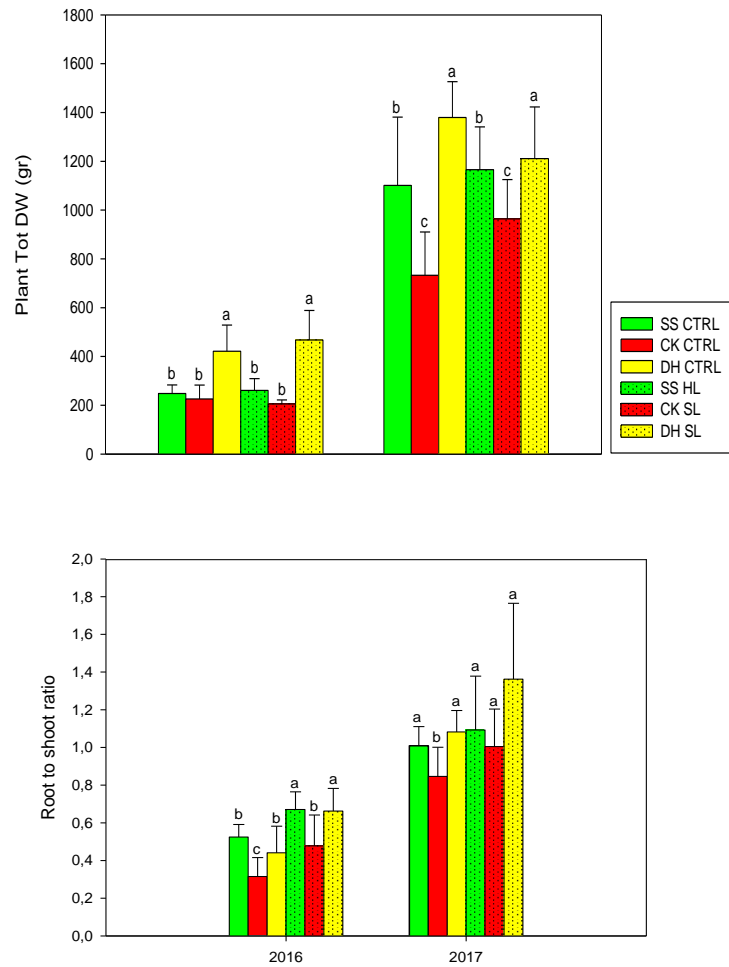
FIGURE 6A AND 6B9. PLANT TOTAL DRY-WEIGHT (GR) AND ROOT TO SHOOT RATIO OF *ACER PLATANOIDES* 'SUMMERSHADE', 'CRIMSON KING' AND 'DEBORAH' DURING THE EXPERIMENT. MEAN VALUES WITH THE SAME LETTERS ARE NOT SIGNIFICANTLY DIFFERENT (P≥0.05).

3.5. Discussion

In this experiment we described the response of three woody cultivars of *Acer platanoides* to a chronic light stress.

The data reported in this work may help to understand how plants are able to cope with excess light. Light is a very dynamic resource. Plants experience excess light during most of their life cycle and under a variety of stress conditions, which ranges in time span from seasons to seconds (Demmig-Adams et al., 2012).

Gas exchange parameters measured during the experiment, in May and July 2017, in leaves of *Acer platanoides* 'Summershade', 'Crimson King' and 'Deborah', showed that big differences existed among the three cultivars.

Generally, under standard light intensity most of the absorbed light can be used in photosynthesis (high photosynthetic efficiency), but under relatively high light intensity, only part of the absorbed light can be used (Long et al., 1994). Indeed, plants possess different mechanisms to cope with light stress and so the three cultivars, grown at different light intensities, showed changes of P_n during the experiment.

The data presented in this study clearly indicate that, under a chronic light stress, the red cultivar appeared an interesting opportunity because cyanic cultivar showed higher value of photosynthesis compared to acyanic cultivars, in particular 'Summershade' (Figure 3).

Chlorophyll fluorescence parameters that related to photosystem II antenna size confirmed that red leaves protected functionality of PSII. Indeed, cyanic cultivar had the highest value when high light is a chronic issue. This is probably due to anthocyanins that contribute protecting the long-term functionality of PSII, particularly when high light combines with heat (during July) (Figure 4). In fact, the decline of maximal quantum yield of PSII as F_v/F_m measured during July 2017 in acyanic leaves clearly indicated damage caused by light stress.

Both P_n and F_v/F_m showed clearly how anthocyanins protect leaves from a chronic high light stress thanks to the ability to adsorb the visible wavelengths of the solar spectrum.

Leaf greenness index was affected by light intensity (Figure 5). Regardless of the species, red cultivar showed higher values than the other cultivars. The lowest readings were found in 'Summershade' HL plants, whereas 'Deborah' HL plants performed intermediately (Figure 5). The higher SPAD-reading of cyanic cultivars may reflect a better nutritional status generated by light intensity.

Moreover, light intensity increased plant biomass dry weight, especially in red cultivars. In fact, cyanic cultivar grown on concrete slabs ('High Light treatment') was much higher than one grown on polypropylene fabric ('Control treatment') (Figure 6).

In root to shoot ratio, data confirmed that 'Crimson King' HL plants had a higher value than CTRL ones of the same cultivar. However, the acyanic cultivars had the highest values compared to cyanic ones and probably this due to anthocyanins that are costly to plants (Figure 6).

3.6. Conclusion

This study aimed to bring new knowledge about mechanism adopted by *Acer platanoides* cultivars to withstand high light stress, especially when it is a chronic issue, which are still largely unknown. Under high light, plants cope with excess light energy by means of different mechanisms of adaptation/acclimation which are mainly directed towards photosynthetic machinery defence.

Results of this study indicate that cyanic cultivars are suitable for sites with very high irradiance through the year because they are better equipped to cope with high light intensity for the presence of anthocyanins, even if they are costly for plants. In fact, they are effective screeners for excess visible light, partly shading the mesophyll beneath, and UV-screeners.

This is particular important for urban sites where *urban plaza* are characterized by reflected sunlight sum up to direct irradiance and this exposed trees experience high irradiance through the year (in urban plazas, irradiance often exceed 2000 mmol m^{-2} s^{-1}).

Our study opens to future experiments, in which the identification of individual anthocyanins, in addition to their location, becomes an objective of primary significance. Further analysis should be carried out to assess anatomical and biochemical plasticity, as well as long-term growth rate, in cyanic and acyanic cultivars to better understand the role of anthocyanins and how they protect plants. This may also help understand the extent to which anthocyanins affect light-induced activation of specific photoreceptors and downstream photomorphogenic responses.

This study presents opportunities for research that will expand our knowledge of how trees acclimate and adapt to their environment.

In conclusion, this study presents new insights on the physiology of three different cultivars of *Acer Platanoides* under high light stresses and should be helpful for choosing the right varieties in urban planning and maximise ecosystem services in the face of future climate change projections.

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General conclusion and future perspective

Because of climate change, drought and high light impose a major environmental threat for plants and their adverse impact has become an urgent problem for urban ecosystem. Some tree species have developed a suite of morpho-anatomical, physiological, and biochemical mechanisms to cope with the severe stress conditions imposed by drought and an excess light. These mechanisms include the ability to synthesise an extraordinary arsenal of secondary metabolites. In particular, some cultivars are generally very rich in polyphenols which, in addition to protect plants from the detrimental effects of solar short-wave radiation, may also counter the negative actions of reactive oxygen species on plant cell metabolism. They are anthocyanins which protect leaves from the stress of photo-inhibiting flows by absorbing excess photons that would otherwise be intercepted by chlorophyll. Furthermore, they serve as a useful optical filter, diverting the excess of high energy amount away from the already saturated photosynthetic electron transport chain. Anthocyanins reduce the oxidative load in a leaf by simply filtering the yellow-green light, since most of the reactive oxygen in plant cells comes from the excitation of chlorophyll. These compoundss are also excellent scavengers of free radicals for which the induction of leaf anthocyanins has been implicated in the acquisition of tolerance to many different types of environmental stressors.

In the present thesis, the objective was to characterize the mechanisms adopted by three cultivars of *Acer platanoides* ('Summershade', 'Crimson King', 'Deborah') under two types of high light stress: one is a sudden excess light and second is a chronic issue. The cultivars differ for the presence of anthocyanins: the first is constitutively green, the second is constitutively red and the third is temporarily (in the juvenile phase) red.

To this end, we carried two field study in which a combination of physiological parameters was observed at different timescales and related to variation in experimental conditions.

In our investigation, we found a wide diversity of physiological responses of the investigated cultivars. Particularly, cyanic cultivars are suitable for urban sites with very high irradiance through the year, for example *urban plazas* where reflected irradiance sum up to direct sunlight.

Moreover, cyanic cultivars are better equipped to cope with sudden, short drought spell, but this had little significance on this cultivar because wilting anticipates photoinhibition. However, anthocyanins are costly for leaves. Under optimal conditions the cultivars with green leaves provided higher benefits, in terms of CO_2 storage and transpirational cooling than cyanic cultivars.

In conclusion, our results largely confirm the central role of anthocyanins in the preservation of

photosynthetic apparatus during high light stress. Furthermore, Green cultivars are better under normal conditions. Consequently, should be helpful for choosing the right varieties in urban planning and maximise ecosystem services in the face of future climate change projections.

Acknowledgments

I would like to thank my PhD advisor, Prof. Francesco Ferrini for giving me the opportunity of carrying on this and many other researches during these three years at the University of Florence.

I am also very grateful to Dott. Massimiliano Tattini for his scientific advice and knowledge and many insightful discussions and suggestions.

I will forever be thankful to my colleague Dott. Alessio Fini for his dedicated help, advice and encouragement throughout my Ph.D. His enthusiasm and love for research is contagious!

I would like to acknowledge Dott. Jacopo Mori for their precious help during my PhD and for spending with me so many days in measuring plants.

I am also very grateful to all my colleagues and friends of the "85 office", especially Dott.ssa Antonella Gori and Dott.ssa Linda Salvi, thank you for listening and supporting me throughout these years.

Finally, I want to thank my family and all of my friends for supporting and encouraging me.