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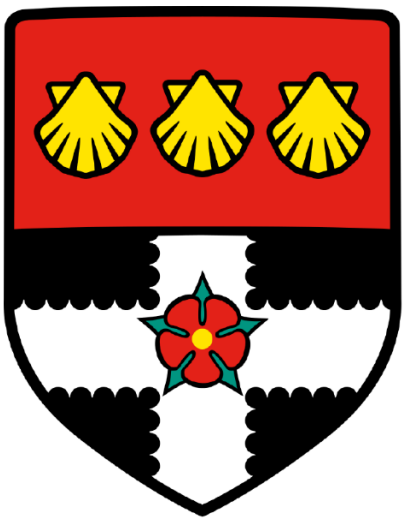

Identification of Drought Tolerant Amenity Trees

Jonathan Michael Banks

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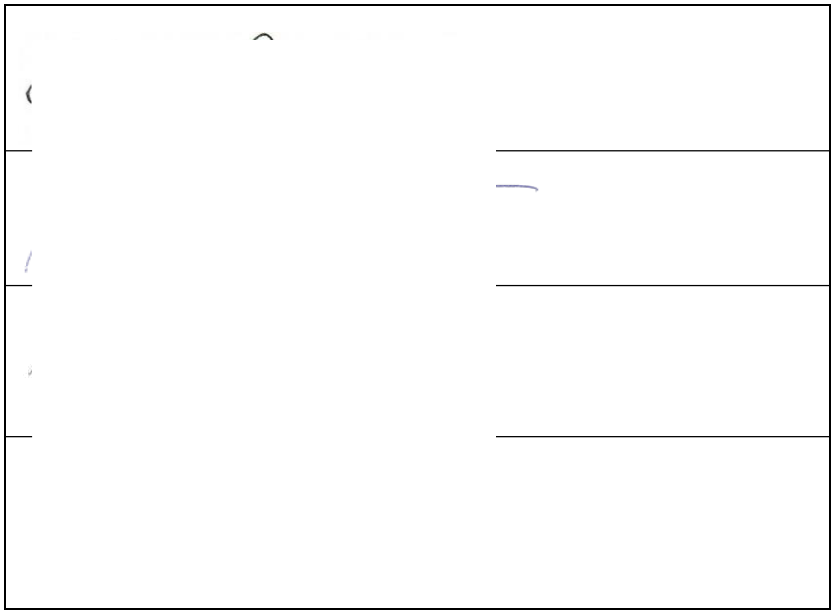
Declaration

I confirm that this is my own work and the use of all material from other sources has been fully acknowledged.

Jonathan Michael Banks

Co-authors Declaration of authorship

The authors listed below confirm that Jonathan Banks was primarily responsible for the design, analysis and writing of each paper with which they are listed as co-authors. Co-authors provided supervisory and editorial assistance to the relevant papers.

Author:	Paper(s):	Signature:
Glynn Percival	a, c	
Martin Lukac	a	
Gillian Rose	a, c	
Andrew Hirons	b	

a: Evaluating the Drought Tolerance of Cultivars Within Acer platanoides, campestre and pseudoplatanus.

b: Alternative Methods of Estimating the Water Potential at the Turgor Loss Point

c: Variations in Seasonal Drought Tolerance Rankings

Abstract

According to climatic models, future drought conditions are expected to increase in severity and frequency. Drought is often cited as the most severe stressor of urban trees. It decreases planting survival rates and increases tree stress, resulting in increased susceptibility to biotic pathogens and reduced lifespan. Selecting trees for increased drought tolerance is thus critical for improving their performance within the urban environment. Drought tolerance is a highly desirable trait, particularly in urban areas where limited rooting space, interception of precipitation and urban heat islands accentuate drought stress. Current information on drought tolerance is imprecise, highly variable and, in some cases conflicting, with some studies reporting a tree species to be drought tolerant whilst other studies contradict this. In addition, cultivar-level tolerance is frequently not reported.

The purpose of this thesis is to assess the current state of amenity tree selection and suggest how the process could be improved by utilising quantitative in vitro and in vivo trials to evaluate genotypic drought tolerance. The drought tolerance of species and cultivars within the same genus is frequently inferred to be very similar, however, this is known to be untrue. Detailed evaluations of drought tolerance are therefore required in order to cover a wide range of species and cultivars.

Eight cultivars from *Acer platanoides, A. campestre* and *A. pseudoplatanus* were used in this study. The photosynthetic response to drought and desiccation was evaluated using chlorophyll fluorescence. Continuous excitation chlorophyll fluorescence parameters F0, PIABS and FV/FM, together with additional less often measured parameters, 1-Vi, Vj, T fm, Sm, V0(Bo) and M0, were used to compare whole-tree response to drought, with foliar dehydration. Double-normalised differential kinetics (Vt, ΔVt, ΔW_{OJ} and ΔW_{OK}) were also utilised to evaluate the underlying reasons for parameter response. Whole-tree chlorophyll fluorescence response to drought was found to be similar to the response to foliage desiccation; however, some differences were observed in differential induction kinetics which require further study to elucidate. The parameters PIABS, Fo/Fm and V0(Bo) are recommended for identifying and monitoring drought stress.

In vitro methods (with potential to rapidly evaluate drought tolerance) were compared to whole tree methods. The methods tested include measurement of chlorophyll fluorescence during foliar dehydration and measurements of water potential at turgor loss point. The latter were undertaken using the classical, but time-consuming, pressure-volume curve method as well as more rapid, direct methods, calculating the turgor loss point from measurements of the water potential at full turgor via a previously devised regression equation. All methods were able to identify drought tolerance differences, with the exception of the pressure-volume curve method which was unable to identify

significant differences within species. Another advantage of rapid in vitro methods is that they facilitated the evaluation of drought tolerance variation by season. Relative tolerance ranking was shown to be consistent between summer and autumn but not spring.

This research demonstrates screening methods for tree drought tolerance which are especially relevant to tree selection in urban environments, where droughts occur during both spring and summer. There is considerable potential for tree selectors to save time and money if they have an increased ability to select trees that are fit for purpose. This research demonstrates that the estimation of water potential at turgor loss point, measured by consistent use of an osmometer or psychrometer, is sufficiently sensitive to correspond with findings from a controlled drought trial. Furthermore, this research also defines and identifies a number of reliable chlorophyll fluorescence parameters suitable for monitoring drought stress in vitro and in vivo.

Preface

This PhD is presented as a thesis by publication; some papers have been published, some submitted for publication, while others are not intended for publication. The format for the central chapters therefore is as journal articles prepared for submission. The status of each chapter (published/accepted/submitted/not intended for publication) at the time of thesis submission, is indicated at the start of each one. Each chapter carries an introduction which expands on its specific purpose. The following general introduction is focused on introducing the motives for undertaking this research as a whole, as well as describing the context and literature of drought tolerance and tree selection.

Acknowledgements

This PhD would not have been possible if it were not for the support and encouragement of my primary supervisor Dr. Glynn Percival, as well as the funding and employment provided by Bartlett Tree Experts during this project. I would also like to thank my supervisors Dr. Martin Lukac and Dr. Gillian Rose for their helpful comments on this manuscript. I would also like to thank Dr. John McKendrik, Nicholas Michael and Martin Reeves for time, effort and advice at the start of this project when I was learning how to use, problem-solve and fix HPLC machines and work out how to efficiently grind up leaves in their very clean analytical chemistry laboratory. Thanks, are also due to the editors and the anonymous reviewers involved in the published papers presented in this thesis and to Dr. Abdallah Oukarroum for advice and encouragement on the interpretation of V_{ip}, L and K band fluorescence.

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My family for their patience, tireless efforts and support especially for their hard work while I was struggling through the school system. My family-in-law for inspiring me with your faith, love and energy.

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1 General Introduction

1.1 Drought Stress and Urban Trees

Drought stress is one of the most common and significant disorders affecting not only crop production (Boyer 1982, Cattivelli et al. 2008, Farooq et al. 2012, Reis et al. 2014), but also tree establishment and growth, particularly in urban and amenity environments (Kaushal and Aussenac 1989, Aranda et al. 2012, Lopez et al. 2012). Drought stress occurs as a result of a low soil water content or availability in relation to the evapo-transpirative demands of the plant (Maccaferri et al. 2009). Drought stress has been shown to "affect almost every aspect of tree growth and development" (Hirons and Percival 2012 p58). Survival of newly planted urban trees is dependent on the availability of water and the ability of a genotype to survive with limited water supplies (Foster and Blaine 1978, Gilbertson and Bradshaw 1990, Ow et al. 2011). Drought resistance can display cross-resistance, where resistance to one stress can induce resistance to another (Taiz and Zeiger 1991). Because of this, the identification and categorisation of drought tolerant trees has potential to prove highly beneficial to those involved in urban tree selection, not only for drought management but also for wider putative abiotic stress tolerance.

Urban trees are particularly predisposed to significant stress factors (Gibbs and Palmer 1994, Fluckiger and Braun 1999), e.g. insufficient soil volume, density and texture, inappropriate pH, and low nutrient content (Jim 1998, Scharenbroch et al. 2017) (Figure 1.1). In addition, there is increased potential for pollutants such as hydrocarbons and de-icing salts to be introduced on the foliage or into the rooting zone (Zhiyanski et al. 2017, Ordóñez-Barona et al. 2018). Furthermore, trees in urban areas are more prone to drought, heat, shade and waterlogging stresses than their parkland, field or forest counterparts (Cregg 1995, Jim 1998, Sjöman and Nielsen 2010). These stresses can result in depressed growth rates, reduced vigour, induce premature decline and inflict additional burdens on tree management (Jim 1998, Vogt et al. 2015). While the focus of this research is drought stress, a single stress factor is rare in the urban environment. Therefore, awareness of the above-mentioned stresses, including synergistic effects, and their interactions with whole-tree stress tolerance is key to the effective management of healthy urban trees.

Wildly differing tree survival figures are reported in the literature (Roman and Scatena 2011). Roman and Scatena (2011) show an average of 63% survival after planting in urban landscapes (data adapted from 16 sources, time scale averages 16.4 years). Additionally, Hirons and Percival (2012), Foster and Blaine (1978) and McPherson and Kendall (2014) discuss survival rates of *ca.*30%. Complexities arise when using survival percentages, as the length of time statistics are collected for or inconsistency in

reporting can drastically change the resulting survival percentage. The commonly quoted life expectancy for street trees is ten years (Foster and Blaine 1978). Roman and Scatena (2011) however, suggest a revised life expectancy from nineteen to twenty-eight years. Genotypic variation however plays a crucial role (Boyer 1982, Sæbo et al. 2005); Table 1.1 demonstrates significant variation in genotypic survival rates.

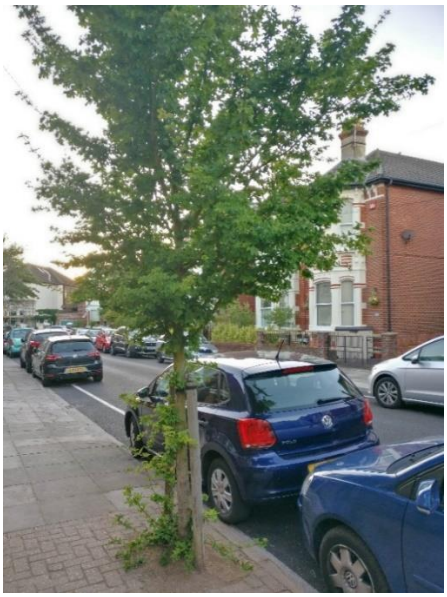

Figure 1.1 Left: Acer campestre tree planted in the urban landscape growing in limited soil volume, displaying nutrient deficiency symptoms and epicormic growth, these symptoms are evidence of the stressful growing environment. Right: Acer rubrum displaying peripheral foliar necrosis indicative of drought stress (photo courtesy of Bruce Fraedrich, Bartlett Tree Experts).

Table 1.1 Data adapted from Roman and Scatena (2011) estimating percentage losses across a ten year life expectancy for trees within a study from Cleveland OH, USA by Sydnor et al. (2010)*. Data derived from linear regression analysis estimating annual survival rates.*

Tree Species.	**Estimated % loss of trees after 10 years**
Acer pseudoplatanus L.	29
Acer rubrum L.	3
Gleditsia triacanthos	3
Liquidambar styraciflua L.	10
Crataegus phaenopyrum Borkh.	98

Many factors are involved in ensuring street tree survival, both prior to and after planting such as soil quality, texture, volume, plant quality, pre and post planting maintenance. Genus and species selection, as will later be discussed, is a highly influential factor. Despite this, in a quantitative analysis of 115 urban tree studies Roy et al. (2012) identified that only 12% of the studies assessed issues pertaining to tree selection. The purpose of this thesis is to contribute to broadening the knowledge

base available to those who select and plant trees in urban environments. This data is particularly important when selecting within a genus or species because these groups are currently often wrongly considered to be consistent in their drought tolerance (Sjöman et al. 2015).

1.2 Tree Selection

Amenity tree selection is performed by a wide spectrum of professionals, enthusiasts and amateurs with widely varying knowledge and experience. Urban tree selection, particularly street trees, is based upon existing local vegetation, size requirements, local context and personal preferences. Additionally, aesthetic traits such as crown architecture, leaf colour, shape, bark colour and texture, flowers, fruits and many others (Harris et al. 2004, Percival et al. 2006) have a significant influence on species choice (Percival and Hitchmough 1995, Pauleit et al. 2005, Vaz Monteiro et al. 2017). The complexity of tree selection criteria can be observed in Figure 1.2 (data property of Keith Sacre, Barcham Trees 2012). The conspicuous absence of drought tolerance within this graph is concerning; despite the wealth of information emphasising the benefits of selecting for abiotic stress tolerance (Pauleit et al. 2005, Sjöman and Nielsen 2010, Vaz Monteiro et al. 2017). Rahman et al. (2015) proposed that a lack of species specific physiological knowledge is inhibiting tree planting. A better understanding of drought tolerance is therefore expected to allow for more informed decisions leading to lower rates of tree death due to poor species selection.

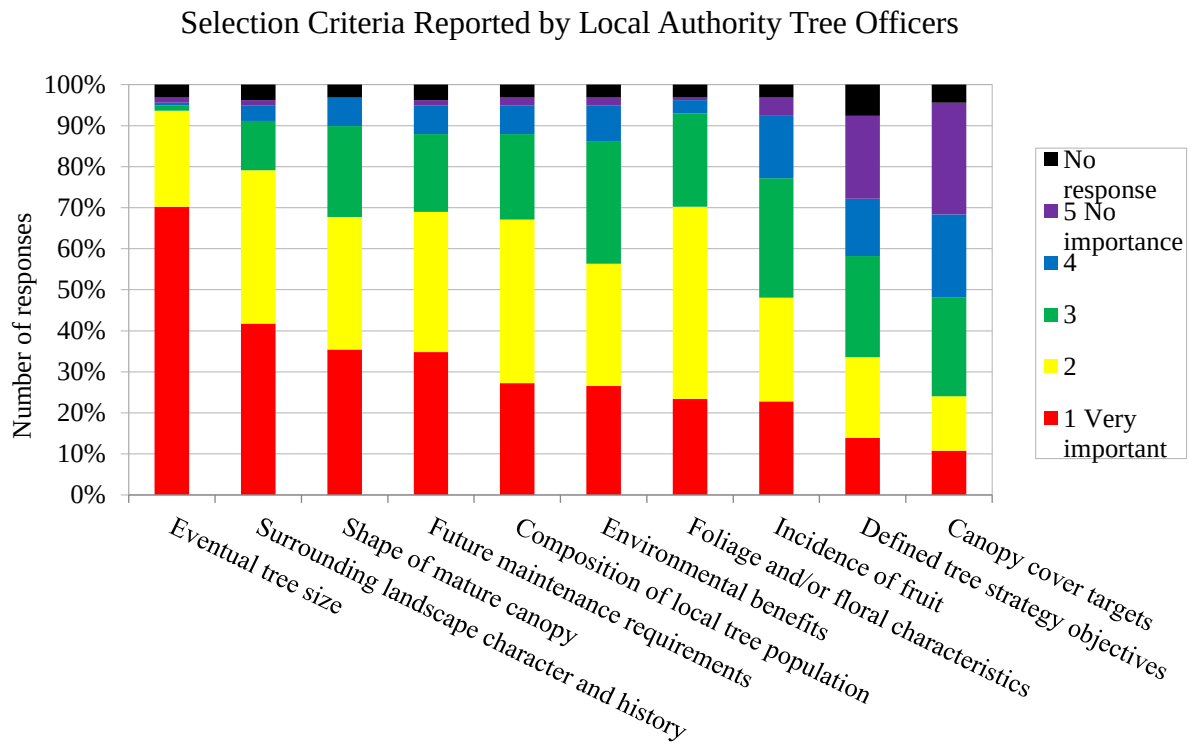

Figure 1.2 Responses of 158 Local Authority tree officers to the question "*Which of the following factors are considered when selecting the tree species to be planted?"* Data presented as percentage of responses. (Data adapted from Keith Sacre, Barcham Trees 2012).

Local borough councils in the UK are facing increasing budgetary cuts (Butler 2017), causing a reduction in the experience and skill levels of tree officers, a factor that is currently highly influential to appropriate tree selection (Hirons et al. 2017). Indeed, it is understood that in some cases the tree officer position is being merged with other positions within the council, leading to a loss of specialist skills and knowledge (David Lofthouse (London Borough of Merton) 2014, personal communication, 28th May). This is not only likely to lead to a reduction in the priority given to trees in the urban environment but a further reduction in appropriate selection of trees within this harsh urban environment.

Future climate models predict greater frequency and intensity of drought events (Allen et al. 2010, Schlaepfer et al. 2017). Additionally, the intensity and speed of drought initiation is predicted to intensify as a result of climate change (Trenberth et al. 2014). Significant predilection for drought tolerance during the tree selection process should therefore occur in all plantings, but particularly in urban areas where intensified climatic extremes occur (Levermore et al. 2018). Reduced mortality among street trees will putatively reduce required replanting and maintenance inputs (Percival et al. 2006), resulting in both monetary and environmental benefits. Increased preference for drought tolerance can be achieved in a number of ways: practical experience, selection for adaptive features and selection based on published tolerance lists. These three approaches will now be discussed.

Most tree officers use practical experience to facilitate appropriate tree selection (D. Lofthouse 2014, personal communication, 28th May); at present this is the most commonly used selection method and the most unreliable, because of inaccurate diagnosis and because of limited experience of genotypes. Occasionally, practical experience is evaluated using scientific methodology, such as work by Solfjeld and Hansen (2004) and Ow et al. (2011) however, these field-based studies generally focus on geographically localised areas and are resource and time consuming (Percival and Sheriffs 2002). Likewise, there is a danger that the information gained may become irrelevant by the time it reaches those involved in its practical application, because of factors such as climate change.

The adaptive features, such as leaf area, thickness, presence or absence of hairs and waxy surfaces are often thought to enable selection for drought tolerance (Blum 1996). However, this is not always the case (Teklehaimanot et al. 1998) and in most cases, little accurate information can be gleaned regarding comparisons between species from the same genera or cultivars. Additionally, information suggests that leaf thickness, leaf anatomical type and leaf area are less correlated with ecological conditions (irradiance and moisture supply) than structural parameters of the photosynthetic tissue (Ivanova 2014). This is also the case for tolerance to biotic organisms (Koch et al. 2016). Therefore,

reliance on leaf anatomical type alone is not a viable option to facilitate appropriate tree selection for drought tolerance.

Tolerance lists such as those of Niinemets and Valladares (2006) and organisations such as the Royal Horticultural Society (RHS) have been compiled in the past (Brickell 1989). These categorise a range of genotypes which are known to tolerate and/or thrive in a particular environment; examples include windbreak plants, coastal plants and plants for pH specific soils (Brickell 1989). However, little information is given as to the sources of this data. Data is often categorised too broadly (e.g. very tolerant, tolerant, not tolerant), preventing detailed comparisons between other species or other genera with similar tolerance types. Sjöman et al. (2015) review a range of tree selection resources and demonstrate significant differences between the reported drought tolerance, for a range of *Acer* species (Table 1.2). Inconsistencies identified within the literature highlight the current difficulty of species selection for the urban environment.

Table 1.2 Inconsistencies in reported drought tolerance for a range of Acer species. Data adapted from Sjöman et al. (2015 p860)

Genotype	Reported Tolerance	Reference
Acer nigrum	Sensitive to heat and drought	(Hightshoe 1988)
	Heat and drought tolerant	(Dirr 2009)
Acer platanoides	Has moderate drought tolerance	(Gilman 1997)
	Drought tolerant	(Stoecklein 2001)
	Resists drought	(Beaulieu 2003)
	High demands of moist and cool growing conditions	(Almgren et al. 2003)
Acer pseudoplatanus	Dislikes excessive moisture and dryness	(Krüssmann 1986)
	Tolerates occasional periods of dry soil	(Bassuk et al. 2009)
	Very adaptable to soil types	(Dirr 2009)

The *Acer* genus was selected as a model genus. The *Acer* genus consists of 129 species of evergreen and deciduous trees and shrubs from Europe, North Africa, Asia and North and Central America (Shu 2008). Harris et al. (2004), Bell et al. (2005) and García et al. (2006) refer to the need of urban trees that provide colour, form, texture and pattern in the landscape (Figure 1.3). Species found within the *Acer* genus provide all these benefits, possessing good autumn colour, interesting and frequently-coloured bark and leaves, as well as provision of shade. The influence of the wide diversity within the *Acer* genus on drought tolerance remains little known. Although, recent work by Sjöman et al. (2015) has elucidated the relative drought tolerance of 27 *Acer* genotypes, encompassing a wide range of

species from around the world. The present study focuses on species which are currently widely available within the UK; as such, data is immediately applicable to those involved in selection within the UK, and other countries using a similar range of *Acers*.

Figure 1.3 Photo collage to show the diversity within Acer foliage colour and texture. Left to right: Acer palmatum, A. platanoides 'Drummondii', A. platanoides 'Spaethii', A. campestre 'Louisa Red Shine'.

1.3 What is Drought Tolerance?

Drought "tolerance" is widely discussed with reference to avoidance and escape strategies as well as to tolerance itself. Escape strategies (ephemeral plants) involve the rapid completion of a plant's life cycle before reproduction-limiting dehydration or desiccation occurs (Bayoumi et al. 2008). This strategy however only occurs in annual herbaceous plants (Kooyers 2015). Drought avoiding plants utilise methods of conserving or capturing water such as: waxy cuticles, water-containing stems, hairy leaves and stems, foliage shedding or investment into extensive root systems (Tyree et al. 1993, Augé et al. 2003, Mauseth 2011, Hirons and Thomas 2018). Extensive root growth and adaptive root morphology is arguably one of the most effective methods of drought avoidance (Manschadi et al. 2008); however, suitable water supply must be present to facilitate survival long enough for a plant to grow an extensive root system (Sharp and Davies 1985, Palta et al. 2007). Not all studies agree however, that a larger root system will provide drought tolerance; a balance point is reached, at which, lowered water-use efficiency (ratio of carbon gain to water loss) and the greater root : shoot ratio reduce any benefits attained from a larger root system (Palta et al. 2011). Additionally in urban areas, soil depth limitations, root pruning practices and human intolerance to leaf detachment reduce the efficacy of drought avoidance and escape strategies in urban plantings and thus the suitability of certain species (Sjöman et al. 2015). The final strategy is true drought tolerance. Tolerant plants employ physiological strategies to tolerate drought periods while maintaining function (McKeown and Summers 2010). A truly drought tolerant plant is able to maintain near-optimal growth during a period of drought (Mart et al. 2016). In response to drought a tolerant plant will adjust gene expression, mobilise or synthesise metabolites such as abscisic acid (ABA) or adjust cellular osmotic potential in order to survive a drought period (Umezawa et al. 2006). These strategies however, simply allow plants to be categorised according to survival/response mechanism and are unlikely to describe all

genotypes in all situations. For example, rapidly induced stress may cause a plant to exhibit a drought avoidance response e.g. to drop leaves quickly, even if in normal circumstances its strategy would be to tolerate the stress; in this example, near zero xylem hydraulic conductivity is likely to have caused complete defoliation, rather than a stage-by-stage tolerance response (Brodribb and Cochard 2009).

1.4 Plant Responses to Drought

Drought is a highly complex stress influenced by a range of differing factors. Alcohols, sugars, proline, glycine, betaine, catalase, dehydrins, transcription factors, protein kinases and phosphatases are all known to be synthesised during drought stress (Akinci and Lösel 2012, Boaretto et al. 2014, Yoshida et al. 2014). The typical understanding of plant drought response involves stomatal closure, in response to *de novo* synthesis of ABA. Many researchers discuss however that ABA is produced in the roots and transported in the xylem stream as a signalling molecule translating soil moisture status to the stomata (Wilkinson and Davies 2002, Cameron et al. 2008). ABA is in fact synthesized in almost all chloroplast containing cells (Taiz and Zeiger 2015). More recently, whole leaf and guard cell synthesised ABA has also been discovered to play an important role regulating stomatal conductance, especially in response to rapid drought stress (Brodribb and McAdam 2013) or high vapour pressure deficits (Wilkinson and Davies 2002, Bauer et al. 2013) independent of xylem ABA flux (Georgopoulou and Milborrow 2012). At the mechanistic level it is widely accepted that ABA-induced stomatal closure involves a decrease in potassium (K), via K_{out} channels, within guard cells, which flank the stoma, in turn, increasing guard cell osmotic potential and as a result of subsequent water-loss through osmosis lowering guard cell turgor, leading to stomatal closure (Wilkinson and Davies 2002, Kim et al. 2007). Cellulose microfibrils also play an important structural role in the regulation of guard cell expansion/contraction characteristics. Horizontal cellulose microfibrils prevent significant widening of guard cells, in addition to microfibrils, attachments at either end prevent increases in length, resulting in the bowing observed when stoma are open (Rui and Anderson 2016). Stomatal sensitivity is highly dependent on both exogenous and endogenous factors. Substrate potassium availability can have a significant influence on stomatal closure (Arquero et al. 2006). Calcium (Ca^{2+}) can also alter guard cell conductance and ABA sensitivity, however, this is dependent on the prior conditions of guard cells (Allen et al. 2002, Zhu et al. 2012). Indeed complexities within stomatal physiology have led investigators to state that it is "one of the most complex issues in plant physiology" (Cochard et al. 1996 p198) which has not been fully resolved to-date (Papacek et al. 2017). Extensive hormonal cross-talk (ABA, cytokinin, ethylene and auxin) has only recently begun to be elucidated (Rowe et al. 2016).

Stomatal closure acts to slow the transport and uptake of water. Drought is known to induce stomatal closure to a greater extent than can be attributed to root-sourced ABA alone, additional factors and non-ABA processes are therefore involved during periods of drought (Wilkinson and Davies 2002).

Leaf level ABA redistribution acts through alterations in pH gradients moving ABA from mesophyll chloroplasts into the apoplast (Taiz and Zeiger 1991, Wilkinson and Davies 2002). ABA may also inhibit root K uptake, increasing guard cell ABA sensitivity (Roberts and Snowman 2000, Wilkinson and Davies 2002). Ca^{2+} influx, prompted by reactive oxygen species (ROS) and mediated by Ca^{2+} channels, promotes stomatal closure and limits opening (Zhu et al. 2012). Evidence also exists suggesting that cytokinins and auxins inhibit ABA-induced stomatal closure through modulation of ethylene synthesis (Tanaka et al. 2006), also known to inhibit ABA-induced stomatal closure (Tanaka et al. 2005, 2006). ABA is also only partly responsible for induction of stress-responsive genes. The ABA-independent dehydration-responsive element-binding proteins (DREBs) are important transcription factors that regulate stress inducible genes (Yoshida et al. 2014). Their expression is also induced by high salinity and heat shock (Reis et al. 2014). Transgenic over production of DREBs has resulted in significantly improved drought tolerance in sugarcane, maize, rice, tobacco, wheat and barley (Reis et al. 2014). Group II late embryogenesis abundant (LEA) proteins such as dehydrins (DHN) also play an important role accumulating in response to stressors which induce dehydration (drought, salinity, cold, heat etc.) (Hanin et al. 2011). Dehydrins stabilize hydrophobic domains of other proteins susceptible to dehydration (Ntuli 2012), acting to stabilize cellular and intracellular membranes (Hincha and Thalhammer 2012). As the name suggests, LEA proteins were originally discovered in developing seeds; however, they have since been discovered to accumulate in vegetative plant tissues (Hincha and Thalhammer 2012). Some potential exists for DHN to also confer pathogen resistance, however further work is necessary to elucidate this (Hanin et al. 2011).

Stomatal response strategies are not consistent between species. Two strategies are widely discussed, isohydric and anisohydric. Isohydric species maintain stable control over leaf water potential by reducing stomatal conductance during drought periods (Attia et al. 2015). Anisohydric species maintain stomatal conductance while leaf water potential is allowed to fluctuate (Nolan et al. 2017). Several compromises are, however, inherent in each strategy. Isohydric species are at risk of increased rates of photorespiration and carbohydrate depletion (Nolan et al. 2017) followed by increased susceptibility to biotic agents caused by a cascade of downstream effects (McDowell et al. 2008). Whereas, anisohydric species are at risk of xylem cavitation and concomitant loss of hydraulic conductivity (Nolan et al. 2017). Anisohydric species tend to tolerate better embolisms in comparison to isohydric species; however, they also have reduced quantities of stored carbon within the sapwood (Saiki et al. 2017) and may recover from drought stress more slowly (Attia et al. 2015). The isohydric species *Quercus petraea* (Martínez-Sancho et al. 2017) however, has been found to regulate stomatal aperture and thus sap flow in order to balance embolism vulnerability (Cochard et al. 1996). The strategies are therefore not binary but are a continuum from anisohydric to isohydric (Meinzer et al.

2014). Species variability has also been reported for hybrid poplar (Attia et al. 2015). No single strategy is widely accepted as resulting in superior tolerance (Sherrard and Maherali 2006). Important external factors, such as the speed and longevity of drought stress and internal, genotypic factors such as tolerance to oxidative stress, embolisms and ability to store and metabolise carbon, play important roles in the relative success of isohydric and anisohydric strategies. Stomata are important early indicators of abiotic stress; however, because of intra-specific response intricacies, characterisation of stomatal response strategies alone is not a viable selection criterion for drought tolerance.

Stomatal closure reduces water loss but causes a concomitant decrease in CO_2 influx, limiting photosynthesis and carbon fixation (Farooq et al. 2012). In C3 plants, photorespiration occurs when ribulose 1,5-bisphosphate (RuBP) carboxylase adds oxygen rather than CO_2 in the Calvin–Benson cycle, which occurs as a result of stomatal closure (Mauseth 2011). Photorespiration can theoretically reduce photosynthesis by 48%; however, other factors such as the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), temperature and CO_2 levels can significantly influence rates of photorespiration (Walker et al. 2016). Photorespiration also depletes nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP) (Walker et al. 2016). Reactive oxygen species (ROS) or reactive oxygen intermediates such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide radical (O_2^-) and hydroxyl radical (HO^-) are produced as a result of photorespiration (Cruz De Carvalho 2008). When ROS accumulation occurs at damaging levels they must be detoxified by antioxidants (Abdallah et al. 2017) to prevent lipid peroxidation (Choudhury et al. 2017). Catalase, superoxide dismutase (SOD) and ascorbate peroxidase (APX) are the major ROS scavenger enzymes (Mittler 2002, Cruz De Carvalho 2008). SOD is often the first ROS scavenger, detoxifying superoxide radical into water and H_2O_2 which then also needs to be detoxified (Mittler 2002, Cruz De Carvalho 2008). H_2O_2 is converted into water and oxygen by catalase exclusively in peroxisomes (Chelikani et al. 2004) to prevent damage. However, many other antioxidants also detoxify H_2O_2 (Mittler 2002). Even in normal growing conditions O_2^- and H_2O_2 are synthesised at high rates, suggesting they are not exclusively a negative by-product (Larkindale and Knight 2002, Cruz De Carvalho 2008). Many ROS including H_20_2 are in fact used as a signalling molecule altering drought resistance genes, antioxidant and metabolic activity (Niu and Liao 2016). They also facilitate biotic stress defence mechanisms (Cruz De Carvalho 2008) and programmed cell death (Mittler 2017). Both O_2^- and H_2O_2 are also thought to be synthesised to prevent formation of the hydroxyl radical (Mittler 2002), the most potent antioxidant which can affect protein synthesis and create lesions in DNA (Bohnert and Jensen 1996). The signalling hormone ABA also plays an interactive role with ROS, modulating acclimation to abiotic stress and oxidative damage (Wang et al. 2015, Choudhury et al. 2017). ROS can influence the speed and efficacy of drought response (Mittler et al. 2011), in turn

influencing a genotypes drought tolerance; they represent an interesting and exciting target for future study; however, ROS are beyond the scope of this study.

1.4.1 Mechanisms of plant death

Long-term water stress has been widely hypothesized as leading to carbon starvation (McDowell et al. 2008), resulting in exhausted carbon stored in sapwood (Saiki et al. 2017). However, many complexities surround the carbon starvation hypothesis (McDowell and Sevanto 2010), notably that not all carbohydrates can be used or metabolised equally during periods of drought (Hartmann 2015). Indeed, phloem transport is a related cause of carbon starvation (Sevanto 2014). Technical difficulties are also prevalent when determining carbohydrate concentrations (Quentin et al. 2015). An additional cause of mortality is the loss of hydraulic conductivity (xylem embolism) (Saiki et al. 2017). Loss of hydraulic conductivity has been found to occur without carbon starvation in tropical rainforest (Rowland et al. 2015). McDowell et al. (2008) suggest that the speed of drought imposition and duration of stress dictates whether a plant will succumb to either carbon starvation or loss of hydraulic conductivity, or indeed both mortality mechanisms, as observed by Sevanto et al. (2014). Biotic agents can also amplify, or be amplified by the mortality process (Figure 1.4) (McDowell et al. 2008).

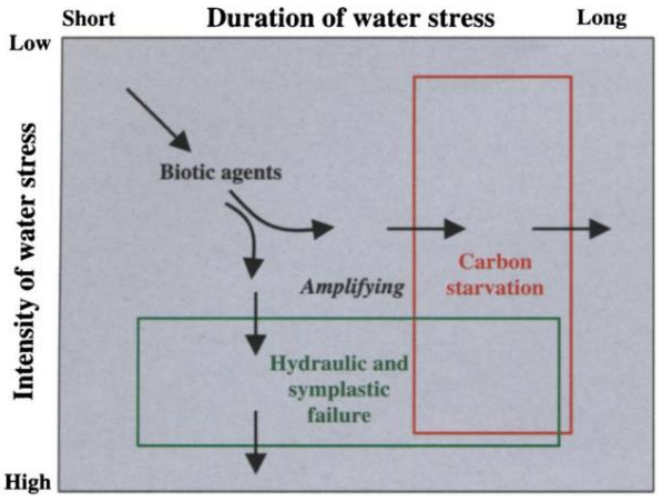

Figure 1.4 Schematic adapted from McDowell et al. (2008). Describing the theoretical relationship between duration of water stress, the relative intensity of imposition and the mechanisms underlying mortality.

Non-structural carbohydrates, including soluble sugars are also involved in stomatal closure and guard cell sensitivity to ABA (Wilkinson and Davies 2002, Kelly et al. 2013) and have been seen to be highly influential in the drought survival of tropical tree seedlings (O'Brien et al. 2014). Non-structural carbohydrates have also been linked to the repair processes of xylem embolisms through aquaporin regulation and creation of osmotic gradients within embolised conduits (Salleo et al. 2009, McDowell 2011). Exogenous sugar applications have been shown to provide protective properties to the photosynthetic apparatus (Sulmon et al. 2004, 2007), as well as acting to mitigate damage with respect

to hypoxic and anoxic root systems (Huang 1997, Vartapetian and Jackson 1997), replant stress (Percival and Fraser 2005), heat tolerance (Robertson et al. 1994), salt tolerance (Al-Habsi and Percival 2006) and also to increase nitrogen fixation (Jones 1974, Owen 1980). However, Koch (1996) discusses how gene responses to changing carbohydrate status can vary markedly. Therefore, a link between a plant's survival of adverse conditions and the ability of a plant to acquire carbohydrates may exist; however, specific circumstances (e.g. species, carbohydrate availability, temperatures and rate of desiccation) need to be taken into account. This potential variation can be seen when comparing the response to exogenous sugar applications; Capellades et al. (1991) found a decrease in photosynthetic rates occurs, whereas Martinez-Trinidad et al. (2009) show no photosynthetic inhibition and Furbank et al. (1997) show significant increases in photosynthetic rates.

This introduction aims to present the need for an empirical drought tolerance ranking system. It does not attempt to provide an exhaustive physiological account of the drought response processes; reviews such as McDowell et al. (2008), Akinci and Lösel (2012), Aranda et al. (2012), Farooq et al. (2012) provide further detailed information on this subject.

1.4.2 Drought Mitigation

Many researchers point out that plants cannot simply relocate themselves (Klee 2008, Haswell and Verslues 2015), in order to ensure survival and are therefore required to tolerate or avoid drought. Mitigation of drought stress can be achieved by plant species selection or management of current plant stock. As discussed, drought mitigation by plant selection is a result of the plant's dynamic internal and external processes mediated by endogenous systems which prevent lethal damage (Ramanjulu and Bartels 2002). Management strategies designed to combat drought most frequently involve irrigation; however, an application of landscape mulch is also an effective drought mitigation strategy (Chalker-Scott 2007, Percival et al. 2009) although Gilman and Grabosky (2004) dispute this for mulches placed over tree root balls. Techniques of irrigation adaptation such as regulated deficit irrigation and partial root drying have also been shown to increase drought resistance (Cameron et al. 2008). The application of plant protection products such as anti-transparents have also shown promise in reducing plant water loss (Cochran et al. 2013), as reduced foliar water loss putatively increases drought tolerance.

1.4.3 Drought vs desiccation

A number of authors have raised fundamental differences between drought and desiccation tolerance. Desiccation is the loss of free or bulk protoplasmic water, leaving only water bound within the cellular matric (Ramanjulu and Bartels 2002). As Ntuli (2012) and Alpert (2005) explain, desiccation tolerance exists when a plant can withstand drying to equilibration with the air, more severe damage

is therefore caused when the air is dry (<50% RH). Drought tolerance "is survival of low environmental water availability while maintaining high internal water content" (Ntuli 2012 p29). A drought-tolerant plant may therefore, not be tolerant of desiccation. Dehydration is a less severe form of drying than desiccation. Dehydration tolerance may be more similar to drought tolerance than desiccation tolerance. The process of foliar dehydration is also less likely to counteract many drought tolerance adaptations (e.g. presence of hairs, foliar ABA redistribution, and structural foliage adaptations) and it may therefore be possible to consider them viable indicators of drought tolerance. Results of studies investigating leaf level dehydration have shown promise (Smillie and Hetherington 1983, Percival and Sheriffs 2002, Augé et al. 2003), and suggest that leaf dehydration correlates with whole plant drought tolerance (Percival and Sheriffs 2002). Ögren (1990) suggests a mechanistic difference between drought and rapid dehydration in willow leaves, but were unable to identify the mechanism within the scope of their study. Augé et al. (2003) point out that research, such as their own and those mentioned previously, misrepresents species which rely on dehydration avoidance strategies, such as species which send roots further and deeper or those with sensitive stomata. These species may be more able to retain water (dehydration avoidance) but incapable of enduring dehydration (dehydration tolerance). In vitro dehydration experiments are therefore expected to elucidate dehydration-tolerant species but not species which employ some avoidance strategies, such as leaf shedding or deep rooting strategies. In vitro dehydration experiments may therefore be of benefit when selecting for urban plantings in root limited environments.

1.4.4 Rehydration and recovery

For a plant to recover following drought, rehydration and in some cases xylem structural repair must take place. Xylem cavitation occurs when negative xylem pressure results in air being introduced through the pores of the pit membrane (Venturas et al. 2015) and causes a concomitant reduction in water transport and carbon assimilation (De Baerdemaeker et al. 2017). The extent of damage is dependent on the severity of stress and vulnerability to damage, which is species specific. Some species limit drought-induced cavitation damage by actively altering water-use efficiency (ratio of carbon gain to water loss) through leaf abscission (Tyree et al. 1993), while others mobilize non-structural carbohydrates in order to lower vulnerability (De Baerdemaeker et al. 2017). Even in drought tolerant species, reinstatement or repair of the water column is essential following severe drought and is usually achieved by two mechanisms; refilling of embolized conduits and/or building-up new conduits (Vilagrosa et al. 2013). Building new conduits by seasonal replacement occurs in almost all plant species (Vilagrosa et al. 2013). When xylem is near atmospheric pressure, refilling of embolisms is achieved by spontaneous bubble dissolution (Klein et al. 2018), enabled by positive root pressure and/or capillary action following the cessation of drought stress (Alpert 2005). Root pressure

or capillary action are not, however, considered to be influential beyond three meters high and so this method of embolism repair is not thought to occur at greater heights (Alpert 2005). Active refilling must therefore occur at negative xylem pressures (relative to atmospheric pressure) and at heights greater than three metres. Great controversy, however, exists in the current understanding surrounding active refilling of embolised conduits. Most sources are in agreement that osmotic gradients are used to decrease the osmotic potential of an embolized conduit, in combination with local phloem unloading to parenchyma cells and ABA-induced upregulation of aquaporins and metabolization of carbohydrate and starch (Vilagrosa et al. 2013, Sperry and Love 2015, Klein et al. 2018). Rapid rehydration may occur as a result of significant osmotic adjustment, possibly leading to cell damage or rupture if cell rigidity or rehydration is not appropriately controlled (Patakas et al. 2002, Hessini et al. 2009). Therefore, cell wall thickening and changes in cellular elastic modulus (Bartlett, Scoffoni, and Sack 2012) are implemented during drought exposure to prevent drought-induced and rehydration-induced structural damage (John et al. 2018). A genotype's capacity to recover is an important factor determining survival, and has been associated with the drought tolerance of 89 species (John et al. 2018). However, selection based on recovery characteristics alone is unlikely to be broadly beneficial for selection criteria in urban areas, as the leaf losses and aesthetic damage associated with some recovery strategies are likely to be problematic.

1.5 Tolerance categorisation

Since the development of agricultural and horticultural breeding, selection for specific traits is pervasive. In the amenity sector however, tree selection is primarily based on aesthetic qualities (Percival and Hitchmough 1995, Percival et al. 2006, Vaz Monteiro et al. 2017); few studies have assessed the impact of this aesthetic bias against stress tolerance (Percival et al. 2006). As Percival and Sheriffs (2002 p219) discuss the "selection of robust trees and shrubs that will survive into maturity" will reduce "replacement costs and give permanence to the landscape".

A number of experiments have assessed abiotic stress tolerance in vitro, based on excised leaves (Smillie and Hetherington 1983, Percival and Sheriffs 2002, Percival et al. 2003). Percival and Sheriffs (2002) and Faraloni et al. (2011) compared the impact of dehydration in vitro with drought in vivo, and their results identified a connection with leaf dehydration tolerance and whole-plant drought tolerance, following chlorophyll fluorescence measurements. Rong- Hua et al. (2006) suggest that classical methods of photosynthetic evaluation based on CO_2 and water exchange are not sufficient to determine the effect of water stress on photosynthesis without ambiguity. Percival and Sheriffs (2002) and Faraloni et al. (2011) identified chlorophyll fluorescence as a viable tool to rapidly screen and categorise species for drought tolerance. The advantages of this in vitro chlorophyll fluorescence method are two-fold, speed of categorisation (24 hours vs 70 days), coupled with the costs associated

with purchasing plant stock in order to carry out drought experiments. However, the disadvantages may also be potentially significant, as whole plant mechanisms such as rooting depth and structure, aquaporin density (Chaumont and Tyerman 2014), carbohydrate metabolism, and the presence and capacity of water storage organs are not accounted for in experiments which only utilise leaf chlorophyll fluorescence data. Despite these significant potential issues relating to the use of chlorophyll fluorescence, Percival and Sheriffs (2002) discuss its suitability with reference to wider studies assessing freezing, chilling, and heat stress tolerance as indicated by chlorophyll fluorescence measurements on excised leaves (Smillie and Hetherington 1983, Brennan and Jefferies 1990, Yamada et al. 1996, Hakam et al. 2000). Additional work successfully assessing drought tolerance using chlorophyll fluorescence data has been conducted using the excised leaves of olive (Faraloni et al. 2011) and barley (Rong- Hua et al. 2006).

Alternative, foliar based, drought tolerance identification measurements have been utilised more recently, the most prevalent of which are measurements of leaf water potential at the turgor loss point (π_{tlp}) (Sack et al. 2003, Bartlett, Scoffoni, and Sack 2012). This measurement was originally derived from pressure-volume (P-V) curves which take significant time and attention to adequately produce; it is now, however, possible to calculate π_{tlp} from the osmotic potential at full turgor (π_0) (Bartlett, Scoffoni, Ardy, et al. 2012), a rapidly determinable parameter when measured using osmometry. Estimating π_{tlp} by osmometry avoids the necessity for practitioners to visually determine the point at which the exponential portion of a P-V curve becomes linear, discussed critically by Jane and Green (1983). From a practical perspective, both methods have elements of potential inaccuracy; however, the osmometry approach reduces inconsistency in the estimation by use of a regression calculation which remains consistent between measurements. Benchmarks have recently been published using this technique within a wide range of genotypes, including wheat (Mart et al. 2016), *Acer* (Sjöman et al. 2015), *Magnolia* (Sjöman et al. 2018a) and an additional 45 tree species (Sjöman et al. 2018b).

Some caveats are important with all measurements of drought tolerance. As with almost any biological measurement, drought tolerance will vary within a genotypic range. Provenance (Teklehaimanot et al. 1998, Bauerle et al. 2003) and drought priming (Wang et al. 2015) are known to cause significant drought tolerance variation. Provenance is even known to alter foliar anthocyanin content (Chalker-Scott 2002). Plasticity in π_{tlp} is thought to be reduced following successive drought events (Bartlett et al. 2014). The degree of potential tolerance variation has, however, not been fully elucidated. It is therefore difficult to ascertain from evaluations of one population if measurements are truly reflective of the potential variation and range of drought tolerance of the genotype. Ascertaining the relevance of any drought tolerance ranking between different geographic regions

could therefore be considered problematic. Drought exposure history should therefore be maintained as a consistent factor within experimental plant material, resulting in consistent tolerance rankings between genotypes, and restoring the validity of measurement. This is true as long as relative tolerance rather than numeric values are considered, i.e. one genotype compared against another and rankings cross-calibrated against species-in-common.

The aim of this research is to:

1. Identify and quantify the drought tolerance within a range of *Acer* (maple) species and cultivars.
2. Evaluate a range of empirical in vitro categorisation methods to allow comparison within and between *Acer* species.
3. Quantify the level of drought tolerance to facilitate future comparison with other taxa.
4. Identify drought variation within and between species.

1.6 Conclusion

Plant responses to drought and drought tolerance strategies are well described; however, the tolerance implications of strategies and interrelations between them are poorly understood. Therefore, knowledge of tolerance strategies or morphological adaptations will not necessarily help to identify drought tolerant genotypes (Teklehaimanot et al. 1998). In urban areas it is widely assumed that drought tolerant genotypes will have superior health to their drought sensitive counterparts (Pauleit et al. 2005, Percival et al. 2006). Trees in good health are better able to provide greater benefits, especially important in urban areas (Nowak et al. 2002). These benefits include mitigation of the urban heat island effect, reduced storm water runoff, particulate filtration and carbon sequestration (Rogers et al. 2015). Drought and the intensity of drought is predicted to increase as a result of climate change (Allen et al. 2010, Trenberth et al. 2014, Schlaepfer et al. 2017). Use of an empirical drought tolerance benchmark therefore facilitates tree selectors in choosing species with drought tolerance, in drought-prone environments. However, several of the current resources tree selectors utilise are in disagreement with one another (Sjöman et al. 2015), and therefore a repeatable, defendable, tolerance measurement, which enables comparison against species-in-common, is superior to arbitrary tolerance categories such as “very tolerant”, “somewhat tolerant”, “sensitive”.

1.7 Reference List

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2 Continuous Excitation Chlorophyll Fluorescence Parameters: a review for practitioners

Jonathan Michael Banks[1, 2, 3]

[1] The University of Reading, School of Agriculture Policy and Development, Reading, Berkshire, RG6 6AR UK; [2] Bartlett Tree Experts, Research Laboratory (UK) Reading, Berkshire, RG2 9AF, UK; [3] Corresponding author:

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Abstract: This review introduces, defines and critically reviews a number of chlorophyll fluorescence parameters with specific reference to those derived from continuous excitation chlorophyll fluorescence. A number of common issues and criticisms are addressed. The parameters fluorescence origin (F_0) and the performance indexes (PI) are discussed as examples. This review attempts to unify definitions for the wide range of parameters available for measuring plant vitality, facilitating their calculation and use.

Keywords: chlorophyll fluorescence, photosynthesis, fluorescence origin, performance index, continuous excitation, fluorescence induction kinetics.

2.1 Introduction

Chlorophyll *a* fluorescence (CF) is one of three fates of light, following interception by a leaf (and other photosynthetic organs). Alternative fates of light following interception are dissipation (i.e. heat or non-photochemical quenching) and photosynthesis (photochemistry) (Roháček and Barták 1999, Maxwell and Johnson 2000). Importantly, these fates are linked; a change in one results in a change in the another two (Maxwell and Johnson 2000); it is this phenomenon which allows measurements of CF to be particularly useful to practitioners. Chlorophyll fluorescence measurements provide information on the status and function of photosystem II (PSII) reaction centres and antenna on the donor (P680) and acceptor (pheophytin) sides (Kalaji et al. 2016). While this information may seem highly specialist it has been used to measure and in some cases categorise a range of stresses impacting the photosynthetic processes (Mohammed et al. 2003). It has been used extensively to identify stress and stress responses in plants (Baker and Rosenqvist 2004), algae (Kodru et al. 2015) and other photosynthesising organs (DeEll and Toivonen 2003). A number of researchers have

identified highly suitable CF measurement parameters for specific plant stresses. However, as suggested by Lazár (2015) the high number of parameters available from CF is likely to confuse researchers. The inconsistency and haphazard method by which some parameters are defined and referred to within the literature provides further difficulties for researchers (Baker and Rosenqvist 2004). Parameters are often disconnected from their calculable foundations, in some cases making interpretation difficult and/or preventing the utilisation of these parameters. The aim of this paper is to define and outline CF parameters used within a wide range of studies; however, first we must introduce the physiological basis behind the measurement itself.

Chlorophyll *a* fluorescence is a form of photoprotective energy dissipation, when a very small amount of excess light energy (usually about 1-2% of total absorbed light), absorbed by chlorophyll molecules, is reemitted as light (Maxwell and Johnson 2000). The widely discussed and generally accepted physiological basis of chlorophyll *a* fluorescence is known as the Q_A model (Schansker et al. 2014). The Q_A model explains the fluorescence rise from minimum (F_0) to maximum (F_M) fluorescence, occurring within 200-300ms following dark to light exposure of photosynthetic material. Chlorophyll fluorescence was originally discovered visually by Kautsky & Hirsch (1931) and is now widely described as the Kautsky effect. However, it was not until Duysens & Sweers (1963) that the Q_A model was first proposed. The fluoresence rise from F_0 to F_M indicates the reduction of the PSII downstream acceptor quencher, plastoquinone, primarily Q_A (Stirbet and Govindjee 2011, Schansker et al. 2014). Q_A is unable to accept more than one electron from PSII therefore it must first pass on the electron to the subsequent two electron carrier Q_B (Figure 2.1). The resulting delay causes the reaction centres to be in what is known as a "closed state" reducing the efficiency of photochemistry and subsequently increasing photoprotective energy dissipation, resulting in both an increase in fluorescence yield and non-photochemical dissipation (i.e. heat) (Maxwell and Johnson 2000). Importantly the rate and relative magnitude of the fluorescence yield increase is reflective of PSII efficiency. The Q_A model makes a number of assumptions: 1.) that the PSII unit is homogeneous, 2.) that the physiological state remains the same during the measurement from F_0 to F_M (rate constant) and 3.) that the rise from F_0 to F_M reflects the reduction of Q_A (to Q_A^-); i.e. at F_M all Q_A molecules are completely reduced (Stirbet and Govindjee 2011, Schansker et al. 2014). Additionally during measurements made at room temperature the contribution of photosystem I (PSI) is considered to be constant and very low (Stirbet and Govindjee 2011).

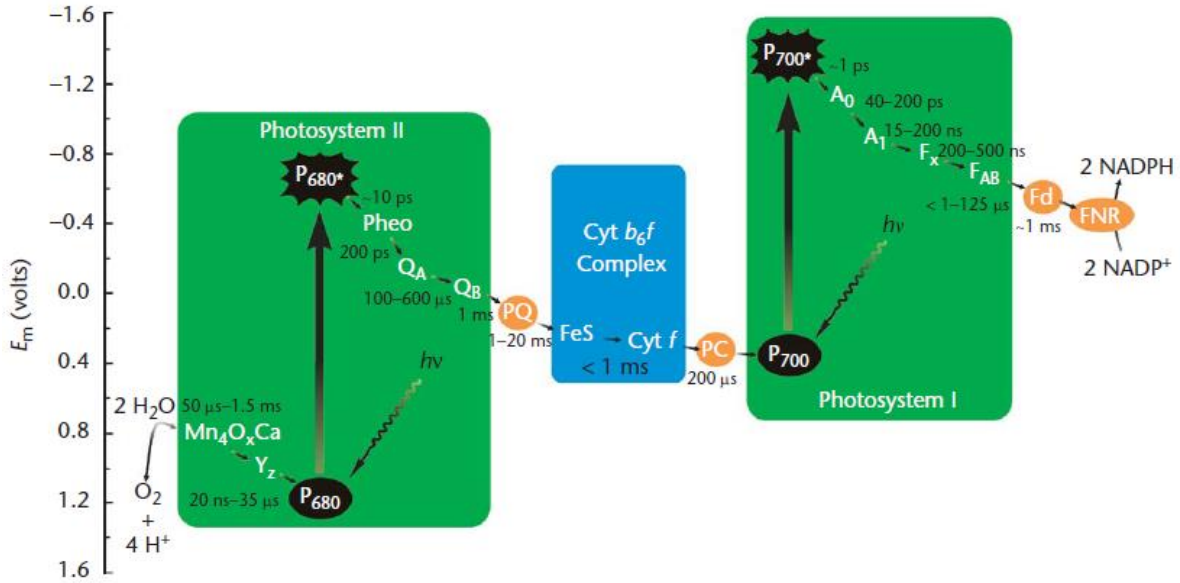

Figure 2.1 Z scheme showing the energetics of oxygenic photosynthetic electron transport from (Govindjee et al. 2010)

2.2 Intermediate steps: OJIPSMT

When viewed on a logarithmic scale a fast polyphasic response (widely known as OJIP, Figure 2.2) is observed, with intermediary steps labelled F_J and F_I. The F_0 to F_J stage is known as the photochemical phase (or single turnover phase (Strasser et al. 2004, Bussotti et al. 2011)) and is influenced by the intensity of the exiting light; F_J to F_I and F_I to F_P are known as the thermal phases (Stirbet and Govindjee 2011, Schansker et al. 2014). The Q_A model, although the "*most credible interpretation*" (Stirbet & Govindjee 2012 p47) does not fully elucidate the cause of these intermediary steps in the fast fluorescence rise, multiple interpretations exist (see table 1 in Stirbet & Govindjee (2012) and Schansker et al. (2014)). Other processes have been proposed during the fast induction kinetics besides Q_A quenching (see Stirbet & Govindjee (2011) for more information) however currently it is still accepted generally that Q_A photoreduction is the primary cause of fluorescence yield change from F_0 to F_M. The thermal phase (JIP) is known to be influenced in some part by PSI activity (Ceppi et al. 2012), however, this contribution is ignored when measuring at room temperature and at wavelengths below 700nm (Murchie and Lawson 2013). Despite this Oukarroum et al. (2009) determines that relative loss of the I-P phase (ΔV_{IP}) is reflective of PSI in varieties of drought stressed barley and chickpea.

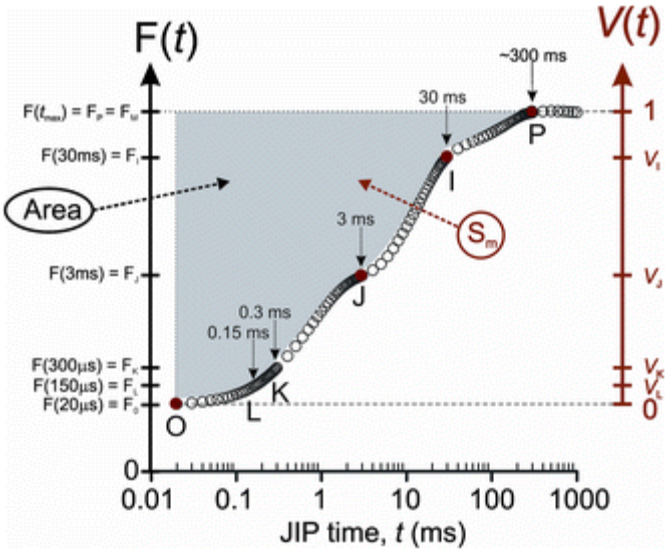

Figure 2.2 typical O(L)(K)JIP transient adapted from Kalaji et al. (2014) *On the left hand y axis, the unnormalized F scale associated with the complementary "Area" and on the right y axis, the V scale double normalized between O and P associated with the normalized area Sm*

Following the rapid OJIP rise, the fluoresence level begins to reduce (decay) across the course of a number of minutes. This is called SMT; where: S = Semi-steady state, M = maximum, and T = terminal steady state. This slow phase is termed fluorescence quenching and is thought to be caused by two quenching mechanisms (Stirbet and Govindjee 2011). First, photochemical quenching is achieved by an increase in the efficiency of electron transportation away from PSII due to light induced activation of a number of enzymes involved in carbon metabolism and stomatal opening. Second, none-photochemical quenching, where energy is converted to heat (Maxwell and Johnson 2000).

Two main types of chlorophyll fluorimeters are available, pulse amplitude modulated (PAM) and continuous excitation fluorimeters (CEF). This paper will focus on CEF which may represent greater suitability for use by practitioners owing to their relative lower cost, speed of sample throughput and simplicity of use. PAM fluorimeters do however have the potential to provide specialists with more information pertinent to those involved in physiological research. Other measurements of chlorophyll fluorescence such as passive, laser-induced, solar induced, multi-signal and delayed fluorescence are not covered in any detail in this review, although some overlap in parameters and calculation may exist.

PAM fluorimeters are perhaps the most widely used fluorimeters within research studies (Roháček and Barták 1999, Lazár 2015); they are also the subject of a number of excellent reviews for practitioners (Maxwell and Johnson 2000, Baker and Rosenqvist 2004) and will therefore only be introduced briefly here. PAM fluorimeters will in most cases measure the fast (up to 1s) chlorophyll fluorescence induction kinetics (FIKs), slow fluorescence decrease and steady state fluorescence; additionally, most instruments are capable of measuring dark and light adapted samples (Roháček and

Barták 1999, Lazár 2015). PAM fluorimeters get their name from the pulse of light which comprises one of the three sources of light used during the PAM measurements. These are, far-red, continuous and pulse light. Far-red light is used to oxidize the electron transport chain (ETC) (Lazár 2015). Actinic and saturating light (this can be white, red or blue light) is used to drive photosynthesis, whereas the pulse light is used to measure photosynthesis (Lazár 2015). "Amplitude" comes from the method the PAM fluorimeter uses to derive the output, which is the difference between the signal during and after the pulse light (Lazár 2015). This, importantly allows PAM fluorimeters to measure under natural lighting conditions. Many of what might be considered the fundamental parameters of PAM fluorometry also overlap with CEF.

Continuous excitation fluorimeters measure ultra-fast high resolution FIKs that occur in less than one second (Kalaji et al. 2016). Techniques have been applied to take advantage of the high data acquisition capabilities of these devices, thereby expanding the range, complexity and potential suitability and specificity of subsequent parameters. The JIP-test (Strasser et al. 2000) is perhaps the most widely cited within research studies. Additionally, there are lesser utilised calculations such as the estimation of Q_B and non-Q_B-reducing reaction centres by using the "double tap" method (Strasser et al. 2004, Mathur et al. 2011) or the V_{oj300} parameter used to indicate a "K" peak, commonly associated with heat stress (Desotgiu et al. 2012). It is important to note the huge step forward in the suitability of this method which occurred following the development of JIP-test parameters; reviews such as Maxwell & Johnson (2000) occurred prior to this and references to continuous excitation measurements are now out of date. It is however important to be aware that CEF are currently unable to provide parameters capable of measuring under background illumination; resulting in an inability to provide measurements of photochemical and non-photochemical quenching (Maxwell and Johnson 2000). Additionally, some parameters, claiming to provide detailed information of PSII activity, antenna size and electron transport have gained criticism (Murchie and Lawson 2013). Despite the criticism Murchie & Lawson (2013) acknowledge that the technique is highly valuable for crop phenotyping and stress detection. With correct data normalisation and, if necessary, corroboration of data with supplimentary methods of vitality assessment, highly robust measurements can still be attained. Additional empirical support is however welcomed to facilitate further meaningful interpretation of some parameters. However, clear parameter definition to a foundational fluorescence basis is essential in order to facilitate additional research.

It is common for practitioners to attempt to use CF measurements to suggest specific impacts on the whole organism and put measurements into context. This is commonly desired in research studies where PAM fluorimeters are commonplace. The PAM parameters, photochemical efficiency (F_q'/F_M' =

(F_M'-F')/F_M) and electron transport (ϕPSII x irradiance) have been found to correlate with the rate of $C0_2$ assimilation in controlled conditions (Maxwell and Johnson 2000, Govindjee 2004, Murchie and Lawson 2013). However, in field conditions (in C_3 plants) this is unlikely to be the case as the multitude of potential stressors (primarily: photoinhibition, stomatal closure, temperature, and the Mehler reaction) will alter this relationship (Maxwell and Johnson 2000, Murchie and Lawson 2013). Although CF is a valuable tool, it cannot currently replace direct measurements of photosynthetic rates if these measurements are required. It may be necessary to connect measurements of CF with additional measurements in order to build a whole picture of the photosynthetic system. This is therefore commonly undertaken in order to further elucidate the extent and type of damage or adaptations within the photosynthetic system (Percival and Sheriffs 2002, Smethurst and Shabala 2003, Guo et al. 2005, Murchie and Lawson 2013). Recent developments in infra-red gas analysers (IRGA) have included the capacity to take readings of CF in parallel with gas exchange measurements for this reason (Li-COR 2017).

The widespread, almost ubiquitous inclusion of CF measurements into screening and physiological studies has led many authors to point out the complex theoretical background to this practically simple technique. Chlorophyll fluorescence is typically reviewed from a biophysicist's or molecular plant physiologist's point of view compounding difficulties interpreting data (Maxwell and Johnson 2000). It is for this reason that information sharing and clear explanation is critical to facilitate understanding among researchers and practitioners.

2.3 Foundational parameters

The foundational basis for all FIK parameters (utilising a single flash [often 1 second], most common among studies) involves four steps: 0, J, I and P. These can also be described in a time format: 0µs 2ms (J), 30ms (I) and max (M, or P is used if full saturation is not achieved). "F" precedes letters or time points to denote fluorescence stages. Additional parameters used by the JIP-test includes 150µs (L step), 300µs (K step), 60ms, time to maximal fluorescence (Tfm, also widely called Tf(max)) and the area above the fluorescence curve (Area or A_{MAX}) (Strasser et al. 2000). However, 150µs and 60ms are not used in any parameter subsequent to Strasser et al. (2000) (see Strasser et al. (2004)). One additional parameter which is worth including here because of its pervasive nature is variable fluorescence or F_V ($F_v = F_M$-F_0). OJIP transients are occasionally referred to, including the L and K steps also (OLKJIP) however L and K peaks have been shown to only respond to specific stressors (Oukarroum, El Madidi, et al. 2016).

Table 2.1 showing basic extracted and technical parameters from which all JIP-test parameters are devised (Srivastava et al. 1997, Strasser et al. 2000, 2004, Hansatech Instruments 2006)

Parameter	Synonym	Description
F_0	$F_{50\mu s}$, $F_{\hat{0}}$, ≈ABS/CS_0	F_0 is the level of fluorescence emission when all the primary quinone acceptors (Q_A) are in the oxidized or open state. Current Hansatech devices extrapolate from points 16-4 to point zero alternatively 50µ seconds is used (Hansatech Instruments 2006, Lazár 2006). (≈ Minimum absorption flux). "An increase in Fo has been attributed to the physical separation of the PS II reaction centers from their associated pigment antennae resulting in blocked energy transfer to PS II traps" (Srivastava et al. 1997 p97)
F_L	F2, $F_{100\mu s}$, $F_{150\mu s}$	Position of L step (either 100µs or 150µs are used) (Oukarroum et al. 2007).
F_K	F3, K peak, $F_{300\mu s}$	Position of K peak (300µs). This peak occurs in response to heat stress.
F_J	F4, F_{2ms}	Position of J step (2ms).
F_i	F5, F_{30ms}	Fluorescence intensity of I step (at 30ms).
F_m	≈ ABS/CS_M	Maximal fluorescence, where all reaction centres are closed. (≈ Maximum absorption flux)
F_p	Peak fluorescence	F_P is used in place of F_M to differentiate when light levels are not at fully saturating intensities. This value is more commonly used with older devices that are not capable of reaching Fm.
F_v	F_M-F_0	Variable fluorescence (F_M-F_0).
Tfm	T_{Fmax}	Time to maximal fluorescence.
Area	A_{MAX}	Area above the fluorescence curve.

Unfortunately, many differing descriptors exist to identify a number of commonly used parameters, all of which are fundamentally based on those listed above. It is for this reason that clarity is required, authors should be mindful to cite the fundamental bases of parameters in order to facilitate an understanding of those replicating or comparing articles. In most cases the literature does not clarify if differing descriptors serve a particular function. A lack of clarity here results in potential uncertainty within research studies. Numerous, apparently identical descriptors are encountered with the parameters F_V/F_M, M_0 and 1-V_J see Table 2.2.

Table 2.2 Clarification of parameters with many synonyms

Parameter	Synonyms	Source
F_V/F_M	φPo, TRo/ABS, ΦPSII/qP, J_0^{TR}/J^{ABS} 1-F_0/F_M, (ϕ_{Fm}-ϕ_{F0})/ϕ_{Fm},	(Maxwell and Johnson 2000, Rosenqvist and Kooten 2003, Hansatech Instruments 2006, Stirbet and Govindjee 2011)
M_0	dV/dt_0, $(\Delta V/\Delta t)_0$ and $V_{300\mu s}/250\mu s$	(Tsimilli-Michael et al. 2000, Strasser et al. 2004)
1-V_J	J-Phase, ETo/TRO, Ψ_{ET20} and Ψ_{EO}	(Stirbet and Govindjee 2011, Desotgiu et al. 2012)

2.3.1 Fluorescence Origin

Differing maximum fluorescence intensities are distinguished in the form of saturating maximum (F_M) and non-saturating maximum/peak fluorescence (F_p); however, no distinguishing notation exists to identify if F_0 is calculated using a regression model to predict so called true F_0, or if 50µs is used (Hansatech Instruments 2006). F_0 and $F_{50\mu s}$ are the most commonly used F_0 time points; however, Srivastava et al. (1997) use 40µs, Kalaji et al. (2016) uses 30µs and Stirbet & Govindjee (2011) uses 20µs as F_0. Additionally other authors do not define which F_0 is used (Mathur et al. 2011). One potential reason for this apparent oversight is that early fluorimeters such as the original Plant Efficiency Analyser (PEA), launched in 1989, were electronically incapable of accurately estimating F_0 and therefore $F_{50\mu s}$ was used as a standard reliable "close-to-zero" point. Owing to rapid electronic developments since 1989 it may be generally accepted that true F_0 can be reliably estimated (Richard D. Poole 2016, pers. comm. 29th April). Knowing which F_0 is being used is important when calculating almost all parameters but of great importance when dealing with parameters such as M_0 ($\equiv(dV/dt)_0 \approx (\Delta V/\Delta t)_0 = V_{300\mu s}/250\mu s$) (See Equation 2.2 for calculation) (Tsimilli-Michael et al. 2000, Strasser et al. 2004), or calculating parameters dependent on M_0 such as: N, ABS/RC, RC/CS_0, RC/CS_M and PI, to name but a few. The severity of the problem is because M_0 is in part derived using a function calculating the slope between "F_0" and 300µs. In the cases of estimated true F_0, $3.\dot{3}$ should be used; however, if using $F_{50\mu s}$, 4 should be used (see Equation 2.1). Errors of this type can cause up to 20% variation in PI_{ABS}. For clarity and repeatability we suggest that F_0 or $F_{\hat{0}}$ are appropriate to identify only estimated F_0. $F_{50\mu s}$ should be used when 50µs is used.

$$\frac{T1000\mu s}{T300\mu s - TF_{0(\mu s)}} = x$$

Equation 2.1 Initial calculation used in the calculation for M_0. Where $TF_{0(\mu s)}$ is the time at F_0 in µs. Adapted from Strasser et al. (2004) *and Metrope Tsimilli-Michael (2016, pers. comm. 29th April).*

$$dV/dt_0 = M_0 = x \times \frac{F_k - F_0}{Fm - F_0}$$

Equation 2.2 M_0 calculation adapted from Tsimilli-Michael et al. (2000); Živčák et al. (2008); Gravano et al. (2004); Strasser et al. (2004) Strasser et al. (2000) *(F_K = F300µs).*

Conversely to the above equations Strasser et al. (1995) defines M_0 as $(F_{300\mu s} - F_{50\mu s})/(F_M\text{-}F_{40\mu s})$ and Srivastava et al. (1997) define M_0 as $(F_{150\mu s} - F_{50\mu s})/(F_M\text{-}F_{50\mu s})$ both leaving out x and varying the equation above slightly. Krüger et al. (1997) does not define the M_0 calculation in foundational terms. It appears that at some point between Strasser et al. (1995) and Strasser et al. (2000) $x \times$ was included in the calculation for M_0.

The importance of appropriate parameter definition and citing the source of origin is therefore clear when using CF for research studies and screening trials. For this reason a number of the most widely utilised parameters will be defined, providing synonyms, calculation, a brief description and source where possible.

2.4 Performance index

Many differing forms of calculation are cited for the chlorophyll fluorescence parameter known as the performance index (PI). Performance index is multi-parametric as well as multi-typal, PI can be expressed as either: total (PI_{tot}), cross section (PI_{CS0} or $_{CSM}$) or absorbance (PI_{ABS}) basis. However, no information currently exists to explain which expression is most appropriate for differing situations. In fact, a number of papers do not define which PI is being used (see Clark et al. 2000). PI_{ABS} is the most frequently utilised form of the parameter and shall therefore be discussed here. PI_{ABS} also forms the foundational basis of all PI parameters where: PI_{CS0} and PI_{CSM} includes the multiplication of F_0 and F_M respectively, (ABS/CSm is often substituted in this circumstance to denote estimated absorption flux), PI_{tot} includes $\delta_{R0}/(1\text{-}\delta_{R0})$ (δ_{R0} may also be referred to as δ_{RE10}) δ_{R0} is equal to $(1\text{-}V_I)/(1\text{-}V_J)$ (Stirbet and Govindjee 2011).

Table 2.3 Performance index forms and calculation. F_x may equal either F_0 or F_M. (Strasser et al. 2004, Stirbet and Govindjee 2011)

Parameter	Calculation
PI_{ABS}	$\frac{RC}{ABS} \times \frac{\varphi_{po}}{1-\varphi_{po}} \times \frac{\psi_0}{1-\psi_0}$
PI_{CSx}	$PI_{ABS} \times F_x$
PI_{TOT}	$PI_{ABS} \times \frac{(1-V_I)}{(1-V_J)}$

PI_{ABS} is a complex parameter derived in analogy to the Nernst equation of redox potential (Oukarroum et al. 2007). PI_{ABS} is often referred to as burgeoning from three independent parameters encompassing the active reaction centres per absorption (RC/ABS), the yield of primary photochemistry (φ_{PO}) and the efficiency of electron movement into the electron transport chain (ψ_0) (Appenroth et al. 2001). In simplistic terms the parameter is all encompassing, identifying perturbations within the fast FIK. It

identifies alterations in FIK with and importantly between F_0 and F_M and therefore provides more information than parameters containing only combinations of F_0 and F_M. Explanations of performance index are highly varied and often only referred to briefly and in some cases using only parameter which have not been defined (Desotgiu et al. 2012). Although no calculable error exists in the equations below it is clear a unified approach is needed to prevent convoluted, disconnected and none-specific explanations. Varying example descriptions are shown below.

$PI_{ABS} = \frac{RC}{ABS} \times \frac{\varphi_{po}}{1-\varphi_{po}} \times \frac{\psi_0}{1-\psi_0}$ (Appenroth et al. 2001, Strasser et al. 2004, Gravano et al. 2004, Liu et al. 2006, Mathur et al. 2016)

$PI_{ABS} = \frac{\gamma_{RC2}}{1-\gamma_{RC2}} \times \frac{\varphi_{po}}{1-\varphi_{po}} \times \frac{\psi_{ET20}}{1-\psi_{ET20}}$ (Stirbet and Govindjee 2011)

$PI_{ABS} = \frac{RC}{ABS} \times \frac{F_V}{F_0} \times \frac{1-V_J}{V_J}$ (Clark et al. 2000)

$PI_{ABS} = \frac{(F_M-F_J)\times(F_M-F_0)^2}{4\times(F_K-F_0)\times F_M\times F_0}$ (Dao and Beardall 2016)

We suggest, where possible, foundational fluorescence terms should be used:

$$PI_{ABS} = \left(\frac{1}{3.\dot{3} \times \left(\frac{F_k - F_0}{F_v}\right)} \times \frac{F_j - F_0}{F_v} \times \frac{F_v}{F_m}\right) \times \left(\frac{F_v}{F_0}\right) \times \left(\frac{Fv - Fj + F0}{Fj - F0}\right)$$

Equation 2.3 performance index expressed in foundational terms

Mathematical simplifications can be made which improve on the convoluted nature of Equation 2.3. However, further simplification reduces Equation 2.3 from the three stage nature of most explanations of PI_{ABS}. It is however possible to retain the foundational basis of the equation and further optimize the equation to facilitate efficiency of description.

$$\frac{F_v^2 \times (F_v - F_j + F_0)}{3.\dot{3} \times (F_k - F_0) \times F_m \times F_0}$$

Equation 2.4 PI_{ABS} equation simplification

Table 2.4 Fluorescence parameters available from continuous excitation fluorimeters.

Parameter	Synonym	Calculation	Description	Reference
F_v/F_m	φPo, TRo/ABS, J_0^{TR}/J^{ABS}, Φ_{PSII}/qP	(Fm-Fo)/Fm, 1-Fo/Fm	Maximum quantum yield of PSII. Indicates the probability that a trapped photon will end up in the reaction centre and cause a photochemical event.	(Srivastava et al. 1997, Maxwell and Johnson 2000, Rosenqvist and Kooten 2003, Hansatech Instruments 2006, Stirbet and Govindjee 2011)
F_v/F_o	$\frac{\psi_{po}}{1-\psi_{po}}$	(Fm-Fo)/Fo, 1/(1-Fv/Fm)-1, 1/(Fo/Fm)-1	Has been shown to decrease with frost hardening from about 6.0 to 4.0/2.0 (upper/lower surface). It also increases quickly in healthy tree foliage.	(Mohammed et al. 2003)
F_0/F_m		1-Fv/Fm	Ratio of extrema.	(Strasser et al. 2000)
Area		$\int_0^{{}^tF_{max}} (F_M - F_t)dt$	The area above the fluorescence curve. It is proportional to the pool size of the electron acceptors Qa on the reducing side of PSII.	(Roháček and Barták 1999, Strasser et al. 2004, Hansatech Instruments 2006)
PI_{ABS}	PI	$\frac{RC}{ABS} \times \frac{F_v}{F_o} \times \frac{1-V_j}{V_j}$	Performance Index (PI) is an indicator of sample vitality. It is an expression indicating the internal force of the sample to resist constraints from the outside. Further discussion below.	(Clark et al. 2000, Percival and Fraser 2001, Strasser et al. 2004, Hansatech Instruments 2006);
$\frac{RC}{ABS}$	$\varphi Po \times (\frac{V_J}{M_0})$, $\frac{\gamma}{1-\gamma}$	$\frac{F_V}{F_M} \times \left(\frac{\frac{F_M - F_J}{F_V}}{4 \times \frac{F_K - F_0}{F_V}} \right)$	The density of active PSII reaction centres expressed based on the quantity of light absorbed by the antenna.	(Clark et al. 2000, Desotgiu et al. 2012)
TR_0/RC		M_0/V_J	Maximal trapping rate of PSII	(Force et al. 2003)
V_t		$\frac{F_t - F_0}{F_M - F_0}$	Relative variable fluorescence is used to normalize fluorescence measurements at set time points (F_t), to facilitate comparison.	(Srivastava et al. 1997, Strasser et al. 2000)
$1-V_J$	J-Phase, ET_o/TRO, Ψ_{EO}, $\frac{\psi_0}{1-\psi_0}$, $\frac{ET_0}{TR_0}$	$\frac{F_M - F_J}{F_M - F_0}$	Describes the efficiency that a trapped excitation can move an electron into the ETC from Q_A^- to the intersystem electron acceptor.	(Force et al. 2003, Desotgiu et al. 2012)

1-V_I	IP-Phase, ΔV_{IP}	$\frac{F_M - F_I}{F_M - F_0}$	Relative contribution of the I-P phase.	(Oukarroum et al. 2009, Desotgiu et al. 2012)
V_K	$V_{oj}300$, K band	$\frac{F_K - F_0}{F_J - F_0}$	Relative fluorescence at 300µs. It expresses the breakdown of the oxygen evolving complex (OEC).	(Srivastava et al. 1997, Desotgiu et al. 2013)
V_L	V_{OK100}, L band	$\frac{F_L - F_0}{F_K - F_0}$	Relative fluorescence at 100µs.	(Desotgiu et al. 2013)
M_0	dV/dt_0, $(\Delta V/\Delta t)_0$, $V_{300\mu s}/250\mu s$	$\frac{1000\mu s}{300\mu s - TF_{0\mu s}} \times \frac{F_K - F_0}{F_V}$	Slope of the origin of the fluorescence rise. Maximal rate of accumulation of the fraction of closed reaction centres.	(Tsimilli-Michael et al. 2000, Strasser et al. 2004)
N	$S_M \times \left(\frac{TR_0}{RC}\right)$ $S_M \times M_0 \times (1/V_J)$	$\left(\frac{Area}{F_V}\right) \times \left(\frac{M_0}{V_J}\right)$	Time dependent turnover number of Q_A	(Strasser et al. 2000, Force et al. 2003)
Vo(Bo)	B_0,	$\frac{\left(\frac{F_V}{F_M}\right) - \left(\frac{F_V^*}{F_M^*}\right)}{\frac{F_v}{F_m}}$	Relative amount of Q_B non-reducing PS II centres. (1-Vo(Bo) = Q_B reducing centres). F_t^*= F_t during a second flash (see citation for further information.)	(Mehta et al. 2010)
OEC		$1 - (\frac{V_K}{V_J})$	Fraction of O_2 evolving centres. This can be used alone or used in comparison with control sample e.g. OEC treated / OEC control.	(Appenroth et al. 2001, Liu et al. 2006)
S_m		Area/F_V	Normalized area. Also regarded as the energy needed to close all reaction centres. (assumed proportional to the number of reduction and oxidation of one QA-molecule during the fast OJIP transient, and therefore related to the number of electron carriers per electron transport chain).	(Appenroth et al. 2001, Stirbet and Govindjee 2011)
α, β and γ centres		See (Mathur et al. 2011)	Heterogeneity of PSII from fluorescence rise.	(Mathur et al. 2011)

2.5 Future methods

Recent methods of data interpretation have transitioned away from a single parameter approach and attempted to view multiple parameters in relation to each other, often displayed as a radar plot, the shape of the resulting plot has been coined the fluorescence fingerprint or fluorescence barcode. Interpretation is possible when visualised as radar plots (Figure 2.3), however, computational pattern recognition facilitates quantitative recognition and has been shown to identify plant species (Tyystjärvi et al. 1999, Codrea et al. 2003, Keränen et al. 2003), drought stress (Goltsev et al. 2012) and both virulent and avirulent strains of *Pseudomonas syringae* (Berger et al. 2007). The potential

practical implications of this method of interpretation have not yet been exploited for practitioners. Keränen et al. (2003) discusses the implications of species identification for precision farming, using the technology for targeted herbicide applications. However, a system capable of specific stress identification could have wider implications for plant diagnostics in agriculture, horticulture and arboriculture, identifying stressors prior to visual symptoms becoming apparent. While the potential for such systems is large, current research is limited, this is most likely owing to the requirement for large, highly controlled training datasets. Recent developments in CEF devices, such as multi-signal instruments and delayed fluorescence are also in their infancy, with further research needed to provide a sound physiology basis to the readings (Stirbet and Govindjee 2011, Oukarroum, El Gharous, et al. 2016).

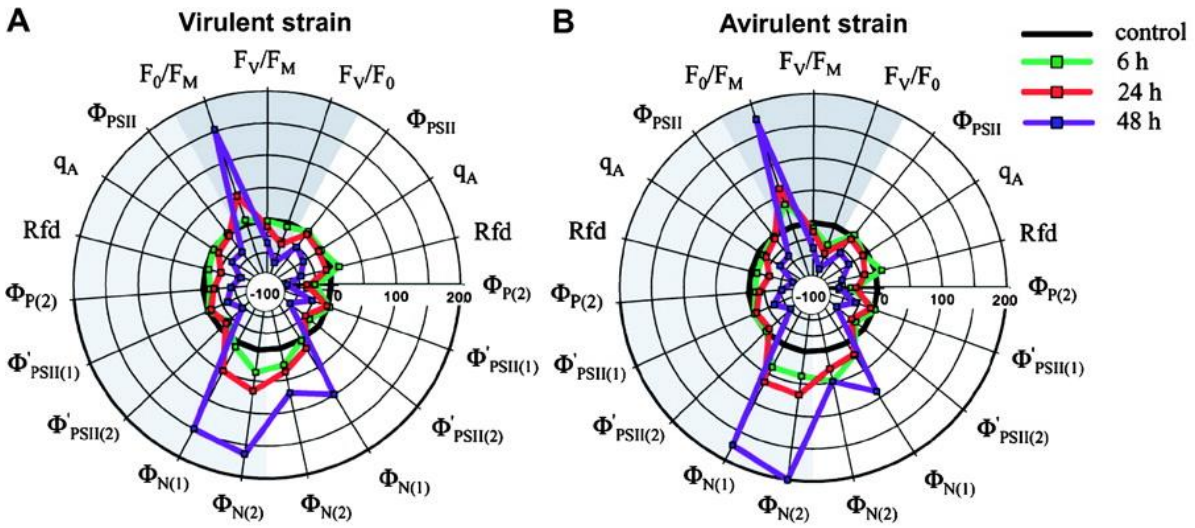

Figure 2.3 Example of fluorescence fingerprint visualisation adapted from Berger et al. (2007) *Relative changes of mean values of selected fluorescence parameters in A. thaliana infected by the virulent strain (A) and by the avirulent avrRPM1 strain (B) of P. syringae. The dark grey-shaded segments show parameters measured with dark-adapted plants. The fluorescence parameters measured during Kautsky induction and quenching analysis in low actinic light are shown in the lightly shaded segments. The non-shaded white segments show fluorescence parameters collected with high actinic light.*

2.6 Conclusion

The degree to which fluorescence parameters have become convoluted is clear. A unified approach is required to facilitate improved understanding and utilisation of this technology by researchers and practitioners not knowledgeable in the areas of biophysics or molecular plant physiology. Widely utilised foundational parameters have been defined here as well as some additional parameters which may prove promising, further work is however required to identify and clarify parameter response to specific plant stresses. Further work is also required to evaluate the potential of fluorescence fingerprinting as a potential future direction of this technology.

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3 Chlorophyll Fluorescence as a Tool to Identify Drought Stress in *Acer* genotypes

Jonathan M. Banks[a,b]

a The University of Reading, Department of Agriculture Policy and Development, Reading, Berkshire, RG6 6AR UK; b Bartlett Tree Experts, Research Laboratory (UK), Reading, Berkshire, RG2 9AF, UK

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Abstract: The effect of drought stress on continuous excitation chlorophyll fluorescence parameters and the OJIP transient is examined in cultivars of *Acer campestre, A. platanoides* and *A. pseudoplatanus*. Comparisons between whole tree level drought and desiccation of detached leaves under laboratory conditions is evaluated using both fluorescence parameters and differential kinetics. Data presented in this study suggests similarities exist between drought and desiccation. Chlorophyll fluorescence parameters which are both suitable and unsuitable at identifying drought stress are discussed and evaluated. New or uncommon fluorescence parameters and methods of analysis which may prove beneficial as drought detection tools are assessed. The over utilisation of the parameter Fv/Fm is also discussed. Results suggest utilisation of the parameters PI_{ABS}, Fo/Fm and V0(Bo) is recommended in preference to Fv/Fm, in studies aiming to identify drought stress in trees.

Key words: Acer, chlorophyll fluorescence, continuous excitation, drought, Fv/Fm, quantum efficiency.

3.1 Introduction

Chlorophyll fluorescence (CF) is one of the most common methods used to measure and in some cases categorise a range of stressors impacting the photosynthetic processes (Maxwell and Johnson, 2000; Mohammed et al., 2003). It has been used extensively to identify stress and stress responses in plants (Baker and Rosenqvist, 2004), algae (Kodru et al., 2015) and other photosynthesising organs (DeEll and Toivonen, 2003). However, authors disagree on parameter suitability and in some cases parameters are discussed as being both suitable and unsuitable to identify drought stress. Little information exists to aid practitioners in deciding which parameter is most appropriate. Additionally,

chlorophyll fluorescence is typically reviewed from a biophysicist's or molecular plant physiologist's point of view compounding the difficulties for practitioners when interpreting data (Maxwell and Johnson, 2000). For this reason, information sharing and clear explanation is critical to facilitate understanding among researchers and practitioners. The focus of this paper is on continuous excitation fluorimeters (CEF) which currently represents greater suitability for use by practitioners owing to their relative lower cost and simplicity of use. With recent technological developments however, a greater number of parameters are now becoming available on multiple fluorimeter types, meaning clear information sharing between methods is increasingly important. The purpose of this study is to evaluate the suitability of a range of parameters available from CEF and evaluate if similarities exist between drought and foliar desiccation. Additionally, the response of non-parameter based methods (OJIP, Vt, ΔW_{JO}, ΔW_{OK}) are also investigated for both drought and foliar desiccation.

Drought stress is known to initiate a range of physiological processes including turgor loss, reductions in leaf water potential (Ψ) and reduced stomatal aperture, in addition to increased abscisic acid (ABA) concentrations and exacerbated energy imbalances in the chloroplast (Bray, 2001; Vanlerberghe et al., 2015). Turgor loss is the primary reason for reduced growth in drought stressed plants as cellular expansion is dependent on cellular turgor (Bray, 2001). The primary cause of drought induced reduction in photosynthetic function is however disputed. It is accepted that a reduced $C0_2$ concentration as a result of either stomatal closure or reduced internal $C0_2$ diffusion decreases carbon fixation (Tang et al., 2002). This leads to chloroplast damage through photoinhibition (Nakamura and Izumi, 2018). However, reductions in foliar water content and subsequent increases in ion concentrations have been linked to reductions in photosynthetic metabolism, independent of stomatal aperture (Flexas et al. 2012). During severe drought, feedback effects, metabolic and structural changes may also become prevalent (Kalaji et al., 2016). Drought is particularly damaging when combined with other stressors, such as high light intensities which induce an imbalance in the reaction centre leading to oxidative damage and photoinhibition (Goltsev et al., 2012). Photosynthetic responses to low Ψ and low relative water content (RWC) have been discussed as being dependent upon genotype (Tardieu and Simonneau, 1998), environmental conditions and the velocity of drought imposition (Flexas et al., 2002). However, and perhaps owing to these complexities, further research is required in order to fully elucidate the CF responses to drought.

Fast induction kinetics (FIK) such as those measured by continuous excitation chlorophyll fluorescence are known to provide information primarily about photosystem II. Photosystem II is however considered highly resistant to water shortages (Cornic and Fresneau, 2002; Desotgiu et al., 2012; Havaux, 1992), resulting in parameters investigating primary photochemistry, such as the quantum

efficiency of PSII (Fv/Fm), often cited as unreliable. It is clear however, from comparison between studies such as Ögren (1990) and LI et al. (2006) that Fv/Fm may be suitable in some circumstances but not in others (Table 3.1) (also discussed by Ow et al. (2011)). Fv/Fm has been cited to only respond in extreme desiccation conditions with *Quercus petraea* and *Arabidopsis thaliana* (Epron and Dreyer, 1992; Mishra et al., 2014). However, Percival et al. (2006) identified significant reductions in Fv/Fm, varying depending on *Fraxinus* species. Fv/Fm was 11% to 65% lower than in control plants following two weeks of drought (Percival et al., 2006). Ow et al. (2011) also identify some species which exhibit both Fv/Fm sensitivity (*Conradina grandiflora* & *Chlorophytum cosmosum*) and insensitivity (*Euonymus cochinchinensis* & *Talinum triangulare*) to soil water status. These studies suggest Fv/Fm may be a useful measure of drought in drought-sensitive species or severe desiccation but is unlikely to be useful in drought-tolerant species or under minor drought.

Photosystem I is however less resistant to water deficit in comparison to PSII (Kalaji et al., 2016). Ceppi et al. (2012) identified that the IP amplitude ($1\text{-}V_{IP} = \Delta V_{IP}$) reflects changes in PSI content, and $1\text{-}V_{IP}$ has been found to be a suitable parameter to identify drought and drought tolerance (Oukarroum et al., 2009). Additional alternate parameters have been identified within the literature which may provide greater applicability to studies investigating drought stress (Kalaji et al., 2017b, 2016, Strasser et al., 2004, 2000). The multi-parametric parameter, performance index (PI_{ABS}) is capable of identifying drought stress and its decline has been used to identify drought tolerance in wheat (Živčák et al., 2008). However, the complexity behind its calculation and relative under-utilisation within the literature may deter some practitioners. Strasser et al. (2000) however, suggested six parameters which can be used in screening tests. These are: F_0/F_M, $(dV/dt)_0$, V_J, V_I, t_{Fmax} and S_m. The authors explain that F_0/F_M, $(dV/dt)_0$ ($\equiv M_0$) and V_J refer to the structure and function of PSII whereas V_I, t_{Fmax} and S_m refer to the activity of the electron transport chain (ETC) beyond Q_A (Strasser et al., 2000). The ETC is however thought to also be relatively insensitive to drought stress (Niinemets et al., 1999). Strasser et al. (2000) also do not comment on the impact of species or genotype on the expected sensitivity of these parameters. Table 3.1 identifies that few researchers have utilised the six parameters discussed by Strasser et al. (2000). The parameter Fv/Fm is more frequently occurring and widely used (Su et al., 2015); putatively because it is widely used and commonly available from early pulse amplitude modulated (PAM) fluorimeters (Osinga et al., 2012). The ratio-metric normalisation of Fv/Fm also facilitates ease of interpretation. Clear evidence exists however, strongly suggesting against a reliance on Fv/Fm alone (Force et al., 2003; Menezes-Silva et al., 2017). There is additionally an under-utilisation of fluorescence parameters not containing combinations of F_0 and F_M (F_V/F_M, F_0/F_M and F_V/F_0), which are known to carry the same information as each other, but differ in scaling (Strasser et al., 2000; Sun et al., 2015). Recently some investigators have been not solely analysing parameters but

additionally using relative variable fluorescence or double normalised differential kinetics, which have shown promise identifying drought (Desotgiu et al., 2012; Kalaji et al., 2017a; Oukarroum et al., 2007), salt (Kalaji et al., 2017a) aluminium (Jiang et al., 2008) and ozone (Desotgiu et al., 2012) stresses.

Table 3.1 utilisation of fluorescence parameters within drought stress studies. Arranged according to studies conclusion of suitability.

Parameters deemed effective	**spp. and source**
Fv/Fm	*Fraxinus spp*. (Percival et al., 2006), *Racomitrium sp, Anomodon sp. & Rhytidiadelphus sp.* (Proctor and Smirnoff, 2000), *Hordeum vulgare* (Rong- Hua et al., 2006), *Salix sp.* (Ögren, 1990), *Quercus petraea* (Epron and Dreyer, 1992), *Citrus spp*. (García-Sánchez et al., 2007)
Fv/F_0	*Hordeum vulgare* (Rong- Hua et al., 2006)
F_0	Five woody perennials (Percival and Sheriffs, 2002), *Hordeum vulgare* (Rong- Hua et al., 2006)
PI_{ABS}	*Triticum aestivum* (Živčák et al., 2008), *Parmelina tiliacea* (Oukarroum et al., 2018)
PI_{tot}	*Fagus sylvatica* (Pflug et al., 2018), *Parmelina tiliacea* (Oukarroum et al., 2018)
1-Vi	*Hordeum vulgare* and *Cicer arietinum* (Oukarroum et al., 2009)
DFI	*Hordeum vulgare* (Oukarroum et al., 2007)
DIo, ETo, Tro & ABS	*Fraxinus spp*. (Percival et al., 2006)
Parameters deemed ineffective	**spp. and source**
Fv/Fm	*Tilia* sp. & *Acer platanoides* cultivars (Fini et al., 2009), Triticum aestivum (Živčák et al., 2008), *Populus maximowiczi* (Desotgiu et al., 2012), Zea mays (Cornic and Fresneau, 2002), coffee (Menezes-Silva et al., 2017), Fagus sylvatica (Pflug et al., 2018)
Fv/F_0	*Tilia* sp. & *Acer platanoides* cultivars (Fini et al., 2009)
Fp/Fm	*Salix sp.* (Ögren, 1990),
F_0	*Tilia* sp. & *Acer platanoides* cultivars (Fini et al., 2009)
qP & qNP	*Populus maximowiczi* (Desotgiu et al., 2012)

The fluorescence response to drought is broad and variable but has been discussed in the literature (Doneva et al., 2017; Oukarroum et al., 2009), the response to leaf detachment and subsequent desiccation however requires further study (Angelopoulos et al., 1996). Despite this, a number of studies have used detached plants, rosettes, leaves or leaf discs as a proxy for drought in order to evaluate genotypic tolerance (Catala et al., 2007; Ögren, 1990; Percival and Sheriffs, 2002). Percival & Sheriffs (2002) and Faraloni et al. (2011) identify that relative drought tolerance rankings of five woody perennial genotypes and cultivars of Olive (*Olea europaea*) respectively do not vary between whole

plants and detached leaves. However, they do not elucidate if the fluorescence response to detachment is similar to that of whole plant drought stress. Conversely Ögren (1990) suggest that "dehydration is different from drought at the mechanistic level" however, this was assessed using primarily slow phase (S to M) fluorescence provided by a PAM fluorimeter. A PAM fluorimeter was used by Faraloni et al. (2011) but not by Percival & Sheriffs (2002). Foliar dehydration studies are relatively uncommon, however they have potential to provide benefits over currently common in vitro methods of genotypic tolerance ranking, such as PV or psychrometric methods (Bartlett et al., 2012; Sjöman et al., 2018). One major advantage is that desiccation studies can account for foliar morphological adaptations (highly cut foliage, foliar pubescents, foliar water storage organs) which may reduce the rate of desiccation and thus more accurately determine genotypic tolerance in some scenarios. Desiccation studies can also be performed with relatively inexpensive equipment and because tolerance scales are relative to the species tested the necessity for stringent environmental controls can be reduced. This study aims to evaluate the response of CF and CF parameters to whole tree drought and foliar desiccation using continuous excitation fluorescence.

3.2 Materials and Methods

3.2.1 Plant material

Seven-year-old 4.2 m (± 0.3) tall trees with a DBH of 50.7mm (±7.9) growing in 45 litre Light Pots™ (white, mypex woven grow bags) were used for this experiment. Trees were grafted onto their respective species-type rootstocks. In all, 80 trees belonging to the following species were used: *A. campestre, A. campestre* 'Louisa Red Shine', *A. platanoides* 'Drummondii', *A. platanoides* 'Emerald Queen', *A. platanoides* 'Princeton Gold', *A. platanoides* 'Royal Red', *A. pseudoplatanus* 'Negenia', and *A. pseudoplatanus* 'Spaethii'. For the duration of this experiment, all trees were kept outside, at Barcham Trees nursery, Ely, Cambridgeshire (52.366923° N, 0.315864° W). All experimental trees were arranged in a single row, either side of which was bordered by two rows of nursery trees of equal size to minimise edge effects. Experimental trees were arranged in 5 repeat blocks, each containing a drought and well-watered treatment, specimens were randomly placed representing all aforementioned *Acer* genotypes. A watering treatment (irrigated or non-irrigated) was alternated between blocks, and all trees allocated to the irrigated treatment were drip irrigated with *ca.* 5 litres of water per day. Drought treatment was initiated by detaching irrigation lines from non-irrigated blocks. Drought was augmented by installing black plastic rain covers over all root balls (irrigated and non-irrigated) to exclude rainfall and by raising all pots ca. 75mm off the ground to avoid water uptake from the ground, which was covered in pea gravel.

Chlorophyll fluorescence measurements of the drought treatment were recorded on the 18th July 2016 (-216 hours since irrigation removal), 4th August 2016 (192 hours) and 9th August 2016 (310 hours).

3.2.2 In vitro Desiccation

Three replicate leaves from each of the 80 experimental trees were collected on 20th June 2016. Immediately after removal, all leaves were sealed in ziploc plastic bags and kept in the dark while being transferred to the laboratory. CF was measured on these leaves between 21st and 29th June 2016. Measurements were initiated immediately upon arrival at the laboratory (approximately 20 hours later). Immediately after the first measurements (hour zero), leaves were laid out across the laboratory bench and left to desiccate. Leaves were arranged in a completely randomised block design ensuring each leaf had >1cm peripheral zone to reduce effects on nearby leaves. The laboratory was kept at an average vapour pressure deficit of 0.65kPa (± 0.14) (Temp 17.5°C (± 0.89) Humidity 67.6% (± 6.59)). The windowless laboratory was kept in darkness at all times apart from when measurements were taking place (<3 hours per day). Darkness was maintained in order to avoid accentuated water-loss and photooxidative damage, darkness of this duration was not expected to cause appreciable stress (Sui et al., 2012). Measurements of chlorophyll fluorescence were taken each day at 0, 23, 47, 71, 143 and 191 hours of desiccation.

3.2.3 Relative water content

Because of the size of the trial it was not possible to measure relative water content for each leaf. Time was therefore used as a substitute for relative water content (RWC). In order to confirm this was an appropriate assumption in a subset of this trial RWC was plotted against time and a strong correlation was observed (Figure 3.1), this was in agreement with data displayed by Rastogi et al. (2002) and Faraloni et al. (2011).

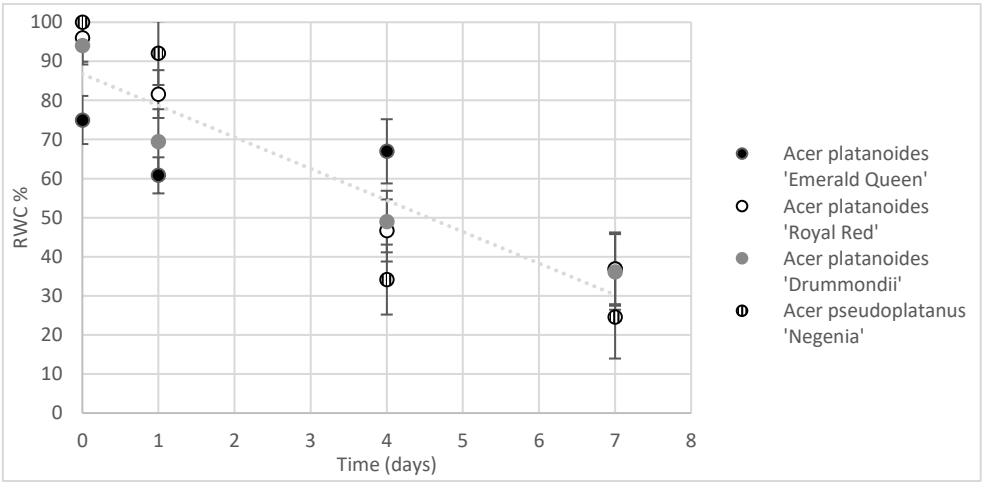

Figure 3.1 Relative water content (RWC) (%) plotted against time (days) for four selected species. n = 15. Error bars show standard error. Linear regression expressed for all four species was y = -8.0701x + 86.733 R² = 0.808.

3.2.4 Visual Index

General tree health was visually assessed for each tree; crown structure, density and degree of dieback was assessed together with size, colour, density and necrosis of foliage. Visual indexing facilitates tree vitality, to be compared with CF measures, and provides a rapid assessment of plant health useful to the tree selector. Trees were assessed in accordance with the following 1-5 scale:

1. 66-100% of leaves affected, severe foliar discoloration and necrosis
2. 36-65% of leaves affected, significant defoliation and/or leaf yellowing
3. 5-35% of leaves affected with some yellowing but little or no defoliation
4. less than 5% of leaves affected and little aesthetic impact
5. No necrosis or aesthetic impact observed

On 9th August (310 hours) an additional surveyor was asked to rank the visual vitality of the trees independently to ascertain any bias present in the recording of visual indices.

3.2.5 Soil moisture

Soil moisture content was recorded for all trees on each measurement day using a Delta-T Devices SM150 soil moisture sensor kit (Delta-T Devices Ltd, Cambridge) generating volumetric soil moisture content (VMC) data with ±3% accuracy. The probe permits soil moisture readings to be taken with minimal root or soil disturbance by gently pushing the two 51x2.5mm probes through the Light Pots™ 10-15cm from base of pot. Repeated measures were performed by utilising the same holes in the growing bags while ensuring sufficient soil:probe contact.

3.2.6 Chlorophyll fluorescence

All leaves were dark adapted (~30 minutes) before a fluorescence response was induced by a one second flash of 650nm light at an intensity of 1500µmol/m^2/s, provided by an array of three light-emitting diodes covering a 4mm diameter circle of leaf surface. Chlorophyll fluorescence parameters were calculated according to the calculations described in Banks (2017), while double-normalised differential kinetics (L- and K-band, ΔW_{OK} and ΔW_{OJ} respectively) were calculated in accordance with Oukarroum et al. (2007):

$$\Delta W_{0X} = \frac{F_t - F_{\hat{0}}}{F_x - F_{\hat{0}}}(treated) - \frac{F_t - F_{\hat{0}}}{F_x - F_{\hat{0}}}(control)$$

Equation 3.1 ΔW_{ox} where F_t is the fluorescence at time (t) across the induction curve. F_x is replaced with either F_J or F_K for W_{OJ} or W_{ok} respectively.

Relative variable fluorescence (V_t) was calculated in accordance with Oukarroum et al. (2009):

$$V_t = \frac{F_t - F_{ô}}{F_m - F_{ô}}$$

Equation 3.2 Calculation of relative variable fluorescence V_t

ΔVt x 10 was calculated to aid interpretation of Vt as:

$$\Delta V_t = V_t(treated) - V_t(control) \times 10$$

Equation 3.3 Calculation of relative variable fluorescence V_t

In the whole tree drought trial, measurements on 18th July 2016 (-216 hours) and on 4th August (192 hours) were performed using a Pocket PEA device (Hansatech instruments Ltd., Norfolk). Measurements made on 9th August (310 hours) utilised the Handy PEA device (Hansatech instruments ltd., Norfolk). The use of the Handy PEA facilitated measurement of V0(B0).

In vitro measurements were made using the Handy PEA device. Measurements were made at 0, 23, 47, 71, 143 and 191 hours following detachment. Hour zero was the first time consistent desiccation could occur following the removal of leaves from the sampling bags (at ca. -20 hours).

CF measurements made using the handy PEA device were corrected for optical and measurement gain discrepancies between Handy and Pocket PEA, by multiplication of foundational parameter ($F_{ô}$, F_J, F_I, F_M) by 16 and 0.679 (Hansatech Instruments personal communication). PI_{ABS} values were corrected for a similar discrepancy in Handy PEA measurements using a linear regression calculated using 10 identical leaves measured with both devices (y = 3.7569x+0.0355, where Y= Handy PEA, x = Pocket PEA) (R^2=0.972).

3.2.7 Statistical Analysis

Statistical analysis was performed using GenStat 17. Data was pooled per tree prior to analysis. Following tests for normality, a residual maximum likelihood (REML) model was used to analyse data in time series. Analysis of variance (ANOVA) was used when data was not in time series. Duncan's multiple range test was used to identify differences between genotypes. Additional interpretations were performed using Microsoft Excel 2013.

3.3 Results

Percent volumetric water content (VWC) was confirmed to be significantly different between watered and droughted trees (p = <.001). Results are displayed per cultivar in Figure 3.2.

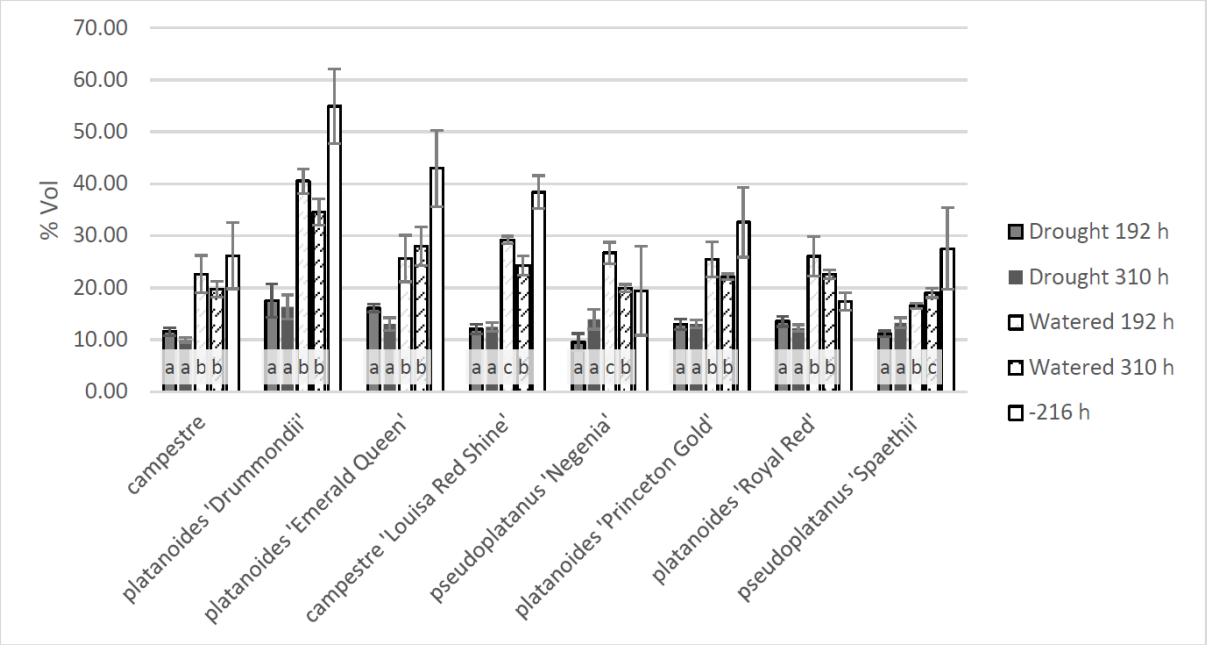

Figure 3.2 Percent volumetric water content for droughted and control cultivars, 192 hours and 310 hours following irrigation removal. Error bars shows standard error, letters denote significant differences within cultivars (n = 5). -216 hours prior to drought initiation includes both droughted and watered groups it is included for comparison but omitted from statistical analysis (n = 2).

A strong rank correlation was observed between, time for visual index to decline to 2 on the 5 to 1 scale and Fo/Fm, Fv/Fm, PI_{ABS}, V0(Bo) and Vj for drought (at 310 hours) and desiccation (71 hours) (Table 3.2). This data strongly suggests that these parameters are capable of identifying the differing deleterious effect of drought stress within the genotypes tested. The parameters Vi and 1-Vi did not correlate with the visually determined rank; additionally, t for Fm, Sm, and M0 were inconsistent, identifying drought but not desiccation, or desiccation but not drought.

A strong agreement (R^2 = 0.63 and Rs = 0.62) was observed between drought and desiccation when assessed using Fv/Fm (Figure 3.3). Average reductions in this parameter ranged between -92.8% (*A. platanoides* 'Drummondii') and -33.0% (*A. platanoides* 'Princeton Gold') in response to desiccation and drought respectively (Figure 3.3). A significant influence of cultivar was observed with all parameters tested following desiccation: 1-Vi, Vj, Fo/Fm, Fv/Fm, PI abs, Sm, t for Fm, V0(Bo) (p=<0.001) and M0 (p=0.002). Following drought however, only the parameters Fo/Fm, Fv/Fm, PI_{ABS}, t for fm (p = <0.001) and Sm (p = 0.011) identified a significant influence of cultivar. The parameters 1-Vi, Vj, M0 and V0(B0) were unable to identify significant differences at a cultivar level. Cultivars were therefore assessed individually following 71 and 310 hours of desiccation and drought respectively. These time points were selected as a response to drought was observed both visually and by observation of florescence transients. Utilization of earlier timepoints gave unclear results as little similarity to visual index had developed particularly in drought tolerant genotypes and slow-reacting

parameters which had not responded until 71 hours (See Figure 3.4 A. *platanoides* 'Drummondii' parameters Fv/Fm and t for Fm).

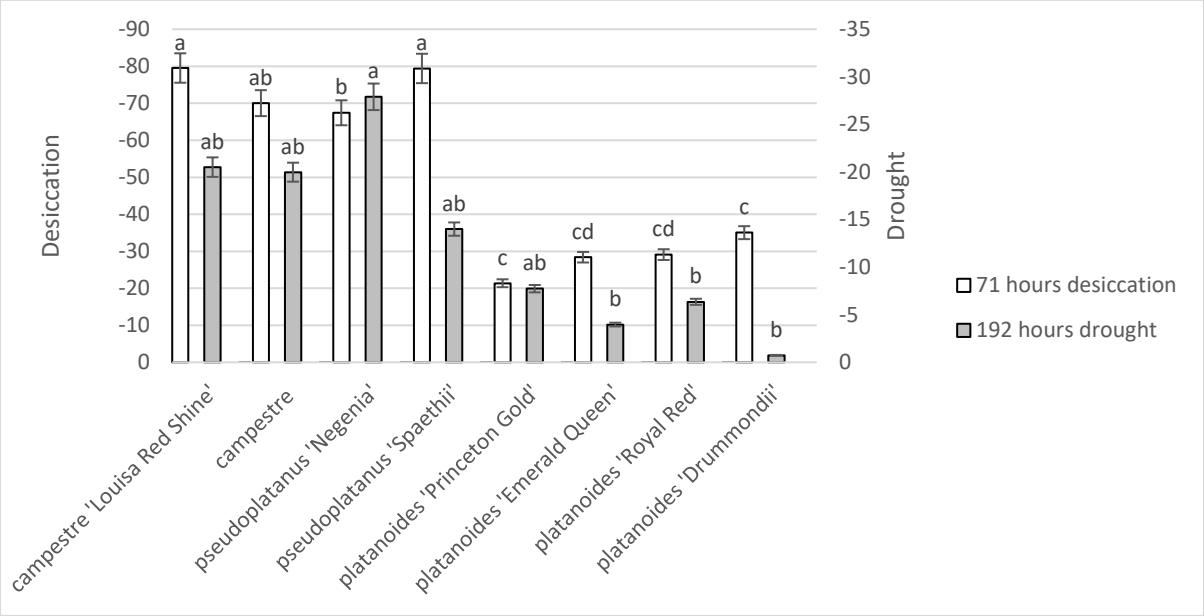

Figure 3.3 Percentage change in Fv/Fm after 192 hours of drought or 71 hours of desiccation. Letters denote significant difference between cultivars at the 95% confidence interval. Error bars indicate standard error.

Table 3.2 Rank correlation (Rs) between time for visual index to decline to 2 and CF parameters following 310 and 71 hours of drought and desiccation respectively. Light grey cells indicate values ≥0.5 and ≤-0.6. Dark grey cells indicate values ≥0.8 and ≤-0.8.

	Drought (310 hours)	Desiccation (71 hours)
1-VI	-0.476	-0.429
Fo/Fm	-0.667	-0.571
Fv/Fm	0.667	0.571
M0	-0.381	0.738
PI abs	0.548	0.524
Sm	-0.667	0.167
V0(Bo)	-0.690	-0.500
Vi	0.476	0.429
Vj	0.857	0.548
t for Fm	0.571	0.333

Based on this trial *A. platanoides* 'Drummondii' (relatively tolerant) and *A. campestre* 'Louisa Red Shine' (relatively sensitive) were used to demonstrate the fluorescence response to drought and desiccation at differing tolerance levels from within this study. In order to efficiently describe parameter response, parameters were normalised; percent change from the initial reading provides a unified point and gives parity to parameters on differing scales.

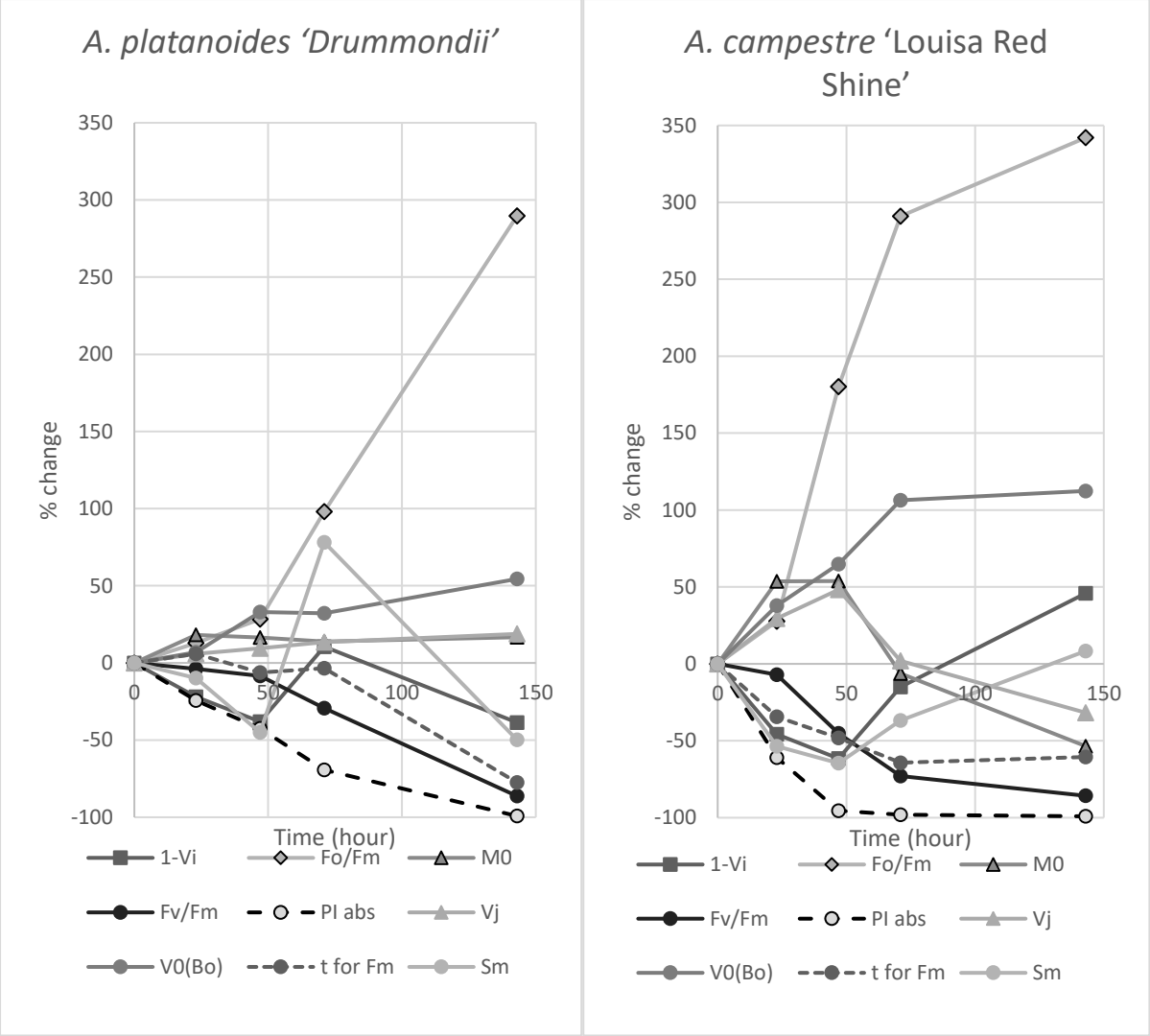

Figure 3.4 Percentage change in fluorescence parameters in response to desiccation. Results shown for A. platanoides 'Drummondii' (drought tolerant) and A. campestre 'Louisa Red Shine' (drought sensitive). Error bars removed for clarity.

Parameters were also displayed for *A. platanoides* 'Drummondii' (tolerant) and *A. campestre* 'Louisa Red Shine' (sensitive) at both 23 and 71 hours. This allowed the consistency of parameter response across time and between genotypes of differing sensitivity within this trial to be compared. Figure 3.5 highlights the consistency of PI_{ABS}, F_0/F_M and V0(Bo) at differing time points and drought tolerances.

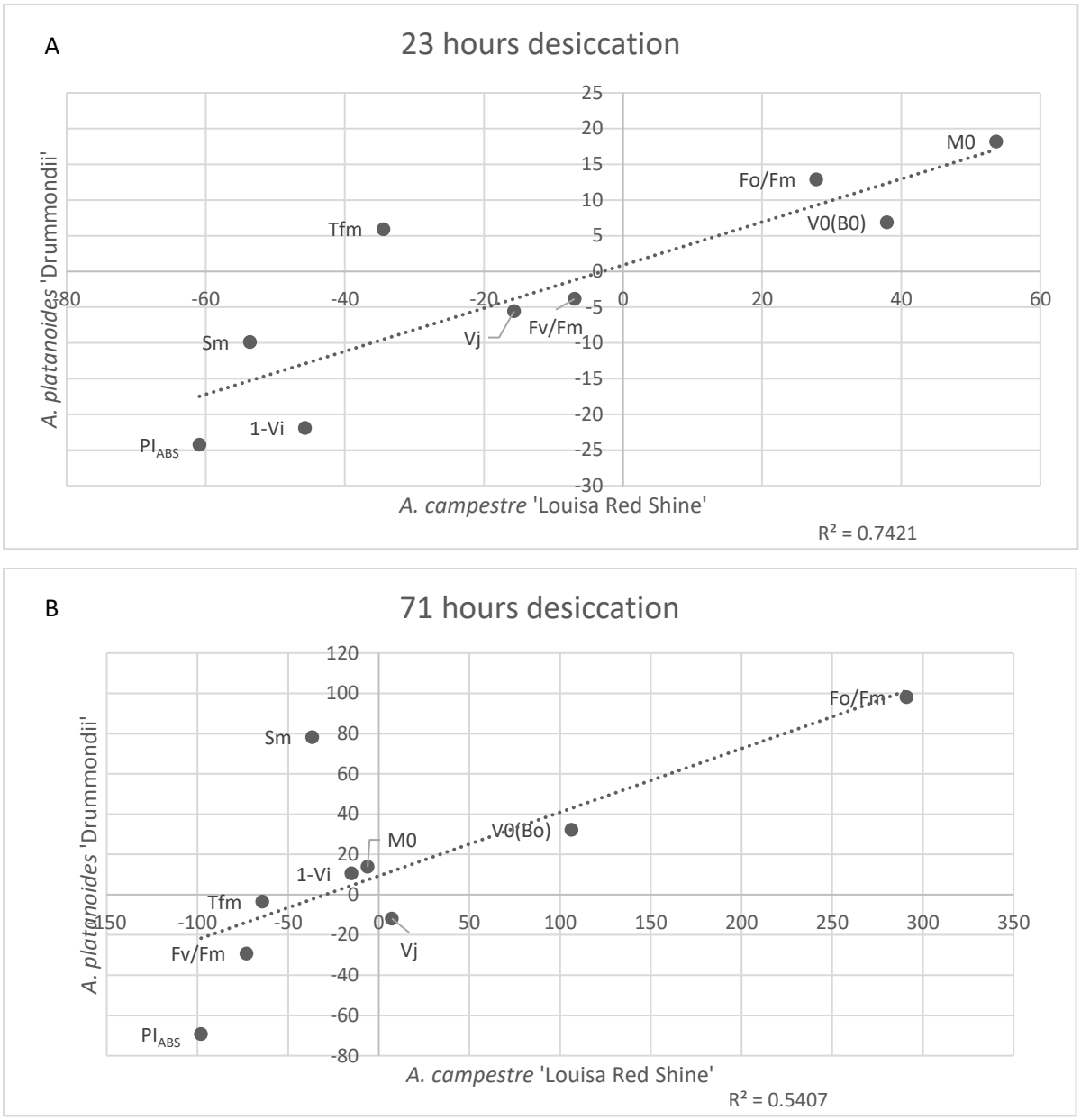

Figure 3.5 Parameter response (%) following 23 (A) and 71 (B) hours of desiccation, for A. platanoides 'Drummondii' (drought tolerant) and A. campestre 'Louisa Red Shine' (drought sensitive). (R2 = 0.74 at 23 hours. R2 = 0.54 at 71 hours).

Comparative OJIP transients and differential kinetics were also included for *A. platanoides* 'Drummondii' (drought tolerant) and *A. campestre* 'Louisa Red Shine' (drought sensitive) in order to aid understanding of parameter response to desiccation and dehydration. Figure 3.6 and Figure 3.7 clearly identify differences in both drought and desiccation tolerance between *A. p.* 'Drummondii' and *A. c.* 'Louisa Red Shine'. Genotypic differences in drought response can be observed in OJIP graphs, however, no observable difference is present between drought and desiccation in OJIP, ΔW_{OJ} and ΔW_{OK} response at the cultivar level. ΔVt does however identify a difference in the fluorescence response between drought and desiccation (Figure 3.7).

A. platanoides 'Drummondii'

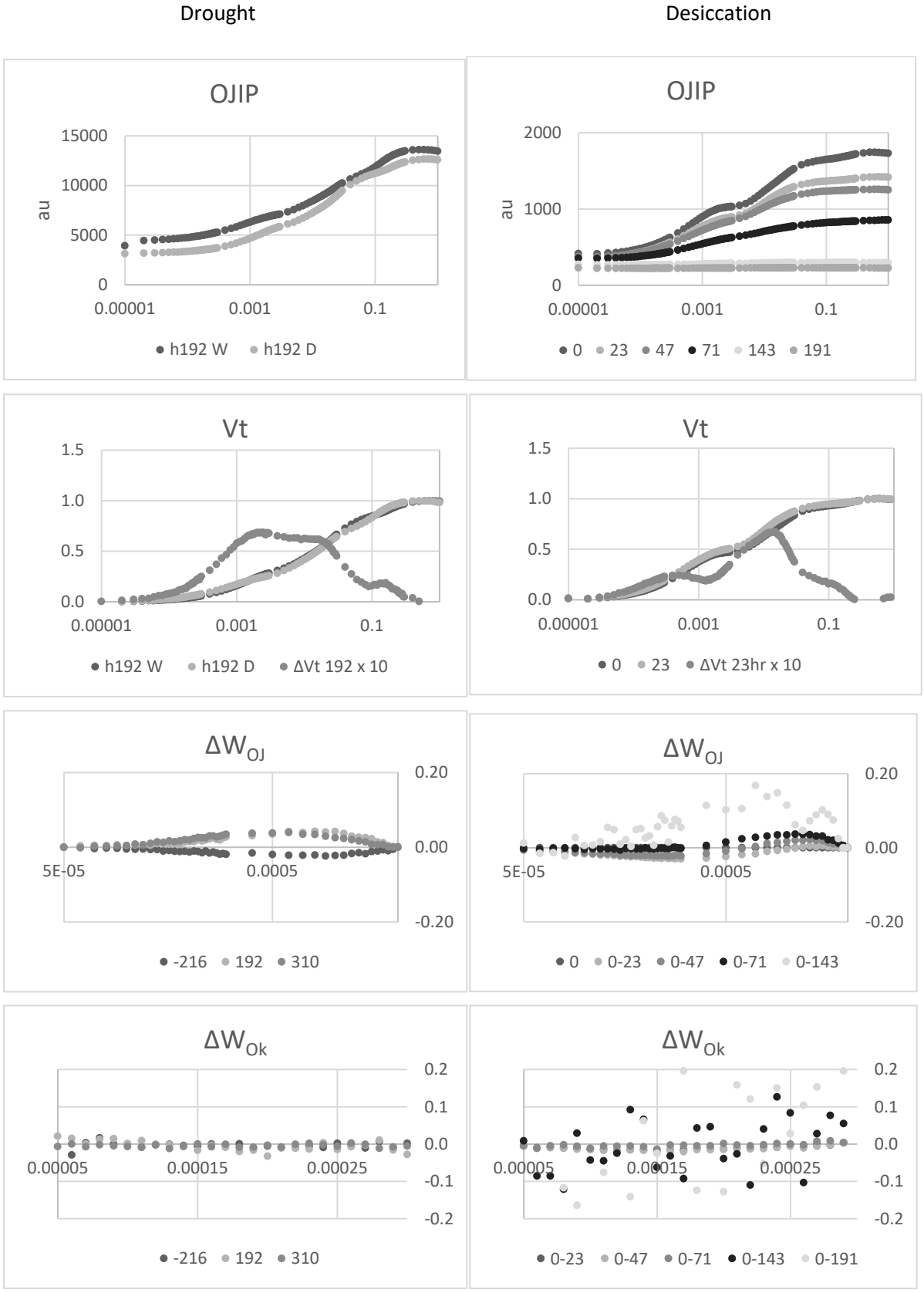

Figure 3.6 OJIP response (fluorescence intensity (a.u.)) and double-normalised differential kinetics (Vt, ΔW_{OJ}, ΔW_{Ok}) of A. platanoides 'Drummondii' in response to drought (left column) and desiccation (right) plotted for selected time points.

A. campestre 'Louisa Red Shine'

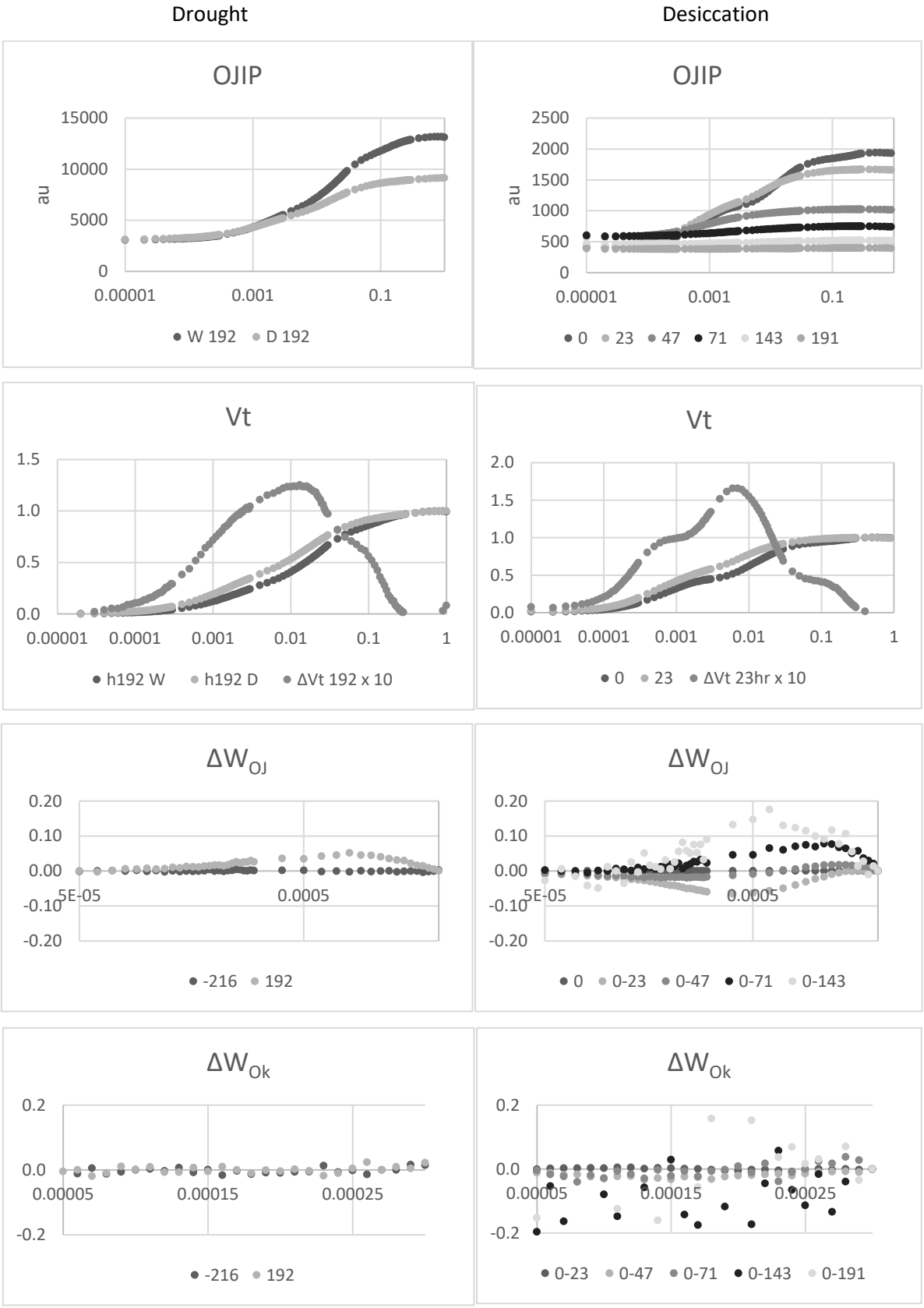

Figure 3.7 OJIP response (fluorescence intensity (a.u.)) and double-normalised differential kinetics (Vt, ΔW_{OJ}, ΔW_{OK}) of A. campestre 'Louisa Red Shine' in response to drought (left column) and desiccation (right) plotted for selected time points.

3.4 Discussion

Similarities between genotypic tolerance to whole tree drought and detached leaf dehydration under laboratory conditions strongly suggest that foliar dehydration can be used as a proxy for relative drought tolerance. However, parameter sensitivity to drought tolerance can differ between drought and desiccation (Table 3.2). Desiccation studies provide significant practical and efficiency advantages over whole tree drought studies in that they are faster and less resource-intensive to undertake. Additionally, greater response resolution and larger sample sizes are generally possible, in comparison to whole tree drought studies. However, the possibility of holistic tree responses (aquaporin mediated water uptake, hormone and signalling compounds such as abscisic acid, ethylene and salicylic acid (Aroca et al., 2012), and hydraulic lift (Wan et al., 2000)), must still be considered, especially if evaluating trees which significantly vary in their physiological drought tolerance responses.

Extracting the parameter response displayed in Figure 3.4 for both cultivars at 23 hours of desiccation allows rapidly responding parameters to be compared between genotypes (Figure 3.4). In this case M0 and PI_{ABS} responded most significantly, in a positive and negative direction respectively. Tfm responded positively in *A. p.* 'Drummondii' but negatively in *A. c.* 'Louisa Red Shine', suggesting that impairment of the electron transport chain (ETC) occurred only in the more drought-sensitive (within this trial) *A. c.* 'Louisa Red Shine' (Strasser et al., 2000).

Following 71 hours of desiccation, the parameters Fo/Fm and PI_{ABS} respond most positively and negatively respectively. The slowest parameter to respond was Fv/Fm adding to the evidence that reliance on this parameter alone is not appropriate in drought studies (Force et al., 2003). Fv/Fm however, acts as a stable metric for measuring the impact of drought on the maximal quantum yield of PSII. Additionally, Fv/Fm only responds to stress negatively, allowing easy interpretation for practitioners. The data presented here does not, however, support the suggestion that Fv/Fm only responds in drought sensitive species (Ow et al., 2011); however, foliar desiccation is expected to represent severe drought. In mild to moderate dehydration events, more sensitive parameters are likely to be of putative benefit. Although Strasser et al. (2000) suggest Fv/Fm and Fo/Fm differ only in scaling, Kalaji et al., (2014) define Fo/Fm with reference only to heat dissipation of the PSII antenna; conversely Fv/Fm is widely defined as the maximum quantum yield (Kalaji et al., 2014; Pflug et al., 2018; Strasser et al., 2000). No study to our knowledge has utilised F_0/F_M in drought studies (Table 3.1) a clear response however can be observed in Figure 3.4.

The data presented here strongly suggests that the parameters PI_{ABS}, Fo/Fm and V0(Bo) are valuable to practitioners because of their rapid and consistent response. The parameters Sm and 1-Vi responded stochastically and as such are not recommended to practitioners. Both parameters were

less sensitive in comparison to PI_{ABS} and are therefore also not recommended as a screening tool where further interpretation is required. As discussed by Banks (2017) PI_{ABS} is a complex multi-parametric parameter which describes alterations within and between F_0 and F_M. PI_{ABS} has previously been shown to respond rapidly to drought stress (Živčák et al., 2008). V0(Bo) describes the relative changes of Q_B non-reducing centres (Mehta et al., 2010) by measuring the change of F_V/F_M between two successive CF measurements in a double flash protocol. Previous research has currently only utilised this parameter for the identification of salt stress (Mehta et al., 2010). However, evidence suggests the accumulation of Q_B-non-reducing centres occurs in response to water stress (Lu and Zhang, 1999). V0(Bo) and F_0/F_M are currently underutilised therefore further work is recommended to describe their performance and uniformity between genotypes, stressors and stress intensities.

Observations of OJIP transients identifies deterioration only in the IP phase for *A. c.* 'Louisa Red Shine' in both desiccation and drought; whereas, *A. p.* 'Drummondii' deteriorates much more evenly across the entire OJIP transient. OJIP graphs do not however identify an observable difference between drought and desiccation. Differences between drought and desiccation are only observed in measurements of ΔVt. Drought stress resulted in a consistent bell-like shape between J and I peaks on the graph. However, the response following desiccation occurred in bimodal phases corresponding to the J and I time points, in this case the I peak had a higher intensity. Bussotti et al. (2011) observe a similar shape in Poplar response to ozone stress, and discussed that the J peak was caused by accumulation of the reduced Qa pool and that both peaks represented "a transient block at the acceptor side of PSI" (Bussotti et al., 2011). While Strasser et al. (1995) points to a reduction in heterogeneity between fast- and slow-reducing plastoquinone centres, this may however be negated by appropriate dark adaptation (Tomek et al., 2001). Few drought studies have used and displayed the characteristics of ΔVt transients, and to our knowledge no study has evaluated differences in ΔVt between drought and foliar desiccation. These differences require further study in order to evaluate their significance.

Severe drought and desiccation resulted in the presence of ΔW_{OJ} (K-band) peaks; however, no ΔW_{OK} (L-band) peaks were observed (Figure 3.6 and Figure 3.7). The absence of the L-band suggests no change in excitation energetic transfer took place, until significant photosynthetic deterioration had occurred. By this point, desiccation induced reduction in Fv/Fm had reached 29% and 73% for *A. p.* 'Drummondii' and *A. c.* 'Louisa Red Shine' respectively. This is contrary to the findings of Oukarroum et al. (2007) who observed both L and K bands in drought-sensitive genotypes but no L and K band in a drought tolerant genotype. Responses in L and K band expression have however been reported to vary in response to ozone stress as a result of light or age graduations in leaf material (Desotgiu et al.,

2012). The presence of a K-band in both species suggests disruption of the oxygen-evolving complex (Bussotti et al., 2011; Kalaji et al., 2016; Srivastava et al., 1997). L and K peaks appear to not be viable as early indicators of drought stress, as the response was less sensitive than a parameter approach to stress identification. L and K peaks are also not specific to drought stress as they have been found to occur in response to salt (Kalaji et al., 2017a), ozone (Bussotti et al., 2011), and nitrogen (Zhao et al., 2017) as well as drought (Kalaji et al., 2017a, 2016; Oukarroum et al., 2007). The absence of L and K peaks may however be useful indicators for viable recovery (Kalaji et al., 2016; Oukarroum et al., 2007). No differences in L and K bands are observed between drought and desiccation; however, excessive desiccation (>71 hours) resulted in a stochastic response for both species exposed to it. This has not been measured in droughted trees, possibly because leaf abscission occurred prior to this point.

3.5 Conclusion

Similarities exist between drought and desiccation tolerance rankings, and the fluorescence response to these stressors. Data presented here adds to evidence against an over-reliance on the parameter Fv/Fm alone. In this study, significant advantages over Fv/Fm were achieved by the utilisation of the parameters PI_{ABS}, Fo/Fm and V0(Bo). Observations of double normalised differential kinetics identified similarities between drought and desiccation when observing ΔW_{OJ} and ΔW_{OK}. However, drought and desiccation differences were observed in ΔVt transients which require further study in order to elucidate their significance.

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4 Evaluating the Drought Tolerance of Cultivars Within Acer platanoides, campestre and pseudoplatanus.

Jonathan Michael Banks[ab*], Martin Lukac[b], Glynn Clive Percival[a], Gillian Rose[b]

[a] *Bartlett Tree Experts, Research Laboratory (UK) Reading, Berkshire RG2 9AF, UK.* [b] *School of Agriculture Policy and Development, The University of Reading, Reading, Berkshire RG6 6AR, UK.*

Abstract: An investigation into the drought tolerance of pot-grown *Acer platanoides, campestre and pseudoplatanus* cultivars was carried out to compare visual and physiology-based indexes of the drought tolerance. The study identified significant drought tolerance differences between and within selected species. The relative order of species tolerance was found to be contrary to the findings of other researchers; *A. platanoides* was significantly more drought tolerant than *A. pseudoplatanus* and *A. campestre*. No significant differences were observed between cultivars belonging to *A. pseudoplatanus* and *A. campestre*. However, *A. platanoides* cultivars demonstrated significant variation in drought tolerance. These observations indicate that species level drought tolerance rankings cannot be used exclusively as an indicator of cultivar drought tolerance.

Keywords: drought, tolerance, chlorophyll fluorescence, soil water, visual index

4.1 Introduction

Drought is a significant and common stress in trees, particularly in urban environments, where water infiltration and soil volumes are limited (Cattivelli et al. 2008). Despite the importance of drought stress, knowledge and ranking of cultivars according to drought tolerance are often inconsistent and of a poor quality (Niinemets and Valladares 2006, Sjöman et al. 2015). A number of studies appear to provide conflicting information regarding the drought tolerance of the same species (Sjöman et al. 2015). Drought is a complex plant stress factor, known to conflict and interact with other stressors. Polytolerance (simultaneous, multiple stress tolerance) is uncommon, owing to trade-offs inherent in differing tolerance strategies, particularly drought-shade, cold-waterlogging adaptations (Niinemets and Valladares 2006, Laanisto and Niinemets 2015). Therefore, stress specific tolerance rankings are necessary in order to appropriately select species or cultivars for urban sites.

Numerous studies use arbitrary scales to rank genotypic drought tolerance. Bassuk et al. (2009) rank soil moisture tolerance from 1: tolerance of "occasionally saturated or very wet soil", to 12: tolerant of "prolonged periods of dry soil". It is however not clear how these values were calculated. Niinemets & Valladares (2006) rate tolerance from 1: very intolerant to 5: very tolerant; this is one of the largest studies of genotypic tolerance to-date, including 806 species. The ranking is based upon site characteristics of species dispersal and physiological potentials from five main studies using primarily North American species (Niinemets and Valladares 2006). A high correlation was found between the sources utilised in the study (R^2=>0.80). Differing tolerance scales were cross-calibrated using species in common within the data sets and an average tolerance score used (Niinemets & Valladares 2006, Appendix B). These methods provide significant advantages for those in the field, in that they are uncomplicated and limited prior knowledge regarding the influence of drought on trees is required to understand and interpret the significance of differences within the ranking system. Disadvantages also exist, namely, it is difficult to compare and cross-validate rankings, this may be necessary in different geographical areas or with genotypes of differing provenance. Sjöman et al. (2015) discuss that 'drought tolerance' may be conferred by avoidance, tolerance or a combination of the two. Niinemets and Valladares (2006) system may therefore consider genotypes as highly tolerant if they avoid drought by dropping leaves or invest in deep roots. However, it is generally advisable to avoid these species in urban areas where such traits are undesirable (Sjöman et al. 2015). The value of such methods may also be reduced where rankings include species with extreme tolerances; species around the median of tolerance are likely to be compressed into similar or the same rank, reducing the sensitivity of tolerance identification. The sensitivity of tolerance rankings is therefore inherently dependent on the species and cultivars included within the rank.

Many studies utilise the chlorophyll *a* fluorescence (CF) response to drought as an indicator of the presence and degree of drought stress response in plants (Percival and Sheriffs 2002, Oukarroum et al. 2007, Fini et al. 2009). The fast CF rise exhibits a characteristic polyphasic shape, timepoints during the rise are labelled from minimum (F_0) to peak (F_P or F_M) with intermediary (F_J, F_I) steps; the whole CF rise is widely known as OJIP (Strasser et al. 1995, Banks 2017, Stirbet et al. 2018). A range of CF measurement parameters are accepted methods of screening for stress tolerance (Hakam et al. 2000, Percival and Sheriffs 2002, Kalaji et al. 2016). A so-called drought factor index (DFI) has been devised using CF measurements made at multiple periods across a drought trial for both droughted and watered plants; this has been shown to be an indicator of species tolerance (Oukarroum et al. 2007). Additionally, a lesser utilised parameter describing the latter IP phase of the polyphasic OJIP rise has been discussed to respond to drought stress in barley and chickpea (Oukarroum et al. 2009). A more specific parameter may reduce "noise" from other stressors acting in the natural environment.

Despite potential advantages of CF, when communicating data to practitioners, visual index (VI) is recommended as it is a practically useful applied metric which is scientifically-defensible to determine drought tolerance (Vahdati et al. 2017). Therefore, in this study CF was compared with a VI based on overall tree vitality, crown dieback, foliar symptomology (wilting, necrosis, and chlorosis) and foliar crown cover. Independent quantification of vitality was achieved by measuring continuous excitation CF.

This study utilises an approach combining visual characteristic with measurements of CF (Clark et al. 2000) to provide a drought tolerance rank for several cultivars belonging to the *Acer* genus. The genus consists of 129 species of evergreen and deciduous trees and shrubs from Europe, North Africa, Asia and North and Central America (Shu 2008). Harris (1992), García et al. (2006) and Bell et al. (2005) refer to the need for urban trees that provide colour, form, texture and pattern in the landscape. Species found within the *Acer* genus provide all these benefits, possessing good autumn colour, interesting and often coloured bark and leaves.

The *Acer* genus was selected for experimental purposes for several reasons:

i.) Acers have a high economic value to the horticultural/landscape industry;

ii.) They provide contrasting forms which are highly desirable to local authority tree officers;

iii.) They present a broad range of cultivars among single species, thus facilitating comparison.

Previous studies have revealed that Acer species span a wide drought tolerance range, significant intraspecific (within species) tolerances also exist (Sjöman et al. 2015) making appropriate selection very difficult. Clarification is therefore needed as to what level of both intraspecific and species variation exists. Additionally, few studies have applied a range of drought tolerance identification methods, this is a major drawback as it is essential to have a reliable benchmark of drought tolerance. We hypothesise that tolerance can be determined by visual indexing however, greater sensitivity and accuracy could be achieved when using CF, particularly timepoint specific parameters such as the IP phase.

4.2 Materials and Methods

4.2.1 Experimental design and sampling

All *Acer* species represented within this trial are commonly utilised as urban street trees, a wide range of genotypes with contrasting intraspecific foliar and crown characteristics was selected. All tested cultivars were grafted onto their respective species-type rootstocks. Eighty, seven-year-old 4.2 m (± 0.3) tall trees with a DBH of 50.7mm (±7.9) growing in 45 litre Light Pots™ (white, mypex woven grow

bags) were used for this experiment. The potting substrate consisted of a 50:50 green waste and pine bark compost mix, with a pH of 7.7. Throughout the experiment, all trees were kept outside at Barcham Trees nursery, Ely, Cambridgeshire (52.366923° N, 0.315864° W). The trees were arranged in a single row, bordered by two rows of nursery trees of an equal size on either side of the experimental row to eliminate possible edge effects. Experimental trees were arranged in 5 repeat blocks, each containing a drought and well-watered treatment, specimens were randomly placed representing all aforementioned *Acer* genotypes. All irrigated trees were drip irrigated with *ca.* 5 litres of water per day. The species and cultivars selected, characteristics and relevant tolerance information is included in Table 4.1.

Drought treatment was initiated by detaching irrigation lines from non-irrigated blocks. Drought was augmented by installing black plastic rain covers over each root ball to exclude rainfall and by raising all pots ca. 75mm off the ground to avoid water uptake from the ground (pea gravel). Drought was initiated on the 27th July 2016 and continued until the 9th of August. A range of measurements were performed on the 18th July (-216 hours prior to irrigation removal), 4th August (192 hours) and 9th August (310 hours).

Table 4.1 Available information on the species used within this study

Species 'Cultivar'	Common Name	Key Features	Ecology/Drought tolerance
A. pseudoplatanus	Sycamore	Fast growing deciduous spreading tree Considered a British non-native, introduced to the UK in ca. 1250 (Southwood 1961).	Recommended for an exposed position (Brickell 1989). Drought sensitive in comparison to *F. sylvatica, F. excelsior, Q. petraea, T. platyphyllos* and *P. avium.* Scherrer et al. (2011). Tolerant of prolonged periods of dry (cat. 10) and very wet soil (cat. 3) (Bassuk et al. 2009). Very adaptable to soil types, preferably well drained (Dirr 1990). 2.75 ±0.16 (Niinemets and Valladares 2006). π_{TLP} -2.70MPa (permanent wilting point) (Sjöman et al. 2015)
'Spaethii' ('Atropurpureum', 'Purpureum')		Leaves dark green above, rich purple below (Dirr 1990)	Thought to be less tolerant than the straight species to insect and disease factors, leaf scorch, sunscald and borer infestations (Bassuk et al. 2009).
'Negenia'		Vigorous conical shaped tree with red-stalked, large dark green leaves (Hillier 1993).	Tolerant to poor soil conditions (van den Berk and van den Berk 2015).
A. campestre	Field Maple	Low branching compact tree commonly found in field hedgerows and brownfield sites Native to the UK (Southwood 1961).	Tolerant of prolonged periods of dry (cat. 12), and consistently moist soil (cat. 4). (Bassuk et al. 2009). Cat. 2.93 ± 0.32 (Niinemets and Valladares 2006). Tolerant of any soil except dry infertile and sandy (van den Berk and van den Berk 2015). π_{TLP} -3.00MPa (permanent wilting point) (Sjöman et al. 2015)
'Louisa Red Shine'		Leaves have a slight red coloration in spring.	Tolerant to all soil types except dry infertile and sandy (van den Berk and van den Berk 2015).
A. platanoides	Norway Maple	Vigorous deciduous spreading tree with clusters of yellow flowers in mid spring, before leaves appear. Considered an invasive plant in parts of the United states of America (Martin 1999). Considered a British non-native. Native to continental Europe (Webb and Kaunzinger 1993).	Tolerant of prolonged periods of dry (cat. 10) and very wet soil (cat. 3) (Bassuk et al. 2009). Cat. 2.73 ± 0.16 (Niinemets and Valladares 2006). π_{TLP} -3.09MPa (permanent wilting point) (Sjöman et al. 2015)
'Drummondii'		Variegated cultivar, leaves have creamy-white edges turning yellowish in autumn (Brickell 1989, Bassuk et al. 2009).	Supposedly the best of its class (Dirr 1990).
'Emerald Queen'		Upright when young, leaves have a reddish tint in spring and are bright yellow in autumn (Hillier 1993)	Less tolerant than 'Summershade' similar to 'Deborah' (Fini et al. 2009). One of the best Norway Maples for urban plantings (Dirr 1990).
'Royal Red'		Deep reddish-purple leaves (Brickell 1989) Underlying suspicion that 'Royal Red' and 'Crimson King' are the same tree (Dirr 1990)	Considered more susceptible to pest problems than the straight species, but more tolerant and slower growing than 'Crimson King' (Bassuk et al. 2009). Supposedly hardier than 'Crimson King' (Dirr 1990).
'Princeton Gold'		Shows yellow spring and summer foliage and may fade to a darker yellow in autumn (Bassuk et al. 2009).	No source available

4.2.2 Soil moisture

Soil moisture content was recorded for all trees on each measurement day using a Delta-T devices SM150 soil moisture sensor kit (Delta-T Devices Ltd, Cambridge) generating volumetric soil moisture content (VMC) data with ±3% accuracy. The probe permits soil moisture readings to be taken with minimal root or soil disturbance by gently pushing the two 51x2.5mm probes through the Light Pots™ 10-15cm from base of pot. Repeated measures were performed by utilising the same holes in the growing bags while ensuring sufficient soil:probe contact.

4.2.3 Chlorophyll fluorescence

Measurements were made on three leaves per tree, on the 18th July 2016 (-216 hours prior to irrigation removal) and 4th August (192 hours) measurements were performed using a pocket Plant Efficiency Analyser (PEA) device (Hansatech instruments Ltd., Norfolk), while on the 9th August (310 hours) a handy PEA device was used (Hansatech instruments Ltd., Norfolk). A known inconsistency in measurement gain between devices was corrected by multiplication of foundational parameters ($F_{\dot{O}}$, F_J, F_I, F_M) by 16 and 0.679 (Hansatech Instruments 2016, personal communication). PI_{ABS} values were corrected by applying a linear regression constructed from measurements on 10 leaves with both devices (HandyPEA = 3.7569PocketPEA+0.0355, R^2=0.972).

Following dark adaptation (30 minutes), the fluorescence response was induced by a one second flash of light (650nm, 1500µmol/m^2/s) provided by an array of three light-emitting diodes over a 4mm diameter of leaf surface. The ratio of variable (F_V=F_M-F_0) to maximal fluorescence (F_M) was calculated. The multi-parametric parameter known as performance index (PI_{ABS}) was also calculated (Equation 4.1).

$$PI_{ABS} = \frac{F_V \times (F_V - F_J + F_0)}{3.\dot{3} \times (F_K - F_0) \times F_M \times F_0}$$

Equation 4.1 Calculation of performance index (PI_{ABS}) F_J = fluorescence intensity at 2ms, F_K= fluorescence intensity at 300µs (Banks 2017).

Time for the CF parameters Fv/Fm and PI_{ABS} to decline by 50% was calculated per tree and results per tree were subsequently analysed (n=5).

4.2.3.1 Drought Factor Index

Drought factor index (DFI) was calculated per tree. The DFI is based on the CF parameter performance index (PI) expressed as an absorption basis (ABS) (Oukarroum et al. 2007) Equation 4.2.

$$DFI = Log(PI_{ABS}^{1}) + 2Log(PI_{ABS}^{2})$$

Equation 4.2 Drought factor index (DFI) adapted from Oukarroum et al. (2007) where PI_{ABS}^{1} is the relative PI_{ABS} ($PI_{ABS}^{treated}/PI_{ABS}^{control}$) following one week of drought and PI_{ABS}^{2} is the relative PI_{ABS} following two weeks of drought.

4.2.3.2 IP-Phase

Drought stress has been shown to influence the latter stage of the OJIP polyphasic chlorophyll a fluorescence rise (Oukarroum et al. 2009). Characterisation of this phase may be beneficial for drought stress specific studies. This is possible by utilisation of the parameter known as the IP-phase (also known as 1-V_I) (Desotgiu et al. 2012).

$$IP - phase = 1 - V_I = \frac{F_M - F_I}{F_M - F_0}$$

Equation 4.3 IP-phase calculation. $F_I = F_{30ms} = F5$ (Oukarroum et al. 2009, Desotgiu et al. 2012, Banks 2017)

Characterisation of the differential IP phase has also been discussed to provide tolerance categorisation information (Oukarroum et al. 2009).

$$\Delta IP = 1 - V_I(control) - 1 - V_I\ (droughted)$$

Equation 4.4 IP-phase calculation. $F_I = F_{30ms} = F5$ (Oukarroum et al. 2009)

4.2.4 Visual Index (VI)

General tree health was visually assessed for each tree; crown structure, density and degree of dieback were assessed together with size, colour, density and necrosis of foliage. Visual indexing facilitates tree vitality, to be compared with other quantitative measures, and provides a rapid assessment of plant health useful to the tree selector. Trees were assessed in accordance with the following 1-5 scale:

1 66-100% of leaves affected, severe foliar discoloration and necrosis

2 36-65% of leaves affected, significant defoliation and/or leaf yellowing

3 5-35% of leaves affected with some yellowing but little or no defoliation

4 less than 5% of leaves affected and little aesthetic impact

5 No necrosis or aesthetic impact observed

On 9th August (310 hours) an additional surveyor was asked to rank the visual vitality of the trees independently to ascertain any bias present in the recording of visual indices.

4.2.5 Statistical analysis

Statistical analysis was performed using GenStat 17. Time series fluorescence data (Fv/Fm, 1-V_I and PI_{ABS}) was analysed using a residual maximum likelihood (REML) model. Following this, analysis of variance (ANOVA) was performed on processed data (processed data included: time for VI to reduce to 2, Fv/Fm, PI_{ABS}, and DFI^{PIABS}) to assess for differences between eight *Acer* genotypes. Cultivar was

nested within species for overall analysis. Post-hoc analysis was performed using a Bonferroni multiple comparison test at the 95% level.

Additional data interpretation and regression analysis were performed using Microsoft Excel 2013.

Spearman's rank correlation coefficient (ρ [rho] or r_s) was calculated to determine the similarity in ranking between each categorical variable on a per tree basis. The strength of rank correlations was interpreted using the following thresholds:

R_s	**Interpretation**
.00 - .19	very weak
.20 - .39	weak
.40 - .59	moderate
.60 - .79	strong
.80 – 1.0	very strong

4.3 Results

4.3.1 Soil Moisture

Percent volumetric water content (VWC) was confirmed to be significantly different between watered and droughted trees (p = <0.001). Results are displayed per cultivar in Figure 4.1.

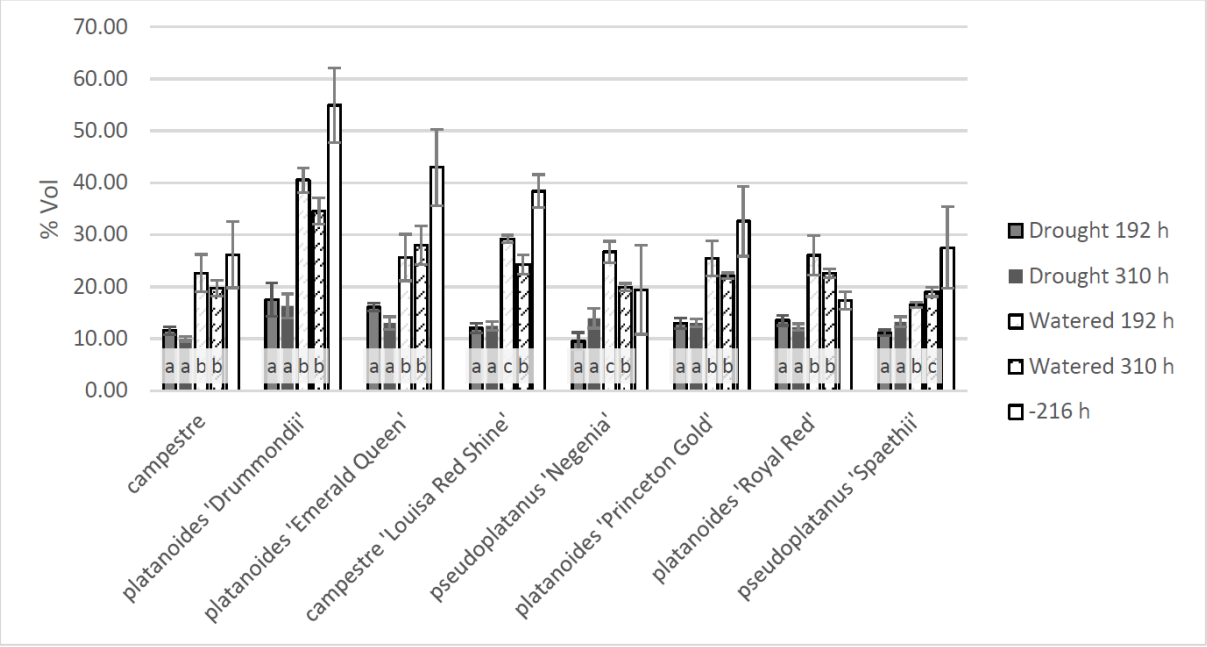

Figure 4.1 Percent volumetric water content for droughted and control cultivars, 192 hours and 310 hours following irrigation removal. Error bars show standard error, letters denote significant differences within cultivars (n = 5). -216 hours prior to drought initiation includes both droughted and watered groups it is included for comparison but omitted from statistical analysis (n = 2).

4.3.2 Visual index (VI)

There was a significant impact of drought on VI (p = <0.001). Regression analysis was used to predict the time taken for a genotype to decline to a VI of 2. A linear regression was fitted to data from each droughted tree in the trial, returning an average R^2 of 0.82. A significant interaction between species and cultivar (p = 0.007) was discovered. Species sensitivity was in the order *pseudoplatanus* (243.5) < *campestre* (296.1) < *platanoides* (647.1) (values in parentheses show average calculated hours to reach a VI of 2). Cultivar sensitivities are displayed in Figure 4.2.

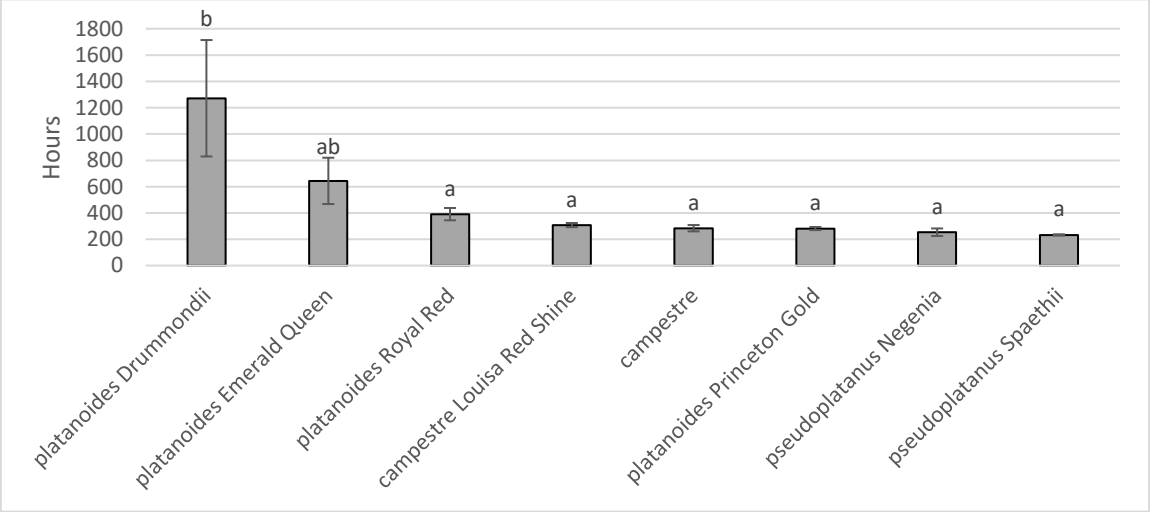

Figure 4.2 Mean time for visual index to reduce to 2 per tree. Error bars show standard error (n = 5). Letters denote significant differences between cultivars at the 95% confidence interval. Overall mean time = 458 hours. (Values including and above A. platanoides 'Royal Red' were calculated by extrapolation.)

4.3.3 Chlorophyll Fluorescence

A quadratic (order two polynomial) and exponential regressions were used to calculate the time for PI_{ABS} (R^2 = 0.76) and Fv/Fm (R^2 = 0.85) respectively to drop, from their origin, by 50%, this was performed for each droughted tree; and confirmed visually against the time-course graph for each tree. For this, we assumed no difference between hour -216 and hour zero, as it was not possible to measure at hour zero. The polynomial regression facilitated the model to account for the over-compensation effect reported to occur in PI_{ABS} in response to stress (Yordanov et al. 2008).

Significant species:cultivar interactions were discovered when using DFI-PI_{ABS} (p = 0.002) and time for Fv/Fm and PI_{ABS} to decline to the 50% (p = <0.001 and p = 0.043 respectively). Significant interactions suggest adequate sensitivity suitable to evaluate closely related genotypes. An alternative DFI using Fv/Fm did not identify significant species:cultivar interactions (p = 0.485) (data not shown).

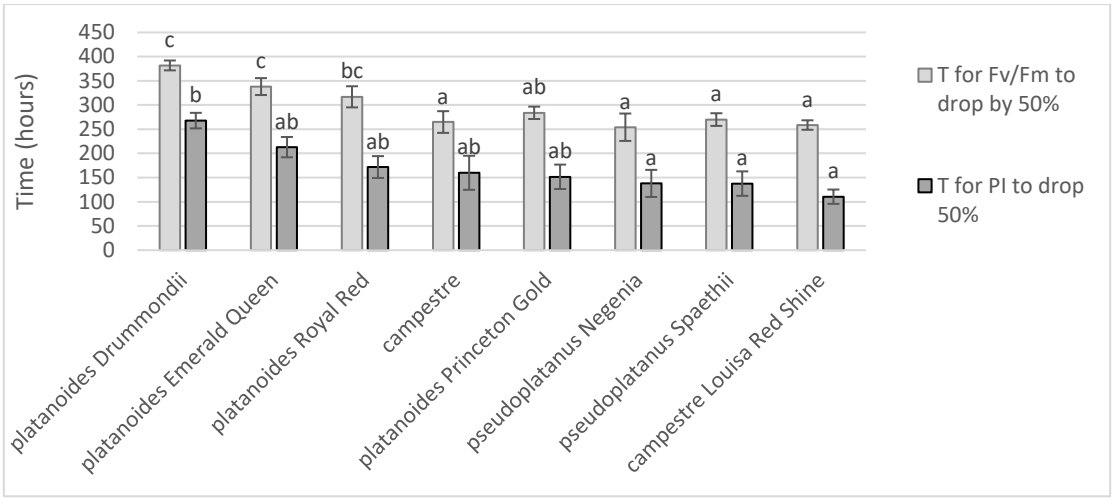

Figure 4.3 Time taken for Fv/Fm and PI to drop by 50%, calculated per tree. Error bars show standard error (n = 5). Letters denote significant difference between cultivars at the 95% confidence interval.

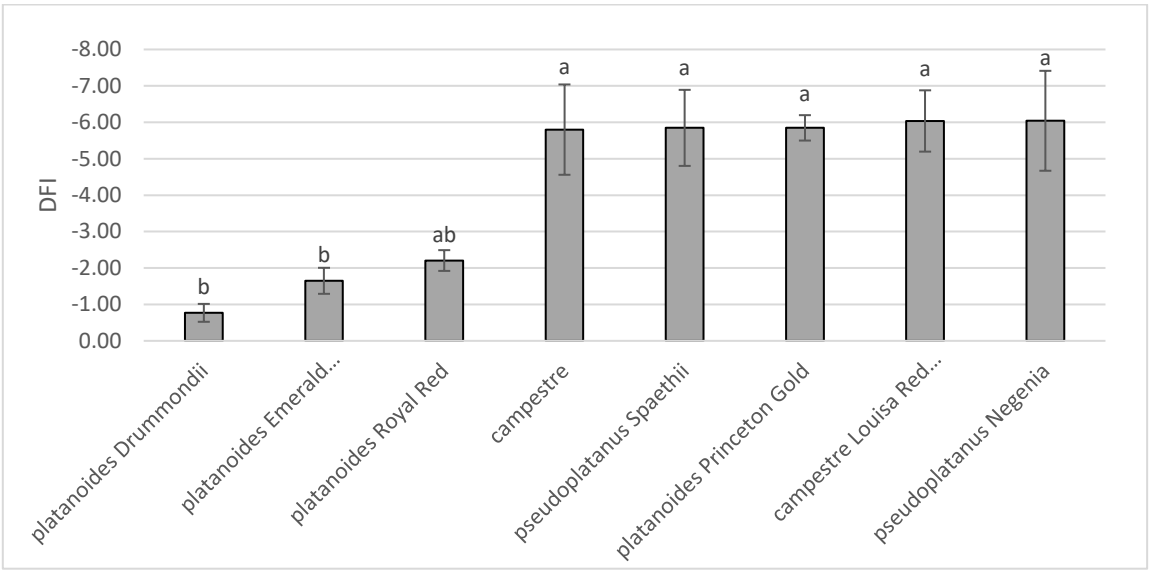

Figure 4.4 Drought Factor Index (DFI) calculated using PI_{ABS} per tree. Error bars show standard error (n = 5). Letters denote significant difference between cultivars at the 95% confidence interval.

4.3.4 Summary of Rankings

Table 4.2 Spearman's rank correlation coefficient matrix comparing results per tree for visual measurements against fluorescence data. Cells marked with identify values >0.8, bold cells identify values >0.6, VI = visual index, T= time, DFI = drought factor index.*

		1	2	3	4	5
T for VI to reduce to 2	1	1				
DFI	2	**0.71**	1			
ΔIP Phase	3	0.52	0.55	1		
T for Fv/Fm to drop by 50%	4	**0.62**	**0.82***	**0.65**	1	
T for PI_{ABS} to drop by 50%	5	0.55	**0.74**	0.35	**0.78**	1

A poor rank correlation was achieved when using ΔIP phase with all measurements other than time for Fv/Fm to drop by 50%. The highest rank correlation overall was achieved using time for 50% reduction in Fv/Fm. Therefore, this metric was compared with time for visual index to reduce to 2 in order to rank the genotypes tested. Based on these criteria, tolerance was ranked in the order: *A. platanoides* 'Drummondii' > *A. platanoides* 'Emerald Queen' > *A. platanoides* 'Royal Red' > *A. campestre* ≈ *A. campestre* 'Louisa Red Shine' ≈ *A. platanoides* 'Princeton Gold' ≈ *A. pseudoplatanus* 'Spaethii' ≈ *A. pseudoplatanus* 'Negenia'.

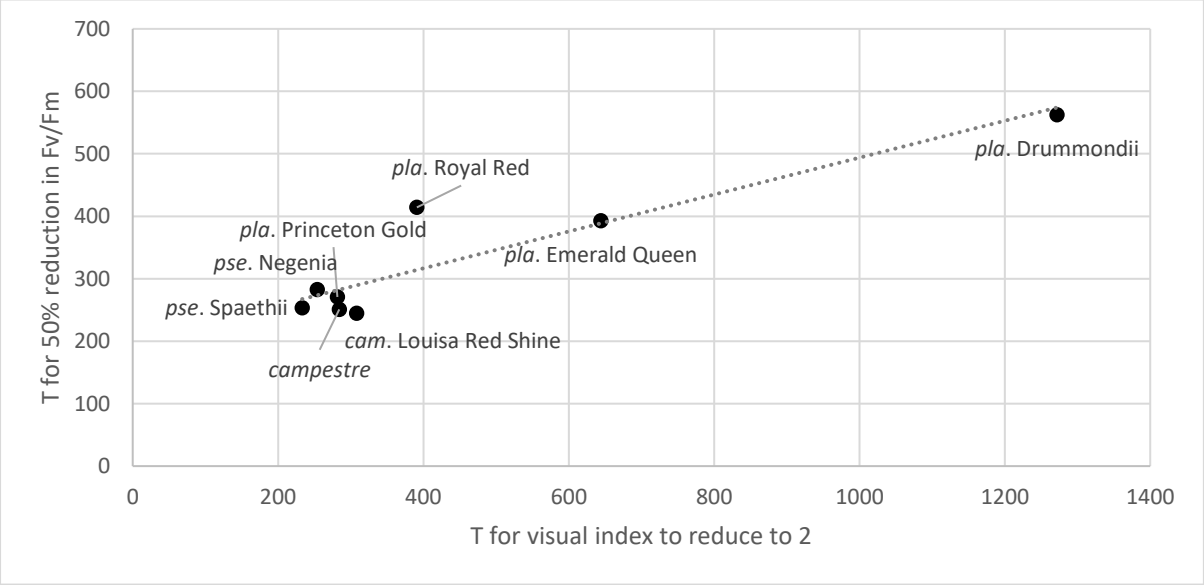

Figure 4.5 Average position of cultivars, relative to visual and chlorophyll fluorescence drought tolerance rankings. (y = 0.2953x + 198.5 R² = 0.8492). Species are: *pla. = A. platanoides, cam = A. campestre, pse. = A. pseudoplatanus.*

4.4 Discussion

According to the literature, *A. campestre* is considered the most drought tolerant species tested in this study (Niinemets and Valladares 2006, Bassuk et al. 2009). However, little information exists on the relative tolerance of cultivars present within this genus, with the exception of *A. pseudoplatanus* 'Spaethii' which is considered less tolerant than the straight species (Bassuk et al. 2009) and A. *platanoides* 'Royal Red' thought to be more tolerant than *A. platanoides* 'Crimson King' (Dirr 1990, Bassuk et al. 2009). Owing to the scarcity of information available, tree selectors are thought to use species as the tolerance determining factor and select cultivars thereafter based on aesthetic value (Vaz Monteiro et al. 2017) and other selection criteria relevant to the intended planting site. Results from this trial however, indicate significant drought tolerance variability within the species *platanoides*. Conversely, cultivars of *A. campestre* and *A. pseudoplatanus* exhibited limited intraspecific variation. Cultivars can be pooled to provide a species ranking of A. *pseudoplatanus* and *A. campestre* as drought sensitive and *A. platanoides* as drought tolerant based on this trial. However,

when pooling cultivars to estimate species tolerance it is important to clarify that this ranking is relative to the genotypes tested within this study.

Photosystem II is considered highly resistant to water shortage (Cornic and Fresneau 2002, Desotgiu et al. 2012). Despite this, chlorophyll fluorescence was shown to be more sensitive than visual indexing; a 50% reduction in PI_{ABS} took ca. 150 hours (Figure 4.3) whereas the same reduction in visual indexing took ca. 450 hours (Figure 4.2). The chlorophyll fluorescence parameter F_V/F_M has been used extensively to measure drought stress in: *Fraxinus spp.* (Percival et al. 2006), *Salix* (Ögren 1990), *Quercus petraea* (Epron and Dreyer 1992), and *Hordeum vulgare* (Barley) (Rong- Hua et al. 2006). The performance index has similarly been shown to provide a viable indication of drought and drought tolerance in: transgenic *Oryza sativa* (rice) (Redillas et al. 2011) *Hordeum vulgare* (barley) (Oukarroum et al. 2007) and *Triticum aestivum* (winter wheat) (Živčák et al. 2008). In outdoor experimental trials, parameters attempting to encompass the whole, or the extremes of the fluorescence transient, such as PI_{ABS} and Fv/Fm respectively, are likely to provide benefits where additional response intricacies and potential stressors may also be involved. Owing to the significant impact of genotype on non-treated values, data must be normalised, in this case, percent change was used to facilitate interpretation between genotypes. A strong (R_s 0.78) corroboration was achieved between ranks attained using Fv/Fm and PI_{ABS} (Table 4.2). The IP phase of the polyphasic chlorophyll fluorescence OJIP rise has been shown to respond more acutely than earlier phases in response to drought stress (Oukarroum et al. 2009). However, the parameter IP-phase and ΔIP-phase, discussed to identify drought tolerant varieties of barley and chickpea (Oukarroum et al. 2009), identified no correlation or similarity in ranking with all other ranking methods (Table 4.2). Additionally, no advantage in sensitivity, in comparison to other fluorescence methods, was observed when using IP-phase as a parameter alone (data not shown).

The DFI is based on the parameter PI_{ABS} and has been used to rank barley (*Hordeum vulgare*) cultivars tolerance following a controlled drought (Oukarroum et al. 2007). The origin of the DFI is the chill factor index described by Strauss et al. (2006). It is based on the assumption that tolerant genotypes are capable of maintaining a higher PI_{ABS} for longer than a sensitive genotype, therefore the relative importance of the response variable is increased later in the experiment. The time points chosen for inclusion in the DFI are dictated by the rate of decline in the given experiment. Values are therefore only relative to the specific trial; however, relative differences are comparable. DFI had a very strong and strong corroboration with time for Fv/Fm and PI^{ABS} to decline by 50% respectively, as discussed by Oukarroum et al., (2007) it is an efficient method of screening for drought tolerance in controlled trials. Comparing Figure 4.3 and Figure 4.4 however a lower sensitivity can be observed when using DFI in comparison to metrics using time to decline by 50%.

The speed of decline in this study was more severe than expected; in a study by Munns et al. (2010) in wheat and barley, Fv/Fm began to be impacted by drought at 25 days (600 hours), similarly Auge et al. (1998) identified lethal soil drying took between 26 and 87 days (624 – 2088 hours). Data is therefore representative of severe drought stress. Because of the speed and severity of drought, measurements of stomatal conductance were unsuitable for inclusion in this study; because of equipment limitations it was also not possible to measure water potential during this trial; however, these metrics are strongly recommended for monitoring future studies (Auge et al. 1998). From an urban tree selector perspective however, the aesthetic and survival implication of severe drought imposition is of primary importance, these characterises can be identified using visual indexing and chlorophyll fluorescence (Percival 2002). Under severe drought stress both avoidance and tolerance mechanisms are highly important. Tolerance mechanisms tend to maintain cellular turgor which is essential in maintaining metabolic processes (Touchette et al. 2007). Some drought avoidance strategies such as reductions in stomatal conductance and adaptations to foliage orientation will also lengthen the range of foliar function (Farooq et al. 2012). However, as previously mentioned, some avoidance strategies (leaf abscission and extensive rooting systems) are undesirable or ineffective in the urban environment.

4.5 Conclusion

The results of this study indicate the drought tolerance of the genotypes tested were in the order: *A. platanoides* 'Drummondii' > *A. platanoides* 'Emerald Queen' > *A. platanoides* 'Royal Red' > *A. campestre* ≈ *A. campestre* 'Louisa Red Shine' ≈ *A. platanoides* 'Princeton Gold' ≈ *A. pseudoplatanus* 'Spaethii' ≈ *A. pseudoplatanus* 'Negenia' (Figure 4.5). Significant cultivar variation was discovered in the species *A. platanoides*. These results strongly suggest that tree selectors should utilise empirical tolerance evaluations of cultivars before selecting cultivars for sites which are likely to experience drought stress. Fv/Fm and PI_{ABS} proved useful in the measurements of drought stress in this study, IP-phase did not provide any additional sensitivity during this study.

4.6 Acknowledgements

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4.7 Reference List

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5 Alternative Methods of Estimating the Water Potential at the Turgor Loss Point:

Evaluating closely related *Acer* genotypes using pressure-volume curves in comparison to vapour-pressure osmometer and dewpoint hygrometer measurements.

Jonathan M. Banks[A,B*], Andrew D. Hirons[C]

*[A] School of Agriculture Policy and Development, The University of Reading, Reading, Berkshire RG6 6AR, UK; [B] Bartlett Tree Experts, Research Laboratory (UK) Reading, Berkshire RG2 9AF, UK; [C] University Centre Myerscough, Bilsborrow, Preston, Lancashire PR3 0RY, UK. *correspondence author. E-mail:*

Abstract: Selecting for drought tolerance in urban tree species can have a significant influence on survival rates, aftercare requirements and performance. The water potential at turgor loss point (π_{tlp}) is gaining popularity as a trait to help determine drought tolerance to aid tree selection. Therefore, it is important to understand if differing methods used to measure or calculate π_{tlp} deliver consistent results. The sensitivity of three methods used to determine this valuable selection parameter were evaluated. A classical pressure chamber, pressure-volume (P-V) curve method was compared with vapour-pressure osmometer (Vapro®) and dewpoint hygrometer (WP4C) methods. These methods were evaluated using closely related cultivars of *Acer platanoides* and *A. pseudoplatanus* 'Negenia'. Both the osmometer and hygrometer methods ranked genotypes with a very high similarity (R_s = 1, R^2 = 0.96) and were able to identify significant differences between cultivars. This is the first study to demonstrate suitability of the dewpoint hygrometer in comparison to the vapour-pressure osmometer to measure π_{tlp}. The P-V method was unable to identify differences between the cultivars tested. The Vapro and WP4C provide greater applicability than the conventional P-V method to studies requiring both high throughput and high sensitivity. Consistency of measurement type is however highly recommended in future studies as some differences were observed between Vapro and WP4C.

Key-words: turgor loss point, pressure-volume curve, drought tolerance, pressure-bomb, osmometer, hygrometer

5.1 Introduction

Trees within the urban environment often experience abiotic stresses (Gibbs and Palmer 1994, Jim 1998, Scharenbroch et al. 2017); that in-turn, can increase susceptibility to pest and diseases (Cregg and Dix 2001). Selecting for drought tolerance in urban tree species can have a significant influence on survival rates, aftercare requirements and future aesthetic and environmental benefits (Bassuk et

al. 2009, Roman et al. 2014, Sjöman et al. 2015). Tree selection is often focused on aesthetic characteristics (Vaz Monteiro et al. 2017), however, when tolerance is considered it is often based on personal experience and observation. Data from plant-use literature and scientific studies is frequently inconsistent between sources and often lacks specificity (Sjöman et al. 2015, 2018a). Increases in the frequency and severity of drought events are expected as a result of climate change (IPCC 2007, Bartlett et al. 2014, Pflug et al. 2018). Informed tree selection based on physiological or genetic drought tolerance traits is therefore increasingly desirable, facilitating selection for current and future environmental demands (Cattivelli et al. 2008). Foliar physiological traits are gaining popularity as they can determine physiological drought tolerance as opposed to drought avoidance strategies (Sjöman et al. 2015). Genotypes which avoid drought may shed leaves in response to drought stress or rely on extensive root systems to gather water (Mauseth 2011, Hirons and Thomas 2018), these strategies are not desirable for urban sites. Urban tree selection is clearly more nuanced than simply consideration of functional traits; however, improvements to current tolerance information is essential to aid and encourage appropriate selection (Desclaux et al. 2000). One physiological trait capable of identifying drought tolerance is the measurement of leaf water potential at permanent wilting or turgor loss (π_{tlp}) (Sack et al. 2003, Bartlett, Scoffoni, and Sack 2012). This trait is capable of characterising intraspecific drought tolerance (Sjöman et al. 2015). Techniques are now available to increase the speed of this measurement (Bartlett, Scoffoni, Ardy, et al. 2012) facilitating ecological scale studies (Maréchaux et al. 2015) and studies to aid appropriate tree selection between and within genera (Sjöman et al. 2015, 2018a, 2018b). Therefore, a range of approaches are currently being used to determine π_{tlp}. However, no study has evaluated the sensitivity of these alternative methods among closely related cultivars. In this study, the so-called direct measurements, using a vapour-pressure osmometer and dewpoint hygrometer to measure water potential are compared with a classical pressure-volume (P-V) curve method, measured on adjacent leaves. In this study, closely related genotypes are used to allow the sensitivity of measurement method to be evaluated.

P-V curves are the classical method of inferring a range of plant-water relation parameters (Tyree and Hammel 1972) and can provide information on genotypic drought tolerance using the parameter π_{tlp} (Jane and Green 1983, Baltzer et al. 2008, Bartlett, Scoffoni, and Sack 2012). A more negative π_{tlp} lengthens the functional range of foliar water potential (Lenz et al. 2006) and is thought to be achieved by a combination of osmotic adjustment (solute accumulation to increase cell hydration) and elastic adjustment (decreasing the point at which turgor loss occurs) (Dreyer et al. 1990, Sanders and Arndt 2012). π_{tlp} is now considered the dominant determining factor of drought tolerance (Dreyer et al. 1990, Bartlett, Scoffoni, and Sack 2012). The production of P-V curves has one significant disadvantage; they are time-consuming to produce, meaning adequately large scale studies and

genetic screening are impractical (Corcuera et al. 2002, Callister et al. 2006, Sanders and Arndt 2012). Additionally, despite P-V curves being widely regarded as the classical method for determining water relation parameters, the comparative accuracy between methods has been frequently criticised (Parker and Pallardy 1988, Parker and Colombo 1995). This warrants further studies investigating alternative methods of measuring water potential (Ψ) in plant tissue (Richter 1978, Callister et al. 2006, Martínez et al. 2013).

Direct measurements (vapour-pressure osmometer and dewpoint hygrometer), are rapid methods used to determine water potential (Callister et al. 2006). The dewpoint hygrometer (such as the WP4C, decagon devices Inc. München, Germany) uses the chilled-mirror dewpoint technique (Campbell et al. 1973) measuring water potential from zero to -300 MPa on *ca.* 35 mm diameter leaf discs (Decagon Devices 2007, Nardini et al. 2008). The dewpoint hygrometer measures the sum of osmotic and matric potential; it has been used successfully on leaves of tobacco and ivy (Nardini et al. 2008) and flowers of slipper orchid (Zhang et al. 2017). Relative difference between dewpoint hygrometer and pressure chamber measurements of water potential have been shown to be very similar (R^2=0.84) (Martínez et al. 2013). The vapour-pressure osmometer (Vapro®, Wescor, Logan UT, USA) measures solute concentration (osmolality) which can be converted to water potential using the Van 't Hoff equation; it can measure leaf discs of ca. 8 mm diameter (Callister et al. 2006, Maréchaux et al. 2015) or expressed sap (Turner 1981, Callister et al. 2006). Callister et al. (Callister et al. 2006) show osmometer osmotic potential (π) measurements of expressed sap are comparable with those of parallel π pressure chamber readings. Bartlett et al. (Bartlett, Scoffoni, Ardy, et al. 2012) show that measurements of π on rehydrated freeze-thawed leaf discs can rapidly determine the osmotic potential at full rehydration or full turgor (π_0). Bartlett et al. (Bartlett, Scoffoni, Ardy, et al. 2012) also demonstrated that π_0 correlates to the π_{tlp}. They used vapour-pressure osmometer measurements taken from plants which had pressure chamber derived P-V curves, determined within four weeks of each other, for sixteen species. However, for fourteen additional species, the P-V curves had been calculated within the previous two years (Bartlett, Scoffoni, Ardy, et al. 2012). Significant adjustment of π_{tlp} is known to occur across a single season (Sjöman et al. 2015); however, additional meta-analysis has also shown a good correlation between π_0 and π_{tlp} (Bartlett, Scoffoni, and Sack 2012), adding further validity to the measurement despite potential issue with the timing of the initial data collection. Sufficient evidence now exists to warrant large scale evaluations of π_{tlp}, calculated from π_0, using a vapour-pressure osmometer (Maréchaux et al. 2015, Sjöman et al. 2015, 2018a, 2018b). However, it is not yet clear if a dewpoint hygrometer can be used to evaluate π_{tlp}. Therefore, this study aims to evaluate the accuracy of osmometer and hygrometer measurements in direct parallel to P-V curves using very closely related *Acer* genotypes.

5.2 Materials and Method

5.2.1 Plant material

Thirty-two seven-year-old, 4 m tall trees were used for this experiment arranged across three completely randomized linear rows. The following *Acer* genotypes were measured during this trial: *A. platanoides* 'Drummondii', *A. p.* 'Emerald Queen', *A. p.* 'Royal Red', *A. p.* 'Princeton Gold' and *A. pseudoplatanus* 'Negenia'. All measured cultivars were grafted onto their respective species-type rootstocks. Trees were potted during the winter of 2013/14 and grown at Barcham Trees nursery, Ely, Cambridgeshire, UK (52.366923° N, 0.315864° W) prior to being planted outside in March 2017 at the Bartlett Tree Research laboratory, Shinfield, Reading, Berkshire, UK (51.412393°N, -0.937909°W). Encircling roots were cut on all trees to aid establishment during the planting process. Trees were arranged across three rows, each measured cultivar was randomized within each row.

5.2.2 Sample preparation

Two visually healthy leaves were removed *ca.* 30 cm below a terminal bud on the lower limb (ca. 2 m high) of each tree; opposite leaves were selected to ensure the closest similarity in physiological age. Leaves were collected between 16:00 and 17:00 on the 24th July to the 9th of August 2017. Leaves were removed from the tree by snapping at the axil union and immediately returned to the laboratory (within <2 minutes). In the laboratory, leaves were immediately weighed and petioles re-cut underwater (ca. 1 cm away from the petiole base), petioles and cut petiole portions were left in water to fully hydrate in the dark for ca. 12 hours. Hydrating leaves were left in an insulated container during this time kept near 100% relative humidity (average vapour-pressure deficit (Allen et al. 1998) equalled 0.01 (±0.03)). Individual, fully hydrated leaves were removed from the container, patted dry and immediately weighted and processed using either the pressure chamber P-V curve method or direct methods.

5.2.3 Pressure-Volume Curves

Pressure-volume curves were calculated in accordance with the sap expression method; the method was similar to that used by Parker and Pallardy (Parker and Pallardy 1988). Whole undamaged leaves were sealed inside a pressure chamber (model 600D, PMS instruments Co., Albany, USA) with a piece of damp filter paper to reduce water loss. The average initial balance pressure was -0.13 MPa (± 0.007). Leaves which did not hydrate to an initial Ψ of >-0.2 MPa were discarded (Lenz et al. 2006). Incremental pressures of 0.2 MPa were applied to the leaf, beginning at 0.2 MPa. P-V curves were halted at -2.4 MPa or when greater than three data points were in the linear portion of the graph. Total expressed sap at each pressure was absorbed in pre-weighed 1.5 ml Eppendorf tubes filled with dry low-lint absorbent tissue paper (Kimtech Science, Kent, UK). Tubes were handled and opened for

the minimum possible time during sap collection to prevent evaporation. Leaves were weighed immediately following the final measurement, facilitating determination of the average uncollected water (4.7%). Leaves were then dried for >48 hours at 60°C. P-V curves were plotted as 100-RWC (relative water content) (D) on the x axis, against -1/MPa (y axis). RWC was calculated following the method of Lansac et al. (1994). Overhydration, or plateau effects were corrected where appropriate in accordance with the method described by (Dichio et al. 2003). Water potential at the turgor loss point (π_{tlp}) was calculated based on a method developed by Schulte and Hickley (Schulte and Hinckley 1985), obtained from: landflux.org/resources/PV_Curve_Fitting_5.6.xls. This method has also been used by (Bucci et al. 2004, Mitchell et al. 2013, Arndt et al. 2015).

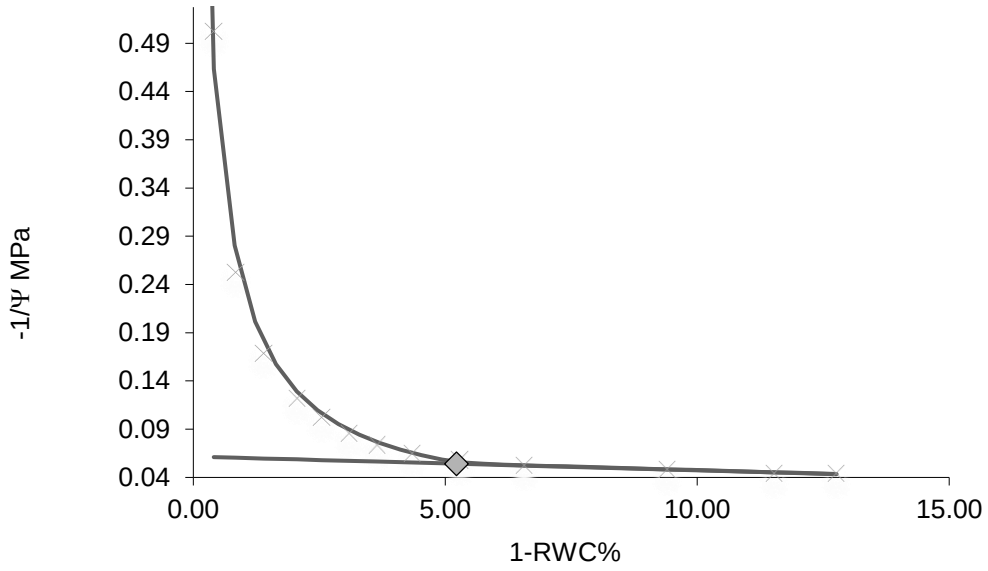

Figure 5.1 Representative Pressure-Volume curve. The square indicates the turgor loss point.

5.2.4 Direct Measurements

Two leaf discs, 35 mm and 8 mm diameter, (dewpoint hygrometer and vapor pressure osmometer respectively) were taken between the mid-rib and margin on the lower quartile of the opposing leaf used in the P-V curve. Leaf discs were foil wrapped and submerged in liquid nitrogen. Prior to the measurement, leaf discs were punctured 10-15 times with sharp-tipped forceps to improve equilibration times (Maréchaux et al. 2015).

Dewpoint hygrometer (WP4C, decagon devices Inc. München, Germany) measurements were taken with the device in its continuous mode, connected to the AquaLink data logging software (decagon devices) on a laptop computer. One measurement per leaf was recorded when values became stable (ca. 15-20 min.). Stability was assessed graphically for each leaf disc. The WP4C measures total water potential which is the sum total of gravitational, matric, osmotic and pressure potentials. In freeze

thawed leaf discs it is putatively assumed that gravitational, matric and pressure potentials are all zero or negligible, therefore, in this study, osmotic potential is the considered component.

Osmometer measurements were taken with a vapour pressure osmometer (Vapro 5600, Wescor, Logan UT, USA) using the standard 10µl chamber. Measurements were made in accordance with the method detailed by Sjöman et al. (Sjöman et al. 2018a).

For measurements made using the Osmometer, solute concentration (mmol kg^{-1}) was converted to water potential using Van 't Hoff's equation:

$$\pi_0 = -CRT$$

Equation 5.1 Van 't Hoff Equation, where C is the molar solute concentration (mmol kg^{-1}), R is the universal gas constant (8.3144598E-0.6) in m^3 MPa K^{-1} mol^{-1}, T is the temperature (K) (Khare 2015).

Dewpoint hygrometer and vapor pressure osmometer are hereafter referred to as WP4C and Vapro for simplicity.

Both direct measurements were converted into $\hat{\pi}_{pv}$ using the equation determined by Bartlett et al. (Bartlett, Scoffoni, Ardy, et al. 2012).

$$\hat{\pi}_{pv} = 0.587\pi - 0.546$$

Equation 5.2 conversion from osmometer measurement (π) to predicted P-V (π_{pv}) measurement (Bartlett, Scoffoni, Ardy, et al. 2012).

π_{tlp} was calculated from π_0 using the regression equation adapted for temperate species by Sjöman et al. (Sjöman et al. 2015) originally calculated from the supplementary data published by (Bartlett, Scoffoni, and Sack 2012):

$$\pi_{TLP} = -0.2554 + 1.1243 \times \pi_0$$

Equation 5.3 Adapted equation facilitating prediction of π_{TLP} (Ψ_{P0}) from π_0 ($\Psi_{\pi 100}$) (R2=0.91) (notation in parentheses is the notation used by Sjöman et al. (Sjöman et al. 2015). *The notation used here correspond to Bartlett et al.* (Bartlett, Scoffoni, and Sack 2012).

5.2.5 Statistical analysis

Statistical analysis was performed using GenStat 17th edition. Following tests for normality, analysis of variance (ANOVA) was used to test for differences between means. Linear regression (R^2) and Spearman's rank correlation coefficient (r_s) was also calculated in order to describe the relationship between readings. Post-hoc analysis was performed using a Tukey's 95% confidence interval.

5.3 Results

A significant effect of both genotype and measurement method (p = <0.001 for both) was observed. However, a significant interaction between genotype (cultivar) and method was observed following a

two-way ANOVA (p = <0.001). Data was therefore compared overall with cultivars nested within measurement method.

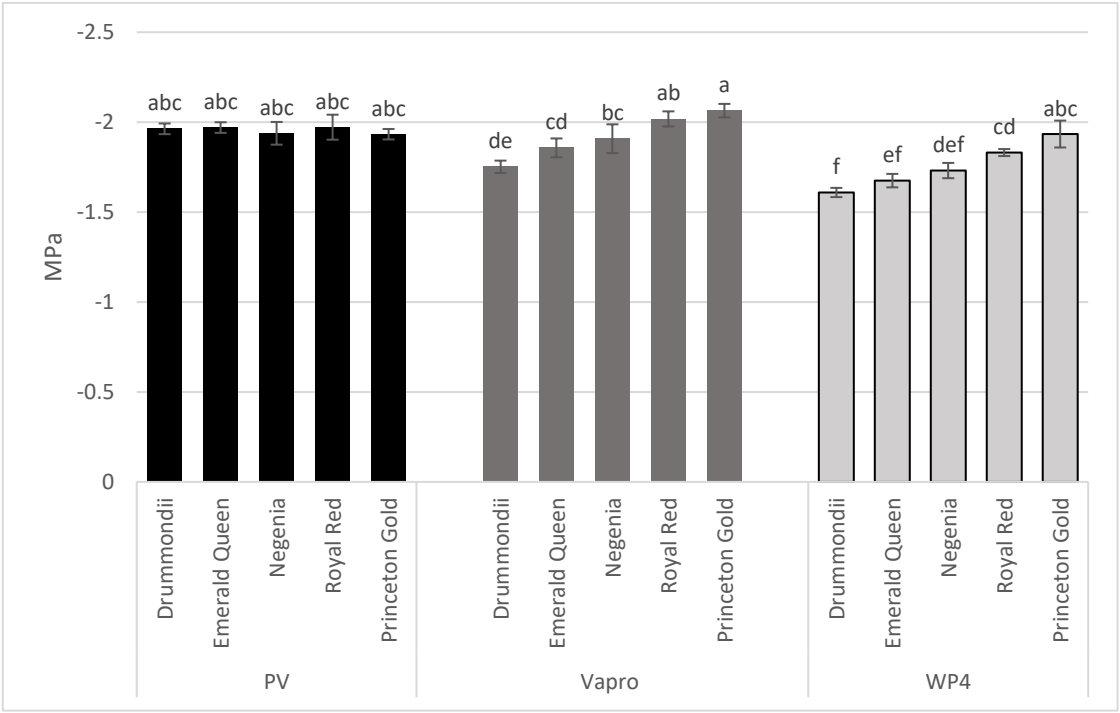

Figure 5.2 Showing πtlp for each method. Error bars show standard error. Letters denote significant differences (Duncan multiple range test) between cultivars nested in method at the 95% confidence interval. A. platanoides 'Drummondii', A. p. 'Emerald Queen', A. p. 'Royal Red', A. p. 'Princeton Gold' and A. pseudoplatanus 'Negenia'. Between species analysis for each measurement method P = <0.001 for Vapro and WP4C, P = 0.938 for P-V πtlp.

Similarities between measurements was determined using a correlation coefficient (R^2) and Spearman's rank correlation coefficient (R_s). P-V measurements were excluded from correlation comparisons as no significant differences were discovered between cultivars. The Vapro and WP4C provided the same rank (R_s = 1) and highly similar correlation coefficient (R^2 = 0.96). Values of π_0 provided the same comparative ranking as π_{TLP}.

Correcting measurements using Equation 5.2 is highly important, especially if values are to be compared against P-V curve data. Equation 5.2 improved similarity to P-V curves by an average of ca. 5% for both Vapro and WP4C. However, in this study significant and species-specific differences occurred with both devices when compared to the P-V method (Figure 5.2).

5.4 Discussion

In this study, the pressure chamber pressure-volume (P-V) curve method was unable to identify significant differences between the closely related cultivars tested (p = 0.938). However, both direct measurements tested (WP4C and Vapro), identified highly significant differences between cultivars (p = <0.001). This is the first study to our knowledge to demonstrate the suitability of the dewpoint hygrometer (WP4C) in comparison to the P-V curve and vapour-pressure osmometer (Vapro) methods

when measuring π_{tlp}. Significant differences between measurement methods were present for all cultivars except *A. pseudoplatanus* 'Negenia' ($p = 0.092$) and *A. platanoides* 'Princeton Gold' ($p = 0.112$) despite the use of the correction factor described by Bartlett et al. (Bartlett, Scoffoni, Ardy, et al. 2012) (Equation 5.3). No difference in rank however was observed between the WP4C and Vapro ($R_s = 1$). The Vapro returned results comparably closer to those from P-V curves. The Vapro and WP4C differed from P-V values at an average 0.04 MPa (±0.055) and 0.20 MPa (±0.057) respectively, these differences are not however thought to be practically significant for species selection. Therefore, either device can be utilised for tolerance studies.

As suggested by Zhang et al. (Zhang et al. 2017) and Martínez et al. (Martínez et al. 2013) more negative values (average -26.5%, without correction, Equation 5.3) were observed using both devices in comparison to the pressure chamber. Many theories exist to explain why thermocouple and hygrometer devices measure more negatively than pressure chambers, including water loss during leaf excision as well as active accumulation of solutes by neighbouring undamaged tissue (Barrs and Kramer 1969, Martínez et al. 2013). Zhang et al. (Zhang et al. 2017) however, also discuss simply that the measurement is of the air above the sample, thus a more negative Ψ is returned. It is however imperative that the air above the sample is in equilibration with the sample, consequently we assume Zhang et al. (Zhang et al. 2017) discussion is based on the assumption that losses in water potential may occur in locations where sample water potential is more negative than ambient humidity. Therefore, a decrease in sample water potential would occur in order to reach equilibration. If this was the case, more negative values would be expected from the WP4C owing to the greater leaf to chamber volume (0.27 $mm^3\ ml^{-1}$ vs 2.3 $mm^3\ ml^{-1}$ for WP4C and Vapro respectively); in this trial this did not occur.

In some circumstances, utilisation of the Vapro device can be recommended; the larger leaf disc size required by the WP4C, reduces the ability to evaluate plants with smaller or more complex leaf areas without adaptation of the method. Previous studies have also utilised the Vapro to evaluate relatively large genotypic selection (Sjöman et al. 2015, 2018a, 2018b). In future studies, we recommend a process of cross calibration with previous studies using species in common in order to place genotypes within the drought tolerance continuum.

5.5 Conclusion

The Vapro and WP4C provide greater applicability than the conventional P-V method to studies requiring high throughput and high sensitivity. Data presented here reveals the sensitivity of the vapour-pressure osmometer and dewpoint hygrometer methods to measure π_{tlp} characterising the drought tolerance of closely related genotypes. Data identifies no difference in rank between results

from both WP4C and Vapro. Some significant differences were however observed between Vapro and WP4C (Figure 5.2) therefore consistency of measurement type is recommended in future studies. Poor sensitivity was observed when using the P-V method, therefore, future studies should utilise either the vapour-pressure osmometer or dewpoint hygrometer in order to provide rapid and sensitive genotypic drought tolerance quantifications.

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6 Variations in Seasonal Drought Tolerance Rankings

Jonathan M. Banks[1,2], Glynn C. Percival[1], Gillian Rose[2]

[1] *Bartlett Tree Experts, Research Laboratory (UK) Reading, Berkshire RG2 9AF, UK.*

[2] *School of Agriculture Policy and Development, The University of Reading, Reading, Berkshire RG6 6AR, UK.*

Abstract: Drought is one of the most common and significant disorders affecting tree establishment and growth, in the urban environment. Consequently, improving tree selection by the provision of quantifiable tolerance data by which to evaluate genotypes is an important area of scientific research. A number of in vitro studies address drought tolerance in isolation, at a single time point during the growing season. Using an in vitro foliar dehydration method to evaluate closely related cultivars of *Acer platanoides, A. pseudoplatanus* and *A. campestre* drought tolerance rankings were not found to be consistent throughout the growing season (spring, summer and autumn). i.e. A. *pseudoplatanus* 'Negenia' was found to be comparatively drought tolerant in spring, but displayed moderate to low comparative tolerance in summer and autumn; as measured using in vitro foliar dehydration as a proxy for drought tolerance. Drought conditions are most severe and common in summer; however, this study discusses the need to consider spring tolerance in addition to summer or autumn, particularly when selecting trees for tough urban sites, prone to drought stress across all three seasons.

Keywords: drought, drought tolerance, foliar dehydration, turgor loss point, *Acer*

6.1 Introduction

Drought is one of the most common and significant disorders affecting tree establishment and growth (Aranda et al. 2012). The frequency and intensity of drought events is likely to increase with climate change (IPCC 2007, Bartlett et al. 2014, Pflug et al. 2018); this is particularly true within urban areas, where root zone limitations (Kopinga 1991, Volder et al. 2009) and the urban heat island effect (Cregg and Dix 2001) decrease water availability and increase vapour pressure deficit and evapotranspiration respectively. Species selection can significantly affect survival prospects (Roman et al. 2014). Low-value and short-lived plants can be rapidly replaced with plant material that exhibits more appropriate tolerance characteristics. However, for longer-lived and higher-value plants, such as urban trees, replacement is undesirable. Appropriate tree selection for future climatic conditions is therefore of

increasing importance to prevent losses due to inappropriate selection (Roman et al. 2014). Tree selectors however, have been criticised for selecting trees based on aesthetic criteria, rather than tolerance traits (Vaz Monteiro et al. 2017). Additionally, the information available to tree selectors is often ambiguous, conflicting and lacking in appropriate specificity (Sjöman et al. 2015). A number of in vitro empirical methods are currently available which can address some of the problems associated with the current paradigm of tree selection. However, the seasonal specificity of such tolerance evaluation methods requires further study. It is clear that drought tolerance is a trait which develops (Sjöman et al. 2015) as environmentally imposed drought risk increases. However, it is not yet clear if, or at what point, tolerance rankings maintain consistency throughout the course of a growing season.

Large scale drought trials are important (Ryan 2011) but not generally practical to undertake on a wide range of trees owing to cost and time needed to reach maturity in comparison to most crop plants. In vitro experimentation is therefore essential to evaluate whole tree genotypic drought tolerance without undertaking whole tree drought trials. Percival & Sheriffs (2002) and Faraloni et al. (2011) show dehydration of excised foliage can act as a viable proxy for whole-plant drought experiments. Plant root detachment and dehydration has also been used to represent drought stress in *Arabidopsis* rosettes (Catala et al. 2007). Leaf dehydration studies account for foliar anatomical and morphological adaptations such as trichomes, sunken stomata, thick cuticles and reduced leaf size. Another method currently gaining traction is the measurement or estimation of water potential at the turgor loss point (π_{tlp}) (Maréchaux et al. 2015, Sjöman et al. 2015, 2018); this measure is indicative of the permanent wilting point (Bartlett, Scoffoni, and Sack 2012). The π_{tlp} is known also to reflect the point below which a plant cannot take up sufficient soil water, and is therefore considered a 'higher-level' drought tolerance trait (Bartlett et al. 2014). Foliar-focused measurements are unlikely to account for whole plant adaptations, such as foliage shedding, stem water storage mechanisms or structures (e.g. succulents or cacti) and deep rooting strategies, further discussed by Bartlett et al. (2012). Experimentation has however revealed the validity of foliar-focused measurement's when compared against whole plant studies, either directly or via meta-analysis (Percival and Sheriffs 2002, Bartlett, Scoffoni, and Sack 2012, Bartlett et al. 2014). Additionally, many drought avoidance mechanisms (foliage shedding) are undesirable for trees planted in the urban environment (Sjöman et al. 2015). Measurements which exclude these avoidance factors, such as measurements of π_{tlp} and foliar dehydration studies, are therefore of potential benefit to those involved in the selection of urban trees.

A tree's ability to adapt to drought stress events throughout a season facilitates a balance between the costs and benefits of maintaining tolerance traits (Postma and Jaramillo 2008, Bigler 2016). Most water is lost from the leaves of woody plants (Pallardy 2007); therefore, adaptations are primarily

located in the foliage. Stomatal closure is the primary means of reducing water loss, maintaining turgor and extending the range of foliar function under minor to moderate drought (Arndt et al. 2001). However, this reduces the rate of photosynthesis while increasing the risk of photoinhibition (Takahashi and Murata 2008). Under more severe drought, developmental water deficit tolerance mechanisms involve the maintenance, and extension of cellular tolerance to reduced relative water content (RWC). Plants can alter their drought tolerance by reallocation of root/shoot dry matter and other molecular changes (Kozlowski and Pallardy 2002). True tolerance mechanisms however, involve the maintenance of cellular turgidity through cellular osmotic potential (osmoregulation) and tissue elasticity (Kozlowski and Pallardy 2002), both mechanisms and are capable of fluctuating throughout the course of a season.

Osmoregulation involves the local synthesis of osmotically active metabolites, resulting in osmotic adjustment (Arndt et al. 2001, Burg and Ferraris 2008), increasing osmotic gradients and maintaining cellular turgidity (Farooq et al. 2012). Turgor maintenance is required in order for growth to continue during drought events (Clifford 1998). Increases in inorganic ion concentrations (sodium, calcium and potassium) for this purpose however, can perturb protein function (Burg and Ferraris 2008). Therefore organic osmolytes are also used, such as proline and glycine betaine (Bohnert 1995). Significant growth period, seasonal, environmental and species-specific fluctuations in osmolytes are known to occur (Bohnert and Jensen 1996, Murakeözy et al. 2003, Regier et al. 2010, Ryan 2011). Current evidence suggests osmolyte levels in foliage are highest in spring and lowest in the summer months (Lansac et al. 1994, Murakeözy et al. 2003, Bandurska et al. 2009). Conversely, inorganic ions and carbohydrates tend to accumulate across the seasons (Murakeözy et al. 2003); however, significant seasonal and intraspecific variations in stored and utilised carbohydrates in response to drought are known to occur (Regier et al. 2010, McDowell 2011, Ryan 2011, Brunner et al. 2015). Osmoregulation is not however a universal strategy (Pallardy 2007); *Acer saccharum* (Bahari et al. 1985), *Fagus sylvatica* and *Quercus petraea* (Backes and Leuschner 2000), for example, were reported to exhibit little or no osmotic adjustment.

Tissue elasticity or elastic modulus (ε) can also influence drought tolerance variation throughout a season. Elastic modulus is defined as the pressure required to cause a unit change in cell volume (Verslues et al. 2006). It is well documented that (other variables being equal) a more elastic tissue, low ε, is more capable of maintaining turgor under relatively large water losses (Kozlowski and Pallardy 2002, Verslues et al. 2006, Pallardy 2007). However, highly elastic cells which exhibit significant osmotic adjustment may be at risk of damage upon rehydration (Clifford 1998). Decreased elasticity (greater rigidity, high ε) tends to maintain RWC as water potential and cell volume decrease (Clifford 1998, Pallardy 2007). This is observed in scleromorphic and coriaceous (texture of leather) leaves

which are often considered drought resistant (Ogaya and Peñuelas 2006, De Micco and Aronne 2012). Both increases and decreases in elasticity therefore have been associated with tolerance to drought (Clifford 1998, Pallardy 2007). *Acer saccharum* trees have been shown to display seasonal variations in ε from ca. 5 MPa in mid-May to 18 MPa in July, lowering again to 7 MPa in October (Tyree et al. 1978). However, differences in elastic response to drought have been noted to diverge even in closely-related species of the same genus (Pallardy 2007). Divergence may in some cases however, be as a result of other ecological strategies such as resistance to herbivory (Powell et al. 2017).

Acer saccharum has been shown to exhibit little osmoregulation through the seasons (Bahari et al. 1985). However, significant variation in elasticity is recorded by Tyree et al. (1978). They also noted seasonal variations in π_{tlp} (incipient plasmolysis), identifying values ca. 1 MPa in May rising rapidly to ca. 1.8 MPa by June before gradually rising to ca. 2 MPa by October (Tyree et al. 1978). Sjöman et al. (2015, 2018) also identify seasonal change in π_{tlp} within a range of *Acer* and *Magnolia* species and cultivars. They identify that spring tolerance is lower and the species tested rank differently in comparison to summer tolerance (Sjöman et al. 2015, 2018). Little information is however available to suggest if tolerance levels are maintained or decline following summer tolerance acquisition. Additional data regarding seasonal variation is not available for other species and cultivars within the *Acer* genus. A range of information is important as differences in both osmoregulation and elastic adjustment have been noted in closely-related species (Pallardy 2007). This study uses species and cultivars from the *Acer* genus as a model for the drought tolerance of very closely related deciduous tree species. The seasonal consistency of foliar desiccation is determined and compared to summer measurements of π_{tlp} and whole tree drought tolerance.

6.2 Materials and Methods

6.2.1 Plant Material

All *Acer* species represented within this trial are commonly utilised as urban street trees. A range of genotypes with contrasting intraspecific foliar and crown characteristics were selected. All cultivars used were grafted onto their respective species-type rootstocks. Eighty, seven-year-old 4.2 m (± 0.3) tall trees with a DBH of 50.7mm (±7.9) growing in 45 litre Light Pots™ (white, mypex woven grow bags) grafted onto their respective species-type rootstocks were used for this experiment. The potting substrate consisted of a 50:50 green waste and pine bark compost mix, with a pH of 7.7. Eighty trees from the following genotypes were used: *A. campestre, A. campestre* 'Louisa Red Shine', *A. platanoides* 'Drummondii', *A. platanoides* 'Emerald Queen', *A. platanoides* 'Princeton Gold', *A. platanoides* 'Royal Red', *A. pseudoplatanus* 'Negenia', and *A. pseudoplatanus* 'Spaethii'. For the duration of this experiment, all trees were kept outside, at Barcham Trees nursery, Ely,

Cambridgeshire (52.366923° N, 0.315864° W). All experimental trees were arranged in a single row, either side of which was bordered by two rows of nursery trees of equal size to minimise edge effects. Experimental trees were arranged in 5 repeat blocks, each containing 8 randomly placed specimens representing all aforementioned *Acer* genotypes.

6.2.2 Whole Tree Drought Treatment

A watering treatment (irrigated or non-irrigated) was alternated between blocks, and all trees allocated to the irrigated treatment were drip-irrigated with ca. 5 litres of water per day. Drought treatment was initiated by detaching irrigation lines from non-irrigated trees. Drought was augmented by installing black plastic rain covers over all root balls (irrigated and non-irrigated) to exclude rainfall and by raising all pots ca. 75mm off the ground to avoid water uptake from the ground, which was covered in pea gravel.

Drought was initiated on the 27th July 2016 and continued until the 9th of August. Measurements were made on three leaves per tree on the 18th July (-216 hours prior to irrigation removal), 4th August (192 hours) and 9th August (310 hours).

6.2.3 In Vitro Dehydration Treatment

Three leaves from each of the 80 experimental trees were collected. Leaves were collected from the second and third node region as these displayed appropriate maturity and would putatively provide greater consistency. Spring foliage collection commenced once foliage was deemed to have reached maturity following leaf flush i.e. developed mature colouration and structure. This occurred on the 11th May 2016 (hereafter referred to as spring). Summer foliage collection took place on 20th June 2016 (summer). Autumn collection occurred immediately as the first signs of senescence (leaf colour change (Ougham et al. 2005)) were visible in nearby healthy early indicator species (*Prunus* spp.), this occurred on the 23rd September 2015 (autumn). Initial measurements for each season were confirmed to demonstrate a "normal" polyphasic OJIP fluorescence rise to ensure immaturity or senescence would not influence results (data not shown) (Holland et al. 2014). Immediately after removal, all leaves were sealed in Ziploc plastic bags and kept in the dark while being transferred to the laboratory. Chlorophyll fluorescence (CF) was measured on these leaves between 11th and 16th May 2016 (spring), 21st and 29th June 2016 (summer), 23rd and 26th September 2015 (autumn). Measurements were initiated immediately upon arrival at the laboratory. Immediately after the first measurements, leaves were laid out across the laboratory bench and left to dehydrate. Leaves were arranged in a completely randomised block design ensuring each leaf had >1cm peripheral zone to reduce effects on nearby leaves. Laboratory conditions were measured using Tinytag plus TGP-4500 (Gemini Data Loggers Ltd.

Chichester, West Sussex, UK). Vapour pressure deficit (VPD) was calculated as the saturation vapour pressure (e_s) multiplied by the inverse of relative humidity (%) divided by 100:

$$e_s = 6.1078 \times \mathrm{e}\left[\frac{a(T - 273.16)}{T - b}\right]$$

Equation 6.1 Calculation of the saturation vapor pressure where T = temperature in °C, a = 17.2693882, b= 35.86, e is the mathematical constant ≈ 2.71828. (Kirkham 2014, Shamshiri et al. 2017)

The laboratory was kept at an average VPD of: 0.34 (±0.13) spring, 0.57 (±0.14) summer, 0.34 (±0.15) autumn. The windowless laboratory was kept in darkness at all times apart from when measurements were taking place (<3 hours per day). Measurements of CF were taken each day at: 8, 22, 30, 46, 72 and 120 hours after removal from the trees (Spring); 20, 43, 67, 91, 163 and 211 hours (summer); 5, 25, 29, 46 and 79 hours (Autumn).

6.2.4 Pressure-Volume Curves

Pressure-volume curves were calculated between the 12th and 22nd of July 2016 in accordance with the sap expression method; similar to that used by Parker and Pallardy (1988). Six healthy, representative leaves were collected from each cultivar at random from the 80 experimental trees throughout the measurement period. Leaves were collected and remove to the laboratory in sealed plastic bags within 15 minutes. Petioles were re-cut underwater and left to rehydrate overnight (>15 hours). Leaves were then sealed inside a pressure chamber (model 1505D EXP, PMS instruments Co., Albany, USA). The average initial balance pressure was -0.07MPa (±0.017). Leaves which did not hydrate to an initial water potential of <-0.2MPa were discarded (Lenz et al. 2006). Incremental pressures of 0.2MPa were applied to the leaf, beginning at 0.2MPa. P-V curves were halted at 2.2MPa if at least three data points were in the linear portion of the graph. Total expressed sap at each pressure was absorbed in pre-weighed 1.5ml Eppendorf tubes filled with dry low-lint absorbent tissue paper (Kimtech Science, Kent, UK). Tubes were handled and opened for the minimum possible time during sap collection to prevent evaporation. P-V curves were plotted as accumulated expressed sap on the x axis, against -1/applied pressure (MPa) (y axis). Osmotic potential at full turgor (π_0) was determined by extrapolation of the linear portion of the graph to zero expressed sap (Tyree and Hammel 1972). Water potential at the turgor loss point (π_{tlp}) was calculated as the Y axis value at the intersection point of the linear and exponential models (i.e. Y = when Δ x-axis lin. & exp. = 0) (Jane and Green 1983).

6.2.5 Chlorophyll Fluorescence

Chlorophyll fluorescence has been shown to be a viable quantitative indicator of drought stress and survival (Woo et al. 2008, Jedmowski et al. 2015, Kalaji et al. 2017). Measurements were performed

on leaf material from both the whole tree drought and foliar dehydration. Following dark adaptation (30 minutes) measurements were performed using a Pocket Plant Efficiency Analyser (PEA) device (Hansatech instruments ltd., Norfolk). Dark adaptation was still necessary despite the laboratory being kept in near-complete darkness as light was necessary when working in the laboratory. Chlorophyll fluorescence was induced by a one second flash of light (650nm, 1500µmol/m^2/s) provided by an array of three light-emitting diodes over a 4mm diameter of leaf surface. The ratio of variable (F_V=F_M-F_0) to maximal fluorescence (F_M) was calculated. F_0 at $T_{\hat{0}}$ was used during this study (Banks 2017).

Time for the chlorophyll fluorescence parameter Fv/Fm to decline by 50% was calculated per tree, using a linear regression and results per tree were subsequently analysed (Drought n = 5 R^2 = 0.85 ± 0.21, Dehydrated n = 10 average R^2 = 0.79 ± 0.11).

6.2.6 Relative water content

Because of the size of the trial it was not possible to measure relative water content for each leaf. Time was therefore used as a substitute for relative water content (RWC). In order to confirm this was an appropriate assumption in a subset of this trial (n = 15) RWC was plotted against time and a strong correlation was observed, this was in agreement with data displayed by Rastogi et al. (2002) and Faraloni et al. (2011). This data confirms time can be used as a proxy in this experiment, it is important to note however that RWC should not be predicted usig this data.

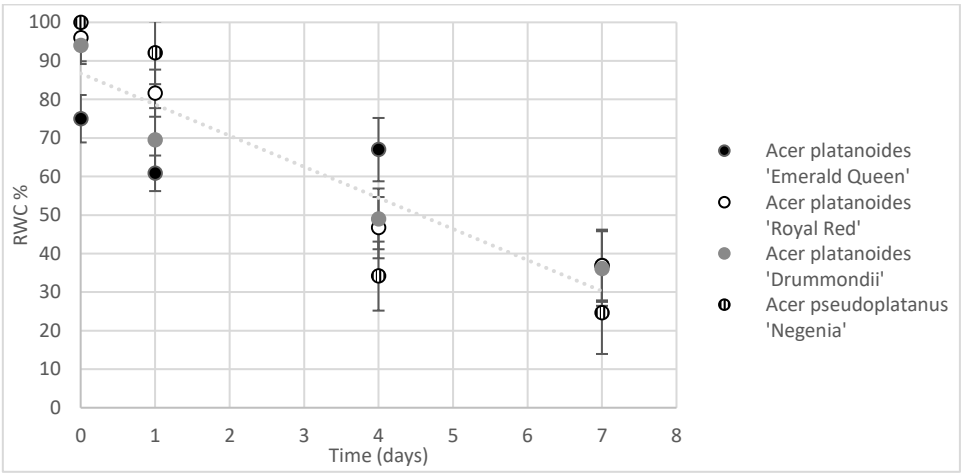

Figure 6.1 Relative water content (RWC) (%) plotted against time (days) for four selected species. n = 15. Error bars show standard error. Linear regression expressed for all four species was y = -8.0701x + 86.733 R² = 0.808.

6.2.7 Statistical Analysis

Statistical analysis was performed using GenStat 17. Time series data was analysed using a residual maximum likelihood (REML) model. Following this, analysis of variance (ANOVA) was performed on transformed data (time for 50% reduction in Fv/Fm) to assess for differences between *Acer* genotypes

for each measurement season individually. Post-hoc analysis was performed using a Tukey's multiple comparison test at the 95% level.

Spearman's rank correlation coefficient (ρ [rho] or r_s) was calculated to determine the similarity in ranking between each categorical variable on a per tree basis. The strength of rank correlations was interpreted using the following thresholds:

Table 6.1 Spearman's rank correlation coefficient interpretations (Weir 2018).

r_s	**Interpretation**
.00 - .19	Very weak
.20 - .39	Weak
.40 - .59	Moderate
.60 - .79	Strong
.80 – 1.0	Very strong

6.3 Results

Significant differences (p = <0.05) were observed within whole tree drought, π_{tlp}, and dehydration for all seasons between the *Acer* cultivars evaluated. Table 6.2 identifies the time taken for *Acer* genotypes to decline by 50%, with π_{tlp} included for comparison. It was not possible to achieve consistent environmental conditions between seasons, resulting in dehydration occurring more rapidly in summer owing to a higher VPD (ca. +4°C in comparison to autumn and spring), this difference prevents direct comparison of raw values. However, ranking genotypes, relative to the rank-position within the other seasons facilitates comparison between seasons. In order to provide this, a Spearman's rank correlation coefficient (r_s) was performed (Table 6.3) on the data displayed in Table 6.2. This allows the relative rank of each season to be compared. Results identify a progression in conformity towards the drought tolerance ranking; a negative weak (r_s -0.21), strong (0.62) to moderate (0.52) rank correlation (Table 6.3) can be observed developing from spring to summer and autumn respectively in comparison to the rank observed in the summer whole tree drought.

Table 6.2 Sumary of means including, time (hours) for 50% reduction in Fv/Fm and mean π_{tlp} (MPa) for the Acer cultivars tested. Letters denote significant differences between cultivars at the 95% confidence interval.

	In vitro								Whole Tree	
Sp. Cultivar	**Spring Dehydration (hours)**		**Summer Dehydration (hours)**		**Autumn Dehydration (hours)**		**π_{tlp} (MPa)**		**Drought (hours)**	
pseudoplatanus 'Spaethii'	146.7	a	94.0	a	71.6	ab	-1.45	ab	269.7	a
campestre 'Louisa Red Shine'	85.8	a	96.9	a	49.5	a	-1.33	ab	259.3	a
campestre	177.4	ab	106.0	ab	56.9	ab	-1.28	a	260.4	a
pseudoplatanus 'Negenia'	301.4	bc	112.4	b	84.5	ab	-1.44	ab	265.8	a
platanoides 'Drummondii'	94.3	a	121.4	bc	64.9	ab	-1.49	ab	449.7	c
platanoides 'Royal Red'	113.1	a	127.9	cd	103.0	b	-1.39	ab	382.7	bc
platanoides 'Emerald Queen'	134.4	a	130.7	cd	96.7	ab	-1.55	ab	342.9	abc
platanoides 'Princeton Gold'	346.7	c	140.5	d	219.9	c	-1.67	b	286.7	ab
P =	<0.001		<0.001		<0.001		0.031		<0.001	

Note that comparison between measurment methods should not be attempted using raw data, rather, comparing relative cultivar positions facilitates assessment between seasons and methods.

Table 6.3 Spearman's rank correlation coefficient matrix comparing the time for 50% Reduction in Fv/Fm between dehydration seasons, π_{tlp} and a controlled drought event. All trials conducted during the 2016 season with the exception of the autumn dehydration conducted in 2015.

		1	2	3	4	5
Leaf dehydration (spring)	1	1				
Leaf dehydration(summer)	2	0.17	1			
Leaf dehydration (autumn)	3	0.45	0.81	1		
π_{tlp}	4	-0.19	-0.64	-0.74	1	
Whole tree drought	5	-0.21	0.62	0.52	-0.60	1

A more negative π_{tlp} infers greater drought tolerance, therefore the corresponding r_s values are negative. In this study the π_{tlp} conforms strongly to the rankings achieved for summer and autumn foliar dehydration (-0.64 and -0.74 r_s respectively) and summer drought (-0.6 r_s) (Table 6.3).

This data suggest that spring foliage dehydration cannot be used to evaluate summer drought tolerance in the Acer genus. However, very strong similarity does exist between summer and autumn (r_s 0.81) suggesting that tolerance is maintained from summer into autumn.

6.4 Discussion

Spring drought tolerance is lower in both annual plants (Farooq et al. 2012) and deciduous trees (Sjöman et al. 2015, 2018) when compared to summer tolerance. Sjöman et al. (2015) identifies, however, that *Acer* species which are most drought tolerant in spring are not necessarily the most tolerant in summer. It is therefore important to clarify if tolerance ranks are maintained throughout the season. This will determine the applicable seasonal range for this type of categorical data.

Data presented here adds further evidence that foliar dehydration and π_{tlp} can identity whole-plant drought tolerance (Jane and Green 1983, Percival and Sheriffs 2002, Baltzer et al. 2008, Faraloni et al. 2011, Bartlett, Scoffoni, Ardy, et al. 2012, Sjöman et al. 2015, 2018). However, the timing of dehydration studies is highly important. Spring dehydration tolerance is not concurrent with summer π_{tlp}, drought or summer dehydration tolerance (Table 6.3). Tolerance ranks approach parity with whole-tree summer drought tolerance and π_{tlp} after the spring season. A very strong correspondence exists between autumn and summer dehydration ranks, suggesting tolerance levels are maintained into the autumn. The autumn dehydration experiment however, occurred during the previous year, and therefore this data suggests that consistency of rank is present in successive years. The timing of drought events in perennial plants is clearly an important factor determining species tolerance or survival. Sjöman et al. (2015) rank tolerance based on summer estimates of π_{tlp} while also reporting spring values. Water deficit is most severe in summer (Lansac et al. 1994) therefore species with a greater summer tolerance are likely more desirable. However, a combined high spring and summer tolerance is also desirable if a risk of spring drought is present. Drought stress is more likely to occur in all seasons in urban areas where limited rooting volumes and increased root injury are likely (Clark and Kjelgren 1990, Savi et al. 2015). Data presented by Sjöman et al. (2015) identifies *Acer monspessulanum* as the most summer drought tolerant (π_{tlp} ca. -4.2MPa) species in comparison to the twenty seven other *Acer* genotypes evaluated. However, *Acer rubrum* 'Northwood' would be significantly more tolerant in a spring drought event, π_{tlp} ca. -2.6MPa vs -1.7MPa for *A. monspessulanum* while also maintaining a relatively low summer π_{tlp} of ca. -3.5MPa (Sjöman et al. 2015). In urban areas therefore, *A. rubrum* 'Northwood' may be recommended over the more summer drought tolerant *A. monspessulanum*. In this study, *A. platanoides* 'Princeton Gold' was consistently the most dehydration tolerant (Table 6.2). However, in the whole tree drought study the variegated *A. platanoides* 'Drummondii' was significantly more drought tolerant (Table 6.2). Soluble carbohydrates in white non-photosynthetic tissue have been shown to not respond to drought to the

same degree as green tissue (Pattanagul and Madore 1999). Additionally white foliage has a lower stomatal conductance (Aphalo and Sánchez 1986) and higher reflectance (Smith 1986) than its green counterpart, irrespective of drought treatments. This putatively suggests that the variegation in this genotype facilitates an avoidance mechanism which was not identified in detached leaves or when using π_{tlp}. Variegation may have reduced total photoinhibition because of the lack of photosynthetic activity and reduced water loss because of the lower stomatal aperture and higher reflectance in the white portions of these leaves. Ecological evidence supports this assumption; Smith (1986) identifies an advantage of increased variegation in clearings with higher drought risks but additionally high availability of photosynthetically active radiation.

The progression in conformity to the summer drought rank observed here suggests that drought tolerance is required to develop, in order to provide an accurate summer ranking. However, some evidence from well-watered experiments suggests tolerance acclimation may be genetically "programmed" (Bahari et al. 1985, Kozlowski and Pallardy 2002) and not necessarily as a result of the plant's water deficit history. It is clear from this data, however, that there is a seasonal or developmental element for tolerance acclimation. Later season evaluations may provide more opportunity for drought exposure or tolerance development; however, an undesirable, slight reduction in conformity to a summer drought rank would be expected to be observed later in the season. Seasonal tolerance patterns within evergreen plants is an area requiring further study.

6.5 Conclusion

This study adds to evidence suggesting that foliar dehydration tolerance can act as a proxy for whole tree drought tolerance, evaluated in this study using deciduous genotypes from the genus *Acer*. We suggest species which exhibit both high spring and summer drought tolerances are more suitable for planting in urban areas than species which only exhibit summer tolerance. Caution is however recommended as other factors are associated with drought tolerance and tree selection more generally. However, the increase in measurements detailing drought tolerance selection metrics is essential in order to progress from the current paradigm of tree selection.

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7 Extraction and Quantification of Compounds with Potential to Characterise Drought Tolerance

Jonathan Michael Banks[1, 2, 3]

[1] The University of Reading, School of Agriculture Policy and Development, Reading, Berkshire, RG6 6AR UK; [2] Bartlett Tree Experts, Research Laboratory (UK) Reading, Berkshire, RG2 9AF, UK; [3] Corresponding author:

Subject specific supervisor: Dr John Mckendrick

Abstract: Extraction and quantification of β-carotene was attempted in order to test the impact of a range of foliar compounds relevant to drought stress tolerance within foliage of highly pigmented *Acer* (Maple) species and cultivars. B-carotene was selected as a target initially, in order to develop a reliable analytical protocol which had potential to be expanded to include a range of other compounds of interest. Compatible osmolytes are of significant interest, and as such are introduced in this paper. However, the identification of such analytes requires a good working knowledge of the HPLC technique. During this study, the time taken for the development of an appropriate extraction and chromatography protocol was significant, preventing further investigation. Further development of the protocols and high-performance liquid chromatography methods used in this study is required in order to provide viable quantifiable data for β-carotene. For the identification of β-carotene spectrophotometric methods are putatively more suitable for the analysis of chlorophylls and carotenoids in foliar tissue, however, spectrophotometers and HPLC techniques alone currently lack the potential to conduct simultaneous identification of compounds such as proline, and glycine betaine.

The influence of a plant's molecular physiology on drought stress adaptation and tolerance is significant. Some compounds show potential to elucidate, monitor and categorise genotypic drought tolerance (Cicevan et al. 2016). The potential for genotypic characterisation based upon a range of relevant molecules may provide valuable information to those involved in plant selection. However, first, characterisation of compounds and development of an extraction and measurement technique is necessary to facilitate compound evaluation at a scale that is practical for plant evaluations.

Compounds of interest include glycine betaine (GB) and proline. These are two amino acids known to act as osmolytes, which accumulate in response to environmental stresses such as drought, salinity,

extreme temperatures, UV radiation and heavy metals (Ashraf and Foolad 2007, Szabados and Savouré 2010). Osmolytes are thought to act also as 'compatible solutes' replacing water in biochemical reactions (Bohnert and Jensen 1996), but also involved in protection from the effects of reactive oxygen species (Farooq et al. 2012). Glycine betaine is most abundant in chloroplasts where it is involved in thylakoid membrane protection and adjustment (Tian et al. 2017). Glycine betaine is known to accumulate in response to stress in a range of plant species however, a small number of other species (rice, mustard, *Arabidopsis* and tobacco) do not produce GB (Ashraf and Foolad 2007). Cruz et al. (2013) identified that foliar applications of GB significantly increases the antioxidant enzymes ascorbate peroxidase and catalase, thus reducing oxidative stress. Proline is involved in stabilising sub-cellular structures, scavenging free radicles and buffering cell redox potential under stress conditions (Ashraf and Foolad 2007, Szabados and Savouré 2010, Rejeb et al. 2014). Proline levels also increase dramatically in response to drought stress and have been identified as a reliable marker for stress (Cicevan et al. 2016). Genetically engineered overproduction has been shown to increase tolerance to osmotic stress in transgenic plants, proline production has also been further enhanced through applications of nitrogen fertiliser (Kishor et al. 1995). Proline and GB are therefore of significant interest for monitoring drought stress and may have potential as markers for evaluating genotypic drought tolerance (Bayoumi et al. 2008). It is possible to measure proline and GB individually using high-performance liquid chromatography (HPLC) equipment (Rutledge and Rudy 1987, Airs and Archer 2010).

Anthocyanins and carotenoids are also known to be involved in drought tolerance; both essential for photosynthesis and photoprotection (Zhao et al. 2014). Anthocyanins are commonly discussed as solar screens because of their UV absorbing properties (Alexieva et al. 2001). Anthocyanins have also been shown to decrease leaf osmotic potential, resulting in increased water uptake and reduced transpiration losses (Chalker-Scott 2002). The carotenoid β-carotene is of interest in drought studies because of its photoprotective and antioxidant properties but also because it is associated with zeaxanthin production, a precursor of abscisic acid (Iuchi et al. 2001, Du et al. 2010, Wright et al. 2011, Bauer et al. 2013). A rise in ABA triggers stomatal closure and in-turn reduces transpiration rates (Iuchi et al. 2001). B-carotene hydroxylase has been shown to induce over-production of zeaxanthin, which improves the drought tolerance of tobacco plants (Zhao et al. 2014). Stomatal closure has been identified as a cavitation avoidance strategy in *Quercus petraea* (Cochard et al. 1996) and *Zea mays* (Cochard 2002); therefore, ABA induced stomatal closure may further reduce long term drought stress.

These compounds are known to influence a plants ability to tolerate drought stress (Bayoumi et al. 2008). However, no study to our knowledge has quantified or evaluated these compounds in *Acer*

trees or assessed if these compounds can be used to determine genotypic drought tolerance in trees. It is for this reason that the extraction and quantification of these compounds was investigated.

7.1 Method

This study initially targeted the extraction and quantification of β-carotene using high performance liquid chromatography (HPLC) in order to act as a future "launch pad" for the analysis of differing compounds using similar methods. β-carotene was selected because of its stability (Craft and Soares 1992) and the availability and low cost of high quality standards. The HPLC method was investigated as it has potential to provide simultaneous measurement of a wide range of compounds from a single extraction and analytical process. A simultaneous measurement method is desirable as it required less solvent, minimizes waste, is less labour intensive and inexpensive in comparison to individual extraction and analysis methods (Kalsoom et al. 2016). This study was used to gain familiarity with the HPLC technique, in addition to extracting 31 compounds simultaneously, including: chlorophylls *a,b,c,d*, as well as α and β carotene (Hegazi et al. 1998).

7.1.1 Plant Material

Five-year-old trees growing in 45 litre Light Pots™ (white, mypex woven grow bags) were used for this experiment. All trees were grafted to their respective species-type rootstocks. The tree species evaluated were from the following seven genotypes (ten trees per genotype): *A. campestre, A. campestre* 'Louisa Red Shine', *A. platanoides* 'Emerald Queen', *A. platanoides* 'Princeton Gold', *A. platanoides* 'Royal Red', *A. pseudoplatanus* 'Negenia', and *A. pseudoplatanus* 'Spaethii'. The potting substrate consisted of a 50:50 green waste and pine bark compost mix, with a pH of 7.7. The trees were arranged in a single row, bordered by two rows of nursery trees of an equal size on either side of the experimental row to eliminate possible edge effects. Experimental trees were arranged in 5 repeat blocks, each containing a drought and well-watered treatment, specimens were randomly placed representing all aforementioned *Acer* genotypes. A watering regime was designed to impose a contrasting drought stress environment (irrigated or non-irrigated) and was alternated between randomised blocks. All irrigated trees were drip irrigated with *ca.* 5 litres of water per day. Irrigation was removed from half of the trees on the 18th of July 2014. Following visual symptoms of drought (foliar necrosis, abscission and wilting) two representative leaves per tree were collected on the 7th August 2014 from both droughted and irrigated trees.

7.1.2 Chlorophyll Fluorescence

In order to monitor the progression of drought, chlorophyll florescence measurements were made on three leaves per tree, on the 18th, 24th, 30th, of July, 7th, 13th, 20th, of August and 4th September 2014.

Following dark adaptation (30 minutes), the florescence response was induced by a one second flash of light (650nm, 1500µmol/m^2/s) provided by an array of three light-emitting diodes over a 4mm diameter of leaf surface. The ratio of variable (F_V=F_M-F_0) to maximal (F_M) fluorescence was calculated as Fv/Fm (F_0 at time base-zero was used). The multi-parametric parameter known as performance index (PI_{ABS}) was also calculated.

$$PI_{ABS} = \frac{F_V \times (F_V - F_J + F_0)}{3.\dot{3} \times (F_K - F_0) \times F_M \times F_0}$$

Equation 7.1 Calculation of performance index (PI_{ABS}) F_J = fluorescence intensity at 2ms, F_K= fluorescence intensity at 300µs, F_O= fluorescence intensity at time base 0, calculated using least squared regression (Banks 2017).

7.1.3 Extraction

For pigment extraction a method was adapted from Ahamad et al. (2007). Leaf samples were ground in a pestle and mortar using liquid nitrogen before being suspended in 30mL acetone with 0.1% butylated hydroxytoluene (BHT), as an antioxidant. Anhydrous sodium sulphate (20 g) was added to these extracts for five minutes before the solid material was removed by filtration into vials. The solvent was removed from these vials using a sample concentrator (Techne, Staffordshire, UK) until complete solvent removal was evident. Vials were weighed and acetone (100% HPLC grade) plus BHT was added to bring the final concentration to 0.1g/ml (+/-0.005g). 1 ml of supernatant was aliquoted into HPLC vials via a 0.20µl syringe filter (Minisart®) for immediate analysis.

7.1.4 HPLC method

Two HPLC methods were evaluated using a HP Agilent 1100 series HPLC system. The isocratic mobile phase described by Ahamad et al. (2007) of acetonitrile, dichloromethane and methanol in the ratio of 70:20:10, respectively was evaluated. The method of Hegazi et al. (1998) was also evaluated using a gradient method. Ammonium acetate was removed from the method described by Hegazi et al. (1998) owing to its risk of precipitation in an analytical HPLC system as opposed to the semi-preparative HPLC system it was originally developed for. The gradient was therefore adapted to an initial 90:10 for ten minutes, rising to 70:30 methanol acetone respectively after 35 minutes with a stop time at 43 minutes.

The flow rate was set to 0.7 ml/min. Detection wavelength was fixed at 452 nm. A Brownlee analytical 5µm 150x4.6 mm C18 HPLC column was used for both methods. Three 5 µl injections were performed per sample with one blank (acetone) injection every ca. 5 samples.

7.1.5 Quantification

Quantification was attempted via inclusion of β-carotene (Sigma—Aldrich, Germany, purity ≥97.0% [UV]) as a ‘spike’ in tandem with a sample run, and as a calibration curve using dilutions of 1:3, 1:4,

1:8, 1:10, 1:16, using a stock solution of 1mg and 5mg β-carotene to 1ml, 10ml and 30ml 100% Acetone. β-carotene precipitation was discovered in all stock solutions and in all dilutions (Figure 7.1), therefore it was necessary for quantification to be disregarded.

Figure 7.1 β-carotene mixed with acetone, showing precipitation of β-carotene at all concentrations; concentrations increase from left to right.

7.1.6 Statistical Analysis

Statistical analysis was performed using GenStat 17. Following tests for normality, analysis of variance (ANOVA) was used to test for differences between groups. Tukey's multiple comparison was used to identify differences between genotypes.

7.2 Method Development

The gradient method described by Hegazi et al. (1998) provided superior separation and resolution in comparison to the isocratic method described by Ahamad et al. (2007). The gradient method was therefore used for this experiment. Example chromatograms for both methods are shown in Figure 7.2 and Figure 7.3.

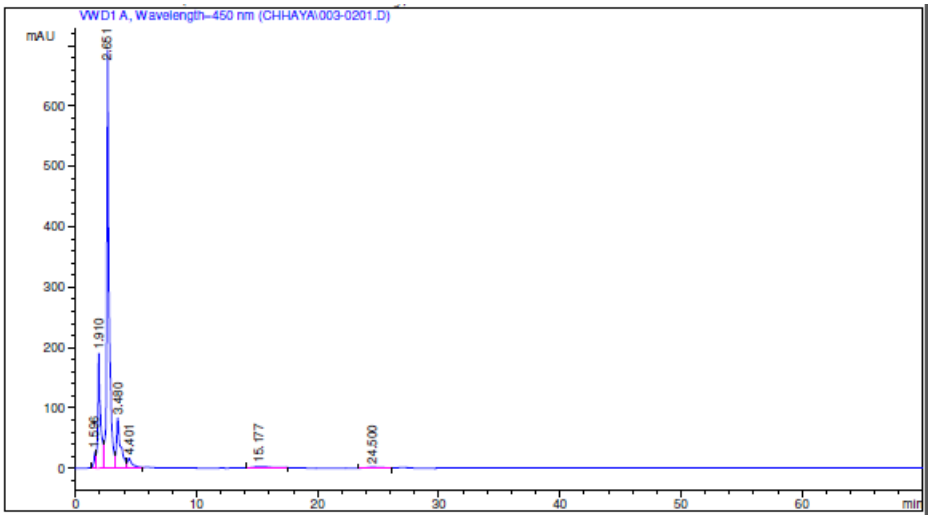

Figure 7.2 Example of typical chromatogram from the isocratic HPLC method described by Ahamad et al. (2007)*. Poor separation of compounds prevented identification of peaks.*

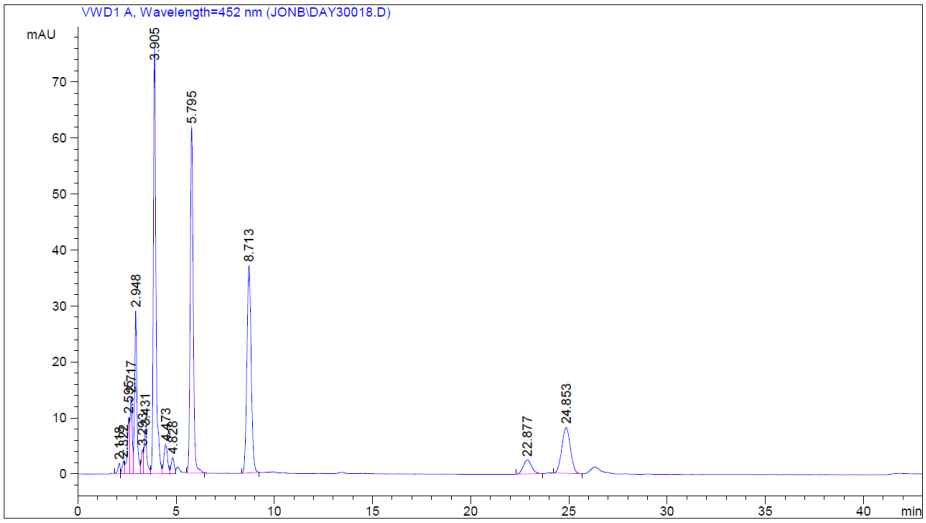

Figure 7.3 Example of a typical chromatograms from the gradient method described by Hegazi et al. (1998)*. Superior compound separation was consistently observed using this method. The Peak at 24.9 minutes was found to be β- carotene, the peak at 22.8 minutes is putatively α-carotene, peak at 8.7 minutes is putatively chlorophyll a. Further peak identification is not possible as retention times differ from that described by* Hegazi et al. (1998)*.*

Samples were "spiked" with BHT to assess if inclusion of this antioxidant affected the chromatogram. No change in peaks were observed when measured at 452nm. Butylated hydroxytoluene was observed to eluted at 2.9 minutes when measuring at 220nm only (Figure 7.4).

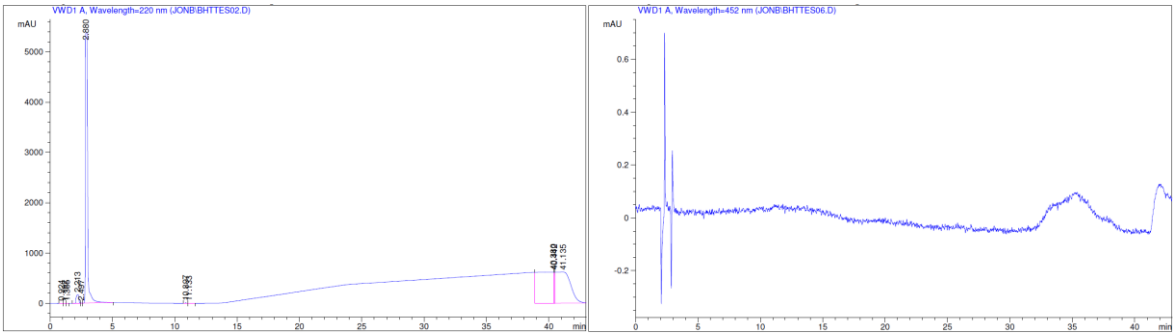

Figure 7.4 Butylated hydroxytoluene (BHT) spiked samples measured 452nm (left) and 220nm (right). (note differing Y axis scales max 5500mAU left vs 0.7mAU right).

The leaf extraction protocol was adapted owing to precipitation which became evident in the samples visually (Figure 7.5). Precipitation was observed before and after filtration and resulted in blockages in the HPLC column causing pressures >10MPa (100bar). Additions of Hexane, methanol and dichloromethane at varying concentrations was evaluated, however, this did not prevent precipitation. Sample purification by evaporation or centrifugation prior to filtration was also investigated. The solvent evaporation stage was discovered to cause precipitation; therefore, this stage was removed from the sample extraction. Centrifugation reduced the size and number of peaks in comparison to non-centrifugation, therefore syringe filtration alone was deemed suitable (Figure 7.6). Sample quantification was performed by carefully measuring ground samples. This technique may however result in unwanted variability owing to the leaves having variable moisture contents throughout the course of the drought trial.

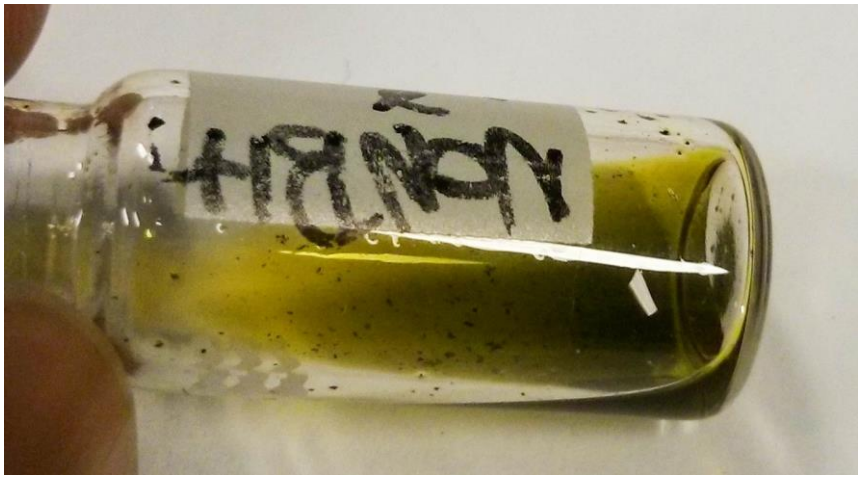

Figure 7.5 Sample precipitation observed in samples during analysis. Found to be caused by the sample concentration process.

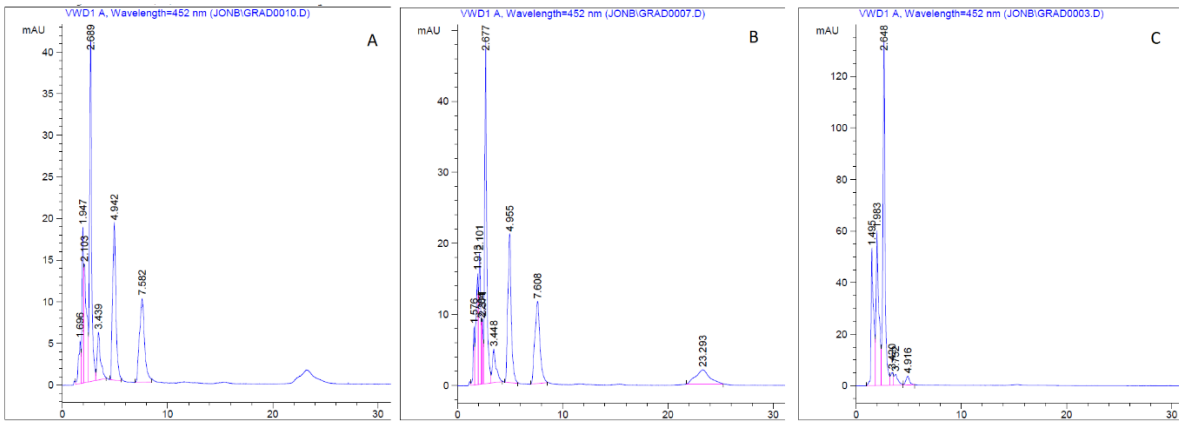

Figure 7.6 Example chromatograms showing results of sample purification and concentration methods. Samples were: A: centrifuged, B: unprocessed, C: evaporated and rehydrated, prior to analysis.

β-carotene was confirmed using samples spiked with ≥97.0% β-carotene (Sigma—Aldrich, Germany) (Figure 7.7). A retention time (rt) of *ca.*23 minutes was observed using the method of Hegazi et al. (1998).

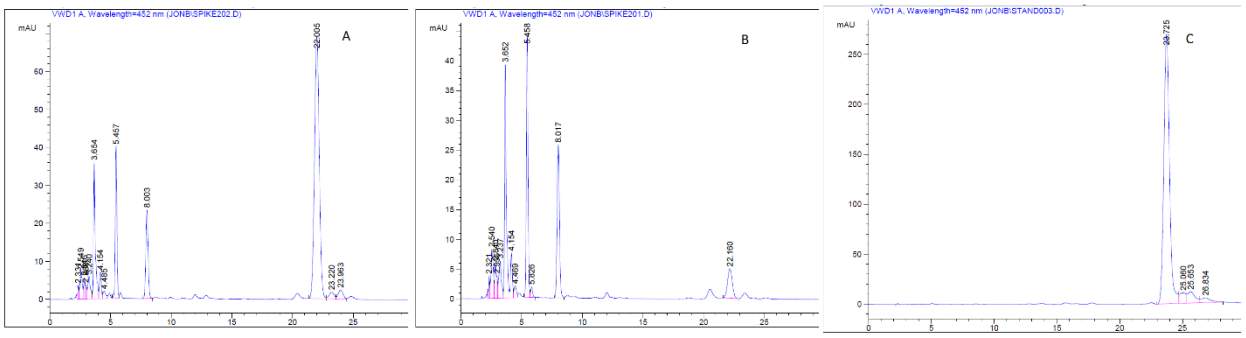

Figure 7.7 Example chromatograms showing: A: β-carotene spiked sample, B: non-spiked sample, C: pure β-carotene. β-carotene was eluted from the column at ca.23 minutes.

7.3 Results

Because quantification was not possible analysis on the peak area was conducted.

Significant differences occurred between genotypes and between droughted and watered groups (p= <0.001). For ease of interpretation data presented is the difference between the droughted (D) individual and the mean of the species-type watered (W) trees (ΔArea = D-$\bar{x}$W).

The impact of watering regime (droughted vs watered) measured by chlorophyll fluorescence were not significantly different from the watered controls p= 0.11 Fv/Fm and p= 0.32 PI_{ABS}.

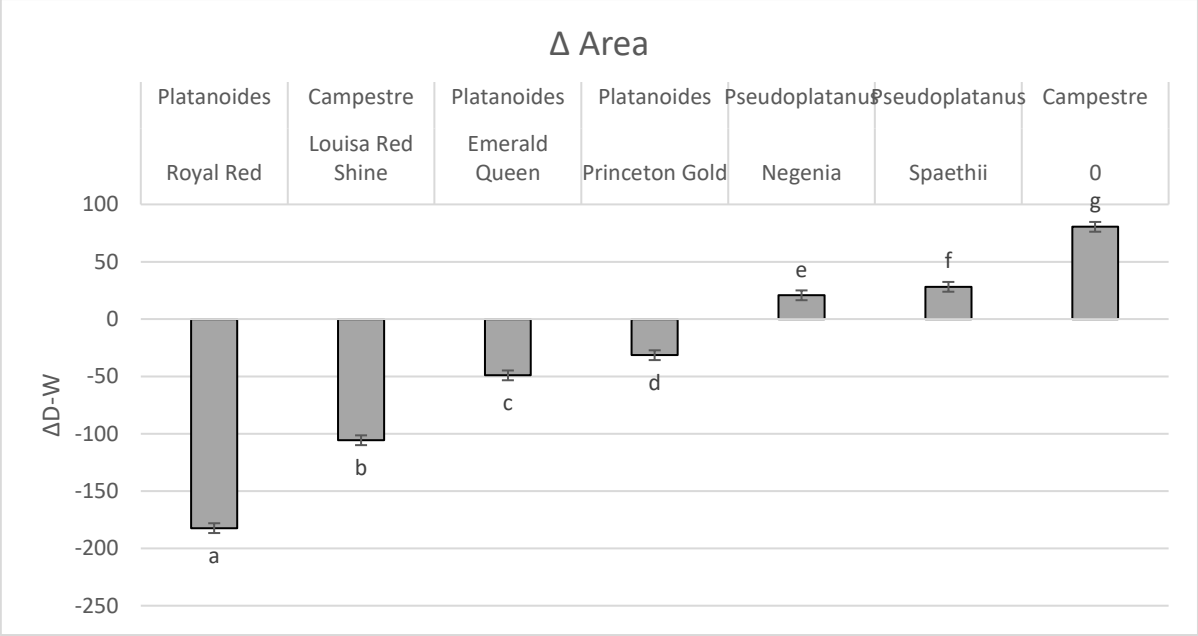

Figure 7.8 Variation in ΔD-W relative absorbance (452nm) between cultivars. Letters indicate significant differences at the 95% confidence interval. Error bars show the least significant difference. (n = 3).

7.4 Discussion

Data in this study identifies significant genotypic variation in β-carotene response to drought stress (Figure 7.8). A varied but universal decrease in β-carotene concentration in response to water stress was observed by Mibei et al. (2017), Cicevan et al. (2016) and Al Hassan et al. (2015). Cicevan et al. (2016) show between -69% and -6% decrease in total carotenoids. However, no record of carotenoid increase in response to drought stress has been reported. Despite significant symptoms of drought stress chlorophyll fluorescence did not identify a significant impact of the drought treatment (p= 0.11 and 0.32, Fv/Fm & PI_{ABS} respectively). It was therefore not possible to ascertain if genotypic drought tolerance was proportional to the decrease in β-carotene in this trial.

Owing to precipitation within foliar extracts and β-carotene standards during the extraction protocol and technical issues with the HPLC systems used for this experiment, the time taken to further develop an acceptable broad scale analytical protocol was considered too high to warrant further investigation. The HPLC method used in this study may, with some development, be suitable to identify a wide range of chlorophyll and carotenoids. However, it is suggested that spectrophotometer methods using relatively crude leaf extracts are less time consuming and more accurate for these pigments (Lichtenthaler and Buschmann 2005). A spectrophotometer method has been utilised to identify proline (Sleimi et al. 2015) following an extensive extraction procedure. However, no such method is capable of identifying glycine betaine. Kalsoom et al. (2016) evaluated simultaneous methods of studying osmoregulants in plants including proline and GB. Simultaneous extraction and quantification of osmolytes is challenging (Kalsoom et al. 2016) and might not be possible using HPLC alone. Indeed

the only successful simultaneous extraction and quantification was conducted using mass spectrometer coupled high-performance ligand-exchange liquid chromatography (MS-HPLEC) (Oufir et al. 2009). Therefore, further investment in HPLC methods were deemed inappropriate.

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8 Technical Challenges

Several technical challenges were encountered during this project. For many of these challenges it was possible to adjust or adapt in response and still maintain the integrity of the data (see optical and measurement gain discrepancies, p50). Additional challenges have, however, prevented the extraction of viable data, as previously discussed for HPLC Quantification (p112). Another challenge was encountered during 2014. A drought trial set up in a very similar way to that described in Plant material (p48) and using the same *Acer* genotypes was unsuccessful in imposing a significant, drought-induced difference from control values, as measured by the chlorophyll fluorescence (CF) parameters Fv/Fm (P= 0.114) and PI^{ABS} (P = 0.317) (Figure 8.1). Significantly different visible impacts between droughted and control trees were, however, observed (p=<.001). At the cessation of the study it was discovered that the visual indexing data did not provide sufficient statistical power to characterise differences between genotypes, as not enough datapoints were available.

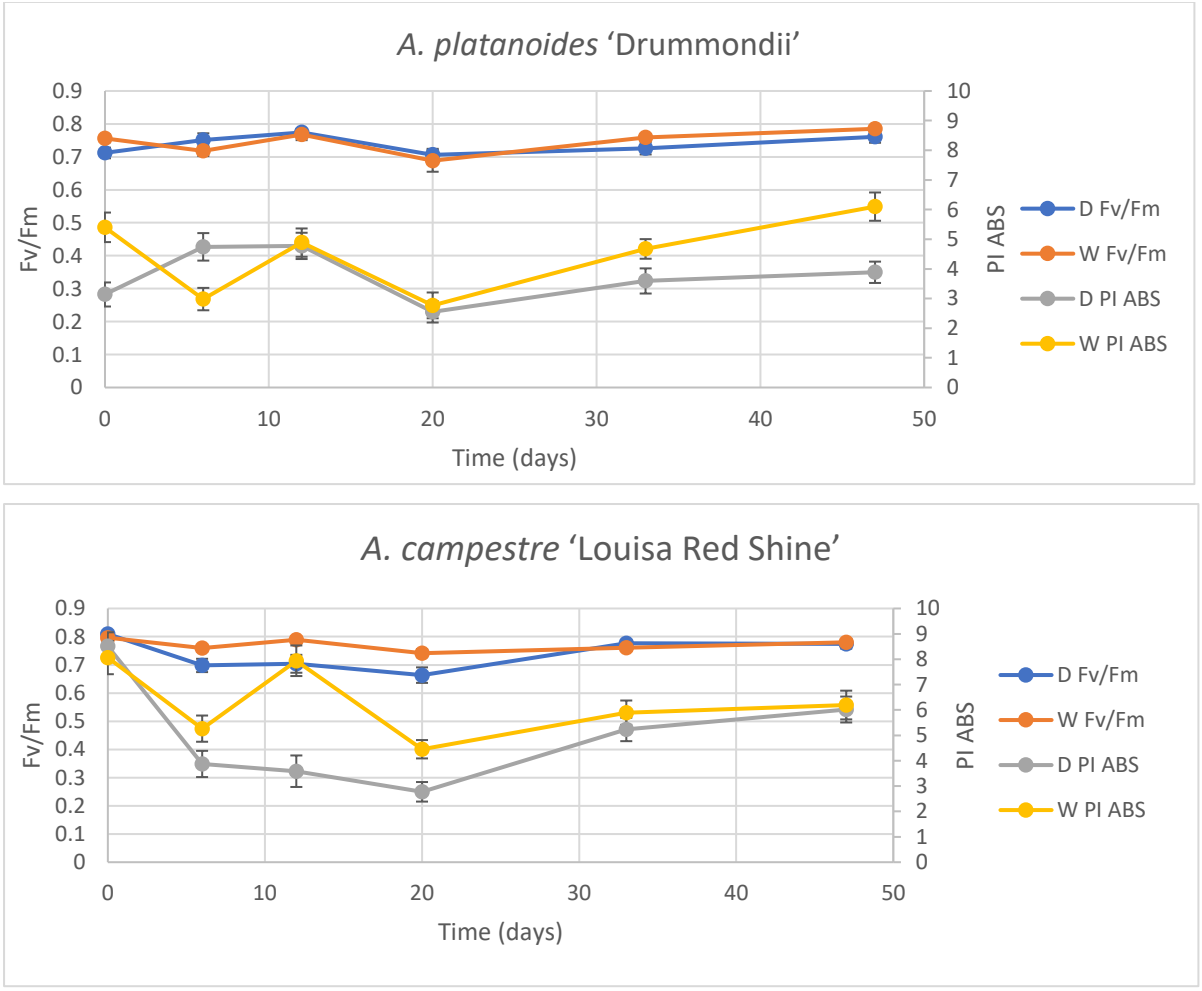

Figure 8.1 Example time series graphs, Fv/Fm and PI^{ABS}, from 2014 whole tree drought showing stochastic response to the imposed drought (day zero) in droughted (D) vs control (W) trees.

Water uptake from the ground (pea-gravel) and rain intercepted by the growbag was thought to be preventing consistent water stress; this may have contributed to the lack of significant differences between CF readings. All trees were therefore returned to the irrigated system and left to recover for two full growing seasons. Following this period of recovery, no significant difference between the previous drought and control treatment groups was recorded when measuring: Fv/Fm (p=0.661), PI^{ABS} (p=0.42) and visual index (p=0.562). Further drought treatments were therefore imposed starting on 27th July 2016. Trees were raised from the ground and rain covers were installed over all root balls (Figure 8.2) to ensure that it was only the irrigation drippers which were providing irrigation to the trees. Additional measurements were collected during the 2016 drought including: stomatal conductance, soil moisture, visual index (increased sample size), precipitation and temperature data. Additional CF measurement protocols (such as: 1-Vi, Vj, V0(Bo), Vt, ΔVt, ΔW_{OJ} and ΔW_{OK}) were also conducted to facilitate an evaluation of novel parameters.

Figure 8.2 Left: schematic diagram of plastic rain cover (black shaded area), bricks (brown squares) raising the pot off the ground and irrigation arrangement (blue lines) installed on all trees for 2016 drought. Right: photograph of the rain cover in situ.

8.1 Foliar Water Potential

Measurements of foliar water potential were also attempted during the 2016 drought in order to determine drought impacts at the genotypic level. No suitable device was available within Reading University. Consequently, Bartlett Tree Experts purchased a Sky Instruments PMS device (Sky Instruments Ltd., Llandrindod Wells, Wales). However, difficulties achieving a sufficient seal between the petioles and the device were encountered during the 2016 season. Therefore, a Plant Moisture Stress (PMS Instrument Co., Albany, Oregon) device was borrowed and later purchased. This was

unfortunately after the 2016 drought trial had been completed. The device was later utilised in the study investigating differences between pressure chamber-derived and psychrometric-derived measurements of water potential at the turgor loss point.

9 Conclusion

Studies undertaken in this thesis defined chlorophyll fluorescence (CF) parameters and evaluated CF parameter selection for monitoring, evaluating and quantifying drought and dehydration stress in the genotypes, *Acer campestre*, *A. platanoides* and *A. pseudoplatanus*. CF was then combined with other physiological measurements in order to evaluate these genotypes in vivo and in vitro under differing imposed drought and dehydration events. Additionally, methods of calculating the water potential at the turgor loss point (π_{tlp}) from leaf material were also assessed, including evaluations of the sensitivity of these methods. Finally, the implications of seasonality on relative drought tolerance rankings was evaluated.

Two in vitro methods proved viable for determining genotypic drought tolerance. One method utilised a technique measuring CF to determine the rate of decline after foliage was detached and allowed to desiccate over time. The P-V method used foliar water potential at the turgor loss or wilting point (π_{tlp}). This was determined by applications of incremental mechanical pressure on a leaf, or predicted from osmotic potential at full turgor (π_0) by using a regression approach based on a previously published meta-analysis. A potential advantage of the CF method is that it is rapid and inclusive of foliar architecture. Data presented in this thesis, however, identified a high similarity between CF and the pressure-volume (P-V) method in terms of tolerance rankings (R_S = -0.64). Seasonality, however, proved to be a significant factor; whole tree drought tolerance rankings showed a strong similarity to rates of detached foliar dehydration when assessed during the summer (R_S = 0.62). However, such strong correlations were not observed when compared with in vitro measurements conducted in autumn (R_S = 0.52) and a weak correlation was observed in spring (R_S = -0.21).

A comparison of multiple in vitro methods reveals that significant drought tolerance differences exist between cultivars of the same species. It is therefore strongly recommended that tree selectors understand the implications of cultivar selection. More information on drought tolerance has the potential to influence genotypic diversity, especially within urban areas. In the current tree selection paradigm, tree selectors use past planting experiences to determine the environmental tolerance of genotypes (D. Lofthouse 2014, personal communication, 28th May). Reliable information on genotypic drought tolerance can be combined with tree selectors' experience, presenting them with a range of similarly tolerant genotypes from which to choose, facilitating greater potential genetic diversity, as tolerance similarities between known and unknown plants can be rapidly assessed. Increased diversity can also facilitate reductions in pest and disease risk, commonly associated with monocultures (Kendal et al. 2014). A recent publication from the industry group Trees and Design Action Group (TDAG)

(Hirons and Sjöman 2018) has attempted to meet the above aims using scientifically grounded selection criteria to broaden the range of species used in urban plantings, thereby facilitating more appropriate urban tree selection. This information may well prove to be an excellent resource for the industry as a whole; however, the full impact of this document remains to be seen.

In field trials *A. platanoides* 'Drummondii' provided an additional complexity when compared between studies. This genotype was determined to be the most drought-tolerant (Figure 4.5). However, in vitro trials consistently highlighted *A. platanoides* 'Emerald Queen' and *A. platanoides* 'Princeton Gold' as the most drought tolerant genotypes (Table 6.2). It was observed that *A. platanoides* 'Drummondii' had a lower initial CF reading relative to the other genotypes evaluated. Although still considered within the range associated with unimpaired functioning of the leaf photosynthetic system, this may have resulted in a slower rate of observed decline in comparison to the other cultivars. This was, however, corrected for by using the method of "time taken to decline by 50%". Superior tolerance was also observed when using both raw visual index data (Table 6.2) and "time for 50% reduction" in visual index data (Figure 4.5). White foliage is known to have a lower stomatal conductance (Aphalo and Sánchez 1986) and higher reflectance (Smith 1986) than its green counterpart, irrespective of drought treatments. The variegation in *A. platanoides* 'Drummondii' may therefore have facilitated an avoidance mechanism which was not identified in detached leaf dehydration or pressure chamber derived π_{tlp}. Further work, including additional in vivo experimentation is required to elucidate this discrepancy.

Quantified genotypic drought tolerance systems, such as those investigated within this thesis, can be used to match genotypes to sites with particular stress characteristics or requirements. For example, on sites with limited soil volume or free draining soil, tolerant genotypes can be planted; more sensitive genotypes can be used on sites with larger soil volumes or increased watering regimes. Rahman et al. (2015) highlight that knowledge of species-specific stress tolerance will allow planners to appropriately select species to maximise ecosystem services. It should however be stressed that a highly drought resistant tree is not a panacea for all the challenges of the urban environment. Selecting stress tolerant trees does not facilitate savings on soil and site preparation or aftercare. A planting strategy pairing genotypes of high tolerance with reduced aftercare is not advised. Practicable quantities of trees, planted and maintained appropriately, is the only viable method of achieving resilient trees which can bring wide-ranging benefits to the urban landscape.

9.1 Further works

Urban trees are subjected to a multitude of stressors, while drought tolerance has been thought to infer some degree of cross-resistance (Bussotti 2008), also known as polytolerance. Polytolerance is

however rare, it is therefore worth considering drought tolerance within the context of other stressors. Future evaluations are therefore encouraged, categorising other stressors especially for promising genotypes tested in other tolerance studies. Stressors common within the urban environment such as: salt, pollution, waterlogging, high and low light and temperature are also worthy targets for further tolerance categorisation.

It is recommended that future studies include additional measurements to monitor and measure drought stress and water deficit. Increased frequency of measurement is also strongly recommended. Plant water potential and stomatal conductance measurements are known to provide a reliable and sensitive metric of water deficit (Mccutchan and Shackel 1992, Martínez-vilalta and Garcia-forner 2017). These measurements are expected to provide more reliable drought response data than that obtained from CF. It would also be valuable to include and compare pulse amplitude modulated (PAM) florescence measurements with the continuous excitation technique used throughout this thesis. The parameter V0(Bo) has some potential to compare favourably to parameters from the PAM technique. Improving soil moisture measurements, including probe burial and inclusion of soil water potential measurements in parallel to leaf or stem water potential is also highly valuable data for future drought studies.

During the investigations in this thesis, inconsistencies within the scientific literature have been highlighted. These are examined for CF parameters but are also present with plant-water relation parameters. Results also emphasise that it is vital to detail the time of year when plant-based studies are conducted. Clear and consistent calculation of parameters is essential for the fields of CF and plant water relations to progress efficiently (Nahlik et al. 2012).

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