JOÃO PAULO PENNACCHI

A SYSTEMIC APPROACH TO THE QUANTIFICATION OF THE PHENOTYPIC PLASTICITY OF PLANT PHYSIOLOGICAL TRAITS

LAVRAS – MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia / Fisiologia Vegetal, para a obtenção do título de Doutor

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UMA ABORDAGEM SISTÊMICA PARA QUANTIFICAÇÃO DA PLASTICIDADE FENOTÍPICA DE PARÂMETROS FISIOLÓGICOS DE PLANTAS

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia / Fisiologia Vegetal, para a obtenção do título de Doutor

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RESUMO

A avaliação da plasticidade fenotípica de plantas é uma parte importante do processo de mapeamento da performance e adaptação de materiais vegetais. È também crucial para o entendimento da formação da vegetação natural, recomendação de espécies para recuperação de áreas de conservação e genótipos para programas de melhoramento genético, bem como para modelagem da resposta de plantas em condições climáticas futuras. Os métodos atuais e passados para quantificação de plasticidade fenotípica em plantas apresentam múltiplas limitações, principalmente relacionadas à normalização de dados, repetições, análises de múltiplos ambientes e integação de variáveis diversas. Este estudo propõe um novo método de avaliação de plasticidade fenotípica de plantas, baseado em uma técnica de estatística multivariada, a Análise de Componentes Principais (PCA) e no cálculo de distâncias euclidianas entre escores do PCA. O índice Multivariado de Plasticidade (MVPi) foi aplicado a dados fisiológicos foliares coletados para dois estudos de casos experimentais composto de: a) quatro espécies nativas de Cerrado cultivadas em ambientes com diferentes incidências luminosas e disponibilidade de água e b) quatro variedades comerciais de cana-de-açúcar cultivadas sob diferentes regimes hídricos. Os resultados sugerem que o método foi eficiente em explicar o comportamento das diferentes espécies/variáveis em ambientes contrastantes e informar a plasticidade de parâmetros fisiológicos a nível foliar. O método também se mostrou capaz de sanar algumas das limitações apresentadas por métodos anteriores e permitiu uma análise integrada da plasticidade fenotípica de plantas. Recomenda-se o uso do MVPi como uma ferramenta para análise de plasticidade fenotípica como uma característica emergente de plantas, no contexto de uma avaliação sistêmica e integrada de atributos fisiológicos de plantas.

Palavras-Chave: Análise de Componentes Principais. Cana-de-Açúcar. Cerrado. Ecofisiologia.

ABSTRACT

The evaluation of plant phenotypic plasticity is an important part of mapping plant performance and adaptation. This is crucial to the understanding of natural vegetation formation, indication of species for recovering of conservation areas and genotypes to crop breeding programs, as well as for modelling of plant behaviour in future climatic conditions. The past and current methods for quantification of plant phenotypic plasticity present multiple limitations, mainly in what concerns data normalization, replication, analysis of multiple environments and coupling of multiple varieties. This study proposed a new method, based on a multivariate statistics technique, the Principal Component Analysis (PCA) and in the calculation of Euclidian distances between PCA scores to evaluate plant plasticity. The Multivariate Plasticity index (MVPi) was applied to leaf physiology data collected from two experimental study cases composed of: a) four Cerrado native species grown under environments of contrasting light and water and b) four sugarcane commercial varieties under different water regimes. The results suggest that the method was efficient in explaining species/varieties behaviour in the different environments and to inform the plasticity of leaf physiological traits. It has also showed potential to overcome the main limitations of other methods and allow an integrated analysis of plant phenotypic plasticity. We recommend the use of the MVPi method as a tool for analysis of phenotypic plasticity as an emergent plant characteristic in the context of a systemic evaluation of plant physiological traits.

Keywords: Cerrado. Ecophysiology. Principal Component Analysis. Sugarcane.

SUMMARY

1 GENERAL INTRODUCTION

1.1 Scope and background

Phenotypic plasticity is by definition the capacity of a given genotype to express different phenotypes under different environmental conditions being its measurement, the amount of change in a given trait in different environments (BRADSHAW, 1965). Phenotype can be defined as any morphological, physiological or phenological feature, from the cell to the whole-plant level, which can be measured (VIOLLE *et al.*, 2007). However, this definition is controversial mainly in what concerns the different plant levels in which it is expressed and/or measured (MAHNER & KARY, 1997).

Plasticity is thought to be an evolutionary process defined according to the specific conditions at which each species evolved, characterizing it as a species-specific characteristic (WHITMAN & AGRAVAL, 2009). As the formation of a phenotype is a combination of the influence of its genotype (G), the environment it is exposed to (E) and their interaction (GxE), it is expected that individuals that share a more similar genetic background would have a closer trend to phenotypic plasticity, reinforcing its species-specific nature (BRADSHAW, 1965).

If this is true, the phenotypic plasticity is a pre-determined genetic character and so it should be defined mathematically as a constant or as a coefficient among genotypes. However there are phenotypic changes, or changes in the gene expression which are motivated by the environmental effects without changing the individual DNA (GRATIVOL *et al.*, 2012). Despite of the strong genetic influence to phenotypic plasticity, the interactions between the individual and the environment make plasticity a flexible character and not just of a species-specific nature (SCHLICHTING &WUND, 2014).

That said, phenotypic plasticity of plant physiological traits, or, for definition, physiological plasticity, can be mathematically defined as a variable that can be properly measured and modelled as any variable. The measurement of physiological plasticity based on the adjustments of physiological characteristics of plants and their interaction for genotypes and environments can be a good alternative to quantify such characteristic.

This study proposes a new systemic approach to quantify and evaluate phenotypic plasticity of plant physiological traits based on an integrated method and multivariate analysis of multiple traits coupling different genotypes and environments. This method is applied to two case studies involving: a) native Cerrado species growing under different light and water conditions and b) sugarcane varieties facing contrasting irrigation regimes. Plant plasticity and the physiological bases of leaf adjustment to contrasting conditions if then discussed for the two cases.

1.2 Bibliography review

1.2.1 The Plasticity Theory

Lubiner (1975), was the first to formalize the theory of plasticity for solid materials. Of course, the term plasticity and its concepts were older than that and commonly used not just to describe material properties but also used for plants and other leaving being. However, never before quantified or properly formalized.

The plasticity theory is originated of the study of solid materials exposed to a load pressure and its response when the load is removed. There are two main ways of a material to respond to the removal of a load that was previously imposed to it: totally reversing to its normal form or undergo some level of permanent, irreversible, deformation. Elastic materials are the ones which present the capacity to return to their initial state and form after the load is removed and are not the most common. Plastic materials are the ones which present a permanent deformation and which initial state can just be re-achieved by the expenditure of extra energy. The load can be understood as a stress factor and the critical value where permanent deformations start to happen is named yield stress. This is a material property (LUBINER, 1975).

After the theory of plasticity proposed by Lubiner (1975), the definition of phenotypic plasticity to plants by Bradshaw (1965) started to be used in a “plant stress” context. That is say that plant phenotypic plasticity was a property related to stress-strain responses. However, the proper formalism and definition of the theory of plasticity in plants are still lacking. If one considers some aspects of living systems related to phenotype adjustments to environmental conditions the elasticity term would emerge as the most adequate.

What are the differences in the plastic and elastic approaches considering the deformation of solids? The answer to such question should consider that the formalism based on thermodynamics will provide the best approach. Thermodynamic laws must be satisfied in all natural phenomena, while, needless to mention, an elasticity or plasticity formalism is not an exception. However, the thermodynamic explanation is hardly described in any literature on plasticity. On the other hand, the formalisms on the aspects of thermodynamics are described in the formalisms of damage and transformation phenomena related to elasticity formalisms (LUBARDA, 2001).

As being a material property it is expected that different material would present different levels of plasticity/ elasticity when reacting to the same stresses. This theory of elastoplastic responses to stresses can be also applied to plant adjustment to environmental conditions and will be the focus of this study.

1.2.2 Phenotypic plasticity: definition and importance in plants

The definition of phenotypic plasticity, as the capacity of a given genotype to express different phenotypes under different environmental conditions, infers the ability of plants to respond to different environments by changing their phenotype (BRADSHAW, 1965), or, in other words, the capacity of different genotypes (G) to respond to different environments (E) in different ways, according to their interaction (GxE) (Fig. 1.1 - (a)). This ability reflects the capacity to maintain their fitness even when facing conditions of biotic or abiotic stresses (KULHEIM *et al.*, 2002). Plant fitness reflects the capacity of plant to pass its genes to the offspring, being higher fitness related to plants that are more likely to survive and reproduce in specific environments (GEBER & GRIFFEN, 2003).

Phenotypic plasticity studies assume an important role in plants mainly because their incapacity to move across environments in a single generation. While animals can migrate for different habitats when the conditions turn particularly severe, plants have to adapt themselves to survive (PALMER *et al.*, 2012). The indeterminate development characteristic enables plants to maintain greater plasticity during the life cycle, allowing them to grow or discard organs after embryogenesis. The presence of meristems and cell pluripotency are primordial characteristics that boost plant phenotypic plasticity (PALMER *et al.*, 2012). The bigger phenotypic plasticity in plants helps to explain the big gap in terms of animals and plants

species numbers. As plants can assume multiple phenotypes from the same genotype, the number of different genotypes, or species, is reduced (DE JONG & LEYSER, 2012).

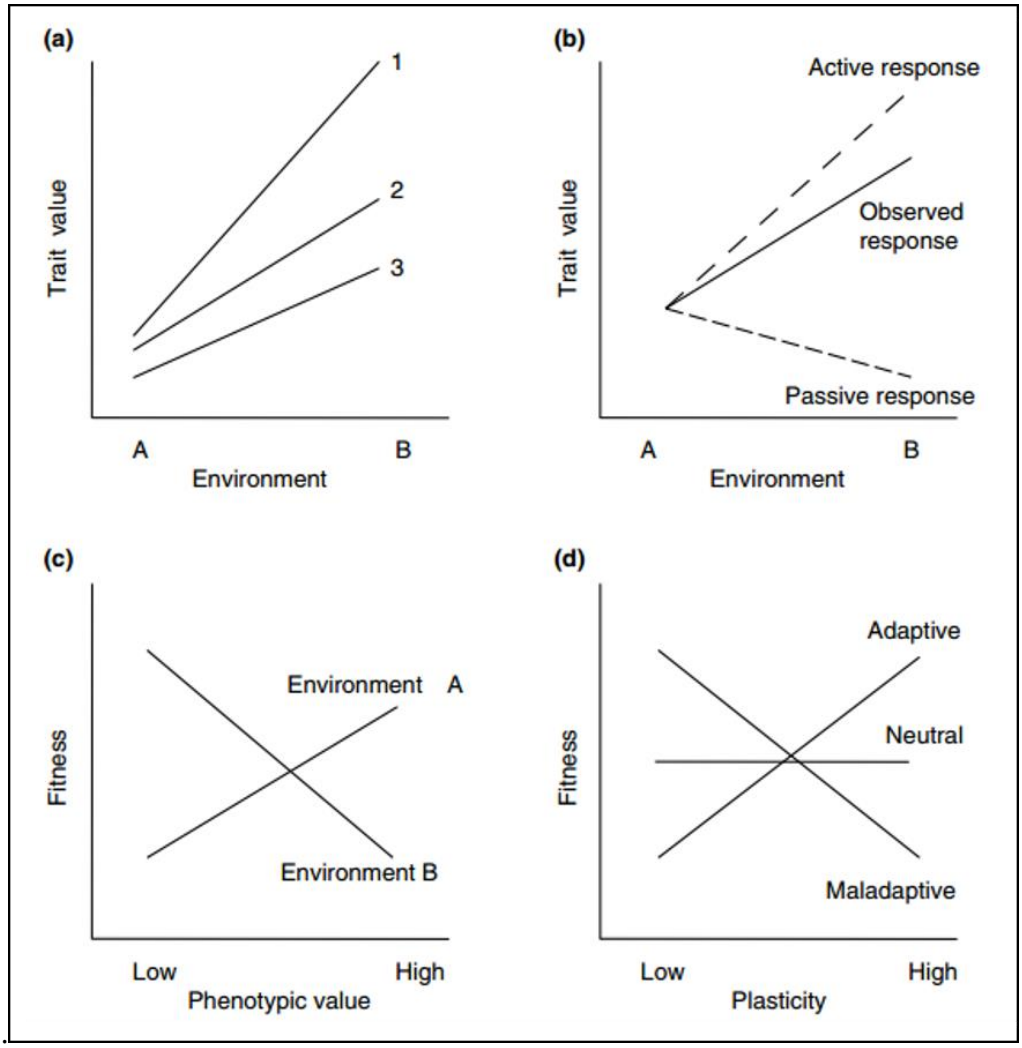
1.2.3 Phenotypic plasticity: adaptive or non-adaptive?

From the exposed before, it is common to think in phenotypic plasticity as adaptive and contributing to reproductive success and plant fitness. Although phenotypic plasticity has important roles in the evolutionary diversification of plants (SCHEINER 1993), not all the phenotypic variation in plants are adaptive and related to improved fitness and in some occasions can even be related to fitness loss (Fig. 1.1 – (c)) (DE JONG & LEYSER, 2012). The loss of fitness may be a remarkably response to a complex trait which is a result of mutations or selection of multiple interconnected traits (ALPERT & SIMMS, 2002) or a negative developmental effect motivated by stress conditions or resource limitation (VAN KLEUNEN & FISCHER, 2005). The observed phenotype in response to an environmental condition is a resultant of multiple interactions of passive and active responses. For instance, the stem elongation in response to shade is pushed by an active response, as hormonal balance, although it might be limited by a passive response, as resource limitation to growth, due to plant competition (Fig. 1.1 – (b)) (VAN KLEUNEN & FISCHER, 2005). Depending on the balance of active and passive responses, the observed phenotype may be linked to a lack of fitness and be understood as adaptive or non-adaptive (Fig. 1.1 – (d)).

In an ecological point of view, when plants face novel environmental conditions that lead to phenotypic changes with reduced fitness, selection will favour genetic arrangements that counteract these changes to restore the phenotype to its previous state (GRETHER, 2005). Thus, non-adaptive plasticity reduces the likelihood of persistence in a new environment that is stressful and increases the strength of selection (DE JONG & LEYSER, 2012).

It is important to highlight the critical difference between adaptation, in an evolutionary point of view, and acclimation or adjustment to environmental conditions. While the first a long term process that lead to specific changes and the differentiation of species the second is related to the quick adaptation of plants to environmental changes that are more related to plant fitness and are effectively drivers of plant plasticity.

Figure 1.1 – Plasticity, fitness and phenotypic value in multiple environments



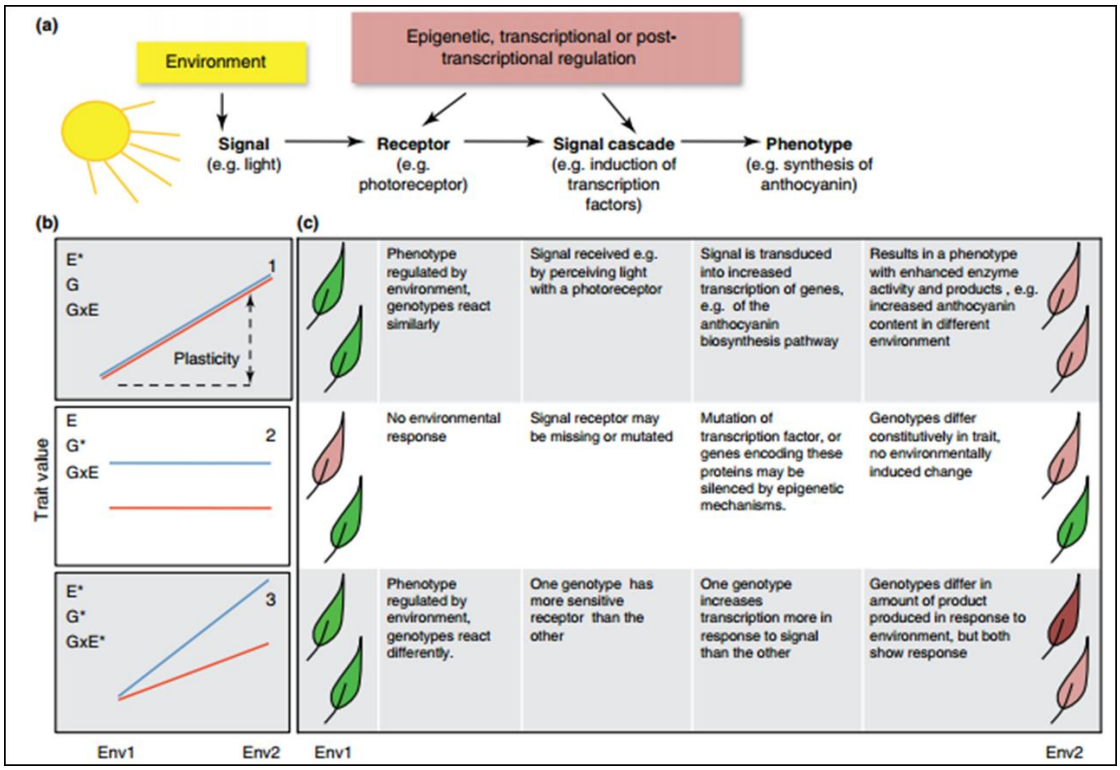
Generally, plasticity studies aims to assess genotype (G) (or alternatively population or line) and environmental (E) effects and their interactions (G x E). The interaction term is used to determine whether contrasting genotypes differ in their ability to alter phenotype in response to environmental signals. (a) The response of three 'lines' (1–3) to two environments (A and B). Line 1 shows the greatest phenotypic plasticity, line 3 the least. (b) An illustration of how an observed plastic response can be the result of active and passive responses occurring at the same time. For example, the passive response can reflect resource limitation, whereas the active response changes in hormonal balance and allocation factors. Adaptive plastic responses are generally, but not necessarily, those that are active and that require a specific signal perception-transduction system allowing plants to change their development. (c) & (d) show tests of adaptive plasticity. In (c), fitness is maximized at a high value of the phenotypic trait in environment A and at a low value in environment B, so that the ability of the genotype to alter its phenotype depending on the environment will itself be adaptive. (d) A representation of a different approach to assessing adaptive plasticity in which a measure of plasticity (absolute or an index) is regressed against average fitness; the relationship could be adaptive, neutral or even maladaptive. Adapted from Nicotra *et al.* (2010)

1.2.4 The basis of phenotypic plasticity and the importance of physiological traits

According to Nicotra *et al.* (2010) as a phenotypic change, the basis of phenotypic plasticity is genetic. However, some changes in phenotype may not be related to changes in the DNA constitution or expression, as epigenetic changes (GRATIVOL *et al.*, 2012). Thus, the formation of a phenotype, and consequently plasticity, is turned into a complex figure that can be manifested in different levels as physiological and morphological changes (GEBER & GRIFFEN, 2003). Physiological mechanisms underlying gene response as transcription and translation and also enzymatic and hormonal regulation affect the phenotypic changes, producing local to systemic responses (WHITMAN & AGRAVAL, 2009). An example of different responses and interactions on the formation of a phenotype related to leaf colour is presented in Fig. 1.2.

Considering the complexity and variability of phenotypic responses that plants traits can assume under different environmental conditions, the decision of what to measure/monitor to inform plant performance related to plant phenotypic plasticity is very challenging. Gratani (2014) has highlighted the different time responses of physiological and morphological plasticity and the importance of each of them in understanding and tracking phenotypic plasticity. Physiological plasticity is a faster response of plants to changes in the environment if compared to morphological plasticity. While gas exchange measurements can vary in minutes' scale, the changes in leaf colour or stomatal number, for instance, will be manifested in a scale of days or even generations. Although all of them have their importance to plant acclimation to environments (SULTAN, 2000), physiological parameters may be a strong choice of traits to explain plant performance and general fitness. It is mainly because their importance to phenotype definition, the short term response in contrasting environmental conditions and also because the capacity to explain plant performance in an integrated way and in different hierarchical levels. They are also responsive to the different levels of control of the gene expression as transcriptional, post-transcriptional or epigenetic.

Figure 1.2 – Plasticity influence to phenotype formation



Anthocyanins are produced in leaves in response to excess light and temperature, and serve as a reversible plastic mechanism for the protection of photosynthetic machinery. Here, we use an anthocyanin example to illustrate (a) the points in the molecular machinery, which translate an environmental signal (excess light in this case) into a phenotype. (b) In the evolutionary and ecological literature, these responses are commonly presented as reaction norms. Here, the blue and red lines indicate the reaction norms of two different genotypes responding to a change from a low light environment (Env1) to a high light one (Env2). The extent of phenotypic change in response to a signal is its phenotypic plasticity. Asterisks in the panels denote whether there is a significant effect of environment (E) or genotype (G), or genotype by environment interaction (GxE). (c) Likely examples of the mechanisms underlying the cases depicted in panels 1–3 are given separately for each point in the signal pathway. The leaves on the left and right represent the phenotypes in Env1 and Env2, respectively. Reproduced from Nicotra *et al.* (2010).

1.2.5 The costs and limits of phenotypic plasticity

Phenotypic plasticity of plants emerges from inherent strategies for efficient use of resources in order to fit environmental conditions adjust, in most cases allowing them a bigger flexibility and capacity to survive in a bigger range of conditions. However, this phenotypic plasticity is limited by a range of factors; one of them is of course the energetic cost of changing.

The limits of plasticity were classified by de Witt *et al.* (1998) as: a) developmental-range limits, associated to a limitation of plastic organisms to produce extreme phenotypes when compared to fixed organisms; b) information-reliability limits, associated to imperfect sensory mechanisms or changing environmental conditions that prevent plastic organisms from accurately assessing environmental conditions; c) lag-time limits, associated to a sub-optimal intermediate phenotype in plastic organisms due to the requirement of an adaptation time; d) epiphenotype limits, associated to a lack of phenotypic change in plastic organisms due to a late detection of environmental changes; e) plasticity-history limits, associated to disconnected phenotypic responses in plastic organisms due to early influence of ontogenetic responses; and f) ecological limits, associated to the influence of previous environmental factors, restricting the range of phenotypic responses to current environmental factors.

The costs of phenotypic plasticity were classified as: a) maintenance costs, associated to internal sensing and regulatory systems needed to detect environmental conditions; b) production costs, associated to the extra costs that plastic organisms incur to express a certain trait in comparison to fixed organisms; c) information-acquisition costs, associated to sampling the environmental conditions at a determined frequency; d) developmental-instability costs, associated to penalties of imperfect phenotype–environment matching; and e) genetic costs, associated to linkage between loci affecting plasticity and loci with negative fitness effects, pleiotropic effects of loci affecting plasticity and other traits, or (negative) epistatic interactions among loci affecting plasticity and other loci (DE WITT *et al.*, 1998).

In a simpler way, there are multiple factors that could constrain and limit plasticity, as observed for any other traits, such as: lack of genetic variation, allometric relationships and trade-off among traits, environmental limitations to the expression of a specific phenotype or a phylogenetic history that may restrict plasticity to reach its limits (SCHLICHTING & PIGLIUCCI, 1998).

Cryptic Genetic Variation (CGV), defined as ‘standing genetic variation that does not contribute to the normal range of phenotypes observed in a population, but that is available to modify a phenotype that arises after environmental change, mutation induction or the introduction of novel alleles’, can open the spectrum of plant plasticity studies by unrevealing new possibilities of phenotypes and extending the plasticity limits as currently known

(GIBSON & DWORKIN, 2004). In crops, the use of the Targeting Induced Local Lesions IN Genomes (TILLING) has been successfully used to create a broad range of possible phenotypes and also to understand gene and allele effect in phenotypic variation (KUROWSKA *et al.*, 2011).

Despite of the multiple methodologies and experiments designed to measure and/or infer plasticity costs and limits, there is still a lot of uncertainty about their practical effects on plant performance and its application to plant adaptation studies (VALLADARES *et al.*, 2007), although measuring the impact of each single cost/limit doesn't look to be the most appropriate method (AULD *et al.*, 2010).

1.2.6 Phenotypic Plasticity in biomes and crops

Plastic responses of plants to environmental factors may be placed in an ecological context by regarding them as components of sets of traits which are predictably related to habitat stability and productivity (GRIME *et al.*, 1986). In naturally grown vegetation areas, the ecological process of species adaptation and survival have been straight related to their fitness and phenotypic plastic (SULTAN, 1995). Understanding intraspecific phenotypic plasticity can be useful to predict the behaviour of plants species in natural and human-built biomes in future predicted environmental scenarios. It can also help to understand the mechanisms of plant plasticity and expand their use to improve the breeding of forests species and crops (ASPINWALL *et al.*, 2015).

From the agricultural point of view, phenotypic plasticity can define the viability of plants to be grown in a specific geographical location or their capacity to keep their productivity under pressure conditions. Past efforts in the breeding process were concentrated in increasing yield potential and were focused in generating genotypes to be grown under specific field conditions, with a decreased importance to GxE interaction (PALMER *et al.*, 2012). Current efforts in crop breeding are related to increasing crop productivity as well as decreasing the yield gap, throughout yield resilience to the fluctuating environmental conditions (POWELL *et al.*, 2012).

Selecting target traits to be used in the breeding process of high and stable yielding genotypes is a laborious part of the job. According to Mir *et al.* (2012), the decision of traits

to include in a breeding process to enhance crop yield potential and stability needs to be made based on: their relative correlation to yield, the extent of genetic variation, heritability and genotype x environment interactions. Phenotypic plasticity is a crucial part of it as it is straight related to the capacity of the plant to respond to contrasting environments and in defining crop yield potential and stability (DE JONG & LEYSER, 2012). Despite of its importance, phenotypic plasticity has not been largely exploited in the breeding process (NICOTRA *et al.*, 2010).

1.2.7 Phenotypic plasticity and global changes

The effect of the human activities affecting global features has been noticed and tracked in more details since the nineteenth century, although, the rate of change has been raised quite drastically in the last decades. These global changes are usually merged to the definition of climate change, albeit there are more players but climate change in the equation. The global change components were listed by Matesanz *et al.* (2010) as: climate change, land use change, overexploitation, pollution, and invasive species.

All the above mentioned components have been affecting the way that plants are grown, naturally or commercially, in the planet and will continue to affect in the future. Phenotypic plasticity plays a big role in defining and understanding the interaction and adaptation of plants to the current and future global scenarios. There are multiple levels of plant response to global changes as presented in Table 1.1.

Although the science behind plant modelling has evolved recently, it is difficult to predict the plant response to current extreme or future predicted climatic conditions (CHIOU *et al.*, 2015). The understanding of the capacity of plants' phenotypic plasticity is still very limited, mainly due to a lack of an appropriate method of evaluation of plant plasticity in a systemic way. Some of the cited global changes may create unique conditions that might create new genetic diversity and unravel unexploited phenotypes, increasing the plastic responses of plants and bringing new light to plant adaptation to future scenarios (MATESANZ *et al.*, 2010).

Table 1.1 – Functional traits expected to be affected by different global change components. Ps, photosynthetic rate; gs, stomatal conductance; WUE, water use efficiency; R, respiration; C:N, carbon:nitrogen ratios.

Global change	Traits components expected to be affected
Land use change	Growth traits Phenology Reproductive traits
Climate change	Biomass allocation Phenology Physiological traits (Ps, gs, WUE, R) Reproductive traits Specific Leaf Area
Invasive species	Biomass allocation Flowering morphology Herbivore defences Phenology Physiological traits (Ps, gs, WUE, R) Reproductive traits Tolerance to allelopathy
Pollution (including elevated CO_2 and N deposition)	C:N ratios, leaf N content Growth traits Phenology Physiological traits (Ps, gs, WUE, R) Plant biomass and allocation
Overexploitation	Growth traits Survival

Reproduced from Matesanz *et al.* (2010)

1.2.8 Approaches to measure phenotypic plasticity

Since the importance of phenotypic plasticity on plant behaviour started to be studied, multiple methods were developed to try to estimate plant phenotypic plasticity. Valladares *et al.*, 2006 have listed and compared a number of phenotypic plasticity estimators as well as

pointed their limitations and advantages. They also proposed new methods to overcome the limitations mapped for the methods previously released. They compared the different methods of phenotypic plasticity quantification by using each of the indices to evaluate data of final biomass and shoot to root ratio for 4 species (*Quercus robur*, *Quercus pyrenaica*, *Pinus sylvestris* and *Pinus pinaster)* grown under different light regimes. In general the indices proposed for evaluating plant plasticity for a single trait are based on the difference between the trait measured under different environmental conditions.

Schlichting & Levin (1984) and Valladares *et al.* (2002) proposed the use of indices based on the ratio of the standard deviation and the mean for each single trait and genotype in different environments. This type of index accounts for the variability between environments and also uses normalization, allowing the comparison of different traits. The main weak point of this approach is the limitation in comparing the plasticity of different genotypes, due to the lack of replication for the plasticity measurements.

Another variation of the plant plasticity indices involves the use of covariates, as plant biomass, in the analysis of allometric traits. This has been recommended by multiple studies (CHEPLICK, 1995; VALLADARES *et al.*, 2000; BALAGUER *et al.*, 2001; NAVAS & GARNIER, 2002; GRATANI *et al.*, 2003) mainly when evaluating traits that have a strong link with plant biomass or height, for instance. The use of covariates assumes normality and can improve plant plasticity evaluation albeit it has a limited statistical use when comparing genotypes or species.

Valladares *et al.* (2006) proposed a number of indices, of different complexities, aiming to overcome the limitations found in the previous indices presented in the literature. The use of the trait's median, instead of the mean, and covariates allowed an improved analysis of data without normal distribution. They reported advantages in using phenotypic distances between genotypes in multiple environments as a technique to overcome the limitations of comparing genotypes, commonly presented in other methods. However, there are plenty of new limitations related to the choice of environments, number of replicates, complexity of calculation and interpretation of the indices.

Although these methods presented different approaches, the great majority of them is based on the analysis of single traits in different environmental conditions. There are a

multitude of plant traits in different levels interacting in a systemic way for which phenotypic plasticity can be hardly estimated. The biggest limitation of the current proposed methods is related to the linked interpretation of all these parameters and their trade-offs. It also goes against the claim of the needs of an integrative and systemic approach of plant physiology studies to understand plant behaviour and performance as pointed by Souza *et al.* (2016).

Plant scientists commonly tends to focus on vegetative and phenology-related traits to inform plant fitness and plasticity. Geber & Griffen (2003) have highlighted the importance of physiological traits to understand and model plant plasticity and have pointed to the lack of use and interpretation of physiological traits by plant scientists. It is also crucial to consider the importance of the timing, specificity and speed of the plastic responses (WHITMAN AND AGRAVAL, 2009). Modern high-throughput phenotyping methods could be of great value to increase the phenotyping capacity and the monitoring of plant plasticity (ARAUS AND CAIRNS, 2014). However, a multi-trait approach to quantify the plasticity as an emergent characteristic is needed to accomplish this goal.

Coupling physiological parameters and multivariate statistics may help to address the challenge of understanding plant performance on a systemic and integrated way, increasing the possibilities of mapping and predicting their performance in current or future environmental conditions. Moreover, it may overcome the limitation of comparing species or genotypes when evaluating plant plasticity.

1.3 Method's proposition

The use of indices to evaluate plant plasticity presents multiple limiting factors. The needs of normalization in order to compare multiple traits, the comparison of multiple environments, the lack of replication which limits genotypes comparison and the needs of an integrated method for analysing multiple traits are a number of them. Solutions for the two first limitations were commented above. The use of an average of plasticity for multiple traits is proposed as a technique to overcome the lack of replication by generating an estimator of dispersion of statistical meaning (CASTRO-DÍEZ *et al.*, 2006). However, coupling different traits without using proper statistic methods may generate a lack of mathematical and biological meaning (VALLADARES *et al.*, 2006). The Principal Component Analysis (PCA)

is a multivariate approach that has the potential to overcome the two last limitations mentioned above.

PCA (Principal Component Analysis) is a multivariate statistics technique used to simplify the analysis and interpretation of big sets of data. It is based on the use of orthogonal transformation to convert a set of variables into new axes, known as principal components, defining eigenvectors and eigenvalues. This form of defining new orthogonal axis (eigenvectors) and score (eigenvalues) is of valuable use in understanding the sources of variation and their influence to the dataset. The PCA analysis is greatly applied to studies of plant ecology, including environmental characteristics and their influence in species distribution (KENT & COKER, 1994), but can also be exploited in analysis of plant physiological responses.

A Multivariate Plasticity Index (MVPi) is proposed in this study. Its calculation is carried out using the Euclidian distance of scores from a PCA analysis. The method is based in the assumption that in a multivariate dataset projected in *n* dimensions, where *n* is the number of axis and variables, meaning 1 axis per variable, the PCA can be used to inform a projection of the scores on a unidimensional plan. The scores' projection value would represent the real state of the object, as a high informative index that integrates the whole group of measured values and tested environments.

Each score represents a position on the covariance matrix formed by the individual and the measured variables. Thus, the score is formed of coordinates (PC1, PC2, PC3,..., PCn) in the multiple orthogonal axis of the PCA. The score's position for each species will vary within the different environments in the *n* dimensions defined by the PCA. Scores with smaller values to the coordinates (close to zero) represent a smaller correlation to the PC component and, consequently, present a smaller contribution to the variance of the specific component. Therefore, the states that are most responsive to environmental variations, indicating higher plasticity, will present bigger distances between them in any, or all, *n* directions.

The Euclidian distance between scores, projected in *n* directions would represent the changes in the phenotypic states (norm of reaction) motivated by the variation sources.

Thereby the MVPi is calculated as the absolute deviation between different phenotypic states (Equation 1).

$$MVPi = [(S_{PC1}-Z_{PC1})^2 + (S_{PC2}-Z_{PC2})^2 + (S_{PC3}-Z_{PC3})^2 +...+(S_{PCn}-Z_{PCn})^2]^{0,5} \quad \text{(Equation 1)}$$

where S and Z are scores with coordinates S (PC1, PC2, PC3,…, PCn) and Z (PC1, PC2, PC3,…, PCn).

1.4 Thesis' structure and objectives

The main objective of this research was to improve the understanding of phenotypic plasticity quantification in plants by understanding the main limitations of current methods and proposing solutions to overcome them. This was made through the proposal of a new method of phenotypic plasticity evaluation based on multivariate statistics, more specifically, principal component analysis of leaf physiological traits. The application of this method to two case studies was also on the scope of this research.

The thesis structure is based on:

a) a general introduction of the topic, followed by a literature review and method's proposal (Chapter 1);

b) the presentation of the method and its evaluation by the studies of two practical cases: one with Cerrado native species under different conditions of light and water availability and one with sugarcane varieties under contrasting irrigation regimes. This allows the investigation of the method's application to the scope of research for biomes and crops (Chapter 2, case studies 1 and 2);

The results, achievements and limitations of this work are discussed in terms of the current and future possible contributions to the field of study and general research area (Chapter 3).

2 QUANTIFYING THE PHENOTIPIC PLASTICITY OF PHYSIOLOGICAL TRAITS USING A SYSTEMIC APPROACH: METHOD PROPOSAL AND CASE STUDIES

2.1 Introduction

The environment can induce changes in the physiological and morphological characteristics of plants without altering its genotype, what is defined as phenotypic plasticity (PP). Such phenotypic changes are essential for the survival of individuals since bigger plasticity is understood to be related to the species fitness to different environmental conditions (DE JONG & LEYSER, 2012). Recently, there is an increased interest in understanding the PP in plants as it can help on the prediction of plant behaviour in future climate scenarios (GRATANI, 2014). Understanding the capacity of plants to alter their physiological and morphological characteristics when facing contrasting environmental conditions is urgent to predict the future composition of natural biomes and crop productivity (BARBOSA *et al.*, 2012).

Despite of the vast data generated by phenotyping plant traits in different environmental conditions, there are few conclusive studies with effective practical applications of PP. The complexity of trait responses to environmental drivers limits precise interpretation of PP results. This limitation is mainly related to the emergent characteristic of plasticity, where the phenotypic changes motivated by the environment can be observed in multiple organizational levels. Alterations in lower organizational levels may be or not related to changes in higher organizational levels (NOVOPLANSKY, 2002; SCHLICHTING, 2002; SCHLICHTING & SMITH, 2002). Because of the complexity of PP, a standardized method for its quantification is complex and somehow abstract. This further limits the effective use of PP results in breeding programs, models' simulation for plant behaviour in future climatic scenarios or to explain why some species do not occur in specific environmental conditions.

The plasticity, at the leaf level, involves modifications in the standard leaf metabolism to adjust the biochemical processes to fit the environmental conditions. Changes in the activity of antioxidant enzymes (SPERRY, 2000) and gas-exchange characteristics (FOYER & NOCTOR, 2002) are important controllers to maintain higher photosynthetic rates and

lower water loss, at the leaf level, contributing to a higher homeostasis. Leaf physiological balance is also affected by modifications in light intensity and quality and in the soil, air and leaf water content. Combinations of the mentioned factors build a multitude of conditions to which leaves need to acclimate (LARCHER, 1995).

Aiming to standardize the quantification of the phenotypic plasticity of plant traits, Valladares *et al.* (2007) proposed an index based on the maximum and minimum values for a trait in different conditions. This index is calculated by the ratio of the difference between maximum and minimum value of the trait and the maximum value of the trait. Thus this index varies from 0 to 1, where higher values indicate higher PP. However, this approach presents several limitations for its application and interpretation. The main concern about the use of the index is related to the fact that each evaluated trait presents a single value what limits the understanding of PP as an emergent, systemic and complex phenomenon.

Another weak point of the use of the method proposed by Valladares *et al.* (2007) can be a misinterpretation of the PP as illustrated by the following physiological example: the maintenance of CO_2 assimilation rate over bigger variations in values of stomatal conductance in contrasting environmental conditions would result in a lower PP index for the assimilatory process and a higher PP index for the mechanisms controlling stomata movement. However, the plasticity would have the function to maintain the homeostasis in a hierarchized way, according to the importance of each of the process to the plant. Thus, it does not mean that the plant has a low PP for CO_2 assimilation but, that the assimilation process was maintained by adjustments in other concurrent processes.

Only a model with an integrated analysis of diverse variables can improve the interpretation of PP and overcome the limitations found in previous approaches. This research proposes an integrative index for evaluating and quantifying PP based on Principal Component Analysis (PCA) and systemic approaches. The index is evaluated in two study cases using Cerrado species (Study case 1) and sugarcane varieties (Study case 2) as models. The use of native woodland species and a crop aims to exploit and test the dynamism of the index and highlight its potential use and limitation.

On the Cerrado, a seasonally dry Savannah biome, the different physiognomies form several environments with contrasting light and water availability. The species presenting higher PP are usually more spread across Cerrado's physiognomies as they are more able to acclimate to multiple environments. Because trees have long-life cycle, an important trait related to their fitness is their trade-offs between root-to-shoot growth, which emerge from an inherent strategy for efficient use of resources in order to fit environmental conditions. We hypothesise that water and light availability determine shifts between carbon allocation to shoot increment or to leaf and branch growth and that different species present different strategies. We also correlate these traits with PP of leaf traits.

For this, we studied four Cerrado species. These species showed different physiological responses, at the leaf level, to the environmental variations, although the response is dependent of the species and of the specific environmental conditions. In diverse Cerrado species, leaf PP itself and how PP affects plant behaviour in different phitophysiognomies, is not well known. This information is crucial to be mapped as Cerrado species with higher leaf PP can contribute to a bigger resilience of the Cerrado domain to current and future environmental changes.

For the sugarcane, a very important crop for food and energy production, drought conditions during the crop cycle can reduce yield in 60% (JANGPROMMA *et al.*, 2012). In Brazil, drought stress is a common condition in multiple areas, including the Southeast region, which corresponds to more than 66% of the total production in the country (CONAB, 2017). Varieties with contrasting PPs may respond differently to drought stress revealing patterns of tolerance to low water availability. This can be exploited in crop management and reduced the losses related to drought stresses.

Four sugarcane varieties with contrasting characteristic for drought-tolerance were grown under glasshouse conditions. A comparison between well-watered plants and plants in a specific drought regime revealed pattern of response for the MVPi and contrasting leaf physiological balances.

2a CASE 1: STUDY OF FOUR CERRADO WOOD SPECIES IN RESPONSE TO LIGHT AND WATER VARIATIONS

2a.1 Material and methods

2a.1.1 Plant material

The species studied were: *Pimenta pseudocaryophyllus* (Gomes) Landrum, *Machaerium opacum* Vogel, *Tabebuia serratifolia* (Vahl) Nich and *Zeyheria montana* Mart. *M. opacum* and *P. pseudocaryophyllus* are found in habitats where the understory radiation is reduced due to the formation of a denser and higher canopy above (MACIEL *et al.*, 2002). *T. serratifolia* and *Z. montana* are more abundant in environments where there are predominantly sparse trees and herbaceous stratum at higher altitudes in the landscape with lower water and higher radiation availability (ROSSATTO *et al.*, 2010).

Seeds of *P. pseudocaryophyllus* were harvested in a wooded area in Ijaci county (21°10'12'' S e 44°55'31'' W GRW) in the south of Minas Gerais. Seeds of *M. opacum* were harvested in a wooded area of Monte Carmelo county (18°43'29'' S e 47°29'55'' W GRW) in the region of the Triângulo Mineiro. Seeds of *T. serratifolia* and *Z. montana* were harvested in wooded areas in Lavras county (21º14'45'' S e 44º59'59'' W GRW) and Ingaí county (21º 24' 04" S e 44º 55' 02" W GRW) respectively, both located in the south of Minas Gerais state.

After harvesting, the seeds were cleaned in 70 % ethanol for one minute. Afterwards, they were immersed in a solution of sodium hypochlorite 2% for two minutes, rinsed in tap water for five minutes and sown in a plastic tray in a sand substrate (non-autoclaved).

2a.1.2 Environmental variations: light and water availability

Trays containing the seeds were moved into a glasshouse and distributed in three environments: a) Full Sun (FS); b) shade by undercover with Shade Net (SN) Sombrite® with 50% interception of the short wave and long wave radiation; c) shade by undercover with a Shade Film (SF) Insufilm® (SPfilm, Brazil) with 75% interception of ultra-violet to yellow wavelengths, but with no interception effect over longer waves as red or infrared, allowing a shade environment simulating an understory condition.

Two months after the seedlings emergency, the most uniform individuals were selected, being transplanted into 4 litres Citropotes® (33.5 x 14 cm) using sand as substrate and kept in the same environments in the glasshouse. The seedlings were fertilized with a one-fourth strength nutritive solution, throughout the experimental period, according to Malavolta (2006). The seedlings were acclimated to the new recipient in the three different light environments for 30 days after the transplant, then, submitted to two water treatments.

The Photosynthetic active Radiation (380nm – 720 nm) in the different environments was measured using a portable spectroradiometer USB-650 RED TIDE (Ocean Optics) at 8, 12 and 16h solar time once a week. Air temperature (T, in °C) and air relative humidity (RH, in %) were measured by a thermo hygrometer by Extect Instruments model RHT10 sampling every 3h. Vapor pressure deficit (VPD) was calculated from the temperature and relative humidity data.

Substrate field capacity (FC) of the sand substrate was determined (0.21 $m^3.m^{-3}$) using a volumetric humidity probe (ML2x TetraProbe, Delta Devices, UK). Citropotes® were coupled in seven-liter buckets wherein irrigation was conducted every three days, aiming to keep water availability between 65 % FC and 70% FC (high water availability treatment) and between 35% FC to 40% FC (low water availability treatment) using the ML2x TetraProbe to estimate the amount of water needed to keep constant water availability. Since ML2x TetraProbe measure soil volumetric water content in 10-15 cm, the height of the water column in the bucket was also measured.

Air temperature and DPV were similar for the different treatments in both measurement periods: 30 and 60 days after treatment imposition (DATI). Only PAR and water availability in both measurement periods differed significantly among the imposed treatments, with a decreasing PAR from FS>SN>SF (Table 2a.1). Thus, any change in leaf physiological traits in a specific species, was expected to be a result of light and water availability.

Table 2a.1 – Experimental conditions of full sun, Shade by shade net and shade film environments in 30 and 60 days after water treatment induction. VPD - Vapor pressure deficit; PAR –photosynthetic Active Radiation; T – average air temperature; θ – water volumetric content; % FC – percentage of field capacity.

Environment conditions		30 DATI			60 DATI		
		Sun	Shade Net	Shade Film	Sun	Shade Net	Shade Film
VPD (kPa) n=240		2.27±0.34a	2.46±0.24a	2.50±0.22a	2.00±0.42a	2.04±0.49a	2.10±0.37a
PAR (μmol $m^{-2}s^{-1}$) n=12		592.5±64.7a	280.7±14.1b	201.2±21.6c	556.8±87.8a	275.6±19.7b	196.2±17.0c
T (°C) n=240		30.69±1.75a	31.49±1.61a	31.57±1.72a	28.18±2.65a	29.13±1.98a	28.79±1.34a
θ (m^3 m^{-3}) n=60	High (% FC)	0,137±0,012 a (65.5±5.7)	0,147±0,022a (69.8±8.5)	0,142±0,024a (67.6±8.8)	0,138±0,013a (65.7±6.2)	0,149±0,018a (71.0±9.1)	0,146±0,015a (69.5±6.7)
	Low (% FC)	0,075±0,009 a (35.7±7.0)	0,088±0,012a (42.1±5.5)	0,090±0,012a (42.9±6.7)	0,072±0,010a (34.3±4.8)	0,087±0,010a (41.5±5.0)	0,089±0,012a (42.4±4.7)

Means with the same letter, between Sun, Shade Net and Shade Film, are not significantly different (Tukey, $p<0.005$) in the same evaluation time. Values are the means ± standard error.

2a.1.3 Leaf physiological traits

Leaf gas exchange

Leaf gas exchange measurements were conducted during the morning period between 9 and 10 a.m. using an IRGA model LI-6400XT (LI-COR, Lincoln, USA). Values of net CO_2 assimilation (*A* - μmol CO_2 m^{-2} s^{-2}), stomatal conductance (*gs* - mol H_2O m^{-2} s^{-1}) and transpiration (*E* – mmol H_2O m^{-2} s^{-1}) were obtained. From these values, the intrinsic water use efficiency (IWUE - μmol CO_2 mmol H_2O^{-1}) and the instantaneous water use efficiency (WUE - μmol CO_2 mmol H_2O^{-1}) were calculated. The measurements were conducted on three leaves per individual in ten plants in each environmental condition (for each species and each time of evaluation, 30 and 60 days, in a total of 180 samples). Analysed leaves were completely expanded and without any visible injuries caused by insects or diseases.

H_2O_2 content

H_2O_2 was determined according Velikova *et al.* (2000): a sample of 200 mg of leaf fresh weight was macerated in liquid nitrogen, added to 20% of PVPP (m/m) and homogenized in 1500 µL of trichloroacetic acid (TCA) 0,1% (m/v). The homogenate was centrifuged at 12000 g for 15 minutes at 4 °C. H_2O_2 was determined by measuring the absorbance at 390 nm in a reaction medium containing 500 µL of extract, 500 µL of 10 mM (pH 7.0) potassium phosphate buffer and 1000 µL of 1M potassium iodide.

Ascorbate content

Ascorbate concentration was determined as described by Arakawa *et al.* (1981): a sample of 50 mg of leaf fresh weight was macerated in liquid nitrogen, added to 20% of PVPP (m/m) and homogenized in 1500 µL of trichloroacetic acid (TCA) 5% (m/v). The homogenate was then centrifuged at 13000 g for 15 minutes at 4 °C. Aliquots (40 µL) of the supernatant were added to the reaction medium composed of TCA 5% (m/v), ethanol 99,8% (v/v), phosphoric acid (H_3PO_4) 0,4% in ethanol (v/v), bathophenantrolina 0,5% ethanol (m/v) and $FeCl_3$ 0,03% in ethanol (m/v). The mix was homogenized thoroughly and incubated at 30 °C for 90 minutes. Readings were performed at 534 nm.

Lipids peroxidation

Lipid peroxidation was determined by quantification of thiobarbituric acid reactive species, as described by Buege and Aust (1978). A sample of 200 mg of leaf fresh weight was macerated in liquid nitrogen, added to 20% of PVPP (m/m) and homogenized in trichloroacetic acid (TCA) 0.1% (m/v). The homogenate was centrifuged at 10000 g for 10 minutes at 4 °C. Aliquots of the supernatant (250 µL) were added to the reaction medium [thiobarbituric acid (TBA) 0.5% (m/v) and TCA 10% (m/v)], and then incubated at 95°C for 30 minutes. The reaction was stopped by rapid cooling on ice and readings were determined in a spectrophotometer at 535 nm and 600 nm. TBA form complexes of red color with low molecular weight aldehydes, such as malondialdehyde (MDA), a by-product of the peroxidation process. Concentration of MDA/TBA complex was calculated by the following

equation: [MDA] = (A535 – A600) / (ξ.b), where: ξ (extinction coefficient = 1.56 x 10-5 cm-1); b (optical length = 1).

ROS Enzimatic Scavenger System

Antioxidant enzymes were obtained by grinding a sample of 0.3 g of roots in liquid nitrogen, following the protocol proposed by Biemelt *et al.* (2000) with modifications. The supernatants were collected and used in the quantification of the activity of superoxide dismutase (SOD) (Giannopolitis and Ries 1977), catalase (CAT) (Havir and Mchale 1987) and ascorbate peroxidase (APX) (Nakano and Asada 1981). Specific activity was determined through the quantification of proteins (Bradford, 1976).

2a.1.4 Root-to-shoot ratio

At the end of the experimental period (60 days after treatment imposition) the plants were harvested, the parts (leaves, roots and shoots) where separated and oven dried for 72h at 60 °C. After this period, the parts were weighted and the results were expressed as the ratio between root-to-shoot dry mass.

2a.1.5 Data analysis and multivariate plasticity index

Data for all the phenotyped traits was organized for each of the species in each environment (light and water treatments variation) and for the 2 time points (30 and 60 DATI).

A PCA analysis was performed for the leaf physiological traits for each species and for each evaluation period. A PCA was performed considering the evaluation period for each species aiming to observe the plasticity over time. The MVPi was calculated from the PCA scores as mentioned in the session, using the Equation 1. All the analyses were carried out using the RStudio and Excel softwares.

2a.2 Results

2a.2.1 MVPi quantitatively approached the phenotypical plasticity of physiological leaf traits

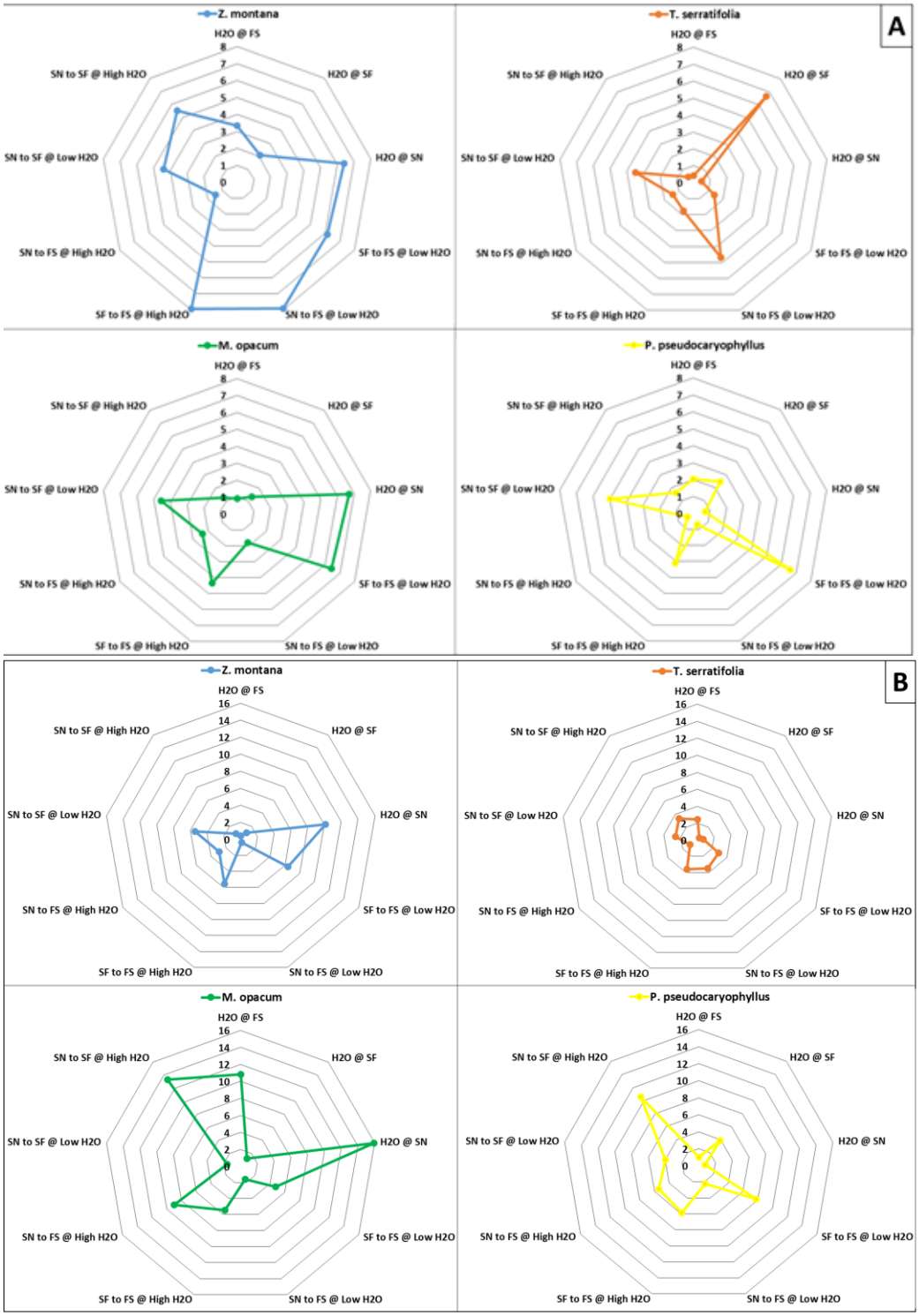
In general terms, at 30 DATI, *Z. Montana* presented higher plasticity to the tested environments, followed by *M. opacum*, *P. pseudocaryophyllus* and *T. serratifolia*. This can be observed by the areas formed in Fig. 2a.1-A, with bigger areas meaning bigger plasticity. The response to water was more prominent on the SN environment for *Z. Montana* and *M. opacum*, while *T. serratifolia* presented higher plasticity to water in the SF environment. *P. pseudocaryophyllus* presented low plasticity to water in all light environments (Fig. 2a.1-A).

For the light environments, *M. opacum* and *P. pseudocaryophyllus* presented higher plasticity when the plants were grown under SF. *Z. montana* presented the same pattern but also high plasticity between SF and FS at low water levels. *T. serratifolia* presented higher plasticity when grown at the SN environment (Fig. 2a.1-A).

At 60 DATI, *M. opacum* presented higher plasticity, followed by *Z. montana* and *P. pseudocaryophyllus* with *T. serratifolia* presenting the lowest plasticity levels. For the water effect, *M. opacum* presented high plasticity to water at FS and SN. *Z. montana* presented high plasticity to water on the SN environment. The other two species presented low plasticity to water (Figure 2a.1-B).

In terms of the light effects, for *Z. montana* and *P. pseudocaryophyllus*, the biggest plasticity was observed to plants grown at the SF environment. *M. opacum* presented higher plasticity to plants grown on the SN environment (Fig. 2a.1-B). For *T. serratifolia* plasticity was higher at the SN environment, however, in general, this species presented the lower MVPi values.

Figure 2a.1 – Plasticity for four Cerrado species in contrasting light and water environments

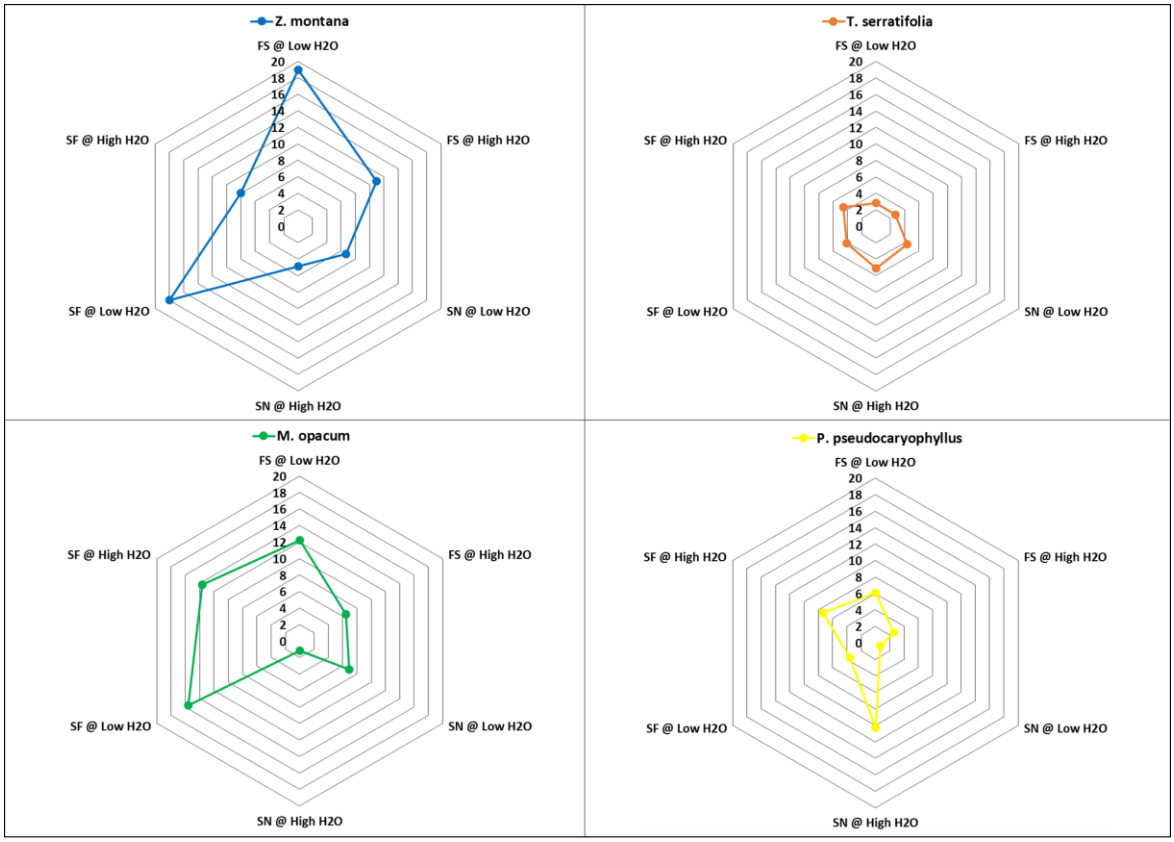


MVPi values at A) 30 and B) 60 days after treatment induction. FS, Full Sun; SN, Shade Net; SF, Shade Film; H2O, water levels.

2a.2.2 MVPi quantitatively approached the temporal variability of physiological leaf traits

Within each environmental condition, the stability of leaf physiological traits was estimated as the temporal MVPi (Fig. 2a.2). In general, after 30 days from the first evaluation *T. serratifolia* presented lower MVPi values, while *Z. montana* presented the higher values followed by *M. opacum* and *P. pseudocaryophyllus*.

Figure 2a.2 – Plasticity difference over time

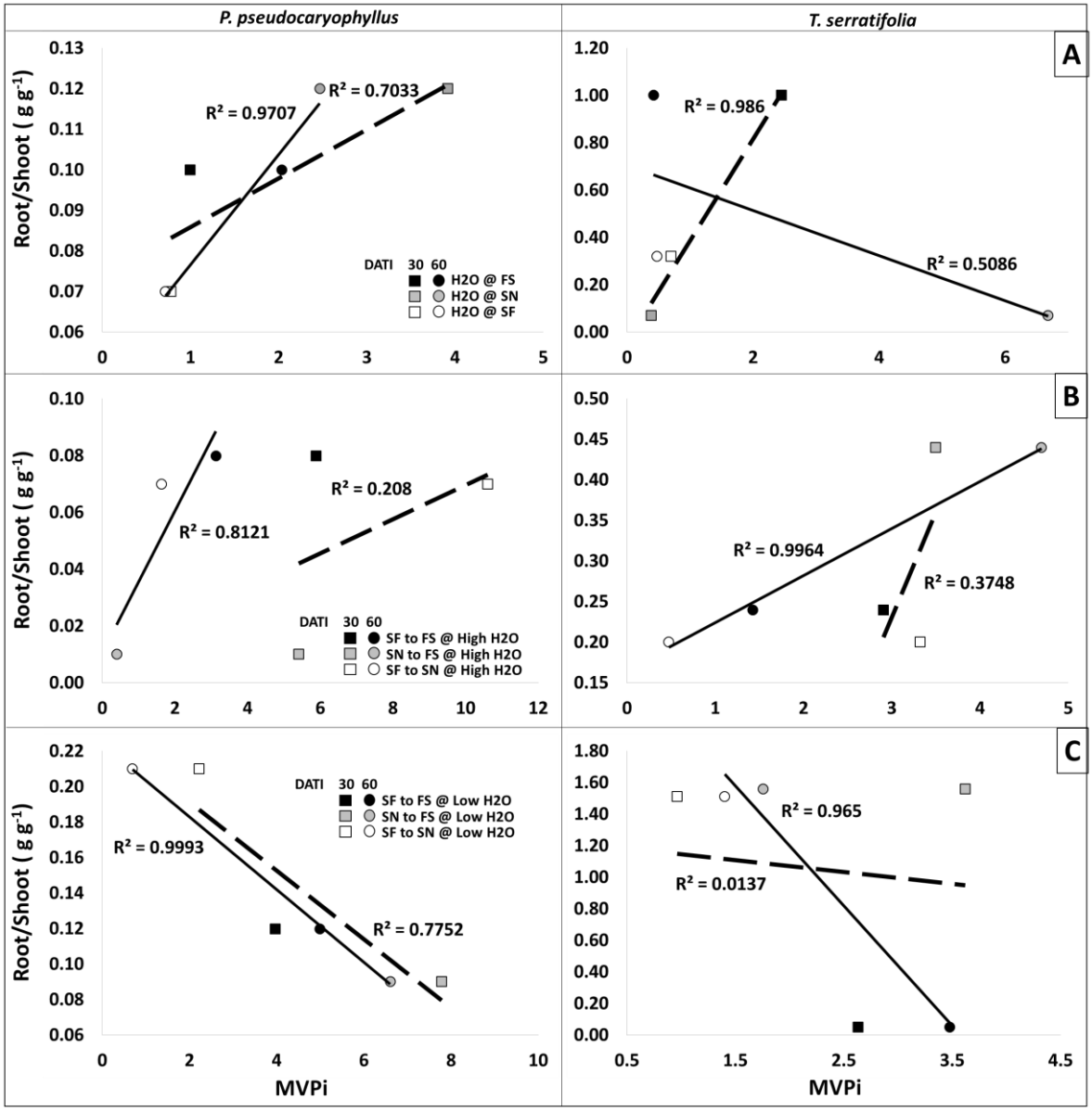


MVPi between 30 and 60 DATI. FS, Full Sun; SN, Shade Net; SF, Shade Film; H2O, water levels.

2a.2.3 MVPi as a predictor for plant fitness

The effect of leaf physiological plasticity to the adjustment of plant biomass allocation in response to the environmental conditions was analysed by plotting the MVPi versus the final root-to-shoot ratio (RSR) to *P. pseudocaryophyllus* (higher MVPi values at 60 DATI) and *T. serratifolia* (lower MVPi values at 60 DATI) (Fig. 2a.3) at 30 and 60 DATI.

Figure 2a.3 – Plasticity vs root-to-shoot ratio



Variation of root to shoot ratio in function of MVPi. Circles and Solid line – 30 DATI; Squares and Dash line – 60 DATI. FS, Full Sun; SN, Shade Net; SF, Shade Film; H2O, water levels.

For the effect of water, increased plasticity at 60 DATI was related to higher RSR for *P. pseudocaryophyllus* and *T. serratifolia*. The same pattern was observed for *P. pseudocaryophyllus* when comparing MVPi at 30 DATI and RSR at 60 DATI. For *T. serratifolia* higher plasticity to water at 30 DATI was linked to lower RSR at 60 DATI ((Fig. 2a.3-A).

For the effect of light at well-watered conditions, higher MVPi at 30 DATI was linked to higher RSR for both species. The correlation between MVPi at 60 DATI and RSR was lower than at 30 DATI (Fig. 2a.3-B). For the effect of light at low water conditions, higher MVPi levels at 30 DATI were related to lower RSR for both species. The same pattern was observed for *P. pseudocaryophyllus* when analysing MVPi at 60 DATI and RSR, but not for *T. serratifolia* (Fig. 2a.3-C).

2a.3 Discussion

Four Cerrado species were grown under different light conditions and water regimes in glasshouses aiming to understand their leaf physiological plasticity to different environments and its relation to growth and allocation patterns. Plasticity was measured by the MVPi method, proposed in this study. Results indicated contrasting patterns of plasticity in the multiple environments and different impacts of leaf physiological plasticity to the allocation patterns. Results are discussed in terms of the adaptation of species and the impact of their original habitat and their impact in the leaf physiological plasticity.

Leaf plasticity can play an important role in adjusting leaf metabolism to fit the environmental conditions in order to keep whole plant functional homeostasis through the adjustment of important process as carbon uptake and hydration (Thompson, 1991). In the specific case of the leaf physiological traits examined in our study, the species tend to modify their gas-exchange and antioxidant system performance as a response to water conditions. The decrease in leaf plasticity quantified by MVPi in general could be associated to plants under conditions of limited water availability, especially at the 30 DATI. The water shortage influenced the adjustment of photosynthesis to light conditions at this evaluation period, in general decreasing carbon assimilation and increasing the antioxidant system. This general response seems to be less specie-specific than the response to light when water availability was kept constant. This is because the fact that plants germinated and acclimate to the light environment after the imposition of the water regimes. Therefore, after 30 DATI the new physiological balance of the new leaves was still under the impact of the water-shortage.

Under diverse light conditions, the capacity of changing leaf physiological traits seems to be more specific for the Cerrado species. When the plants were well-watered, the adjustment of the antioxidant system played an important role to fit to light availability. This was observed especially for the plants growing under SF and for *M. opacum* and *Z. Montana*, which are, naturally, more abundant in open physiognomies.

The capacity of the leaf to change its metabolism in response to environmental variations can vary over time. This pattern was observed for the Cerrado species in the present study. *P. pseudocaryophyllus* and *M. Opacum* presented, in general, an increase in leaf plasticity over the evaluation periods, associated with an improvement in photosynthesis and water use efficiency, with a special highlight for *P. pseudocaryophyllus*. This is an evergreen understory tree and so the leaf physiological traits adjustment to environmental variation could be a crucial mechanism to fitness maintenance.

For *T. serratifolia*, maintaining some leaf traits more constant along time might be the best adjustment strategy. This is a deciduous species and the adjustment to environmental conditions could be more related to leaf phenology than to leaf physiology.

Considering the differences of functional group between *P. pseudocaryophyllus* and *T. serratifolia*, and its association with leaf physiological plasticity, we could identify diverse whole plant allocation patterns. *P. pseudocaryophyllus* allocates biomass to above ground parts when light is the limiting factor and to root growth when water is the limiting factor. Inversely, *T. serratifolia* always tends to allocate more biomass to root growth, with small shifts in leaf physiological traits. This observation confirms that leaf plasticity is species dependent, related to whole plant behaviour and adjustment strategies to the environmental changes. Thus, the concept that species with high leaf physiological plasticity and improved capacity to maintain the adjustment of variables in diverse environmental conditions tend to be less vulnerable to environmental changes is in check since, as demonstrated in the present study, leaf phenology and allocation strategies integrates the plant leaf response.

For *Z. montana* we observed in general a decrease in the MVPi values related to decreases in photosynthesis and stability of antioxidant system performance. When an

increase in plasticity results in a decrease in the traits' adjustment, and a decrease in their values, this is said to be due to the plasticity costs; or, in other words, when the modifications in plant traits do not cooperate to overcome the acclimation challenges imposed by the environment (VALLADARES *et al.*, 2007).

In the other hand, among the studied species, *Z. Montana* was the only one that presented improved photosynthetic levels under conditions of limited water. However it presented reduction in leaf plasticity to light, being more sensitive to light variability. Considering that water availability is a common limitation in the Cerrado domain, *Z. Montana* presented characteristics that can be highlighted as advantageous to adaptation in water-shortage conditions. Furthermore, *Z. Montana* presented the highest leaf plasticity to water in the full sun environment. This higher plasticity allowed a better adjustment of leaf metabolism and the maintenance of higher levels of photosynthesis and WUE when facing water variation in a high light environment.

2a.4 Conclusions and final comments

The MVPi was responsive to the different environments combining contrasting light and water conditions. The contrasting amplitudes of plasticity response to the environments and its different stability over time indicates different capacity and rates of physiological adjustment among the species and were partially linked to the species natural habitats inside the Cerrado biome. Reported correlations between the MVPi and root-to-shoot rate may reflect the impact of adaption in survival rate, fitness and capacity of facing stresses for each species. It is also important to highlight the link between leaf physiological traits and growth and allocation patterns.

2b CASE 2: STUDY OF FOUR SUGARCANE VARIETIES UNDER DIFFERENT IRRIGATION REGIMES

2b.1 Material and methods

2b.1.1 Plant material and experimental conditions

The study was conducted with 4 sugarcane (*Saccharum spp*) varieties breed by RIDESA (Rede Interuniversitária de Desenvolvimento do Setor Sucrooalcoleeiro). The chosen varieties are largely used in plantations in the Southeast region of Brazil, normally presenting high sucrose content in the stem as a characteristic. They present contrasting characteristics for drought tolerance and physiological maturity (Table 2b.1).

Table 2b.1 –Variety characterization for physiological maturity and drought-tolerance

Varieties	Physiological Maturity	Drought-Tolerance
RB72454	Medium	Sensitive
RB867515	Late	Tolerant
RB835486	Early	Tolerant
RB835453	Early	Sensitive

Plants were grown from gems obtained from the germplasm bank of the Universidade Federal de Lavras (UFLA). They were planted in propylene pots with volume of 8 liters. Natural substrate was collected and analyzed by the Laboratório de Análise de Solo do Departamento de Ciência dos Solos (UFLA). Fertilizers were added in order to meet the crop requirements as recommended by Alvarez *et al.* (1999). The propagation material was treated with fungicide (Derozol® - 1ml/L) and four propagation units were planted per pot. Two weeks after planting, two plants were discarded from each pot and two were kept for the experiment.

The experiment was conducted from February to June, 2014, in a glasshouse on the Plant Physiology Depart of UFLA in a completely randomized design. Plants were grown for

70 days under normal irrigation before the imposition of drought cycles. After that plants were split in two treatments: well-watered (WW – control) and water stress (WS). WW plants were kept under normal water irrigation regimes for all the experiment cycle with soil moisture between 80-100% of field capacity. WS plants had no irrigation from day 0 until day 8; re-irrigation from day 9 to day 37 (with soil moisture between 80-100% of field capacity, as WW); no irrigation from day 37 to 53.

Measurements were taken at days 0, 5, 8, 9, 16, 37, 46, 50 and 53 days after treatment imposition. Three replicates of each variety and each of the water treatments were used, totalizing 24 experimental units per time point. Different plants were used for the measurements at each time point. The leaf +1 from the main stem (DILLEWIJN, 1952) was used for the physiological measurements.

Environmental conditions, as air temperature and vapor pressure deficit, were monitored at each 30 minutes in the glasshouse using a HT 500 (Instrutherm) data logger.

2b.1.2 Physiological measurements

Leaf water potential and relative water content

The leaf water status was evaluated by the leaf relative water content. The leaf water potential (*LWP*) was measured using a Scholander pressure bomb (PMS Instruments - Modelo 1000). Maximum *LWP* was obtained between 4 and 5 am and the minimum *LWP* between 12 and 2 pm.

The relative water content (*RWC*) was determined for 5 leaf discs. Each disc was instantly weighted for fresh weight (*FW*) after cutting and then placed in a petri dish for with distilled water for 24 hours for the turgid weight (*TW*). It was finally dried in an oven at 70ºC for 48 hours for determining dry weight (*DW*) Relative water content was calculated as per Equation 2:

$$RWC\ (\%) = (FW - DW) \times 100 / (TW - DW) \qquad \text{(Equation 2)}$$

Gas-exchange analysis and Chlorophyll content

The analysis of gas-exchange parameters was conducted using an infra-red gas analyzer (IRGA) LI-6400 (Licor Inc., Nebraska, USA). Instantaneous values for net photosynthesis rate (*A*) stomatal conductance (*gs*) and transpiration (*E*) measurements were performed under saturating light conditions (1200 µmol photons m-2 s^{-1}), at leaf temperature of 25ºC and 400 µmol mol^{-1} of CO_2. Measurements were taken between 9 and 11 am. For dark respiration (*Rd*), measurements were taken from 9 to 11 pm using the same leaf and equipment. Chlorophyll content measurements were made using a SPAD meter.

Carbohydrate quantification

For the carbohydrate quantification, a sample of 200 mg of dried leaf material was homogenized in 5 mL of potassium phosphate buffer (100 mM, pH 7). It was placed in a 40ºC water bath for 30 minutes. The homogenate was centrifuged at 5000g for 10 minutes and supernatant was collected. Leaf sucrose concentration was determined by mixing 800 µL of the leaf extract and 800 µL of 30% KOH in an Eppendorf tube. The tube was placed in a 37ºC water bath for 15 minutes.

For starch extraction, the pellet was re-suspended in 8 ml of acetate potassium buffer (200 mM, pH4.8). Sixteen units of the amiloglucosidase enzyme were then added to the solution and left in a 40ºC water bath. It was centrifuged at 5000g for 20 minutes, supernatant was collected and the volume toped up to 15 mL.

For the quantification of starch and sucrose, the method of Antrona (DISCHE, 1962) was used. For the reducing sugar, fructose, the DNS method was used (Miller, 1959).

2b.1.3 Data analysis and MVPi calculation

Data was organized for each of the time points (0, 5, 8, 9, 16, 37, 46, 50, and 53) for the three replicates of the 4 varieties, in WW and WS treatments. The available data was: carbohydrate content (sucrose, fructose and starch); leaf water conditions (LWP_{max}, LWP_{min} and *RWC*), chlorophyll content; gas-exchange parameters (*A*, *gs*, *E* and *Rd*).

A Principal Component Analysis (PCA) was run using the R software for each variety in each time point. The results collected from the PCA analysis were: the eigenvalue, the percentage of variance explained (PVE) and the accumulated percentage of variance explained (Acc PVE) for each of the principal components; the scores for each of the 6 replicates (3 WW and 3 WS) in each of the PCs; the vectors coordinates for each of the 11 measured/calculated traits for each of the PCs. The MVPi was calculated as the Euclidian distance between WW and WS, for each of the replicates, using the Equation 1.

2b.2 Results

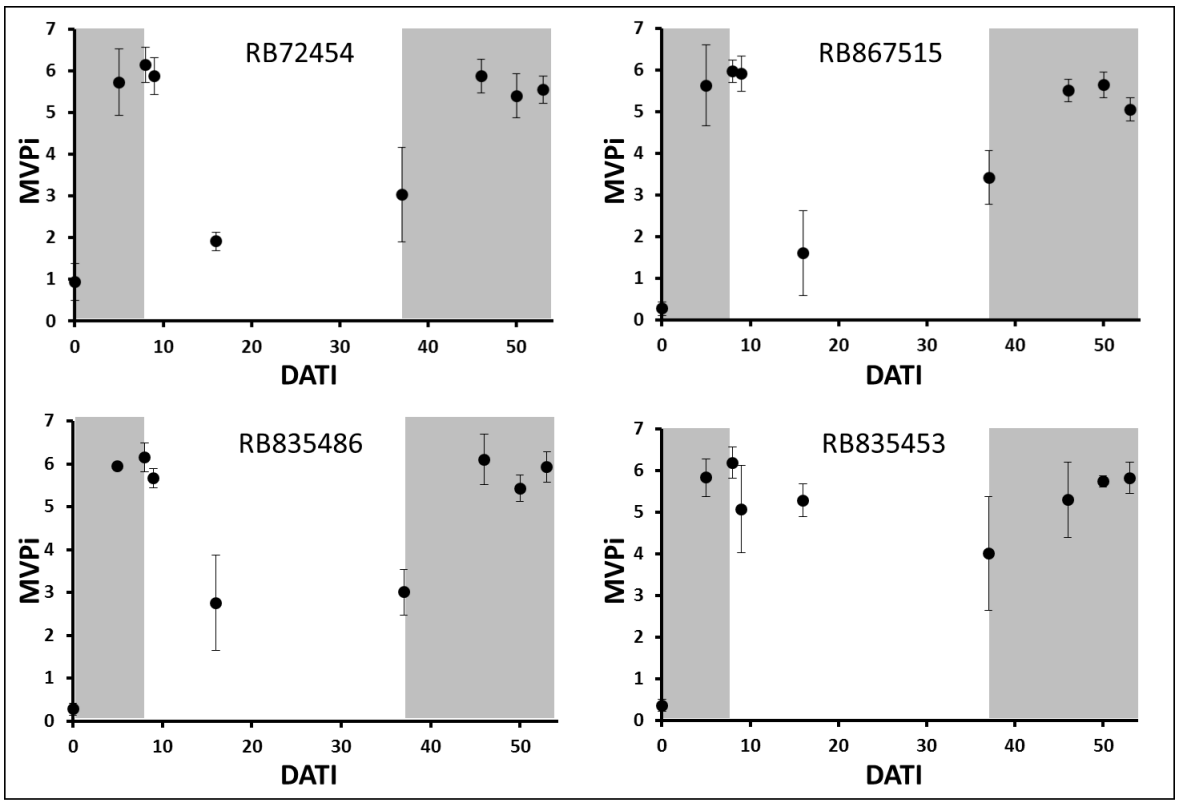
2b.2.1 The MVPi was responsive to the water treatments

The distance between scores of the PCA for well-watered (WW - control) and water stress (WS) treatments were calculated by the MVPi for four sugarcane varieties. The distance varied along the crop growth cycle showing the response of the plants to the water treatments and drought imposition (Fig. 2b.1).

Higher values of the MVPi represent a bigger distance between the scores of the WW and WS plants, for each variety and time. This reflects the change in the physiological balance of their leaves in response to the water conditions. Varieties with higher MVPi values tend to present higher leaf phenotypic plasticity to drought stresses.

A general pattern of increase in the MVPi was observed after the two drought stress impositions (day 0 and day 37) which represent an increase in the distance between WW and WS scores in the PCA. A recovery pattern was observed after the irrigation was returned to the WS treatment (day 8), representing a decrease in the distance between WW and WS scores in the PCA (Fig. 2b.1).

Figure 2b.1 – MVPi four 4 sugarcane cultivars under different water regimes



Multivariate Plasticity index (MVPi) response to water regimes in 4 sugarcane varieties grown in glasshouses, in Brazil. Grey areas represent the stress imposition intervals. The white area represents the interval when irrigation was the same for both treatments (well-watered and water stress). DATI, days after treatment imposition.

2b.2.2 Varieties behaved differently to drought stress imposition cycles

For the day 0 after treatment imposition, the index presented a low initial value to all the varieties, showing consistency in the response to the well-watered conditions which were applied to all plants before the drought was imposed for the plants in the WS treatment. When irrigation was suspended for the WS treatment, the MVPi increased, as observed for 5 and 8 days after treatment imposition for all the varieties (Fig. 2b.1). MVPi reached a value around 6 for all the cultivars after 8 days of drought imposition. The slope was similar to all the varieties, as well. This increase in the MVPi shows the index response to the physiological changes at a leaf level between WW and WS treatments.

Normal irrigation was returned to the WS treatment by day 8. Plants did not respond automatically to the irrigation as by day 9 the MVPi was similar to the day 8 for almost all the varieties. Only RB835453 presented a reduction in the distance for day 9. A recovery pattern was observed by day 16, represented by a decrease in MVPi. RB72454 and RB867515 presented values between 1 and 2, RB835486 between 2 and 3. RB835453 was an exception and did not show a decrease pattern, presenting MVPi around 5 by day 16, as by day 9 (Fig. 2b.1). These contrasting behaviours represent different capacities of the varieties to return to their normal physiological balance after facing a period of drought stress.

During the following days of normal irrigation (from day 16 to day 37), varieties behave differently. RB72454 and RB867515 presented a tendency of increase in MVPi, RB835486 did not change and RB835453 has presented a tendency of reduction. When the second drought regime was started, by day 37, the varieties presented and increased in the MVPi, reaching, by day 46, values in the same range as after the first stress. These values, around 6, were generally maintained for the following days of drought stress (Fig. 2b.1).

The rate of increase for MVPi was different for the two impositions of drought stress. From day 0 to day 5 there was an increase of around 5 units in MVPi, while from day 37 to day 46, there was an average increase of 2.5 units (Fig. 2b.1). This may be related to a memory of stress or a capacity of the plant to respond to a condition that was faced before. The fact that the plants cannot return to their base physiological balance after the first stress can also help to explain the difference between the slopes of MVPi change at the two different stress imposition times.

2b.2.3 Multiple traits influenced MVPi during the crop cycle

The leaf physiological balance was evaluated by multiple traits during the crop cycle. Carbohydrates' levels (starch, sucrose and fructose) levels, leaf water potential (maximum and minimum) and relative water content, chlorophyll, gas exchange parameters (photosynthesis, stomatal conductance and transpiration) and dark respiration). Those parameters were analysed using a principal component analysis (PCA) approach. From the PCA, just the two first principal components (PC1 and PC2) were significant (eigenvalue > 1)

and, in general they could, together, explain from 69 to 97% of the total variance in the data (data not shown).

The differences between the WW and WS plants that caused the variation in the MVPi were motivated by different traits along the cycle. In general, by day 8, the difference between WW and WS plants was highly explained by PC1 for the 4 varieties (84.7, 61.9, 79.5 and 85.3 for RB72454, RB867515, RB835486 and RB835453, respectively). By this day, for instance, in all the varieties the MVPi (or the difference between WW and WS) was pushed by reductions in gas-exchange parameters and chlorophyll content. However, the pattern of change for the varieties was not exactly the same for the carbohydrates profile, leaf water content or dark respiration for instance (Figure 2b.2).

By day 16, 8 days after re-irrigation, RB72454, RB867515 and RB835486 presented a reduction of MVPi (between 1 and 3), representing a bigger proximity to the WW plants in their leaf physiological balance. However, for RB835453, MVPi was still high (5.3) representing some contrasting behaviour for some traits between WW and WS plants. This behaviour was motivated by an increased content of starch and chlorophyll, higher photosynthesis and maximum leaf water potential in the WS plants and a decreased content of sucrose and fructose and a lower minimum leaf water potential and dark respiration (Figure 2b.2).

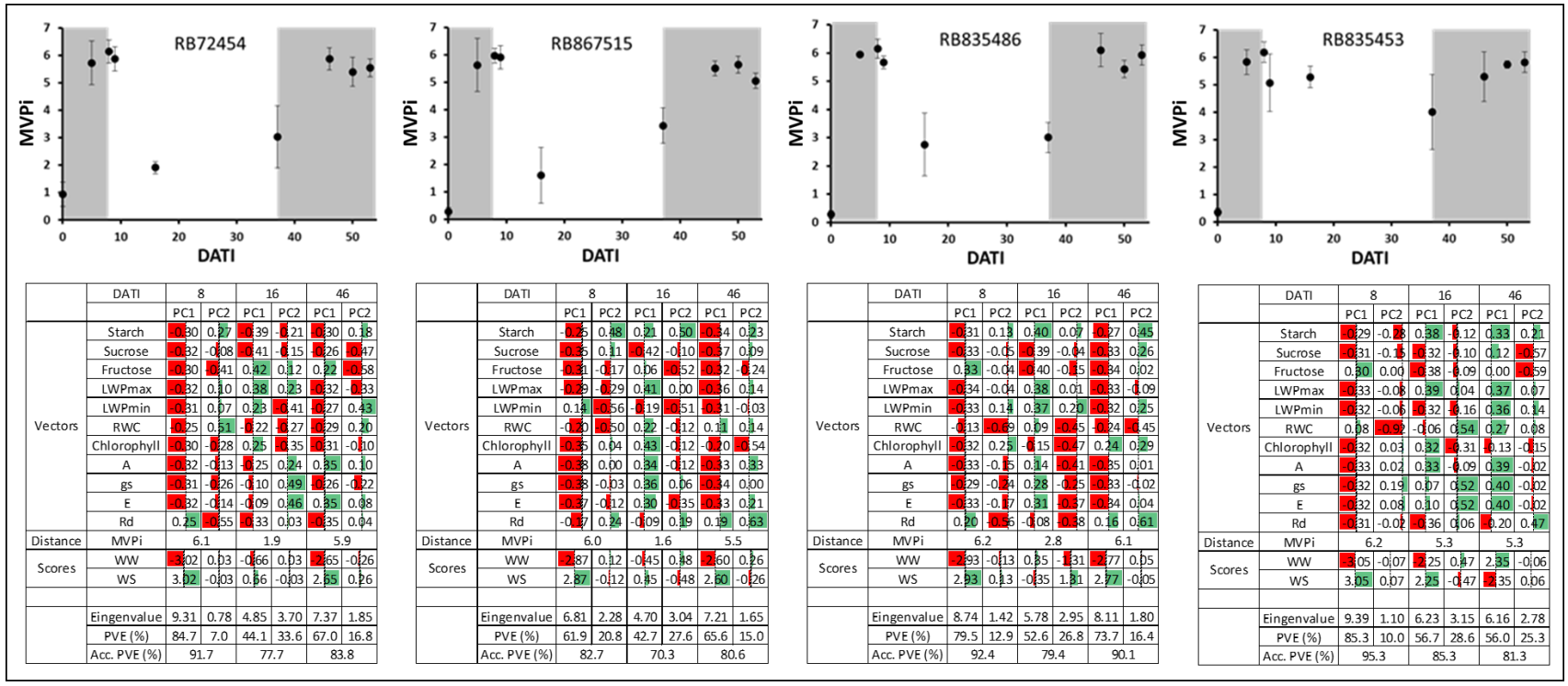
Nine days after the imposition of the second drought regime, by day 46, all the varieties presented an increase in MVPi, representing an increase in the difference between the leaf physiological balance between WW and WS regimes. RB835453 presented a smaller increase in the MVPi, as the previous values of MVPi (at day 37) were more elevated than the other three varieties. RB835486 and RB835453 presented a similar physiological balance, with the plants on WS presenting higher chlorophyll levels and higher *Rd*. RB867515 also presented *Rd* and also higher RWC. For RB72454, plants on the WS treatment presented higher fructose content and higher *A* and *E* than the WW plants (Figure 2b.2).

2b.2.3 Leaf physiological balance correlated to root growth under water stress

In general the varieties presented a reduction in root and shoot biomass from WW to WS conditions, however there was not a common pattern for the RSR (Figure 2b.3).

The accumulated MVPi was calculated as the area below the curve on Fig. 2b.1 and represents the accumulated difference in MVPi between WW and WS over the crop cycle. Plants with higher leaf physiological plasticity (higher accumulated MVPi) tended to present less biomass allocated to roots when growing under WS conditions (r = -0.95, p < 0.5). There is not a significant correlation between accumulated MVPi and shoot growth and RSR at WS and for any of the biomass allocation traits at WW conditions (Figure 2b.3).

Figure 2b.2 – MVPi and PCA results for 4 sugarcane varieties under different water regimes



	DATI	8		16		46	
		PC1	PC2	PC1	PC2	PC1	PC2
Vectors	Starch	-0.30	0.27	-0.39	-0.21	-0.30	0.18
	Sucrose	-0.32	-0.08	-0.41	-0.15	-0.26	-0.47
	Fructose	-0.30	-0.41	0.42	0.12	0.22	-0.58
	LWPmax	-0.32	0.10	0.38	0.23	-0.32	-0.33
	LWPmin	-0.31	0.07	0.23	-0.41	-0.27	0.43
	RWC	-0.25	0.31	-0.22	-0.27	-0.29	0.20
	Chlorophyll	-0.30	-0.28	0.25	-0.35	-0.31	-0.10
	A	-0.32	-0.13	-0.25	0.24	0.35	0.10
	gs	-0.31	-0.26	-0.10	0.49	-0.26	-0.22
	E	-0.32	-0.14	-0.09	0.46	0.35	0.08
	Rd	0.25	-0.55	-0.33	0.03	-0.35	0.04
Distance	MVPi	6.1		1.9		5.9	
Scores	WW	-3.02	0.03	-0.66	0.03	-2.65	-0.26
	WS	3.02	-0.03	0.66	-0.03	2.65	0.26
	Eingenvalue	9.31	0.78	4.85	3.70	7.37	1.85
	PVE (%)	84.7	7.0	44.1	33.6	67.0	16.8
	Acc. PVE (%)	91.7		77.7		83.8	

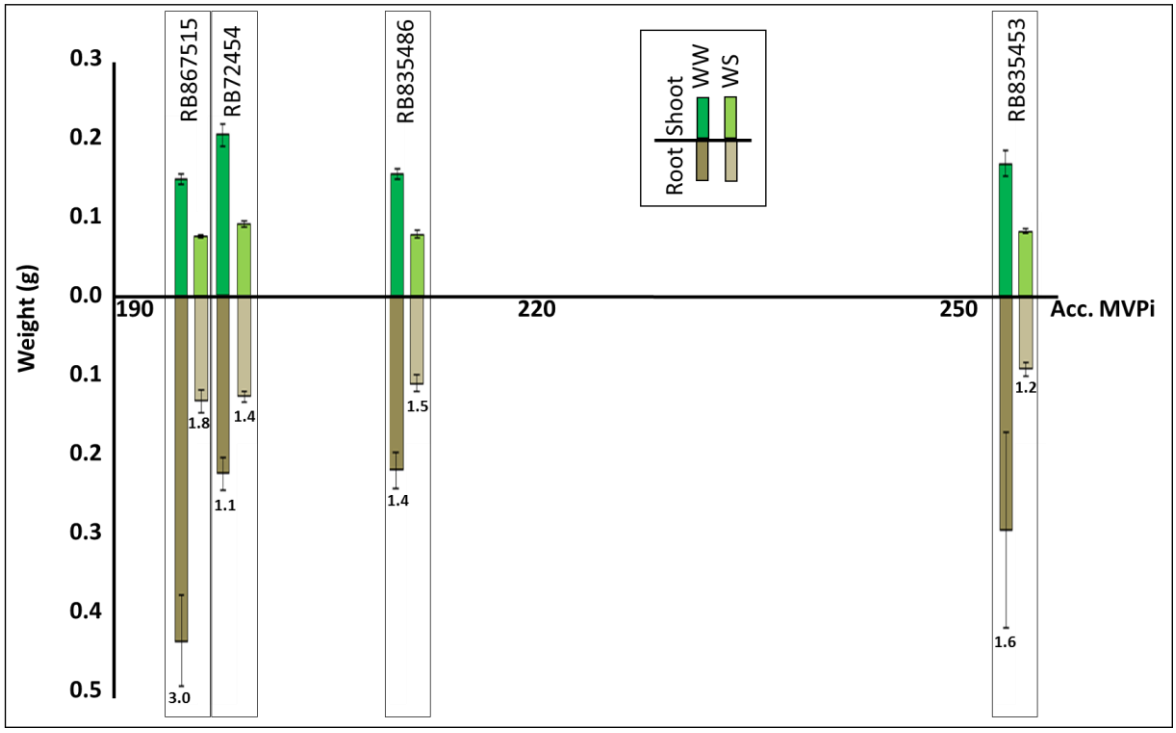
	DATI	8		16		46	
		PC1	PC2	PC1	PC2	PC1	PC2
Vectors	Starch	-0.25	0.48	0.21	0.50	-0.34	0.23
	Sucrose	-0.35	0.11	-0.42	-0.10	-0.37	0.09
	Fructose	-0.31	-0.17	0.06	-0.52	-0.32	-0.24
	LWPmax	-0.29	-0.29	0.41	0.00	-0.36	0.14
	LWPmin	0.14	-0.56	-0.19	-0.51	-0.31	-0.03
	RWC	-0.20	-0.50	0.22	-0.12	0.11	0.14
	Chlorophyll	-0.35	0.04	0.43	-0.12	-0.20	-0.54
	A	-0.38	0.00	0.34	-0.12	-0.33	0.33
	gs	-0.38	-0.03	0.36	0.06	-0.34	0.00
	E	-0.37	-0.12	0.30	-0.35	-0.33	0.21
	Rd	-0.17	0.24	-0.09	0.19	0.19	0.63
Distance	MVPi	6.0		1.6		5.5	
Scores	WW	-2.87	0.12	-0.45	0.48	-2.60	0.26
	WS	2.87	-0.12	0.45	-0.48	2.60	-0.26
	Eingenvalue	6.81	2.28	4.70	3.04	7.21	1.65
	PVE (%)	61.9	20.8	42.7	27.6	65.6	15.0
	Acc. PVE (%)	82.7		70.3		80.6	

	DATI	8		16		46	
		PC1	PC2	PC1	PC2	PC1	PC2
Vectors	Starch	-0.31	0.13	0.40	0.07	-0.27	0.45
	Sucrose	-0.33	-0.05	-0.39	-0.04	-0.33	0.26
	Fructose	0.33	-0.04	-0.40	-0.15	-0.34	0.02
	LWPmax	-0.34	-0.04	0.38	0.01	-0.33	-0.09
	LWPmin	-0.33	0.14	0.37	0.20	-0.32	0.25
	RWC	-0.13	-0.49	0.09	-0.45	-0.24	-0.45
	Chlorophyll	-0.32	0.25	-0.15	-0.47	0.24	0.29
	A	-0.33	-0.15	0.14	-0.41	-0.35	0.01
	gs	-0.29	-0.24	0.28	-0.25	-0.33	-0.02
	E	-0.33	-0.17	0.31	-0.37	-0.34	0.04
	Rd	0.20	-0.56	-0.08	-0.38	0.16	0.61
Distance	MVPi	6.2		2.8		6.1	
Scores	WW	-2.93	-0.13	0.35	-1.31	-2.77	0.05
	WS	2.93	0.13	-0.35	1.31	2.77	-0.05
	Eingenvalue	8.74	1.42	5.78	2.95	8.11	1.80
	PVE (%)	79.5	12.9	52.6	26.8	73.7	16.4
	Acc. PVE (%)	92.4		79.4		90.1	

	DATI	8		16		46	
		PC1	PC2	PC1	PC2	PC1	PC2
Vectors	Starch	-0.29	-0.28	0.38	-0.12	0.33	0.21
	Sucrose	-0.31	-0.15	-0.32	-0.10	0.12	-0.57
	Fructose	0.30	0.00	-0.38	-0.09	0.00	-0.59
	LWPmax	-0.33	-0.08	0.39	0.04	0.37	0.07
	LWPmin	-0.32	-0.06	-0.32	-0.16	0.36	0.14
	RWC	0.08	-0.92	-0.06	0.54	0.27	0.08
	Chlorophyll	-0.32	0.03	0.32	-0.31	-0.13	-0.15
	A	-0.33	0.02	0.33	-0.09	0.39	-0.02
	gs	-0.32	0.19	0.07	0.52	0.40	-0.02
	E	-0.32	0.08	0.10	0.52	0.40	-0.02
	Rd	-0.31	-0.02	-0.36	0.06	-0.20	0.47
Distance	MVPi	6.2		5.3		5.3	
Scores	WW	-3.05	-0.07	-2.25	0.47	2.35	-0.06
	WS	3.05	0.07	2.25	-0.47	-2.35	0.06
	Eingenvalue	9.39	1.10	6.23	3.15	6.16	2.78
	PVE (%)	85.3	10.0	56.7	28.6	56.0	25.3
	Acc. PVE (%)	95.3		85.3		81.3	

MVPi variation and PCA results for four sugarcane varieties at 8, 16 and 46 DATI. DATI, days after treatment imposition. PC1 and PC2, principal components 1 and 2. LWP max/min, leaf water potential max/min; RWC, relative water content; A, photosynthesis; gs, stomatal conductance; E, transpiration; Rd, dark respiration. WW, well-watered; WS, water stress. PVE, percentage of variation explained. Acc PVE, accumulated percentage of variation explained. Red and green colours represent negative and positive coordinates on the PCs' axes for the vectors of the measured parameters and the score of the varieties under WW and WS regimes. The size of the bar means represents the value of the coordinates.

Figure 2b.3 – Accumulated MVPi and biomass allocation for 4 sugarcane varieties under different water regimes



Accumulated MVPi and biomass accumulation on roots and shoots for four sugarcane varieties. Accumulated MVPi was calculated as the area below the curve on fig. 2b.1. Error bars are the standard error of the mean. Values below the error bars are the root-to-shoot ratio (RSR).

2b.3 Discussion

Four sugarcane commercial varieties with different patterns of tolerance to drought were grown under glasshouse conditions aiming to understand the impact of drought cycles in their leaf physiological balance. A new method of integrated evaluation of plant plasticity and performance was also applied to the collect data. The MVPi was responsive to the changes in irrigation patterns and reflected the differences between WW and WS plants, by calculating their Euclidian distances in a PCA analysis. The physiological traits that influence the MVPi response varied along the cycle. The discussion is based on the different physiological balances of the cultivars, its implication in the MVPi and its influence in plant response.

A clear pattern of increases in MVPi was observed after the two occasions where drought stresses were imposed to the sugarcane plants, by days 0 and 37 (Fig. 2b.1). This

pattern reflected the changes in leaf physiological traits between the WW and WS. Considering the two areas where MVPi presented increase (grey areas in Fig. 2b.1, between day 0 and 8 and between day 37 and 53), the slope and the amplitude of the first was bigger than the second. The smaller slope and amplitude in the second stress cycle could have been influenced by the first drought cycle. This can be observed by comparing the physiological profile of the plants by day 8 (8 days after the first stress imposition) and by day 46 (9 days after the second stress imposition). For all the cultivars, MVPi was around 6 at both days, reflecting the same distance between WW and WS in the PC Cartesian plan. The physiological profile for RB867515, RB835486 and RB835453 were pretty similar in the two time points (PC1 vectors by day 8 and 46 in Fig. 2b.2). However RB72454 plants presented considerable changes in their physiological profile by day 46 if compared to day 8. For instance, the vectors for *A* and *E* were positively linked to the WS plants average score by day 46, in opposition to the observed by day 8. These differences between the varieties may be related to different stress memory from each of the varieties.

The other common pattern on the index changes was the decrease in MVPi after the restart of irrigation on the WS plants, by day 8 (Fig. 2b.1). While RB72454, RB867515 and RB835486 decreased to values between 1 and 3 by day 16, RB835453 presented a higher value for the same day. This behaviour was mainly related to the WS plants behaviour in LWP_{max}, *A* and chlorophyll content. The different performances of the varieties highlight the contrasting capacities of each of them to return to their base metabolism after facing a period of stress. Even the WS plants in the varieties that presented a quicker decrease in MVPi did not return to values closer to the WW or to MVPi values similar to those on day 0.

In terms of the differences of tolerance to drought stress between the varieties, apparently, there is not a strong link between the tolerance and the physiological leaf profile or the MVPi for this experiment. The two drought-tolerant cultivars, RB867515 and RB835486, did not perform much differently than the others. In some cases, the drought-sensitive varieties, RB72454 and RB835453, presented an improved physiological profile in terms of carbon and water balance than the tolerant ones, as reported in the above paragraphs. This may be related to the fact that the qualification in drought-sensitive or tolerant is based

mainly in the biomass production under stress conditions and not in the physiological balance of the plant as a whole under those constraints.

The MVPi index accumulated over the cycle correlated negatively to the biomass allocation under WS conditions. In this specific case, being more plastic did not result in an advantage for plant growth, specifically for root biomass accumulation. Usually under drought conditions, allocation of biomass is common to exploit lower layers in the soil. Further investigation on root elongation and final productivity could help to understanding if the increased plasticity could have resulted in higher final biomass production.

2b.4 Conclusions and final comments

The different patterns of MVPi revealed different plasticity to water regimes among the sugarcane varieties. The MVPi method showed itself sensitive to the irrigation regimes. The PCA vectors and scores were useful to understand which physiological parameters played important role during the drought and re-irrigation cycles and at the same time allowed an integrated view of plant plasticity and the disparity between WW and WS plants in the same species.

The MVPi was correlated to allocation patterns on plant showing coupling between leaf physiological balance and biomass production. The use of the method for evaluating leaf physiological plasticity in combination to productivity data could enhance the understanding of the different physiological adjustments to sugar content and final biomass production.

3 GENERAL DISCUSSION AND CONCLUSIONS

The Multivariate Plasticity index (MVPi) was used to integrate a set of variables, measured at the leaf level, defining the leaf plasticity for 4 species of the Cerrado biome and 4 sugarcane varieties. By using this approach, phenotypic plasticity, moreover than a measurement of specific changes in punctual traits, can be evaluated as an integrative characteristic of plant species that can be fragmented in different hierarchical levels. It is important to highlight that the MVPi was not compared to other methods of evaluation of plasticity in the research. The reason is the fact that, of our knowledge, until the date of publication of this thesis, there is not an index for evaluation of plant plasticity that integrates multiple traits. A comparison trait by trait could be applied although it would not represent the main improvement of the method which is the integrative analysis of multiple traits.

The MVPi was proved to be a useful tool for evaluating phenotypic plasticity, at the leaf level, on an integrated way and not fragmented to each individual measured variable. It was also correlated to biomass allocation patterns showing connection between leaf physiological balance and yield drivers or components.

The amplitude of change observed in each of the case studies reinforces the capacity of the MVPi to respond to different levels of phenotypic variation. This can be observed by its range of variation between species in the case study 1 (around 16; Fig. 2a.1) and between varieties of the same species in the case study 2 (no more than 3; Fig. 2b.1).

An advantage of the use of the MVPi is the facility of evaluating phenotypic re-adjustment of genotypes after a period of stress. This could be observed in the case of study 2 when after a period of drought, WS plants re-adjusted their leaf physiological balance but did not return to the same state as the WW ones (Fig 2b.1). As some WS plants presented higher photosynthesis than WW plants 7 days after irrigation was re-stablished, a single trait analysis could bring an idea of complete recuperation of the WS plant, although it is not observed when plasticity is evaluated in an integrative analysis.

In summary, the MVPi showed potential of use to the evaluation of plant phenotypic plasticity in a leaf level. Coupling the plasticity data with plant growth and development and

mainly productivity indices may help to clarify the physiological process driving plant adjustment in multiple environments and how it can influence crop productivity and species adaptation. The data presented in both case studies reinforces the link between plasticity analysed as a systemic attribute and biomass allocation. The use of MVPi may be useful in the process of choosing species for recover of conservation areas or of genotypes for breeding programs.

Integrating the use of the MVPi to other fields of study could help to enhance the understanding of the plasticity process and its potential impact in plant behaviour under contrasting environmental conditions. Molecular biology studies could help to unravel the role of the intrinsic plasticity in species; metabolomics and proteomics to identify the mechanisms of plasticity; modelling for predict phenotypic plasticity in different future scenarios and so on.

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