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Marco Zibordi, Pierre-Eric Lauri, Jean-Luc J.-L. Regnard, Luca Corelli
Grappadelli

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Fast and reliable phenotyping of leaf functions: a tool for water stress tolerance evaluation

P. Losciale¹, L. Manfrini², B. Morandi², E. Pierpaoli², M. Zibordi², P.E. Lauri³, J.L. Reignard³ and L. Corelli Grappadelli²

¹Consiglio per la Ricerca in Agricoltura e l'analisi dell'Economia Agraria, Bari, Italy; ²Dipartimento di Scienze Agrarie, Università di Bologna, Italy; ³INRA, UMR AGAP, 34398 Montpellier, Cedex 05, France.

Abstract

A protocol and a related physiological tool to discriminate drought tolerant apple genotypes in a reliable, fast way were assessed. Trials were carried out in two years and two sites: Bologna (Italy) and Montpellier (France). Potted trees from 17 genotypes grafted on Pajam 2 rootstocks were used. After sufficient shoot development, three pots genotype⁻¹ were maintained at field capacity, while three other plants were subjected to water stress, by keeping their soil water content at about 40% of field capacity. Leaf net photosynthesis, stomatal conductance, electron transport rate via chlorophyll fluorescence, air and leaf temperature were measured on each plant. By a multi-variate semi-mechanistic approach, a new physiological index (I_{PL}) was developed, tested and validated, which appeared strongly and linearly related to net photosynthesis. All the validation tests suggest I_{PL} can be used as a reliable and fast (about 30" leaf⁻¹) photo-assimilation index. The response of apple genotypes to water shortage was evaluated considering the percent reduction in leaf parameters when plants were subjected to water stress. These percentages were subjected to a Principal component analysis (PCA) and the case coordinates of the main components F1 and F2 were clustered via a K-means clustering. Each genotype was placed in one of 3 pre-defined classes: drought susceptible, intermediate, tolerant. The same clustering was performed using only the I_{PL} percent reduction. Using either F1, F2 or the less time consuming I_{PL} as discriminant factors, about 53% of the genotypes were assigned to the same class in the four cases (2 years and 2 sites). I_{PL} could be useful to assess leaf functioning under varying environmental conditions, like water availability. However, as only half of the genotypes behaved in the same way in the 4 cases, further research should be performed in order to set up a replicable water stress imposition protocol.

Keywords: fluorescence, drought, PCA, phenotyping, photosynthesis

INTRODUCTION

Water shortage is one of the pivotal societal problems of the 21st century and agriculture can contribute to savings via increased efficiency of its use with minimal negative consequences in terms of quality and productivity (Shideed, 2004). Selection of stress tolerant genotypes is one strategy to cope with water scarcity, although genetics of fruit tree species render this approach more complicate and expensive, given their perennial nature and the long non-fruited period. Considering this limitation and the polygenic nature of drought tolerance, a phenotyping pre-selection based on quick and easy identification of putative water stress tolerant genotypes could be useful to concentrate research efforts on the most promising ones. Although leaf photosynthesis and stomatal conductance are considered some of the most reliable variables, as they give information about leaf functionality and water consumption, their determination is quite time consuming (≈ 3 min leaf⁻¹), thus incompatible with the huge amount of progenies and individuals breeders must work with. Stem water potential is surely one of the most used proxy for evaluating plant water status (Naor et al., 1995), however its detection could be time consuming as leaves should be pre-conditioned by covering with an aluminium bag for 90-120 minutes. Other



proxies like leaf temperature or chlorophyll fluorescence are quicker but not always accurate and reliable when considered separately (Edwards and Baker, 1993; Flexas et al., 2002; Jones et al., 2002).

This paper describes the state of the art of a “research path” started during the EU Fruit Breedomics project. One of the main purposes of the project was to set up a physiological tool and a water stress protocol usable for the reliable and quick discrimination of water stress tolerant apple and peach genotypes. Previous work pointed out that chlorophyll fluorescence and leaf temperature could have been candidate variables for building up a new index describing plant performance (Losciale et al., 2014). Starting from this preliminary findings the present study reports the results of a multi-year and multi-site trial aiming at assessing a replicable water stress protocol and discriminating drought tolerant from susceptible apple genotype through a newly developed physiological index.

MATERIALS AND METHODS

The trial was carried out in Bologna (Italy) and Montpellier (France) in 2011 and 2012 using 15 apple genotypes from the ‘Granny Smith’ (GS) × ‘Stark Delicious’ (STK) cross, plus the two parents (ID of each genotype: 7, 23, 26, 35, 37, 38, 40, 41, 48, 54, 70, 96, 117, 121, 125, GS, STK), supplied by INRA (France) in the context of the Fruit Breedomics European Project. Grafts comprising two-three buds for each genotype were grafted on Pajam rootstocks, and grown in pots containing a mix of 20:80 sand:soil, in Bologna and a mix composed of 40% brown peat, 30% composted pine bark, 20% disinfected soil and 10% pouzzolane (2/6 mm), in Montpellier. Six shoot plants genotype⁻¹ (whose terminal buds were still growing) were transferred into a greenhouse, where maximum temperature and relative humidity were set at 28°C and about 60%, respectively. The experiment was started after the shoots had grown in the greenhouse for a length exceeding 80-100 cm. For each genotype 3 plants were well watered, maintaining the soil at field capacity (WW); the remaining 3 plants were subjected to water stress maintaining the soil moisture at about 40% of field capacity (WS). Previous work performed on the same genotypes had shown that this level of soil humidity could be considered as a threshold at which the pots should be maintained to be sure that they were under stress conditions, although not visible yet (Losciale et al., 2014). Plants were maintained at this new condition for 2 weeks. After this adaptation period, leaf gas exchange and fluorescence parameters were measured on the leaf at Plastic Index (PI) of 12 for each shoot, at midday. At the same time, stem and leaf water potential measurements were performed on the leaf with PI of 11. For each leaf net photosynthesis (P_n , $\mu\text{mol m}^{-2} \text{s}^{-1}$) stomatal conductance (g_s , $\text{mol m}^{-2} \text{s}^{-1}$), leaf and air temperature and their difference (T_l , T_a , DT , °C), the electron transport rate exiting from Photosystem II (J_{PSII}) were measured using an open circuit infrared gas exchange system fitted with a leaf fluorometer and a LED light source (Li-COR 6400, LI-COR inc., Lincoln Nebraska, USA). The actinic light was set at $600 \mu\text{mol m}^{-2} \text{s}^{-1}$. Leaf (PI) and stem (Ps) water potential (MPa), were measured at the same time with a Scholander pressure chamber according to Naor et al. (1995). Genotype response to water limitation was evaluated considering the reduction in the variables measured (P_n , g_s , J_{PSII} , T_l , PI, Ps). For each genotype, the analysis was carried out on the ratio between the average of the values in WS and in WW shoots (i.e. $P_n_R = P_n_WS/P_n_WW$). A principal component analysis (PCA) was performed for all ratios among variables for each genotype. The case coordinates of the main factors explaining more than 80% of the total variance (at least two factors) for each genotype, were subjected to a K-means clustering in order to assign each genotype to one of the 3 pre-defined drought tolerance classes: susceptible, intermediate, tolerant.

The same multi-year and multi-site dataset was used to develop a quick and reliable index (I_{PL}) able to estimate accurately leaf photo-assimilation (Losciale et al., 2015). A subset of the whole dataset of 2011 (75%) was randomly chosen and, using a multi-variate, semi-mechanistic approach a step-wise multiple linear regression analysis was performed. The regression considered P_n as dependent variable and J_{PSII} , the Michaelis Menten constants for carboxylation (K_c) and photorespiration (K_o , aggregated with J_{PSII} forming a new variable

$P_{KOKC} = J_{PSII} \times K_O / K_C$), and the leaf to air temperature difference (ΔT) as predictors. The obtained and parametrised equation was internally cross validated on the remaining 25% of the 2011 dataset, then externally cross validated on the whole 2012 dataset. As for all the variables listed above, also for I_{PL} the response to water shortage was evaluated on the ratios I_{PL_WR} / I_{PL_WW} for each genotype and the values were subjected to a K-means clustering assigning each genotype to one of the three pre-defined classes.

RESULTS AND DISCUSSION

The water stress protocol was quite effective. As in 2011 (Losciale et al., 2014) also in 2012 a general decrease of leaf net photosynthesis was recorded when plants were subjected to water restriction, even if no visible symptoms of stress occurred. Nevertheless, it was quite difficult to reach the same level of stress in the 2 years and 2 sites. For example in 2012, water restriction reduced Pn in genotype 117, however the reduction was 70 and 30% in Italy and France, respectively (Figure 1). A possible reason for this discrepancy could be the different soil composition used in the 2 sites as well as the different microclimate in the 2 greenhouses. Even if maximum temperature and relative humidity were maintained at about the same level between the 2 sites, other variables such as temperature, light intensity and quality, might have changed. A study carried out on *Arabidopsis* (*Arabidopsis thaliana*) involving different laboratories pointed out this difficulty even in those particularly controlled conditions (Massonnet et al., 2010).

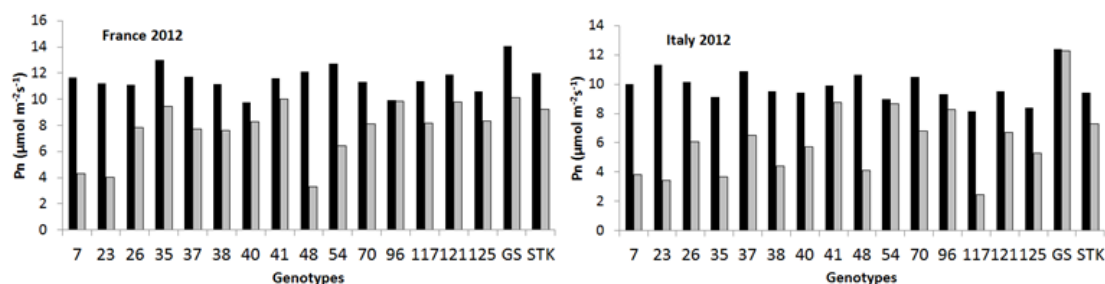


Figure 1. Net photosynthesis (Pn) recorded on 17 apple genotypes, well-watered (black bars) or subjected to water restriction (grey bars), in Italy and France in 2012.

The PCA analysis performed on the ratio between the average of the values in WS and WW shoots for the 6 variables taken into account showed that the first 2 factors were able to explain more than 80% of the total variance in all of the 4 cases (2 sites \times 2 years). In general, Factor 1, explaining from 65 to 90% of the total variance depending on the site and year, was mainly related to leaf functionality variables (Pn, gs, J_{PSII} , DT), followed by leaf and stem water potential. Leaf functionality variables and water potential were negatively correlated confirming the linkage between gas exchange and water potential: the more the increase of water potential (in absolute value) the more the reduction of stomatal conductance, leaf photosynthesis and J_{PSII} . Stem and leaf water potential mainly contributed to determine Factor 2 (Figure 2a). The projection of the cases on the factor plane revealed that the 17 genotypes under investigation were quite spread along Factor 1 and the K means clustering performed on F1 and F2 case coordinates ranked the genotypes in 3 pre-defined classes: drought tolerant, intermediate, drought susceptible. In 2012 in Italy, genotypes with the lowest reduction of their performance when subjected to water restriction (tolerant) were: 41, 54, 96, GS; followed by 26, 37, 38, 40, 70, 121, 125. The most drought susceptible genotypes were: 7, 23 and 35 (Figure 2b).

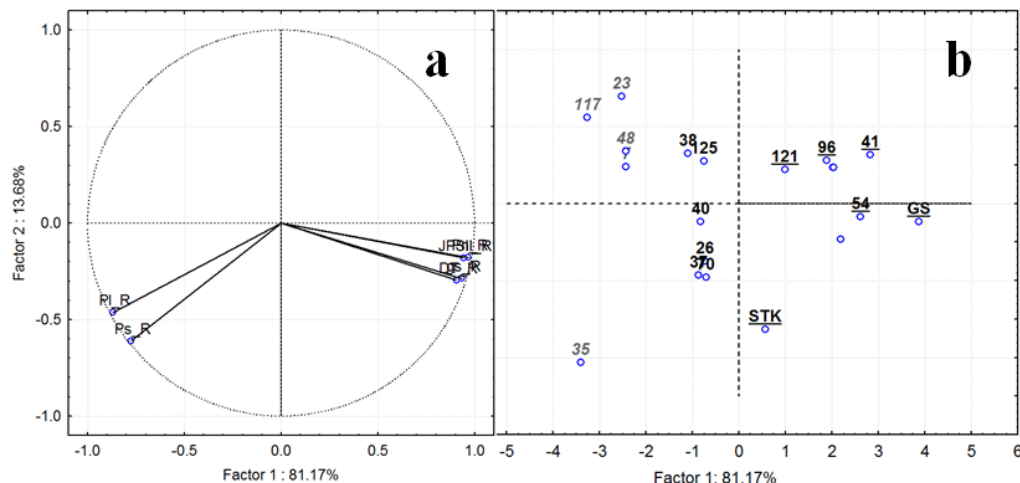


Figure 2. PCA analysis performed on the ratio between the average of the values in WS and WW shoots for each genotype for the following variables: net photosynthesis (Pn_R); stomatal conductance (gs_R); electron transport rate exiting from Photosystem II (JPSII_R); leaf to air temperature difference (DT_R); leaf (PL_R) and stem (Ps_R) water potential (a). Projection of the cases on the factor plane. Susceptible, intermediate and tolerant genotypes in grey, black and underlined black, respectively. Discrimination was performed by means of a K-means clustering using the Factor 1 and Factor 2 case coordinates of each genotype (b). Data refer to the Italian site in 2012.

Linear multiple regression analysis, used to obtain a quick index strictly related to net photosynthesis and performed on 75% of the 2011 dataset, revealed that using $P_{KO/KC}$ and ΔT as predictors and Pn as dependent variable, the relation between the observed Pn and the estimated Pn (I_{PL}) was linear with an adjusted r^2 of 0.86, residual prediction deviation (RPD) of 2.62 and a 1 to 1 relation. All these parameters (Chang et al., 2001; Bellon-Maurel et al., 2010) suggested that I_{PL} and the model behind were reliable and able to give an accurate estimation of the leaf photo-assimilation (Losciale et al., 2015). The same results were obtained when the model was validated on the remaining 25% of the 2011 dataset, and the subsequent external cross validation on the entire 2012 dataset (Figure 3).

For each genotype the ratios between the average of I_{PL} values in WS and WW shoots (I_{PL_WS}/I_{PL_WW}) were subjected to a K-means clustering with 3 pre-defined classes. Rankings obtained using either I_{PL} or F1, F2 as discriminating factors were quite similar and in Italy in 2012 the two rankings matched almost totally (Figures 2b and 4). Using either F1, F2 or I_{PL} as discriminant factors about 53% of genotypes were assigned to the same classes in the 4 cases (Table 1).

Table 1. Classification of apple genotypes that were assigned to the same category (Susceptible, Intermediate, Tolerant) in 2011, 2012, in Bologna and Montpellier experimental sites.

Category	Genotype
Drought susceptible	7, 23
Intermediate	26, 37, 70, 125
Drought tolerant	41, 54, STK

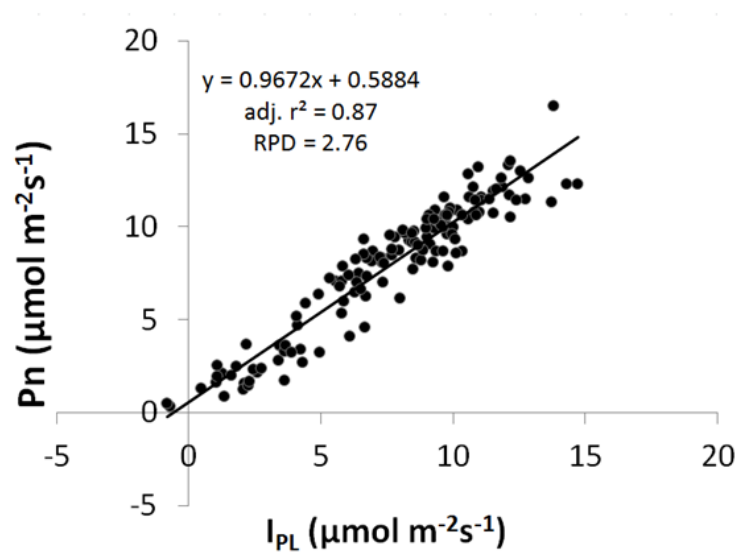


Figure 3. Linear relation between observed leaf net photosynthesis (Pn) and the I_{PL} index during the external cross validation stage performed on the entire 2012 dataset.

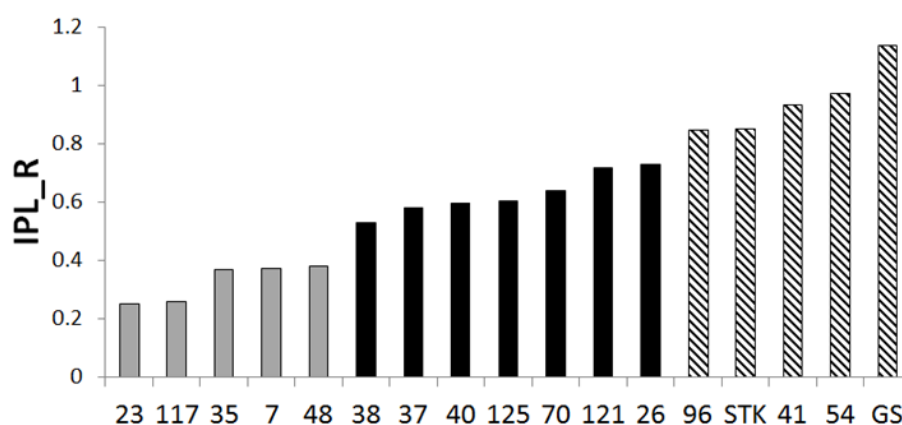


Figure 4. Ranking performed subjecting to K-means clustering with 3 pre-defined classes the ratios between the average of I_{PL} values in WS and WW shoots (I_{PL_WS}/I_{PL_WW}) for each genotype. Drought susceptible, intermediate and tolerant genotypes in grey, black and dashed bars, respectively. Data refer to the Italian site in 2012.

CONCLUSIONS

The water restriction protocol was able to limit plant functionality before visible symptoms appeared. However this protocol proved difficult to replicate, as plant behaviour can be affected by other microclimatic variables hardly manageable in different sites and years.

The I_{PL} index seems to be a promising tool to assess leaf photo-assimilation in a reliable and fast way, as the acquisition time of the parameters needed is about 30 s leaf⁻¹ instead of 3 min required for a gas exchange measurement. Development of this kind of indices could also lead to improved water stress protocols. For example, a less time consuming (even continuous) measure of leaf functionality could allow to analyse the leaf functionality trend as a function of soil dehydration, rather than considering a single point of water restriction.

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