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Research article

Plant stress analysis: Application of prompt, delayed chlorophyll fluorescence and 820 nm modulated reflectance. Insights from independent experiments

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ABSTRACT

Nine short-term independent studies were carried out with two M-PEA units on several plant species differing in their functional traits (woody evergreen, woody deciduous, herbaceous) and exposed to different kind of abiotic stress (drought, salt, ozone, UV radiation). Aim of the study is to check the consistency of plant responses, assessed through three sets of simultaneously measured signals: Prompt Fluorescence (PF), Delayed Fluorescence (DF) and Modulated Reflectance of 820 nm light (MR). The decrease of F_V/F_M and F_0, the increase of V_J and V_I were the most common responses related to PF parameters. The decrease of v_{ox} and v_{red} as well the increase of MR_{min} were common response of MR. DF showed species-treatment specific behaviours. The Principal Component Analysis (PCA) suggests that the combination of PF and MR parameters represents a powerful tool for plant stress phenotyping, whereas MR parameters are linked to physiological strategies, related to different functional groups, to cope with stress factors.

1. Introduction

Chlorophyll fluorescence (ChlF) techniques are widely used in plant stress analysis *in vivo* (Baker and Rosenqvist, 2004; Tsimilli-Michael and Strasser, 2008; Kalaji et al., 2014b). There is a large amount of literature about its use, the physiological significance of several ChlF parameters, both from prompt and modulated fluorescence (see Strasser et al., 2004), and the specific responses to different kind of stresses (Papageorgiou and Govindjee, 2004). This body of theoretical studies and practical experiences makes the ChlF a mature physiological technique, that can be employed in research and in field application, such as forest and ecosystem monitoring (Pollastrini et al., 2014b), arboriculture (Percival et al., 2006), phenotyping (Rousseau et al., 2013) and crop production (Baker and Rosenqvist, 2004).

The concepts and scientific insights related to ChlF are developing quickly, theorizing new parameters for plant stress analysis, and producing new techniques and new commercial instruments. Among them, the multi-channel fluorimeter M-PEA (Multi-

Abbreviations: ChlF, chlorophyll "a" fluorescence; DF, delayed fluorescence; D_2, the second minimum of the DF induction curve; F_0, minimal fluorescence, of the dark adapted leaf, when all RCs are open; F_M, maximal fluorescence of the dark adapted leaf, when all RCs are closed; F_v/F_m, maximum quantum yield of primary photochemistry; I_1, first maximum of the DF induction curve; I_2, second maximum of the DF induction curve, or 'shoulder'; I_4, the final maximum of the DF induction curve; MR, modulated reflectance of 820 nm light; MR_t/MR_0, ratio between modulated 820 nm reflection intensity at time t (MR_t), and value of the 820 nm reflection of the sample at the onset of the actinic illumination (between 0.3 and 1 ms, MR_0); MR_{min}, minimal MR_t/MR_0 value, a transitory steady state, with equal oxidation and re-reduction rates of P700 and PC; PCA, principal component analysis; PF, prompt fluorescence; V_I, the relative variable fluorescence at 30 ms, $V_I = (F_I - F_0)/(F_M - F_0)$; V_J, the relative variable fluorescence at 3 ms, $V_J = (F_J - F_0)/(F_M - F_0)$; v_{ox}, the rate of P700 and PC oxidation, calculated as the maximum slope decrease of MR_t/MR_0; v_{red}, the rate of P700 and PC re-reduction, calculated as the maximum slope increase of MR_t/MR_0.

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Function Plant Efficiency Analyzer, Hansatech Instruments Ltd, Petney, Norfolk, UK) is of particular interest, allowing to measure simultaneously the prompt fluorescence (PF), the modulated reflectance of P700 (MR) and the delayed fluorescence (DF) (Strasser et al., 2010; Kalaji et al., 2012).

Prompt fluorescence (PF) refers to the fluorescence induction curve from F_0 to F_M in dark adapted samples. This curve, called "fluorescence transient" (FT) (Strasser et al., 2000, 2004, 2010), represents the "fast kinetics" of fluorescence emission. Plotted on a logarithmic time scale, the fluorescence transient shows a polyphasic behaviour. The different bands and steps of this polyphasic transient are labelled as: O (20 μs, indicates the minimum fluorescence intensity F_0), L (100 μs), K (300 μs), J (2 ms), I (30 ms) and P (peak). The latter indicates the highest fluorescence intensity (F_M). The analysis of the FT is formalized in the so-called JIP-test (Strasser et al., 2004). The JIP-test parameters link the different steps and phases of the PF transient with the redox states of photosystem II (PSII). In other words, they describe the efficiency of electron transfer in the intersystem chain until to the end electron acceptors at the PSI acceptor side.

The modulated reflection (MR) signal measured at 820 nm provides information about electron transport after the plastoquinone (PQ) and to the photosystem I (PSI) acceptors (Schansker et al., 2003; Strasser et al., 2010), thus indicating changes in the redox state of the PSI reaction centres (RC) and plastocyanin (PC).

Delayed chlorophyll fluorescence (DF) is a form of light emission from plants (Goltsev et al., 2009, 2012; Krasteva et al., 2013) in the red-infrared region of the light spectrum after they have been exposed to light. DF emission occurs for a short time after the prompt fluorescence decay. DF is mainly emitted from PSII. DF decays as a polyphasic function in different time domains: from nanoseconds, microseconds to milliseconds, even seconds and minutes (see Goltsev et al., 2009 and references therein). DF emitted in micro- and millisecond time ranges has been thought to reflect the recombination, in the dark, between the reduced electron acceptor Q_A^- and the oxidized secondary electron donor, Z^+, of PSII. These oxidized electron donors are formed after light-induced charge separation. In the time range of seconds, DF is associated with the recombination of S_2 and S_3 states of the oxygen-evolving complex (OEC) with Q_A^- and Q_B^- (Rutherford and Inoue, 1984).

A first generation of studies about the applications of M-PEA is available (Strasser et al., 2010; Goltsev et al., 2012; Kalaji et al., 2012; Oukarroum et al., 2013), and responses both to drought (Strasser et al., 2010; Goltsev et al., 2012; Krasteva et al., 2013) and heath stress (Oukarroum et al., 2013) were assessed. However, whereas the knowledge about the application of PF analysis and JIP-test in stress analysis is large and exhaustive, as far as MR and DF and relative parameters are concerned, a clear and comprehensive picture of the effects of different kind of stress on photosynthetic apparatus is still at the begin. This state of affairs constitutes a limitation on the wide application of M-PEA for routine stress analysis, as well as for agricultural and environmental monitoring but, at the same time, it is a challenging issue for researchers.

In this paper, we have tested the M-PEA for routine analysis in several independent experiments, where different plant species were subjected to different stress factors. Our aim was to evaluate if the different parameter sets (PF, MR, DF), alone and in combination, are suitable to phenotype stress responses in different plant species, finding common response of these parameters, and verifying consistency and comparability across different instruments, plant material and stress factor.

2. Methods

2.1. Experiments: plants and treatments

The routine analysis of prompt and delayed chlorophyll *a* fluorescence, and modulated reflectance was carried out with M-PEA in the following nine experiments, summarized in Table 1.

2.1.1. Experiment 1 and 2: soil salinity and drought stress on Arbutus unedo L. (Strawberry tree)

Fifteen plants of *A. unedo*, two years old, grown in the nursery of the Castelporziano Estate (RM), were transplanted in 15 l pots filled with a mixture of garden soil (60% of volume), sand (20%) and turf (20%). The plants were placed inside a closed "walk-in" chamber facility (2.5 m × 3.9 m × 3.0 m h) at the Department of Environmental Biology, Sapienza University of Rome (Italy). Air temperature was maintained at 24.2 ± 1.4 °C, and 20.7 ± 1.5 °C during the day and night, respectively. Relative humidity was constantly maintained at 62 ± 4%, and PAR of 500 μE m^{-2} s^{-1} was provided for 12 h per day by means of six metal halide lamps (1000 W, Philips HPI-T). After 20 days of acclimation, 5 plants were kept as controls and regularly irrigated with tap water, 5 plants were salt treated, and for the other 5 plants the irrigation was suspended.

2.1.1.1. Experiment 1: soil salinity. Plants were irrigated twice a week with 150 mM NaCl for 30 days (8 salt doses). In order to avoid salt shock, the first dose of 150 mM was supplied in three different steps of 50 mM NaCl each. Measurements were made after the 8th salt dose, on 2–3 fully developed, current year leaves ($N = 10$ and 11 for treated and control plants, respectively, Table 1).

2.1.1.2. Experiment 2: drought stress. The irrigation was suspended for 30 days, whereafter measurements were made on 2–3 fully developed, current year leaves per plant ($N = 11$ and 11 for treated and control plants, respectively, Table 1). Leaf relative water content (RWC) at the day of measurements was 97 ± 5.2% and 59 ± 12.4% for control and drought stressed plants, respectively.

2.1.2. Experiment 3 and 4: soil salinity and drought stress on Quercus ilex L. (holm oak)

Fifteen *Q. ilex* plants, two years old, were grown in the same conditions as in Experiments 1 and 2, respectively. After 20 days of acclimation, 5 plants were kept as controls and regularly irrigated with tap water, 5 plants were salt treated, and for the other 5 plants the irrigation was suspended.

2.1.2.1. Experiment 3: soil salinity. Salt treatment and measurements were made as in Experiment 1 ($N = 10$ and 8 for treated and control plants, respectively, Table 1).

2.1.2.2. Experiment 4: drought stress. Drought treatment and measurements were made as in Experiment 2 ($N = 13$ and 8 for treated and control plants, respectively, Table 1). Leaf RWC at the day of measurements was RWC of 98 ± 4.6% and 65 ± 9.3% for control and drought stressed plants, respectively.

2.1.3. Experiment 5: ozone stress on Phaseolus vulgaris L. (Snap bean), S156 genotype

Seeds of the ozone-sensitive (S156) *P. vulgaris* genotype were sown in 7 l pots filled with garden soil and sand (1:0.5), and grown in two closed "walk in" chambers (see Experiment 1), one used as control and one for O_3 fumigation (Salvatori et al., 2013). PAR of approximately 350 μE m^{-2} s^{-1} at sample leaf height was provided for 11 h per day, air temperature was 25.7 ± 0.24 °C and 22.9 ± 0.3 °C during day and night, respectively. Relative humidity