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ORIGINAL PAPER

Ronald Maldonado-Rodriguez · Stancho Pavlov · Alberto Gonzalez · Abdallah Oukarroum · Reto J. Strasser

Can machines recognise stress in plants?

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Abstract In this paper we show that chlorophyll *a* fluorescence signals analysed with the self-organizing map (SOM) can be used as a routine tool for the monitoring and classification of pea varieties (*Pisum sativum*) according to their degree of resistance against drought stress. Fluorescence kinetics measurements were obtained from non-stressed plants. The aim of this study is to evaluate the applicability of artificial intelligence techniques in eco-physiological research. Our goal is to provide a fast tool that will contribute to the knowledge needed to develop strategies that would help to decrease the impact of environmental stress in agriculture and forestry.

Keywords Artificial neural networks · Chlorophyll *a* fluorescence · Drought stress · JIP-test · O-J-I-P fluorescence rise · Pea · *Pisum sativum* · Plants · Self-organizing map (SOM)

Introduction

Nowadays there is a need for fast methods in plant health status survey, plant selection and classification tasks such as breeding, somatic embryogenesis, and genetic engineering. One such method is the analysis of the polyphasic chlorophyll *a* fluorescence rise (Strasser et al. 1995) by the JIP-test (Strasser et al. 2000), which has proven to be a valuable tool in plant vitality monitoring. Although the data used are characteristic of the fluorescence transient, the JIP-test does not exploit the whole information stored in the kinetics of the curve. Here we present a novel approach that makes use of the full characteristics of the fluorescence rise. Recently, it has been shown that fluorescence signals can be considered as a built-in “barcode” of the physiological characteristics of plants and can be used for taxonomic purposes (Tyystjärvi et al. 1999). Hence, in this paper we proceeded to classify chlorophyll *a* fluorescence induction curves on the basis of the whole information they carry.

Artificial neural networks are ideal for analysing the vast quantities of data provided by chlorophyll fluorescence measurements, and can make identifications in near-real time. The Kohonen’s self-organizing map (SOM; Kohonen 1982) is an unsupervised artificial neural network that maps the input space data into clusters in a topological form, whose organization is related to trends in the input data, and can be used for reducing dimensionality and revealing clusters in datasets. A plant classifier based on a modified SOM method for classifying reflectance patterns of crops and weeds was recently proposed by Moshou et al. (2001).

In this paper we have used a self-organizing map to classify fluorescence induction curves measured with a portable fluorometer from eight, previously well-determined and physiologically characterized breeding lines of pea plants (Sanchez et al. 1998). These eight lines were classified according to their capability to resist against drought stress into three classes: resistant, intermediate and sensitive. Their capability to resist against drought stress was determined by analysing the osmotic adjustment and turgor maintenance of the studied pea cultivars in order to characterise their physiological response. This is a process that demands much experimental work and consumes a lot of time.

On the other hand, measuring and processing fluorescence data is a fast routine. The fluorescence data from the eight *P. sativum* varieties were used to generate a self-organizing map. The neurons in the map show well-defined patterns reflecting the varieties of peas grouped according to their drought-stress resistance classes. The

R. Maldonado-Rodriguez (✉) · A. Oukarroum · R. J. Strasser
Bioenergetics Laboratory,
10 Chemin des Embrouchis, 1254 Jussy, Switzerland
e-mail: Ronald.Rodriguez@bioen.unige.ch
Tel.: +41-22-7599940
Fax: +41-22-7599945

S. Pavlov
Department of Mathematics, University Asen Zlatarov,
8010 Burgas, Bulgaria

A. Gonzalez
INIA Ctra de la Coruña, 28040 Madrid, Spain

measured in-situ and in-vivo chlorophyll *a* fluorescence signals are registered with a portable fluorimeter which makes the analysis suitable for field applications. We extracted geometric features contained in the fluorescence transient for creating a self-organizing map in order to generate natural clusters of the input data. The plant samples were systematically and objectively identified according to their resistance capability against drought stress. It is noteworthy that the plant material used for the fluorescence measurements was not exposed to drought stress. The major goal of this study was to evaluate the applicability of recently developed artificial intelligence techniques in plant research, in order to provide a fast tool that will contribute to the knowledge needed to develop strategies that would decrease the impact of biotic and abiotic stress in plants. In this frame, it can be used to adapt agricultural and forestry practices to cope with fluctuations of local environmental conditions as a result of predicted global climate changes.

Experimental

Plant material

The experiments were performed with eight varieties of pea plants (*Pisum sativum*). Plants were grown in 15-cm-diameter pots containing vermiculite (three plants per pot, six replicates for each assay) under greenhouse conditions: 23/16 °C, day/night in a 14/10 h photoperiod, HFI Hg lamps 400 W. The plants were watered every 2 days with tap water.

Leaf water relations

When the plants were 21 days old, drought was imposed by withholding water. At the onset and at various times during the water-stress period, usually 4, 7, 10 and 14 days after the treatment was initiated, leaflets from the first and second expanded leaf were sampled. The genotypic variation in *turgor maintenance* was determined as the slope of the correlation line between *turgor potential* and *water potential* of the investigated pea varieties grown under drought-stress conditions. The *osmotic adjustment* was measured as the difference between the osmotic potential at saturation in watered plants and the osmotic potential of plants with 70% relative water content. The fresh weight, dry weight, and turgid weight were used to measure the relative water content. The environmental water potential and osmotic potential was determined using psychrometric chambers connected to a CR7 measurement and Campbell Scientific control unit according to Sanchez et al. (1998). The varieties were characterized and classified into stress resistance classes according to their osmotic adjustment and turgor maintenance response.

Chlorophyll *a* fluorescence

A fast chlorophyll fluorescence screening routine was carried out with fully mature, attached leaves from each plant on the fifth week after germination. No stress treatment was applied for these experiments. All cultivars were measured under identical conditions. The plants were dark adapted for 60 min prior to measurements. The chlorophyll *a* fluorescence kinetics measurements were conducted with a portable fluorimeter. The measurements were done on the top and bottom leaves of the plants and analysed separately. The fluorescence transients O-J-I-P were induced by excitation pulses (1-s duration) of red light (peak at 640 nm), which was provided by an array of six light-emitting diodes (600 W m^{-2}) focused on an area of 4-mm diameter of the sample surface (Strasser et al. 1995). The fluorescence signals were detected using a PIN photodiode after passing through a long-pass filter (50% transmission at 750 nm).

The JIP-test

The JIP-test is a screening procedure that is based on the measurement of the fast fluorescence rise that all oxygenic photosynthetic material investigated so far shows. It provides information about the structure and function of photosystem II (Strasser et al. 2000). From the stored fluorescence points (data acquisition resolution 10 μs for the first 200 points and 1 ms afterwards) during the first second, selected values are retained as data. They are used for the calculation of several phenomenological and biophysical expressions leading to a dynamic description of a photosynthetic sample at a given physiological state. These values are the maximal measured fluorescence intensity, F_P, which can be denoted as F_M since the excitation intensity is high enough to permit the closure of all reaction centres; the fluorescence intensity at 50 μs, considered to be F_0, i.e. the intensity when all reaction centres are open; the fluorescence intensity at 150 μs, 300 μs, and 2 ms (denoted as F_J); the time to reach the maximal fluorescence intensity (t_{Fmax}); the area between the fluorescence transient and the level of maximal fluorescence intensity. Based on the theory of energy fluxes in biomembranes (Strasser 1978), formulae for the specific energy fluxes (per photosystem II reaction centre) and phenomenological energy fluxes (per excited cross section, i.e. per active measured leaf area) as well as for the flux ratios or yields have been derived using the experimental values provided from the JIP-test.

Software

The JIP-test analysis was performed using the BIOLYZER software developed by R. Maldonado-Rodriguez and freely available at www.unige.ch/sciences/biologie/bioen/bioindex.html. For the generation of self-organizing maps, each measured fluorescence curve was divided

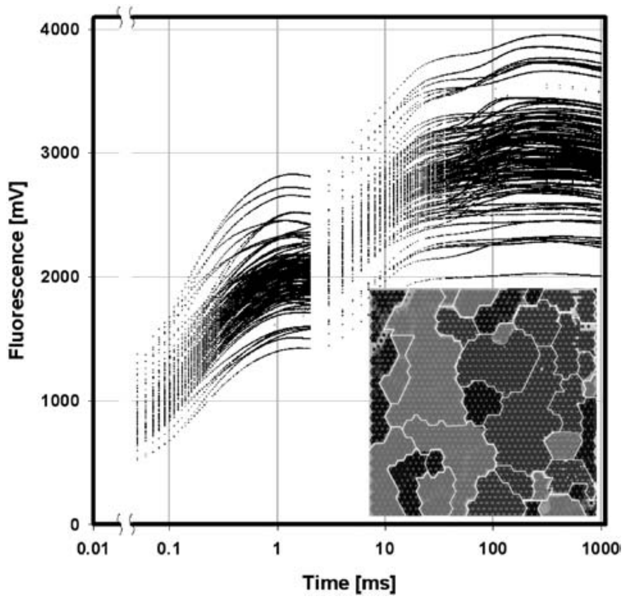

Fig. 1 Chlorophyll *a* fluorescence transients measured from eight pea varieties. The *inset* shows the generated self-organizing map. *Darkly shaded areas* correspond to stress-resistant groups, *lightly shaded areas* to sensitive, and *closed areas* to intermediate-resistance cultivars

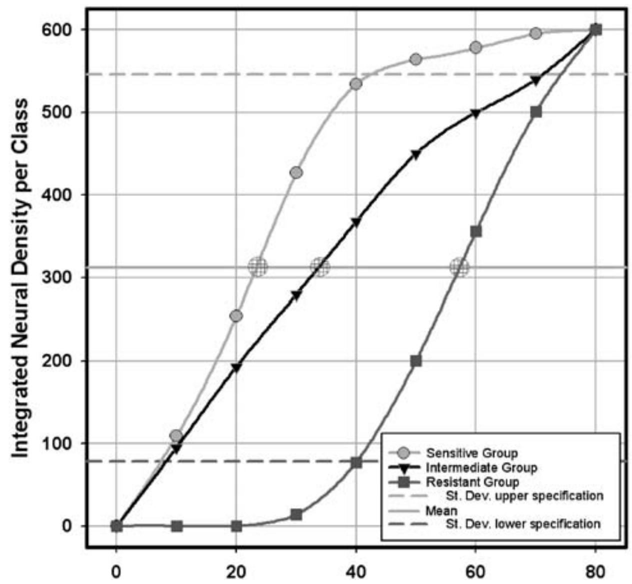

Fig. 2 The figure shows the integrated area of self-organizing map stress clusters according to their density distribution in the topological feature map. The three mean density values are extracted from this plot and used for quantifying stress resistance of the studied pea cultivars

into 10 intervals and the geometric characteristics (slope and intercept) of each interval were used to define a 20-component vector of features for each fluorescence curve using a computer-based program that we wrote specially for this purpose. The feature vectors were fed into the self-organizing map toolbox (T. Kohonen, J. Hynninen, J. Kangas, J. Laaksonen, 2002, SOM_PAK available at www.cis.hut.fi/nnrc/nnrc-programs.html) in order to generate a two-dimensional topological feature map (see inset of Fig. 1). The clusters in the map were quantified by their density distribution according to their stress resistance class (Fig. 2).

Results and discussion

Screening with the JIP-test

Applying the JIP-test analysis to all of the measured O-J-I-P chlorophyll *a* fluorescence transients (Fig. 1), it was found that there is a strong dependency of each of the JIP-test parameters on the energetic state of the photosynthetic material (data not shown). As we observed a differential behaviour of top and bottom leaves with respect to these parameters, we used as criterion the ratio of the performance index (PI) of the top leaves to the performance index of the bottom leaves, and we found two main drought stress-sensitivity classes: $(PI_{top})/(PI_{bottom})$ values around 1.65 correspond to plants with high response (sensitive), and $(PI_{top})/(PI_{bottom})$ values around 1.1 correspond to plants with low response (resistant).

Screening with the self-organizing map neural network plots

Two self-organizing map plots containing 30×30 neurons were generated using the measured fluorescence curves corresponding to two levels of plant height; for illustration purposes we show only top and bottom leaf maps, but the method could give more insights into the self-organizing map response of the leaves at different levels of plant height. Both maps presented highly ordered groups reflecting pea varieties, as shown in the inset of Fig. 1, where a self-organizing map generated with fluorescence curves measured in bottom leaves is presented. It was observed that the pea varieties were distributed in the map according to their pertaining to a stress resistance class. The horizontal axis of the self-organizing map in the inset of Fig. 1 is visually polarized into more darkly shaded zones, showing resistant varieties to the right, and lighter shading zones showing sensitive varieties to the left of the map. Each class consists of 600 neurons, shown in dark shading areas for resistant, closed areas for intermediate, and light shading areas for sensitive. We calculated the distribution of the density of the neurons, and their classification was done based on the polarization of the map in three major blobs.

Figure 2 shows the cumulative appearance of the input data grouped into three stress classes relative to their distribution along the abscissa: lightly shaded circles represent the sensitive group, closed triangles the intermediate group, and darkly shaded squares the resistant group. The position of the mean density value (300 neurons) is marked with big circles, and the standard

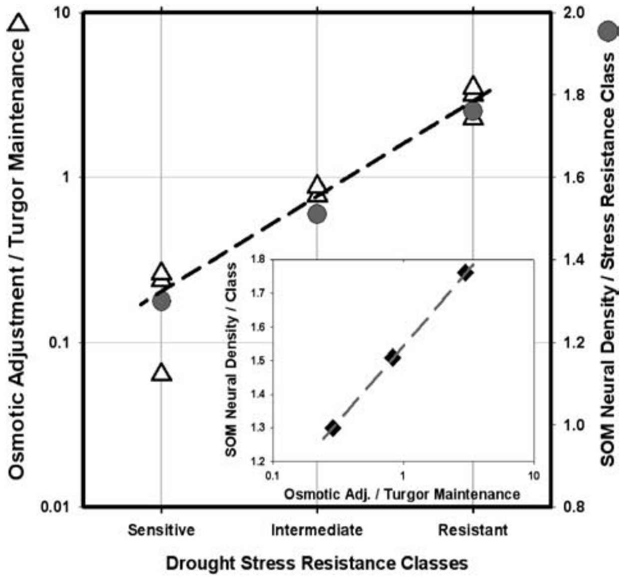

Fig. 3 The osmotic adjustment per turgor maintenance (*open triangles*) and the neural network-based parameter (mean value of the SOM neural density per stress resistance class, *shaded dots*) for three drought-stress resistance classes: sensitive, intermediate and resistant. The *inset* shows the correlation of the physiological response parameter with the mean value of the integrated density determined by the proposed neural network approach

deviation $\sigma=\pm 33\%$ is indicated with horizontal dashed lines at the position 540 and 90 of the vertical axis.

Self-organizing map and leaf water relations

In Fig. 3 we plotted the response of the eight *P. sativum* varieties, quantified by the ratio (osmotic adjustment)/(turgor maintenance) (left vertical axis) versus the degree of their resistance against drought stress. The mean value of the integrated neural density for each drought resistance class from Fig. 1 was also plotted for comparison (see right vertical axis in Fig. 3). The plotted results in the inset of Fig. 3 show a high linear correlation between the drought-stress classes, determined by using the osmotic adjustment and turgor maintenance responses of the studied cultivars, and the mean values of the density distribution of the groups, determined by topological analysis of the self-organizing fluorescence features map. This provides strong evidence of the high potential of the self-organizing map for classifying pea varieties according to their genetically inherited capability to resist against drought stress. We also evaluated here the response of the two sides of the leaves for all of the studied varieties (data not shown). Fluorescence fingerprints of the upper and lower surfaces of a leaf are different in shape and intensity. Despite this fact, we found similar classification results using fluorescence signals sampled from the upper and lower leaf sides.

Our results show that the analysis and quantitative determination of stress resistance in pea varieties is possible using chlorophyll fluorescence techniques combined with an artificial neural network approach. Based on the idea that fluorescence signals intrinsically carry information on the physiological state of vegetation samples, we propose a neural network approach for quantitative determination of resistance against drought stress of pea plants, utilising the polyphasic fluorescence transient of 1 second. Comparative studies for individual samples, e.g. top versus bottom leaves or stressed vs. non-stressed samples, have been conducted. Comparative studies of whole groups of cultivars, e.g. here the varieties 1 to 8, are most efficiently analysed and quantified by self-organizing map neural network. Since the geometric features extracted from the fluorescence curves form the input data for the self-organizing map, the capability of the map to reflect plant varieties can be improved by modifying or extending the experimental protocol for collecting fluorescence signals, e.g. by using actinic light of different duration, applying flash repetitions, and comparing data from dark- and light-adapted leaves.

The photosynthetic potential of a plant can be used as an environmental bioindicator because it reveals the presence of stress factors affecting photosynthesis. In-vivo fluorescence techniques have the advantage that they are not invasive and that they can be done on all plants at any time. In this work we show that the recorded fluorescence signals of a plant reflect functional information about the energetic state of the plant and its genetically inherited capabilities to cope with environmental stress. The actual state of a plant is always influenced by the environment around it. The specific response of different plant species to environmental influences is determined by their genetic, morphological and physiological characteristics.

Artificial neural network for evaluation of plant stress

Our results demonstrate that different varieties of a same plant species present differences in their photosynthetic potential, whose quantification can be achieved with a biophysical approach: chlorophyll *a* fluorescence measurements independently analysed with the JIP-test and the self-organizing map neural network provide well-comparable results. The applicability of this approach was also shown in a recent work, revealing the potential of the self-organizing map to extract quantitative information on nitrogen fixation and formation of nodules in *Vigna unguiculata* plants using fluorescence characteristics as input data. Generally, information on the specific action of different stressors, such as light, water and mineral availability, wind speed, soil and air temperatures, soil compaction and heavy metals, at different plant levels, e.g. roots, stems, buds, flowers, fruits and leaves, is reflected in the functioning of their photosynthetic apparatus at the level of leaf chloroplasts and can thus be detected with the approach here presented.

The neural network approach proposed in this work was based on the extraction of the full geometric characteristics of the fluorescence curves from non-stressed plants. Another approach could include the JIP-test parameters calculated for each fluorescence transient as input data or a combination of the geometric features with the JIP-test parameters. As these parameters are based on a theoretical consideration of the photosynthetic apparatus, a self-organizing map generated with the JIP-test parameters could also help for a better understanding of the proposed mechanistic models of primary photochemistry.

Conclusions

Chlorophyll *a* fluorescence has proven to reflect the activity of primary photochemical reactions. This means that information about stress effects on plants can be gathered with fluorescence signals, provided that a suitable method is applied to extract and reveal this information. In this paper we report a method for quantitative and qualitative evaluation of the stress resistance characteristics of different varieties of one and the same plant species (peas). The presented neural network approach with self-organising mapping seems to be a powerful tool for this purpose. It is fast and can be fully automised. Our results provide new insights towards the understanding of the relationship between the photosynthetic structure-function of plants and their specific, genetically inherited characteristics for coping with drought stress. Further developments of this work include neural network mapping of biotic and abiotic stress effects on different plant varieties, and application of the self-organizing map to the analysis of spatial data obtained by fluorescence imaging of vegetation.

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