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Original Article

Chlorophyll Fluorescence as a Tool to Assess the Regeneration Potential of African Violet Leaf Explants

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Abstract

Micropropagation of many ornamentals has enabled their efficient commercialisation and many problems have been solved by the elaboration of adequate culture protocols. Nevertheless, a non-destructive technique for monitoring the developmental progress of explants would be desirable. The present study focussed on the applicability of chlorophyll fluorescence in leaf explants of African violet (a *Saintpaulia ionantha* × *confusa* – hybrid) explanted onto Murashige and Skoog basic medium. The explants that survived on the medium without additional phytohormones had the capacity to develop further into two different kinds of explants: light green explants, characterized by a non-regular size growth and stiffer appearance, and dark green explants capable of organogenesis. Compared to the source leaves of African violet plants, explants were characterized by reduced chlorophyll (Chl) and carotenoid (Car) contents as well as a tendency towards a higher Car/Chl ratio. The Chl *a*/*b* ratio decreased significantly in the light green explants. A reduction of maximum quantum efficiency of photosystem II (F_v/F_m) accompanied by a high percentage (> 50%) of thermal energy dissipation as a consequence of an elevated light intensity (800 μmol m^{-2} s^{-1} quanta) indicated photoinhibition in the light green explants, whereas in the dark green explants the largest percentage (> 50%) of the light energy was dissipated into the fraction of photon energy trapped by 'closed' photosystem II reaction centres. These results suggest that the capacity of organogenesis of leaf explants of African violet can be monitored using chlorophyll fluorescence, because it is related to modifications of the photosynthetic system.

Keywords: carotenoids, chlorophyll, micropropagation, tissue culture

Introduction

In vitro propagation techniques are routinely used for mass propagation of ornamentals, initiated from apical buds, nodal segments, leaves, petioles, cotyledons or embryos (Jain and Ochatt, 2010). *In vitro* culture potentially shortens and improves the conventional breeding cycle. However, its implementation into horticultural practice requires the development of efficient and reproducible procedures of plant regeneration under controlled conditions. In addition, non-destructive and non-invasive monitoring procedures are desirable that enable predicting the capacity of explants for organogenesis and/or embryogenesis. Image analysis is one of the techniques that fulfil the above requirements, but has been largely limited to suspension cultures (Ibaraki and Kurata, 2001). In contrast, information of non-destructive monitoring techniques for explants is rather limited. Żur *et al.* (2000) proposed microcalorimetric measurements in order to estimate the regeneration potential of callus. Following their concept, the regeneration processes in callus tissue (embryogenesis or organogenesis) manifests itself by a higher metabolic activity, which is accompanied by an elevated heat flow that can be monitored. In addition, Muñoz-Muñoz *et al.* (2007) demonstrated the effectiveness of laser-induced fluorescence (LIF) for monitoring developmental processes in *in vitro* tissue cultures in a non-destructive and non-invasive way. In this approach, the induction of organogenesis resulted in an increase of the F690/F740 ratio in the LIF spectra having its origin in the organogenic explants of *Saintpaulia ionantha*.

During studies on the micropropagation of African violet (a *Saintpaulia ionantha* × *confusa* – hybrid) with leaf discs the present authors noticed that the explants, without the additional application of phytohormones, had the potential of developing into two different kind of morphotypes: either (1) the size of the explants increased distinctly and the leaves exhibited a stiffer appearance, partly resembling callus tissue, or (2) the explants

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more or less maintained their shape and consistency, but developed new shoots and roots on the upper leaf pedicle and the nearby leaf margin (organogenesis). The overall appearance (Fig. 1) of both types of explants, in particular the colour of the leaf pigments, led to the hypothesis of different adaptation strategies of the photosynthetic apparatus. During the present study both types of explants were characterized using chlorophyll fluorescence in order to differentiate between explants with and without the capacity of organogenesis.

Chlorophyll fluorescence has frequently been used as a non-destructive indicator of photosynthetic functioning of *in vitro* cultivated plantlets under *ex vitro* acclimatization (Carvalho *et al*., 2001; Cassana *et al*., 2010), but also during the development of the photosynthetic system under different environmental *in vitro* conditions (Miranda and Williams, 2007). In the present study the hypothesis tested, was pulse-amplitude modulated (PAM) chlorophyll fluorescence can differentiate between African violet explants surviving on Murashige and Skoog (1962) basic medium with or without the capacity of organogenesis. For additional information on current achievements in the field of micropropagation of African violet the interested reader is here referred to as Shukla *et al*. (2013) and Ghorbanzade and Ahmadabadi (2014).

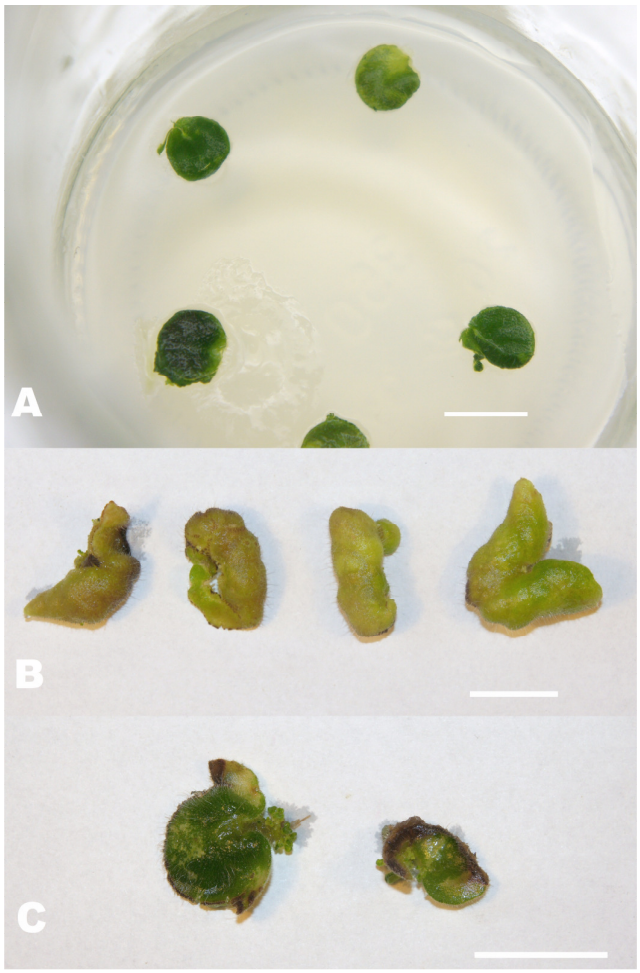

Fig. 1. Leaf discs cut from leaves of African violet placed on Murashige and Skoog medium (Panel A). Light green leaf explants used to measure the chlorophyll fluorescence and pigment content (Panel B). Dark green leaf explants used to measure the chlorophyll fluorescence and pigment content. These explants developed small shoots and roots at the upper part of the pedicle (Panel C). Scale bars in Panels = 1 cm.

Materials and Methods

Plant material

One year old plants of African violet (a *Saintpaulia ionantha* × *confusa* – hybrid) were cultivated *in vitro* in glass jars at the UTP Bydgoszcz in a growth chamber, where they were kept at 25/23 ± 2 °C under a 16/8 h day/light regime and an air humidity of approximately 70-75%. Light was generated by fluorescent lamps (Philips – TLD36 W/840). Light intensity was kept at 90 μmol m^{-2} s^{-1} within the jars.

Tissue culture

Leaf discs (0.8 cm diameter) of young, expanding leaves with a fragment of the petiole were excised from African violet plants, which were also cultivated in the growth chamber. The discs were surface-sterilized and defatted in 70% ethanol for 30 s, followed by a 10% solution of sodium hypochlorite for 20 min, then were rinsed in bi-distilled water and transferred onto Murashige and Skoog (1962) basic medium solidified with 0.7% agar and supplemented with 3% sucrose. Auxins and cytokinins were not added in order not to control the development of the leaf discs towards organogenesis or size increase, despite the consequence that survival of explants on a medium without phytohormones is rather low (Lo *et al*., 1997). Leaf discs were placed with the abaxiale side onto the medium (Fig. 1A). Five explants were placed into each of the 50 tissue culture jars (bottom diameter 70 mm). The experiment was repeated with another 30 jars, resulting as a whole in 400 explants.

During the following five weeks the explants were kept in the growth chamber under the same environmental conditions mentioned above for the source plants. They were not transferred onto fresh MS medium during this period. At the end of the experiment the surviving explants were subdivided into the two morphotypes, the size increase of the leaf blades of explants was rated, and the induction of organogenesis was noted. For chlorophyll fluorescence measurements and pigment analyses only those explants were used that survived the treatment. Seven young, expanding leaves freshly excised from *in vitro*-cultivated African violet plants were used as the control.

Pigment analyses and chlorophyll fluorescence measurements

Chlorophyll (Chl) *a* and *b* as well as total carotenoids (Car) contents of leaves and leaf discs expressed per g fresh weight were determined according to Wellburn (1994). Fresh leaves were weighed, triturated in 80% acetone with a mortar and 80% acetone was added to a volume of 10 cm^3. The absorbance was read with a spectrophotometer (Eppendorf Biospectrometer Basic, Eppendorf AG, Hamburg, Germany) at wavelengths of 663, 646 and 470 nm against 80% acetone as a blank.

For chlorophyll fluorescence measurements with the Portable Chlorophyll Fluorimeter PAM-2500 (Heinz Walz GmbH, Effeltrich, Germany) the selected leaves and explants were dark-adapted for 25 min, then were initially exposed to a

weak, modulated measuring beam with a photon irradiance of less than 1 µmol quanta m^{-2} s^{-1} to determine minimum fluorescence (F_0), then to a saturating pulse of white light with a photon irradiance of about 10,000 µmol m^{-2} s^{-1} to measure maximum fluorescence (F_m). Next, actinic light (800 µmol m^{-2} s^{-1} quanta) was switched on and saturation pulses were applied every 20 s for 5 min in order to follow the adaptation of the photosynthetic system to the applied actinic light intensity by measuring F_m' and F, the fluorescence under the actinic light. An actinic light intensity of 800 instead of 90 µmol m^{-2} s^{-1} quanta was applied in order to elucidate differences between the two types of explants with respect to their light utilisation capacity. Minimum fluorescence of the non-dark-adapted leaf (F_0') was calculated according to Oxborough and Baker (1997). From these values maximum quantum efficiency of photosystem II (PSII) photochemistry (F_v/F_m), maximum efficiency of PSII photochemistry in the light, if all centres were open (F_v'/F_m'), and an estimate of the fraction of open PSII centres (qL) were calculated in line with Murchie and Lawson (2013). According to Liu *et al.* (2011), the light energy absorbed by the photosynthetic system is dissipated into the fraction of photon energy absorbed by PSII antennae (P), a thermally dissipated portion (D) and the remaining fraction (denoted as the 'excess'; E), which were all estimated in percent. However, the P-fraction, trapped by 'open' PSII reaction centres and used in PSII photochemistry was calculated using qL instead of qP, because the relationship between qP and the fraction of open centres is regarded as non-linear (Murchie and Lawson, 2013); hence $P = F_v'/F_m' \times qL$, $D = 1 - F_v'/F_m'$, and $E = 1 - (P + D)$.

Statistical analyses

Analyses of pigment and chlorophyll fluorescence data were compared with IBM SPSS STATISTICS version 21 (2012). Normal distribution was tested with the Shapiro-Wilk test, the homogeneity of variances with the Levene test. In case the variances were not homogeneously distributed, data were transformed. Because the resulting variances were homogeneous, ANOVA was performed and samples were compared using Tukey HSD tests for unequal number of replicates. In addition, the univariate ANOVA was performed with the covariates "explant" and "leaf colour". In case of "explant" both kinds of explants were tested against the control leaves, whereas in case of "colour" the light green explants were tested against the dark green explants and control leaves.

Results

Only 13 explants of the 400 leaves transferred to Murashige and Skoog basic medium survived (c. 3%). These could be divided into two groups. The first group consisted of eight light green explants, which significantly increased the size of the leaf blades. Growth of the leaves was irregular (Fig. 1B). In the second group there were only five explants, which were characterized by a dark green colour and the regeneration of shoots in addition to a few rather small roots. Shoot regeneration occurred on the margin of the leaf surface exposed to air close to the pedicle. Small, tiny roots evolved at the opposite side, without any callus formation (Fig. 1C).

Table 1. Chlorophyll and carotenoid contents of leaves and leaf explants of African violet in mg g^{-1} fresh mass

Variants	Chl *a* [mg g^{-1}]	Chl *b* [mg g^{-1}]	Total Chl [mg g^{-1}]	Chl *a*/*b* ratio	Car [mg g^{-1}]	Car/Chl ratio
Control	0.270 ± 0.033 a	0.073 ± 0.007 a	0.343 ± 0.037 a	3.7 ± 0.4 a	0.069 ± 0.008 a	0.20 ± 0.2 b
Light green	0.093 ± 0.077 b	0.030 ± 0.022 b	0.123 ± 0.098 b	3.0 ± 0.2 b	0.029 ± 0.018 b	0.26 ± 0.3 a
Dark green	0.150 ± 0.061 b	0.038 ± 0.013 b	0.188 ± 0.073 b	3.9 ± 0.4 a	0.043 ± 0.013 b	0.24 ± 0.3 ab
Transformation	none	none	none	none	none	none
$P_{Shapiro\text{-}Wilk}$	0.011	0.031	0.010	0.433	0.088	0.592
P_{Levene}	0.367	0.193	0.278	0.467	0.313	0.799
P_{ANOVA}	0.000	0.000	0.000	0.000	0.000	0.008
$P_{Explant}$	0.003	0.001	0.003	0.355	0.006	0.056
P_{Colour}	0.120	0.400	0.155	0.000	0.104	0.266

Note: Different letters between variants denote significant differences (Tukey test, $p < 0.05$). Moreover, the significance level for tests of normal distribution (PShapiro-Wilk), homogeneity of variances (PLevene), and of the ANOVA (PANOVA) are given, in addition of the significance of the covariates explant versus control ($P_{Explant}$) and leaf colour (P_{Colour}).

Table 2. Minimum fluorescence (F_0), maximum fluorescence (F_m), maximum quantum efficiency of PSII photochemistry (F_v/F_m), the fraction of photon energy absorbed by PSII antennae (P), the thermally dissipated portion (D) and the fraction of photonic energy absorbed by PSII antennae and trapped by 'closed' PSII reaction centres (E) of control leaves, light green and dark green explants of African violet

Variant	F_0	F_m	F_v/F_m	P [%]	D [%]	E [%]
Control	0.190 ± 0.031 a	0.643 ± 0.074 a	0.705 ± 0.033 ab	19.3 ± 4.0 a	40.4 ± 2.9 b	40.3 ± 6.4 b
Light green	0.102 ± 0.039 b	0.248 ± 0.161 b	0.524 ± 0.137 b	11.4 ± 8.0 ab	52.7 ± 11.0 a	35.9 ± 13.3 b
Dark green	0.120 ± 0.021 b	0.504 ± 0.136 a	0.755 ± 0.038 a	11.4 ± 3.4 b	34.9 ± 3.2 b	53.7 ± 2.4 a
Transformation	none	none	$f(x)= \ln(x-0.212)$	$f(x)=x^{2.1}$	none	$f(x)=x^4$
$P_{Shapiro\text{-}Wilk}$	0.678	0.066	0.000	0.093	0.012	0.116
P_{Levene}	0.617	0.415	0.063	0.079	0.104	0.176
P_{ANOVA}	0.000	0.000	0.029	0.032	0.001	0.002
$P_{Explant}$	0.002	0.086	0.743	0.018	0.228	0.002
P_{Colour}	0.367	0.003	0.020	0.593	0.001	0.001

Note: Different letters between variants denote significant differences (Tukey test, $p < 0.05$). Moreover, the significance level for tests of normal distribution ($P_{Shapiro\text{-}Wilk}$), homogeneity of variances (P_{Levene}), and of the ANOVA (P_{ANOVA}) are given, in addition of the significance of the covariates explant versus control ($P_{Explant}$) and leaf colour (P_{Colour}).

Total Chl, Chl *a*, Chl *b* and Car contents of the light green and dark green explants were significantly lower than in control leaves (Table 1) and the univariate ANOVA confirmed that the reduction of pigment contents was a consequence of explanting the leaves onto Murashige and Skoog basic medium. The Chl *a*/*b* ratio was significantly reduced only in case of the light green explants and this effect was related to leaf colour as indicated by univariate ANOVA. The Car/Chl ratio of the light green explants was significantly lower than that of the control leaves and that of the dark green explants was intermediate (Table 1). The univariate ANOVA did not reveal a significant effect neither of the colour nor of explanting, but the effect of explanting was close to the 5% significance level (Table 1).

Chlorophyll fluorescence measurements revealed different responses of F_0 and F_m with respect to the different kinds of explants. F_0 was significantly reduced in both kinds of explants and the univariate ANOVA confirmed an effect of explanting onto this parameter (Table 2). F_m, however, was significantly reduced only in the light green explants and the univariate ANOVA, thus, indicated a significant relationship with leaf colour. Significant differences of F_v/F_m occurred between light and dark green explants, while that of control leaves was intermediate and did not differ significantly from both kinds of explants. The univariate ANOVA revealed a significant effect of leaf colour.

When adapted to actinic light conditions (800 μmol m^{-2} s^{-1} quanta), about 20% of the absorbed light energy was used in PSII photochemistry (*P*) in the control leaves, whereas only about 10% were used in both kinds of explants (Table 2). The univariate ANOVA confirmed a significant effect of explanting. The thermally dissipated portion of energy (*D*) was largest in the light green explants (> 50%), while it was comparable between control leaves and dark green explants (c. 35-40%). As a consequence, the univariate ANOVA revealed an effect related to leaf colour. The light energy trapped by 'closed' PSII reaction centres (*E*) was largest in the dark green explants and smaller in the light green ones and control leaves (Table 2). As a consequence of the fact that the 'excess' fraction E depended on both *P* and *D*, the univariate ANOVA revealed a significantly effect of explanting and colour, which is not immediately evident from the results of the Tukey test (Table 2).

Discussion

When comparing the explants with the control leaves of African violet, it is obvious that the explants underwent significant changes to survive on the growth medium. The size of the light green explants, for instance, increased considerably, but non-regularly (Fig. 1B) and leaf growth was accompanied by a decrease in chlorophyll content by 64% compared to a decrease by only 45% in the dark green explants. In the case of the light green explants the decrease may partly be explained by a dilution effect of chlorophyll due to growth. The dark green explants, apart from partial tissue dieback, almost maintained their size and developed shoots and fine root structures. The changes in both kinds of explants imply the involvement of phytohormones, although they were not added to the present medium. Lo *et al*. (1997) applied a shoot-inducing medium containing 2.0 mg L^{-1} indole acetic acid (IAA) and 0.08 mg L^{-1} 6-benzylaminopurine (BAP) in order to regenerate shoots from leaf discs of *Saintpaulia ionantha* × *confusa* – hybrids. While auxins such as IAA are known to induce root or shoot formation (depending on cytokinin contents), cytokinins such as BAP are involved in size growth by cell division (Drozdowska *et al*., 2004). The adequate balance of both phytohormones allowed the regeneration of shoots.

In case of the light green explants it may be assumed that a phytohormone "imbalance", notably elevated cytokinin content, accompanied by the lack of an adequate IAA level, contributed to their development. In addition to the significance of cytokinin in size growth, this phytohormone is involved in the regulation of photosynthetic rate, source-sink balance, and may stimulate the expression of photosynthetic genes including Rubisco and the light harvesting Chl *a*/*b* binding protein (Paul and Foyer, 2001). Cytokinins delay leaf senescence, and this last aspect might represent a crucial factor for the survival of the light green explants on the medium.

The comparison of pigment contents and chlorophyll fluorescence of leaves and leaf explants is complicated, because the explanted leaves at least partly underwent morphological changes. This is obvious for the light green explants, which partly resemble callus tissue, but may, to a limited extent, also be valid for the dark green explants with incomplete organogenesis. In the case of explants having a callus-like morphology the physiological environment of the plastids should be different to that in the control leaves. All factors influencing photosynthesis like light intensity and spectrum, leaf internal CO_2 concentration, water and nutrient availability might have been modified. As a consequence, the interpretation especially of chlorophyll fluorescence is very sensitive to artefacts in the measured data, which may be one reason, why literature on such kind of studies is not available. In order to overcome the disadvantages due to leaf morphologies, the comparisons are limited to relatively 'stable' parameters of chlorophyll fluorescence such as maximum quantum efficiency of PSII (F_v/F_m) and the relative distribution of absorbed light energy into the fractions 'energy absorbed by PSII antennae (*P*)', 'thermally dissipated energy' (*D*) and the remaining fraction (*E*).

F_v/F_m is generally regarded as a stress indicator of the photosynthetic system, a reduction being typical of photoinhibition (Maxwell and Johnson, 2000). In the present experiment F_v/F_m values of control leaves were comparatively low in the range of c. 0.71 (Table 2), which is distinctly lower than the value of 0.82 commonly reported for non-stressed control leaves. However, the here recorded value may be considered typical for *in vitro* plants under the present environmental conditions, because *in vitro* plants are generally characterized by smaller values (Premkumar *et al*., 2001).

When interpreting the results of maximum quantum efficiency of PSII of control leaves and explants, it should be kept in mind that leaves with a lower chlorophyll packing, such as the explants in the present experiment, are characterised by a modified light gradient. This may influence chlorophyll fluorescence parameters like F_0, F_m, and F_v/F_m (Netto *et al*., 2005). As a consequence, it might be concluded that the decrease of F_v/F_m in the case of the light green explants probably indicates photoinhibition, but with

respect to the callus-like morphology, this conclusion must be taken with reservation (Table 2).

F_v/F_m (= $(F_m-F_0)/F_m$) is calculated from both, F_0 and F_m. Noteworthy, the reduction of F_0 is related to explanting, while that of F_m and F_v/F_m is related to leaf colour as indicated by univariate ANOVA (Table 2). Because changes in leaf pigment contents are also affected by explanting (Table 1), it is obvious that only the modification of F_0 can immediately be related to total Chl content but not F_m and F_v/F_m. The modifications of F_m and F_v/F_m are seemingly related to changes in the Chl *a*/*b* ratio as indicated by the significant leaf colour effects of the corresponding univariate ANOVA (Tables 1 and 2).

In both kinds of explants the fraction of photon energy absorbed by PSII antennae (*P*), is reduced by about 40% when compared to control leaves (Table 2), indicating a less efficient utilisation of light for photosynthetic processes. Differences between both kinds of explants exist, however, in the remaining fractions. In case of the light green explants the highest percentage of energy (c. 53% of the absorbed light energy) is thermally dissipated, whereas in the case of the dark green explants the highest percentage (c. 54% of the absorbed light energy) was found in the fraction of energy absorbed by PSII antennae but trapped by so-called 'closed' PSII reaction centres. The physiological significance of this latter fraction is not yet clear.

With respect to leaf pigments, the Chl *a*/*b* ratio remained similar, when control leaves and dark green explants are compared, whereas it decreased significantly in the case of the light green explants (Table 1). Chl *a* is present in the light harvesting complex and in the PSII reaction centres, while Chl *b* is found in the light harvesting complexes, only. As a consequence, a selective damage of the reaction centres should result in a reduction of the Chl *a*/*b* ratio due to the selective elimination of Chl *a*. This effect would also explain the significant reduction of F_v/F_m in the light green explants.

The Car/Chl ratio is difficult to interpret, but there is an obvious tendency towards a relatively higher Car content in both kinds of explants and a significantly higher relative Car content in case of the light green explants when compared with the control leaves (Table 1). Carotenoids are involved in the response of plants to environmental stresses. They are known to play important roles in plants as accessory light-harvesting pigments and as antioxidants (Havaux, 2013). Furthermore, a small fraction of carotenoids serves as antioxidants in the photosynthetic membranes (Havaux *et al*., 2004). The xanthophyll cycle, for instance, is involved in the regulation of light harvesting in the chlorophyll antenna system of PSII. Violaxanthin, in case of low light levels, serves as an accessory pigment, transferring energy to the PSII reaction centre, while zeaxanthin, in case of high light intensities, stimulates thermal energy dissipation within the light-harvesting antenna proteins by non-photochemical quenching and, as a consequence, reduces the amount of energy that reaches the photosynthetic reaction centres (Cazzonelli, 2011). The high percentage of thermally dissipated energy especially in the case of the light green explants would suggest that the xanthophyll cycle might have been involved (Lu *et al*., 2001). An alternative aspect of carotenoids related to photosynthesis represents its contribution to the defence against reactive oxygen species, especially singlet oxygen, by β–carotene in the PSII reaction centres. Recent results indicate that the reaction centres rather than the PSII chlorophyll antennae are the major sites of singlet oxygen accumulation (Havaux, 2013). As a consequence, an elevated activity of β–carotene should protect the PSII reaction centres, for instance by inhibiting pigment bleaching (Krieger-Liszkay, 2005).

The results of the present study may be summarized as follows: Both kinds of explants are characterized by a reduction of the fraction of light energy used in PSII photochemistry (*P*), hence a reduced photosynthetic efficiency. In both kinds of explants pigment contents are significantly reduced, but more distinctly in case of the light green explants resembling callus tissue, at least partly as a consequence of dilution due to leaf growth. When exposed to elevated light intensities (about nine times higher than ambient), the photosystem of the light green explants was photoinhibited as indicated by the large percentage of light energy thermally dissipated (*D*) and the decreases of F_v/F_m and F_m. In case of the dark green explants the largest percentage of the light energy absorbed by PSII antennae was trapped in 'closed' PSII reaction centres (*E*). When compared to the light green explants, the capacity to get rid of excessive light energy by thermal dissipation seemed limited in dark green explants. However, this may not represent a problem under the low ambient light conditions (90 μmol m^{-2} s^{-1}) during cultivation, but simply reflect the sensitivity of the explant's photosystem during organogenesis.

Conclusions

Two kinds of African violet explants survived and adapted onto the Murashige and Skoog basic medium that differ in their capacity to induce organogenesis, visual appearance, and also in their photosynthetic properties. The observed differences between the light and dark green explants in maximum quantum efficiency of PSII (F_v/F_m) and the relative distribution of absorbed light energy into the fractions 'energy absorbed by PSII antennae (*P*)', 'thermally dissipated energy' (*D*) and the remaining fraction (*E*) may serve to differentiate between leaf explants with and without organogenic potential in African violet, but a generalization of this observation needs to be confirmed in future experiments on further species and under modified *in vitro* conditions.

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