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Light energy management in micropropagated plants of *Castanea sativa*, effects of photoinhibition

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ABSTRACT

The limited development of photoprotective mechanisms, specifically heat dissipation capacity, found in micropropagated plants may be the result of low xanthophyll cycle pigment content and reduced de-epoxidation capacity making them highly susceptible to photodamage. The effects of gradual or sudden increase of light on *Castanea sativa in vitro* cultured and during their *ex vitro* transference was evaluated. The results were compared with those determined in nursery-grown plants. *In vitro* plants responded poorly to gradual increase in irradiance, exhibiting a low electron transport rate (ETR) agreeing with low non-photochemical quenching (NPQ) and a limited de-epoxidation capacity, not synthesizing detectable amounts of zeaxanthin (Z). Regarding a sudden increase in light (photoinhibition treatment, PhT); post-PhT as *in vitro* as well nursery plants showed a significant decrease in their maximal efficiency of PSII (F_v/F_m), but *in vitro* the decrease was very drastic (around 0.2) different from that observed in nursery (around 0.69). *In vitro*, NPQ was mainly determined by the slow relaxing component, NPQ_s (80.8%), concomitant with a pronounced decrease of D1 protein post-PhT, and a lack of de-epoxidation capacity. During *ex vitro* transfer, PhT lead to death of some plants, specifically during root induction. The photoprotective mechanisms were activated over time in *ex vitro* conditions, indicating that micropropagated *Castanea sativa* display a potential for light acclimation, adjusting their photosynthetic apparatus to the ambient growth irradiance. Understanding the mechanisms that micropropagated plants deployed and how they face high light intensity events, will allow us to search for strategies to improve performance to possible light fluctuations that normally occur in *ex vitro* conditions during plant acclimation.
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1. Introduction

Plants have developed a series of mechanisms that allow them to maintain a balance between light energy absorbed through photochemistry and energy utilization through photosynthetic electron transport coupled to carbon reduction. Yet, under certain environmental conditions, the absorbed light energy may exceed the plant's ability to use it, creating an imbalance that can lead to photoinhibition [1]. Photoinhibition is a consequence of either the reversible down-regulation of PSII through the dissipation of excess absorbed energy or the irreversible inactivation of PSII and damage to the D1 reaction center protein [2].

To avoid or minimize photoinhibition, photosynthetic organisms have some constitutive characteristics at the level of photosystems, such as the presence of carotenoids, which inactivate singlet oxygen or trap chlorophyll triplet states [3]. This is combined with long and short-term photoprotection mechanisms which help to dissipate excess absorbed light energy. Among short-term responses, thermal dissipation capacity, analyzed through non-photochemical quenching (NPQ), constitutes the fastest protective strategy and involves the dissipation of excess light energy at the antenna level of PSII [4]. NPQ has been described as being the sum of two components [5] with different relaxation kinetics, the fast and the slow relaxing component. The fast relaxing component (NPQ_f), also called qE, is associated with changes in the pH trans-thylakoid gradient (ΔpH), which has at least two functions: activation of violaxanthin de-epoxidase enzyme (VDE) that catalyzes the reversible de-epoxidation of violaxanthin to zeaxanthin *via* the intermediate antheraxanthin, and driving protonation

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of one or more PSII proteins that are involved in qE, such as the PsbS protein [6,7]. When these changes occur, an excited chlorophyll can transfer its excess energy to *Z*, which returns to its ground state releasing energy as heat [8,9]. The slow relaxing component (NPQ_s), called qI, is usually much smaller than qE, and is attributed to a more prevalent effect of excess light which leads to sustained heat dissipations. Therefore, NPQ_s has been associated with photoinhibition, and was thought to arise from quenching by a photodamaged PSII reaction center [3]. It has been suggested that NPQ is not an effect of excess light stress, but an adaptive response to it [5,10]. Thus, the responsiveness of these mechanisms will depend on the light environment of plant, which in nature is predominantly heterogeneous [11].

Under *in vitro* culture conditions, ambient light is completely different from that in the natural environment, both in intensity and quality, and within the photoperiod, this is preeminently homogeneous [12]. This condition and other factors associated with the chemical culture conditions had produced that *in vitro* plants are characterized by anatomical and functional anomalies making the application of this technique still questioned [12,13]. In particular, the permanent low light conditions *in vitro* have been considered a limiting factor for photosynthesis, and the development of photoprotective mechanisms in *in vitro* cultured plants result in a very limited capacity to develop NPQ *in vitro* [14–16]. This makes *in vitro* plants very susceptible to high light conditions [13], and prone to photoinhibition [17,18]. Several strategies have been developed around the acclimatization of *in vitro* plants, producing plants that are potentially able to develop a functional photosynthetic apparatus [19]. However, this capacity does not prevent the symptoms of photoinhibition that normally appear during acclimatization [17,20].

In previous studies, we discussed that the slow *ex vitro* establishment, observed in micropropagated plants of *Castanea sativa*, could be associated with the poor development of photoprotection mechanisms by heat dissipation (NPQ) compared to nursery grown plants [16]. In this study we postulate that this is associated to a limited xanthophyll cycle pigment content with low de-epoxidation capacity, making *in vitro* plants highly susceptible to photodamage, due to increase of irradiance. We tested the effects of a gradual increase in light and sudden increase light on *C. sativa* microshoots, and we analyze their ability to cope with excess light energy. Additionally, we compared the results obtained with plants grown under nursery conditions. Finally, given that the *ex vitro* transfer is usually accompanied by event of sudden increase of irradiance, which may produce symptoms of photoinhibition, we also evaluated the effects of this events during *ex vitro* transfer stages and the capability to recover photosynthetic efficiency during these stages. Understanding how micropropagated plants adapts to new *ex vitro* conditions, mainly to fluctuations in irradiance levels, and potential excess light events that usually occur in nature, will lead to the development of micropropagation protocols that improve performance and survival of micropropagated plants under natural light conditions with minor risks of photoinhibition.

2. Materials and methods

2.1. Plant material

In vitro and nursery culture plants of *Castanea sativa* were used in the present experiments. *In vitro* plants derived from zygotic embryos were cultured in a MS medium [21] containing 0.22 μM of 6-benzylaminopurine (BAP), 0.024 μM of indolebutyric acid (IBA), 30 g L^{-1} sucrose and 7 g L^{-1} agar, at pH 6.2. The culture conditions were 16 h light photoperiod, photon flux density (PFD) of 50 ± 5 μmol photons m^{-2} s^{-1} and 24 ± 2 °C and 60% relative humidity. Microshoots were rooting according to Vieitez et al. [22] in 15 cm^3 plastic pots with a mixture of peat and *perlite* 3:1 and maintained inside plastic container until rooting. After, microplants were transfer to 45 cm^3 pots with a mixture of organic soil and *perlite* 3:1 and acclimated by 20 days in the same growth chamber where the *in vitro* culture and root induction was made. Subsequently, microplants were transferred to a greenhouse with natural light and irrigated by micro aspersion three times a day. Greenhouse trials were conducted in the month of June; the average maximum temperature inside the greenhouse was 23 °C and minimum 3 °C. Average photon flux density (PFD) varied between 90 and 870 μmol photons m^{-2} s^{-1} (measured at 11:00 h). The nursery plants corresponded to plants of 2 years old cultured in an outdoor nursery in black plastic bags filled with organic soil mixed with pine bark compost, maintained beneath a shade cloth (80% solar interception) and irrigated once a day.

2.2. Experimental approach

To analyze the management of light energy in micropropagated plants and their photoprotective mechanisms, three experiments were performance. In experiments 1, the response to gradual increase of light was analyzed through light response curves obtained in attached expanded leaves of *in vitro* and nursery plants. Dark adapted plants (30 min) were later subjected to 10, 50, 75, 100, 250, 450, 600 and 900 μmol photons m^{-2} s^{-1} of actinic light until steady state fluorescence was reached (average duration per light intensity was 5 min). Fluorescence parameters were obtained under steady state and at the same time samples were taken for pigment analyses as described below (Sections 2.3 and 2.4 respectively). In experiment 2, the behavior of plants to sudden increase light, *in vitro* and nursery plants were subjected to a photoinhibition treatment (PhT) was studied. For this, plants were transferred to a home-made photoinhibition chamber, consisting of a vertical freezer modified with a glass upper door and, on top of it, a 5 cm thick water filter to prevent radiant heat inside the chamber. Plants were exposed for 2 h at 1000 μmol photons m^{-2} s^{-1} and room temperature (18 ± 2 °C). The light was provided by two 450 watts metal-halide lamps and chamber temperature was controlled by a cooling water bath and monitored continuously with a thermocouple. The recovery from PhT was performed under darkness at room temperature and maximal fluorescence signal (F_m') was monitored by 75 h, whereupon NPQ components were determined. Additionally, pigment and protein content were analyzed in predark-adapted leaves, immediately after PhT (post-PhT) and during recovery. In experiment 3, microshoots in the rooting stage (R), acclimation stage (A) and 10 and 40 days following the greenhouse transference (10 T and 40 T, respectively) were subject to the same PhT used in the experiment 2, and their recovery kinetics, NPQ components and de-epoxidation capacity were analyzed. Additionally, the basal pigments and proteins content (in their respective growing conditions) were determined both *in vitro* and nursery conditions.

2.3. Chlorophyll fluorescence analyses

Chlorophyll fluorescence parameters were analyzed with pulse-amplitude modulated fluorimeter (FMS II, Hansatech Instrument, King's Lyn, UK) at actinic light determined in experiment 1 (Section 2.2). Minimal fluorescence (F_0) was determined by applying a weak modulated light (6 μmol photons m^{-2} s^{-1}) and maximal fluorescence (F_m) was induced by a short pulse (0.8 s) of saturating light (9000 μmol photons m^{-2} s^{-1}) according to [23]. Then, different actinic irradiance levels were sequentially applied (Section 2.2, experiment 1) until fluorescence reached the steady state (F_s). To determine the maximal fluorescence under light (F_m') various

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