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Photochemical Efficiency during the Establishment and Consolidation Phases of *in Vitro Pinus radiata* Micrograft Made from Scions of Different Ontogenetic Age

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Abstract

The aim of the present study was to evaluate the applicability of maximal photochemical efficiency of photosystem II (Fv/Fm) as an early estimate of *P. radiata* micrografts viability coming from different position (basal vs. apical) in the ortets. We hypothesize that Fv/Fm variation is a good indicator of micrograft's viability and phenological stage during micrograft development. The micrografts were established in QL medium supplemented whit 0.1 mg·L^{-1} IBA and 1 mg·L^{-1} BAP and cultured at 25˚C ± 2˚C and 80 µmol photons m^{-2}·s^{-1} of photosynthetic active radiation by 16 h per day. During the establishment and consolidation phase, we found significant differences in Fv/Fm with respect to time and buds positions provenience. During establishment, basal shoot tips have

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lower Fv/Fm than apical shoot tips, which agrees with the lowest viability (35%). However, during the consolidation phase, the trend changed and basal shoot tips presented higher Fv/Fm than apical shoot tips and showed an increase in ETR and NPQ, with respect to apical shoots and ortet. Although the measurement of fluorescence parameters implies the insertion of the fluorometer sonde *in vitro*, this implies aseptic considerations, but always conveies a contamination risk. We conclude that fluorescence (Fv/Fm, ETR, NPQ) can be indicators of the micrograft's development according to the shoot tips position in the ortet and can be useful early-indicators of the scions' physiological condition during micrograft transition from establishment to consolidation.

Keywords

Maximum PSII Photochemical Efficiency, Micrograft, *P. radiata*

1. Introduction

Pinus radiata D. Don is one of the most important forestry species in the world in terms of annual wood production, with almost 25% of the world's production [1]. Today, this production depends on the ability to generate selected clones in the short term. However, as in many woody species, in *Pinus radiata*, there is a decline in morphogenetic ability across maturation. Therefore, upgrading forestry programs face the problem of selecting interesting traits during the mature age, while vegetative propagation is only possible during juvenile phases of development [2]. Thus, the decrease of morphogenic capacity with the ontogeny of the source material [3], affect among others the rooting ability of plant tissue [4] being often a barrier for plant multiplication or regeneration. Several studies conducted in *Pinus* sp. indicate an ontogenetic gradient of shoot tips morphogenic competence, determined by the vertical localization in the tree crown, where basal meristematic shoot tips present higher morphogenic capacities than apical ones [5]-[7]. It has been suggested that the decline in morphogenic capacity could be due to the loss in competence at the cellular level and it is highly likely that this phenomenon causes changes in gene transcription [8]. *In vitro* propagation methods may induce reinvigoration of advanced physiological tissue and renewal of ontogenetically adult *P. radiata* [3] [6], and in this way avoid the problems associated to the lost of morphogenic capacity. This reinvigoration includes anatomical, molecular and epigenetic changes which reflect characteristic juvenile individual's protein patterns, DNA methylation and polyamine content [6] [9]. Altogether, the reversion of several adult phenotypic traits towards more juvenile stages and the recovery of tissue morphogenic capabilities allow the cloning of selected adult trees [6] [9] [10]. One tissue reinvigoration techniques is the *in vitro* micrograft, which has been tested to reinvigorate ontogenetically adult vegetative buds on juvenile rootstocks [11] and can be a solution for cloning adult trees of several species, in which sprouts present deficient rooting and lack of vigor [3] [4].

It is known that during plant ontogenetic development, apical meristems exhibit significant morphophysiological changes, which reflect modifications in cellular competence to perceive or respond to external and internal signals, such as growth regulators [12]-[14] and environmental conditions [15]-[17]. These variations in cell sensitivity induce modifications in the capability to form adventitious buds and roots, but also involve changes in leaf anatomy, growth rates and photosynthetic functioning [4] [16]. Photosynthetic capacity has been commonly considered as plant overall performance indicator [18]-[20]. Light energy absorbed by chlorophyll can drive charge separations at photosystem II (PSII) reaction centers, triggering photosynthetic electron transport, which mostly drive carbon uptake. These reactions can be probed by chlorophyll *a* fluorescence measurements through a process called photochemical quenching. In this context, fluorescence measurement has become a physiological tool commonly used to evaluate plant stress responses [15] [21]. Specifically, maximal photochemical efficiency of PSII (Fv/Fm) is a parameter that quantifies the fraction of absorbed light that is used in photochemistry after a dark period long enough to obtain all PSII reactions centers to reach an open state (reaction center reduced and acceptors completely oxidized), allowing the determination of the maximal photochemical light use capacity [22]. Hence, Fv/Fm has been commonly assumed as a proxy of photosynthetic capacity, reflecting the photosynthetic apparatus condition and at the same time is considered an ideal monitor of plant health and viability [18] [20] [21] [23]-[25]. Recently chlorophyll fluorescence has become a common tool to assess the photosynthetic performance of *in vitro* cultured plants [26]-[29]. Thus, [30] reported higher photo-

synthesis rates in juvenile and rejuvenated than adult *in vitro* grown *Sequoia sempervirens* shoots. Photosynthetic performance has also been positively correlated with growth rates and survival of *in vitro* plants and with their viability to establish in *ex vitro* conditions [31] and has been used as an indicator of better *in vitro* acclimatization treatments [26]. We hypothesize that variations in fluorescence parameters such as Fv/Fm, ETR and NPQ are good indicators of micrograft's viability and phenological stage. Accordingly, we inquire into the effects of the position from which the scion is obtained (basal vs. apical) in the ortet and the utility of fluorescence as an early estimate of *P. radiata* micrografts viability. To our knowledge, this is the first study that specifically addresses the early prediction of micrograft viability through the detection of scion's photochemical capacity along *in vitro* reinvigoration process.

2. Materials and Methods

2.1. Plant Material

Fresh rootstocks coming from certified *P. radiata* seeds were obtained. Plantlets were cultivated *in vitro* by germinating seeds in QL medium [32] free of growth regulators and with macroelements diluted to 25% v/v of the original concentration. In order to prepare the rootstock, after 30 days of cultivation, the plantlets were extracted and root system and needles were eliminated at collar and cotyledonary needles insertion level, respectively. The scions were obtained from caulinary apexes coming from the lower and apical quarter portions of 9-year-old and ca. 8.8 m tall *P. radiata* crowns (hereafter basal and apical, respectively. **Figure 1(A)** and **Figure 1 (B)**). Trees used for scions collection were part of the Genetic improvement program of the Fletcher Challenge

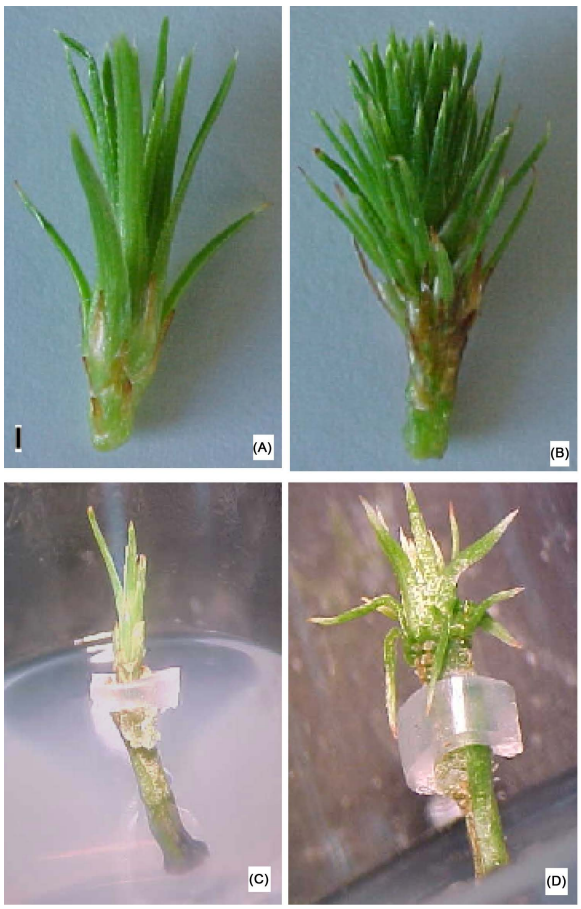

Figure 1. Morphology of vegetative shoot tips used as scions in the micrografts and *in vitro*-micrograft developmental stages. Shoot tips from the apical portion (A), shoot tips from the basal ortetportion (B); micrografts during the establishment (C) and consolidation phases (D). Vertical black bar in A represents 1 mm length.

(New Zealand) and Forestal Bio-Bio (Chile) Forest companies. The superficial scion asepsis began with the immersion of shoot tips in a solution of Captan® (2 mg·L^{-1}) for 15 min, followed by a washing with sterile distilled water. Under laminar flow chamber conditions, shoot tips were submerged in diluted ethanol 20% (v/v) for 10 s, followed by a washing with sterile distilled water. Then, scions were immersed in a solution composed by sodium hypochlorite 2.5% (v/v) and 100 µl of Tween-20 for 20 min, followed by 3 washings with sterile distilled water for 3, 4 and 5 min, respectively. Finally, scions were maintained until their use in a benomile plus cystein solution, both of 50 mg·L^{-1}, acting as a fungicide and antioxidant, respectively.

2.2. Micrograft Technique

The micrografts were made using the wedge grafting method proposed by [3]. Briefly, the rootstocks were beheaded below the insertion point of the cotyledonary needles, and then these caulinary segments were cut by it longitudinal axis 3 mm from the apical part. In laminar flow chamber conditions and under an optical magnifying glass, needles of the scions were cut in order to obtain 2 mm long apexes. Then, in its basal portion, 2 "v"-cuts were made, forming a wedge to insert the scion in the rootstock's crevice. The contact between scion and rootstock was maintained with a sterile silicon rings, obtained from transversal cuts of 5 mm diameter silicon tube (**Figure 1(C)** and **Figure 1(D)**). Finally, micrografts were established in 50 ml test tubes containing 15 ml of QL medium supplemented whit 0.1 mg·L^{-1} indole butyric acid (IBA), 1 mg·L^{-1} bencil aminopurine (BAP) and 30 g·L^{-1} sucrose. Growth chamber conditions during micrografts development were: 24˚C ± 2˚C air temperature, photosynthetic photon flux density (PPFD) at scions level 80 µmol·m^{-2}·s^{-1} by 16 h per day, and 60% air relative humidity.

2.3. Viability, Establishment and Consolidation

The viability was assessed visually according [9] and [3], hence if micrograft remained green without oxidative damage and turgid was considered viable. The establishment phase time was considered from the total micrografts initially introduced *in vitro* and remained viable, before scion-rootstock callus formation. The consolidation phase started with micrografts that remains viable and with scion-rootstock callus formation. The micrografts percentage reaching this phase was determined from those that survived the establishment phase.

2.4. Maximal Photochemical Efficiency of PSII

Fluorescence signals were measured by a pulse-amplitude modulated fluorometer (FMS 2, Hansatech, U.K). According to the terminology of [33], minimal fluorescence (Fo) was determined by applying a weak modulated light (0.4 µmol photons m^{-2}·s^{-1}) and maximal fluorescence (Fm) was induced by a short pulse (0.8 s) of saturating light (9000 µmol photons m^{-2}·s^{-1}). Maximal photosystem II photochemical efficiency (Fv/Fm), was estimated as described by Schreiber *et al*. (1994) where Fv = Fm − Fo. Before fluorescence measurements, the whole ortet was dark adapted for 45 min, with a black 0.2 mm thickness polietilene cover, in order to obtain open reactions centers through the oxidation of PSII primary acceptors. Shoot tips were labeled in both the apical and basal quarter portions of the ortets (0.0 - 2.2 and 6.6 - 8.8 m tall, respectively) and then Fv/Fm was subsequently measured. Once the micrografts were undertaken, Fv/Fm measurements were performed *in vitro* every ca. 2 days for each micrograft until their consolidation assessment (60 days after being cultivated). Additionally, the electron transport rate (ETR) was calculated at PPFD of 75 µmol m^{-2}·s^{-1} according to [24] as: ETR = 0.84 × ΦPSII × PPFD × 0.5. Where the factor 0.84 is the mean value of absorbance for green leaves, and the factor 0.5 assumes that the efficiency of both photosystems is equal and that radiation is equally distributed between them. The non-photochemical quenching (NPQ) was calculated as: (Fm − Fm')/Fm' ([21]).

2.5. Data Analysis

Generalized Lineal Models (GLM) and deviance analyses were used to compare the viability, establishment and consolidation percentages assuming Binomial error distribution [34] of 25 replicates where the experimental unit was the micrograft. The following analyses were conducted in plants that survived, using a completely random design with 10 replicates. For continuous variables, differences between apical and basal shoot tip provenance within each *in vitro* culture phases, were compared by one-way ANOVAs and the post hoc Tukey-test ($P < 0.05$). Normality and homogeneity of variance were evaluated by Kolmogorov-Smirnov test ($P < 0.05$) and Le-

vene ($P < 0.05$) tests. When appropriate, variables were transformed to follow the former assumptions [35]. Analyses were performed using Statistica software (Version 6, 2001, StatSoft, Tulsa, OK).

3. Results and Discussion

The micrograft's viability varied between vertical positions of shoot tips in the ortet. Micrografts made from apical shoot tips shows higher viability than basal ones ($P = 0.029$; **Figure 2**, statistical details in **Table 1** supplementary material). Thus, the 35% lower establishment of basal shoot tips may be due to several reasons; among them the effectiveness of asepsis of plant tissues introduced in *in vitro* conditions usually have a deep impact in their viability [36]. In this context, is important to note the different morphology exhibited between apical and basal scions (**Figure 1(A)** and **Figure 1(B)**). Apical scions present longer and less compact needles (**Figure 1(A)**). This may favor disinfection efficiency inducing greater micrograft viability and therefore establishment. On the contrary, basal scions showed evidently higher abundance, aggregation and shorter needles (**Figure 1(B)**). Otherwise, the concentrations of growth regulators in the shoot tips vary depending on their position in the ortet. [37] Reported higher concentration of auxins and cytokinins in apical shoot tips than basal ones, which may improve tissue responses to the *in vitro* introduction, specifically during *P. radiata* micrograft establishment [38].

Despite apical scions showed a higher micrografts establishment than basal ones, those differences fade out during the micrograft consolidation reaching in average 39%. Commonly, the development of shoot tips and scion meristematic activity expressed as growth rates are key factors that determine micrograft success [3] [5]. The decrease in vigor and adventitious organs formation capacity are responses associated with the tissue maturation stage [4]. Hence, basal scions must present higher morphogenic capacities than apical ones, because their younger ontogenetic age determined by the proximity to the mother cell located at the collar of the ortet [5] [6]. In the consolidation phase we found that juvenile traits of ontogenically young tissues can be preserved in basal scions. The fact that maturity takes place in the periphery of tree crowns; in chronologically young but ontogenically older tissues, was reflected by the decrease of consolidation percentage of apical scions respect to establishment ($P = 0.024$; **Figure 2**). However, the final grafting efficiency for apical scions was 20% and basal scions only 6%. Therefore, this confirms that micrografting is a suitable strategy to reinvigorate old ontogenetic tissues in *P. radiata*.

Regarding to maximal photochemical efficiency, significant differences in shoot tips Fv/Fm values were detected between the establishment and consolidation phases (**Figure 3**, statistical details in **Table 2** supplementary material). Only during the first day of *in vitro* introduction, a decrease in Fv/Fm was observed in basal scions ($P = 0.01$; **Figure 3**). Such small Fv/Fm reduction could be attributable to the physiological stress imposed by the excision from ortet, and then the *in vitro* introduction [39] [40]. Despite this, we find a fast scions Fv/Fm recovery, due to the *in vitro* culture conditions, which also exert the surpass of Fv/Fm values measured in the ortet

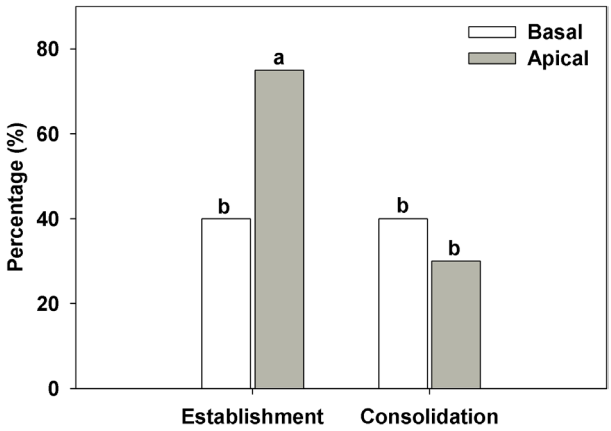

Figure 2. Effect of shoot tips provenance in the ortet on percentage of establishment and consolidation of *P. radiate* micrografts. Shoot tips were collected from the basal and apical quarter of the ortet crown (9-year-old and ca. 8.8 m tall). Different letters indicates significant differences (n = 20, Wald stat, $P < 0.05$).

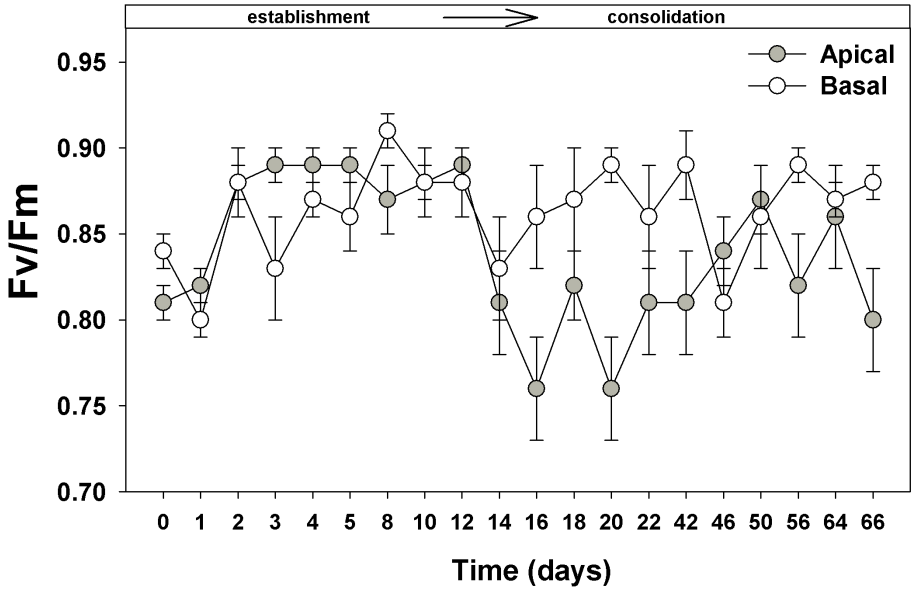

Figure 3. Photosystem II maximal photochemical efficiency (Fv/Fm) of micrografts made from basal and apical shoot tips during *in vitro* establishment and consolidations phases. Shoot tips were collected from the basal and apical quarter of the adult *P. radiate* ortet crown (9-year-old and ca. 8.8 m tall). Mean ± SE (n = 10).

from the second day. During the first two weeks of *in vitro* culture, in average basal scions had a more variable Fv/Fm and sometimes slightly lower than apical ones ($P = 0.045$; **Figure 3**), which agreed with their lowest micrograft establishment (**Figure 2**). Thereafter, during the beginning of consolidation phase (around 13 day), the trend was reversed. Apical scions significantly decreased their Fv/Fm ($P < 0.001$; **Figure 3**), even to lower values than those observed in the ortet. On the contrary, along *in vitro* culture basal scions displayed higher Fv/Fm during consolidation than apical ones ($P < 0.001$; **Figure 3**).

Additionally, during establishment PSII light energy partitioning of apical and basal scions were similar (lowest $P = 0.435$; **Figure 4**, statistical details in **Table 3** supplementary material). Conversely during consolidation these ontogenetic younger (basal) and more plastic scions were able to increase ETR in 43% and NPQ in 100% ($P = 0.046$ and $P = 0.019$, respectively). The latter both are save valves to dissipate higher PSII excitation pressures, which are indicators of higher capacity to handle and take advantage of higher PPFDs. Increments by more than 100% in thermal dissipating capacity of excess light energy has been reported in ventilated vessels respect to traditional *in vitro* condition [29]. Regardless of vertical location in the ortet, shoot tips show similar values of ETR, indicating that in the field they display similar capacities to conduct absorbed light forwards photochemical processes. Interestingly, apical shoot tips in the ortet exhibited higher NPQ, therefore higher capacity to safely dissipate the excess absorbed energy as heat. A commonly response to high light is the increment in xanthophyll pool size (VAZ), allowing them manage the excess of absorbed light by means of heat dissipation [41] [42]. Together, these parameters could determine a greater ability to withstand the stress of transfer to *ex vitro* conditions [31] and may therefore be an indicator of greater potential physiological performance once a micrograft coming from basal section in the ortet is consolidated. Unfortunately, the low efficiency of micrografting of these basal scions is a big constraint for a reinvigoration program. Further studies on the behavior of these micrografted plants during transference to *ex-vitro* conditions are needed to fully address this problem.

The different responses among *in vitro* culture phases suggest that it is more feasible to establish micrografts *in vitro* from apical shoot tips than that from basal shoot tips. Hence they are physiologically younger, and theoretically they are synthesizing auxins and therefore are able to faster cellular differentiation and division, favoring rapid adaptation to *in vitro* conditions [13] [14] [37] [43]. This was reflected in a higher viability and photochemical efficiency during establishment. Basal shoot tips, even though they are ontogenetically younger, they are physiologically and chronologically older, have lower endogenous auxins concentration, and slower cellular de-differentiation rates [13] [14] [37]. However, once basal scions are established, they achieve greater consolidation, concomitant with greater Fv/Fm at the end of this phase, concomitantly with greater photochemical ca-

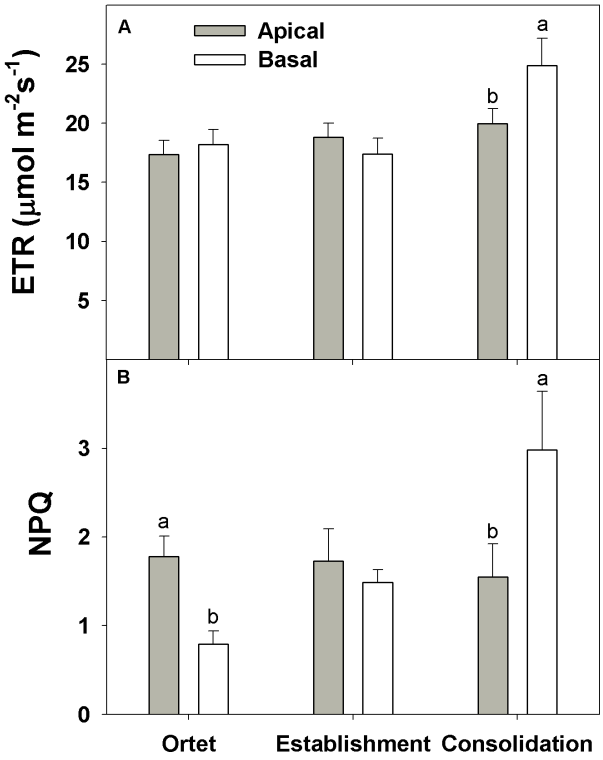

Figure 4. Electron transport rate trough PSII (ETR) (A) and non-photochemical quenching (NPQ) (B) of micrografts made from apical and basal shoot tips collected from adult *P. radiate* (9-year-old and ca. 8.8 m tall). Values showed in the ortet and during it *in vitro* micrograft establishment and consolidation phases. Mean ± SE (n = 10). Different letters indicate significant differences between apical and basal shoot tips within each phase (Tukey test, $P < 0.05$).

pacity, manifested through higher ETR and NPQ values. This higher photochemical activity is a proxy of higher CO_2 assimilation capacity, and therefore of greater photosynthetic performance and potentially faster growth [18] [20] [21]. Additionally higher Fv/Fm reflects an ontogenetic age gradient of shoot tips morphogenic competence, where basal meristematic shoot tips present higher morphogenic capacities than apical ones [5] [6]. Finally, based on our results, we conclude that Fv/Fm can be an indicator of the micrograft's development according to the shoot tips position in the ortet and can be a useful indicator of the physiological stage of scions during micrograft transition from establishment to consolidation. This method is suggested to be suitable at low scale or laboratory characterization. It needs further scaling up if intended to use at larger scale such as productive forest nurseries.

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Supplementary Material

Table 1. Comparison of percentage of establishment and consolidation of *P. radiata* micrografts shoot tips collected from the basal and apical quarter of the ortet crown (9-year-old and ca. 8.8 m tall). Generalized lineal models and deviance analyses were used assuming Binomial error distribution. Significant differences were detected with the Wald statistic $P < 0.05$ (n = 20).

Source of variation	d.f.	Wald Stat.	*P*
Intercept	1	1.011480	0.314548
Provenance within establishment	1	4.762631	**0.029084**
Intercept	1	0.789130	0.374363
Provenance within consolidation	1	0.000000	1.000000
Intercept	1	0.085022	0.770604
Apical between in vitro phase	1	5.097302	**0.023963**
Intercept	1	1.052173	0.305007
Basal between in vitro phase	1	0.000000	1.000000

Table 2. Comparison of Fv/Fm between apical and basal shoot tip provenance within each *in vitro* culture phases, respect to the ortet and between *in vitro* culture phases. Normality and homogeneity of variance were evaluated by Kolmogorov-Smirnov test ($P < 0.05$) and Levene ($P < 0.05$) tests. When appropriate, variables were transformed to follow the former assumptions.

Source	**Df**	**SS**	**MS**	**F**	***P***
Fv/Fm within establisment between provenance (ln transformed)					
Intercept	1	3.176	3.176	1049.05	<0.001
Provenace	1	0.012	0.012	4.09	**0.045**
Error	158	0.478	0.003		
Fv/Fm within consolidation between provenance (ln transformed)					
Intercept	1	7.070	7.070	939.52	<0.001
Provenace	1	0.212	0.212	28.18	**<0.001**
Error	218	1.640	0.008		
Fv/Fm ortet vs. 1thr day in vitro of apical scions					
Intercept	1	13.358	13.358	14851.61	<0.001
in vitro introduction	1	0.001	0.001	1.23	0.281
Error	18	0.016	0.001		
Fv/Fm ortet vs. 1thr day in vitro of basal scions					
Intercept	1	13.561	13.561	13778.41	<0.001
in vitro introduction	1	0.008	0.008	8.41	**0.010**
Error	18	0.018	0.001		
Fv/Fm establisment vs. consolidation of basal scions					
Intercept	1	2.023853	2.023853	442.153	<0.001
Phase of in vitro culture	1	0.004161	0.004161	0.9091	0.343
Error	97	0.443991	0.004577		

Continued

Fv/Fm establisment vs. consolidation of apical scions					
Intercept	1	3.212842	3.212842	598.9765	<0.001
Phase of in vitro culture	1	0.352417	0.352417	65.7019	**<0.001**
Error	97	0.520297	0.005364		
Levene's Test for Homogeneity of Variances		**MS Effect**	**MS Error**	**F**	***P***
Fv/Fm within establisment between provenance (ln transformed)		0.010	0.001	9.029	0.053
Fv/Fm within consolidation between provenance (ln transformed)		0.038	0.002	8.298	0.050
Fv/Fm ortet vs. 1thr day in vitro of apical scions		0.000	0.000	1.027	0.324
Fv/Fm ortet vs. 1thr day in vitro of basal scions		0.000	0.000	0.021	0.886
Fv/Fm establisment vs. consolidation of basal scions (ln transformed)		0.011	0.001	9.329	0.050
Fv/Fm establisment vs. consolidation of apical scions (ln transformed)		0.040	0.002	8.984	0.049
Kolmogorov-Smirnov *test for normality*				**d**	***P***
Fv/Fm within establisment between provenance (ln transformed)				0.105	>0.20
Fv/Fm within consolidation between provenance (ln transformed)				0.200	<0.20
Fv/Fm ortet vs. 1thr day in vitro of apical scions				0.128	>0.20
Fv/Fm ortet vs. 1thr day in vitro of basal scions				0.109	>0.20
Fv/Fm establisment vs. consolidation of basal scions (ln transformed)				0.114	<0.20
Fv/Fm establisment vs. consolidation of apical scions (ln transformed)				0.150	<0.10

Table 3. Comparison of light energy partitioning between apical and basal shoot tip provenance in the ortet and within each *in vitro* culture phases. Normality and homogeneity of variance were evaluated by Kolmogorov-Smirnov test ($P < 0.05$) and Levene ($P < 0.05$) tests.

Source	Df	SS	MS	F	*P*
ETR in the ortet					
Intercept	1	6315.632	6315.632	405.967	<0.001
Provenance	1	3.528	3.528	0.227	0.640
Error	18	280.026	15.557		
ETR in the establishment					
Intercept	1	6553.217	6553.217	414.108	<0.001
Provenance	1	10.083	10.083	0.637	0.435
Error	18	284.848	15.825		
ETR in the consolidation					
Intercept	1	10051.577	10051.577	299.880	<0.001
Provenance	1	118.476	118.476	3.535	**0.046**
Error	18	603.336	33.519		
NPQ in the ortet					
Intercept	1	32.903	32.903	92.567	<0.001
Provenance	1	4.862	4.862	13.680	**0.002**

Continued

Error	18	6.398	0.355		
NPQ in the establishment					
Intercept	1	51.833	51.833	107.537	<0.001
Provenance	1	0.292	0.292	0.607	0.446
Error	18	8.676	0.482		
NPQ in the consolidation					
Intercept	1	102.622	102.622	66.082	0.000
Provenance	1	10.227	10.227	6.585	**0.019**
Error	18	27.953	1.553		
Levene's test for homogeneity of variances		**MS Effect**	**MS Error**	**F**	***P***
ETR in the ortet		0.057	4.257	0.013	0.909
ETR in the establishment		1.709	5.865	0.291	0.596
ETR in the consolidation		36.745	10.202	3.602	0.074
NPQ in the ortet		0.187	0.096	1.954	0.179
NPQ in the establishment		1.831	11.142	4.873	0.051
NPQ in the consolidation		0.829	0.248	3.347	0.084
Kolmogorov-Smirnov *test for normality*				**d**	***P***
ETR in the ortet				0.105	>0.20
ETR in the establishment				0.200	>0.20
ETR in the consolidation				0.128	>0.20
NPQ in the ortet				0.109	>0.20
NPQ in the establishment				0.189	>0.20
NPQ in the consolidation				0.201	>0.20

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