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BRIEF COMMUNICATION

Photosynthesis parameters during acclimatization of *in vitro*-grown olive plantlets

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Abstract

Monitoring some parameters would help to overcome the difficulties that can affect *in vitro*-grown plants during the crucial step of their acclimatization. Thus, after the determination of net photosynthesis and other parameters during acclimatization of *in vitro*-grown olive plantlets, we concluded that three months after the transfer to *ex vitro*, the *in vitro*-grown olive plants become well acclimated. In fact, even though the net photosynthesis, relatively high *in vitro*, recorded low values after 15 d from the transfer, it reverted back to its standard rates after 180 d of acclimatization. Transpiration and stomatal conductance first increased significantly with a maximum of 6.22 mmol(H_2O) m^{-2} s^{-1} and 1.8 mmol(H_2O) m^{-2} s^{-1}, respectively, but they regressed to very low values after 180 d of acclimatization. Some changes in the leaf anatomy were also observed; the reduction of stomata density and inversely, the increase of trichome density, especially on the abaxial side of the leaves, were observed.

Additional keys words: gas exchange; *in vitro* culture; micropropagation; *Olea*.

Acclimatization is a crucial step in the whole process of micropropagation. In fact, the acclimatization is sometimes difficult and delicate to achieve considering changes of physical conditions in the culture and it can have adverse effects on the survival of young seedlings. Therefore, commercial utilization of micropropagation requires a successful acclimatization (Deccetti *et al*. 2008, Dobránszki and Teixeira da Silva 2010). Indeed, it was shown that various structures and parameters in plants can change abruptly during the first days of *ex vitro* transfer and therefore it can influence the survival rate of plantlets (Kozai *et al*. 1997, Pospíšilová *et al*. 1999, Hazarika 2006, Moncaleán *et al*. 2007, Deccetti *et al.* 2008, Dobránszki and Teixeira da Silva 2010). At the leaf level, the density and morphology of stomata are modified by the increase of the light intensity and by the decrease of the relative humidity during the acclimatization phase (Blanke and Belcher 1989, Capellades *et al.* 1990, Pospíšilová *et al.* 1998, Deccetti *et al.* 2008). On the other hand, the net photosynthetic rate (P_N) generally decreases significantly just after the *ex vitro* transfer, while the transpiration rate (E) and the stomatal conductance (g_s) show an important increase.

Furthermore, Pospíšilová *et al.* (1999) and Hazarika (2006) showed the dependence of P_N on the environmental conditions of acclimatization; they observed that an increase in CO_2 concentration causes a significant increase in the photosynthetic activity of plantlets. The E is generally high at the beginning of acclimatization but decreases gradually after adaptation to the external environment (Pospíšilová *et al.* 1999, Hazarika 2006, Chaari-Rkhis *et al.* 2011). The objective of the present study was to determine some indicators associated with photosynthesis in order to find a necessary and minimum acclimatization period to obtain viable micropropagated olive plants.

In vitro shoots from two Tunisian olive varieties (Chemlali and Oueslati), which grew up to 3–4 cm and had 3–4 nodes, were rooted under *in vitro* conditions (Chaari-Rkhis *et al.* 2011). Once rooted, the micropropagated plants (30 per variety) were carefully taken and transplanted into small plastic pots containing a mixture of compost and peat (1:1). The potted plantlets were irrigated once a week with a nutrient solution composed of half concentrations of Murashige-Skoog macro and micronutrients medium (Murashige and Skoog 1962). They were placed in a greenhouse where the relative humidity was adjusted

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Abbreviations: E – transpiration rate; g_s – stomatal conductance; P_N – net photosynthetic rate.

to 80 ± 2% and temperature maintained at 25 ± 1°C under 16-h photoperiod. For the first week after the transplantation, the plantlets were covered with transparent polyethylene to maintain the adequate temperature and high humidity.

The P_N [μmol m^{-2} s^{-1}], E [mmol(H_2O) m^{-2} s^{-1}], and g_s [mmol(H_2O) m^{-2} s^{-1}] of the leaves already formed *in vitro* were measured *in vitro*, and 15, 30, 90, and 180 d after the beginning of acclimatization (DA) using an *LCi* photosynthesis apparatus (*ADC Bioscientific Ltd*., England). The apparatus is equipped by a small chamber for small leaves. For the measurements of *in vitro* leaves, we took the leaves grown under *in vitro* conditions; the measurements were done rapidly in the growth room before the leaves were damaged. At least 30 measurements were made for all parameters on three different leaves.

The number of trichomes, which are the protective structures on the both sides of olive leaves, was computed by taking leaf trichome imprints on self-adhesive bands (Boujnah 1997). These ribbons were then stuck onto the slides and observed using a microscope (*DIALUX 22EB*, *Leitz*, Germany). The measurements were performed using program *UTHSCSA Image Tool Version 3.0*. To collect prints of the stomata on each leaf, clear nail polish was spread on the surface of the leaf after removing the trichomes. Once dried, the varnish layer was removed by scotch tape which was placed on a microscope slide and then a microscope with appropriate software (*UTHSCSA Image Tool Version 3.0*) was used to count the number of both structures (Boujnah 1997).

The parameters were recorded on leaves taken from *in vitro* plants and leaves taken after 15, 30, 90, and 180 DA. At least 30 values were taken to count stomata and trichome numbers. The statistical analyses were performed using *SPSS* 11.0 (Chicago, IL, USA). The results were expressed as average ± standard error (SE). *Duncan*'s multiple range tests was used to determine the significance of differences between the compared mean values at a level of confidence of 0.05.

During *in vitro* culture, we recorded P_N values (Fig. 1), which invalidated the fact that *in vitro* plantlets generally have a very low photosynthetic capacity to provide a positive carbon balance due to the presence of sucrose in the media (Grout and Aston 1977, Amâncio *et al.* 1999, Serret *et al.* 2001, Osório *et al.* 2013). On the contrary, some authors reported significant P_N *in vitro*, mainly if the environment was enriched with CO_2 (Desjardins *et al*. 1987, Fila *et al.* 1998, Pospíšilová *et al.* 2007). However, P_N decreased significantly during the first days of *ex vitro* transfer and the lowest values were recorded (Fig. 1). This has been reported also in other species (Hazarika 2003, Dias *et al.* 2014); it can be attributed to the stress experienced by plant tissues in response to changing environmental conditions or to the partial damage of the photosynthetic apparatus (Hazarika 2003, 2006).

However, in all micropropagated species, P_N reverts back to its standard rates after a certain period of acclimatization which depends on species (Hazarika 2006, Pospíšilová *et al*. 2007, 2009). In olive, after three-month acclimatization, P_N increased to 5.6 and 5.05 μmol(CO_2) m^{-2} s^{-1} for Chemlali and Oueslati, respectively, and reached the values recorded in the adult olive tree (Msallem 2002) indicating autotrophy acquisition. Estrada-Luna *et al*. (2001) and Slavtcheva and Dimitrova (2001) found that P_N of *in vitro*-plantlets in *Capsicum annum* and *Vitis vinifera* increased after 6 and 30 d of acclimatization, respectively.

In terms of g_s and E, our results showed a significant increase in the values of both parameters just after transplantation of *in vitro*-grown olive plants, inversely than P_N values (Fig. 1). This was consistent with the work of Pospíšilová *et al*. (1998) who found E from *in vitro* plants of *Nicotiana tabacum* increased in two weeks after

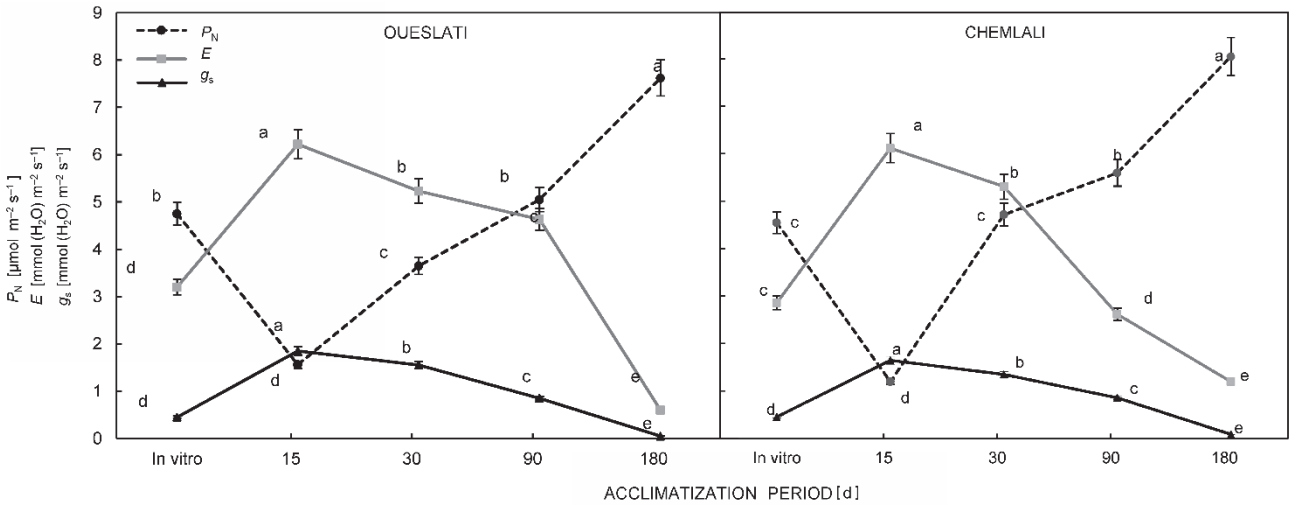

Fig. 1. Net photosynthetic rate (P_N), leaf transpiration (E), and stomatal conductance (g_s) of Chemlali and Oueslati olive varieties *in vitro* and after 15, 30, 90, and 180 d of acclimatization. Means ± SE (n = 30). *Different letters* for each curve indicate a significant difference between values at p<0.05.

the beginning of acclimatization. Fila *et al*. (1998) reported a decrease of g_s one month after *Vitis* transplantation. This phenomenon should be related to the degree of stomatal opening. In fact, some authors mentioned a malfunction of those structures *in vitro* and the nonfunctioning stomata during *in vitro* cultivation (Blanke and Belcher 1989).

According to Pospíšilová *et al*. (1999), the increase in *E* just after transplantation and the nonfunctioning stomata during *in vitro* culture are also due to the late development of the cuticle and epicuticular wax. The decrease in water potential of the atmosphere around the plantlets can also explain the high *E*. Indeed, in *in vitro* culture conditions, plantlets grow under saturating humidity which considerably limits their *E*. For both parameters, a sharp decline in their values was recorded after about six months of acclimatization indicating that originally *in vitro*-grown trees were well acclimatized since P_N values indicated autotrophic behaviour.

Our results showed that *in vitro* stomatal density values were higher than those registered after transplantation, but it was significantly different only 180 DA in Chemlali variety (Table 1). The same decrease was observed in the leaves of several species after *ex vitro* transfer (Blanke and Belcher 1989, Capellades *et al*. 1990, Brutti *et al*. 2002, Deccetti *et al.* 2008). This can be explained by enlargement in leaf area that results in reducing the stomata number per area unit. Deccetti *et al*. (2008) mentioned that *Annona glara* has a higher stomata frequency in leaves developed *in vitro* than those of mother plant leaves of the same species; they concluded that under conditions of higher light and CO_2 availability, the number of stomata per mm^2 increases. The reduction of the stomata number could be a kind of plant adaptation to a hostile environment. Thus, contrary to our results, the stomatal density may increase after *ex vitro* transfer in some cases (Pospíšilová *et al.* 2007).

Unlike stomata structure, which is present only on the underside of the leaf in olive, the trichomes are found on both the upper and lower sides of the leaf. During the acclimatization, there was a significant increase in the number of trichomes on both sides, mainly on the lower side of the leaf for both varieties (Table 1). The densities of stomata and trichomes are inversely proportional, that is, when the stomata number decreases, the trichome density increases. Indeed, trichomes have an important protective role mainly under *ex vitro* conditions. Similar results were also reported by Donnelly *et al.* (1986). In fact, an increase of trichome density is considered as one of the defense responses of plants (Mauricio *et al.* 1997, Agrawal 1998, Steinite and Ievinsh 2003).

Table 1. Densities (per mm^2) of stomata and trichomes of Chemlali and Oueslati olive varieties *in vitro* and after 15, 30, 90, and 180 d of acclimatization. Means ± SE (n = 30). Values not followed by *the same letter* are significantly different at p<0.05.

	Transfer period	Trichome number [mm^{-2}] Oueslati	Chemlali
Leaf upper side	*in vitro*	1.00 ± 0.01^e	3.48 ± 0.10^c
	15 d	1.20 ± 0.50^d	3.44 ± 0.50^c
	30 d	3.00 ± 0.50^c	3.56 ± 0.01^c
	90 d	3.90 ± 0.50^b	4.65 ± 0.01^b
	180 d	6.00 ± 0.15^a	6.85 ± 0.10^a
Leaf lower side	*in vitro*	7.30 ± 0.90^e	6.81 ± 0.20^d
	15 d	8.70 ± 0.50^d	7.21 ± 0.30^d
	30 d	10.10 ± 0.50^c	8.88 ± 0.80^c
	90 d	16.80 ± 0.80^b	11.60 ± 1.10^b
	180 d	28.60 ± 1.10^a	21.59 ± 1.10^a
		Stomata number [mm^{-2}]	
Leaf lower side	*in vitro*	341.22 ± 22.92^a	321.23 ± 30.00^a
	15 d	339.36 ± 20.30^a	320.33 ± 21.00^a
	30 d	334.10 ± 20.10^a	308.33 ± 08.50^b
	90 d	325.20 ± 45.00^a	296.30 ± 25.00^b
	180 d	269.40 ± 7.00^b	249.30 ± 25.00^c

In conclusion, the changes recorded in *in vitro*-grown olive leaves after the three-month period of acclimatization indicated that plantlets could be considered well acclimated. In fact, after this period, the photosynthetic apparatus operated in a normal way and autotrophic capacity was assumed to be properly acquired. Given the obtained results, we can conclude that monitoring P_N on one hand and the densities of trichomes and stomata on the other hand could help us to predict the necessary duration of the acclimatization period.

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