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Cultivating *in vitro* coconut palms (*Cocos nucifera*) under glasshouse conditions with natural light, improves *in vitro* photosynthesis nursery survival and growth

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Abstract

Plantlets of coconut were cultured *in vitro* under three different ambient conditions including a standard culture room, a culture room inside a glasshouse with natural light but controlled temperature, and a standard glasshouse with natural light and natural fluctuations of temperature. Plantlets from the 3 treatments were compared in terms of growth, plant survival as well as net photosynthesis and efficiency of PSII (Fv/Fm ratio) both at the end of the *in vitro* stage and at 3 stages of *ex vitro* acclimatization. At the end of the *in vitro* stage, plantlets cultured *in vitro* under glasshouse conditions showed the best performance showing the highest photosynthesis rate, dry weight and number of leaves. Plantlets from the standard culture room showed the lowest photosynthesis and growth rate. After 6 months of *ex vitro* acclimatization, plantlets originally grown *in vitro* under glasshouse conditions maintained better field survival and growth rates in terms of fresh weight, dry weight and leaf number than plantlets originally grown *in vitro* in the standard culture room. Although more studies are required to define the reason for this effect, it is clear that the conditions of standard culture rooms are not the best for *in vitro* cultivation of coconut and perhaps other tropical species.

Abbreviations: CR – culture room; CR/GH – culture room inside a glasshouse; Fv/Fm – optimum quantum yield; GH – glasshouse; IRGA – infra red gas analyzer; PEA – plant efficiency analyzer; Pn – net photosynthesis; PPFD – photosynthetic photon flux density; PSII – photosystem II; RH – relative humidity

Introduction

The success of micropropagation ultimately depends on the capacity of plantlets to survive and grow when transferred to *ex vitro* conditions. However, some plant species show more difficulties than others adapting to *ex vitro* conditions, and the rate of survival is affected. It has been demonstrated that the low photosynthetic rate, as a result of low light intensity in the culture rooms, is associated to this phenomenon (Lees, 1994). Studies have been performed to determine if culture of plantlets *in vitro* under increased light intensity could improve subsequent *ex vitro* growth of the plantlets (Lees 1994; Pospisilová et al., 1995; Fuentes et al., 2005). In these studies, however, light intensity was increased using artificial lighting. Very few reports exist on the effect of increasing light intensity using natural light on the performance of plantlets cultured *in vitro* (Infante et al., 1994; Kodym and Zapata-Arias, 1999)

In the case of coconut plantlets cultured *in vitro*, the rate of survival to *ex vitro* conditions has been low (about 40%) (Assy-Bah et al., 1987; Rillo 1998), although efforts to increase the survival rate have been made (Karun et al., 2002; Pech et al., 2002), an efficient protocol has not been established and plantlets still show slow growth *ex vitro*. As in banana (Kodym and Zapata-Arias, 1999) it is possible that the conditions of standard culture rooms are not suitable for tropical plantlets like coconut. For this reason we studied the *in vitro* and *ex vitro* survival, growth and physiological performance of coconut plantlets cultured *in vitro* under three different treatments; standard culture room (artificial light and controlled temperature), glasshouse (natural light and un-controlled temperature) and a culture room inside a glasshouse (natural light but controlled temperature).

Methods and materials

Plant material and in vitro *culture treatments*

Zygotic embryos of coconut (*Cocos nucifera* L.) cv. Yellow Malayan Dwarf, were removed from immature nuts (seeds, 12 months post-anthesis) from 15 year-old plants grown in the field. Forty vessels containing one embryo per vessel where placed in each of the three different treatments:

- standard culture room (CR),
- modified culture room inside a glasshouse with temperature control (CR/GH) and
- standard glasshouse (GH).

In the first treatment (CR) plantlets were grown with artificial light using white lamps (Phillips 39 W) with a 12/12 h photoperiod, light intensity (photosynthetic photon flux density; PPFD) of 0/50 μmol m^{-2} s^{-1} (night/day) and maintained at a temperature of 26 ± 2 °C, the relative humidity (RH) oscillated from 90% during the dark period to 40% during the light hours (Figure 1).

In the second treatment (CR/GH), plantlets were cultured in an adapted culture room inside a glasshouse fitted with an air conditioning unit to keep a temperature of 26 ± 5 °C with natural light intensities oscillating from 0–250 μmol m^{-2} s^{-1} (night to day) and RH from 95% at night to 40% at noon (Figure 1).

In the third treatment (GH), plantlets were cultured *in vitro* under natural glasshouse conditions (without air conditioning) with light intensities oscillating from 0 to 250 μmol m^{-2} s^{-1} (night to day), temperatures oscillating from 25–38 °C (night to day) and RH from 95% at night to 40% at noon (Figure 1). Experiments were performed at Mérida, Yucatán, México (20.7° N and 89.62° W, altitude: 10 m), during spring-summer season.

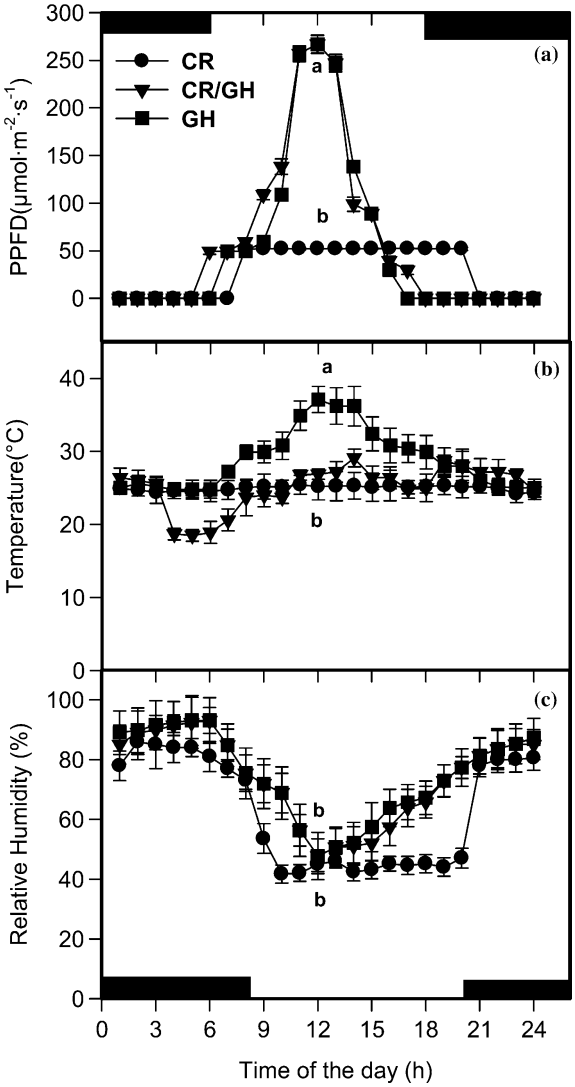

Figure 1. Diurnal variations in: Light intensity (*a*), Temperature (*b*), and RH (*c*) of plantlets grown in the culture room (CR●), adapted culture room inside a glasshouse (CR/GH▼) and glasshouse (GH■). Each point is the mean of ten plantlets. Dark bars in (*c*) correspond to the dark periods in CR while the dark bars in (*a*) correspond to the natural dark periods for GH and CR/GH treatments. Each point is the mean ±SD of 3 independent measurements in each treatment. Treatments with the same letter are not different ($\alpha = 0.05$).

In each of the 3 treatments, after surface-sterilization, embryos were germinated *in vitro* and cultured according to the protocol reported in Pech et al. (2002). First, embryos were germinated in darkness during 8 weeks. Darkening covers were then removed and germinated embryos were transferred to 500 ml vessels containing 50 ml of liquid medium Y3 with 45 g l^{-1} of sucrose (Rillo and Paloma, 1992) for further 16 weeks. Metal lids were then removed from all vessels and they were replaced with transparent polypropylene bag extensions (280 mm height and 130 mm width) to allow more space for plantlet growth for further 16 weeks.

Stages and conditions of the ex vitro *acclimatization process*

After a total of 40 weeks of being cultured *in vitro*, all plantlets from the 3 treatments were subjected the same *ex vitro* three-stage acclimatization process: 8 weeks in a fogging room, 8 weeks in a nursery under shade and 8 weeks in a nursery at full sun.

- Fogging room. Plantlets were removed from the *in vitro* culture vessels, washed with tap water, and dipped in 0.2% (w/v) of fungicide Benlate (Bayer) and transferred to pots (black plastic bags) containing a mixture of peat moss-sand-clay (1:1:1 proportions). Plantlets were then covered with a transparent bag to avoid excessive evapo-transpiration for two weeks and then the bags were removed, keeping the temperature at 26 ± 2 °C, the RH at 80% at noon and 98% at night and a PPFD from 0 at night to 120 μmol m^{-2} s^{-1} at noon (Table 1).
- Nursery (shaded). Plantlets were removed from the fogging room and transferred to outside nursery conditions in larger pots and kept under shade conditions (black mesh polypropylene allowing 30% of sun light and temperatures oscillating from 18 °C at night to 38 °C at noon, RH from 50% at noon to 98% at night and PPFD from 0 at night to 600 μmol m^{-2} s^{-1} PPFD at noon.
- Full-sun. Finally, plantlets were exposed to full-sun nursery conditions at temperatures oscillating from 20 °C at night to 40 °C at noon, RH from 40% at noon to 98 % at night and PPFD from 0 at night to 2000 μmol m^{-2} s^{-1} at noon.

Measurements

All physiological parameters were determined on 4 occasions; at the end of the *in vitro* stage and at the end of each acclimatization stage. Net photosynthetic rate (Pn) was determined using a portable LI-COR 6200 (IRGA, Lincoln, NE, USA). The efficiency of photosystem two (PSII; Fv/Fm) was determined using a PEA: (Hansatech Norfolk, UK) on the youngest fully expanded leaves. Additionally, plant fresh weight (FW), dry weight (DW) and number of leaves were recorded. Ten plantlets from each treatment were collected at the end of each stage for measurements. The experiment was repeated twice and the analysis of variance was performed using Sigma Stat program (Jandel Corporation, San Rafael, CA, USA). Means were separated using Tukey's multiple range test ($p = 0.05$).

Table 1. Duration and environmental conditions at which all plantlets from the 3 treatments (CR, CR/GH, GH) were exposed during the 3 different *ex vitro* acclimatization stages (at the fogging room, at the nursery shaded and finally at the nursery with full-sun).

	Environmental conditions during the *ex vitro* acclimatization stages		
	Fogging	Nursery shaded	Full-sun
Time *ex vitro* (weeks)	0 to 8	8 to 16	16 to 24
Light intensity (night/day) PPFD (μmol m^{-2} s^{-1})	0–120	0–600	0–2000
Room temperature (night/day) (°C)	26 ± 2	18–38	20–40
Relative humidity (day/night) (%)	80–98	50–98	40–98

Results

Photosynthetic capacity and growth of plantlets at the end of the in vitro *stage*

At the end of the *in vitro* stage (week 0 *ex vitro*), the photosynthetic rate (Pn) of *in vitro* coconut plantlets grown in the standard culture room (CR) was low (4.2 ± 0.2 μmol CO_2 m^{-2} s^{-1}) when compared to those grown in CR/GH (5.1 ± 0.5) or in GH (5.5 ± 0.4 μmol CO_2 m^{-2} s^{-1}) (Figure 2a; week 0). The Pn values found in the plantlets grown under glasshouse conditions with natural light are in fact closer to those obtained in coconut seedlings of equivalent age growing in the nursery at full sun (7.0 ± 0.6 μmol CO_2 m^{-2} s^{-1}) as reported by Fuentes et al. (2005).

No difference was found in the efficiency of PSII (Fv/Fm ratios) in the three treatments CR (0.783 ± 0.02), CR/GH (0.789 ± 0.02) or GH (0.790 ± 0.02) (Figure 2b; week 0). These Fv/Fm ratios were close to those reported for seedlings germinated naturally (0.750 ± 0.01) (Fuentes et al., 2005).

In terms of growth, plantlets grown during the *in vitro* stage under GH conditions showed slightly but significantly higher fresh and dry weight, as well as larger number of leaves than those grown in CR or CR/GH (Figure 3a, b and c; week 0).

Photosynthetic capacity, survival and growth of plantlets after being transferred ex vitro *fogging stage*

After 8 weeks of being transferred *ex vitro* into the fogging-room, Pn of CR plantlets decreased while the Pn of the other 2 treatments increased slightly relative to the values shown at the end of the *in vitro* stage (Figure 2a; week 0).

No differences were found in Fv/Fm in any treatment. In general the values of Fv/Fm were slightly lower than those found at the end of the *in vitro* stage (Figure 2b; week 8).

Plantlets grown under natural light, either in CR/GH or GH, showed higher plant survival than those grown under CR (Figure 2c; week 8).

No differences were found in plant growth in terms of fresh weight or dry weight. However, leaf number was higher in plantlets grown in GH than in plantlets from the other two treatments (Figure 3; week 8).

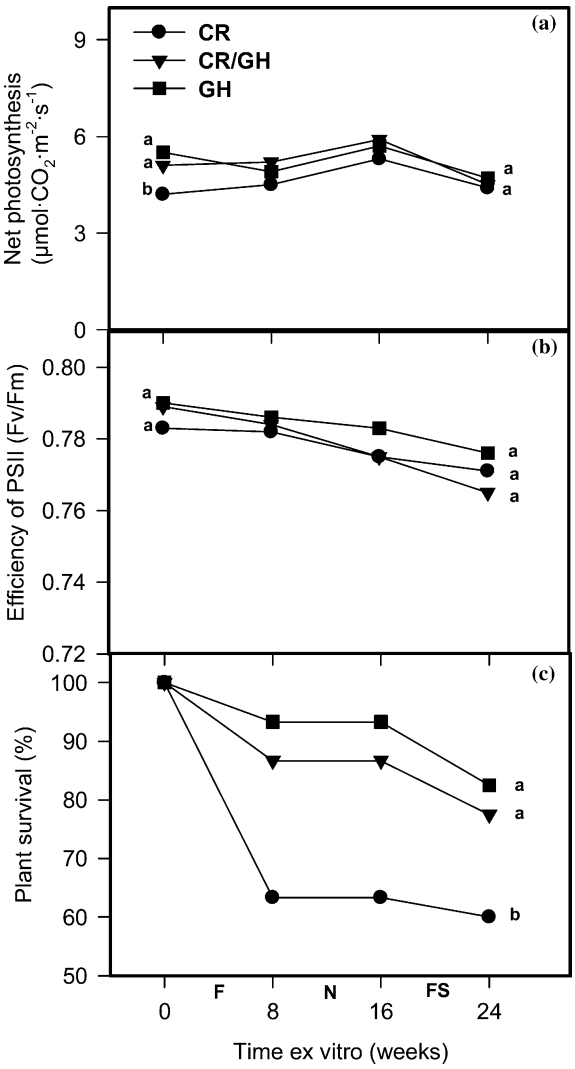

Figure 2. Changes in: Net photosynthesis (*a*), Efficiency of PSII (Fv/Fm) (*b*), Plant survival (*c*) at the end of the 40 weeks *in vitro* stage (week 0), at the end of the fogging stage (*F*, week 8), at the end of the nursery stage (*N*, week 16), and at the end of the full-sun stage (FS, week 24), of plantlets grown *in vitro* in the culture room (CR●), adapted culture room inside a glasshouse (CR/GH▼) and glasshouse (GH■). Each point is the mean of ten plantlets, treatments with the same letter are not different ($\alpha = 0.05$).

Nursery stages (shaded and full-sun stages)

At the end of the shade stage, all plantlets increased photosynthesis rate slightly to values higher than those measured in the fogging room without showing differences among treatments (Figure 2a; week 16). After 8 weeks of exposure to full-sun, all plantlets had slightly lower

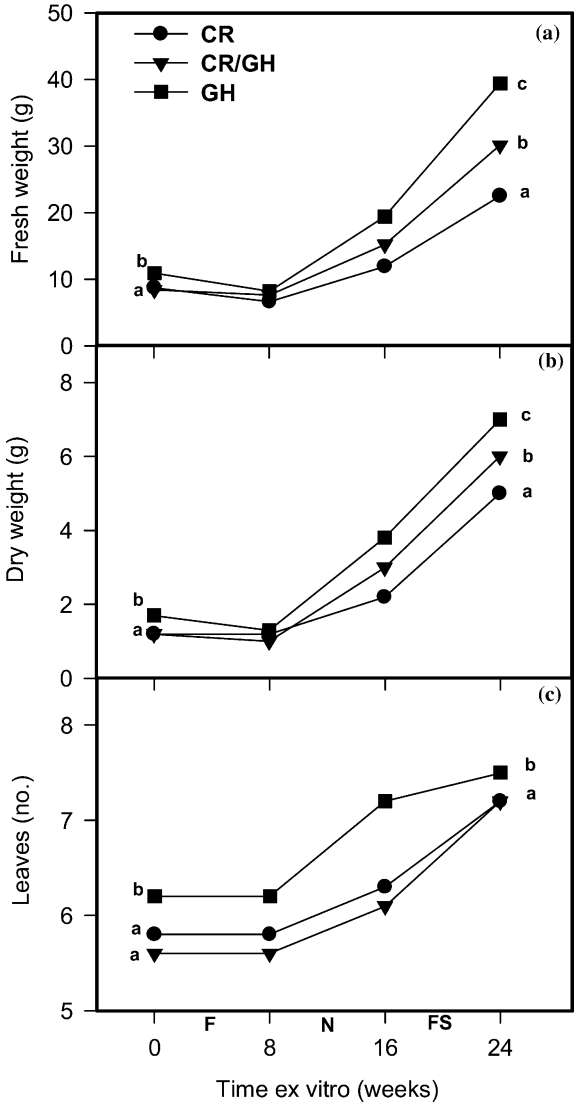

Figure 3. Changes in growth parameters: Plant fresh weight (*a*), Plant dry weight (*b*), No. of leaves (*c*), at the end of the 40 weeks *in vitro* stage (week 0), at the end of fogging stage (*F*, week 8), at the end of nursery stage (*N*, week 16), and at the end of full-sun stage (FS, week 24), of plantlets grown *in vitro* in the culture room (CR●), adapted culture room inside a glasshouse (CR/GH▼) and glasshouse (GH■). Each point is the mean of ten plantlets, treatments with the same letter are not different ($\alpha = 0.05$).

photosynthetic rates than those measured at the end of the shade stage without showing differences among treatments (Figure 2a; week 24).

Fv/Fm values were reduced at these stages relative to the values shown at the end of the *in vitro* stage or fogging stage but no differences were found among treatments (Figure 2b, weeks 16 and 24).

Plantlets survival was similar at the end of the nursery shade stage to that observed at the end of the fogging stage but decreased slightly at the end of the full-sun stage in both CR/GH or GH. At the end of the nursery shaded stage, plantlets survival in CR remained similar to that shown at the end of fogging maintaining the lowest survival of all 3 treatments (Figure 2c, weeks 16 and 24).

Furthermore, at the end of both the nursery shaded and the full-sun stages, plantlets originally grown *in vitro* in GH had higher fresh weight and dry weight than the other two treatments (Figure 3a, b, weeks 16 and 24). GH plantlets also showed more leaves than CR and CR/GH plantlets (Figure 3c; weeks 16 and 24). Plantlets grown in CR showed the lowest fresh weight or dry weight despite showing a similar number of leaves as plantlets in CR/GH.

Discussion

Culturing plantlets *in vitro* in a glasshouse with natural light resulted in improved *in vitro* photosynthesis and growth relative to those cultured in standard culture rooms. The improved *in vitro* photosynthesis in the glasshouse could have resulted from increased light intensity during the *in vitro* growth, as the 2 treatments grown in the glasshouse received 5 times higher irradiance than plantlets grown in the standard culture room. Many studies have shown that by increasing light intensity in the culture rooms it is possible to increase *in vitro* photosynthesis (Lees 1994; Fuentes et al., 2005). However, it cannot be ruled out that the different light quality between treatments could have played a role in this effect. Plantlets in both glasshouse conditions (GH, CR/GH) were exposed to the solar spectrum while those at culture rooms were grown with fluorescent lamps having high blue light but low red light content.

The improved growth *in vitro*, did not result solely from the increased photosynthesis. Despite the fact that both treatments in the GH showed similar high Pn, only the one without temperature control had higher growth. Plantlets grown inside the GH experienced 10 degrees higher temperature, than the plantlets grown in the culture room

(CR) or temperature-controlled culture room inside a glasshouse (CR/GH). This suggests that both the higher light intensity and higher noon temperature experienced by these tropical plantlets during the *in vitro* stage could have contributed to this effect.

An important additional effect of culturing plantlets under natural light in the glasshouse rather than in the culture rooms was the improvement in plant survival after plantlets were transferred to *ex vitro* conditions. For plantlets cultured in the standard culture room one critical stage was the transfer from *in vitro* to fogging conditions where some plantlets died. Once they were transferred to the shade in the nursery, the survival did not change but still remained much lower than if exposed to the other 2 treatments. Better survival on plantlets cultured with natural light occurred whether they were grown in temperature-controlled rooms or not, indicating that the higher light intensity rather than higher temperature, was the factor associated with improved field survival.

The improved *in vitro* growth of plantlets cultured under glasshouse conditions continued during the *ex vitro* stages in terms of biomass production and particularly in leaf number. The superior leaf number obtained after the GH treatment seems to be associated more to increased temperature than to higher light intensity.

In conclusion, similar to the reported effects on the other tropical species such as banana (Kodym and Zapata-Arias, 1999), the exposure of the coconut plantlets during *in vitro* culture to glasshouse conditions (natural light and temperature peaking higher than 35 °C) enhanced their survival and performance when transferred to *ex vitro* conditions.

Although more research is needed to fully understand the negative effect of standard culture rooms on the survival and growth of coconut, the results of this paper on the positive effects of cultivating coconut plantlets *in vitro* under glasshouse conditions with natural light, open opportunities to improve the *in vitro* culture efficiency of this and perhaps other tropical species.

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