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GROWTH OF *IN VITRO* BANANA (*MUSA* SPP.) SHOOTS UNDER PHOTOMIXOTROPHIC AND PHOTOAUTOTROPHIC CONDITIONS

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Summary

In vitro banana (*Musa* spp.) shoots were cultured under photomixotrophic (30 g l^{-1} sucrose and 0.2 h^{-1} number of air exchanges of culture vessels) and photoautotrophic (0 g l^{-1} sucrose and 3.9 h^{-1} number of air exchanges) conditions for 28 d in 370 cm^3 Magenta boxes (GA7-type) containing 70 ml of half-strength Murashige and Skoog (MS) medium with 22.2 μM N^6-benzyladenine (BA). The effects of varying CO_2 concentration (475 or 1340 μmol mol^{-1}) and light intensity (photosynthetic photon flux (PPF) of 100 or 200 μmol m^{-2} s^{-1}) were investigated. Fresh and dry weights of banana shoots grown photomixotrophically were significantly greater on day 28 than those grown photoautotrophically. Photoautotrophic shoots had a larger number of unfolded leaves and greater leaf area than photomixotrophic plants by days 14 and 28, regardless of CO_2 concentration. The shoot fresh and dry weights on day 14 in photoautotrophic conditions were significantly greater at PPF of 200 μmol m^{-2} s^{-1} than at 100 μmol m^{-2} s^{-1}. The increase in net photosynthetic rate of photoautotrophic banana shoots was significant compared with photomixotrophic shoots. The multiplication ratio of *in vitro* banana shoots grown photoautotrophically in a 28-d culture period was the greatest at 100 μmol m^{-2} s^{-1} PPF and 475 μmol mol^{-1} CO_2.

Key words: adventitious shoot; assimilation; microenvironment; photoautotrophic growth; sink demand.

Introduction

Banana and plantains (*Musa* spp.) are the most important tropical fruit crop in the world, with a production of approximately 70 million tons per annum. However, the production is threatened by many diseases, such as Black Sigatoka, *Fusarium* wilt, Banana Bunchy Top Virus, burrowing nematodes and banana weevil borer. Plant breeding by conventional propagation is difficult because of high sterility and polyploidy of the edible banana varieties. Thus, mass propagation of selected genotypes via somatic embryogenesis and micropropagation has been undertaken (Novak et al., 1989; Escalant et al., 1994; Grapin et al., 1996; Smith et al., 1997).

Banana plants regenerated via tissue culture have CO_2 assimilation rates higher than plants from conventional propagation (Eckstein and Robinson, 1995). Banana plants derived from micropropagation can produce suckers earlier and in larger amounts as compared with those from conventional propagation (Israeli et al., 1995). Navarro et al. (1994a) demonstrated that *in vitro* conditions, such as light intensity and vessel ventilation rate, significantly affect the growth of micropropagated banana plants *in vitro* and *ex vitro*, and that a better *in vitro* environment facilitates plant adaptation when *in vitro* banana plants are transferred to the *ex vitro* stage. The increase in CO_2 concentration significantly enhanced the dry weight and leaf area of *in vitro* photomixotrophic banana plants under high light intensity compared with low light intensity (Navarro et al., 1994b). However, young *in vitro* banana plants produced in conventional micropropagation are tender and sensitive to environmental stress after planting out in the field.

Plants are capable of developing on a sugar-free medium with proper control of photosynthetic photon flux (PPF), CO_2 concentration and relative humidity in the vessel, i.e., photoautotrophically. In many cases, this results in increased plant growth in the culture vessels, reduced plant losses due to bio-contamination, simplified and shortened acclimatization, and a higher rate of survival *ex vitro*, compared with those grown on the sugar-containing medium in conventional micropropagation (Kozai, 1991a, b). Nguyen et al. (1999) showed that, at the end of the rooting stage, there were no significant differences in dry weight gain or shoot/root dry weight ratio of *in vitro* banana plants cultured photoautotrophically compared with those cultured photomixotrophically. However, there is no information on banana shoots grown photoautotrophically during the multiplication stage. The objective of the present study was to investigate the effect of the photoautotrophic culture method on new shoot production and growth of *in vitro* banana plants in the multiplication stage as compared with the photomixotrophic culture method.

Materials and Methods

Experimental procedure. Single shoots of *in vitro*-cultured banana (*Musa* spp.) having two unfolded leaves and an average fresh weight of 180 mg and length of 10 mm were used as explants and cultured in 370 cm^3 Magenta boxes (GA7-type). Each vessel contained 70 ml of half-strength MS medium

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TABLE 1

DESCRIPTION OF TREATMENTS FOR THE EXPERIMENT ON *IN VITRO* GROWTH OF BANANA UNDER PHOTOMIXOTOPHIC AND PHOTOAUTOTROPHIC CONDITIONS

Treatment codes	Sucrose concentration ($g\,l^{-1}$)	CO_2 concentration[z] ($\mu mol\,mol^{-1}$)	PPF[w] ($\mu mol\,m^{-2}\,s^{-1}$)	No. air exchanges[y] (h^{-1})
Control	30	475 ± 40	100	0.2
LL[x]	0	475 ± 40	100	3.9
LH	0	475 ± 40	200	3.9
HL	0	1340 ± 100	100	3.9
HH	0	1340 ± 100	200	3.9

[z] CO_2 concentration of the culture room where the treatment was placed.
[w] PPF applied from day 7 to day 28.
[y] Number of air exchanges of culture vessels: $0.2\,h^{-1}$ for vessels without hole used in the photomixotrophic condition; $3.9\,h^{-1}$ for vessels with two holes in the photoautotrophic condition.
[x] For treatment codes, L and H on the left represent low or high CO_2 concentration, respectively; L and H on the right represent low or high PPF, respectively.

(Murashige and Skoog, 1962) with 22.2 μM N^6-benzyladenine (BA) and 7.5 $g\,l^{-1}$ agar (Bacto-Agar, Difco Laboratories, Detroit, MI) for both photomixotrophic (30 $g\,l^{-1}$ of sucrose and MS vitamins) and photoautotrophic (no sucrose and no vitamins) treatments. The clear caps of the culture vessels were either airtight (in the photomixotrophic treatment) or aerated by means of a pair of holes (10 mm in diameter) covered by two gas-permeable membrane filters (Milli-Seal, Millipore, Tokyo, Japan) with a pore size of 0.5 μm (in photoautotrophic treatments). The number of air exchanges was estimated to be $0.2\,h^{-1}$ for the airtight vessel and $3.9\,h^{-1}$ for the aerated vessel according to the method demonstrated by Kozai et al. (1986). The pH of the medium was adjusted to 5.6 before autoclaving. The culture room was set at 24 ± 2°C air temperature with 70 ± 5% relative humidity and 16 h d^{-1} photoperiod.

The CO_2 concentration of the culture room, controlled by a computerized system including an infrared CO_2 analyzer, was either 475 ± 40 $\mu mol\,mol^{-1}$ (low) or 1340 ± 100 $\mu mol\,mol^{-1}$ (high) (Table 1). The PPF for all treatments was 50 $\mu mol\,m^{-2}\,s^{-1}$ from day 0 to day 3, and increased to 100 $\mu mol\,m^{-2}\,s^{-1}$ on day 4. From days 7 to 28, a PPF of either 100 $\mu mol\,m^{-2}\,s^{-1}$ (low) or 200 $\mu mol\,m^{-2}\,s^{-1}$ (high) was applied (Table 1). The light source was cool white fluorescent lamps (National Co., Tokyo, Japan). PPF was measured with a PPF sensor (LI-COR Inc., Lincoln, NE, USA). The control (photomixotrophic) treatment was put under a low CO_2 concentration and low PPF condition (Table 1).

Estimation of net photosynthetic rate in vitro. Net photosynthetic rate on shoot basis, P_n *in situ*, was estimated according to the method developed by Fujiwara et al. (1987) using the following equation:

$$P_n = K\,E\,V\,(C_{out} - C_{in})$$

where K is the conversion factor of CO_2 from volume to molecular weight (40.9 $mol\,m^{-3}$ at 25°C); E, the number of air exchanges per hour (h^{-1}) of the culture vessel; V, the air volume (ml) of the culture vessel; C_{in} and C_{out}, CO_2 concentrations ($mol\,mol^{-1}$) inside and outside the culture vessel under steady-state conditions.

CO_2 concentrations inside and outside the culture vessels were measured 4 h after starting the photoperiod by analyzing gas samples on days 7, 14, 21, and 28 with a gas chromatograph (GC-12A, Shimadzu Co., Ltd., Tokyo, Japan).

Statistical analysis. Increase in fresh and dry weights, number of unfolded leaves, leaf area and multiplication ratio (number of vigorous and normal shoots, with shoot length over 10 mm, available for the next subculture), were measured either on day 14 or 28. Data from three replications, each containing five vessels (four plantlets per vessel), were used for statistical analysis. The net photosynthetic rates on a shoot basis on days 7, 14, 21, and 28 were also estimated. Analysis of variance (ANOVA), performed with the program SIGMASTAT, of a 2^2 factorial design was used to test the effects of four treatments in photoautotrophic conditions: LL, LH, HL, and HH treatments (for treatment codes, L and H on the left represent the low or high CO_2 concentration of the culture room; L and H on the right represent low or high PPF). Student's t-test at $P \leq 0.01$ and 0.05 was used to test for differences between the control (photomixotrophic) treatment and each of the photoautotrophic treatments.

Results

On day 14, there was no significant increase in fresh weight and percent dry matter of photoautotrophic cultures grown under the high CO_2 concentration (HL and HH treatments) as compared with those in the photomixotrophic condition (the control treatment) (Table 2). Increases in fresh and dry weights and percent dry matter in the LL treatment were significantly less than the control. The number of unfolded leaves of all treatments under photoautotrophic conditions on day 14 was approximately twice that of the control except the LL treatment (Table 2). Leaf areas of photoautotrophic shoots were also greater than that of the control. The HH treatment had a significantly larger leaf area than other treatments. The positive effect of high CO_2 concentration and high PPF on the photoautotrophic growth of banana shoots on day 14 was evident. Increases in fresh and dry weights and leaf area of photoautotrophic shoots were significantly greater at a CO_2 concentration of 1340 $\mu mol\,mol^{-1}$ than at 475 $\mu mol\,mol^{-1}$ (Table 2). Shoots cultured under photoautotrophic conditions also showed an increase in fresh and dry weights and leaf area when PPF was elevated to 200 $\mu mol\,m^{-2}\,s^{-1}$, regardless of CO_2 concentration (Table 2).

On day 28, the gains in fresh and dry weights of shoots in the control were significantly higher than all other treatments, yet the number of unfolded leaves and leaf area were the smallest (Table 3). The percent dry matter of shoots in the LL and LH treatments was approximately two-thirds of that in the control; however, there was no significant difference between the control and the HL and HH treatments (Table 3). The increased dry weight, percent dry matter and leaf area of banana shoots growing under photoautotrophic conditions on day 28 were significantly greater at a CO_2 concentration of 1340 $\mu mol\,mol^{-1}$ than at 475 $\mu mol\,mol^{-1}$ (Table 3). Although the increase in shoot fresh weight in the HH treatment was the least of all treatments, there was no significant difference in mean leaf area between this treatment and the LL and HL treatments (Table 3).

Response of adventitious shoot formation to culture conditions differed among the five treatments. In the control, 42% of explants had adventitious shoots after 10 d of culture, whereas this occurred

TABLE 2

EFFECTS OF CULTURE CONDITIONS ON INCREASED FRESH WEIGHT AND DRY WEIGHT, % DRY MATTER, NUMBER OF UNFOLDED LEAVES, AND LEAF AREA OF *IN VITRO* BANANA SHOOTS ON DAY 14

Treatment[y]	Increased fresh weight (mg)	Increased dry weight (mg)	% Dry matter	Number of unfolded leaves	Leaf area (cm^2)
Control	193 ± 12	19 ± 2	7.2 ± 0.5	2.0 ± 0.2	1.9 ± 0.4
LL	$98 \pm 11^{**w}$	$10 \pm 1^{**}$	$5.5 \pm 0.6^{*}$	$3.7 \pm 0.3^{**}$	2.8 ± 0.4^{NS}
LH	$132 \pm 6^{**}$	$12 \pm 2^{**}$	6.0 ± 0.8^{NS}	$4.2 \pm 0.6^{**}$	$3.9 \pm 0.4^{**}$
HL	157 ± 23^{NS}	$14 \pm 3^{*}$	6.3 ± 1.0^{NS}	$3.7 \pm 0.5^{**}$	$3.5 \pm 0.6^{*}$
HH	170 ± 12^{NS}	$17 \pm 1^{*}$	6.5 ± 0.6^{NS}	$3.8 \pm 0.4^{**}$	$5.1 \pm 0.7^{**}$
ANOVA[z]					
CO_2 conc. (A)	**	**	NS	NS	**
PPF (B)	**	**	NS	NS	**
A×B	NS	NS	NS	NS	NS

[y] For treatment codes, L and H on the left represent low or high CO_2 concentration, respectively; L and H on the right represent low or high PPF, respectively.
[w] NS, *, **: nonsignificantly or significantly different from the control treatment at $P \leq 0.05$ or 0.01, respectively, according to Student's *t*-test.
[z] ANOVA was applied for four treatments under photoautotrophic condition. NS, **: nonsignificant or significant at $P \leq 0.01$, respectively.

TABLE 3

EFFECTS OF CULTURE CONDITIONS ON INCREASED FRESH WEIGHT AND DRY WEIGHT, % DRY MATTER, NUMBER OF UNFOLDED LEAVES, AND LEAF AREA OF *IN VITRO* BANANA SHOOTS ON DAY 28

Treatment[y]	Increased fresh weight (mg)	Increase dry weight (mg)	% Dry matter	Number of leaves	Leaf area (cm^2)
Control	369 ± 17	42 ± 3	11.2 ± 0.7	3.0 ± 0.5	3.5 ± 0.6
LL	$282 \pm 9^{**w}$	$22 \pm 3^{**}$	$7.9 \pm 0.9^{**}$	$5.5 \pm 0.3^{**}$	$5.7 \pm 0.4^{**}$
LH	$261 \pm 11^{**}$	$21 \pm 2^{**}$	$7.9 \pm 0.9^{**}$	$5.0 \pm 0.6^{**}$	4.3 ± 0.1^{NS}
HL	$285 \pm 26^{**}$	$29 \pm 2^{**}$	10.3 ± 0.6^{NS}	$4.9 \pm 0.5^{**}$	$5.6 \pm 0.2^{**}$
HH	$226 \pm 7^{**}$	$27 \pm 1^{**}$	11.7 ± 0.4^{NS}	$5.1 \pm 0.7^{**}$	$5.8 \pm 0.2^{**}$
ANOVA[z]					
CO_2 conc. (A)	NS	**	**	NS	**
PPF (B)	**	NS	NS	NS	**
A×B	*	NS	NS	NS	**

[y] For treatment codes, L and H on the left represent low or high CO_2 concentration, respectively; L and H on the right represent low or high PPF, respectively.
[w] NS, **: nonsignificantly or significantly different from the control treatment at $P \leq 0.01$, respectively, according to Student's *t*-test.
[z] ANOVA was applied for four treatments under photoautotrophic condition. NS, *, **: nonsignificant or significant at $P \leq 0.05$ or 0.01, respectively.

in only 20% of explants in the LL treatment, and 8% in the LH, HL, and HH treatments (data not shown). High CO_2 concentration did not promote adventitious shoot formation; therefore, the LL treatment had a higher multiplication ratio than the HL and HH treatments at the end of the culture period. The mean multiplication ratios of LH and HH treatments at PPF of 200 $\mu mol\,m^{-2}\,s^{-1}$ were lower than LL and HL treatments at PPF of 100 $\mu mol\,m^{-2}\,s^{-1}$, respectively (Fig. 1).

The average CO_2 concentrations inside the culture vessels of all photoautotrophic treatments were lower than the average CO_2 concentrations outside the culture vessels (Fig. 2*a*). Net photosynthetic rate on a shoot basis, P_n, was significantly greater at the CO_2 concentration of 1340 $\mu mol\,mol^{-1}$ than at 475 $\mu mol\,mol^{-1}$ (Fig. 2*b*). P_n of banana shoots increased with time from day 7 to day 14 in photoautotrophic conditions. P_n continued increasing from day 14 to day 21 at 1340 $\mu mol\,mol^{-1}$ CO_2, but decreased on day 28. The average CO_2 concentration inside the culture vessel of the control was higher than the CO_2 concentration outside the culture vessel and those of other treatments on day 7, then decreased gradually during the culture period and became lower than the CO_2 concentration outside the culture vessel and

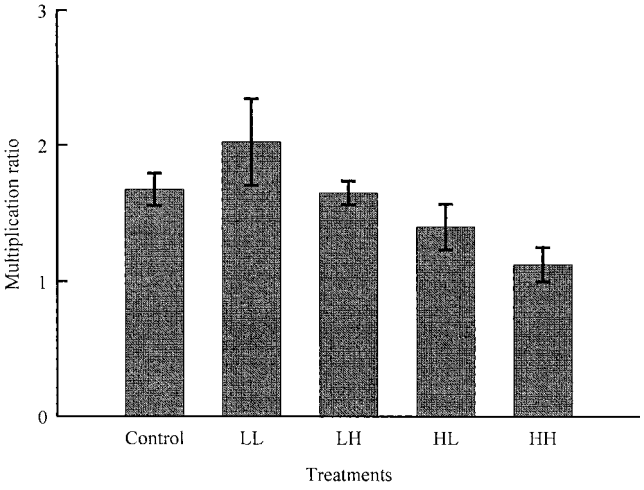

FIG. 1. Multiplication ratio of *in vitro* banana shoots on day 28 as affected by sucrose concentration, CO_2 concentration, and PPF. For treatment codes, L and H on the left represent low or high CO_2 concentration, respectively; L and H on the right represent low or high PPF, respectively.

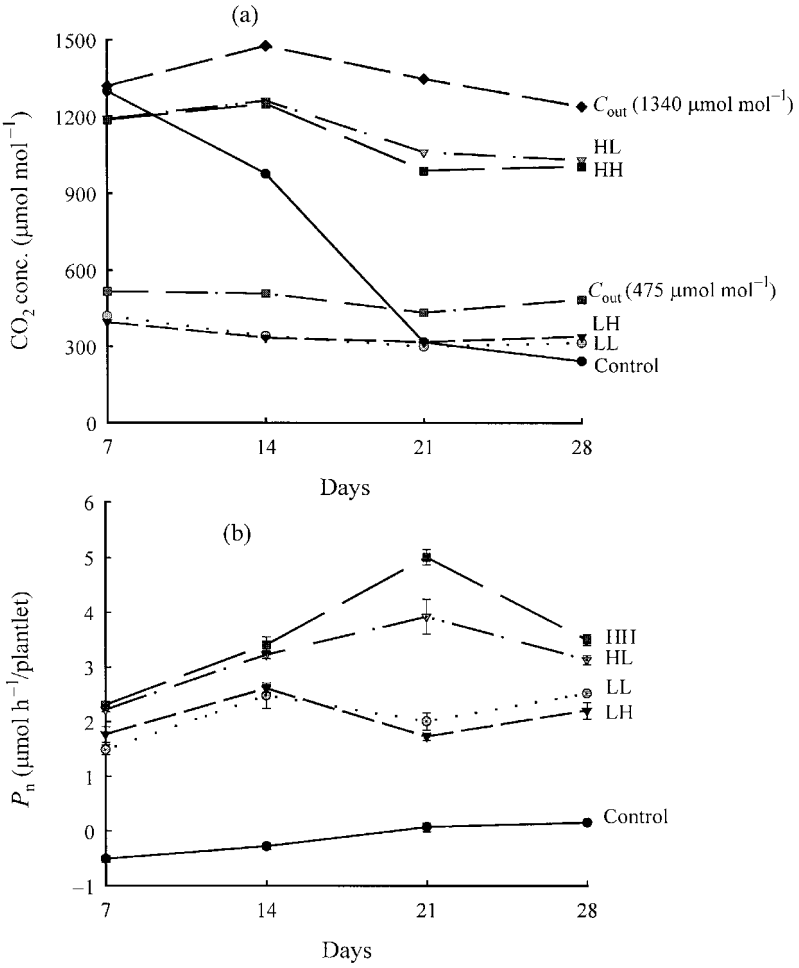

FIG. 2. Time courses of average CO_2 concentration inside the culture vessel (*a*) and net photosynthetic rate (P_n) (*b*) of *in vitro* banana shoots in response to sucrose concentration, CO_2 concentration and PPF levels. For treatment codes, L and H on the left represent low or high CO_2 concentration, respectively; L and H on the right represent low or high PPF, respectively.

those of photoautotrophic treatments on day 28 (Fig. 2*a*). This resulted in a negative P_n for banana shoots grown photomixotrophically on days 7 and 14 (Fig. 2*b*). Net photosynthetic rates on a shoot basis, P_n, of treatments under photoautotrophic conditions were much greater than that of the control during the culture period (Fig. 2*b*). Although P_n of banana shoots in the control increased gradually with time, rising to 0.2 μmol h^{-1} per shoot on day 28, this P_n was approximately one-twelfth that in the LL treatment. Net photosynthetic rate on a leaf area basis in the control was 0.11 ± 0.005 μmol m^{-2} s^{-1} on day 28, whereas it was 2.00 ± 0.12 in the LL treatment, 1.75 ± 0.13 in the LH treatment, 2.50 ± 0.17 in the HL treatment, and 2.79 ± 0.08 μmol m^{-2} s^{-1} in the HH treatment.

DISCUSSION

Our results, showing P_n of *in vitro* banana shoots in the control as low as −0.5 to 0.2 μmol h^{-1} during the culture period, supported the argument of Hdider and Desjardins (1994) that sucrose used as the main carbon source in the culture medium may limit the efficiency of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in the CO_2-fixation cycle, and thus, decrease the net photosynthetic rate of *in vitro* plantlets. Although increases in fresh and dry weights of banana shoots under the photomixotrophic condition were greater than those under photoautotrophic conditions, low P_n with small numbers of unfolded leaves and lower leaf areas of photomixotrophic cultures could predict slow growth and low productivity of *in vitro* banana shoots in successive cultures. Navarro et al. (1994a) also stated that *in vitro* banana plants, cultured conventionally on sugar-containing medium and in airtight vessels, had reduced leaf area and chlorophyll content and accumulated C_2H_4 in the culture vessel during the growth phase.

The growth of *in vitro* banana shoots under photoautotrophic conditions in the multiplication stage was affected by CO_2 concentration and PPF in the present study. The net photosynthetic rate on a shoot basis, P_n, of banana shoots was greatly enhanced under photoautotrophic conditions, especially with high CO_2 concentration, soon after the experiment was started (Fig. 2*b*). Under photoautotrophic conditions, higher P_n on days 7 and 14, as well as higher fresh weight, dry weight and leaf area on day 14 of shoots developed under high CO_2 concentration showed that the increase of CO_2 concentration in the culture vessel promoted

photosynthesis and production of organic compounds necessary for shoot proliferation. The higher P_n and the increase in dry weight, percent dry matter and leaf area under a high CO_2/high PPF regime in photoautotrophic conditions on day 28 is consistent with the suggestion that the enhancement of photosynthesis by high CO_2 concentration, high PPF or both, is responsible for improved performance of *in vitro* plants (Lakso et al., 1986; Kozai, 1991a). Infante et al. (1989) also demonstrated the increase in net photosynthetic rate of *Actinidia deliciosa* proliferating cultures with increasing CO_2 concentration from 330 to 1450 $\mu mol\ mol^{-1}$. *In vitro* rose plants cultured photoautotrophically had greater fresh and dry weights when CO_2 concentration in the culture room was elevated to 3400 $\mu mol\ mol^{-1}$ (Hayashi et al., 1993). The lower increase in shoot fresh and dry weights of the HH treatment from day 14 to day 28 as compared with those of the HL treatment (Table 3) together with the decrease in P_n of HH treatment from day 21 to day 28 (Fig. 2*b*), implied that the supply of CO_2 higher than 1500 $\mu mol\ mol^{-1}$ on day 21 might result in a greater growth of banana shoots on successive days of the culture period.

The decrease in P_n from day 14 to day 21 of LL and LH treatments (Fig. 2*b*) suggested that, in the culture room with ambient CO_2 concentration, adventitious shoot formation may start early at high CO_2 concentration condition, and that, under photoautotrophic conditions, a strong demand by shoot formation could create competition for energy source between existing leaves and new buds for remobilizable nitrogen, leading to a drop in the photosynthetic capacity (Salisbury and Ross, 1992). The presence of plant growth regulators, such as BA, in the culture medium could help to direct translocation of assimilates from leaves to new, reproductive tissues. However, under photoautotrophic conditions, *in vitro* plant leaves may become a new sink, resulting in the delay of adventitious shoot growth. The increase in P_n from day 21 to day 28 of these treatments demonstrated the reactivation of photosynthetic ability by shoot growth. In the environment with ambient CO_2, the availability of CO_2 inside the culture vessel became a limiting factor for photosynthesis after new shoots appeared; therefore, high PPF could not raise P_n of shoots in the LH treatment higher than that of the LL treatment from day 21 to day 28 as it did from day 7 to day 14 (Fig. 2*b*). The same tendency of decrease in P_n of the HL and HH treatments happened from day 21 to day 28, which could be explained by the competition for energy source between leaves and new shoot growth, resulting in low multiplication ratios for these treatments on day 28. However, these ratios were expected to be higher in the successive subcultures based on the greater increased dry weight, percent dry matter and leaf area of banana shoots. The shoots growing in the photomixotrophic condition did not show any depletion in P_n, though this P_n was very low, as the energy source for new tissue formation may come from the breakdown of sucrose existing in the culture medium, instead of from photosynthesis.

In the experiment, air temperature was kept at 24°C on average. Banana is a tropical fruit species, for which the optimum temperature for leaf emergence is about 31°C, and the overall optimal mean temperature for growth (assimilation) and development (leaf emergence) is about 27°C (Robinson, 1996). Therefore, an air temperature of 24°C might have decreased the leaf emergence rate of *in vitro* plants, reducing photosynthetic efficiency. Water evaporation might also have been slow at relatively lower air temperatures, resulting in the inhibition of assimilate manufacture and the promotion of assimilate import in the growth stage (Salisbury and Ross, 1992). This explains why increases in fresh and dry weights of *in vitro* plants in the photomixotrophic treatment on day 28 were greater than those of photoautotrophic treatments.

The low P_n during the last stage of the culture period may be related to the concentration of PO_4^- ion in the culture medium. Kozai et al. (1995) demonstrated that, during the first 2 wk, almost all PO_4^- ion was absorbed by *in vitro* potato plants cultured photoautotrophically, regardless of whether full- or half-strength MS medium was used. Phosphate is known as an essential ion for production of ATP, which is required for CO_2 assimilation in photosynthesis. Lack of phosphorus inhibits CO_2 assimilation by decreasing intermediary metabolism and the formation of assimilates (Lawlor, 1987). Therefore, under photoautotrophic conditions, the decrease in P_n of banana plants in all treatments might result from the relatively low PO_4^- ion in the culture medium, as only 52.5 μmol of phosphate initially existed in 70 ml medium per culture vessel. The effect of deficient PO_4^- ion supply on the photosynthesis and growth of banana plants may be expressed earlier in the treatments under ambient CO_2 during the last 2 wk of the culture period. Yang et al. (1995) also showed that P_n of strawberry plants, cultured *in vitro*, increased when PO_4^- ion of the culture medium was initially supplied two or three times higher than its concentration in MS medium.

Conclusions

The present study shows that *in vitro* bananas could produce new shoots when cultured photoautotrophically. Regarding the photoautotrophic cultures, the multiplication ratio was greater for those grown under ambient CO_2 at PPF of 100 $\mu mol\ m^{-2} s^{-1}$. Photosynthetic activity of banana shoots was improved under photoautotrophic conditions, resulting in a significant increase in net photosynthetic rate of *in vitro* plantlets when the CO_2 concentration inside the culture vessel was increased. The results demonstrated here can be applied to mass micropropagation of banana.

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