BIOLOGIA PLANTARUM 48 (4): 621-624, 2004

BRIEF COMMUNICATION

In vitro growth and shoot multiplication of *Achras zapota* in a controlled carbon dioxide environment

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Abstract

The culture vessels with multiplying shoots of *Achras zapota* L. on Schenk and Hildebrandt (SH) medium containing 8.88 μM 6-benzylaminopurine (BAP) with or without sucrose were kept under varied CO_2 concentrations ranging from 0.6 to 40.0 g m^{-3} using different concentrations of sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), potassium bicarbonate ($KHCO_3$), and potassium carbonate (K_2CO_3) in small acrylic chambers. Complete absence of carbon source caused death of shoots within 20 d. Under elevated concentrations of CO_2 (10.0 and 40.0 g m^{-3}) the shoots grew photoautotrophically on sucrose-free medium. The growth of cultures was better at 40.0 g (CO_2) m^{-3} than on 3.0 % sucrose under ambient air of growth room. However, the best response was obtained at 10.0 g (CO_2) m^{-3} and 3.0 % sucrose where maximum number of shoots, shoot length, fresh and dry mass, total number of leaves and leaf area was observed.

Additional key words: hardening and acclimatization, micropropagation, photoautotrophy, sapota, shoot culture.

Sucrose is the main source of carbon and energy in the nutrient medium during micropropagation of plants (Thompson and Thorpe 1987) but its presence increases the risk of contamination and depresses photosynthetic activity leading to mixo- or heterotrophy (Deng and Donnelly 1993). This and other factors cause morphological and physiological disorders in *in vitro* grown plants resulting into high rate of mortality during hardening, acclimatization and field transfer (Kozai 1991, Vasil 1991). The goal of micropropagation is to mass propagate genetically identical, physiologically uniform and developmentally normal plants that can be acclimatized quickly (Jeong *et al.* 1993). One of the ways to achieve this is photoautotrophic cultivation of plants under CO_2 enriched conditions. Photoautotrophic cultivation not only reduces the loss due to contamination but also leads to development of plants which after transplantation to *ex vitro* conditions are able to acclimate quickly to decreased air humidity. The leaves of photoautotrophically grown cultures have lower stomatal index (Sha Valli Khan *et al*. 2003) and such plants would show higher survival rates and better growth (Langford and Wainwright 1987). Improvement in survival during hardening and acclimatization of tissue culture plantlets has been achieved in number of herbaceous species through *in vitro* growth of shoots/plantlets under CO_2 enriched environment (Mousseau 1986, Fujiwara *et al*. 1988, Kozai *et al*. 1992, Solárová and Pospíšilová 1997). In case of woody plants there are only few reports on such studies (Aitken-Christie *et al*. 1990, Kirdmanee *et al*. 1995, Vyas and Purohit 2003).

Achras zapota L. is an evergreen tree cultivated mainly for its fruits. Micropropagation of *A. zapota* on sugar enriched SH (Schenk and Hildebrandt 1972) medium has been carried out by Purohit and Singhvi (1998) but there is no report about the growth of shoots under controlled and CO_2 enriched environment. The present investigation aims to examine the effect of

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Received 23 February 2004, *accepted* 5 May 2004.

Abbreviations: BAP - 6-benzylaminopurine; SH - Schenk and Hildebrandt; SM - standard multiplication medium; SCSM - sucrose containing multiplication medium; SFSM - sucrose-free multiplication medium.

Acknowledgement: The financial support provided by Council for Scientific and Industrial Research, New Delhi, India to one of the authors (N. Dave) is gratefully acknowledged.

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controlled and CO_2 enriched environment on *in vitro* growth and shoot multiplication in *A. zapota*.

Fruits of sapota [*Achras zapota* L., syn. *Manilkara zapota* (L.) van Royen] cv. Cricket ball were collected from Horticulture Farm of Rajasthan College of Agriculture, Udaipur. Seeds were de-coated and surface sterilized with 0.1 % mercuric chloride for 5 min and washed thoroughly with autoclaved distilled water for 4 - 5 times and inoculated aseptically on water agar for germination. Cotyledonary nodes obtained from 45-d-old seedlings were inoculated on standard multiplication (SM) medium described by Purohit and Singhvi (1998) containing Schenk and Hildebrandt (1972) salts supplemented with 8.88 μM 6-benzylaminopurine (BAP), 3.0 % sucrose and 0.8 % agar. The pH of the medium was adjusted to 5.8 and autoclaved at 1.06 kg m^{-2}, 121 °C for 15 min. Repeated sub-culturing of multiplying shoots was carried out every 3 weeks on fresh SM medium for maintenance of cultures.

Erlenmeyer flasks (100 cm^{-3}) (*Borosil*, Mumbai, India) containing multiplying shoot cultures on SM medium were placed in near-transparent, air-tight acrylic boxes (7500 cm^3), for control of CO_2 concentration as described by Solárová *et al.* (1989). Experiments were conducted to see the effect of different concentrations of CO_2 in presence and absence of sucrose on the growth and multiplication of shoots in culture. Various concentrations of CO_2 were applied in acrylic boxes by placing solutions of sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), potassium bicarbonate ($KHCO_3$) and potassium carbonate (K_2CO_3) in different concentrations and combinations in open Petri plates providing maximum surface area for diffusion of CO_2. The solutions were changed every fifth day. Carbon dioxide-free atmosphere was maintained by keeping a 10 % KOH solution in the chambers. The CO_2 concentration of 0.6 g m^{-3} (0.03 %) in the chamber was controlled by 0.1 M solutions of $NaHCO_3$ and Na_2CO_3 mixed at a ratio of 77/23 (v/v) while higher concentrations (10.0 and 40.0 g (CO_2) m^{-3}; 0.5 and 2.0 %, respectively) were controlled by 3.0 M solutions of $KHCO_3$ and K_2CO_3 mixed in the ratios of 50/50 and 73/27 (v/v), respectively.

For each treatment a cluster of two shoots was inoculated on 40 cm^3 of shoot multiplication medium containing 3.0 % sucrose (SCSM) or without it (SFSM) in an Erlenmeyer flask with non-absorbent cotton plugs. Six culture flasks (replicates) each of SCSM and SFSM, each inoculated with one shoot cluster were carefully placed in separate chambers providing different concentrations of CO_2. Chambers were closed with lids and sealed with packing tape (*Miracle*, 5.0 cm wide). Similarly six culture flasks each of SCSM and SFSM, inoculated with one shoot cluster were placed in the growth room under ambient air environment. The acrylic chambers containing culture vessels were kept in growth room at 28 ± 2 °C under a 16-h photoperiod (irradiance of 45 μmol(photon) m^{-2} s^{-1} provided by cool-white fluorescent tubes) and 40 - 50 % humidity. Shoot clusters were subcultured after 3-weeks without harvesting of shoots and were maintained for 42 d under the same conditions. The experiments were repeated thrice. Various growth parameters like number of shoots, shoot length, fresh and dry mass of shoots produced in each treatment, and the total number of leaves per cluster and average leaf area were studied. The data were recorded after 42 d of growth and were analysed statistically by two-factorial *ANOVA* to define the relative significance of each factor and possible interaction between them.

Shoot clusters derived from cotyledonary nodes of *A. zapota* and inoculated on SCSM medium produced more than four shoots in ambient air of growth room after 42 d of culture. Fresh mass of shoots was four times higher than the dry mass (Table 1). In comparison to these ambient air controls, the shoot cultures grown in CO_2 free atmosphere showed significant decline in all the parameters studied (Table 1). At 0.6 g m^{-3} CO_2, improvement in growth and multiplication of shoot cultures was observed. Under these conditions significant increase in number of shoots, fresh and dry masses and leaf area over the ambient air controls was obtained (Table 1). However, the best response was obtained at 10.0 g m^{-3} CO_2 where maximum number of shoots (average 8.66) with highest shoot length, leaf number and leaf area was obtained. Dry mass of such shoot cultures was almost double than those grown in ambient air (Table 1). The leaves became broad, shiny and dark green in colour. Further increase in CO_2 concentration to 40.0 g m^{-3} was inhibitory for the growth of cultures as it recorded significant decline in shoot length, fresh and dry masses (Table 1). The leaves became small and scaly with reduced leaf area (34.33 mm^2) and cultures had rosette-like morphology.

In the ambient air of the growth room, cultures grown on sucrose-free medium were characterised by thin shoots, with lesser number of leaves, reduced leaf area, and low fresh and dry masses. Yellowing of leaves was also observed. Under CO_2 free air all the cultures died within 20 d. At 0.6 g m^{-3} CO_2, cultures did not show any improvement in shoot proliferation but quality of cultures in terms of shoot length, fresh and dry mass was better in comparison to those grown in ambient air of growth room. Leaf area was also significantly higher in these cultures. Increase in concentration of CO_2 to 10.0 and 40.0 g m^{-3} was found to be stimulatory for the growth of cultures on SFSM. Under elevated concentrations of CO_2 (10.0 and 40.0 g m^{-3}) the cultures were able to grow fully photoautotrophically on sugar free medium. At 40.0 g m^{-3} CO_2 the number of shoots obtained were significantly higher than the cultures grown on SCSM and under ambient air environment. Length of the shoots, fresh and dry mass and total number of leaves per cluster were also significantly higher in these cultures (Table 1).

Table 1. Effect of CO_2 enrichment on different parameters of *in vitro* growth of *A. zapota* grown on sucrose containing and sucrose free shoot multiplication media. Observations are recorded after 42 d of growth. Means followed by different letters differ significantly at 5 %. GR - ambient CO_2 in growth room; CV - coefficient of variation; CD - critical difference.

Sucrose [%]	CO_2 conc. [g m^{-3}]	Number of shoots	Shoot length [cm]	Fresh mass [mg $cluster^{-1}$]	Dry mass [mg $cluster^{-1}$]	Number of leaves	Leaf area [mm^2]
3.0	GR	4.67d	1.88d	696.67c	170.00e	34.00c	67.14c
	0.0	3.67e	1.82e	560.00e	156.67f	26.17e	53.68f
	0.6	6.33b	1.65g	863.33b	243.33c	36.33c	72.89b
	10.0	8.67a	2.80a	1268.33a	396.67a	52.00a	102.09a
	40.0	6.17b	0.70i	480.00f	140.00g	29.67d	34.33h
0.0.	GR	3.00e	1.50h	390.00h	90.00i	19.00f	49.72g
	0.0	0.00f	0.00i	0.00i	0.00j	0.00g	0.00i
	0.6	3.33e	1.72f	460.00g	110.00h	21.00f	54.17f
	10.0	4.50d	2.09c	665.00d	190.00d	31.00d	59.90e
	40.0	5.50c	2.21b	853.33b	273.33b	39.00b	65.05d
CV		10.00	2.51	1.58	5.01	7.07	1.13
SEM		0.187	0.016	4.01	3.62	0.832	0.257
CD [5 %]		0.531	0.047	11.4	10.30	2.36	0.73

Carbon dioxide enrichment during *in vitro* cultivation of shoots of *A. zapota* promoted their growth and multiplication. Shoot cultures grown on sucrose containing medium under CO_2 free condition showed significant decline in all the growth parameters. Shoots grew better when both CO_2 and sucrose were provided. Similar results were obtained in case of tobacco (Tichá 1996), apple (Morini and Melai 2003/4) and carnation (Solárová and Pospíšilová 1997). The growth of shoot cultures in terms of number of shoots, fresh and dry masses and leaf area was better at 0.6 g m^{-3} CO_2 than the ambient air controls. Similar results have been obtained in case of *Wrightia tomentosa* (Vyas and Purohit 2003). Kozai and Iwanami (1988) have explained the comparatively poor growth under ambient CO_2, to be due to decline in CO_2 concentration inside the culture vessel during most of the photoperiod. In controlled environment the continuous availability of CO_2 compensate for the depletion of its concentration and therefore, encourage better shoot growth as compared to ambient air control (Solárová *et al.* 1989, Jeong *et al*. 1993). The combination of sucrose (3.0 %) and 10.0 g m^{-3} CO_2 proved to be the best for shoot multiplication, where maximum number of shoots was obtained. All parameters like length of shoots, their fresh mass, total number of leaves per cluster and leaf area showed best growth among all the treatments tested. Dry mass of shoots was almost double than those grown on SCSM under ambient air environment. Higher concentration (40.0 g m^{-3}) of CO_2 proved to be inhibitory for the growth of shoots of *A. zapota* in culture during present study. Negative effect of CO_2 enrichment on growth of *Magnolia* and *Gerbera* in mixotrophically grown plantlets has been reported (De Proft *et al*. 1985, Woltering 1986).

Multiplying shoots of *A. zapota* grown on sucrose free medium under ambient CO_2 concentration of growth room showed significant reduction in leaf area, fresh and dry mass. This can be accounted to quick consumption of CO_2 present in the vessel and such cultures starved for CO_2 during large part of photoperiod (Fujiwara *et al*. 1987, Doi *et al*. 1989). Increasing the concentration of CO_2 to 10.0 and 40.0 g m^{-3} showed improved rate of shoot multiplication. Cultures grew fully photoautotrophically on SFSM. Similar response has been reported in case of tobacco, carnation and potato plantlets (Solárová *et al*. 1996). Solárová *et al*. (1989) explain that under CO_2 concentrations elevated to 10.0 or 40.0 g m^{-3} the CO_2 in the vessel during light period was never depleted to concentrations lower than those saturating plantlet photosynthesis and therefore could compensate for the lack of carbon supply in the medium. Dry matter accumulation in cultures of *A. zapota* at higher concentrations of CO_2 on SFSM was higher than in SCSM ambient air controls as also observed in case of tobacco (Solárová *et al*. 1989).

Results of the present study revealed that the photoautotrophic growth of shoots of *A. zapota* could be achieved on sucrose free medium under CO_2 enriched conditions. Shoot cultures grew better at 40.0 g m^{-3} of CO_2 on SFSM than on SCSM under ambient air of the growth room. However, when both sucrose and CO_2 were provided, the best response was obtained at 10.0 g m^{-3} CO_2 where optimum growth and multiplication was recorded.

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