Increased CO₂ and Light Promote in Vitro Shoot Growth and Development of Theobroma cacao

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Abstract. Axillary shoots of cacao (Theobroma cacao L.), induced in vitro with cytokinins (BA or TDZ), elongated and produced leaves only in the presence of cotyledons and/or roots. Detached axillary shoots, which do not grow in vitro under conventional tissue culture protocols, rooted with auxin and developed normally in vivo. Detached axillary shoots from cotyledonal nodes and single-node cuttings from mature plants were induced to elongate and produce normal leaves in the presence of 20,000 ppm CO₂ and a photosynthetic photon flux density (PPFD) of 150 to 200 µmol·s⁻¹·m⁻². Subculture nodal cuttings continued to elongate and produce leaves under elevated CO₂ and light levels, and some formed roots. Subculture of microcuttings under CO₂ enrichment could be the basis for a rapid system of micropropagation for cacao. Chemical names used: N-phenylmethyl)-1H-purin-6-amine (BA); 1H-indole-3-butyric acid (IBA); α-naphthaleneacetic acid (NAA); thidiazuron (TDZ).

Cacao has been recalcitrant in tissue culture. Attempts to micropropagate cacao via shoot tip culture have been disappointing. Promotive factors reported include: 1) liquid medium (Adu-Ampomah et al., 1988; Blake and Maxwell, 1984; Dufour and Dublin, 1985; Orchard et al., 1979); 2) physiological stage of the explant source, i.e., either explanted during active flush (Passey and Jones, 1983) or during vegetative rest (Bertrand, 1987; Blake and Maxwell, 1984; Orchard et al., 1979); 3) frequent medium transfer (Adu-Ampomah et al., 1988; Blake and Maxwell, 1984; Legrand and Mississo, 1986); 4) decreased salt concentration (Bertrand, 1987; Dufour and Dublin, 1985); 5) increased culture vessel volume (Dufour and Dublin, 1985); 6) use of activated charcoal (Dufour and Dublin, 1985); 7) use of glucose as a carbon source (Legrand et al., 1984); and 8) explant length of 2 to 4 cm with medial bud placement (Legrand and Mississo, 1986; Litz, 1986). Despite these protocol improvements, only sporadic growth and proliferation of explanted shoots have been achieved. In many cases, shoot growth ceased after 4 to 6 weeks of culture (Adu-Ampomah et al., 1988; Blake and Maxwell, 1984; Legrand and Mississo, 1986; Legrand et al., 1984; Passey and Jones, 1983). No positive results were reported on subculture.

Recently, Flynn et al. (1990) reported bud elongation and leaf development from mature shoots cultured in vitro without exogenous growth regulators, but no data are presented comparing treatments. They reported promotive factors to include flush stage at explant excision, minimization of explant stress through careful handling, orientation of nodal explant within culture vessel, 10 h of light with a maximum of 250 µmol·s⁻¹·m⁻², programmed to reflect diurnal flux changes, high culture vessel relative humidity, and frequent explant transfer.

Previous studies in our laboratory (Janick and Whipkey, 1985) have indicated that shoots can be induced in vitro from cotyledonal nodal tissues of cacao after epicotyl decapitation or supplementation of the basal medium with BA. Shoots elongated and developed leaves in the presence of cotyledons, but proliferated, axillary shoots, when excised from the cotyledonal node, failed to grow under standard tissue culture protocols. The objective of this study was to investigate the growth of cacao shoots in vitro, emphasizing cotyledonal axillary shoots as a model system. Emphasis on CO₂ and light was based on reports by Infante et al. (1989), Kozai (1990), and Lakso et al. (1986).

Materials and Methods

In vivo production and rooting of cotyledonal axillary shoots. Axillary cotyledonal nodal shoots were induced by removing the epicotyl from 1-month-old cacao seedlings grown in the greenhouse. Excised axillary shoots and epicotyls (8 cm long) were dipped for 10 sec in various concentrations of IBA and/or NAA in 50% ethanol as described (Tables 1 and 2). Shoots were transferred to a 1 soil : 1 perlite mixture (v/v) and misted daily with 50% CO₂.

Table 1. Rooting of main and axillary shoots of 1-month-old seedlings of cacao in vivo, 3 weeks after treatment with 4000 ppm IBA plus 4000 ppm NAA in 50% ethanol.

| Shoot type | Rooting (%) | Roots/ cutting | Root length (mm) |
|------------|-------------|----------------|------------------|
| Main       | 50          | 6.8 ± 1.2�     | 21.0 ± 1.8       |
| Axillary   | 80          | 6.7 ± 0.9      | 17.4 ± 1.6       |

*Treatment n = 30.
�±SE.

Table 2. Effects of auxin on in vivo rooting of axillary shoots of seedling cacao.

| Auxin* concn (ppm) | IBA | NAA | IBA + NAAx |
|---------------------|-----|-----|-------------|
| 0                   | 11.1* | --- | ---         |
| 40                  | 37.5 | 20.0| 33.3        |
| 400                 | 33.3 | 33.3| 37.5        |
| 2000                | 62.5 | 77.8| 77.8        |
| 4000                | 83.3 | 70.0| 62.5        |
| 8000                | 100.0| 50.0| ---         |

| Significance        | Linear | Quadratic |
|---------------------|--------|-----------|
|                     | *      | NS        |

*Data obtained after 3 weeks (treatment n = 10).
*Auxin applied in 50% ethanol dip.
*Concentration applies to each auxin.
*Forty-four percent rooting with water alone.
NS = Nonsignificant or significant at P = 0.05 or 0.01, respectively.

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for 8 sec every 8 min for 3 weeks. The soil was maintained at 30°C for 3 weeks with heating cables.

In vitro shoot proliferation from cotyledonary nodes. Mature pods obtained from greenhouse-cultivated trees grown from seed were washed with tap water and flamed with 95% ethanol in a laminar flow hood. Seeds were extracted, and the mucilaginous seed coat was removed. Embryos were germinated on either half-strength MS salts (Murashige and Skoog, 1962) supplemented with 0.3 μM thiamine-HCl, 2.4 μM pyridoxine-HCl, 0.6 mM i-nositol, 4.1 μM nicotinic acid, 26.6 μM glycine, 87.6 mM sucrose, and 8 g agar/liter; or Woody Plant Medium (WPM) (Lloyd and McCown, 1980), supplemented with 88.8 mM fructose and 2 g gellan gum/liter (GelRite). The pH of both media was adjusted to 5.7 before autoclaving.

Seeds were extracted, and the mucilaginous seed coat was removed. Embryos were germinated on either WPM as formulated previously (Lloyd and McCown, 1980), supplemented with 88.8 mM fructose and 2 g gellan gum/liter (GelRite). The pH of both media was adjusted to 5.7 before autoclaving.

Epicoatys were removed 4 to 6 weeks after seeds had germinated. In the first experiment, roots were removed and explants were transferred to fresh WPM as formulated previously and supplemented with 0 or 4.44 μM BA and 0, 0.005, 0.01, 0.05, or 0.1 μM TDZ applied before autoclaving. In the second experiment, cotyledonary nodes were cultured with or without roots and with or without cotoyledons on WPM with 0.05 μM TDZ. Shoots were counted and length measured 4 weeks after treatments were imposed in both experiments.

CO₂ chambers. High CO₂ treatments were conducted in clear acrylic chambers placed in the culture room and received compressed CO₂ and air mixed with a Matheson flowmeter and bubbled through distilled water to increase relative humidity. Final concentration of CO₂ in the chamber was maintained at 20,000 ppm. The chamber was held at a 29/25°C day/night cycle.

Diurnal CO₂ changes in vitro. Test tubes (50 ml headspace) containing 10 ml semi-solid WPM supplemented with 88.8 mM fructose (with or without leafy axillary shoots originated from cotyledonary nodes) were capped with polypropylene closures (Bellco Kaputs, Vineland, N. J.) in which silicone septa had been inserted, and wrapped with flexible plastic (Parafilm). Treatments included 16-h photoperiod of 90 μmol·s⁻¹·m⁻²·PAR from cool-white fluorescent lamps and 800 ppm CO₂ (ambient in culture room = low CO₂) or 200 μmol·s⁻¹·m⁻²·PAR from very high output (VHO) cool-white fluorescent lamps and 20,000 ppm CO₂ (high CO₂). Gas (1 ml) from inside the tubes was extracted with a syringe every 2 h for 48 h. Each of the four treatments (with or without explants, high or low CO₂, and light) consisted of five tubes sampled sequentially every 2 h. Carbon dioxide concentration was measured using a Carle GC 8700 gas chromatography with a thermal conductivity detector.

Effect of high CO₂ and high light levels. Three types of shoots were transferred to tubes with 10 ml semisolid WPM plus 88.8 mM fructose and capped with polypropylene closures (Kaputs): 1) new shoots induced and elongated in vitro under low light and low CO₂ from five-node plagiotropic cuttings from greenhouse-grown trees; 2) axillary shoots (≈5 cm) from cotyledonary nodes cultured in vitro; 3) one-node plagiotropic shoots from mature greenhouse-grown trees. The new secondary shoots were subcultured under either high light (150 μmol·s⁻¹·m⁻²) or high CO₂ levels or low light (45 μmol·s⁻¹·m⁻²) and low CO₂ levels. Axillary shoots or nodal cuttings were placed either under high or low CO₂ (as above) and high (200 μmol·s⁻¹·m⁻²) or low light (45 μmol·s⁻¹·m⁻²) levels obtained either by cheesecloth shading inside the CO₂ chamber or by using non-VHO lamps with a lower lamp : area ratio. All data were obtained 4 weeks after initiating treatment. Leaf area was obtained using a LI-COR (LI-COR, Lincoln, Neb.) leaf-area meter.

Results

In vivo rooting of axillary cotoyledonal shoots. Rooting was obtained from 50% of epicotyls and 80% of axillary shoots dipped in a solution of IBA and NAA, 4000 ppm each (Table 1). In a second study, using different auxin concentrations, optimum rooting was obtained from 8000 ppm IBA (Table 2). Rooted shoots were transferred to soil and grew into normal plants.

In vitro shoot proliferation from cotyledonary nodes. In the first experiment, proliferation of axillary nodal shoots was induced by BA or TDZ. Maximum proliferation was achieved with 0.1 μM TDZ alone (Table 3). Shoots elongated and produced leaves (data not presented) in the presence of cotoyledons at all treatments, although high cytokinin concentrations inhibited shoot elongation.

The second experiment was carried out to determine the effect of cotoyledons and roots on axillary shoot proliferation and elongation. There was little effect of treatment on budbreak. The presence of either roots or cotoyledons promoted elongation; maximum elongation occurred when both roots and cotoyledons were present (Table 4).

Diurnal CO₂ changes in vitro. In tubes containing medium without cacao shoots, CO₂ concentrations were similar to ambient levels found in our culture room, with no appreciable diurnal difference (Table 5). In tubes with cacao shoots, CO₂ was depleted during the day, but returned to ambient levels at night. Carbon dioxide concentration in test tubes in the high CO₂ chamber ranged from 15,000 to 17,000 ppm. No significant diurnal fluctuations in CO₂ levels were detected from tubes in the high CO₂ chamber.

Effect of light and CO₂ on shoot budbreak and elongation. In a preliminary experiment, secondary shoots were cultured in high CO₂ receiving PPFD of 150 μmol·s⁻¹·m⁻², or in low CO₂ receiving 45 μmol·s⁻¹·m⁻². Budbreak per explant in high CO₂/

Table 3. Effect of TDZ and BA on in vitro axillary shoot proliferation from the cotyledonary node of cacao seedlings without epicotyl.*

| BA (μM) | TDZ (μM) |
|--------|----------|
| 0      | 0.005    | 0.01   | 0.05  | 0.1   |
| No. elongated shoots/node |
| 0.0 | 1.0 ± 0.3  | 1.2 ± 0.5 | 1.8 ± 0.2 | 2.0 ± 0.9 | 6.4 ± 1.2 |
| 4.4 | 1.6 ± 0.4  | 1.6 ± 0.8 | 2.2 ± 0.7 | 3.0 ± 0.4 | 5.0 ± 1.2 |
| Shoot length (mm) |
| 0.0 | 27.8 ± 2.7  | 33.5 ± 6.4 | 46.6 ± 7.6 | 32.5 ± 7.3 | 22.0 ± 2.3 |
| 4.4 | 42.8 ± 9.4  | 57.5 ± 6.1 | 43.8 ± 6.5 | 48.5 ± 3.4 | 21.8 ± 3.8 |

*Data obtained after 4 weeks (treatment n = 5).

Table 4. Effect of roots and cotoyledons on budbreak and elongation of cotoyledonal nodal shoots of cacao.*

| Coyledons | Absent | Present |
|-----------|--------|---------|
| Budbreak/node |
| Absent 2.6 ± 0.2  | 3.6 ± 0.5 |
| Present 2.1 ± 0.2 | 2.3 ± 0.4 |
| Elongation/explant (mm) |
| Absent 5.2 ± 0.7   | 27.8 ± 8.8 |
| Present 19.2 ± 4.2 | 77.2 ± 15.3 |

*Data obtained after 4 weeks (treatment n = 10 to 25). Coyledonary nodes were cultured in WPM supplemented with 0.05 μM TDZ.

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Table 5. Diurnal CO₂ changes in test tubes containing WPM with and without cacao axillary shoots and sealed with Kaput closures and Parafilm.

| Cacao shoot | CO₂ (ppm ± se)* |
|-------------|-----------------|
|             | Day             | Night            |
|             | 800 ppm CO₂     | 20,000 ppm CO₂   |
| Absent      | 883 ± 62        | 1,042 ± 212      |
| Present     | 72 ± 35         | 949 ± 192        |
|             | 15,860 ± 600    | 14,906 ± 393     |
|             | 15,709 ± 661    | 17,080 ± 837     |

*CO₂ readings obtained every 2 h for 48 h; 16-h photoperiod.

Table 6. Effect of CO₂ and light levels on budbreak, elongation, and leaf development of axillary shoots from cotyledonary nodes of cacao.

| Light (µmol·s⁻¹·m⁻²) | 800 | 20,000 |
|-----------------------|-----|--------|
|                        | CO₂ (ppm) | CO₂ (ppm) |
|                        | Budbreak/explant | Budbreak/explant |
| 45                     | 3.9 ± 0.6* | 2.2 ± 0.4 |
| 200                    | 4.0 ± 1.2 | 1.9 ± 0.5 |
|                       | Elongation/explant (mm) | Elongation/explant (mm) |
| 45                     | 10.2 ± 2.8 | 18.0 ± 4.2 |
| 200                    | 10.0 ± 2.1 | 14.8 ± 3.0 |
|                       | Leaf area (cm²) | Leaf area (cm²) |
| 45                     | 1.1 ± 0.3  | 2.1 ± 0.4  |
| 200                    | 0.6 ± 0.1  | 1.8 ± 0.2  |
|                       | Leaf area/explant (cm²) | Leaf area/explant (cm²) |
| 45                     | 0.7        | 6.1        |
| 200                    | 1.9        | 6.8        |
|                       | Leaf no./explant | Leaf no./explant |
| 45                     | 0.4 ± 0.2  | 2.9 ± 0.5  |
| 200                    | 2.9 ± 1.0  | 3.9 ± 0.5  |

Shoots were cultured on semi-solid WPM. Data obtained after 4 weeks (treatment n = 8 to 20).

Fig. 1. Response of axillary nodal shoots of cacao to low and high levels of light and CO₂ (see Table 6).

Table 7. Effect of CO₂ and light levels on budbreak, elongation, and leaf development from nodes derived from mature trees of cacao.

| Light (µmol·s⁻¹·m⁻²) | 800 | 20,000 |
|-----------------------|-----|--------|
|                        | CO₂ (ppm) | CO₂ (ppm) |
|                        | Budbreak/explant | Budbreak/explant |
| 45                     | 0.4 ± 0.1* | 0.7 ± 0.1 |
| 200                    | 1.0 ± 0.1* | 1.0 ± 0.1 |
|                       | Elongation/explant (mm) | Elongation/explant (mm) |
| 45                     | 1.3 ± 0.3* | 2.6 ± 0.4 |
| 200                    | 3.9 ± 0.5* | 4.4 ± 0.6 |
|                       | Leaf no./explant | Leaf no./explant |
| 45                     | 0.0 ± 0.0* | 0.1 ± 0.0 |
| 200                    | 0.3 ± 0.1* | 0.8 ± 0.2 |

Shoots were cultured on semi-solid WPM. Data obtained after 4 weeks (treatment n = 30 to 39).

Discussion

The difficulty of getting cacao to grow and proliferate shoots in vitro has been a common observation of many researchers (see introduction). One exception to this generalization has been cotyledonary nodal tissue, but the growth of axillary shoots appeared to be cotyledon-dependent, and subculture cotyledonary axillary shoots failed to grow (Janick and Whipkey, 1985). Our present results indicate that axillary shoots of cacao grow normally in vivo and that roots can partially substitute for cotyledons in promoting elongation of axillary shoots in vitro.
This substitution suggests that the lack of growth of axillary shoots in vitro is due to nutrition rather than the absence of any cotyledonary promotive factor. It seems unlikely that the promotive factor of roots is cytokinin, because treatment with various kinds, concentrations, and times of application of cytokinins has failed to be promotive (Adu-Ampomah et al., 1988; Bertrand, 1987; Blake and Maxwell, 1984; Dufour and Dublin, 1985; Janick and Whipkey, 1985; Legrand and Mississo, 1986; Litz, 1986; Orchard et al., 1979; Passey and Jones, 1983).

A polysaccharide gum is ubiquitous in all tissues of cacao (Adomako, 1972; Blake and Maxwell, 1984; Blakemore et al., 1966; Brooks and Guard, 1952; Legrand et al., 1986; Orchard et al., 1979; Passey and Jones, 1983; Whistler et al., 1956). Lack of growth of cacao in tissue culture under conventional systems may be due to interference (either physical or chemical) by this gum (Figueira et al., 1989). The growth of rooted cotyledonary axillary shoots in vivo and the absence of growth in vitro are compatible with the hypothesis that shoots are not being properly nourished either from the medium or from photosynthesis.

Studies of diurnal changes in CO₂ within culture vessels containing cacao explants indicate that CO₂ is being depleted during the day. The enhancement of shoot elongation and leaf development under a high CO₂/high light regime in this study is consistent with the hypothesis that an increase in photosynthesis by high light levels, high CO₂, or both, is responsible for the improved performance of cacao in vitro (Infante et al., 1989; Kozai, 1990; Lakso et al., 1987).

The positive results for in vitro propagation of cacao axillary buds recently reported by Flynn et al. (1990) are puzzling, because no single factor was implicated as essential. We suggest the main factor in their results is due to a high light level (daily average of 175 µmol·s⁻¹·m⁻²), but we do not rule out some undetected CO₂ effect.

In conclusion, our results indicate that high CO₂ and high light levels enhanced in vitro shoot elongation and leaf development of cacao shoots and microcuttings. Apparently, improved photosynthesis overcomes the inability of cacao to respond to in vitro conditions. The benefits of high CO₂/high light were observed with axillary shoots from cotyledonary nodes and nodel cuttings from mature trees as well as subcultures from these shoots. Enhanced rooting of axillary and mature shoots has been observed under this regime. This study confirms the beneficial effects of CO₂ enrichment reported by Kozai (1990) for various crops, but the CO₂ levels we used for cacao are considerably higher (20,000 ppm vs. 2000 to 3000 ppm). The optimum CO₂ levels for cacao remains to be determined. Our results suggest that a system for rapid micropropagation of cacao should be feasible with high light and high CO₂ levels.

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