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In Vitro Cell. Dev. Biol.—Plant not known:1–7, Not Known 2003
DOI: 10.1079/IVP2002409

1054-5476/03 $10.00+0.00

MICROPROPAGATION OF *THEOBROMA CACAO* L. USING SOMATIC EMBRYO-DERIVED PLANTS

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(Received 21 March 2002; accepted 2 December 2002; editor S. S. Korban)

Summary

A micropropagation protocol was developed using cacao somatic embryo-derived plants as a source for nodal and apical stem explants, and apical microcuttings. Microcuttings were efficiently rooted and developed into plantlets. Axillary meristems within the remaining decapitated plantlets subsequently developed and were used for production of additional microcuttings, with an average 2.4 growing shoots per decapitated stem. The remaining plantlets were maintained as microcutting stock plants. When nodal stem explants were cultured on thidiazuron medium, axillary buds proliferated and developed into shoots, which were excised and rooted. However, the efficiency of this method is lower than rooting of apical microcuttings harvested directly from stock plants. During root induction, short treatment with indole-3-butyric acid (IBA) increased the total percentage of rooted microcuttings up to 89%. Longer exposures to IBA increased the average number of roots per microcutting (from 1.7 to 5.2). Plant acclimatization after rooting was achieved with an average success of 87%. During several months of growth in the greenhouse, the micropropagated plants developed functional taproots. Currently, cocoa plants produced by this micropropagation method have been successfully acclimated to field conditions in Ivory Coast, Ghana, and Saint Lucia.

Key words: cacao; somatic embryogenesis; micropropagation; tissue culture.

Introduction

Theobroma cacao L., the chocolate tree, is mainly propagated by seeds. As a consequence of this and the high heterozygosity of the crop, wide genetic variation is observed among the seed-derived plants, resulting in a large proportion of low-yielding trees in a singe plantation (Irizarry and Rivera, 1999). Therefore, there is a need for development and practical application of fast and highly-efficient systems for vegetative propagation of superior new cacao varieties selected in breeding programs and newly-identified wild germplasm. Clonal propagation of cacao via rooted cuttings and grafting is practiced throughout the cacao-growing regions (Wood and Lass, 1987). Plagiotropic branches are the predominant vegetative material found throughout the life of a mature cacao tree. This is particularly valid in cultivated plantations where pruning practices frequently remove orthotropic shoots. Under these circumstances, plagiotropic shoots are often the only source of cuttings for vegetative propagation. The plagiotropic nature of this material causes the new trees to display morphological characteristics different from normal seedlings, often having a more fibrous root system, instead of a normal seedling taproot system, making them susceptible to drought and wind. Such plagiotropic plants also lack typical cacao dimorphic architecture (orthotropic and plagiotropic), exhibit bushy growth, and require extensive pruning and training during the first few years of establishment (Wood and Lass, 1987; Bertrand and Agbodjan, 1989).

Few attempts have been made to propagate cacao using orthotropic material. One radical technique is the decapitation of mature trees, thus promoting orthotropic growth (Bertrand and Agbodjan, 1989). It has also been observed that young cacao plants could produce orthotropic shoots from axillary buds in the case of death or excision of the apical orthotropic shoot (Wood and Lass, 1987). Promotion of orthotropic shoot growth has been achieved by the bending of mature trees (Glicenstein et al., 1990) or young orthotropic and plagiotropic stems (Amefia et al., 1985; Bertrand and Agbodjan, 1989; Bertrand and Dupois, 1992). The cacao trees produced from the orthotropic cuttings exhibited dimorphic growth. However, these practices have not gained popularity, mainly due to underdeveloped methodology.

As an alternative method for clonal orthotropic plant production, *in vitro* shoot multiplication of cacao has been attempted with limited success (Orchard et al., 1979; Essan, 1981; Passey and Jones, 1983; Janick and Whipkey, 1985; Flynn et al., 1990; Figueira et al., 1991; Adu-Amponah et al., 1992; Lardet et al., 1998). Recent advances in cacao somatic embryogenesis have enabled the production of large numbers of aseptically-grown cacao plants from flower staminodes and petal cultures (Li et al., 1998; Maximova et al., 2002). Despite the high multiplication rate, its application is limited by the indeterminant nature of embryogenic cultures, the long period of time from culture initiation to embryo production (6–8 mo.), and the relatively high cost per plant produced.

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With these limitations in mind, we sought to develop a micropropagation procedure as a part of an integrated *in vitro* propagation system for cacao that would potentially increase the multiplication rate, reduce the propagation period, decrease the dependency on new culture initiation, and avoid multiple somatic embryogenesis. Such a system could increase the efficiency of the system and reduce the associated costs. This study describes the development of such a protocol. Apical and nodal stem explants from primary somatic embryo-derived plants (SEPs) were cultured for production of axillary shoots. Additionally, we established a successful protocol for rooting of microcuttings *in vitro*. We also demonstrated the establishment of stock plants as a continuous source for microcuttings. To our knowledge this is the first report of a cacao micropropagation method that utilizes SEPs as a source for microcuttings.

Materials and Methods

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). The pH for all media was adjusted to 5.5 with 1 *M* KOH before autoclaving at 121°C for 20 min. All *in vitro* cultures were maintained at 25°C with 16 h light/8 h dark photoperiod and a light intensity of 50 μmol m^{-2} s^{-1}.

SEP establishment and bud culture initiation. SEPs were produced from flower staminodes from greenhouse-grown cacao trees (genotypes: Scavina-6, TSH516, and TSH565) (Li et al., 1998). Mature somatic embryos were converted to plants (Traore, 2000) and the SEPs were maintained *in vitro* in secondary embryo conversion medium (Li et al., 1998) until they developed root systems with a taproot and lateral roots, and a shoot with at least six leaves. Only healthy plants with fresh green leaves were used for bud proliferation cultures or as source plants for microcuttings.

For micropropagation by bud proliferation, *in vitro* TSH516 plants were cut into 1–2 cm-long shoot apex and stem explants. Each explant contained one or more leaf primordia (Fig. 1*A*). The majority of leaves were removed leaving only two leaves per explant, trimmed to one-quarter of their original size. The explants were placed horizontally on bud proliferation medium in Petri dishes (15 mm diameter). The media contained half-strength DKW basal medium (Driver and Kuniyuki, 1984), 10 g l^{-1} glucose, 5 g l^{-1} sucrose, 0.3 g l^{-1} potassium nitrate, 1.75 g phytagel, 2.5 μM each of arginine, leucine, lysine, and tryptophan, and various concentrations of thidiazuron (TDZ). Explants were cultured on TDZ medium for 5 d and then transferred to the same medium without TDZ for stem elongation. Each treatment contained three replicate plates with 12–18 explants each. Axillary bud break followed by proliferation was observed and recorded. During bud break, the stipules of previously dormant buds spread apart and the buds swell (Greathouse et al., 1971). The numbers of swollen buds were recorded at 8 d after culture initiation (ACI) (Fig. 1*B*). Proliferated shoots with developed leaves were counted at 6 wk ACI.

In vitro rooting of microcuttings and stock plant maintenance. One to 2 cm apical microcuttings with two to three leaves were harvested from SEPs and leaves were trimmed to one-quarter of their original size. Cuttings were placed upright in G7 Magenta boxes (Sigma) containing 50 ml of root induction (RI) medium, with approximately 2–3 mm of the stems immersed into the medium (Fig. 1*C*). The RI medium composition was the same as the above-described plant growth regulator- (PGR) free medium, but supplemented with 15 μM indole-3-butyric acid (IBA). The effects of the duration of root induction were evaluated for genotype Scavina-6. The different time periods of culture on RI medium were 1, 2, 4, 8, or 16 d. Each treatment included four replicate Magenta boxes with five microcuttings each (total of 20 per treatment). This experiment including all root induction treatments was repeated three times over 6 mo. Additionally, rooting was performed using genotypes THS565 and TSH516, which were cultured on RI medium for 8 d.

Following root induction, the microcuttings were transferred for root development and elongation to 150 ml of PGR-free medium in G7 Magenta boxes and maintained until plants developed at least three roots, 4 cm long. Subcultures using the same medium were performed every 4–6 wk until roots were developed. After 8 wk of culture on PGR-free medium, explants without roots were trimmed at the base of the stems and transferred back to RI media for additional root induction.

After apical microcuttings were excised, the remaining decapitated stock plants were transferred to fresh PGR-free medium. Prior to transfer, the surface of the medium was broken with sterile forceps to facilitate the immersion of the roots into the medium without any damage. The established stock plants were maintained with transfers to the same medium every 3–4 wk. New apical cuttings from the developed axillary shoots were usually harvested for rooting at the time of stock plant transfer.

Plant acclimatization. Plants with 3 cm or longer roots and three to five hardened leaves were transplanted into deep pots (4 cm diameter × 10 cm) containing a moist soil mix consisting of equal parts of promix (Premier Horticulture LTEE, Rivière du Loup, Quebec, Canada G5R4C9) and concrete sand. The plants were then transferred and maintained in a greenhouse at 27–30°C and 80% relative humidity. Natural light was supplemented with high-pressure sodium lamps as needed to maintain a minimum of 250 μmol m^{-2} s^{-1}, while automatically retractable shading limited light levels to a maximum of 1400 μmol m^{-2} s^{-1}. Irrigation was applied daily for the first 3 d at multiple times to maintain adequate pot moisture, and thereafter as needed. Cacao fertilizer was applied once a week (Traore, 2000).

Results

Effects of TDZ on proliferation of axillary buds from apical and nodal stem explants of cacao genotype TSH516. Preliminary observations of cacao stem cultures indicated that it is typical for a single shoot to proliferate and develop from an axillary bud associated with the leaf primordial closest to the apical cut surface. To attempt to increase the number of axillary shoots per primordia/stem explant, we added TDZ at different concentrations to the proliferation medium. Apical and nodal stem explants from TSH516 SEPs were excised and cultured horizontally on media with different concentrations of TDZ for 5 d then transferred to PGR-free medium. Five days after culture initiation, bud break was observed on both apical and nodal explants (Fig. 1*B*). Callus development associated with the leaf primordia and other wounded sites was also observed, and in some cases callus continued to grow even after the explants were transferred to TDZ-free media (Fig. 1*B*). Extensive callus growth was associated mainly with higher TDZ concentrations. In contrast, only a small amount of callus developed at the wound sites of explants grown in the absence of TDZ. TDZ did not increase bud break from nodal explants, but at higher concentrations (0.1–0.4 μM) it caused a minor bud break increase from apical explants ($P = 0.068$) (Fig. 2*A*). In the absence of TDZ, nodal explants exhibited five times higher bud break than apical explants (86% and 16%, respectively) (Fig. 2*A*).

Although bud break was recorded in both types of explants when cultured on shoot elongation medium, only buds from nodal explants developed further and produced actively-growing shoots (Fig. 2*B*). We also observed that TDZ at concentrations higher than 0.1 μM could cause a reduction in shoot numbers (Fig. 2*B*). On shoot elongation medium, the development of swollen buds from apical explants ceased as the primary apical bud continued to grow and elongate to produce a single apical shoot. Approximately 2 mo. ACI, root formation was observed at the base of some apical explants maintained on PGR-free elongation medium.

Rooting of apical microcuttings. Cacao apical shoots from SEPs sporadically produced roots in tissue culture on PGR-free medium. To increase the percentage of rooted plants and the number of roots per plant in this system, we cultured microcuttings for limited periods of time on medium supplemented with IBA, followed by

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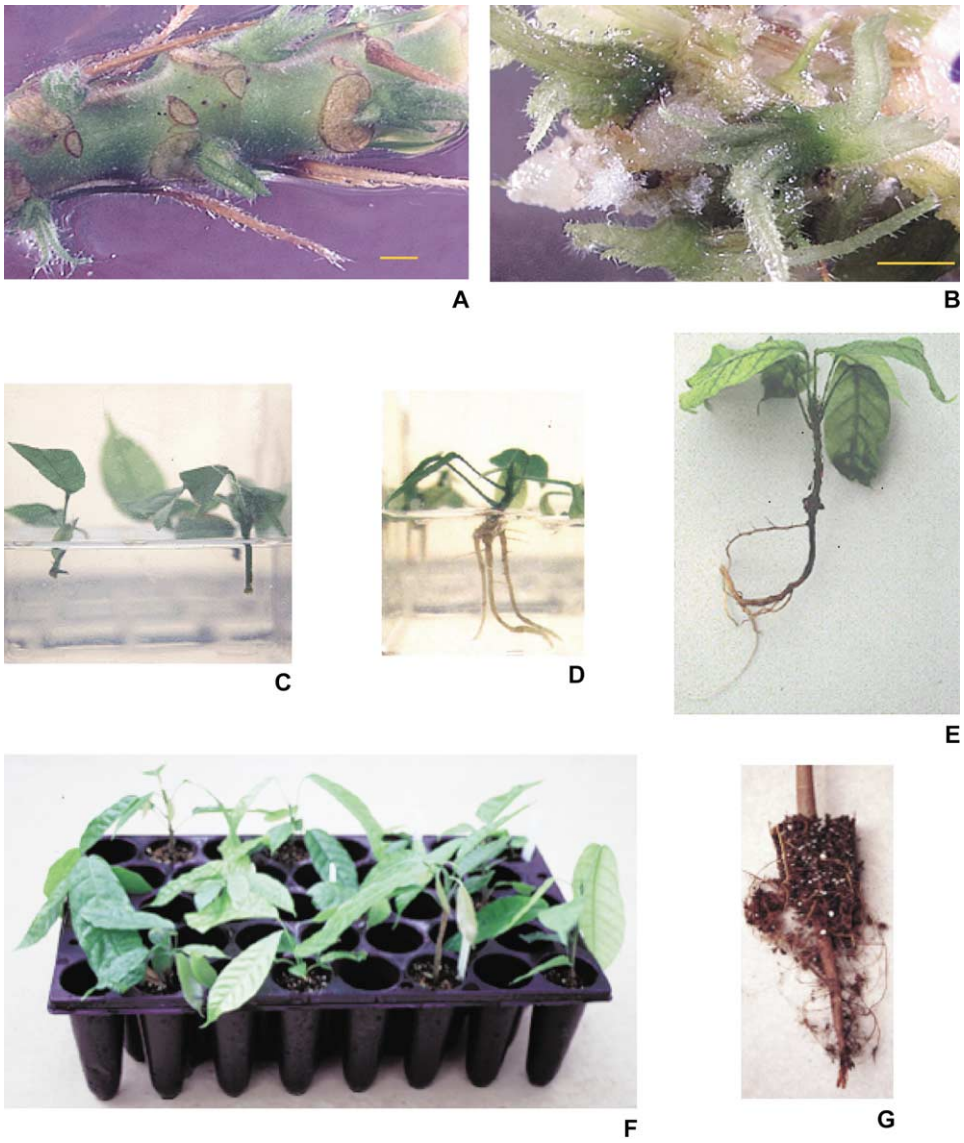

FIG. 1. Shoot induction and bud proliferation of cacao stem explants, and rooting of microcuttings. *A*, Nodal explant prior to culture initiation. *B*, Axillary bud proliferation and callus growth from a nodal explant at 7 d ACI. *C*, Microcutting immediately after culture initiation. *D*, Microcuttings after 8 d of root induction on IBA and 2 d on PGR-free medium. *E*, Micropropagated cacao plant ready for a transfer at 42 d after root induction. *F*, Micropropagated cacao plants 2 mo. after transfer and acclimatization in the greenhouse. *G*, Functional taproot from a micropropagated cacao plant at 8 mo. after acclimitization to the greenhouse.

transfer to PGR-free medium (root induction treatment). Using Scavina-6 shoots, we evaluated different periods of root induction on IBA medium. For most treatments root formation was observed as early as 3 d ACI and the root system was developed by 30 d ACI (Fig. 1*D*). In some cases, the formation of adventitious roots was preceded by callus formation at the base of the stem. At 30 d ACI the percentage of rooted shoots (approx. 90% for all treatments) was not significantly affected by various root induction periods ($P = 0.062$) (Fig. 3*A*). However, a significant difference was observed between the control (no IBA) and all IBA treatments ($P < 0.0001$). A significant effect of IBA exposure time was observed on the number of roots produced per shoot ($P < 0.0001$). The average number of roots progressively increased with the increase in root induction time. Short root induction times (24 or 48 h) produced plantlets with one or two roots (Fig. 3*B*). A significant increase in average root number (five to six roots per explant) was observed after a root induction time of 16 d. However, extended periods of culture on IBA frequently resulted in formation of large callus masses at the base of the stem. Additionally, root induction periods of 16 d appeared to cause a slight reduction in the rooting percentage (Fig. 3*A*) and some of the plants from this treatment were stunted. Shoots continuously cultured on IBA media did not develop roots and their growth was inhibited (data not shown).

The rooting protocol with 8-d root induction was evaluated using three cacao genotypes (Scavina-6, TSH516, and TSH565). High percentages of rooting were recorded for these genotypes (Table 1),

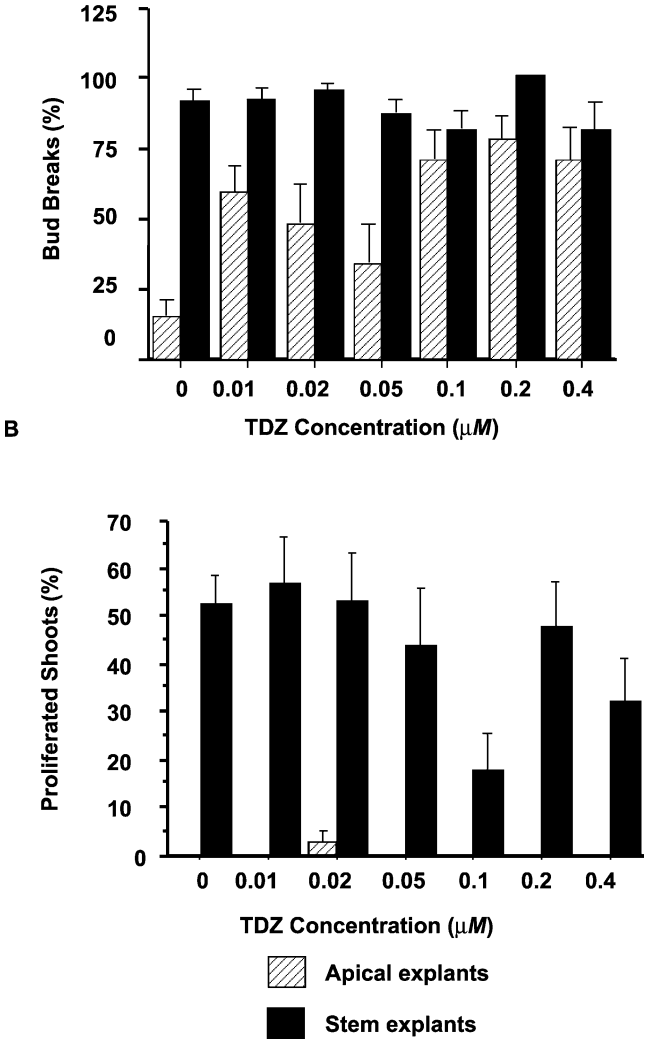

FIG. 2. Effect of TDZ on proliferation of axillary buds from nodal and apical stem explants. *A*, Percentage of swollen buds ± SE at 8 d after culture initiation. *B*, Percentage of proliferated shoots ± SE at 6 wk after culture initiation. Each treatment contained three replicate plates with 12–18 explants each. Significance was determined at $P < 0.05$ using ANOVA.

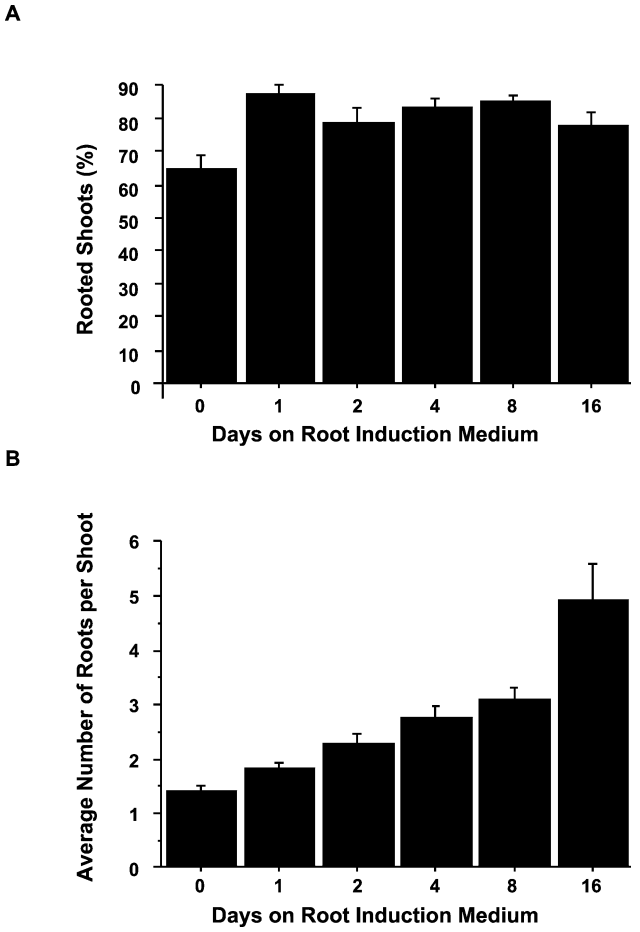

FIG. 3. Effect of IBA time exposure on rooting of cacao microcuttings from Scavina-6 genotype. *A*, Percentage of rooted explants ± SE. *B*, Average number of roots per explant ± SE. Individual experiments included four replicate Magenta boxes with five microcuttings each. The overall data presented is from three identical experiments performed over a period of 6 mo. Significance was determined at $P < 0.05$ using ANOVA.

ranging between 83% and 92% with an average of three to four roots per plant. Only a small difference in the percentage of rooted plants was recorded for TSH516 and TSH565 ($P = 0.013$).

Stock plant establishment and new shoot proliferation. After the excision of apical microcutting from SEPs, the remaining mother plants were transferred to fresh RD medium. The removal of the apical shoots temporarily released the plants from apical dominance and promoted new axillary bud proliferation. The axillary buds proliferated about 10 d after the transfer and the new shoots were harvested for rooting approximately 3 wk after the transfer. An average of 2.4 new and actively growing shoots were obtained per decapitated shoot after the first harvest (Table 2). The new shoots were removed when they were 2–3 cm in height with fully-expanded leaves, leaving a few nodes on the remaining stems. Consequently, after each harvest of microcuttings, the number of decapitated stems increased, creating multiple branches and thus increasing the number of released axillary buds. However, if the harvest is delayed, one of the newly-grown shoots establishes as a leader and restores the apical dominance of the plant, hence inhibiting the growth of the lower shoots on the same stem and reducing new bud proliferation.

Acclimatization of plants produced from rooted microcuttings. After root induction and culture on RD media, most adventitious roots were observed to grow in a manner similar to that of cacao

TABLE 1

IN VITRO ROOTING OF APICAL MICROCUTTINGS FROM THREE DIFFERENT CACAO GENOTYPES

Cacao genotype	Number of microcuttings	Number of rooted plants	Mean number of roots per plant
Scavina-6	50	44	3.1
TSH516	29	24	3.6
TSH565	12	11	4
Total	91	79	3.4

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TABLE 2

AXILLARY SHOOT PROLIFERATION FROM STOCK SOMATIC EMBRYO PLANTS OF CACAO AT 3 WK AFTER FIRST APICAL SHOOT REMOVAL

Cacao genotype	Number of stock plants	Number of stock plants with shoots	Mean number of shoots per plant
Scavina-6	56	56	2.1
TSH516	29	29	2.9
TSH565	12	12	2.3
Total	97	97	2.4

zygotic embryo taproots, extending vertically downward into the media with few branches and producing well-developed root hairs (Fig. 1*E*). Approximately 3 wk after transfer onto RD media, plants were transferred to soil and acclimated to greenhouse conditions. After 1 wk in the greenhouse, development of new leaves was observed, indicating successful establishment of the plants in *ex vitro* conditions. Depending on the genotype, 84–92% of the plants acclimated and survived (Table 3). During the early stages the micropropagated plants exhibited normal orthotropic shoot architecture similar to that of cacao seedlings (Fig. 1*E*). Additionally, all plants developed functional taproots, normal cacao leaves, and normal brown-colored stems (Fig. 1*F*, *G*). Currently we have produce more than 500 micropropagated cacao plants and the oldest ones have formed jorquettes, flowered and produced pods (data not shown).

Discussion

A micropropagation protocol for cacao was developed using somatic embryo-derived plants as a source for stem explants and for microcuttings. The procedure was evaluated using three different cacao genotypes. Earlier studies have documented that factors such as explant type and age, culture conditions, sterilization of the vegetative shoot explants, and media composition could be limiting for establishing an efficient protocol (Orchard et al., 1979; Passey and Jones, 1983; Flynn et al., 1990; Adu-Amponah et al., 1992; Lardet et al., 1998). We described a procedure that utilizes juvenile SEPs as an explant source. The plants were grown and maintained *in vitro*, which eliminated the need for sterilization. Additionally, as a result of advances in cacao somatic embryogenesis (Li et al., 1998; Maximova et al., 2002), DKW medium was substituted for the commonly used Murashige and Skoog or Woody Plant Medium basal media (Orchard et al., 1979; Passey and Jones, 1983; Janick and Whipkey, 1985; Flynn et al., 1990; Figueira et al., 1991; Adu-Amponah et al., 1992; Essan, 1992; Lardet et al., 1998). Li et al. (1998) also demonstrated that TDZ could efficiently induce the regeneration of cacao somatic embryos from floral parts. Hence, we investigated the possibility that the combination of half-strength DKW basal media with TDZ would be beneficial for axillary bud proliferation. TDZ has been previously used to promote shoot development in other woody species (Huetteman and Preece, 1993; Lu, 1993) and to accelerate bud break in apple (Wang et al., 1986). It was also shown to promote shoot proliferation from cotyledonary nodes of decapitated cacao seedlings (Figueira et al., 1991). However, in that study, the shoots did not form roots and did not develop into normal plants. The conclusion from our study was that under the given experimental conditions, TDZ did not contribute to increased production of axillary shoots. We observed that cacao explants cultured on TDZ media were likely to produce callus shortly after culture initiation, which interfered with bud proliferation. Similarly, TDZ was also shown to induce fast callus growth in a variety of plant tissue culture systems (Murthy et al., 1998). Such response is not surprising considering that the exact mechanism of TDZ action is unclear, and that this PGR has been recorded to mimic both auxin and cytokinin effects (Murthy et al., 1998).

Our results indicated that a higher number of axillary buds proliferated from nodal explants than from apical shoot explants. The different responses were likely due to the influence of the apical meristem exercising apical dominance over the axillary buds in the apical shoot explants. In contrast, two to three axillary buds from nodal explants were released from apical dominance, proliferated, and developed quickly into healthy shoots. These results confirmed an earlier observation by Flynn et al. (1990), who also reported that the removal of the shoot apex of micropropagated cacao explants promoted basal bud development. Other studies also described successful shoot proliferation from cotyledonary nodes of decapitated cacao seedlings, but were not able to produce rooted plants (Janick and Whipkey, 1985; Figueira et al., 1991). Similarly, greater bud proliferation from stem cuttings compared to apical cuttings was also observed in cultures of *Ixora coccinea* L. (Lakshmanan et al., 1997).

Rooting of axillary shoots excised from whole SEPs and from nodal explants was successfully achieved after short IBA root induction treatment. The IBA treatment greatly promoted root initiation and roots were fully developed in approximately 30 d ACI. This represents a major improvement over the previously reported protocols for *in vitro* rooting of cacao shoots, which included continuous culture on auxin media (Flynn et al., 1990; Adu-Amponah et al., 1992). In our study, long exposure to auxin contributed to massive callus formation at the bases of the stems and overall inhibition of plant growth and root development.

The absence of dimorphic growth and taproots in cacao plants produced from field plagiotropic rooted cuttings have been one of the main drawbacks of this traditional cacao propagation method (Bertrand and Agbodjan, 1989). The plants appeared to be sensitive to water deficit and high wind during early establishment. An advantage of our *in vitro* rooting system is using juvenile SEPs (still in orthotropic growth phase) as a source for microcuttings and the production of plants with functional taproots, which later exhibit the characteristics of cacao seedlings: above ground dimorphic growth.

TABLE 3

ACCLIMATIZATION OF MICROPROPAGATED CACAO PLANTS TO GREENHOUSE CONDITIONS

Cacao genotype	Number of plants	Number of acclimated plants
Scavina-6	44	37
TSH516	26	24
TSH565	14	12
Total	84	73

Our results are in agreement with those of the previous reports, which state that field orthotropic rooted cuttings produce a dimorphic growth pattern and a functional taproot (Bertrand and Agbodjan, 1989).

The use of primary and secondary somatic embryogenesis provides a powerful tool for fast propagation of a large number of cacao plants from agronomically-valuable genotypes. The addition of this micropropagation technique to the embryogenesis protocol provides several unique advantages. For scale-up in numbers, micropropagation offers a faster, less labor-intensive and thus less expensive method compared to somatic embryogenesis. *In vitro* micropropagation could also be used as a method for the provision of clonal plant material for research purposes, without the need for constant re-initiation of cultures through collection of floral buds, which may be large distances from the research laboratory. This system could also be used for *in vitro* germplasm maintenance to safeguard cacao collections, which must be maintained as living collections.

Acknowledgments

The authors thank Dr Zhijan Li for his help in designing the experiments, Erika Barreto for taking care of the plants in the greenhouse, and Sharon Pishak and Ann Young for their technical support. This work was supported in part by the American Cocoa Research Institute Endowed Program in the Molecular Biology of Cocoa, by grants to M.J.G. from the American Cocoa Research Institute and the United States Department of Agriculture Office of International Research Programs, and support from the Agricultural Experiment Station of The Pennsylvania State University (CRIS 3550).

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Author Queries

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Q1 *JOURNAL:* IVP

Q1 What is RD medium? (not mentioned in Materials and Methods)

Q2 Capelle et al., 1983 not cited in text - please cite. Also changes ok?

Q3 Murthy et al., 1995 - please cite in text.