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INDUCTION OF MULTIPLE SHOOTS IN NODAL EXPLANTS OF FULLY GROWN TREES OF COCOA (*Theobroma cacao* L.)

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ABSTRACT

Microprogation was attempted with the objective of clonal propagation of fully grown trees of cocoa. Multiple shoots were produced from nodal segments of ten year old cocoa trees on woody plant medium (WPM) containing cytokinin and other growth supplements. Increased release of buds up to ten from the pre- existing axillary meristems could be achieved within 10-15 days of culture. Among the different cytokinins, 2ip alone was effective and the best response was obtained at a concentration of 4 mg l^{-1}. The effect of zip on multiple shoot induction depended upon the presence of other media components also. Most consistent results were obtained on WPM supplemented with silver nitrate - the ethylene inhibitor, phloroglucinol - the phenolic hormone synergist, CCC - the antigibberellin and adenine sulphate - the cytokinin precursor along with higher levels of 2ip. The optimum concentrations of these were standardised. The frequency of bud burst varied with genotype, the best response being from the clone GVI - 67. This is the first report of multiple shoot induction in cocoa under *in vitro* conditions. The continued presence of higher levels of 2ip, however, was detrimental to the sustained growth of shoots and was manifested as shoot tip necrosis in such cultures.

INTRODUCTION

Cocoa (*Theobroma cacao*) is a commercially important plantation crop with high genetic variability. Because of its open pollinated habit and heterozygous nature, the seedling progeny need not exhibit the parental characteristics. Variability among the progeny also will be quite high. Therefore, vegetative propagation will be ideal in this crop as this offers the possibiilities of clonal propagation of elite materials. Since the number of propagules obtained from the traditional methods of vegetative propagation are limited it seems profitable to study the possibilities of *in vitro* vegetative propagation in cacao.

There were many reports of micropropagation of cacao during the last two decades but most of them were from explants of axenic seedlings or from glass house grown plants. Until recently, only sporadic growth and proliferation of shoots have been achieved (Orchard *et. al.*, 1979; Passey and Jones, 1983; Legrand *et. al.*, 1984; Dufour and Dublin, 1985; Litz, 1986; Abu Ampomah *et. al.*, 1987; Flynn *et. al.*, 1990 and Mallika *et. al.*, 1992). According to Duncan (1992), cocoa has been recalcitrant to tissue culture propagation and satisfatory shoot proliferation has not been obtained so far. This is more so by using explants from field grown adult trees.

In an economically viable micro-propagation protocol, production of multiple shoots in the axillary bud culture will be ideal as it provides the opportunity for obtaining large number of plantlets from a single explant. Since this objective has not been achieved in cocoa so far,in the present study attempts have been made to induce multiple shoots from pre-existing axillary meristems of nodal segments of field grown trees.

MATERIALS AND METHODS

Explants used in this study were single nodal segments containing the axillary buds. The protocol developed in our laboratory for preparing the plant material for *in vitro* culture is the following.

Green fan shoots at the I_2 stage of flushing (Greathouse *et. al.*, 1971) were collected from ten year old trees in the field. These mother plants were given prior fungicidal protection with systemic fungicide Bavistin 0.2 percent and the contact fungicide Dutgabe 0.3 per cent twice weekly. Major part of the lamina was trimmed leaving about one third near the petiole and the shoots were thoroughly swabbed with cotton dipped in 70 per cent alcohol. In the laminar flow cabinet the shoots were segmented to single node pieces so that each segment is 1.5-2.0 long retaining maximum length of the lower internode and subtended by petiole with a part of the lamina. The explants were surface sterilized in freshly prepared chlorine distilled water. The explants were drained on sterile filter paper kept in a sterile petri plate, dried in the laminar flow for 10 min and then inoculated in medium contained in culture tubes.

All the explants were cultured on a basal medium (Lloyd and McCown, 1980). This medium was fortified with various growth substances, individual and combination.The pH of the medium was adjusted to 5.8 before autoclaving at 121° for 20 min and solidified with 0.7 per cent agar. All the cultures were maintained in a culture room at 28 $\pm$ 2°C and exposed to photoperiod of 12 h light.

The following aspects of study were undertaken during the present innvestigation.

Standardization of best media for induction of multiple shoots

Earlier studies have shown that Woody Plant Medium (WPM) supplemented with amino acids (AA) (Flynn *et. al.*, 1990),silver nitrate ($AgNO_3$) 5.0,ascorbic acid 100, phloroglucinol (PG) 126, and peptone 100 mg l^{-1} was ideal for growh of single shoots from the axillary meristems of field grown trees (Mallika *et. al.*, 1992). The cytokinin 2iP at 1.0 mg l^{-1} was found to be sufficient for bud break and growth of single shoots from the pre-existing axillary meristems. In the presents study we have tried different media additives for induction of multiple shoots (Table II). These include different concentrations of the gibberellin antagonist cytokinin precursor Ad. SO_4 singly and in combination. All these supplements were tried with different levels of 2 iP.

Studies on the effects of different cytokinins on multiple shoot induction

In ordert to indentify the best cytokinin for the induction of multiple shoots in cocoa, four different cytokinins viz., benzyl adenine, kinetin, 2 isopentenyl adenine and zeatin each at four levels were tried in the media (Table II). All the media additives found favourable for multiple shoot induction were tried along with the varying levels of cytokinins

Initial sprouting of buds was observed ten days after culture by which time the original leaf stump was shed. Number and nature of buds developed per leaf axil for each genotype / treatment was recorded after five to six weeks. Mean values of survived as well as sprouted explants were also recorded.

Studies on the response of different genotypes to multiple shoot induction under the most favourable conditions.

Six high yielding cocoa clones viz., GII-12.3., GIV-1.2, GIV-2.5, GVI-51, GBI-59 and GVI-67 were used for the study. Explants from these genotypes were cultured in the most

Table I. Morphogenic response of nodal segments of Cocoa in different media for induction of multiple shoots

Treatment number	Addition to the basal medium * (mg l^{-1})	Mean percentage of surviving explants	Mean percentage of explants showing regeneration	Number of shoot initials regenerated after 30 days	Growth observations at the time of subculture (after 6 weeks)
1.	CCC 0.75	82.6	66.3	Single	Bud growth arrested
2.	CCC 0.75 + PG 200	81.3	69.5	Single	"
3.	CCC 0.75 + AD.SO_4 l	83.0	63.6	One or two	"
4.	PG 200 + AD.SO_4 l	81.7	72.0	One or two	0.5-1 cm shoot with place gren leaves
5.	CCC 0.75 + PG 200 + Ad.SO_4 l	86.6	83.4	Seven to ten	Healthy shoots 2-3cm long with dark green leaves

* Basal medium - WPM + AA + Sucrose 3% + $AgNO_3$ 5 mgl^{-1} + 2iP 4 mgl^{-1}

250 - 300 cultures per treatment. Genotype GVI - 67

favourable media for multiple shoot induction and their response was studied.

RESULTS AND DISCUSSION

Standardization of best media for induction of multiple shoots

Among the different levels of media additives tried singly or in combination, results of the favourable ones are presented in Table I. The basal medium containing macro and micro elements of WPM supplemented with AA (Flynn *et. al.*, 1990), $AgNO_3$ 5.0 and 2iP 1.0 mg l^{-1} has been reported to be ideal for bud break and shoot proliferation in cocoa (Mallika *et. al.*, 1992). The present study has indicated that the axillary bud release from the pre-existing axillary meristem increased with higher levels of cytokinins especially 2iP. A relatively high percentage of cultures (66 to 83 per cent) exhibited shoot regeneration with different media combination. A maximum of 83.4 per cent of the cultures responded in the medium containing CCC 0.75, PG 200 and Ad. SO_4. The major and minor elements of WPM needed other supplements like AA, $AgNO_3$ and 2iP along with carbon source of sucrose at 3.0 per cent. This combination can be considered as ideal for multiple shoot induction since 7 to 10 shoots were formed from each of the primary cultures of axillary bud explants. Sustained growth of the shoots also is obtained only in this combination.

Although all the media supplements in the concentrations listed in Table I have exhibited positive response in shoot regeneration it appears that combined efect of these chemicals is much more ideal with regard to the number of responding cultures as well as the number of shoots per culture. The positive role of the ethylene antagonist $AgNO_3$ in bud culture of cocoa has already been shown in earlier studies (Mallika *et. al.*, 1992). A concentration of 5 mg

l^{-1} has been found to be the most favourable.

The favourable range of biological effects of PG which is known to be a phenolic hormone synergist has been reported earlier. Its stimulatory effects on shoot and root growth in *Mulus sp* has been reported by Jones (1976). It is known that microbial growth is inhibited by PG and hence it is possible that PG in certain plant cultures act in an indirect way by suppressing the contaminants within the tissues (Hunter, 1979). In cocoa, inclusion of 200 mg l^{-1} PG was found to be highly essential for multiple shoot initiation. The shoots put forth in presence of PG were much healthier than in the absence of it. The role of this substance in increasing the number and length of shoots in pixy has been noticed by Jones and Hopgood (1989). As suggested by Jones and Hartfield (1976), the exact role of PG in culture systems remains unclear but it is likely that PG is acting not only as an auxin synergist alone but also as a bactericide in preventing the endogenous bacteria which can suppress culture growth without apparent external symptoms.

The involvement of CCC (0.75 mg l^{-1}) also has been found to be very critical for multiple shoot initiation and growth in cocoa. This chemical was found to be essential for proper expansion and greening of the leaves. In the absence of CCC, the sprounts appeared to be feeble and pale. The growth regulating effect of this compound is reported to be typical of a gibberellin biosynthesis inhibitor (Davis and Curry, 1991). Cycocel treated plants have been shown to have thicker and shorter internodes and have potential to enhance the net photosynthesis. The potential to enhance the net photosynthetic activity is not completely clear but may be related to the increased leaf and mesophyll layer thickness in the treated plants. Leaves of treated plants have been reported to contain increased palisade cell length and spongy parenchyma layer thickness. In addition to directly increasing photosyntheisis, CCC may sometimes prolong the period of normal photosynthetic activity for a leaf by delaying senescence (Davis and Curry, 1991). In the present experiment in cocoa bud cultures also, proper expansion and greening of leaves in presence of CCC may be due to the enhanced net photosynthesis. Being a gibberellin biosynthesis inhibitor, it may be acting by suppressing the action of endogenous gibberellic acid to promote the release of buds from the per-existing meristem and inhibiting shoot elongation. Gibberellic acid (GA_3) is already reported to be inhibitory in cocoa tissue culture (Blake and Maxwell, 1984).

The cytokinin precursor $Ad.SO_4$ was also found to be essential for the initiation of multiple buds from the nodal segments. The mode of action of $Ad.SO_4$ is not clearly understood. This substance is known to act as a substrate for the synthesis of natural cytokinin growth substances. Among the different levels of $Ad.SO_4$ used ranging from 1.0 to 200 mg l^{-1}, the best results were obtained at the lowest concentration indicating probably the possible toxic effect of the chemical at higher levels along with higher levels of added cytokinins. In their studies on vegetative multiplication of chestnut, Vieitez *et. al.* (1983) observed that addition of 1.0 mg l^{-1} of adenine sulphate was useful in preventing apical necrosis in *in vitro* shoots induced by BAP.

Studies on the effects of different cytokinins on multiple shoot induction.

The single node segments of cocoa cultured in WPM supplemented with different growth factors viz., AA, $AgNO_3$ 5.0, CCC 0.75, PG 200 and $Ad.SO_4$ 1.0 mg l^{-1} along with varying levels of cytokinins started bud burst within 10 days. The results of the most effective combinations are presented in Table II. Much differences were observed in the different levels

Table II. Effect of different cytokinins on axillary bud growth in cultured stem segments of Cocoa

Cytokinins added to basal media *	Mean number of buds initiated per culture cytokinin levels (mg l^{-1})			
	1	2	3	4
BAP	1.0	0.9	0.0	0.0
KIN	0.9	1.3	1.2	0.0
2 iP	1.4	1.7	3.0	5.2
Zeatin	2.3	4.6	5.0	2.1

Genotype : G VI 67 60 - 100 Cultures per treatment

* Basal medium WPM + AA + $AgNO_3$ 5 + CCC 0.75 + PG 200 + Ad.$SO_4$1mg/1

as well as with different cytokinins. While benzyl adenine and kinetin were not very effective in inducing multiple shoot production, relatively higher levels of 2iP and zeatin favoured it. The maximum response was noticed with 2iP at 4.0 and zeatin at 3.0 $mg1^{-1}$. The number of buds produced as well as their further growth were more in these combinations of cytokinins. Both benzyl adenine and kinetin seem to have inhibitory effect on bud break at higher concentrations. Benzyl adenine, in general, was found to be not very effective in bud break and shoot proliferation contrary to the observation of Passey and Jone (1983) who obtained shoot elongation and proliferation from nodal segments of cocoa cultured in Murashige and Skoog medium with benzyl adenine. However, proper shoot growth was not achieverd in their studies. Flynn *et. al.* (1990) obtained both shoot growth and rooting from nodal segments of cocoa in the absence of exogenously supplied growth regulators. However, they were unable to produce true proliferation of shoots.

The cytokinin, zeatin was found to be very effective in multiple shoot induction in cocoa. However, the high cost of the chemical as well as its thermolabile nature prevent its common use in cocoa tissue cutlure. Stimulation of axillary bud initials from nodal segements of cocoa has been reported by Litz (1986) in MS medium supplemented with zeatin 2.2 mg l^{-1} but no mention has been made of leaf expansion or shoot proliferation. Passey and Jones (1983) also reported induction of axillary shoot from nodal bud cultures in presence of zeatin, but the rate of proliferation appeared to be very slow.

Studies on the response of different genotypes to multiple shoot induction under the most favourable conditions

Nodal segments from six different genotypes were compared for multiple shoot initiation using the most favourable culture conditions identified in the present study. These genotypes included Amazonians as well as local selections of unknown genotypes, all from elite trees. The rate of multiple shoot initiation from the primary explants showed variation between clones (Table III and Fig. 1). The number of buds initiated per culture as well as the vigour of buds varied with genotype even when cultured under identical conditions. Most favourable and consistent response was obtained in the accession GVI-67. Five to eight buds and rarely upto ten buds were initiated from the single

axillary meristem of the nodal segment within two weeks of culture. Almost similar response was obtained from the accession GIV-1.2 also. In these genotypes, there was good growth of

Table III. Comparative morphogenic response of nodal explants of different genotypes of Cocoa in the media for multiple shoot

Accession number	Genotype / Cultivar	Per cent explants regenerated	Mean numbr of buds initiated per culture	Shoot length (cm) after 6 weeks
G II 12.3	Local	53.4	1.1	0.75
G IV 1.2	Local	85.3	5.8	2.10
G IV 2.5	Local	72.6	2.7	1.55
G IV 51	IMC 67	77.3	3.9	2.15
G IV 59	ICS 6	76.5	2.4	1.80
G IV 67	P 5 C	86.7	6.3	2.30

Media : WPM + AA + $AgNO_3$ 5 + CCC 0.75 + PG 200 + $Ad.SO_4$ 1 mg l^{-1}

200 - 300 culture per treatment

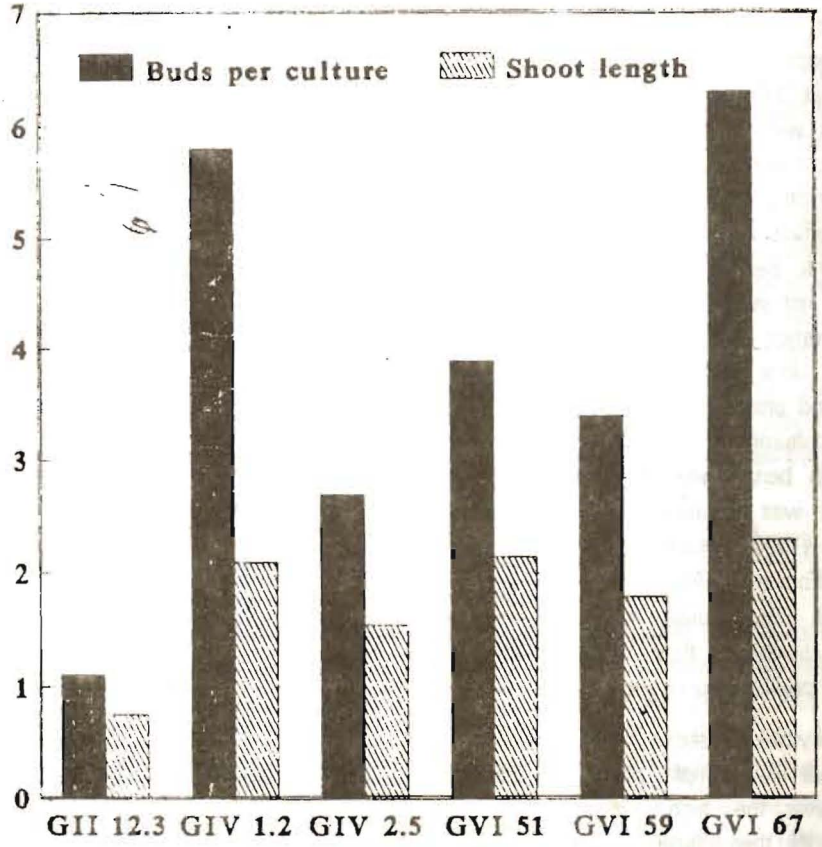

Fig. 1. Comparative morphogenetic response of different genotypes

shoots also and a few of the shoots had grown upto a length of about 2-3 cm. by the time of subculture after six weeks. The shoot growth was found to be satisfactory in the accessions GVI-51 and GVI-59 though the number of buds induced was lesser (3-5 only). Accessions GII-12.3 and GIV-2.5 exhibited relatively poor response with reference to all the three characteristics viz., the per cent explant regenerated, mean number of buds initiated per culture and shoot length at the time of sub culture. These observations strongly suggest clonal variation in their response to *in vitro* culture of cocoa.

Variation in genotypic response to culture has been reported earlier in a number of crops. In cocoa, Pence (1989) has shown that genotypic differences and seasonal effects played a great role in callus and organ forming cultures. Clone difference has been reported to be significant in oak (*Quercus robur*) microprogation also (Junker and Favre, 1989). In mulberry, Jain *et al.* (1990) reported that response of axillary buds as well as the requirement for growth regulators varied with genotype in the five materials tested *in vitro*. Vieitez *et al.* (1983) also observed marked differences in the responses in *in vitro* cultures of three clones of adult chestnut and they attributed the reason to the genetic differences.

Our studies indicate that the most ideal medium for induction of multiple bud initiation in cocoa should have higher levels of the cytokinin 2iP in addition to other media additives like $AgNO_3$, CCC, PG and $Ad.SO_4$. However, continued presence of 4.0 mg l^{-1} of 2iP was found harmful for the sustained growth of shoots which exhibits necrosis under this condition. A reduction in 2iP concentration from 4.0 to 2.0 mg l^{-1} as well as inclusion of 0.25 per cent activated charcoal were round to produce satisfactory growth of shoots upto a certain extent. An ideal medium for shoot proliferation of all the regenerated buds is yet to be standardized.

In conclusion micropropagation by induction of shoots from preexisting meristems is a useful technique in vegetative propagation since it guarantees that the characters of the source plant are conserved. The present study provides a method that ensures a high frequency of multiple shoot induction from adult tree nodal meristems of cocoa. Results so far achieved indicate success in culture establishment, multiple shoot production and further growth of shoots starting from field-grown adult plants. However, more research needs to be undertaken in this line in order to develop a viable protocol of micropropagation in cocoa which is known to be a recalcitrant species with regard to *in vitro* propagation.

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