EFFECT OF CULTURE MEDIA AND EXPLANTS ON CALLOGENESIS OF COCOA

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Introduction

Development of efficient methods for somatic embryogenesis of cocoa was taken up by many research institutes in France and USA (Guiltinan and Maximova, 2001; USDA, 2003). The first report of cocoa somatic embryogenesis was by Esan (1977), who described a method using immature zygotic embryo tissue explants. Various media combination were tried for cocoa by various workers, it includes WPM, Liquid MS, MS-based medium with the addition of 1mg NAA/l, DKW media with potassium nitrate and amino acids. Several reports are available to induce callus from various explants *viz.,* stem, nodal cuttings, petiole, leaf, shoot apex, embryos, anthers, flower parts etc., in different media combination. The present work is focusing on the embryogenic calli production from various explants of cocoa at CPCRI, Kasaragod.

Materials and Methods

Materials used were leaf, nodal cuttings, staminodes and cotyledons from Malaysian clone Jerangau Red Axil.

Field collection

The flower buds (for staminodes), pods (for cotyledon), tender leaves and nodal cuttings were collected non destructively from CPCRI, Regional Station, Vittal, Karnataka. These explants were put into a fresh polythene bag and stored in ice box, kept in refrigerator till the *in vitro* culture initiation.

Surface sterilization

For cotyledons and staminode

Pods were cut to extract the seeds. Flower buds and seeds were cleaned properly to remove mucilaginous mass. Then these were treated with two drops of Tween-20 for 15 minutes. Subsequently these were washed and kept in running tap water for 2-2½ hours. Then these were taken into Laminar air flow and surface sterilized by using 0.01% $HgCl_2$ for 5 minutes. Subsequently these were washed with sterile water for five times.

Surface sterilization of leaf explants

Leaves were taken and washed using tap water to remove the dust particles. Then the explants were washed with Bavistin (1g/L) for 1 hour and treated with two drops of Tween-20 for 15 minutes. Subsequently these were washed and kept in running water for 2-2½ hours and was taken into Laminar air flow. Then these were surface sterilized by using 0.01% $HgCl_2$ for 3-4 minutes and were again washed with sterile water for five times.

Surface sterilization of nodal cuttings

Nodal cuttings were taken and washed using tap water to remove the dust particles. Then these were treated with Bavistin (1g/L) for 1 hour and treated with two drops of Tween-20 for 15 minutes. Subsequently these were washed and kept in running water for 2-2½ hours and were taken into Laminar air flow. These were sterilized with 1:1 sodium hypochlorite (*i.e.,* 50ml of sodium hypochlorite + 50ml of distilled water) by constant stirring for 20 minutes and surface sterilized by using 0.1% $HgCl_2$ for 3-4 minutes. Then these were washed with sterile water for five minutes. Subsequently these were transferred to sterile conical flask with 1% charcoal and were kept in shaker incubator for over night.

Inoculation

Surface sterilized seed was cut open and its seed coat was removed and chopped into small pieces. These chopped bits were inoculated in callus induction medium (Fig. 4). Surface sterilized flower bud was dissected to remove staminodes. These staminodes were inoculated in callus induction medium (Fig. 3). Surface sterilized leaves were cut into 3 mm bits and these were inoculated in callus induction medium (Fig. 2). Surface sterilized nodal cuttings were inoculated in callus induction medium (Fig. 1).

Culture medium

DKW (Driver Kuniyuki Walnut 1984) basal medium supplemented with two types of hormones (2,4-D 1mg/ml stock and Thidiazuron), 20g of sucrose, 5.8g of Agar, Glutamine, Myo-inositol (USDA Protocol) and pH was adjusted to 5.8 with 1N NaOH/1N HCl prior to autoclaving the media at 121°C and 108kp Pressure. MS medium (Murashige and Skoog) supplemented with 2 types of hormones (NAA and BAP), 30 g of sucrose, 5.75g of Agar (CPCRI Protocol-Philomena Abraham, 1990) and pH was adjusted to 5.8 with 1N NaOH/1N HCl prior to autoclaving the media at 121°C and 108kp Pressure.

Culture condition

Initially cultures were incubated in dark, where temperature and RH are maintained at 27±1°C and 80% respectively.

Results

After 15 days

It was noticed that MS basal medium responded better for callus induction about 62% whereas DKW basal medium responded about 25.6%. Leaf explants responded better for callus induction about 68%, (Fig. 6) whereas cotyledon and staminode explants showed less callusing about 28% and 26.6% respectively.

After 30 days

It was noticed that MS basal medium responded better for callus induction about 70% whereas DKW basal medium responded about 42.6%. Leaf explants responded better for callus induction about 73.3%, (Fig. 9) whereas cotyledon and staminode explants showed less callusing about 56.6% and 36.6% respectively.

After 45 days

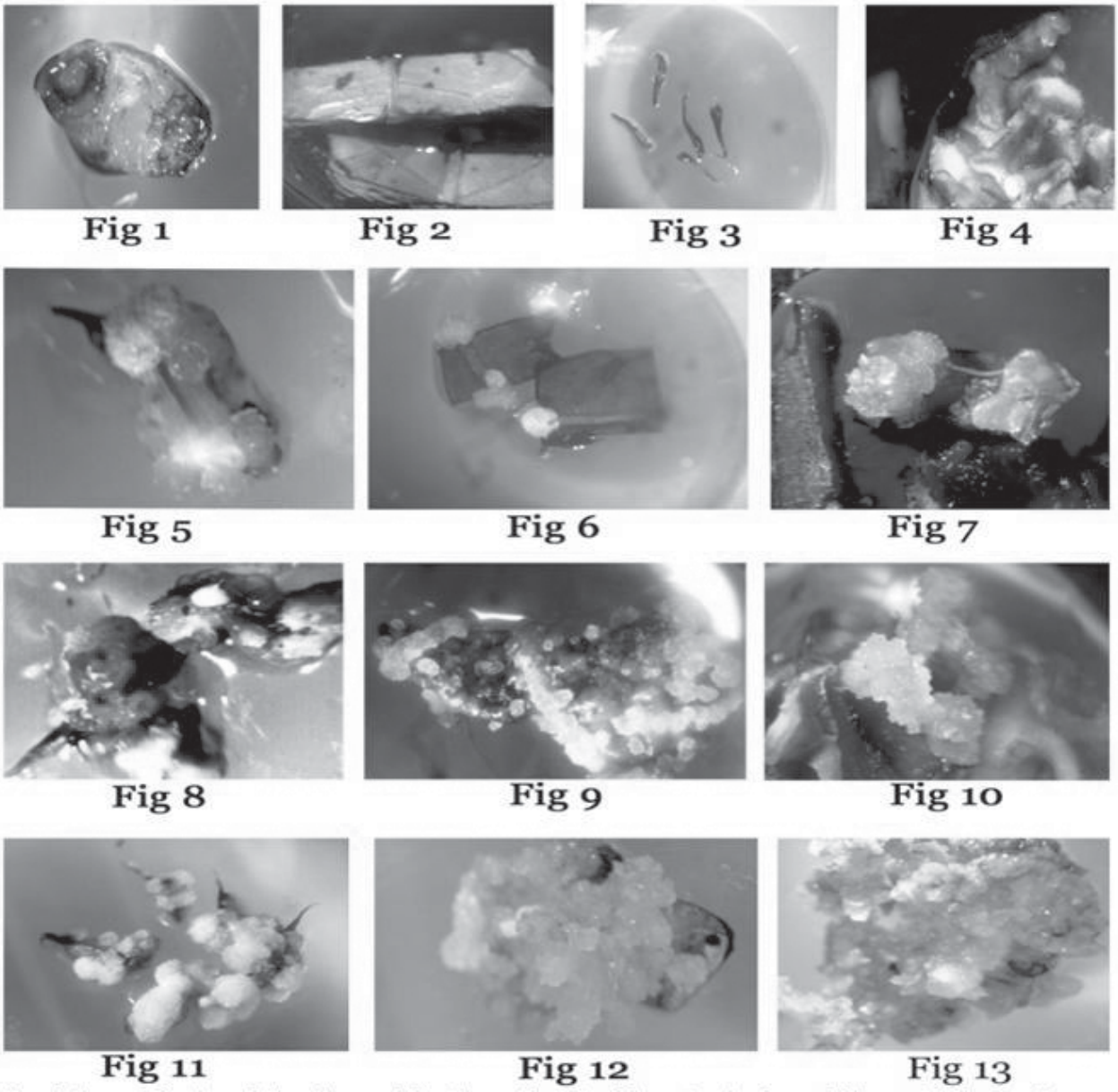
It was noticed that MS basal medium responded better for callus induction about 70% whereas DKW basal medium responded about 59.2% (Table 1). Leaf explants responded better for callus induction about 81.6%, (Fig.12) whereas cotyledon and staminode explants showed less callusing about 71.6% and 60% respectively. In nodal cuttings, due to heavy mucilaginous mass, severe contamination was observed and callus initiation was not observed.

Effect of browning

Because of high phenol content browning was observed in all the explants. Browning was observed more in staminode explants than other explants.

Table. 1. Percentage of response in different explants and media after 45 days of culture initiation

Explants/Media	Percentage of callusing	Percentage of browning
Staminode	60.0	46.6
Leaf	81.6	31.6
Cotyledons	71.6	42.8
MS	70.0	19.8
DKW	59.2	65.0



Fresh tissues after inoculation-Fig. 1. Nodal cutting, Fig. 2. Leaf, Fig. 3. Staminode, Fig. 4. Cotyledon.

15 days observation-Fig. 5. Callus initiation from staminode explant, Fig. 6. Callus initiation from leaf, Fig. 7. Callus initiation from cotyledon.

30 days observation-Fig. 8. Callus formation in staminode, Fig. 9. From leaf explant, Fig. 10. From cotyledon

45 days observation-Fig. 11. Staminode, Fig. 12. Leaf, Fig. 13. Cotyledon.

Discussion

Callus induction was observed in both the medium *i.e.,* DKW and MS medium. MS basal medium supplemented with 0.5mg/l of NAA and 0.5mg/l of BAP responded well for the callus induction than DKW basal medium supplemented with 2,4-D 2mg/l and TDZ 25µl (from 0.2mg/ml). Callus development was observed in staminode, leaf and cotyledon explants. Brown and white coloured callus was observed. Brown coloured callus was observed in cotyledon explant. White friable type of callus was observed more in leaf explant, whereas in case of cotyledon and staminodes only in initial stage friable type of callus was formed later it converted into compact type. More callusing was observed in leaf explants. Callus initiation was observed in leaf explant after ten days of inoculation. Shoot initiation from the embryonic axis was observed in cotyledon explant after thirty five days of inoculation. However axillary shoot induction using cotyledon explant was reported by Figueira *et al.,* in 1991. Callus initiation was not observed in nodal explants because of fungal and bacterial contamination. Browning of explants was observed almost in all explants and in both the medium within fifteen days of inoculation (Alemanno *et al.,* 2003). In DKW basal medium more browning was observed than MS medium. However, the browning had no deleterious effect on induction of callus. Browning was observed more in staminode explant within a week of inoculation due to the presence of phenolic compounds. Contamination from medium and explants was noticed. Both fungal and bacterial contamination was observed in all explants. Different methods of surface sterilization were used. The effectiveness of Domestos a commercial preparation of sodium hypochlorite for thirty minutes was reported by Passey and Jones (1983). However, Dulbin (1984) pointed out that with explants detached from the field, the percentage of infection was 90% regardless of procedure used for surface sterilization. The use of 70% alcohol, 10% Calcium hypochlorite and 10% hydrogen peroxide was suggested for surface sterilization by Legrand and Mississo in 1986. In nodal cuttings high rate of contamination was observed than other explant, due to the presence of mucilage. The result of the present study demonstrated that MS basal medium supplemented with 0.5mg NAA and 0.5mg BAP promoted callus induction after five days of incubation in the dark. It is also demonstrated that leaf explant responded well for callus induction. Initiation of callus was observed within five days of incubation in dark. Root initiation and shoot initiation was also observed from the embryonic axis of the cotyledon.

Conclusion

Among the two basal media studied for *in vitro* multiplication, MS medium responded better for callus induction. Among the four explants studied for *in vitro* multiplication, leaf explant responded better for callus induction. Callus initiation was observed after 10 days of inoculation. Browning was observed more in DKW medium than MS medium. Browning was observed more in staminode explants than other explants.

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