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PERFORMANCE OF COFFEE LEAF EXPLANTS IN SOMATIC EMBRYOGENESIS

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ABSTRACT
Explants of four coffee genotypes: T169,C111, C314 and D57 were cultured and maintained at 27±2oC for 14 to 42 days. Calli observed were then transferred to the embryo development (ED) medium and incubated for 90 days, Matured calli were then cultured in appropriate growth media to generate secondary somatic embryos. Varying concentrations of casein hydrolysate in 1000ml ED medium were thereafter separately tested against the resulting six-leaf explants of each of the clones. Overall results showed that clones C111, C314 and T169 responded well to embryogenesis while D57 did not. Techniques used in this study can therefore be used to develop somatic embryos for the micropropagation of some Nigerian premium coffee genotypes.

Keywords: Coffee, explants, callus, micropropagation, and genotype

Introduction

Coffee is a beverage prepared from seeds(beans) of *Coffea* species after roasting and grinding. It is the second most important commodity in the international market after petroleum. Coffee can be prepared by infusion in boiling water or by hot water under pressure (espresso) (Carneiro,1993).In 1964,Cocoa Research Institute of Nigeria started research work on coffee in Nigeria in the selection for high yielding,improved and high quality coffee,while actual coffee breeding programme commenced in 1969 (William,1971). Plant breeders worldwide have worked on coffee in order to obtain a better quality, higher yielding product,while at the same time seeking genetic resistance to pests and diseases. Vegetative and generative selection methods are employed in the clones with desireable characteristics while generative method aim at selecting clones for seed orchards that may produce favourable progenies when crossed.In Nigeria,selective among varieties of *C.canephora* showed that *Quilloe* is superior in yield to all other *robusta* in different locations (Hermann and Hass1975;Sondahl *et al.*,1980;Yasuda,1995 and Williams,1971). In Colombia, coffee rust caused by*Hamileia vastatrix* has ceased to be a serious threat since the release of a Colombian variety (a mixed line) by the Centre International de Cafe (CENICAFE) (Hermann and Hass,1975).

Snoeck and Petit through a work carried out over a 13-year period at Mulungu Station have shown that the distribution for productivity of individual coffee trees arising from a single mother tree is normal and complies with the law of Gauss (Eira,2002). Instead of making several years' observations,one large selection based on bean size, followed by selection of favourable lines among the progeny which would then be tested in comparative trials is recommended. Selected seedlings are produced in about 12years, compared to 25years with the earlier techniques, based on the transmissability of the productivity characteristics. This method reduces the time required for genealogical selection. Because of the homogenous nature of *C.arabica*, favourable result of improvement is obtained through inter-varietal hybrids. '*MundoNovo'* variety is a hybrid between the two varieties of *C.arabica*: '*Bourbon*' and '*Sumatra*'.

'*Mundo Novo*' is 300% more productive than the original variety(Carneiro,1993).

In Madagascar, Ivory Coast, Central African Rebublic and Cameroun, clonal progenies of cartain selections of *C.canephora* and the hybrids *C.congensis x C. canephora* have produced between 2.5 and 3 tonnes per hectare of coffee with large beans representing a yield of more than 20g or 100 beans. Identification has been made of a natural hybrid: *C.canephora* variety *Ugandae x C.congensis* commonly referred to as '*congusta*' which was first found in Java. It is very hard and flood resistant and the most popular of the interspecific natural hybrid of timor which was identified by portuguese agronomist as a natural hybrid between *Arabica* and *Canephora*. It is fertile and absolutely resistant to different types of rust affecting coffee.

The technique of interspecific hybridization of coffee was initiated in the 60s. The natural hybrid between *C.arabica*, a self –fertile tetraploid (4n=44 chromosomes) and *C.canephora*, a cross-fertilized diploid (2n=22 chromosomes) results in a triploid strain which has three set of chromosomes affecting meotic division; this results in low fertility or sterility. The chromosomal duplication of *C.canephora* which resulted from the application of colchicine made it possible to obtain tetraploid individuals of *C.canephora* that could be crossed with *C.arabica* to obtain fertile hybrids. Ducos (2003), produced the first of these hybrids, called "*Arabussia*.Coffee is grown in 80 countries around the world. In Nigeria, 70% is being produced by small holder farmers, its production has been limited to different susceptibilities to several diseases, photosynthetic efficiency, water utilization and tolerance to soil acidity and aluminium. In Nigeria, conventional breeding efforts in the past decades have been centred on providing solutions to: non-uniform ripening, low yield, biennial bearing, susceptibility to leaf rust and berry disease, susceptibility to insect pest attack, tolerance to drought, bean quality, and, fertilizer requirement.

These efforts have also led to the release of many commercial cultivars of coffee but the techniques have been found to be slow and inadequate at solving many of the crops problems. A further complication in coffee is the lengthy period for fruit development and the bean to bean generation time. Also the two widely cultivated coffee in Nigeria, *robusta* and *arabica* coffee are self-incompatible. As a result, transfer of genetic traits from the wild out bred species of the genus to the cultivated *arabica* cultivars is difficult, thus, traditional plant breeding techniques have been unsuccessful. Hence, the need for non-conventional methods/tissue culture through somatic embryogenesis developmentas a valuable tool for the clonal propagation of coffee breeding species and for the breeding of disease resistance, stress tolerance and low caffeine coffee for multiplication and possibly germplasm conservation, as embryo could also contribute to further improvement, distribution and preservation of germplasm. This study was therefore designed to develop somatic embryos from coffee leaf explants and generate plantlets for the micropropagation of Nigeria's coffee clones.

Materials and Methods

Collection of Samples and Preparation of Cultures

Healthy leaves of fourcoffee genotypes: T169, C111, C314 and D57 were obtained from coffee plots within the premises of Cocoa Research Institute of Nigeria (CRIN), Ibadan, Nigeria. The leaves were collected in clean McCartney bottles containing distilled water early in the morning (between 8-9am).The leaf samples were then surface sterilized in 4% calcium hypochlorite solution, rinsed in three changes of sterile water and transferred into separate sterile Petri dishes (according to their respective genotypes) until ready for dissection.Each of the leaves in the Petri dishes was sliced along the mid region, using a sterile scalpel No. 11 blade with the mid-ribremoved and the other strips cut into units. The coffee leaf explants were separately transferred into Petri dishes containing 25-30ml of primary callus growth (PCG) medium. The plates were then sealed with parafilm, labelled and kept in a sterile box. Cultures were maintained in the dark at 27 $\pm\mathbf{2}^0$C for fourteen days. Four coffee leaf explants were cultured per Petri dish per genotype and each genotype was replicated thrice.

Explants Transfer

After the first 14 days on the PCG medium, the explants were transferred into Petri dishes containing 30ml of the secondary callus growth medium (SCG1) and maintained in the dark for another 14 days. At the expiration of 28days, the explants were again transferred to the secondary callus growth medium (SCG2) and maintained in the dark for 28 days. Both developed callus and undeveloped explants were transferred to the embryo development (ED) medium and kept in the dark for another 90days. These calli were maintained on this medium in the dark and recultured at interval of 14 days for 4 months. After 4 months of incubation, matured calli (about 2cm in length) were selected and further cultured on ED medium supplemented with casein hydrolysate for another 4 months. They were subsequently subcultured onto fresh SCG 2 medium in the dark at 25^{o}C until a next generation of primary somatic embryos developed. These were again transferred to the ED medium with subculture at every 30 days for three months to generate secondary somatic embryos. The number of coffee leaves producing callus as well as those producing embryos were evaluated. Mature somatic embryos with an extended radicle were selected for embryo conversion.

Secondary Embryo Production

Somatic embryos were cut with a scalpel into approximately 4mm^2 pieces together with immature somatic embryo. Each distinctive cotyledon and an extended axis were placed on 30ml of the SCG2 medium at 90ºC to the base of cotyledon at a density of 6-10 embryos per Petri dish. The Petri dishes were sealed with parafilm and maintained under light 16/8 hr photo period at 25-30ºC. Embryos were subcultured to fresh ED medium every 30 days for four months.

Response of Genotype with Respect to the Frequency of Somatic Embryogenesis

Five, 10, 15 and 20g of casein hydrolysate were separately added to embryo development medium (1,000ml). The mixtures were separately tested on the resulting 6 leaf explants of each of the four genotypes used in earlier culturing. The untreated embryo development medium was also tested on the 6 leaf explants of the four genotypes.

Results and Discussion

Callus Induction

Callus induction on coffee leaf explants of genotype C111 is shown in Plate 1. Leaf colour changed from green to yellow on the primary callus growth (PCG) medium, as observed in Plate 1.The callus formation was evident (though scanty) from the 4th day after culture. Genotype D57 (Plate 2) produced friable callus and leaf colour changed from green to brown. Plate 3 showed the massive calli formation on genotype C314. The colour also changed first from green to yellow. Genotype T169 (Plate 4) produced compact whitish callus and leaf colour also changed from green to yellow. Variations existed on genotypic and explant basis among the studied genotypes of coffee, as genotypes C314 and C111 callused faster than genotypes T169 and D57. Genotype D57 callus formation was the smallest but friable. In general, genotype C111 had higher regenerative potential among the studied genotypes at the callus induction stage at two weeks.

Callus Enlargement

Callus enlargement was observed with different morphogenic appearance on SCG1 at 4 weeks on the four studied genotypes; C111, C314, D57 and T169 at 4weeks of culture (Plate 5). The callus enlargement appearance was brownish-yellow in colour from three genotypes; C111, C314 and T169 while genotype D57 (Plate 6) callus enlargement was yellowish. Furthermore,callus enlargement from these three genotypes; C111,C314 and T169 appeared more compact than D57 friable callus. In general, morphogenic appearance differed among the studied coffee genotypes on SCG1 medium at the callus enlargement stage (Plates 1-5).

Plate 1:Callus induction on coffee leaf explant of genotype C111

Plate 2: Friable callus formation by genotype D57

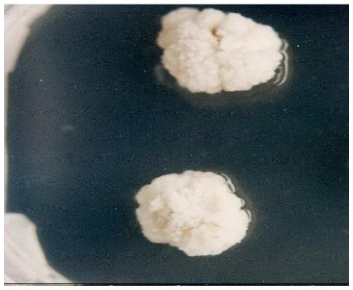

Plate 3: Whitish Callus massive callus formation on genotype C314

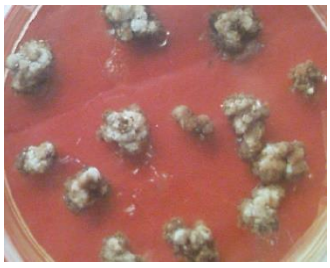

Plate 4: Brownish callus produced by genotype T169

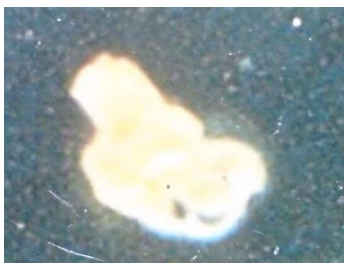

Plate 5:Yellowish callus enlargement formed by D57 with different

Embryogenic Callus Enlargement

Embryogenic callus enlargement was noticed at 98 weeks on the secondary callus growth medium SCG2. Callus formation on genotypes C111, C314 and T169 appeared to be embryogenic with pinkish to yellowish while genotype D57 callus formation remained dormant without further growth on SCG2 medium. Variation in colour was also observed at the embryogenic callus enlargement stage on SCG2.

Primary Embryo Induction

At 122 weeks, on ED medium, primary somatic embryos emerged from the genotypes C111and C314. These embryos were highly embryogenic, well synchronized and of high quality. They are also pinkish to yellowish in appearance. Genotype T169 embryo was not compact as compared to the other two genotypes but also yellowish in appearance. Genotype D57 did not produce any embryos on further culturing as the explant remained dormant. The embryos did not go beyond callus enlargement stage.It was also observed that embryo production of genotypes C111 and C314 was faster than that of genotype T169. The descriptive structures of the primary embryos of the three genotypes of coffee are presented in Table 1.

Secondary Embryo Induction

After 24 weeks of culturing on SCG2 medium, the three genotypes that went through primary somatic embryos; C111, C314 and T169 produced secondary somatic embryos (Plate 6). On further culturing on ED medium at 16 weeks, it produced secondary somatic embryos with elongated hypocotyls and leaf like structures (Plate 7) while genotype D57 on further culturing, still remained dormant at the secondary somatic embryos stage. On further culturing to stimulate their further growth development, the three genotypes; C111 C314, T169 remained dormant without further growth on ED medium. In general, genotype C111 and C314 were the best in terms of callus and embryo production out of the four genotypes explored. The descriptive responses of the secondary embryos are shown in Table 2.

Response of Casein Hydrolyate on the Embryo Development Medium

Results in Table 3 show the number of somatic embryos formed on ED medium. This ranged between 1-30 embryo per genotype when casein hydrolysate was used. In general, out of the 4 concentrations of 5,10,15 and 20g,casein hydrolysate embryo production at 20g was the best among the fourconcentrations tested, while genotype C111 had the highest embryo production.

Table 1: The descriptive structures of the primary embryos of the genotypes

Genotype	Developmental Phase	Embryogenic Structure
C111	Intermediate phase (Callus)	Elongated hypocotyls
C314	Intermediate phase (Callus)	Leaf-like structure
D57	Intermediate phase (Callus)	Dormant
T169	Intermediate phase (Callus)	Elongated hypocotyls

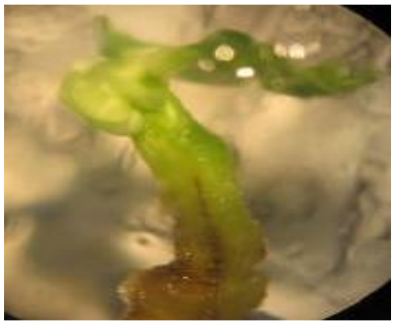

Plate 6: Secondary somatic embryos formed by C111, C314 and T169 at 24wks

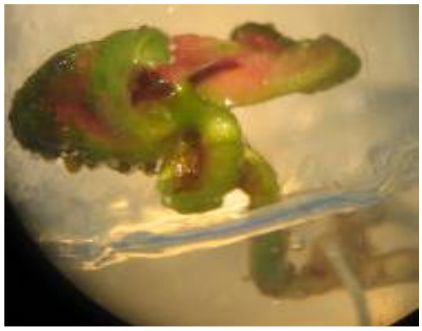

Plate 7: Secondary somatic embryos formed with an elongated hypocotyl and leaf like structures by genotypes C111, C314 and T169

Table 2: The descriptive responses of the secondary embryos of the genotypes

Genotype	Developmental Phase	Embryogenic Structure
C111	Intermediate phase (Callus)	Active leaf-like structure
C314	Direct	Improved embryo production
D57	Direct	Dormant
T169	Direct	Improved embryo production

Table 3: Response of casein hydrolyate on the embryo development medium

Genotype	Medium	Conc. of Casein Hydrolysate (Gm)	Embryo Production (Numbers)
C111	ED	5	-
		10	-
		15	-
		20	30
C314	ED	5	-
		10	-
		15	-
		20	20
T169	ED	5	-
		10	-
		15	-
		20	15
D57	ED	5	-
		10	-
		15	-
		20	4

The availability of planting materials with high genetic potentials is an improvement factor for increased and sustainable crop production. Coffee cloning by conventional method of vegetative propagation is inefficient and expensive (Barton *et al*.,1991). Planting materials for coffee are insufficient because the process of raising the materials is usually affected by the slow process of producing them through the conventional methods of budding, grafting and hybridization. Production of somatic embryos for micropropagation of planting materials is superior to the conventional methods of vegetative propagation (Murashige *et al*., 1962). Therefore, micropropagation of coffee should be an essential programme to ensure wider and increased production of clonal materials for propagation.

Results from the PCG medium showed differences in callus production from greenish to yellowish to whitish friable callus formation after two weeks of culture of the four studied genotypes; C111, C314, T169 and D57. This was observed two weeks after culture; the result showed that callus formation from coffee is genotype dependentas variations that exists among the studied genotypes. Genotype D57 callus formation was small, brownish and friable. Callus formation from the three other genotypes were greenish to yellowish. Genotypes such as C311 and C314 exhibited different morphogenic development but same colourations. They also yielded callus faster than the other genotypes on PCG medium. Afterfour weeks of culture,callus enlargement was evident from the four genotypes cultured on SCG1. Each was diverse in their morphogenic appearance, shapes and colouration. Callus enlargement from genotypes C111, C314 and T169 were bigger, compact and yellowish compared to genotypes D57 which produced yellowish but less compact friable callus on SCG1 medium. The observed variation might be due to the differences in the sources of explants or biochemical reaction. This agrees with the observation of Startisky (1970) who observed varied embryogenic callus from seven different coffee genotypes cultured.

After 28 weeks of culture, embryonic shaped callous were viewed under high power stereozoom fluorescent microscope. Embryonic structures attained different stages in embryology. Torpedo and globular embryoids were observed. Ninety-eight weeksafter, culture on the ED medium, primary somatic embryos were produced through callus intermediary from three out of the four genotypes cultured. This showed that differentiation is generally irreversible in plants.The degree of regression a cell can undergo would depend on the cytological and physiological stages it has reached (Vasil, 1972). When not dividing, equiscent cells from differentiated tissues are grown on a nutrient medium that support their proliferation as the cells first undergo certain stages to achieve the meristematic state. All the embryos produced varied in their degrees,modes and characteristics.The conversion of explants from callus to embryos established the ontogenetic pathway of somatic embryogenesis.

The embryogenic characteristics of genotypes C111 and C314 were distinct,producing more embryos per explant than all the others.However, higher somatic embryo frequency in genotypes C111 and C314 are in agreement with the work of Dussert (2003), Spiral (1999) and Eira (2002).

After 56 weeks of culture on both SCG2 and ED media, three genotypes; C111,C314 and T169 produced secondary somatic embryos,with genotype T169 having less secondary embryos and less superior to genotypes C111 and C314, while genotype D57 remained dormant. These secondary embryos were highly embryogenicwith elongated hypocotyl and leaf-like structure that were well synchronized and of better quality than the primary embryos.

Results also showed that more than 70% of the total somatic embryos were from secondary embryogenesis. Similar results have been obtained by Etienne (2002), Barton (1991), Carneiro (1995), Leroy (2000), VanBoxlel (1996), Sondahl (1977), Ganesh (1999), and Yasuda (1995). As much as 50 secondary embryos could be observed from 20 explants in one Petri dish of highly embryogenic genotypes such as C314 and C111. A significant variation was observed between the different coffee genotypes used for embryogenesis. Genotypes with high percentage of embryogenic explant such as C111 and C314 also exhibited a higher number of embryos per explant. This showed that in coffee,genotypic differences also have pronounced influence on the ability to embryogenesis.

The secondary embryos were more formed and they developed in shorter time than in primary embryos. In the overall result,the regenerative potential of the three out of the four genotypes studied showed that somatic embryo production is genotype dependent. Plants derived from somatic embryos are in essence genetically identical to their parental donor cells (Leroy *et al.*, 2000). This assures the conservation of clonal genetic constitution.

Nutritional requirements for optimal growth of a tissue *in vitro* vary from plant to plant. Even tissues from different parts of plants may have different requirements for satisfactory growth (Sondahl *et al*., 1980).This implies that no single medium can be suggested to be satisfactory for all types of plants, tissues and organs. In addition to the nutrients,it is generally necessary to add one or more growth substances such as auxin or cytokinin to support growth of tissues and organs.

Various scientists have tried various growth hormones to support cultures *invitro* (Sharp *et al*., 1973; Kumar *et al*., 2006; Hermann *et al*., 1975; Dufour *et al*., 2000; Eira *et al*., 2002). From the present study, the effect of casein hydrolysate on the frequency of embryogenesis were evaluated on embryo development medium(ED). Results obtained showed that casein hydrolysate at 20g had the best embryo production compared to other concentrations. This shows that casein hydrolysate is a suitable homone for coffee somatic embryos production (Barton,1991). Results obtained from this study comfirmed the possibility of generally large numbers of embryo that may reach plantlet.

Contribution to knowledge

The present study has established that callus formation can be developed from leaves of coffee genotypes. It has also established that somatic embryos can be produced from coffee genotypes. From the study, the different embryogenic structures depicted unique variations as different embryos produced were highly embryogenic and of different sizes and shapes. The study has also shown that regeneration is possible through the use of somatic embryo production.

Conclusion

The present research achieved somatic embryo production potentials for plantlet regeneration of some Nigerian coffee clones using leaf explants. Secondary somatic embryogenesis was also achieved for the first time from coffee leaves. Culture media: PCG, SCG1, SCG2 and ED, had been substantially significant *in vitro* effects on the explants of the studied coffee genotypes at every culturing stage. Only two out of the four genotypes produced highly embryogenic, well synchronized and bigger embryos. Moreover, embryo production in the two was fast and resulted in secondary embryogenesis. The two genotypes; C111 and C314 are promising germplasms (genetic resources) for coffee breeding and improvement. Since Somatic embryogenesis is possible from the leaf explants of coffee plants, one of the tasks for the immediate future is to subject all the known coffee accessions in Nigeria's germplasm to *in vitro* manipulations. This will go a long way to identifying high yielding and disease resistant varieties through *in vitro* culture for germplasm conservation. However, establishment of micro propagation garden(s) is/are solicited for all the coffee genotypes raised through *in vitro* culture and the use of these somatic embryo derived-plants as clones to reduce labour, time and cost associated with conventional breeding techniques.

Future research in coffee tissue culture will need to focus on the optimization of tissue culture conditions for other genotypes of interest. The use of integrated vegetative propagated systems (whereby the original plant will come through tissue culture and vegetative propagation systems via rooted mini cuttings) is hereby solicited. These two methods will lead to mass propagation of disease-free, high yielding and high bean quality genotypes.

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