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Full Length Research Paper

Tissue culture in *PINUS CARIBAEA* Mor. var. *HONDURENSIS* barr. and golf. II: Effects of two auxins and two cytokinins on callus growth habits and subsequent organogenesis

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The growth habits of calluses of *PINUS CARIBAEA* Mor. were investigated to determine their correlation with organogenesis for micropropagation purposes. Two forms of callus growth habits were observed; friable and compact calluses. Their development occurred over a range of auxin-cytokinin combinations. In naphthalene acetic acid (NAA) x 6-furfuryl amino purine (kinetin) cultures, 100% friable calluses were obtained while in NAA x 6-benzyl aminopurine (BAP) cultures, 100% friable callus was formed only with 1:2 NAA/BAP ratio. Many compact calluses were also formed by the various combinations of NAA and BAP. Friability of calluses was further promoted by the interaction of indole butyric acid (IBA) and BAP, but organogenesis was not achieved. However, different degrees of greening were observed in some of the cultures (both compact and friable type). Thus, greening was not associated with a particular type of callus growth habit. Anatomical studies indicated that the differences between the compact and friable calluses were in the distribution of the meristematic cells. The histological studies also revealed some important and unexpected features. These were the presence of embryo-like structures, tracheary elements, lignification and starch-grain like structures. These results have further demonstrated the potential totipotency of callus cells of *P. CARIBAEA.*

Key words: Auxins, cytokinins, callus, growth habits, organogenesis.

INTRODUCTION

Heterogeneity exists both between calluses and within a callus whatever be the control imposed on callus maintenance in culture (Allan, 1991). Also in established calluses, the author noted that this heterogeneity could be observed as differences in colour, morphology, structure, growth, metabolism and even in ploidy levels. The pattern of growth of a callus determines its morphology. Allan (1991) reported that as callus grows, cells are pushed upwards and outwards from the surface of the medium thus leading to the establishment of nutrient gradients between the cells and the growth substrate. The presence of these gradients may result in the establishment of microenvironments. Furthermore, growth habits or patterns are affected by such gradients and micro-environments through for instance, the promotion or inhibition of cell expansion and division."

Conditions which favour rapid cell division can lead to the formation of a soft friable callus which is composed of undifferentiated and loosely attached cells (George and Sherrington, 1984). However, typically cells are densely packed (compact callus) and such cells by extension are formed under conditions, which do not favour rapid cell division. Many authors have reported that callus growth habits can be correlated with organogenesis in tissue cultures. Zamora et al. (1987) observed two types of calluses (friable and compact) in callus cultures of *Dendrocalamus latiflorus* cv. *Machiku*. However they noted shoot-like structures only on compact calluses developed on 1 ppm 2,4-dichloro phenoxyacetic acid (2,4-D) and benzyl adenine (BA) medium. Ozias-Akins and Vasil

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(1982) reported that in wheat, callus with embryogenic potential was compact in nature.

Friability of callus tissue is highly desirable because it enables easy establishment of liquid (suspension) cultures (Noguchi et al., 1977; Allan, 1991). Allan (1991) further suggested the testing of different media in order to obtain friable callus before the initiation of suspension cultures. When compared with callus cultures, suspension cultures grow faster and media alterations or additions can be easily made than in callus cultures (George and Sherrington, 1984).

The importance of friable nature of calluses and suspension cultures had earlier been emphasized by Krikorian (1987). If truly large plantings of agri-forestry species, tropical timber trees and other woody species are going to be needed, the approach or strategy of choice will necessarily implicate suspension cultures that are capable of yielding somatic embryos or related propagules. No other means can fulfill this requirement of large numbers in a timely and cost effective manner (Krikorian, 1987).

In our previous paper (Akaneme and Ene-Obong, 2005), we reported that many plantations of *Pinus caribaea* Mor. var. *Hondurensis* were established in Nigeria to supply long fibres to the pulp mills. Unfortunately, the country has been unable to achieve this objective as a result of difficulties associated with seed supply such as high cost of imported seeds. Most of the older plantations established with these imported seeds also produce many empty seed cones (Okoro and Okali, 1987). We, therefore, decided to investigate the possibility of by-passing the seed supply problem with the use of plant tissue culture technique. This paper is a report of further investigations on callus cultures of *P. caribaea* where the growth habits of calluses were studied.

MATERIALS AND METHODS

Calluses obtained from two-week old aseptically germinated seeds were used in this study. The procedures of callus initiation, maintenance and culture conditions were the same as described previously (Akaneme and Ene-Obong, 2005). The growth regulators whose effects on callus growth habits were studied were naphthalene acetic acid (NAA), Indole butyric acid (IBA)., Benzylamino purine (BAP) and kinetin. Each of the auxins, IBA and NAA was added in four concentrations; 0, 0.5, 1.0, 2 mg/l. And each of the cytokinins, kinetin and BAP was also added in four concentrations; 0, 0.5, 2 and 5 mg/l.

NAA was combined in all possible combinations with kinetin and BAP. Thus 16 treatment combinations were obtained for NAA x kinetin and NAA x BAP respectively. IBA levels were combined only with the levels of BAP and 16 treatment combinations were also obtained. Each treatment combination was replicated five times. Calluses weighing 50 mg (fresh weight) were inoculated onto Von Arnold and Eriksson (AE) (1977) medium supplemented with the various combinations of NAA x kinetin and NAA x BAP. While callus mass weighing 500 mg was inoculated onto the same medium (AE) but supplemented with the combinations of IBA and BAP. All the calluses were incubated at 25 ± 2^{O}C in a 12 h photoperiod provided by white fluorescent tubes.

At the end of the culture period (30 - 32 days) the calluses were observed for signs of organogenesis and also visually rated for the nature of its growth habit. The sense of touch was also applied to some of the calluses to determine their texture. This was done across the various auxin-cytokinin combinations and the results recorded. For histological examinations of friable and compact calluses, representative callus pieces were fixed in FAA (formaldehyde 10 ml, glacial acetic acid 5 ml, ethanol 50 ml, water 35 ml) according to the method employed by Ameh (1993).

The callus tissues were those maintained on AE medium + 0.5 mg/l NAA + 0 mg/l BAP (for compact callus), AE + 1 mg/l NAA + 0.5 mg/l kinetin (for friable callus) and also a piece of the stock callus left on Murashige and Skoog (1962) (MS) medium supplemented with 2 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) (for friable callus). The calluses were left in the same medium for 5 months, 3 months and 4 months respectively without subculturing, and before fixing in FAA. The lengths of time that elapsed before fixation, however, were not deliberate. Subsequently, the callus tissues were dehy-drated for one day each in the following: 90% ethanol, absolute ethanol, propanol-tertiary butyl alcohol mixture (50:50, v/v) and tertiary butyl alcohol.

Metal gauze was prepared and placed to carry wax at the top of t-butyl alcohol in the specimen tube. Wax pellets were added to top of t-butyl alcohol on the float and infiltrated in 35 – 40^{O}C oven until wax crystals appeared all over the alcohol. The specimen tube was transferred to 57 – 62^{O}C and the wax – alcohol mixture decanted several times until all alcohol was removed and the tissue fully infiltrated with wax. The tissues were later embedded in tissue mat.

The embedded material was prepared as small blocks and fixed on wood blocks using molten wax. This was allowed to cool in a basin of ice-cold water. Serial sections, 10 µ thick were made using a rotary 820-spencer microtome and the ribbons were floated on drops of water on slides smeared with egg albumen to prevent washing off during staining. The slides with affixed sections were dried at 40^{O}C in the oven, dewaxed in xylene and stained with safranin and fast-green, using the schedule described by Anon (1968). The sections were then mounted in a Depex (D.P.X.) mountant and later photographed at different magnifications using a photomicroscope.

RESULTS

Two types of calluses were observed; compact and friable calluses (Figures 1a and b). Physically, the friable calluses were very soft, and brittle. They were also very watery. The compact calluses on the other hand, were hard to touch and required some pressure to break into pieces. Their development occurred over a range of auxin-cytokinin combinations.

In NAA x kinetin cultures, 100% friable calluses were obtained for all the treatment combinations (Table not shown). With respect to NAA x BAP cultures, Table 1 reveals that 100% friable calluses were formed only at the interaction between 1 mg/l NAA and 2 mg/l BAP. The rest of the treatment combinations formed varying degrees of both compact and friable calluses. The interaction between IBA and BAP stimulated more friable callus formation than NAA x BAP cultures. Almost all the treatment combinations formed 100% friable calluses especially 0.5 mg/l BAP in combination with all the levels of IBA (Table 1). Various degrees of greening were also observed in some of the calluses (both compact and fri-

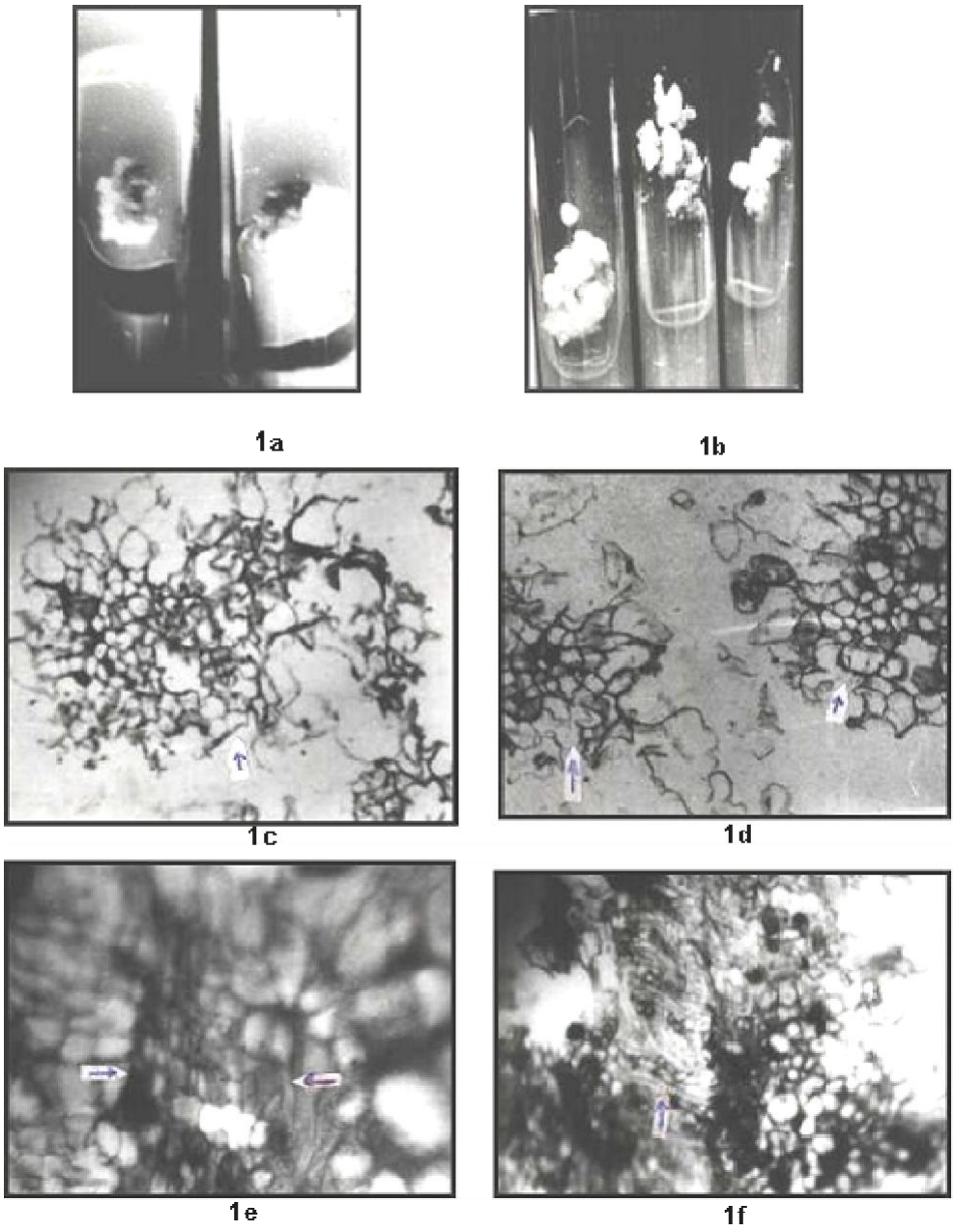

Figure 1. a) Compact calluses x1.04. b) Friable calluses x1.04. c) and d) Sections of friable calluses. Arrows indicating meristemoids x320. e) and f) Sections of compact calluses. Arrows indicating meristematic cells x320.

friable). Thus, greening (a sign of organogenesis) was not associated with a particular type of growth habit.

Anatomical studies of the compact and friable tissues showed that they differed in the distribution of the meristematic cells. In the friable tissues, the arrangement of the cells could be seen to be very loose (Figures 1c and d). It was also observed that localized groups of cells (growing centers) otherwise called meristemoids were scattered throughout the callus. On the other hand, cells of the compact calluses were more closely packed and cambium-like cells could be seen within the callus and also enveloping the callus (Figures 1e and f).

Unexpected observations were also made in the paraffin sections of the calluses (both friable and compact). Firstly, structures resembling early stages of zygotic embryogeny were observed. The stages seen were more of globular and few heart-shaped and torpedo-stage embryoids (Figures 2a and b). The embryoids were generally aggregated at the periphery of the calluses and were free-floating (Figures 2a and c). A structure which was

Table 1. Frequency in percentage of friable and compact calluses (five callus pieces per treatment combination).

BAP levels	NAA levels								IBA levels							
	Friable				Compact				Friable				Compact			
	0	0.5	1	2	0	0.5	1	2	0	0.5	1	2	0	0.5	1	2
0	-	60	40	80	100	40	60	20	20	100	100	100	80	-	-	-
0.5	40	20	-	80	60	80	100	20	100	100	100	100	-	-	-	-
2	20	-	100	80	80	100	-	20	100	60	60	80	-	40	40	20
5	40	20	60	80	60	80	40	20	100	60	80	100	-	40	20	-

difficult to call an embryoid was also observed (Figure 2d). Subsequent observation of the friable calluses that were sectioned revealed the manner of growth of the calluses (Figures 2e and 2f). Some definite structures could be seen at the surfaces of the calluses when compared with Figures 1a and b.

Secondly, tracheary elements were observed in the callus sections. The tracheids occurred in groups with bigger cells at the center and the smaller ones surrounding them (Figures 3a, b and c). They were not found dispersed in the callus tissues and they were distributed in a longitudinal manner.

Thirdly, lignification was observed as deposition of wall thickenings in the tracheary elements (Figures 3a, b and c) and in some of the parenchyma cells which were found to be empty and dead (Figure 3d). And finally, many of the parenchyma cells were seen to have accumulated starch grain-like structures within their cytoplasms (Figures 3e and f). The grains were mainly seen within the callus tissues especially in meristemoid areas.

DISCUSSION

The use of suspension cultures to establish plantations of woody species could save cost and time (Krikorian, 1987). This is because somatic embryos (George and Sherrington, 1984) can be formed in suspension cultures in very large numbers more than in callus cultures. Such embryoids according to the authors could be induced to develop into somatic seedlings when transferred to a basal medium or to a medium with lower levels of auxin than used in the initiation medium.

Results of the present study provide evidence that, friable calluses, which are the main prerequisite for establishing suspension cultures, could be produced in *P. caribaea*. This result, however, was dependent on the hormonal composition of the culture medium. Bhojwani and Razdan (1983) reported that genes determine the texture of a callus and that often it may be difficult to obtain a good dispersion of cells under any condition. However, they noted that by changing the composition of the media such as the auxin-cytokinin ratio and the subculture routine, it has been possible to improve the tissue dissociation.

Thus in the present investigation, various auxin-cytokinin combinations produced some friable calluses especially the NAA x kinetin interactions. One hundred percent friable calluses were obtained from all the treat-ment combinations. This is similar to the report of Ameh (1993) on *P. caribaea* who also obtained 100% friable calluses when MS basal medium was supplemented with IBA, BAP and kinetin in various combinations. Reynolds and Murashige (1979) have reported obtaining better cell dispersion in tobacco when the concentration of 2,4-D was increased from 0.3 to 2.0 mg/l and also when the callus medium was supplemented with additional vitamins and casein hydrolysate. Torrey and Reinert (1961) noted that auxins activate the enzymes that dissolve the middle lamella of plant cell walls, thus leading to cell dissociation (From George and Sherrington, 1984). Narayanasawamy (1977) concluded that increase in cell dispersion could be brought about by a combination of relatively high concentration of auxin and low concentration of cytokinin in the culture medium.

The structural differences observed between the friable and compact calluses have also been reported by Blakely and Steward (1964). The loosely arranged nature of friable calluses may have been the reason for their more water retention capacity as earlier reported by Reddy and Narayana (1974). Compact calluses are also important in the *in vitro* cultures because they have been associated with *in vitro* organogenesis (Kirkham and Holder, 1981; Zamora et al., 1987). The reason for this has been substantiated by Street (1977) who indicated that compact calluses have more ability to develop chlorophyll than friable calluses from the same explant. This is because chloroplast formation and integrity are favoured by cell aggregation (cited by George and Sherrington, 1984). The greening of calluses has also been associated with organogenesis (Shepard et al., 1980).

Furthermore, George and Sherrington (1984) noted that cell differentiation and morphogenesis may be pro-moted where oxygen is insufficient such as in compact calluses. On the other hand, they reported that, friable calluses admit more oxygen because the cells are loosely arranged and full respiration occurs under this condition. It may thus be concluded that friability of calluses is most often associated with somatic embryogenesis while compact calluses are readily associated with organogenesis although the reverse may be the case on some occasions. In the present study, however, greening (dev-

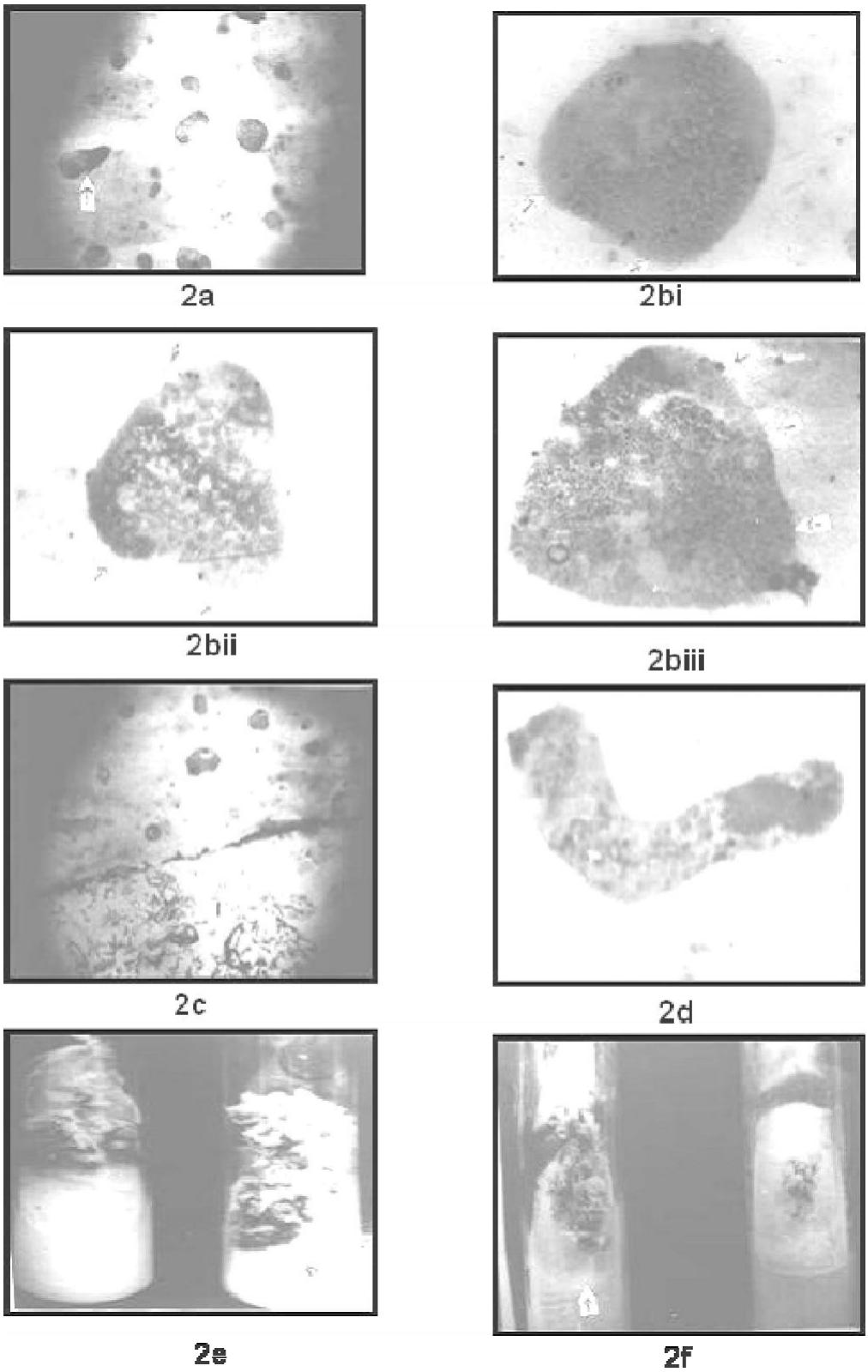

Figure 2. a) Many globular embryoids. Arrow indicates torpedo-stage embryoid, x120. bi, bii, biii) Heart shaped embryoids. Arrows indicate cotyledonary initials separated from the root poles. c) Aggregation of embryoids at the periphery of friable callus (I), x120. d) Peculiar structure observed in the callus sections, x120. e) and f) Definite structures on the surfaces of the friable calluses which were sectioned, x1.04.

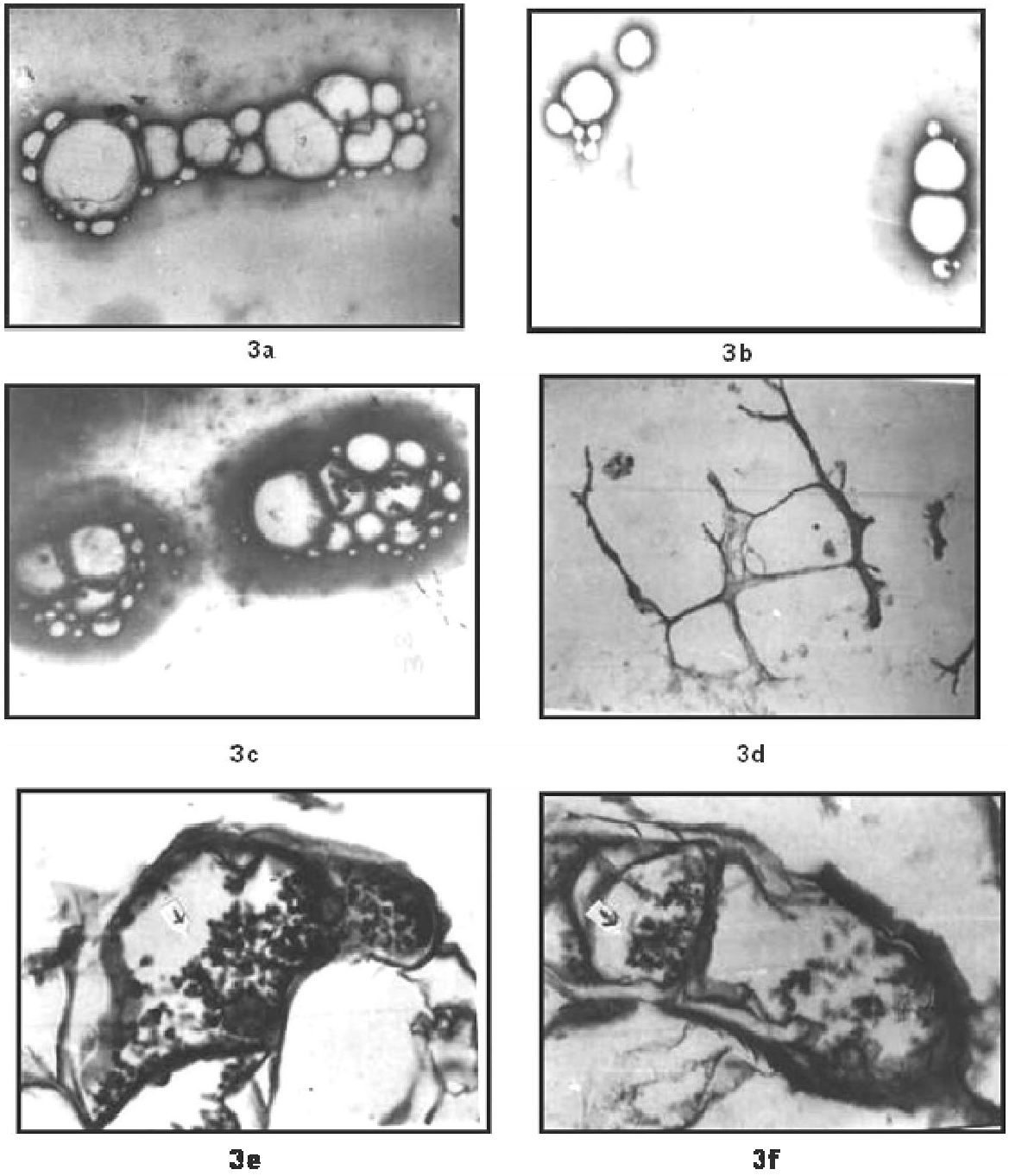

Figure 3. a, b, c) Trachaery elements with lignified cell walls, x320. d) Dead and empty parenchyma cells with lignified cell walls, x320. e) and f) arrows indicate starch grain-like structures within parenchyma cell, x400.

elopment of chlorophyll) was not confined to any type of callus. Both types (compact and friable) showed some degrees of greening.

The fact that embryo-like structures were observed in paraffin sections of calluses of *P. caribaea* indicates that the conditions for callus induction and subsequent maintenance were conducive to embryoid initiation. It has, however, been reported that it is often difficult to observe embryogenesis in unorganized structures and that in many species it has been observed unexpectedly (George and Sherrington, 1984).

Bennici (1992) also reported that it has been difficult to observe regeneration of plantlets *in vitro* through organogenesis or somatic embryogenesis in woody plants. Fortunately, results from this study have given evidence that *P. caribaea* (a woody plant) should be listed as a

species with the ability for somatic embryogenesis.

What needs clarification are the conditions for somatic embryogenesis, which were met, in the present investigation. Several authors among whom are Reynolds and Murashige (1979), Evans et al. (1981), Ammirato (1983), Raghavan (1986) have isolated a number of conditions for the induction of somatic embryos *in vitro* and these include:

a). That a high auxin concentration is often required for embryo induction but for further development of the embryos, this should be lowered or in some cases, it should be completely eliminated from the medium. 2,4-D particularly has proven extremely useful. Effective concentrations ranges are 0.5 – 27.6 µm for 2,4-D, 0.5 – 10.7 µm for NAA and 0.5 – 5.0 µm for kinetin.

b). That a substantial supply of reduced nitrogen was required for both embryo initiation and maturation.

c). That a sucrose concentration of about 2 - 3% was optimal.

d). That it is possible to increase the embryogenic potential of the callus by aging it in the medium.

e). That the chances of embryogenesis are greater with a callus that has originated from a juvenile plant.

The protocol that was used in this investigation agrees with the above criteria. Firstly, the initial calluses induction were from two weeks old seedlings, which were cultured on MS medium, supplemented with 2 mg/l 2,4-D, 0.4 g/l glutamine, 0.1 g/l casein hydrolysate (CH) and 3% sucrose (Akaneme and Eneobong, 2005). The uses of CH and L-glutamine as sources of reduced nitrogen are confirmed by the reports of Ammirato and Steward (1971) and Wetherell and Dougall (1976). According to Kamada and Harada (1979), L-glutamine is very important for somatic embryogenesis. They reached this conclusion after comparing the performance of individual amino acids in carrot cultures and they discovered that carrot somatic embryos were best promoted by L-glutamine.

Secondly, the concentrations of the growth regulators used during present study compare favourably with the effective concentrations given by previous authors. Furthermore, the important component of MS and other media for somatic embryogenesis (Ammirato, 1983) is the presence of chelated iron, which often occurs in the form of iron EDTA. According to Heberle-Bors (1980) experiments with anther cultures of *Atropa belladonna* showed that iron EDTA was superior to other iron chelates and to non-chelated iron salts. In the absence of iron, there was no development from globular to heart-shaped stage of the embryo. AE medium, which was used as the callus maintenance/regeneration medium in this study, has iron-EDTA as the sole component of group 4 (inorganic microelements). Thus, the iron-EDTA in the two media used in this investigation (MS and AE) could have led to the induction of the somatic embryos.

Thirdly, the fact that the three callus mass remained on their respective media for 5, 3 and 4 months without subculturing could also have contributed to the appearance of somatic embryos. Chang and Hsing (1980) had earlier observed that eight months of culture of callus of *Panax ginseng* without subculturing led to the production of many globular and heart-shaped embryoids.

In callus tissues, somatic embryos can be produced within or outside the tissues (Tisserat et al., 1979). According to Ammirato (1983), histological sectioning should be able to reveal that there is no vascular connection between a true zygotic or somatic embryo and the mother tissue. The observations made in this study agree with the above reports because the embry-oids were found at the periphery of the callus cells and they also showed no vascular connections whatsoever with the callus tissues. Furthermore, Haccius (1978) reported that the characteristic nature that really distin-guishes an embryo is its distinct radicular end, a fact easily discernable from the heart-shaped embryoids obtained in this study.

Early stages of embryogenesis were found to be more frequent in this study. Raghavan (1986) reported that the failure of embryoids to go beyond the early stages were only observed when callus was initiated and maintained in a single formulation of a medium containing 2,4-D and kinetin. He also noted that the concentrations of auxins and kinetin need to be reduced progressively in other to obtain full-term embryoids in culture. Chang and Hsing (1980) also observed that normal plantlets were not formed from the embryoids of *P. ginseng* when they were maintained in the old medium or transferred to a fresh medium with the same nutrient components. These reports suggest that the conditions conducive to embryo initiation in this study were not compatible with their further development.

In this study, tracheary elements were observed in paraffin sections of calluses. Webb (1981) also observed tracheary element formation in cell suspension cultures of *Pinus concorta*. According to George and Sherrington (1984), the formation of tracheids may represent or be associated with an early stage in the development of shoot meristem. Cassels (1979) observed that in callus of *Pelargonium*, the nodules, which contained xylem elements, developed into shoots when transferred to a medium devoid of auxin.

Several substances and conditions have been reported to have profound effects on vascular tissue differentiation. These include the age of culture (Torrey, 1968), auxin-cytokinin balance and the organic nitrogen constitution of the medium (Havranek and Novak, 1973), auxins and sucrose (Bhojwani and Razadan, 1983).

According to Torrey (1968), tracheary elements were only formed when agar and cell suspension cultures were maintained without sub-culturing. Wilbur and Riopel (1971) observed that sclereids were found within 700 µm of the periphery of the callus tissue of *Pelargonium horto-*

rum. They concluded that extracellular conditions of the system may have led to the sclereids occupying this peripheral position. This report could give an explanation to the position in which the tracheary elements seen in this present study occupied.

In plant cultures it is common to observe differentiation of lignified elements from parenchyma cells (Chen and Galston, 1967). According to Schroeder (1961) the effect of maintaining cultures on agar containing medium without subculturing is that active growth practically ceases. The author, however, noted that such cultures still remain alive but may develop a great amount of lignified tissue, and concluded that in this condition there is activation of genes that code for cytodifferentiation and this leads to the observation of many differentiated cells. Wetmore (1955) reported that auxins have been implicated in the synthesis of lignin.

Of interest again in this study was the accumulation of starch grain-like structures in some of the parenchymatous cells. Thorpe and Murashige (1968) reported that in tobacco callus there was accumulation of starch prior to the initiation of shoot but that they subsequently disappeared. They concluded that the starch may have provided energy during the formation of shoot primordia. Investigations of Thorpe and Meier (1972) support the results of the above authors (as indicated by George and Sherrington, 1984). They observed that periods of heavy starch accumulation occur at the time of appearance of meristemoids, which are associated with shoot initiation. Wilbur and Riopel (1971) also observed the presence of starch accumulation and sclereid formation in the same group of cells.

Inspite of these observations, George and Sherrington (1984) noted that the accumulation of starch and morphogenesis are sometimes not correlated. Citing Van Huystee (1977), they reported that prolonged culture of cells on sucrose containing medium may lead to such cells losing the capacity to synthesize chlorophyll and ultimately converting the plastids into amyloplasts packed with starch.

In conclusion, the starch-grain like structures seen in this study was found in the same callus tissues in which tracheary elements and embryo-like structures were observed. Whether their presence was as a result of prolonged culture on sucrose – containing media remains to be confirmed. This study has clearly demonstrated the inherent totipotent nature of the somatic cells of *P. caribaea*.

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