Arch. Biol. Sci., Belgrade, 63 (4), 1135-1145, 2011 DOI:10.2298/ABS1104135O

PLANT REGENERATION VIA SOMATIC EMBRYOGENESIS FROM IMMATURE LEAVES IN *TETRAPLEURA TETRAPTERA* (SCHUM. & THONN.) TAUB.

J. T. OPABODE*, O. A. AKINYEMIJU and O. O. AYENI

Department of Crop Production and Protection, Obafemi Awolowo University, Ile-Ife, Nigeria

Abstract - Plant regeneration via somatic embryogenesis was assessed using immature leaf, petiole and apical meristem explants in *Tetrapleura tetraptera.* Somatic embryos were induced in the immature leaf using MS basal medium supplemented with 2,4-D and matured on MS basal medium containing BAP. Medium supplemented with 12 mg/l 2,4-D had the highest (43.1%) percentage of embryogenic calluses from immature leaf explants. Conversion of embryogenic callus to mature primary somatic embryo occurred in the medium that contained 1.2 mg/l BAP. Development of secondary embryogenic calluses to matured secondary embryos was highest (98.0%) in the medium with 0.4 mg/l BAP, while the highest average number of mature secondary embryos (6.0) was obtained in the same medium. Medium supplemented with 1.0 mg/l BAP and 0.5 mg/l IBA had the highest (38.7%) percentage of explants with shootbuds. The highest (18.1%) percentage of shoot elongation was obtained in medium with 1.0 mg/l BAP and 20 mg/l IBA. Shootbuds survived and produced roots on medium free of plant growth regulators. Shoots obtained on medium supplemented with 1.0 mg/l BAP and 20 mg/g IBA recorded the highest number of roots per plantlet (7.5) with no apparent morphological abnormality.

Key words: Tetrapleura tetraptera, woody legume, Fabaceae, somatic embryogenesis, plant regeneration

Abbreviations - 2,4-D – 2,4-Dichlorophenoxyacetic acid; BAP – 6-Benzylaminopurine; IAA – Indole-3-acetic acid; IBA – Indole-3-butyric acid; MS – Murashige and Skoog medium; PES – ; NAA – α-Naphthaleneacetic acid; PES – Pre-embryogenic structure;

UDC 582.736.3:581.143.5

INTRODUCTION

Tetrapleura tetraptera (Schum. & Thonn.) Taub is a woody legume and native of West Africa. The trees are widespread in tropical Africa, especially as secondary forest, and they are at their best in the rainforest belt (Opeke, 1982; Keay, 1989). The tree is deciduous, reaching 20 – 25 m in height, with a girth of 1.5 – 3 m. The trunk is slender and older trees have very small, low, sharp buttresses. In the forest, the crown is fairly small, thin and rounded, becoming flat when old, but it tends to spread when in the open. Leaves are sessile, glabrous or minutely hairy with a common stalk 15 – 30 cm long, slightly channeled on the upper surface (Opeke, 1982). Flowers are pinkish-cream turning to orange and are densely crowded in spike-like racemes 5 – 20 cm long, usually in pairs in the upper leaf axils; individual flowers have slender stalks and 10 short stamens, the anthers carrying a gland at the apex. The fruit is very persistent, hanging at the ends of branches on stout stalks 25 cm long. In Nigeria, the tree is deciduous in December, flowering begins towards the end of February and is over in early April. The indehiscent pods are ripe from September to December. When the pods fall, their smell attracts small animals that probably disperse the seeds. Trees regenerate from seeds, and germination begins on the 6th day. Seed

storage behavior is orthodox, and there are about 6290 seeds/kg (Keay 1989).

In West Africa, *T. tetraptera* is used as a spice, a medicine and as a dietary supplement rich in vitamins. The fruit pulp is rich in sugars and tannins, and may be used in flavoring. The leaves, bark, roots and kernels are used for medicinal purposes (Keay, 1995), and fruits and flowers are used as perfumes and in pomades prepared from palm oil. It has found use as timber for the production of reddish to brown, fairly hard heartwood and white sapwood. Recently, it was found that the methanol extract of *T. tetraptera* tois be effective in reducing the population of snails, the causative organism of schistosomiasis. An ethnobotanical survey of medicinal plants in Ghana confirmed the use of *T. tetraptera* in the treatment of hypertension through its diuretic actions. Other therapeutic uses include management of convulsions, leprosy and rheumatic pain (Ojewole, 2005: Uchechi et al., 2010).

Despite the numerous uses of *T. tetraptera*, limited attention is given to its production and associated constraints. *Tetrapleura tetraptera* grows in the wild in both forests and savanna, and has a long growth cycle. There is no documented evidence of modern cultivation and genetic improvement of the tree. The seeds of *T. tetraptera* suffer from seed dormancy. The tree is exposed to annual bush fires which is reducing its population rapidly across West Africa. Several pests and diseases have been observed on the trees (Opeke, 1982). Application of biotechnology is a viable means of changing the status of *T. tetraptera* from a local plant on the verge of extinction to an internationally economically significant plant. *In vitro* propagation of *T. tetraptera* has been attempted (Ibikunle, 1998). *In vitro* shoot cultures were established and maintained on MS basal medium supplemented with 1.0 mg/l BAP and 0.5 mg/l IBA from shoot tip explants (Ibikunle, 1998). Somatic embryogenesis has a great potential for mass propagation and exchange of germplasm of *T. tetraptera*. In addition, somatic embryogenesis will provide materials for the cryopreservation and development of artificial seed technologies for the tree. Furthermore, establishment of somatic embryogenesis in *T. tetraptera* will open the tree to modern approaches of genetic improvement by transgenic technology. This report describes the production of somatic embryogenesis and *in vitro* regeneration in *T. tetraptera*.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of 20- to 30-year old *Tetrapleura tetraptera* trees grown on the Obafemi Awolowo University campus were collected and chemically scarified with sulfuric acid as described by Ayeni (2005). Healthy and vigorous seedlings were raised from the scarified seeds in plastic pots at the screen house, Faculty of Agriculture, Obafemi Awolowo University, Ile-Ife, Nigeria. The *T. tetraptera* seedlings were maintained by daily manual watering.

Basal medium and culture conditions

Basal medium (BM) which consisted of full-strength MS (Murashige and Skoog, 1962) salt (Sigma, USA) along with 0.8% (w/v) agar (Oxoid Ltd., England) and 30 g/l sucrose, was used in all experiments unless otherwise stated. The pH of the medium was adjusted to 5.8 by HCl (1 N) or NaOH (1N) prior to autoclaving at 121°C for 15 min at 1.05 kg cm^{-2} pressure. Growth regulators were filter-sterilized through 0.22-µM Millipore filters and added to media after autoclaving. For all experiments, cultures were maintained in a 16 h photoperiod with 20 *l*mol m^{-2} s^{-1} light intensity provided by cool-white fluorescent tubes at 25 ± 2°C.

Induction and maturation of primary somatic embryos from apical meristem, petiole and immature leaves

Explants (apical meristem, immature leaf and petiole) were collected from actively growing 3-month old *T. tetraptera* seedlings. The explants were disinfected by immersion in 1.5% sodium hypochlorite solution for 20 min and rinsed six times with ster-

ile water. Disinfected explants were blotted on dry paper before being placed on medium. Immature leaf explants (about 4-6 mm long) were prepared by first removing the mid-rib to release the leaflets and further cut the apex and base of disinfected leaflets. Petiole explants were prepared by cutting the excised disinfected petiole into 1-2 cm pieces while shoot apical meristems were obtained from disinfected apical buds. For induction of primary somatic embryos, explants (apical meristem, immature leaf and petiole) were incubated in darkness on basal medium supplemented with different (2.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 16.0 mg/l) concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma, USA). Each 100 x 20 mm^2 Petri dish (Corning Inc., USA) contained 10 explants and a total of 100 explants were used per treatment. Petri dishes containing explants were sealed with parafilm. Responses of the explants to treatment were monitored daily using a microscope. At 4 and 6 weeks after incubation, plates were scored for the formation of callus and presence of pre-embryogenic structure (PES), respectively. For the maturation of embryos, explants with developing pre-embryogenic structure were transferred to basal medium supplemented with different (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 mg/l) concentrations of 6-benzylaminopurine (BA) (Sigma, USA) and incubated in the dark. At 3 weeks after dark incubation, plates were scored for the presence of matured (cotyledonary-stage) primary somatic embryo.

Induction and maturation of secondary embryos

Primary somatic embryos obtained from immature leaves were used for secondary embryogenesis. Cotyledons of primary somatic embryos were fragmented into pieces of about 5 mm in size which served as explants for the induction of secondary somatic embryos. Primary somatic embryo cotyledon fragments were cultured on basal medium supplemented with different (2.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 16.0 mg/l) concentrations of 2,4-D. Reponses of the explants were monitored daily with the aid of microscopes. After two and three weeks of incubation, plates were scored for the presence of calluses and pre-embryogenic structures, respectively. Explants with embryogenic structures were transferred to basal medium supplemented with different (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 mg/l concentrations of BAP for maturation. Plates were scored for the presence of matured (cotyledonary-stage) secondary somatic embryos after four weeks of dark incubation. Each Petri dish contained 20 explants and a total of 200 explants were used per treatment.

Shoot induction and elongation

Cotyledons of matured secondary embryos were fragmented into 6 mm pieces. The cotyledon pieces were cultured on basal medium supplemented with 1.0 mg/l BAP and different (0.1, 0.3, 0.5, 0.7, 0.9, 1.1 and 1.3 mg/l) concentrations of indole-3-butyric acid (IBA) (Sigma, USA). Plates were scored for the presence of shoot buds after 14 days of incubation in the light. Shoot buds were detached from the explants and transferred to basal medium supplemented with 1.0 mg/l and different (1.0, 5.0, 10.0, 15.0, 20.0 and 25.0 mg/l) concentrations of IBA for shoot elongation. Plates were scored for the presence of elongated shoots per treatment.

Hardening of plantlets and soil establishment

About eight weeks after successful root formation by the elongated shoots, plantlets were removed from the culture tubes and placed in plastic bags half-filled with peat and vermiculite (50:50). Immediately after transplanting, the plantlets were placed in a humidity chamber and gradually exposed to a source of natural light. At six weeks after transplanting, the plants were transferred to plastic cups filled with rich soil.

Experimental design and statistical analysis

All experiments were arranged in a completely randomized design with four replications, and experiments were repeated at least thrice. Percent and count data were subjected to arcsine and square root transformation to normalize variances. Data were further subjected to analysis of variance to detect differences among treatments. Means were separated by Duncan's Multiple Range Test at 5% level of probability.

Table 1. Effect of concentrations of 2,4-D on induction of primary embryo in immature leaves explant of *T. tetraptera*.

Concentration of 2,4-D (mg/l)	% of immature leaf explants that formed callus	% of calluses with pre-embryogenic structure	Average number of pre-embryogenic structures/ calluses
0.0	0.0d	0.0d	0.0d
2.0	35.3c	0.0d	0.0d
4.0	38.4b	0.0d	0.0d
6.0	40.0b	0.0d	0.0d
8.0	41.3b	12.3b	1.2c
10.0	83.1a	12.4b	1.3c
12.0	85.5a	43.1a	3.2a
14.0	84.7a	8.4c	2.7b
16.0	80.2a	8.2c	2.4b

Means within the same column followed by same letters are not significantly different by Ducan's Multiple Range Test at 5% probability level. The values are means of four replications.

RESULTS

Induction of primary embryos from immature leave explants

Calluses started developing at 9 days after incubation from the cut edge of the leaves and extended into the interior in the responded leaves (Fig.1C). The green color of the leaves disappeared as the calluses were developing. The concentrations of 2,4-D had significant ($P<0.05$) effect on the formation of calluses and the presence of pre-embryogenic structures (PES) in the calluses (Table 1). The PES is globular in shape with bipolar structures and light-brown in color (Fig.1D). The basal medium supplemented with 10 -14 mg/l 2,4-D recorded the highest percentages (83 - 85%) of calluses. Pre-embryogenic structures were not developed in medium supplemented with 0-6 mg/l 2,4-D. The highest (43%) percentage of calluses with embryogenic structures and the number of embryogenic structures per callus was obtained in the medium that contained 12 mg/l of 2,4-D (Table 1).

Maturation of primary somatic embryos

When explants (calluses with embryogenic structures) were transferred to medium supplemented with different concentrations of BAP, they all survived. However, the concentrations of BAP had a significant ($P<0.05$) effect on the percentage of matured somatic embryos and number of matured embryos per explant (Table 2). The basal medium supplemented with 1.2 mg/l BAP had the highest percent (37.4%) of matured primary somatic embryos; however, the medium that contained 1.0 mg/l recorded the highest number of matured embryos per explant. The embryos of media that were supplemented with 0.2-0.8 mg/l BAP emerged from the base of explants and had yellow coloration, while the embryos in media with 1.0-1.6 mg/l BAP were located on the top of the explant and were whitish-yellow in color (Fig. 1F).

Induction and maturation of primary embryos from apical meristem explants

After 6 days of incubation in the dark, calluses started appearing on responding explants. The calluses were yellowish-white in media containing 2-8 mg/l 2,4-D while calluses in media supplemented with 10-16 mg/l 2,4-D were light-brown (data not shown). The concentration of 2,4-D had a significant ($P<0.05$) effect on the formation of calluses and the presence of embryogenic structures in the calluses (Table 3). The highest percentage (43.2%) of apical meristem explants that formed calluses was obtained in medium containing 14 mg/l 2,4-D, while no calluses formed in the medium that contained no 2,4-D. The highest percentage (70.2%) of calluses that contained pre-embryogenic structures occurred in medium that

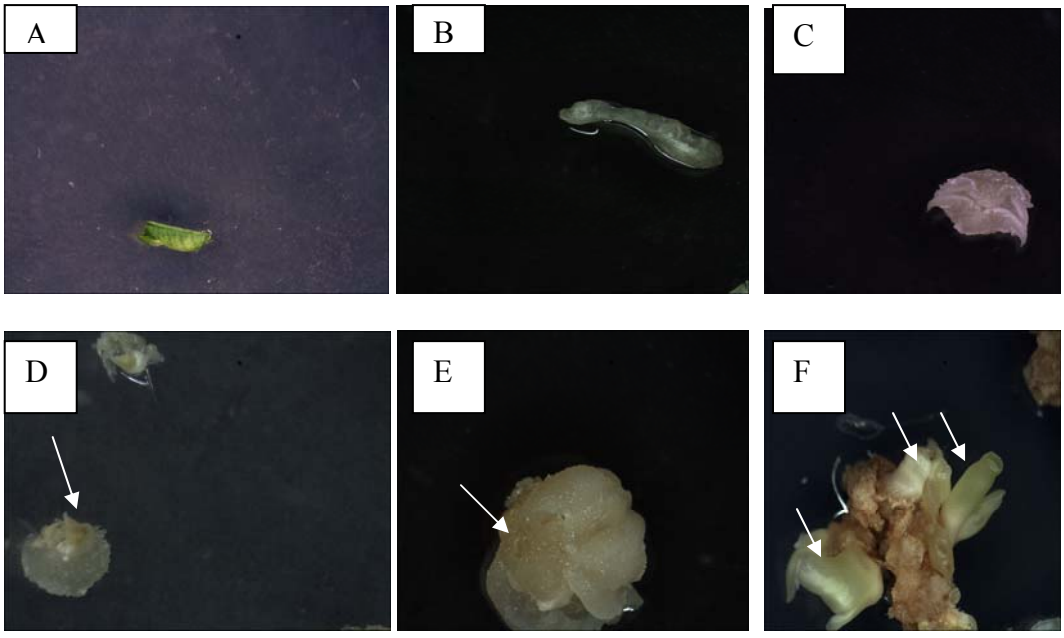

Fig. 1. Primary somatic embryogenesis in *Tetrapleura tetraptera***.** (A) a trimmed immature leaflet explant (x3); (B) 4-day old immature leaflet cultured on MS basal medium supplemented with 12 mg/l 2,4-D (x3); (C) two-week old callus on medium supplemented with 12 mg/l 2, 4 – D (x6); (D) four-week old embryogenic callus with pre-embryogenic structure (arrowed) on medium containing 12 mg/l 2,4-D (x6); (E) five-week old embryogenic callus with globular embryogenic structure (arrowed) on medium supplemented with 1.2 mg/l BAP (x6), (F) cotyledonary-stage primary somatic embryos (arrowed) on medium containing 1.2 mg/l BAP (x6).

was supplemented with 10 mg/l 2,4-D (Table 3). Calluses in media supplemented with 10 and 12 mg/l had two pre-embryogenic structures per explants. Calluses obtained from the apical meristems were larger than calluses obtained from immature leaves and petiolar explants. When developing pre-embryogenic structures were transferred to basal medium supplemented with different concentrations of BAP and incubated in the dark for maturation, none matured; they turned dark brown (data not shown).

Induction and maturation of primary embryos from petiole explants

In those explants that responded to treatment, calluses started forming 8 days after dark incubation from the edge of the petiole (Table 3). Initially, the calluses were whitish-yellow and later turned brown. The concentration of 2,4-D had significant ($P<0.05$) effect on the formation of calluses (Table 3). Only media that contained 8-16 mg/l 2,4-D produced calluses in the range of 5-23.4%, while other media with lower 2,4-D concentrations formed no calluses. Only calluses formed on media that were supplemented with 10 and 12 mg/l 2,4-D had pre-embryogenic structures (3.2-3.5%) with one pre-embryogenic structure per explant. The PES obtained from petiolar explants was a mass with limited distinct bipolar structure and dark brown. As in the apical meristem explants, when developing pre-embryogenic structures were transferred to basal medium supplemented with different concentrations of BAP and incubated in the dark for maturation, none matured; they shrivelled and turned dark brown (data not shown).

Induction and maturation of secondary somatic embryos

There was a significant ($P<0.05$) influence of the concentration of 2,4-D on the percentage of primary somatic cotyledon explants that formed calluses. Similarly, concentrations of 2,4-D had significant

Table 2. Effect of concentrations of BAP on maturation of primary embryos

Concentration of BAP (mg/l)	%PES that formed matured embryos	average number of matured embryo/ explant	Location of embryo on explant	Color of the embryo
0.0	0.0e	0.0c	-	-
0.2	5.3d	2.3b	base	yellow
0.4	6.7d	2.2b	base	yellow
0.6	15.4c	2.3b	base	yellow
0.8	15.3c	2.2b	base	yellow
1.0	35.3a	4.4a	top	Whitish yellow
1.2	37.4a	2.1b	top	Whitish yellow
1.4	30.6b	2.3b	top	Whitish yellow
1.6	30.3b	2.3b	top	Whitish yellow

Means within the same column followed by same letters are not significantly different by Ducan's Multiple Range Test at 5% probability level. The values are means of four replications.

Table 3. Induction of primary somatic embryos from apical meristem and petiole explants as affected by 2,4-D concentration.

Concentration (mg/l)	Apical meristem explant			Petiolar explant		
	% explant that formed callus	% callus with PES	Average number of PES/explant	% explant that formed callus	% callus with PES	Average number of PES/explant
0.0	0.0c	0.0e	0.0d	0.0c	0.0b	0b
2.0	7.5d	15.3d	1.0c	0.0c	0.0b	0b
4.0	7.6d	10.2d	1.0c	0.0c	0.0b	0b
6.0	10.2c	23.2c	1.5b	0.0c	0.0b	0b
8.0	10.4c	21.8c	1.3b	5.0b	0.0b	0b
10.0	25.3b	70.2a	2.4a	5.3b	3.5a	1a
12.0	37.2a	68.2a	2.2a	8.3b	3.2a	1a
14.0	43.2a	43.6b	1.0c	10.5b	0.0b	0b
16.0	40.1a	45.4b	1.0c	23.4a	0.0b	0b

Means within the same column followed by same letters are not significantly different by Ducan's Multiple Range Test at 5% probability level. The values are means of four replications.

Table 4. Effect of concentration of 2,4-D on induction of secondary somatic embryos

Concentration of 2, 4 –D (mg/l)	% explants with calluses	% calluses with PES	Average number of PES per callus
0.0	0.0d	-	-
2.0	82.4c	100.0a	3.1a
4.0	80.4c	100.0a	4.5a
6.0	85.7b	73.0b	3.2a
8.0	90.4b	42.3c	2.5b
10.0	100.0a	37.2c	1.0c
12.0	100.0a	15.2d	1.0c
14.0	100.0a	6.2e	1.0c
16.0	100.0a	6.7e	1.0c

Means within the same column followed by same letters are not significantly different by Ducan's Multiple Range Test at 5% probability level. The values are means of four replications.

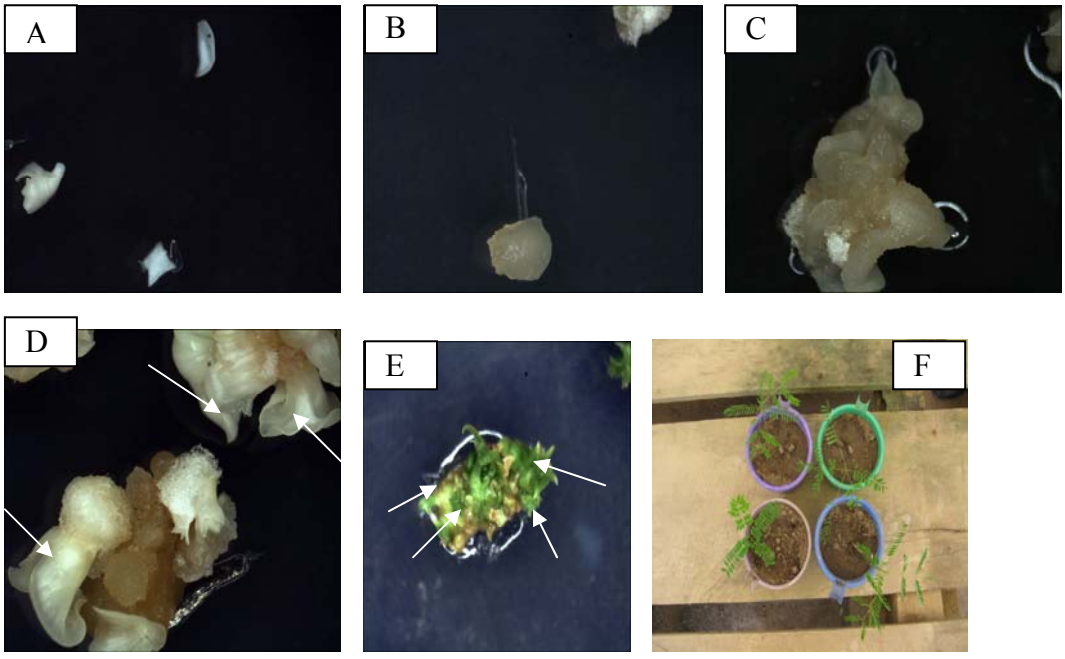

Fig. 2:Secondary embryogenesis and regeneration in *Tetrapleura tetraptera* (A) primary somatic embryo cotyledonal fragment (x3); (B) two-week old callus (1/2); (C) three-week old embryogenic callus (x1); (D) cotyledonary-stage secondary somatic embryos (arrowed) (x1); (E) Multiple shootbuds (arrowed) from secondary embryo cotyledon explant on medium supplemented with 1.0 mg/l BAP and 0.9 mg/l IBA (x2); (F) Acclimatized regenerated plantlets from immature leaf-derived somatic embryo (x2).

($P<0.05$) effect on the percentage of calluses that contained PES (Table 4). All culture media containing 2 and 4 mg/l 2,4-D had 100% calluses with PES, while medium supplemented with 6-16 mg/l 2,4-D had between 6.7-73% calluses with PES. The highest number of PES per callus was obtained in media with 4 mg/l 2,4-D. Concentrations of BAP significantly ($P<0.05$) affected the percentage of PES that developed into cotyledonary-stage embryos (Table 5). The largest percentage (98%) of cotyledonary-stage embryos was obtained in medium supplemented with 0.4 mg/l BAP, while the least (32.2 %) was obtained in medium supplemented with 111.6 mg/l BAP. The concentration of BAP had a significant effect on the number of matured somatic embryos. The highest number of matured embryo per explant (6.7 per explant) was obtained in medium containing 0.4 mg/l, while the least (2 per explant) was produced in media containing 1.4 and 11.6 mg/l BAP. Embryos on media containing 0.2-0.8 mg/l were evenly distributed on the calluses and yellow, while those of media containing 1.0-1.6 mg/l BA were located on the top of the explants and whitish in color (Fig. 2D).

Shoot induction

To evaluate the ability of the somatic cotyledon to form shoots, cotyledonal explants were cultured on basal medium supplemented with 1.0 mg/l BA and various concentrations of IBA. When cotyledonal explants were cultured on basal medium supplemented with 0.1 – 0.7 mg/l IBA, all the explants survived. However there was a decrease (90.5-95.3%) in explant survival when transferred to medium supplemented with 1.0-1.5 mg/l IBA (Table 6). The concentration of IBA had a significant ($P<0.05$) effect on the formation of shootbuds. Medium supplemented with 0.5 mg/l IBA had the highest percentage (38.7%) of shootbuds. There was a significant ($P<0.05$) effect of IBA concentration on the number of shootbuds per explant (Table 6). The medium supplemented with 1.0 mg/l BAP and 0.9 mg/l IBA produced vigorous and green shootbuds,

Table 5: Maturation of secondary somatic embryos as affected by BAP concentrations

Concentration of BAP (mg/l)	% matured embryos	Average number of matured embryos per explant	Location of embryo	Color of embryo
0.0	0.0e	-	-	-
0.2	96.4a	5.4b	even	yellow
0.4	98.1a	6.7a	even	yellow
0.8	80.3b	4.3b	even	yellow
1.0	65.2c	3.2c	top	whitish
1.2	37.2d	3.1c	top	whitish
1.4	37.6d	2.0d	top	whitish
1.6	32.4d	2.0d	top	whitish

Means within the same column followed by same letters are not significantly different by Ducan's Multiple Range Test at 5% probability level. The values are means of four replications.

Table 6. Effect of BAP and IBA on shoot induction of *T. tetraptera*

BAP + IBA (mg/l)	% explant survival	% explant with shootbuds	Average number of shootbud/explant	Appearance of shoot-buds
1.0 + 0.0	100a	0.0c	0.0c	-
1.0 + 0.1	100a	35.3a	2.3b	Green, fragile
1.0 + 0.3	100a	37.3a	2.4b	Green, fragile
1.0 + 0.5	100a	38.7a	3.2a	Green, fragile
1.0 + 0.7	100a	35.6a	3.4a	Green, fragile
1.0 + 0.9	95.3b	10.3b	2.4b	Green, vigorous
1.0+ 1.1	93.2b	9.3b	2.0b	Pale, callused
1.0+ 1.3	90.5b	9.2b	2.0b	Pale, callused

Means within the same column followed by same letters are not significantly different by Ducan's Multiple Range Test at 5% probability level. The values are means of four replications.

Table 7. Effect of BAP and IBA on shoot elongation and root formation in *T. tetraptera.*

BAP + IBA (mg/l)	Explant survival (%)	% elongated shoots	Appearance of shoots	% explants with roots	Average number of roots per explant	Description of plantlets
1.0 + 0.0	100.0a	0.0d	-	0.0c	0.0c	-
1.0 + 1.0	100.0a	3.4c	fragile	100.0a	3.4b	Wrinkled leaves
1.0 + 5.0	100.0a	3.6c	yellowish	100.0a	3.4b	Callused base
1.0 + 10.0	100.0a	12.3a	yellowish	100.0a	4.2b	Curled stem
1.0 + 15.0	100.0a	15.3a	fragile	100.0a	4.2b	Curled stem
1.0 + 20.0	100.0a	18.1a	vigorous	100.0a	7.5a	normal
1.0 + 25.0	100.0a	9.3b	pale	100.0a	6.2a	Thick stem
1.0 + 30.0	93.2b	5.2c	pale	85.0b	6.2a	Thick stem

Means within the same column followed by same letters are not significantly different by Ducan's Multiple Range Test at 5% probability level. The values are means of four replications.

while shootbuds in media containing other concentration of IBA were either fragile or pale in appearance.

Shoot elongation and root formation

Green and vigorous shootbuds produced in the medium supplemented with 1.0 mg/g BAP and 0.9 mg/l IBA were used for shoot elongation study. All explants survived in media containing different concentrations of IBA except the medium containing 30 mg/l IBA which recorded 93.2% (Table 7). The concentration of IBA had significant ($P<0.0$) effect on shoot elongation, while the medium with 20 mg/l IBA had the highest percentage (18.1) of shoot elongation. In addition, medium supplemented with 20 mg/l IBA also produced vigorous shoots while others were fragile, yellowish or pale in appearance (Table 7). When transferred to medium free of plant growth regulators, all explants survived and produced roots, while shoots obtained in medium supplemented with 20 mg/g IBA recorded the highest number of root per plant (7.5) and appeared normal.

DISCUSSION

Somatic embryogenesis has been described for several woody leguminous plants (Lakshmanan and Taji, 2000; Vidoz et al., 2006; Corredoira et al., 2008). The results of these studies suggested that somatic embryogenesis response is determined by several factors including plant species, genotype, age, explant type, season, carbon source, type and concentration of exogenous growth regulators. In the present study, we investigated the production of somatic embryos and *in vitro* regeneration in excised immature leaves, apical meristems and petiolar segments from actively growing 3-month old seedlings of *T. tetraptera* at various concentration of 2,4-D. Our study showed that immature leaves were the most responsive explants out of the three explants examined. Successful induction and maturation of somatic embryos in woody plants using leaf explants have been documented (Cid et al., 1999; Fisichella et al., 2000; Kumar et al., 2002; Vidoz et al., 2006; Liu et al., 2007; Corredoira et al., 2008; Lim et al., 2010). Similarly, immature leaves have been used as explants to induce somatic embryos capable of regeneration in woody leguminous plants such as *Arachis correntina* (Vidoz et al., 2006), *Paulownia tomentosa* (Corredoira et al., 2008) and *Cladratis lutea* (Weaver and Trigiano, 1991). Young meristematic tissues, such as developing leaves and immature embryos, have become widely used explants for the establishment of somatic embryogenesis in both herbaceous and woody leguminous plants. This is attributed to the fact that meristematic cells are capable of reprogramming their activities under stress imposed by exogenous factors to produce organized pro-embroyogenic structures. Jimenez and Thomas (2006) suggested that high levels of endogenous auxin might make immature leaves more responsive than other explants. Furthermore, molecular evidence has suggested that somatic embryogenesis occurs if the genes responsible for the embryogenic developmental program are released from chromatin-mediated gene silencing in vegetative cells. This can happen in response to strong aspecific signals, such as high dose and/or sublethal stress, which evoke the activation of large chromatin regions according to Feher (2005). This hypothesis may explain why less differentiated cells (e.g. immature leaves) are more amenable to somatic embryogenesis and why various aspecific signals can evoke similar embryogenic response (Feher, 2008).

Embryogenic calluses were induced with 2,4-D and the growth regulator affected the number of embryogenic calluses obtained from immature leave explants. 2,4-D alone and in combination with other plant growth regulators, has been widely used to induce somatic embryos in leguminous woody plants (Lakshmanan and Taji, 2000). This is because 2,4-D is a good agent for the establishment of auxin synthesis and polar auxin transport which is a key step in the meristem formation underlying embryo development (Nawy et al., 2008). Imposition of stress on explants is another role of 2,4-D by which the somatic embryo is inducted (Zavattieri et al., 2010). It has been suggested that the embryogenic effect of 2,4-D probably derives from its methylating action on the nuclear DNA (De Klerk et al., 1997; Sharma et al., 2007).

Using the petiole as an explant, we obtained calluses with pre-embryogenic mass in media contain-

ing 5-8.3 mg/l 2,4-D which could not develop to a mature embryo. Like embryogenic calluses from petiolar explants, embryogenic calluses obtained from apical meristem explants also failed to produce mature embryos. Although PESs were observed in calluses that failed to produce mature somatic embryos, the induction phase of the somatic embryos could not be considered to be completed because conversion of PESs to mature embryos is not a major challenge in plant species that are amenable to somatic embryogenesis (Feher, 2008). Several reasons could be adduced to the failure of PES in petiolar and apical meristem explants to convert to matured somatic embryos. It is possible that the PES did not synthesize all the gene products necessary to complete the globular stage of embryogenesis at the time of transfer from 2,4-D medium. Also, the removal of PES from 2,4-D might not inhibit proteins and mRNAs whose continued presence generally inhibits the continuation of the embryogenic program. Other factors which might have contributed to the failure of PES to become mature embryos are structural factors such as the initiation of polarity in embryos, cell walls and microtubule organization (Jimenez, 2001), physiological factors which include secretions of proteins and cellular factors into culture medium exogenous plant hormones, endogenous plant hormones in culture medium, endogenous plant hormones in initial explants and their interactions (Kobayashi et al., 2000).

The primary somatic embryos obtained from immature leaves went into developmental arrest and refused to produce shootbuds on media containing basal medium that was supplemented with either BAP alone or in combination with different concentrations of IBA. As a result, we attempted secondary somatic embryogenesis and successfully produced secondary embryos using primary somatic embryo cotyledon explants. The induction and maturation of secondary somatic embryos was achieved at lower concentrations of 2,4-D and BA, respectively, than for the production of primary somatic embryos. In addition, the induction and maturation of secondary somatic embryos occurred in greater percentages than in primary somatic embryo production. Secondary embryogenesis has been reported in woody plants like black locust (Arrillaga et al., 1994), *Quercus ilex* L (Mauri and Manzanera, 2002) and sandalwood (Rai and McComb, 2002). We obtained green, vigorous shootbuds and elongated shoots on medium supplemented with BA and IBA. Germination of somatic embryos took place on medium fortified with plant growth regulators in woody plants (Kumar et al., 2002; Vidoz et al., 2006; Lin et al., 2010). Somatic embryos of *Pulsatilla koreana* Nakai developed shoots on MS medium supplemented with 0.25 mg/l Zeatin and 0.5 mg/l IAA (Lin et al., 2010). Also, shootbuds were formed on somatic cotyledons cultured on medium containing MS fortified with 10.74μM NAA and 0.044 μM BAP in *Arachis correntina* (Vidoz et al., 2006). Similarly, in *Hippophae rhamnoides*, somatic embryos germinated on Schenk and Hildebrandt (SH) medium supplemented with 1.0 mg/l kinetin and 0.5 IAA. In contrast, somatic embryos have been reported to germinate on hormone-free media in *Cladratis lutea* (Weaver and Triagiano 1991) and in *Calliandra tweedii* (Kumar et al., 2002). In our study, somatic embryos refused to produce shootbuds and elongated shoots on hormone-free medium. Root formation was achieved on media free of hormones. The formation of roots by *in vitro* regenerated shoots have been reported in hormone-free media in *Cladratis lutea* and *Calliandra tweedii* (Weaver and Trigiano, 1991; Kumar et al., 2002)

About 76% of the plantlets successfully passed through the hardening process. After transfer to the potted soil, the plants grew well with no abnormal morphological structures.

In conclusion, we have developed primary and secondary somatic embryos from immature leaf explants using 2,4-D which are capable of *in vitro* regeneration through the combination of BAP and IBA to normal plantlets. The *in vitro* regeneration system will open the woody legume to improvement by biotechnological approaches.

Acknowledgement - The authors are grateful to Mr S. A. Ojo for his excellent technical assistance.

REFERENCES

Arrillaga, I., Tobolski, J. and S. *A. Merkle* (1994). Advances in somatic embryogenesis and plant production of black locust (*Robinia pseudoacacia* L.). *Plant Cell Rep*. **13**, 171-175.

Ayeni, O.O (2005) *Breaking seed dormancy in Tetrapleura tetraptera* (Schum. & Thonn.) Taub. Unpublished B. Agric. Thesis, Obafemi Awolowo University, Nigeria, 20pp.

Cid, L.P.B, Machado, C.M.G, Carvalheira, B. R .C. and *A. C. M. Brasileiro* (1999). Plant regeneration from seedling explants of *Eucalyptus grandis* x *E. urophylla*. *Plant Cell Tiss. Organ Cul.* **56**, 17-23.

Corredoira, E., Ballester., A. and *A. M. Vietez* (2008) Thidiazuron-induced high-frequency plant regeneration from leaf explants of *Paulownia tomentosa* mature trees. *Plant Cell Tiss. Organ Cul*. **95**, 197-208.

De klerk, G-J., Arnholdt-Schmitt, B., Lieberei, R. and *K. H. Neumann* (1997). Regeneration of roots, shoots and embryos: physiological, biochemical and molecular aspects. *Bio. Plant*. **39**, 53-66.

Fehér, A. (2008). The initiation phase of somatic embryogenesis: what we know and what we don't. *Acta Biol. Szeged.,* **52**, 53-56.

Fehér, A. (2005). Why somatic plant cells start to form embryos? In: *Somatic Embryogenesis* (Eds A. Mujid and J. Samaj) Plant Cell Monographs, vol. **5**, 85-101 Springer, Berlin/ Heidelberg.

Fisichella, M., Silvi, E. and *S. Morini* (2000). Regeneration of somatic embryos and roots from quince leaves cultured on media with different macroelement composition. Plant Cell *Tiss. Cul*. **63**, 43-51

Ibikunle, A. A. (1998). Micropropagation of *Tetrapleura tetraptera* (Schum. & Thonn.) Taub. Unpublished B.Sc Thesis University of Uyo, Nigeria, 27pp

Jiménez, V. M. (2001). Regulation of *in vitro* somatic embryogenesis with emphasis on the role of endogenous hormones. *Rev. Brasil. de Fisiol. Veget.* **13**, 196-223.

Jiménez, V. M. and *C. Thomas* (2005) Participation of plant hormones in determination and progression of somatic embryogenesis. In: *Somatic Embryogenesis* (Eds A. Mujid and J. Samaj) Plant Cell Monographs, vol. 5, 103-118 Springer, Berlin/Heidelberg.

Keay, R. W. (1989). Trees of Nigeria. Clarendon Press, Oxford

Kobayashi, T., Higashi, K., Sasaki, K., Asami, T. and *H. Kamada* (2000). Purification from conditioned medium and chemical identification of a factor that inhibits somatic embryogenesis in carrot. *Plant Cell Physio* **41**, 268-273.

Kumar, S., Agrawal, V. and *S. C. Gupta* (2002). Somatic embryogenesis in the woody legume *Calliandria tweedii. Plant Cell Tiss. Organ Cul*. **71**, 77-80.

Lin, G. Z., Zhao, X-M., Hong, S-H. and *Y. J. Lian* (2010) Somatic embryogenesis and shoot organogenesis in the medicinal plant *Pulsatilla koreana* Nakai. *Plant Cell Tiss. Organ Cul.* DOI:10.1007/s11240-010-9897

Liu, C. Q., Xia, X. L., Yin, J. H., Zhou, J. H. and *H. R. Tang* (2007) Direct somatic embryogenesis from leaves, cotyledons and hypocotyls of *Hippophae rhamnoides*. *Plant Cell Tiss. Organ Cul*. **51**, 635-640.

Lakshmanan, P. and *A. Taji* (2000). Somatic embryogenesis in leguminous plants. *Plant Bio* **2**, 136-146.

Mauri, P. V. and *J. A. Manzanera* (2002). Induction, maturation and germination of holm oak (*Quercus ilex* L.) somatic embryos. *Plant Cell Tiss Organ Cul*. **74**, 229-235

Murashige, T. and *F. Skoog* (l962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* **15**, 473-493.

Nawy, T., Lukowitz, W. and *M. Bayer* (2008). Talk global, act local – patterning the Arabidopsis embryo. *Curr. Opin. Plant Biol.* **11**, 28-33.

Ojewole, J. A. (2005). Analgesic and anticonvulsant properties of *Tetrapleura tetraptera* (Taub) (Fabaceae) fruit aqueous extract in mice. *Phytother. Res*. **19**, 1023-9.

Opeke, L. K. (1982). Tropical tree crop. John Wiley & Sons Ltd

Rai, V. R. and *J. McComb* (2002). Direct somatic embryogenesis from mature embryos of sandalwood. *Plant Cell Tiss. Organ Cul*. **69**, 65-70.

Uchechi, N.E. and *C. Okorie* (2010). Antibacterial activity of *Tetrapleura tetraptera* Taub. Pod extracts. *Int. J. Pharm. Bio. Sci* **1**, 734-742.

Sharma, S. K., Bryan, G. J., Winfield, M. O. and *S. Millam* (2007). Stability of potato (*Solanum tuberosum* L.) plants regenerated via somatic embryos, axillary bud proliferated shoots, microtubers and true potato seeds: a comparative phenotypic, cytogenetic and molecular assessment. *Planta* **226**, 1449-1458.

Vidoz, M. L., Klusacek, P., Rey, H.Y. and *L. A. Mroginski* (2006). *In vitro* plant regeneration of *Arachis correntina* (Leguminosae) through somatic embryogenesis and organogenesis. *Plant Cell Tiss. Organ Cul*. **86**, 111-115.

Weaver, L. E. and *R. N. Trigiano* (1991). Regeneration of *Cladrastis lutea* (Fabaceae) via somatic embryogenesis. *Plant Cell Tiss. Organ Cul.* **10**, 183-186

Zavattieri, M. A., Frederico, A. M., Lima, M., Sabino, R. and *B. Arnholdt-Schmitt* (2010). Induction of somatic embryogenesis as an example of stress-related plant reactions. *Electronic J. Bio* 13, 1 DOI: 10.22225/vol 13-issue 1-fulltext-4