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FACTORS INDUCING MULTIPLE SHOOT FORMATION FROM EMBRYO EXPLANTS OF *PINUS CARIBAEA* VAR. *HONDURENSIS IN VITRO.*

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ABSTRACT

Embryo explants of *Pinus caribaea* development multiple shoots when cultured on von Arnold and Eriksson basal medium supplemented with benzylamino purine. The concentration of the basal medium and cytokinin (benzyalmino purine) strongly influenced shoot formation The development and elongation of shoots were enhanced by reducing the strength of mineral salts, vitamins and sucrose in a medium containing activated charcoal but devoid of plant growth regulator. Excised shoots rooted within four weeks in a medium containing reduced strength of mineral salts, vitamins and sucrose, supplemented with indolebutyric acids. Rooted explants were transferred to potted soil hardened and grown under ambient conditions.

INTRODUCTION

The importance of *in vitro* culture techniques for micro propagation of forest tree species, for reforestation and tree improvement has been widely recognised (Biondi and Thorpe, 1982; Durzan and Campbell, 1974; Farnum, *et al.*, 1983). Although successful stimulation of adventitious buds *in vitro* has been achieved in explants of several species of forest trees, only a few studies have resulted in complete plant regeneration (Aitken, *et al.*, 1981; Patel and Thorpe, 1984). According to Bornman (1983), the use of embryo or seeding explants of conifer species has potentials for regeneration and multiplying plants *in vitro* from scarce and costly seeds derived through artificial pollination.

Pinus caribaea is an economically important tree species and grows abundantly in the temperate regions but can also be found in the tropical and sub-tropical regions of the world (Amobi, 1976; Kaufman, 1989). This paper describes the various factors affecting multiple shoot formation from embryo explants and regeneration of plantlets in *P.caribaea.*

MATERIALS AND METHODS

Plant Material

Seeds of *P. Caribaea* collected from the plantations of the Forestry Research Institute of Nigeria, (FRIN) Ibadan, Oyo State of Nigeria, were used for this study. Seeds were surface sterilized with half strength laundry bleach (Jik) containing 5.25% sodium hypochlorite for 40 minutes, with two drops of Tween – 20 added to act as a wetting agent. The bleach was decanted and the seeds were rinsed five times with sterile distilled water. The sterilized seeds were kept in sterile water for 24 hours in aseptic Petri dishes to allow imbibition. This facilitated the dissection of the embryos. Embryos were aseptically dissected out of the surrounding endosperm inside a sterile Laminar-flow chamber and placed on the Surface of the nutrient medium. Only white firm embryos embedded in white and semifluid endosperms were selected.

Culture Medium

Von Arnold and Eriksson (AE) medium (1977) was selected for this study, from the result of screening various media formulations. Full and reduced concentrations of mineral salts and vitamins in the medium were tested for the bud induction process.

Concentrations of various sugars were used in the medium as recommended by von Arnold and Erikson (1977). These included sucrose 34.2g l^{-1}, glucose 0.18 g l^{-1}, xylose and arabinose 0.15 g l^{-1} each.

Table 1: *Effect of the strength of AE medium and IBA concentrations on the rooting of shoot explants of P. Caribaea. Data from 20 shoots per treatment.*

Media Strength	Response % IBA Concentration (mg l^{-1})			
	0	0.1	0.2	0.5
⅛	0	25	10	0
¼	0	60	35	0
½	0	0	0	0
1	0	0	0	0

Shoot Induction and Development

Twenty replicate embryos were cultured in test tubes containing full or reduced strengths of AE medium supplemented with 0.1-0.6mg l^{-1} *benzylamino purine* (BAP) for 3 weeks. The cultures were incubated at 25 ± 2^0C in growth room under white fluorescent light of lux 80μ E. $m^{-2}.s^{-1}$ for 12 hours each day.

After 3 weeks of culture of the isolated embryos on the medium containing cytokinin, the growing embryos that have shoot initials were transferred to the following strengths of AE mineral salts and vitamins (⅛, ¼, ½, 1) each supplemented with 2% sucrose and 0.5% activated charcoal, without plant growth regulator

Elongation of Shoots

After about 12 weeks of culture, shoots of about 1-2cm in length were separated and excised from the mother explants using sterilized forceps and scapels. The excised shoots were cultured individually in test tubes containing various strengths of AE mineral salts and vitamins (⅛, ¼, ½, 1), each supplemented with 1% sucrose and one of the various concentrations of activated charcoal (0.0, 0.1, 0.5, 0.6%) for elongation. The final shoot length was determined after 8 weeks.

Rooting of Shoots

After the shoots had undergone elongation for 8 weeks in the elongation medium, they were given a rooting treatment. Twenty shoots were cultured on each of the following strengths of AE mineral salts and vitamins (⅛, ¼, ½, 1) each supplemented with 2% sucrose, and one of the following concentrations (0.0, 0.1, 0.2, 0.5 mg l^{-1}) of *indolebutyric acid* (IBA) for 4 weeks.

Hardening of Plantlets

After washing off the agar surrounding the roots with sterile distilled water, the plantlets were transferred to pots containing sterile garden soil. The soil was autoclaved at 1.05kgcm^{-2} pressure at 121 ^0C for 20 minutes, to kill any micro-organism that might attack the roots during the hardening process. The pots were kept in the humidity chamber in a greenhouse. The atmosphere of the plantlet was kept misty by spraying the humidity chamber with water once every morning. After hardening the plantlets, they were then planted out in the field.

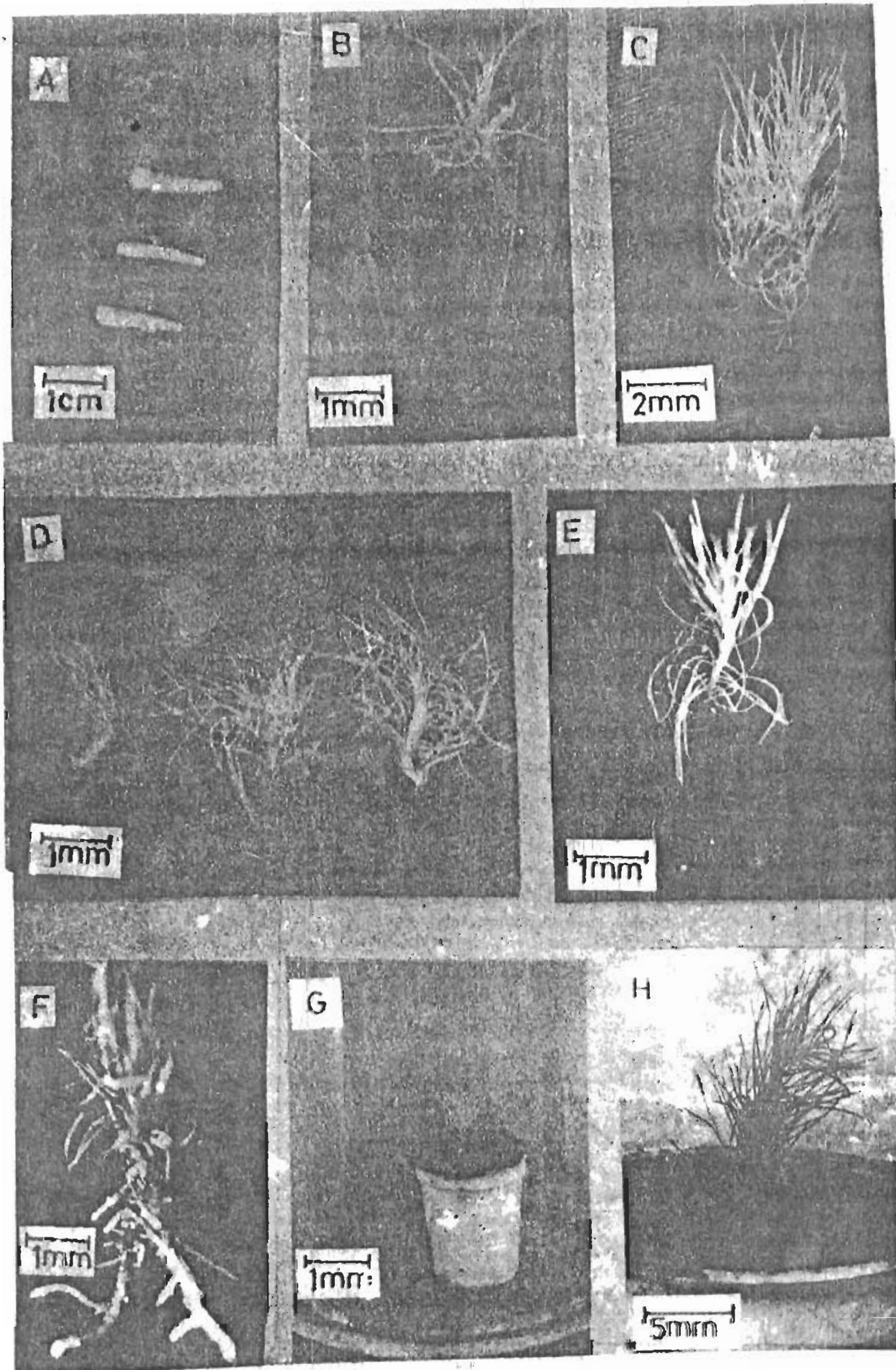

Plate 1: Stages of shoot formation and regeneration from embryo explants of *Pinus caribaea* (A) Excised embryos, (B) Germinated embryo bearing shoot primordia. (C) Development of the induced shoots, (D) Separated shoots from the mother explant, (E) Shoot elongation, (F) Rooting of shoot, (G) Hardened plantlet, (H) Potted plantlet exposed to outside environment.

RESULTS

Shoot Initiation and Development

The initial explant was an isolated embryo which measured 4 mm in length (Plate 1A) Within 5 days of culture of the isolated embryos in AE medium supplemented with 0.1-0.6mg l^{-1} BAP, the embryos elongated slightly. Elongation of the embryos stopped after 7 days. The cotyledons and hypocotyl of the growing embryos became green and swollen but their surfaces were smooth. The radicle did not grow and the root cap cells showed purple pigmentation. Within 14-20 days, it was observed that in apex of the growing embryo developed shoot primordia. The highest number (10±0.6) of shoot primordia was produced when full strength AE medium was supplemented with 0.5mg l^{-1} BAP while supplementing the medium with 0.1 mg l^{-1} BAP resulted in the lowest number (1.3±0.2) of shoots (Fig. 1).

The transfer of the growing embryos to BAP – free medium after 3 weeks of culture led to the development of shoots (Plate. 1B). Shoot development (Plate. 1C) was enhanced (17±0.4) when the growing embryos bearing the shoot primordia were cultured on ¼ strength AE medium supplemented with 2% sucrose and 0.5% activated charcoal (Fig. 2). The lowest number of shoots (10±0.6) was recorded at full strength AE medium.

Elongation of Shoots

The separation and excision of shoots from the mother explants and culture of the isolated shoots on reduced strengths of AE medium devoid of BAP further stimulated the process of elongation (Plate. 1D). The inclusion of 0.5% activated charcoal in ⅛ strength AE medium resulted in the best shoot elongation (Fig. 3). Shoots of 1.3±0.1cm in length reached 5.3±0.2 cm in 8 weeks (Plate. 1E) giving an elongation of 4 times the initial length.

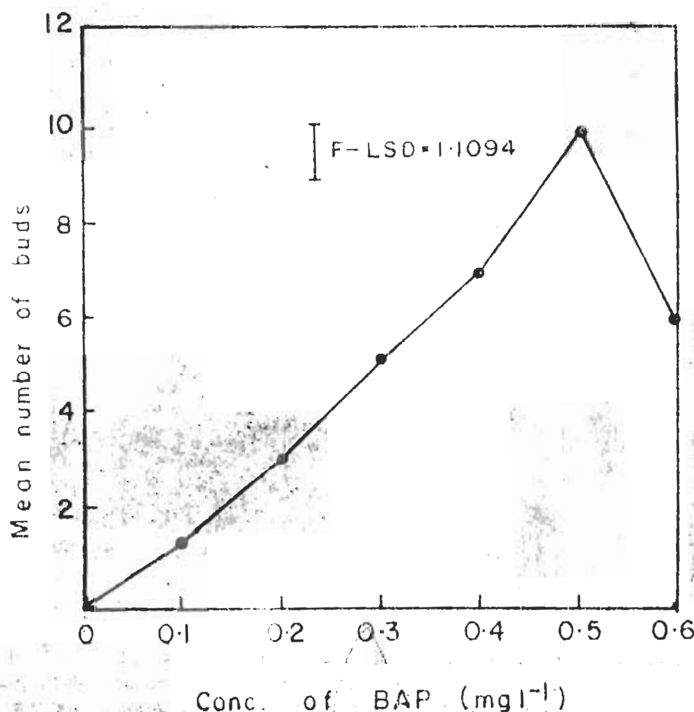

Fig 1: Effects of BAP Concentrations on Shoot Induction from Excised Embryos of *P. Caribaea*.

Rooting of Shoots

Shoots of about 5.5 cm in length rooted on ¼ or ⅛ strength of AE mineral salts and vitamins supplemented with 2% sucrose, 0.1 or 0.2 mg l^{-1} IBA after 4 weeks. This was without any callus in between the root and shoot (Plate IF). On ¼ strength AE medium supplemented with 2% sucrose and 0.1 mg l^{-1} IBA, 60% rooting was achieved while 35% of the shoots rooted on ¼ strength AE mineral salts and vitamins containing 2% sucrose and 0.2mg l^{-1} IBA. When ⅛ strength AE medium was supplemented with 2% sucrose and 0.1 mg l^{-1} IBA, 25% of the shoots rooted while 10% rooted on ⅛ strength AE medium containing 0.2 mg l^{-1} IBA (Table 1). Shoots failed to root when 0.5 mg l^{-1} IBA was added to any of the strengths of AE medium tested. Also rooting did not occur when the shoots were cultured on full or reduced strength AE medium without growth regulator.

Hardening and Transfer to Soil

Growing of plantlets in sterile soil during the hardening process was found to be necessary to prevent damping-off diseases. The plantlets transferred to pots showed signs of wilting within 5 days of their stay in the humidity chamber, and 53% survival was achieved after 4 weeks (Plates. IG and II). The established plantlets transferred to the field recorded 60% survival.

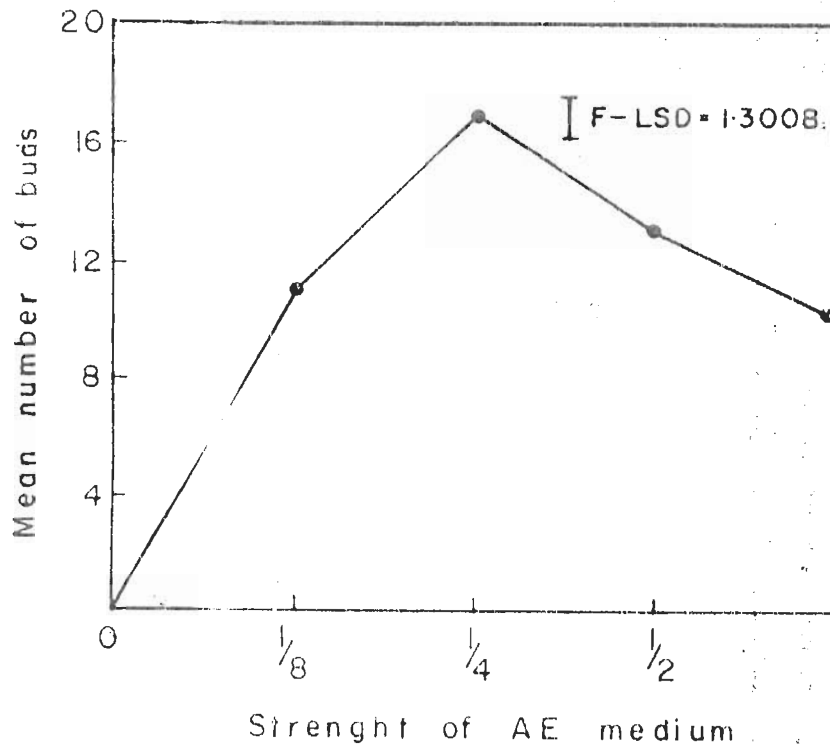

Fig 2: Effects of the strength of AE medium on the development of buds from embryos of excised P. *Caribaea*.

DISCUSSION

The mode of shoot formation from the cultured isolated embryos of *P. Caribaea* was similar to the patterns observed in embryo explants of other coniferous species (Reilly and Brown, 1976; von Arnold and

Eriksson, 1981; Aitken-Christie, 1984). Regeneration of plantlets from the embryo explants of *P.caribaea* could be divided into 3 stages:

1) shoot inductiog on isolated embryos;
2) development and elongation of shoots;
3) rooting of shoots.

From the results obtained, each stage has its own nutritional and hormonal requirements during culture as reported from other studies (Biondi and Thorpe, 1982; Thorpe, 1987).

The process of shoot induction was influenced by the strength of AE mineral salts and vitamins and the concentration of BAP. This is similar to the reports of Patel and Thorpe (1984) on *Pinus contorta*. The optimum concentration of AE medium for shoot induction was ¼ strength and this is in line with the observations of von Arnold and Eriksson (1981) on *P.contorta*. At the optimum concentration of AE medium and BAP, an average of 17 shoots could be formed per embryo. Similar result was obtained on *P.contorta* by Patel and Thorpe (1984). The best level of BAP for shoot initiation and subsequent shoot development was 0.5 mg l^{-1}, which was within the successful range for conifers (Biondi and Thorpe, 1982). In agreement with the studies on *picea engelmanii* (Patel and Thorpe, 1986), the culture of isolated embryo on AE medium containing BAP for 21 days before transfer to BAP- free medium was necessary for shoot development since prolonged stay in BAP containing medium led to the browning of the explants, while exposure of the embryos to BAP for 1-2 weeks resulted in a lesser number of shoots. The dilution of AE medium to ¼ strength, sucrose to 2% and addition of 0.5% activated charcoal to the medium enhanced the development of shoots. This is in accordance with the reports of Aitken-Christie (1984) on *Pinus radiata*.

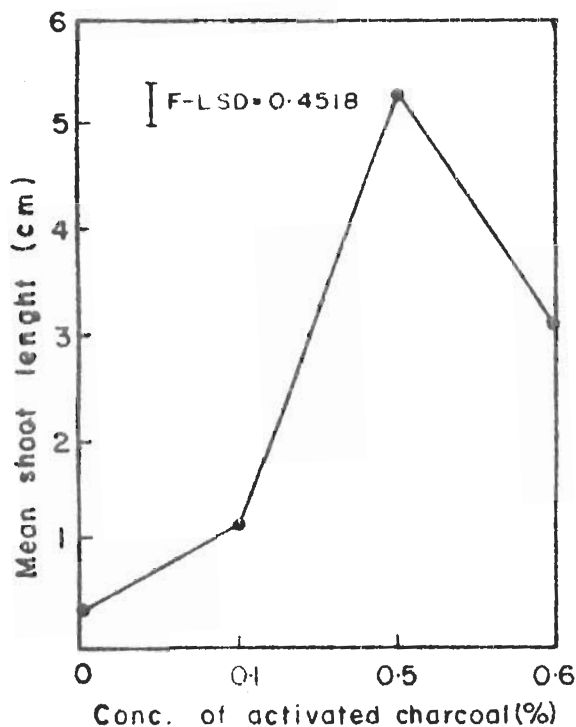

Fig 3: Effects of activated charcoal on the elongation of shoot explants of *P Caribaea*.

Shoot elongation was enhanced by the transfer of the explanted shoots to ⅛ strength AE mineral salts and vitamins containing 1% sucrose and 0.5% activated charcoal. Similar observation was made by Patel and Thorpe (1984) on *P.contorta*. Addition of activated charcoal to AE medium has been shown to stimulate the development and elongation of shoots of *Sequoia sempervirens* (Boulay, 1979). The role of charcoal in culture is not clearly understood, but part of its action is suggested to be based on adsorption of phytohormones, phenolics and toxins created during autoclaving and culture (Weatherhead, Burdon and Henshaw, 1979).

Generally, rooting in conifers is a troublesome and slow process, compared to rooting in angiosperm plants (George and Sherrington, 1984). However, rooting of explanted shoots of *P.caribaea* was achieved on ¼ or ⅛ strength of AE medium containing 2% sucrose and either 0.1 or 0.2 mg l^{-1} IBA. Similarly, Arnold and Eriksson (1981) observed that adventitious shoots of *P.contorta* formed roots on diluted AE medium and that rooting was stimulated by a supply of IBA to the medium. Although rooting of *P.caribaea* shoots was achieved under sterile conditions, Horgan and Aitken (1981) reported that shoots of *P.radiata* formed roots under non-sterile conditions in humid environment with occasional misting. The results of this study improve prospects for the micropagation of commercially valuable *P.caribaea* tree species considered difficult to propagate vegetatively. Although a maximum of 17 shoots were induced from one isolated embryo, and 60% rooting achieved, efforts are needed towards increasing these levels of regeneration.

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