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In Vitro Culture of *Pittosporum pentandrum* (Bl.) Merr.

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ABSTRACT

In vitro culture of *Pittosporum pentandrum* was done in glass containers with nutrient medium, under aseptic conditions. Several explants were tried. The most successful was the very young leaf blade with midrib, cut 6 x 5 millimeters, grown in an agar culture medium of 1 liter Revised Murashige and Skoog Medium supplemented with the cytokinin benzyl adenine (10 and 15 milligrams) and a combination of two auxins, namely, naphthalene acetic acid (0.25 milligram) and dichlorophenoxyl acetic acid (0.25 milligram), producing an initial stage of development (callus) up to organogenesis (plantlet formation).

INTRODUCTION

Pittosporum pentandrum (Bl.) Merr. locally known as mamalis is endemic in the Philippines, found in secondary forests and parang vegetations at low and medium altitudes (1,400 to 2,300 m) from Northern Luzon to Palawan and Mindanao.

It is a small tree or shrub and grows up to 20 meters (Merrill 1912). Morphologically, the leaves are simple, lanceolate (6–12 cm. by 2.3 cm.) glabrous gradually narrowing at both ends, especially towards the acute to acuminate tips, and with undulate margin. The petioles are short and slender. The inflorescence is panicle and either terminal or axillary, with dense white flowers becoming yellow as they mature. The flowers are aromatic, with resinous and strong, turpentine-like odor. The fruit is a capsule with six to eight brown and flattened seeds.

Brown (1921, cited by Quisumbing in 1951) reported that this species contains the hydrocarbons dihydroterpene and heptane which, when frac-

tionated, have similar components as gasoline. Bacon (1929) reported that the oil in mamalis consisted primarily of the same dihydroterpene found in *P. resiniferum*. Blanco (1929) concluded that the dihydroterpenes are those cited by Quisumbing in 1978.

P. pentandrum has therapeutic properties; its fruit juice and docoction are used for cleansing wounds (Quisumbing 1951). Aqueous leaf-extracts possess a high antibacterial effect against *Mycobacterium aureus* and show a slight indication of inhibition for *Escherichia coli* (Masilungan 1963). Calcium oxalate and amygdalin were isolated from leaves, amygdalin and fats from stems (De Padua 1982).

The use of tissue culture techniques has opened new vistas in plant research (De Guzman 1978; Valmayor 1983; Del Rosario and Zamora 1988). By using tissue culture to propagate *P. pentandrum*, a large number of plants can be derived. Timing of plantlet production can be controlled. Moreover, the existing quality of genotype is preserved, hence uniform plants of a selected genotype can be obtained. The oils can be extracted from callus growths instead of seeds (Miel 1991). This shortens the developmental stage of the plant species for purposes of extracting the oil.

Although *P. pentandrum* is a potential source of renewable energy, it has not been mass-propagated by tissue culture. The seeds are difficult to germinate, and there is an absence of fruit-setting in most cases, hence this study on its in-vitro culture.

The specific objectives were: to determine: (a) the optimum culture medium and conditions for the induction of callus growths and organogenesis, (b) the optimum conditions for the establishment and maintenance of callus growths and establishment of plantlets.

MATERIALS AND METHODS

Vegetative organs of *P. pentandrum* were tried as explants, namely very young (vy) and young (y) leaf tip, blade, blade with midrib, petiole, and base, cut 6 by 5 mm, and vy and y-internode cut 8 by 10 mm Before culturing, these were sterilized as follows:

The leaf and stem explants were washed once with tap water, twice with double-distilled water, soaked in 70% ethanol for 30 minutes, then transferred to sterile erlenmeyer flasks and soaked for 20 minutes in a sterilant consisting of 200 ml of 3% Zonrox solution, and 5 drops of Tween-20. The sterile explants were next washed three times with sterilized distilled water, for 10, 5, and 5 minutes, then cut to size 6 x 5 and 8 x 10 mm, respectively.

The explants were then cultured in sterile test tubes with basal agar medium of the Revised Murashige and Skoog (RMS) supplemented as follows: Culture Medium (A)—1 liter of RMS (composition given in Appendix II) plus the auxins naphthalene acetic acid (NAA, 0.25 to 1.25 mg) and dichlorophenoxy acetic acid (2,4-D, 0.25 to 1.25 mg.), and the cytokinin benzyl adenine (BA, 10 mg). Culture Medium (B)—1 liter of RMS plus NAA (0.25–1.25 mg), 2,4-D (0.25–1.25 mg), and BA (10 mg). Four grams of sucrose was added to each medium, the pH was adjusted to 5.7, then solidified with 0.7% of a Japanese agar of unknown brand.* For each culture treatment the number of explants was either 10, 50, or 100, as detailed in Table 1.

After inoculation in culture medium (A) and medium (B), the explants were stored in an aseptic air-conditioned room with temperature at about 22°C. Light was provided by a white 40-watt fluorescent lamp mounted 30 cm (measured from the agar surface) above the row of test tubes spaced 5 cm apart. The initial explants were observed for initial callus growth at intervals of 3 and 7 days and 8 and 21 days.

After 35 and 40 days the shoot and stem initial stages of differentiation in the callus were isolated and subcultured in sterile culture bottles in medium (A) and medium (B), described earlier. This was intended to preclude excessive brown pigmentation and to facilitate the observation of organogenesis (caulogenesis and rhizogenesis) in the expected plantlets.

The plantlets that developed were withdrawn from the culture bottle, and the roots were washed in tap water followed by distilled water, to remove the agar, then soaked in 0.03% Banlate fungicide for 30 seconds. They were next transferred to soil in plastic cups and allowed to stand in the laboratory for one week to acclimatize, then to clay pots.

RESULTS

Observable developments of culture were first noted on the 21st day, when callus appeared on the explant from very young leaf blade with midrib (vylbm). As will be gleaned in Table 1, the explant vylbm proved to be the most viable beyond the callus stage. Its progress after 21 days in culture medium (A) was duplicated by the other explants much later, viz., 25 days

* This Japanese agar costs 1/6 that of refined Bacto agar, hence, Difco brand was used in order to preserve the research fund. Its quality was assured by dry runs with Bacto agar. The dealer is RTC Supply House, 11 Rimas, Project 2, Quezon City Philippines.

for very young leaf blade, 35 days for the other four explants: vy leaf tip, vy leaf base, vy leaf petiole, and vy and y internodes.

After 25–40 days in culture medium (B), vylbm had developed to plantlets while the other explants were still in the callus stage after 35 and 40 days. The latter did not progress to the plantlet stage (Table 1).

The vylbm subcultured in medium (A) showed shoot and root formation, although some were stunted. (The photographs could not do justice to the visual observation because the roots were short, nearly translucent, and of the same hue as the agar. The pentel markings do not make for photogenic presentation; they testify to the authenticity of the photographs.)

DISCUSSION

In vitro culture is done on nutrient media under sterile or aseptic conditions. This occurs on microscale, that is, on a relatively small surface area, as in glassware like test tubes, small and wide-mouth bottles with covers. Physical as well as nutritional and hormonal factors are optimized. This helped us identify the best explant, culture medium, and other culture techniques for the tissue culture of *P. pentandrum*.

Vegetative parts (shoot apex, leaf, stem, both young, very young and mature leaves) were tried as explants since these were available in quantity. Ethyl alcohol (70%) was used for surface sterilization of the explants; it was observed that 95% EtOH is toxic to its tissues, and affected seriously with 95% ethyl alcohol. Initial explants from very young leaf became fleshy, enlarged and green on the 21st day culture since proplastids and parenchyma cells in very young leaf divide profusely.

Very young leaf (vyblm) cut 6 by 5 mm was the most effective explant in a culture medium A (Table 1). Vyblm was observed to have anatomical features such as procambium, mesophyll, storage parenchyma cells, and provascular tissue (Zamora 1990). These tissues are prone to active cell divisions and are typically used in tissue culture.

Explants do not have precisely the same requirements for normal growth development of intact plants. In addition to the essential minerals, the explants also require the addition of a certain organic substances to the culture medium which, in the intact plants, these plant organs (stem, leaf, and shoot apex) are endogenously supplied (synthesized and transported) within the intact plant. In this research, these substances are all exogenously supplied in the explant by the culture medium A or B. With these substances the in-vitro tissue culture was able to cope with the selective biosynthesis

activity of the isolated explants and of the culture system which may change in their metabolic pathways over a period of time. These changes require adaptations in nutritional supply (Zamora, C.V. 1990).

After 21 to 40 days culture, an amorphous mass of loosely arranged parenchyma cells (callus) was observed arising from the proliferating cells of the explant tissue. This shows that the culture medium used stimulated callus growth and development. But only the very young leaf explant (vylbm) formed callus in 21 days, formed plantlets in 40 days. After subcultures of all the callus growths of the different explants in the same medium, only the vylbm explant continued to produce profuse plantlets. This shows that the morphogenetic potential in vylbm was maintained, and that an appropriate balance of auxin-cytokinin combination used in the culture medium stimulated continued caulogenesis (shoot initiation) and rhizogenesis (root initiation).

In this study, subcultures of callus growth in a fresh culture medium was very favorable for plantlet formation due to the fresh supply of essential nutrients. Subculturing precluded dessication of the agar, and prevented the metabolites secreted by the growing callus from accumulating to toxic levels and causing necrosis.

Skoog and Muller (1957) reported that by adjusting the concentrations of exogenous growth regulators in the medium, organogenesis can be controlled. It has also been reported that the relative concentration of auxin and cytokinin is important in initiating organogenesis. In this study, it was observed that the most effective culture medium was the auxin-cytokinin concentration of 0.25 mg. NAA and 0.25 mg. 2,4-D BA with 10 and 15 mg per liter of RMS. The cytokinin BA is more active than naturally occurring (endogenous) cytokinins in shoot proliferation according to Dodds and Lorin (1982).

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Table 1. Callus and plantlet formation observed in explants of *Pittosporum pentandrum* (Bl.) Merr. cultured in different media

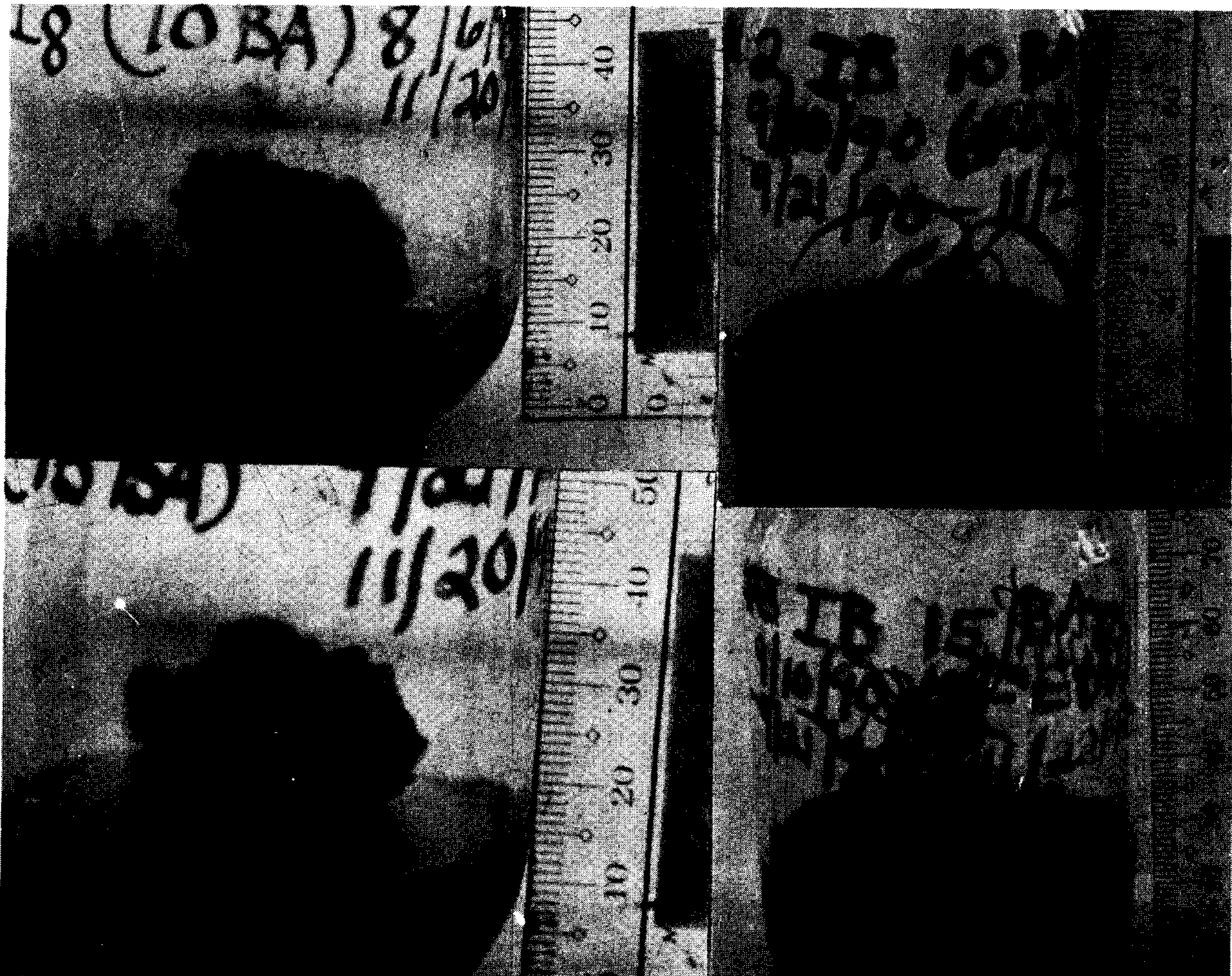
Culture media 1 li. RMS + growth regulators (mg./li.)	Explant	No. of Samples (Explants)	No. of Days Callus Produced	No. of Plantlets per Explant
(A) 10.00 BA+ 0.25 NAA+ 0.25 (2,4–D)	Very young leaf blade with midrib	50	21 to 30*	with callus 3
	Very young leaf blade with midrib producing plantlets	50	31 to 210**	with callus 5–8
	Very young leaf blade	10	25 to 40*	with callus 0
	Very young leaf tip	10	35 to 40*	with callus 0
	Very young leaf base	10	35 to 40*	with callus 0
	Very young leaf petiole	10	35 to 42*	with callus 0
	Very young internode	10	35 to 42*	with callus 0
	Very young leaf blade with midrib	10	160 to 180**	with differentiated shoots, stem, & roots
	Young leaf blade with midrib	10	35 to 38*	with callus 0
	Young petiole	10	40 to 45*	with callus 0
	Young leaf tip	10	40 to 45*	with callus 0
	Young leaf blade	10	40 to 45*	with callus 0
(B) 15.00 BA+ 0.25 NAA+ 0.25 (2,4–D)	Very young leaf blade with midrib	100	25 to 40* and***	with callus 2–5
	Young blade	10	25 to 40*	with callus 0
	Young leaf tip	10	25 to 35*	with callus 0
	Young leaf base	10	25 to 35*	with callus 0
	Young petiole	10	25 to 35*	with callus 0
	Young internode	10	25 to 25*	with callus 0

Legend:
* Callus formation
** Plantlet formation
*** Plantlet formation after subculture

APPENDIX I

Revised Murashige and Skoog's Medium (RMS)

Macronutrients	mg/l
Ammonium nitrate	1650
Potassium nitrate	1900
Calcium chloride dihydrate	400
Magnesium sulfate heptahydrate	310
Potassium dihydrogen phospate	170
Ferrous sulfate heptahydrate	27.80
Disodium ethylenediamine tetra acetic acid (EDTA)	37.20
Micronutrients	
Boric acid	6.20
Manganese sulfate monohydrate	17.50
Zinc sulfate heptahydrate	8.60
Potassium iodide	0.83
Sodium molybdate dihydrate	0.25
Cobalt chloride hexahydrate	0.025
Copper sulfate pentahydrate	0.025
Organic Constituents	
Biotin	0.05
Folic Acid	0.50
Glycine	2.00
Nicotinic acid	5.00
Pyridoxine hydrochloride	0.50
Thiamine hydrochloride	0.50
Myoinositol	10.00
Growth Regulators	
Benzyl Adenine (BA)	10.00–15.00
Naphthaline Acetic Acid (NAA)	0.25–1.25
Dichlorophenoxy Acetic Acid (2,4-D)	0.25–1.25
Japanese Agar (Commercial)	10.50 g
Sucrose	60.00 g



Extensive calli growth in culture medium A (top left) *and in culture medium B* (bottom left). *Plantlet formation (organogenesis) in culture medium A* (top right) *and in culture medium B* (bottom right).