Tissue Culture of *Pittosporum resiniferum* Hemsl. (Petroleum nut tree)

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Abstract

It has been reported that Pittosporum *species are very difficult or even unsuccessful to be propagated by seeds (Veneracion and Costales, 1982) and the seeds of* P. resiniferum *are very difficult to germinate (Balcos, F., 1987). So this plant will be best propagated by tissue culture.*

Explants (blade with midrib or without midrib and leaf tip, 6 x 5) mm) from very young leaf of P. resiniferum *produced more significant results about 90–100% compared to the 20–80% of explants from young leaf. However, leaf blade with midrib (from very young leaf) produced culture growth earlier than any of the explants tried; and plantlets were formed after subcultures of 5–8 weeks old growing explants in 1l of Revised Murashige and Skoog's supplemented with 10 mg Benzyl adenine (BA), 0.25 naphthalene acetic acid (NAA), and 0.25 dichlorophenoxy acetic acid (2,4-D).*

The explants (blade with midrib) from very young leaf became enlarged, fleshy and green after 21 days culture and later produced an excellent and remarkable cabbage-like callus growths. After 30 days culture, these calli became green and friable to very friable creamy-greenish-yellow and roots (whitish structures) were observed. These culture growth characteristics were also observed after 40 days. Whereas, these morphological callus characteristics were observed in explants from blade and leaf tip of very young leaf, only after 40 days culture.

The best culture medium is 1l of Revised Murashige and Skoog's (RMS) agar medium supplemented with 10 mg benzyl adenine (BA), 0.25 naphthalene acetic acid (NAA), and 0.25 dichlorophenoxy acetic acid (2,4-D) at pH 5.7 exposed to light laboratory condition.

Other treatments were tried in the tissue culture of its plant parts, such as: agar media with 1l RMS supplemented with different concentration, of growth regulators such as Benzyl adenine (BA), Kinetin (Ki), naphthalene acetic acid (NAA), indole-butyric acid (IBA), (2,4-D) and gibberellic acid (GA_3) and with or without coconut water as adju-

vant from mature fruits (CW) and young fruits (cw) from 20, 25, 30, 35, 50, and 100 ml. In these treatments, some did not form any callus growth.

Introduction

Pittosporum resiniferum Hemsl. is a native of Malaysia. In the Philippines, it was first discovered as an epiphyte on small trees in 1907 at Mount Sto. Tomas, Benguet, which has an elevation of about 1,500 meters. It is endemic in the Philippines; it is not very abundant in any province of the country, but it is widely distributed as a small tree (2.5 to 6m tall), along mountain ridges and mossy forest with an altitude of from 900 to 2,400 meters in Bontoc, Sorsogon, Catanduanes, Palawan, Iloilo (Guimaras), Laguna, and Mindoro.

Studies on germination of seeds have shown poor viability and germination rate under field conditions (Noble, 1978). But germination rate of freshly-collected seeds from ripe fruits was observed to be 59–75% (Veneracion and Costales, 1982; Noble and Orallo, 1983). Different treatments of the seeds with varied growth regulators and concentrations in culture media also proved unsuccessful seed germination (Balcos, 1987). Thus, the ultimate objective of this research is to propagate *Pittosporum resiniferum* by tissue culture. The specific objectives are to determine (a) the optimum culture medium and conditions for the induction of callus growths and organogenesis; (b) the optimum conditions for the establishment of plantlets under greenhouse and field conditions; and (c) to be able to maintain cultured seedling bank of *Pittosporum resiniferum*, for dissemination at a commercial scale and for research purposes.

This research is very significant because the principles derived from this study will be used in expanding further research in the mass propagation of other Philippine *Pittosporum* species and other hydrocarbon-producing plants noted as potential energy source of petroleum-like products. These products when fractionated could be a very good substitute for gasoline.

It has been reported that *Pittosporum resiniferum* contains pleasant smelling oil with high percentage of heptane, octane, and dihydroterpenes which, when fractionated, have similar components of gasoline. Also, petroleum nut oil contains a very slight amount of sulfur (0.07%), an advantage it has over gasoline (0.1%) (Brown, 1957);

(Fernandez, 1982). It has also gained popularity as a potential, non-conventional energy source. Fresh-cut fruits and stems, when lit, will burn brilliantly. This proves its suitability as direct fuel substitute. The blending of petroleum nut oil with other substances improves its fuel property. Also it has other economic values: the wood is an ideal source for pulp and paper; used in the manufacture of bobbins, tennis rackets, toothpicks, lollipop sticks, shuttles, and spindles. It has therapeutic uses: its leaf decoction is used for bathing women who had just given birth; fruit infusion is a remedy for intestinal and muscular pains, its oleoresin is used as a cure for leprosy and other skin diseases (Quisumbing, 1951).

Materials and Methods

Vegetative parts: shoot apex, stem, blade with and without midrib, leaf base, petiole of very young, young and mature leaves, were tried as explants. These were aseptically cut into varied sizes (Appendix I); and were cultured in sterile test tubes with basal agar medium (RMS), supplemented with growth regulators (mg/1 RMS), either singly to a combination of two to three at different concentrations and with or without the addition of coconut water. Four grams of sucrose were added to RMS and the culture medium was adjusted to pH 5.7 and solidified with 0.7% Bacto-agar. After 5 to 8 weeks, growing explants with callus, shoot and stem differentiation were isolated and subcultured in culture bottles with appropriate medium (Appendices II and III) to avoid excessive brown pigmentation. These were then incubated in aseptic air-conditioned room at +/–22°C, subjected to light about two inches apart to insure uniform distribution of light.

Results and Observations

The study showed that initial explants (tip, blade with and without midrib) (6 mm x 5 mm) of very young leaf produced more remarkable culture growths compared to the explants from leaf base, petiole, shoot tip and stem of very young, young, and mature plant parts (Table I). They produced excellent friable to very friable callus morphological characteristics and plantlets in 1 li RMS agar medium supplemented with 10 mg BA; 0.25 mg NAA and 2,4-D at pH 5.7 under light.

The explants (leaf blade with midrib) of very young leaf immediately became green, enlarged, and fleshly on the 21st day culture and, later, exhibited an excellent and very remarkable growth (cabbage-like). After 30 days culture, friable to very friable, creamy-greenish-yellow calli with numerous whitish structures were observed (Fig. 1). These morphological growth characteristics of the culture explants were still observed in all the 10 samples even after 40 days. Three culture samples produced three plantlets per sample after 44 days (Tables 1 and 3).

Culture growth characteristics were initiated in explants from the blade without midrib in 28 days, but after 40 days, remarkable calli growth (cabbage-like) were also observed (Tables 1 & 3; Fig. 1). Then in some cultures, after 45 days, the green explants became brownish-green and moderately enlarged with very friable creamy-yellowish calli with least growth of whitish structures were observed in all the 10 samples. The leaf tip explants became brownish-green after 7 days culture and cabbage-like callus growths were formed in the nine culture samples after 40–45 days (Tables 1 & 3; Fig. 1).

The explants from leaf base (6 mm by 5 mm) of very young leaf turned green, enlarged, and fleshy only after 30 days which produced remarkable calli growth (cabbage-like) in the seven culture samples after 35 days culture (Tables 1 & 3; Fig. 1); while the petiole explants (7 mm by 1 mm) of very young leaf after 30 days culture produced moderate growth of friable green to yellowish-green calli with whitish structures, and produced lollipop-like and mushroom-like growths in eight culture samples after 35 days (Table 1 & 3; Fig. 2_a). Also some cultures produced least growth of friable creamy calli and the green explants became brownish-green. The morphological characteristics of cultured explants from "cabbage-like," "mushroom-like," and "lollipop-like" callus growths were observed only after 30–45 days culture (Figs. 1, 2_a & 2_b). The study showed that calli formation and development, although significant, were delayed in leaf tip, leaf base, and petiole explants.

Subcultures of all growing explants and calli from test tubes to culture bottles using the same culture medium, 10 BA + 0.25 NAA + 0.25 (2,4-D), produced shoot, stem, and roots (whitish structures) after 20-day subculture; however, it was observed that the explant (blade with midrib) showed more remarkable results. Four plantlets were pro-

duced per culture samples of three, developed from the 10 culture samples (Table 1). Explants of very young leaf blade without midrib produced nine calli culture growths in 10 culture samples, while the explants of very young leaf tip produced eight calli culture growths from 10 culture samples (Tables 1 & 3).

Explants from mature leaf and stem did not produce even callus, but explants from young leaf, stem, and shoot apex were observed to give less remarkable results. Explants of young leaf in RMS supplemented with 10 BA; 0.25 NAA + 0.25 (2,4-D) became greenish and brownish-green produced moderate to least calli growth of friable green to yellowish-green calli with least growth of whitish structures. Cultures of young leaf blade with midrib and petiole (Tables 1 & 3; Fig. 2b) produced calli culture growth of seven while young leaf blade produced calli growth of six per 10 culture samples after 35 days culture. Likewise, young leaf tip and leaf base cultures produced least calli culture growths of five per 10 culture samples after 40 days, (Tables 1 & 3).

Explants of shoot apex, young leaf, and stem in RMS supplemented with 5 BA; 0.25 NAA + 0.25 (2,4-D) became greenish, brownish-green to light brown and produced good to moderate calli growths of very friable to friable greenish-yellow, creamy-green to green with numerous whitish structures. The morphological characteristics were very distinct in young leaf blade with midrib, young stem, young leaf blade, and shoot apex calli cultures. Cultures of young stem and leaf blade with midrib produced calli culture growths of eight young leaf blade, seven and six of shoot apex cultures per 10 culture samples after 25–35 days culture. No plantlets were formed from the calli cultures, while cultures of young petiole, leaf base and leaf tip became slightly compact and produced calli culture growths of five and four per 10 culture samples after 35 days (Tables 1 & 3).

Likewise, it was observed that explant cultures in treatments (Appendix IV) using RMS supplemented with combination of two to three growth regulators in varied concentrations and with coconut water did not give significant results only on some of the young leaf explant cultures of leaf blade with midrib (3–7); leaf blade (2–7); leaf tip (4–7), leaf base (1–6), and petiole (4–6) calli culture growths per 10 culture samples. The explants became brownish-green to light brown with

very least friable to slightly compact creamy calli growths and less creamy-whitish structures after 45 days (Tables 2 & 4).

Discussion

In the tissue culture of *Pittosporum resiniferum* Hemsl., the vegetative parts—shoot apex, leaf, stem, both young and mature and very young—were tried as explants, since these are always available in quantity. Ethyl alcohol (70%) was used for surface sterilization of the explant because it was observed that 95% ETOH is toxic to its tissues and also seriously affected the establishment of callus. The explant turned brown readily 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. Schimper (1958), and Kirk and Tilney-Bassett (1978) also reported that plastids increase in number by the division of proplastids and are passed on from cell to daughter cell during cell division. This division is followed by enlargement and differentiation of proplastids and parenchyma cells.

Of the different explants tried, the blade with midrib (6 x 5 mm) was most effective in the tissue culture of petroleum nut tree. Using tissue culture techniques, callus formation can be induced in plant organs and tissues. Plant materials typically cultured include procambium, storage parenchyma cells, mesophyll, and provascular tissue Yeoman and Macleod (1977). These anatomical features which are prone to active cell divisions are all present in the explant (leaf blade with midrib) of this plant (Fig. 4).

Explants do not have precisely the same requirements for normal growth and development of intact plants. In addition to the essential minerals, the explants also require the addition of certain organic compounds to the culture medium which, in the intact plants, these organs (stem, leaf, and shoot apex) are provided with, synthesized elsewhere and transported to all plant parts. In this study, these compounds are all supplied by the RMS agar medium (Appendices II and III), and supplemented with 10 mg of BA; 0.25 NAA, and 0.25 (2,4-D) per liter RMS, to cope with the selective biosynthesis activity of the isolated explants and also of the culture system which may exhibit changes in their metabolic pathways over a period of time. These

changes in metabolism require corresponding changes in nutritional requirements (Dodds and Roberts, 1982).

The nutritional requirements for the induction of callus vary considerably with primary explants of different origin. In this research, the addition of three growth factors such as NAA, 2,4-D, and BA to the basal culture medium (RMS) stimulated callus formation and development. This remarkable callus development was observed in the form of cabbage-like, mushroom-like to lollipop-like growths. Yeoman and Macleod (1977) reported that majority of excised tissues require the addition of one or more growth factors to stimulate callus development. In petroleum nut tree explants, it is the combination of auxins (NAA and 2,4-D) and cytokinin (BA) in RMS which induce callus formation. After 40–50 days, callus growths in association with the original tissue, subcultures of the callus to a fresh medium or reversal transfers were done. At this time, growth on the same medium leads to a depletion of essential nutrients and to a gradual dessication of the agar because of water loss. Likewise, this will prevent the metabolites secreted by the growing callus to accumulate the toxic levels in the medium and to prevent the tissues to be necrotic.

After 20 days subculture or reversal transfer, shoot, stem, and roots (whitish structures) were observed emanating from the callus. This shows that the morphogenetic potential of the explant callus was maintained and appropriate balance of auxin-cytokinin combination used in the culture medium stimulated caulogenesis (shoot initiation) and rhizogenesis (root initiation).

Low concentration of auxin (0.25 NAA and 0.25, 2,4-D) favors root development in this study and high concentration of auxins inhibits root development. Wareing and Phillips (1981) reported that at high auxin concentration, this root inhibition may be due to the enhancement of ethylene biosynthesis in the root tissues.

Good culture growth was observed in explants (base of young leaf) as compared to least growth of explant (tip of the young leaf) in RMS supplemented with BA; NAA + 2,4-D or no callus growth in petiole explant. These effects may be attributed to variation in the endogenous auxin level of buds in different regions of the stem (Lane, 1978). It was found that BA is more effective than kinetin (Ki) on the proliferation and development of the shoots of jackfruit. This effect was also observed in this study which is similar to what has been observed in

apple by Lundergan and Janick (1980). Synthetic cytokinin such as BA is more active than naturally occuring cytokinins in shoot proliferation. BA at a concentration of 5 mg/l proved to be optimum for shoot proliferation and development in jackfruit (Wareing and Phillips, 1981).

Conclusions

1. This research showed that leaf-blade with midrib of very young leaf is the most effective explant in the tissue culture of *Pittosporum resiniferum* Hemsl.
2. The appropriate size of the identified best explant (leaf-blade with midrib) is 6 mm by 5 mm
3. The best culture medium is 1 liter of Revised Murashige and Skoog's (RMS agar medium) supplemented with BA 10 mg, NAA (0.25 mg), and 2,4-D (0.25 mg) maintained at pH 5.7 under direct light incubated at +/–22°C.
4. Aseptic conditions and subcultures are very important to successful tissue culture.
5. Combination of three growth factors (auxins, NAA, 2,4-D, and cytokinin, BA) with RMS is most effective in inducing callus formation and development.
6. Appropriate balance of auxin-cytokinin supplement in the RMS culture medium of *Pittosporum resiniferum* Hemsl. stimulates caulogenesis and rhizogenesis.
7. The use of IBA, Ki, in addition to NAA, BA, 2,4-D, as supplements to RMS and the use of coconut water as adjuvant gave unsatisfactory results.
8. The culture medium used in my previous research on watermelon (an herbaceous plant) is not recommended for tree species (*Pittosporum resiniferum* Hemsl.).

Recommendations

1. This research should be continued to include histological and cytochemical studies as well as biochemical techniques to obtain some data on differentiation.

2. Studies on ultrastructure of cells before and after they have been stimulated to divide should be done to understand the process of growth induction.
3. Further studies on appropriate culture media that will stimulate more plantlet formation up to mature stage in the field should be done.

Furthermore, experiments of both Philippine species of *Pittosporum* are still being undertaken to visualize, analyze, and compare which of the species will produce favorable and more significant results as callus growth for the determination of hydrocarbon contents as energy source.

Acknowledgments

I wish to acknowledge the financial assistance given by the Natural Science Research Institute (NSRI), and the research assistance of Miss Nerissa S. Vargas.

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Fig. 1. Cabbage-like callus growths produced from very young leaf (blade with midrib as explants).

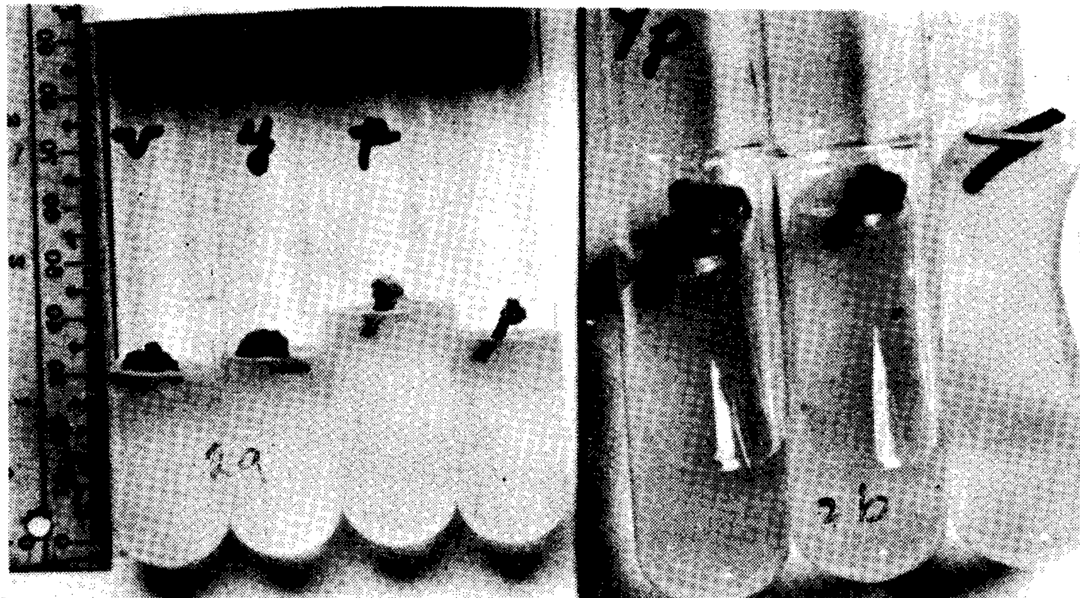

Fig. 2a. Mushroom-like and lollipop-like callus growths from petiole explants of very young leaf after 45 days culture.

Fig. 2b. Callus growths from petiole explant after 20 days culture.

Fig. 3. Transverse section of leaf blade showing mesophyll tissue, vein and epidermal parenchyma cells (x 100).

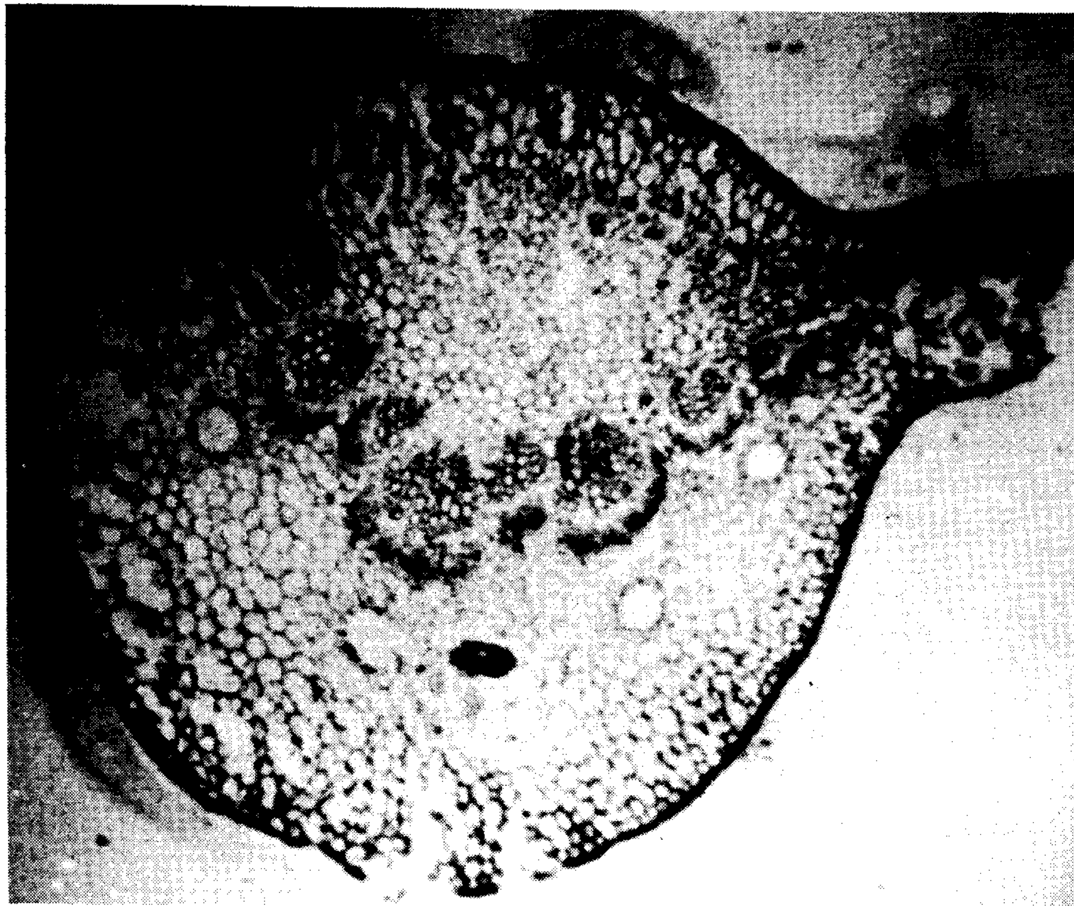

Fig. 4. Transverse section of midrib showing collenchyma, parenchyma, procambium and vascular bundle (x 100).

Table 1. The effect of the different growth regulators and source of explants on the callus and plantlet formation of *Pittosporum resiniferum* Hemsl.

Treatment RMS + Growth Regulators (mg/L)	Source of Explants	No. of Samples	No. of Samples Producing Plantlets	No. of Explants Producing Callus (%)
10 BA + 0.25 NAA 0.25 (2,4-D)	Very young leaf blade with midrib	10	*3/3	10(100)
	Very young leaf blade	10	0	10(100)
	Very young leaf tip	10	0	9(90)
	Very young leaf base	10	0	7(70)
	Very young petiole	10	0	8(80)
	Young petiole	10	0	5(50)
10 BA + 0.25 NAA 0.25 (2,4-D)	Very young leaf blade with midrib	10	**3/4	10(100)
	Very young leaf blade	10	0	9(90)
	Very young leaf tip	10	0	8(80)
10 BA + 0.25 NAA 0.25 (2,4-D)	Young leaf blade with midrib	10	0	7(70)
	Young petiole	10	0	7(70)
	Young leaf base	10	0	5(50)
	Young leaf tip	10	0	5(50)
05 BA + 0.25 NAA 0.25 (2,4-D)	Young stem	10	0	8(80)
	Young leaf blade with midrib	10	0	8(80)
	Young leaf blade	10	0	7(70)
	Shoot apex	10	0	6(60)
	Young leaf base	10	0	5(50)
	Young petiole	10	0	5(50)
	Young leaf tip	10	0	4(40)

Legend:

* 3 samples but each sample produced 3 plantlets

** 3 samples but each sample produced 4 plantlets

Table 2. The effect of coconut water and the different growth regulators on callus formation of *Pittosporum resiniferum* Hemsl.

Treatment RMS + Growth Regulators (mg/L)	Source of Explants	No. of Samples	No. of Explant-Producing Callus (%)
5.00 BA +	Young leaf tip	10	no growth
0.25 NAA	Young leaf blade	10	no growth
30.00 ml CW	Young leaf blade with midrib	10	no growth
	Young leaf base	10	no growth
	Young petiole	10	no growth
5.00 BA +	Young leaf tip	10	no growth
0.25 NAA	Young leaf blade	10	no growth
0.25 IBA 25.00 ml CW	Young leaf blade with midrib	10	no growth
10.00 Ki +	Young leaf tip	10	no growth
2.00 (2,4-D)	Young leaf blade	10	no growth
2.00 GA_3 100.00 ml cw	Young leaf blade with midrib	10	no growth
10.00 Ki +	Young leaf blade	10	5(50)
7.00 (2,4-D) 100.00 ml cw	Young leaf blade with midrib	10	7(70)
10.00 Ki +	Young leaf base	10	6(60)
8.00 (2,4-D) 2.00 GA_3 100.00 ml cw	Young petiole	10	4(40)
10.00 Ki + 9.00 (2,4-D) 2.00 GA_3 100.00 ml cw	Young leaf blade	10	6(60)
10.00 Ki + 10.00 (2,4-D)	Young leaf tip	10	no growth
2.00 GA_3	Young leaf blade	10	no growth
100.00 ml cw	Young leaf blade with midrib	10	no growth
	Young leaf base	10	no growth
	Young petiole	10	no growth

Treatment RMS + Growth Regulators (mg/L)	Source of Explants	No. of Samples	No. of Explant-Producing Callus (%)
10.00 BA +	Young leaf tip	10	no growth
2.00 (2,4-D)	Young leaf blade	10	no growth
100.00 ml cw	Young leaf blade	10	no growth
	Young leaf blade with midrib	10	no growth
10.0 BA +	Young leaf tip	10	4(40)
2.0 (2,4-D)	Young leaf blade with midrib	10	6(60)
1.0 GA_3			
100.0 ml cw	Young leaf base	10	2(20)
10.0 BA +	Young leaf tip	10	7(70)
2.0 (2,4-D)	Young leaf blade	10	6(60)
2.0 GA_3			
100.0 ml cw	Young leaf base	10	5(50)
10.0 BA +	Young leaf tip	10	6(60)
7.0 (2,4-D)			
2.0 GA_3			
100.0 ml cw			
2.5 Ki +	Young leaf tip	10	5(50)
1.0 (2,4-D)			
50.0 ml cw	Young leaf blade	10	4(40)
	Young leaf blade with midrib	10	5(50)
	Young petiole	10	6(60)
2.5 Ki +	Young leaf tip	10	no growth
3.0 (2,4-D)	Young leaf blade	10	2(20)
100.0 ml cw	Young leaf blade with midrib	10	3(30)
	Young leaf base	10	1(10)
	Young petiole	10	no growth
5.0 BA +	Young leaf blade	10	7(70)
5.0 (2,4-D)			
100.0 ml cw			

Table 3. Callus and plantlet formation observed in explants grown in different media with out coconut water.

Media RMS + Growth Regulators (mg/L)	Explants	No. of Days	
10.00 BA +	Very young leaf blade with midrib	21	#
0.25 NAA	Very young leaf blade with midrib producing plantlets	44	##
0.25 (2,4-D)	Very young leaf blade	28	#
	Very young leaf tip	40	#
	Very young leaf base	30	#
	Very young leaf petiole	30	#
	Very young leaf blade with midrib (subcultured plantlets—shoot, stem, and root differentiation)	20	###
10.00 BA +	Young leaf blade with midrib	35	#
0.25 NAA	Young petiole	35	#
0.25 (2,4-D)	Young leaf blade	35	#
	Young leaf tip	40	#
	Young leaf base	40	#
5.00 BA +	Young leaf blade with midrib	25	#
0.25 NAA	Young leaf blade	25	#
0.25 (2,4-D)	Young leaf tip	30	#
	Shoot apex	35	#
	Young stem	35	#
	Young leaf base	35	#
	Young leaf petiole	35	#

Legend:

Callus formation
Plantlet formation
After subcultures

Table 4. Callus formation observed in explants grown in different media with coconut water

Media RMS + Growth Regulators (mg/L)	Explants	No. of Days
10 Ki +	Young leaf blade with midrib	40
7 (2,4-D)		
100 ml cw	Young leaf blade	40
10 Ki +	Young leaf base	45
8 (2,4-D)		
2 GA_3	Young petiole	45
100 ml cw		
10 Ki +	Young leaf base	45
9 (2,4-D)		
2 GA_3		
100 ml cw		
10 BA +	Young leaf blade with midrib	40
2 (2,4-D)		
1 GA_3	Young leaf tip	40
100 ml cw	Young leaf base	45
10 BA +	Young leaf tip	40
2 (2,4-D)	Young leaf blade	40
2 GA_3	Young leaf base	40
100 ml cw		
10 BA +	Young leaf tip	40
7 (2,4-D)		
2 GA_3		
100 ml cw		
10 BA +	Young leaf blade with midrib	40
2 (2,4-D)	Young leaf tip	40
1 GA_3	Young leaf base	45
100 ml cw		

Media RMS + Growth Regulators (mg/L)		Explants	No. of Days
10	BA +	Young leaf tip	40
2	(2,4-D)	Young leaf blade	40
2	GA_3	Young leaf base	45
100	ml cw		
10	BA +	Young leaf tip	40
7	(2,4-D)		
2	GA_3		
100	ml cw		
2.5	Ki +	Young petiole	45
1	(2,4-D)	Young leaf blade with midrib	45
50	ml cw	Young leaf tip	45
		Young leaf blade	45
2.5	Ki +	Young leaf blade with midrib	45
3	(2,4-D)	Young leaf blade	45
100	ml cw	Young leaf base	45
		Young leaf tip	no growth
		Young petiole	no growth
5	BA +	Young leaf blade	45
5	(2,4-D)		
100	ml cw		

Appendix I

Legend:

Color:	G, g	Green, greenish
	Br, br	Brown, brownish
	C, c	Cream, creamy
	L	Light
	Y, y	Yellow, yellowish
	W, w	White, whitish
Consistency:	fr	friable
	vfr	very friable
	slfr	slightly friable
	slcomp	slightly compact
Explant:	vy	very young
	M	Mature
	SA	Shoot apex

1.	YLt	=	Young leaf tip	MLt = Mature leaf tip
2.	Ylbl	=	Youngleafblade	MLbl = Mature leaf blade
3.	Ylblm	=	Young leaf blade w/ midrib	MLblm = Mature leaf blade w/ midrib
4.	YLba	=	Young leaf base	MLba = Mature leaf base
5.	Yp	=	Young petiole	Mp = Mature petiole
6.	Yst	=	Young stem	Mst = Mature stem

Steriland:	$Ca(0Cl)_2$	—	Calcium hypochlorite – 1.0%, 1.5%
	Na0Cl	—	Sodium hypochlorite – 1.5%, 2.0%
Surfactant:	Tween 20	—	polyoxyethlene sorbitant monopalmitate
		—	1 drop/100 ml Na0Cl Solution
Culture Medium:	RMS	—	Revised Murashige & Skoog's
	BA	—	Benzyl Adenine
	IBA	—	Indolbutyric Acid
	GA_3	—	Gibberellic Acid
	NAA	—	Naphthalene Acetic Acid
	Ki	—	Kinetin
	CW	—	Coconut water (Mature fruits)
	cw	—	coconut water (young fruits)
Callus Growth:	+++++	—	Excellent
	++++	—	
	+++	—	
	++	—	Moderate growth
	+	—	Least growth
	x	—	No growth

Explant Size:

8 mm x 7 mm – Young leaf and stem
7 mm x 1.5–2.0 mm – Young petiole
4 mm x 1.0 mm – very young petiole
6 mm x 5.0 mm – very young leaf

Appendix II. Revised Murashige and Skoog's Medium (RMS)*

MACRO NUTRIENTS	mg/liter
Ammonium nitrate (NH_4N0_3)	1650
Potassium nitrate ($KN0_3$)	1900
Calcium chloride dihydrate ($CaCl_2.2H_20$)	440
Magnesium sulfate heptahydrate ($MgSO_4.7H_20$)	310
Potassium dihydrogen phosphate (KH_2PO_4)	170
Ferrous sulfate hepta-hydrate ($FeSO_4.7H_20$)	27.80
Di-sodium ethylene-diamine tetra acetic acid	37.20
MICRO NUTRIENTS	
Boric acid (H_3B0_3)	6.200
Manganese sulfate mono-hydrate ($MnS0_4.H_20$)	17.500
Zinc sulface hepta-hydrate ($ZnS0_4.7H_20$)	8.600
Potassium iodide (KI)	0.830
Sodium molybdate di-hydrate ($NaMo0_4.2H_20$)	0.250
Cobalt chloride hexa-hydrate ($CoCl_2.6H_20$)	0.025
Copper sulfate penta-hydrate ($CuS0_4.5H_20$)	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
Myo-inosital	10.00

GROWTH REGULATORS (Different concentrations)

Bacto-agar (0.7%)
Sucrose (2.0% & 4.0%)

*Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiologia Plantarum* 15: 473–497.

Appendix III

GROWTH REGULATORS

Benzyl adenine (BA)	2.0, 5.0 & 10.0
Kinetin (Ki)	2.5, 5.0 & 10.0
Naphthalene acetic acid (NAA)	0.25, 0.50 & 10.0
Indole Butyric acid (IBA)	0.25
Gibberellic acid (GA_3)	2.0 & 2.0
Dichloro-phenoxy acetic acid 2,4-D	0.25, 1, 2, 3, 5, 7, 8, 9, & 10.0

ADJUVANT

Coconut water (CW) (from Mature fruits)	20, 25 & 30.0 ml.
Coconut water (cw)	50 & 100 ml.
pH 5.7	

Appendix IV. Different treatments (mg/L) of Murashige and Skoog's (RMS) basal agar medium

5 BA + 0.25 NAA + 30 ml CW
5 BA + 0.25 NAA + 25 ml CW + 0.25 IBA
10 Ki + 2(2,4-D) + 100 ml cw + 2 GA_3
10 Ki + 7(2,4-D) + 100 ml cw
10 Ki + 8(2,4-D) + 100 ml cw + 2 GA_3
10 Ki + 9(2,4-D) + 100 ml cw + 2 GA_3
10 Ki + 10(2,4-D) + 100 ml cw + 2 GA_3
10 BA + 2(2,4-D) + 100 ml cw
10 BA + 2(2,4-D) + 100 ml cw + 1 GA_3
10 BA + 2(2,4-D) + 100 ml cw + 2 GA_3
10 BA + 7(2,4-D) + 100 ml cw + 2 GA_3
2.5 Ki + 1(2,4-D) + 50 ml cw
2.5 Ki + 3(2,4-D) + 100 ml cw
10 BA + 0.25 NAA + 0.25 (2,4-D)
5 BA + 0.25 NAA + 0.25 (2,4-D)
5 BA + 5(2,4-D) + 100 ml cw