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Tissue Culture of Dillenia Philippinensis Rolfe

Belen T. Lumeran

Faculty, Department of Mathematics and Sciences, AMA International University-Bahrain

Abstract: A protocol was established for mass propagation of Dillenia philippinensis Rolfe. by tissue culture using mature seeds as initial explants inoculated in vitro on solid Knudson C with 0.5 mg/L benzyladenine (BA) and naphthalene acetic acid (NAA) (T_1). Root calli from the cultured seeds were grown in vitro using solid Murashige and Skoog (MS) culture medium with varying concentrations of BA and NAA. Shoot formation was high in 1.0 mg/L BA and NAA (T_2) at 140-175 days of culture. Microcuttings were rooted in semi-solid MS medium with different concentrations of NAA and BA. The use of NAA only in semi-solid MS medium at 0.186 mg/L initiated rooting of microcuttings. Plantlets were harvested and transferred to pots with coco coir dust for acclimatization and hardening under greenhouse condition and later grown in the field. Periodic monitoring proved 100% survival of in vitro plantlets under greenhouse and field conditions.

Keywords: Tissue culture, Dillenia philippinensis, in vitro, calli

I. INTRODUCTION

Plant tissue and organ culture had been used in morphogenesis [1] of plantation crops of economic and medicinal importance including hard to-germinate plants. Dillenia philippinensis Rolfe is an endemic evergreen tree in the Philippines [2]. The many uses of Philippine dillenia include food sources, medicinal value, red dye from the bark, and wood [3]. The fruits are utilized as hair cleanser, an excellent source of jam, sauce, and fish flavourings. The acid juice of the fruits when mixed with sugar is used to cure cough [4]. However, due to logging and shifting cultivation, this economically important tree species is now depleted. Furthermore, seeds do not germinate due to its hard seed coat. In its natural habitat, no seedlings grow beneath the tree. Thus, propagating the plant requires in vitro culture technique for its mass production to conserve this endemic tree species. Plant tissue culture is an efficient technique for the conservation of endemic plant species using Murashige and Skoog (MS) [5].

Tissue culture or micropropagation is the multiplication of plants in sterile condition with the use of nutrient solution or culture medium [6]. As an eco-friendly technology, this leads to mass propagation of true to type, high quality medicinal plantation crops and forest tree of economic value within a limited period [7]. The growth of plant tissue is achieved via culture media composition, mineral nutrients, and added plant regulators [8, 9]. Plant growth in culture is described as a fascinating process [10]. The whole individual plant is obtained from a single cell through the methods of plant tissue culture. The foundations for the laboratory procedure were already established by the earlier workers on this field including the plant to be used, the nature of the required media, growth regulators, and physical conditions suitable for its growth. Seeds can be germinated in vitro and plant parts- the root, stem, and leaves which are cut to serve as explants . The internodes of new cultivar Long-ma No. 1 of Cannabis sativa L. were used as explants for tissue culture [11].These are cultured in sterilized bottles containing culture media with varying concentrations of the preferred hormones to include shoot, root, and callus formation. MS medium with different concentrations of benzyladenine (BA) and naphthalene acetic acid (NAA) was utilized as an optimal initiation medium for Ardisia mamillata Hance [12]. This is a rare ornamental plant of medicinal importance, hence, optimal initiation, shoot proliferation and shoot elongation were established as reliable mass propagation technique for this particular plant. In vitro culture is considered a reliable technique for mass production of plant materials. The pattern of growth of the culture is principally determined by plant growth regulator composition of the tissue culture media- auxin and cytokinin concentrations. Hence, this study was conducted to establish protocol for in vitro culture of D. philippinensis and determine the type of explants for its mass propagation. Today, tissue culture has developed into a process of great importance to plant propagators, producers, and users of high quality planting materials in addition to discovering plant clones and plant science researchers. With the advancement of biotechnology and breeding program, endangered and economically important plants can now be conserved and preserved through mass production of plantlets by tissue culture.

II. METHODOLOGY

The following pre-experimental and experimental procedures were conducted for the establishment of culture and regeneration of cultures.

A. Preparation of Explants and Culture Medium

The seeds from the mature fruits harvested from the field were used as initial explants. The seeds were inoculated in vitro using Knudson C culture medium with varying concentration of Benzyladenine (BA) and Naphthalene Acetic Acid (NAA). The following treatments were used:

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T_0 (0 mg/L BA + 0 mg/L NAA) as control, T_1 (0.5 mg/L BA + 0.5 mg/L NAA), T_2 (1.0 mg/L BA + 1.0 mg/L NAA), and T_3 (2.0 mg/L BA + 2.0 mg/L NAA). The seeds were prepared following the sterilization procedure below. (1) Seeds were washed with soap and water, (2) Washed in running water for 30 minutes, (3) Soaked in 50% Teepol detergent for 20 minutes, (4) Inside the laminar flow, rinsed with sterilized distilled water 3x, (5) Soaked in 70% ETOH for 10 minutes, (6) Soaked in benomyl solution for 10 minutes, (7) Rinsed 3x with sterilized distilled water, (8) Soaked in 10 NaOCl + 1 drop of Tween 20 for 10 minutes, (9) Rinsed with sterilized distilled water 3x, (10) Blotted dry in sterile Petri dish with sterile paper and cover, (11) Removed the hilum, and (12) Inoculated. The established cultures were placed in the Tissue Culture Laboratory growth room. Each treatment concentration was replicated 15 times laid in a Completely Randomized Design (CRD) with a total of 60 inoculated seeds.

Full grown seedlings from inoculated seeds were harvested after 30 days. Roots, stems, and leaves were cut following aseptic technique. Aseptically-derived explants were inoculated in Murashige and Skoog (MS) medium with the same concentration of BA and NAA as in the basal medium for the seeds. The set-up was laid in a Completely Randomized Design (CRD) with each treatment replicated 9 times for a total of 36 cultures. From among the explants used, callus formation was determined following 28-day cycle from inoculation.

III. RESULTS AND DISCUSSION

The following tables show the results of in vitro germinated seeds and inoculated parts for callus formation.

A. In Vitro Germinated Seeds on Knudson C

Table I. Total Number of In Vitro Cultured Seeds on Knudson C

Treatment Concentration	^{NS} Number of Seeds Inoculated	^{NS}(%)In Vitro Germination
T_0 (Control, 0 mg/L BA + 0 mg/L NAA)	5/15	8.0
T_1 (0.5 mg/L BA + 0.5 mg/L NAA)	11/15	18.0
T_2 (1.0 mg/L BA + 1.0 mg/L NAA)	7/15	12.0
T_3 (2.0 mg/L BA + 2.0 mg/L NAA)	9/15	15.0
Total	32/60	53.0%

NS-means not significant at 0.05 level

Table I presents the total number of in vitro cultured seeds on Knudson C medium with varying concentrations of BA and NAA. A total of 60 seeds were inoculated in vitro. Treatment 1 (T_1) with 0.5 mg/ BA + 0.5 mg/L NAA showed the most number of in vitro germinated seeds (11) with 18% germination. It can be inferred that T_1 is the suitable medium for in vitro germination of dillenia seeds with 0.5 mg/l of BA and NAA based on the number and percentage of in vitro germinated seeds. Statistical analysis using Analysis of Variance (ANOVA) revealed insignificant difference in the number and percentage of in vitro germinated seeds uses different treatment concentrations. It can be observed that even in vitro, seeds are difficult to germinate. Seeds can be germinated in vitro [10] as explants and sources of explants using varying concentrations of nutrient solution or culture medium in sterile condition [6].

Varying concentrations of Benzyladenine (BA) and Naphthalene Acetic Acid (NAA) were also used as initiation medium for a rare ornamental plant of medicinal value [12]. Hence, the combination of 0.5 mg/L BA and NAA is considered the best initiation medium for dillenia seeds to grow in vitro.

B. Callus Formation from In Vitro Culture-Derived Shoot Tips Inoculated on MS Medium

Table II. Callus Type and Percent (%) Callus Formation from Inoculated Shoot Tips on MS Medium

Treatment Concentration	^{NS}Cultures with callus growth	^{NS}Callus Type (%)	
		Loose	Compact
T_0 (Control, 0 mg/L BA + 0 mg/L NAA)	0/36	0.0	0.0
T_1 (0.5 mg/L BA + 0.5 mg/L NAA)	5/36	0.0	14.0
T_2 (1.0 mg/L BA + 1.0 mg/L NAA)	3/36	0.0	8.3
T_3 (2.0 mg/L BA + 2.0 mg/L NAA)	3/36	0.0	8.3
Total	11/36	0.0	31.0

NS-means not significant at 0.05 level

As shown in Table II, Treatment 1 (T_1) with 0.5 mg/L BA and NAA had the most callus growth (5/36) or 14% of cultures having compact type (Fig 1). A total of 11 cultures showed successful callus growth or 31.0%. Of the treatment concentrations, T_1 can be considered the best treatment considering the number of cultures with callus growth.

Statistical analysis using ANOVA depicted insignificant difference in the number and percentage of callus growth using different concentrations of BA and NAA in MS medium. Mass propagation of D.philippinensis through tissue culture is an efficient technique to conserve an endemic plant species using MS medium [5]. The growth of plant tissue is achieved with the desired culture media composition [8, 9, 10].

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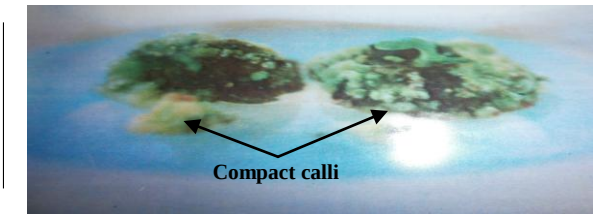


Fig. 1 Compact calli 112 days of culture

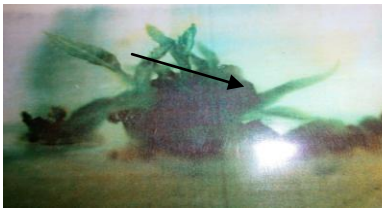
C. Number of Shoots Derived from Established Root Calli of In Vitro Cultured Seedlings

Table III. Number of Callus Culture and Shoots Formed from Root Calli-Derived Cultures 140-175 Days on MS Medium

Treatment Concentration	Total Number of Callus Culture		^{NS}Total Shoots Formed
	^{NS}Initial	^{NS}Final	
T_0	0	0	0
T_1	2	10	7
T_2	4	18	49
T_3	0	0	0
Total	6	28	86

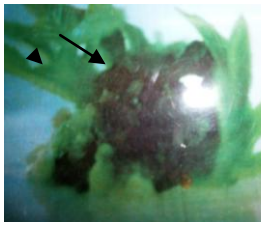
NS- means not significant at 0.05 level

Table III shows the number of callus culture and the shoots formed from root calli-derived cultures, 140 – 175 days culture on MS medium with the same concentration of BA and NAA as in initial medium. Results show increasing number of callus cultures and the total number of shoots formed (Fig 2). Only T_1 (0.5 mg/L BA and NAA) and T_2 (1.0 mg/L BA and NAA) formed shoots. It can be inferred that Treatment 2 is the best culture medium for shoot formation considering the highest number of shoots formed (49) compared to Treatment 1 with only 7 shoots. A total of 49 shoot were formed from an initial culture of 4 and final culture of 18. Hence, a higher concentration of BA and NAA (1.0 mg/L) on MS medium is necessary for shoot formation of the root calli-derived cultures of in vitro germinated seeds. The establishment of shoot proliferation as a reliable mass propagation technique depends on the plant growth regulator composition of the culture medium such as auxin and cytokinin [1]. It also includes the plant to be used and the physical conditions suitable for its growth [10]. Therefore, the desired culture medium and its composition had been established for shoot formation of this particular plant.



140 Days Culture

160 Days Culture



175 Days Culture

Fig 2. Shoots (pointed by arrows) from In Vitro Cultured Root Calli

D. Rooting and Plantlet Establishment

Microcuttings regenerated from root calli cultures were rooted in vitro on semi-solid MS medium with varying levels of NAA and BA. The following treatments were used: T_1 (0.186 mg/L NAA + 0 mg/L BA), T_2 (0.186 mg/L NAA + 0.225 mg/L BA, and T_3 (1.0 mg/L NAA + 0 mg/L BA).

Twenty cultures were established in a Completely Randomized Design (CRD) with 5 replications for each treatment. Results showed that 25% of the microcuttigs rooted in T_1 with 0.186 mg/L NAA. A total of 12 plantlets were transferred to pots with coco coir dust and grown under greenhouse condition for hardening for 28 days.

One hundred percent (100%) of the plantlets survived under greenhouse condition with an average of 5 leaves and an average of height of 59 mm. Plantlets were transferred in the field after 7 days. Periodic monitoring was done under field condition in terms of the indicators of growth and development.

Hence, organ cultured in vitro is considered a reliable technique for mass production of planting materials [1] for economic and hard to-germinate plants specifically D. philippinensis, a forest species of economic and medicinal value.

IV. CONCLUSION

Dillenia philippinensis can be propagated using mature seeds cultured in vitro as initial explants on Knudson C with 0.5 mg/L BA and NAA. Root calli from cultured seedlings established on MS medium with 1.0 mg/L BA and NAA formed shoots.

Rooting of microcuttings at 0.186 mg/L NAA generated viable plantlets for acclimatization under greenhouse condition and later grown in the field. Hence, a protocol for organ culture had been established for mass propagation of this economically important plant.

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