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RESEARCH ARTICLE

Optimization and Initiation of Meristematic Tissue in Melicope Lunu-Ankenda for Organ Culture Establishment

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Abstract

The meristematic tissue or callus induction was observed in culture medium consisted MS basal medium, 3% (w/v) sucrose, 3 g/L phytogel, supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (0, 0.5, 1.0, 3.0 or 5.0 mg/L) and α-Naphthalene acetic acid (NAA) (0, 1.0, 3.0, 5.0 or 10 mg/L) either on its own or combined. At 1.0 mg/L 2,4-D with combination of 10.0 mg/L NAA, dark green meristematic tissue was produced with the greatest weight, 8.45 g. This treatment showed the best characteristics which is dark green colour, highest in weight of and the low number of shoots produced. Meristematic tissue that cultured in a liquid shake media also established. The combination of BAP (0.5 – 1.0 mg/L) and NAA (0.5 – 1.0 mg/L) was superior to other treatments for producing fresh weight of cultures with a maximum of 12.67 g. More meristematic tissue with unorganized cells was produced rather those plantlets in this treatment which can be utilized further in organ culture establishment.

Key words: Micropropagation, Melicope lunu-ankenda, BAP, cytokinin and auxin

Introduction

Melicope lunu-ankenda is a species belonging to the family *Rutaceae*. The leaves are pinnate, trifoliolate, or unifoliolate but less commonly simple; and alternate or spirally arranged, opposite, whorled (rare), and are frequently dotted with oil glands, which appear as dark green spots beneath surface while as translucent spots when they are exposed to light. Genus of *Melicope* is commonly known by Malaysian as "tenggek burung", "pauh-pauh", "medang beberas", "cabang tiga", "tapak itik" while Javanese people called it "sampan" and Siamese called is as "Uam", "Sam Ngam". This plant grows wildly in open area, shrub edge, paddy field and turf areas of land and also grows well in peat and sulphate acid soil. [1]. "Tenggek burung" are popular for their traditional benefits in rejuvenating the body and lowering hypertension. However, Indian usually used this plant to relieve fevers, and as a tonic and for improving complexion [2]. Advanced biotechnology created new potential for *Melicope* genetic improvement and metabolites generation. The best alternative to perform mass production of this plant is tissue culture technique which is micropropagation. Micropropagation is defined as the true-to-type propagation of selected genotypes using in-vitro culture technique. Currently, enhanced axillary branch proliferation from cultured meristems is the most often applied micropropagation technology for commercial production. This is because this approach ensures genetic stability and is simple to use for a wide range of plant species. [3]. The present research is to develop an efficient callus initiation system *Melicope lunu-ankenda* of through shoot micropropagation. This study is to establish a stable callus induction and organ culture with optimum concentration of plant growth regulators.

Materials and Methods

In Vitro Shoot Cultures and Green Meristematic Tissue

The *M. lunu-ankenda* plants grown and maintained in a glass house was used as explant. Lateral shoots segments were used to initiate shoot cultures. Explants were cleaned in a detergent (Teepol) solution for 20 min and rinsed in distilled water. Surface sterilization was preceded by immersed in 20% (v/v) Clorox® containing several drops of Tween-20 for 20 min and repeated for another 20 min in 5% (v/v) concentration. Subsequently, rinsed with sterilized distilled water few times. The dead tissues were excised and shoot explants were inoculated onto MS medium supplemented with 3% sucrose and 1.0 mg/L BAP. The medium was adjusted to pH 5.8 prior

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solidified with 0.3% (w/v) gelrite and sterilized by autoclaving at 121˚C and 104 kPa for 15 min. All cultures were incubated in a culture room under white light provided with white fluorescent light at intensity of 3000 lux at a photoperiodic 16 h. The room temperature was maintained at 25˚C ± 2˚C. The established *in vitro* plantlets were sub-cultured every three months interval. These plantlets (Figure 1a) were used as explant for callus induction.

Under aseptic condition, stem of the *in vitro* plantlets was excised in 1 - 2 cm length and inoculated onto callus initiation medium. The medium consisted of MS basal medium, 3% (w/v) sucrose, 3 g/L phytogel and 2,4-D at concentrations of 0, 0.5, 1.0, 3.0 or 5.0 mg/L or NAA at concentrations of 0, 1.0, 3.0, 5.0 or 10 mg/L, or combination of both phytohormones (Table 1). All cultures were maintained under light (1200 lux) and monitored weekly. The number of plantlets produced and also green meristematic tissue (fresh weight) formed were recorded. Any further changes in the colours of the callus were also recorded.

Meristematic Tissue and Establishment of Organ Culture in Liquid Culture

The yellow-green and dark-green calli were used for induction of meristematic tissue. A small piece of callus (app.3 gram) that has been cut were aseptically transferred onto the liquid medium consisted of MS basal medium, 3% (w/v) sucrose, and phytohormones; BAP at concentrations of 0, 0.5, 1.0, 3.0 or 5.0

Figure1: Establishment of organ cultures in liquid shake culture of *Melicope lunu-ankenda*. (a) *In vitro plantlets,* (b, c) green meristematic tissue, (d) more plantlets produced when cultured on 5 mg/L NAA, (e, f, g) Shoot produced in liquid culture containing 0.5 mg/L BAP, (h, i, j, k) unorganized cells and (l) organ cultures.

Table 1: Effect of 2,4-D and NAA on initiation of plantlets and green meristematic tissue in light condition. [Color of callus: * Yellow- green, **dark-green, *** yellowish, necrotic- green]

NAA Conc.(mg/L)	2,4-D concentrations (mg/L)					
	0	**0.5**	**1.0**	**2.0**	**3.0**	**5.0**
	[Number of plantlets#]					
0	0	3±0.3	4±0.7	0	0	0
0.5	3±0.5	23±5.1	17±6.1	24±4.8	16±2.3	5±0.4
1.0	3±0.7	32±5.3	61±11.2	25±5.7	17±3.1	13±2.8
3.0	4±1.1	41±9.5	49±9.3	34±12.3	18±2.4	12±3.5
5.0	6±0.3	21±6.1	19±4.1	25±5.2	29±1.1	6±0.9
10.0	3±0.5	7±0.9	10±2.2	7±0.6	7±1.4	3±0.7
[Weight of green meristematic tissue produce (gram)]						
0	-	0.16*	0.33*	0.56*	2.11*	0.97*
0.5	-	0.12 *	0.34*	1.12*	4.23*	1.34*
1.0	-	0.34 *	0.56*	2.56*	4.98*	2.12*
3.0	-	0.45*	0.97*	5.23*	7.81**	2.34*
5.0	-	4.56**	5.49**	6.44**	6.45**	1.89***
10.0	-	7.5	8.45**	7.21**	6.54 ***	0.56***

Table 2: Effect of NAA and BAP on meristematic tissue initiation and plantlets produced in a liquid shake flask after 45 days of culture.

Plant growth regulator(mg/L)		Fresh weight (g)	Remarks
BAP	NAA		
0	0	13.4±3.5	More plantlets
0.5	0	17.28±2.5	More plantlets
1.0	0	16.67±4.8	More plantlets
3.0	0	9.34±1.0	-
5.0	0	5.34±1.6	-
10.0	0	3.45±0.5	-
0	0.5	2.45±0.7	-
0	1.0	4.56±0.2	-
0	3.0	3.23±0.3	-
0	5.0	2.91±0.5	-
0	10.0	1.98±0.1	-
0.1	0.1	6.78±1.3	
0.5	0.5	12.45±3.3	unorganized cell
0.5	1.0	12.67±4.1	unorganized cell
1.0	0.5	11.45±0.7	unorganized cell
1.0	1.0	8.56±0.9	-
2.0	2.0	5.67±2.5	-

mg/L singly or in combination with NAA at concentrations of 0.5 or 1.0 mg/L, or with 2,4-D at concentrations of 0.5 or 1.0 mg/L, respectively (Table 2). The cultures were then placed on orbital shaker at 120 rpm. The fresh weight of plantlets or unorganized cell were recorded after 45 days of culture. The unorganized cell was transferred into similar medium liquid medium for further development, then placed on a rack culture with no shaking.

Result and Discussion

Isolation of Meristematic Tissue

The center of plant growth are the meristems and it is situated in the apical and lateral buds of plants and therefore the isolation of meristematic tissue is essential in providing actively dividing tissues [4]. Moreover, meristematic tissue are tissues that are disease free and hence the isolation of the tissue is crucial for future development of *Melicope lunu-ankenda*. However, the optimum concentration of 2,4-D and NAA to produce meristematic tissue in relation for in vitro organ culture are not yet known from past researches. Hence this research provide necessary infromation regarding the cocentration of phytohormones such as 2,4-D, NAA and also BAP.

The effects of different concentration of 2,4-D (0.5, 1.0, 2.0, 3.0 and 5.0 mg/L) and NAA (0.5, 1.0, 3.0, 5.0 and 10.0 mg/L) on green meristematic tissue and plantlets initiation were investigated. Table 1 shows the efficiency of meristematic and plantlets initiation after one month in culture. Different concentrations and combinations of growth regulators showed different responses. In general, MS medium supplemented with lower 2, 4-D (1mg/L) combined with higher NAA (10 mg/L) were more effective in promoting green meristematic tissue (Figure 1b, c). The callus turned yellow-green with some necrotic/brown when 3mg/L 2, 4-D or more were applied in the media. More plantlets produced (Figure 1d) when treatments 5 mg/L NAA or less combined with 0.5 mg/L 2,4-D were used. Media that produced a higher number of plantlets are not superior quality for meristematic tissue initiation. Based on multiple observations, treatment of 1mg/L 2, 4-D combined with 10 mg/L NAA was the optimum medium selected for the purpose. The treatment showed the best characteristic; meristematic tissue with dark-green color, highest in weight and the number of plantlets produced was comparatively lower than other treatments. The entire organized cell developed turned into organ cultures after it was placed on rack culture without shaking. All the collected organ culture is showed in Figure 1l.

1.0 mg/L of 2, 4-D is sufficient to produce good quality meristematic tissue which can be seen stated by [5] and further reduction of 2, 4-D concentration leads to lower callus production in terms of quantity and quality. However in a study by [6], 2 mg/L of 2, 4-D produce the highest callus as expected but the callus produced were whitish and yellowish whitish despite the study used *Citrus reticulata* L. which is in the same family as *Melicope lunu-ankenda.* As for NAA, according to [7], NAA is prominent in producing green, compact callus parallel with this research that uses NAA to produce green callus in the isolation of meristematic tissue. Other than that, 2.0 mg/L of NAA which is the highest concentration used in a research conducted by [8] with the combination of BAP instead of 2, 4-D produce the highest percentage of callus in meristem culture. In contrast, low concentration of NAA that is 1.0 mg/L produce soft yellowish callus [5] which suggested that high concentration of NAA contribute in producing superior callus.

Not only that, it can be seen also in a study by [9] that high concentration of NAA able to produce green callus but also 10 mg/L of NAA can promote efficient plant regeneration. However, [1] obtained a crucial information whereby the combination of higher amount of 2, 4-D (3.0 mg/L) and lower concentration of NAA (1.0 mg/L) was the best for callus formation in *Melicope lunu-ankenda* in context for direct shoot regeneration. On the other hand, in this research, the optimal condition for *Melicope lunu-ankenda* callus formation from isolation of meristem for initiation of organ culture is with a lower amount of 2, 4-D (1.0 mg/L) and a combination of higher amount of NAA (10 mg/L). This suggested that for initiation of organ culture in producing green callus, higher amount of NAA with a combination of lower amount of 2, 4-D is preferable.

Establishment of Organ Culture

The experiment was carried out with the aim of evaluating *in vitro* effects of plant growth regulators; auxin (NAA) and cytokine in (BAP) combined at different levels on meristematic tissue initiation of *Melicope lunu-ankenda*, cultured in a liquid shake media. After 30 days of culture, results showed that the concentration of both hormones had effect on meristematic tissue initiation. Hormonal application of BAP alone stimulated the production of shoots (Figure 1e, f, g). BAP at 0.5 mg/L (17.28) recorded the highest mean in fresh weight (Table 2), followed by treatment of 1.0 mg/L BAP (16.67). The same trend was also observed in number of leaves and plantlets produced. It was noted that the treatments produced taller plantlets than other treatments.

Other than that, the heights in other concentrations series produced plantlets with reduced heights and short internodes when the BAP level was increased. Therefore, we concluded that BAP is unsuitable for meristematic tissue initiation. On the other hand, result showed that the MS media containing BAP (0.5 – 1.0 mg/L) in combination with NAA (0.5 – 1.0 mg/L) were optimal in producing higher fresh weight of cultures (with no shoot). These media produced more meristematic tissue with unorganized cell (Figure 1h, i, j, k). The entire unorganized cell developed turned into organ cultures are showed in Figure 1l. Since the media produce more meristematic tissue which is actively dividing tissues, hence it will be further used as manipulation for future in organ culture researches.

As seen in Table 2, combination of 0.5 mg/L of BAP and 0 mg/L NAA produce the heaviest (17.28 g) fresh weight and despite being the heaviest, the are more plantlets in the flask which is not optimal in this research. Therefore, it can be suggested that the presence of NAA combined with BAP produce unorganized cells that are preferable in order for manipulation. The combination of BAP and NAA in producing unorganized cells can also be seen in a study by [10] that showed 1.0 mg/L of NAA regardless the amount of BAP (0.1 – 0.4 mg/L) contribute to callus production while 0 mg/L of NAA and 0 mg/L of BAP produce tall plantlets parallel with the results seen in Table 2 where similar concentration of BAP and NAA produce more plantlets instead of callus which can be notified as unorganized cells. Other than that, another study by [11] proved that the combination of BAP, NAA but with the addition of 2, 4-D tend to produce superior callus compared to other combination such NAA and kinetin or even only 2, 4-D. Furthermore, [12] stated that increasing concentration of NAA will cause callus formation to decrease parallel with the results that can be observed in Table 2.

Conclusion

As a conclusion, the combination of 1.0 mg/L of 2,4-D with 10 mg/L NAA which are both synthetic auxins produce dark

green callus which is optimal for isolation of meristematic tissue. Other than that, 0.5 mg/L NAA and also 1.0 mg/L of BAP which is a combination of synthetic auxin and cytokinin are found to be suitable in producing unorganized cells which can be further used in future researches. Other than that, the optimal condition to produce green meristematic tissue and also unorganized cells were also established and this not only ease future researches in organ culture establishment but also provide necessary information regarding *Melicope lunu-ankenda* in effort to discover endless posibilities of this species.

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