

IN VITRO CULTURE OF *Pogostemon cablin* BENTH. (NILAM PLANT): THE EFFECT OF NAA AND BAP ON EMBRYOGENIC CALLUS PROLIFERATION AND SUBSEQUENT SOMATIC EMBRYOGENESIS

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Abstract

An experiment to investigate the somatic embryogenesis from shoot-derived callus of *Pogostemon cablin* (nilam plant) has been conducted at the Plant Biotechnology Laboratory, Agricultural Faculty, University of Jambi from January through to July 2004. Callus proliferation was induced on explants taken from young shoots cultured on solid MS medium supplemented with phytohormones NAA (0.8, 1.1, 1.4, and 1.7 ppm) and BAP (1.1, 1.4, 1.7, and 2.0 ppm) under *in vitro* conditions. Cultures were maintained at 25 ± 1 °C, light intensity $50 \mu\text{mol m}^{-2} \text{s}^{-1}$, and 16 hours photoperiod. The results indicated that all cultured explants showed positive responses on callus proliferation on all treatments within two weeks of culture initiation. The effect of phytohormones, however, was unspecific as all callus showed similar properties, from non-embryogenic to embryogenic. The addition of NAA and/or BAP to the culture medium was not significantly affected the number of days to callus proliferation. Callus fresh weight was significantly affected by NAA ($P = 0.01$) or BAP ($P = 0.05$), but the interaction of these phytohormones resulted in a non-significant effect on callus fresh weight ($P = 0.18$). Also, BAP significantly affected callus dry weight ($P = 0.03$). However, neither NAA nor its interaction with BAP significantly affected callus dry weight ($P = 0.07$ and 0.16 , subsequently). Embryogenic and non-embryogenic callus were subcultured separately onto new fresh media with the same composition as for callus induction. Following this subculture, embryogenic callus regenerated somatic embryos within ten days, whereas non-embryogenic callus did not show any symptom of embryogenesis, and lost their proliferative capacity after six weeks of subculture. The regenerated somatic embryos continued to grow to form profuse mass of young plantlets ready for *in vivo* acclimatization.

Keywords: *Pogostemon cablin*, nilam, patchouli oil, tissue culture, *in vitro* culture, NAA, BAP

1. Introduction

Nilam (*Pogostemon cablin* Benth.) has been known as one of important patchouli oil-producing plants in Indonesia. Along with other twelve patchouli oil-producing plants, this species has been developed since many years. Indonesian nilam oil has been recognized as one of the best patchouli oil in the world, having high market price in the international trade [1]. Nilam oil is commonly used as a fixative in perfume industry to keep the fragrance lasts longer. Besides, nilam oil may also be used in soap, hair tonic, and aroma therapy industries.

For years nilam propagation has been through conventional vegetative method using cutting from young stems sizing 20 – 30 cm with at least three axillary buds [1]. Although plant propagation by cuttings offer an advantage to obtain plants with similar genetic and phenotypic properties to the stock plants, the limited number of available stock plants has not made this method of propagation economically sound for a large scale industry [2], especially for nilam plantation that require long-size cutting materials. In addition, this method of plant propagation may reduce the productivity due to various fungi, bacteria, and viruses infections accumulated from one generation to the next [3]. As a consequence, the

number of resulted propagules will be very limited, and having poor health status. Therefore, plant propagation by cutting is not reliable for mass propagation of nilam.

With new approach in plant propagation, i.e. by the use of *in vitro* technique, technical or physiological constraints in cutting propagation could be overcome. Recently, various medicinal and aromatic substance-producing plants such as *Allium sativum* [4] and *Nathopodytes foetida* [5] have successfully been propagated using *in vitro* method via somatic embryogenesis. Plant regeneration via somatic embryogenesis is an *in vitro* process that is used to speed up multiplication rate, and is an efficient system for mass clonal propagation [6-10]; and therefore, it offers advantages in developing artificial seedlings [11]. Many authors have reported the induction of somatic embryogenesis affected by the different types and concentrations of auxins and cytokinins used in culture medium [12, 13], though in certain species the presence of these exogenous phytohormones was not always necessary [14].

We report here the *in vitro* conditions required for the induction of somatic embryogenesis from shoot-derived callus of nilam plant, with the focus on the effect of naphthaleneacetic acid (NAA) in combination with benzylamino purine (BAP) at various concentrations.

2. Methods

Stock plants were from *in vitro* culture provided by Plant Tissue Culture Laboratory, Bogor University of Agriculture. They were then propagated at Plant Biotechnology Laboratory, Agricultural Faculty, University of Jambi to produce more culture materials. Explants used for culture initiation were shoot segments of approximately 1 cm long.

The culture medium used was Murashige and Skoog (MS) [15], supplemented with vitamins, *myo*-inositol, 30 g l⁻¹ sucrose, and 8 g l⁻¹ agar (Bacto-agar, Difco, Fisher Scientific), and medium pH was adjusted to 5,8 ± 0,02 prior to heating to dissolve the agar. The homogenous medium solution was dispensed into culture flasks (jam jars) each of 20 mL per flask, sealed with aluminum foil before being autoclaved at 1.06 kPa, 121 °C, for 15 minutes.

The tested variables were auxin naphthaleneacetic acid (Sigma Chemical) at concentrations of 0.8, 1.1, 1.4, and 1.7 ppm, and cytokinin benzylamino purine (Sigma Chemicals) at concentrations of 1.1, 1.4, 1.7, and 2.0 ppm. There were sixteen combination of treatments, each of ten replicates. Each replicate consisted of one culture flask containing three shoot explants. This experiment was conducted using a completely randomized design.

All cultures were placed on the shelves in a culture room. Temperature in the room was set to 25 ± 1 °C, light intensity of approximately 50 μmol m⁻² s⁻¹, and 16 hours photoperiod resulted from cool white fluorescent lamps (Phillips Indonesia). Cultures were maintained for eight weeks with weekly observations.

The parameters observed were the number of days for callus proliferation, average explant fresh weight, and average explant dry weight. The assessment of explant fresh weight was done by weighing the whole explants (initial explant plus proliferated callus) from sample cultures. Having had the fresh weight, the sample cultures were dried in an oven at 70 °C for 72 hours, and cooled down to room temperature prior to weighing.

The proliferated callus were grouped, based on their morphological appearances, into embryogenic and non-embryogenic, which were then subcultured separately onto new fresh media with the same composition as for callus induction. The observations on further growth and development of the callus, as well as somatic embryo regeneration, were done for another eight weeks on both callus groups.

The Analysis of Variance (ANOVA) was used in data analysis using Microsoft Excel [16] to see the effect of NAA and/or BAP on explant growth. In addition, the differences among treatment means were analyzed using Least Significant Difference test [17] at 5% probability.

3. Results and Discussion

All cultured explants showed positive response to callus formation. Callus started to proliferate on the explant surface, particularly at the cut end, after two weeks of culture initiation. However, the effect of NAA, BAP or their interactions on the number of days to callus proliferation was not significant (*P*-value = 0.09, 0.89, 0.92, subsequently). The fastest

callus proliferation occurred on 0.8 ppm NAA + 1.4 ppm BAP (10.3 days on the average), and the slowest callus proliferation was on 1.4 ppm NAA + 1.7 ppm BAP (29 days on the average) (Figure 1). The ability to obtain embryogenic callus from cultured explants is an advantage for clonal propagation via *in vitro* system [18]. Medium composition is particularly important for embryogenic callus formation [19].

The growth increase in cultured tissues were assessed based on their fresh weight by measuring the whole tissues (initial explant plus proliferated callus) of an experimental unit. The ANOVA showed that the addition of NAA to culture medium significantly affected the average fresh weight of explants ($P = 0.01$). Explant fresh weight was also affected by the addition of BAP ($P = 0.05$). However, the interaction of NAA and BAP did not significantly affected the fresh weight of the explants ($P = 0.18$). The highest fresh weight was obtained on 1.1 ppm NAA + 1.7 ppm BAP (239 mg on the average), whereas the lowest fresh weight was obtained on 1.7 ppm NAA + 1.1 ppm BAP (114 mg on the average) (Figure 2).

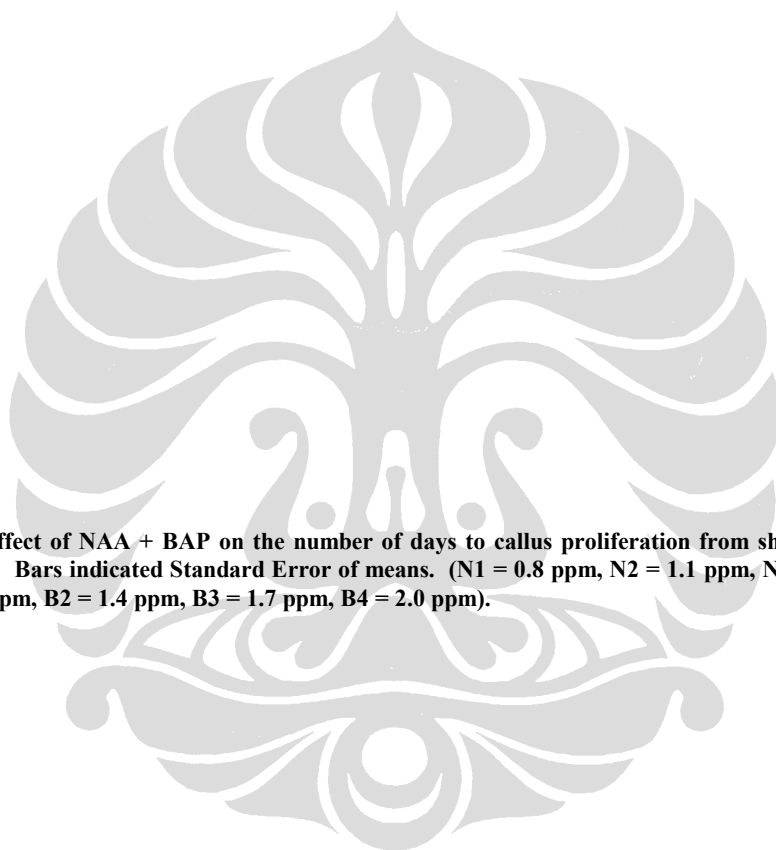


Figure 1. The effect of NAA + BAP on the number of days to callus proliferation from shoot explants of nilam cultured *in vitro*. Bars indicated Standard Error of means. (N1 = 0.8 ppm, N2 = 1.1 ppm, N3 = 1.4 ppm, N4 = 1.7 ppm, B1 = 1.1 ppm, B2 = 1.4 ppm, B3 = 1.7 ppm, B4 = 2.0 ppm).

Figure 2. The effect of NAA + BAP on the fresh weight of nilam explants following 4 weeks of *in vitro* culture. Bars indicated Standard Error of means. (N1 = 0.8 ppm, N2 = 1.1 ppm, N3 = 1.4 ppm, N4 = 1.7 ppm, B1 = 1.1 ppm, B2 = 1.4 ppm, B3 = 1.7 ppm, B4 = 2.0 ppm).

Explants with known fresh weight were dried in an oven at 70 °C for 72 hours, and cooled down prior to weighing. The ANOVA showed that the presence of NAA in the culture medium was not significantly affected the explant dry weight ($P = 0.07$). On the other hand, the explant dry weight was significantly affected by the presence of BAP in the culture medium ($P = 0.03$). Meanwhile, the interaction of NAA and BAP did not show any significant effect on the explant dry weight ($P = 0.16$). There was a positive correlation between fresh weight and dry weight of the explants. Explants with higher fresh weight were liable to have higher dry weight (Figure 3). The difference in weight after the explants were dried varied from 28 to 43%. This implies that approximately 35% of explant weight was contributed by water. Taji *et al.* [20] suggested that changes in water content within the tissue might result in the fluctuation in explant fresh weight in *in vitro* culture system. Therefore, the measurement of fresh weight should be coupled with the measurement of dry weight to obtain a more appropriate assessment of growth rate of the tissue.

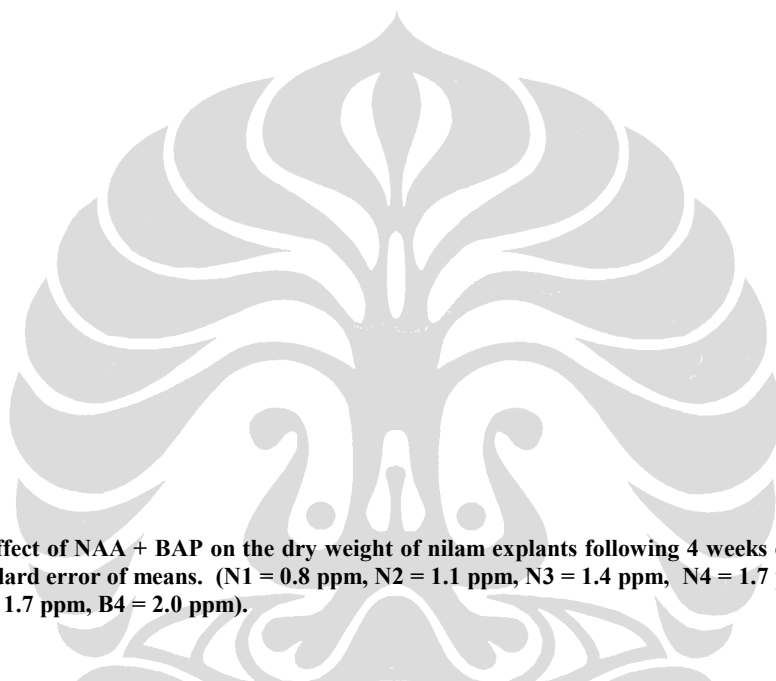


Figure 3. The effect of NAA + BAP on the dry weight of nilam explants following 4 weeks of *in vitro* culture. Bars indicated standard error of means. (N1 = 0.8 ppm, N2 = 1.1 ppm, N3 = 1.4 ppm, N4 = 1.7 ppm, B1 = 1.1 ppm, B2 = 1.4 ppm, B3 = 1.7 ppm, B4 = 2.0 ppm).

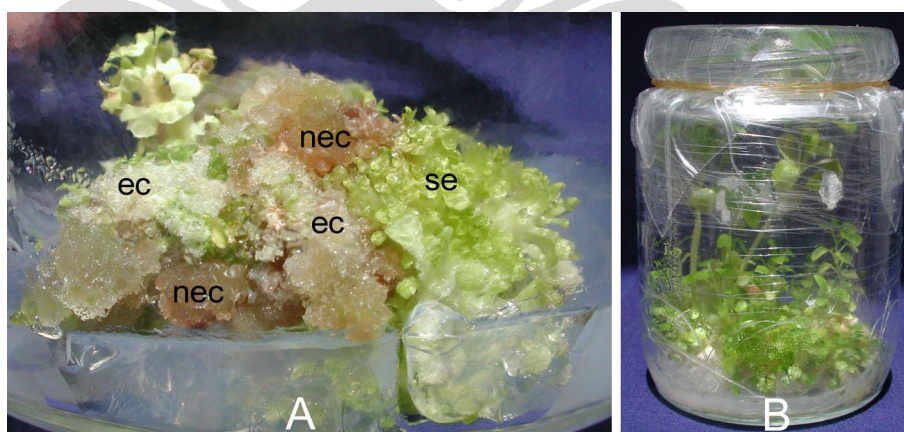


Figure 4. The induction of somatic embryogenesis on *Pogostemon cablin*. Callus proliferation and somatic embryos development (A) Plantlets raised from somatic embryos 8 weeks after subculture (B). (se=somatic embryos, ec=embryogenic callus, nec=non-embryogenic callus).

All treatments produced callus with similar characteristics. The proliferated callus were classified into two groups: embryogenic and non-embryogenic (Figure 4A), with no specific relationship to the levels or types of phytohormones

tested. The embryogenic callus were characterized by white-colored, friable and grew rapidly to form massive somatic embryos on the surface. In contrast, the non-embryogenic callus were light brown or greenish white in color with compact structure, and tended to be hyperhydrated. Following the ten days subculture onto new fresh medium, callus from the first group regenerated somatic embryos with the same growth pattern as in *Hybanthus enneaspermus* [10], i.e. globular, heart, torpedo, and mature embryo stages, which were then developed into plantlets (Figure 4B). In meantime, callus from the second group turned brown, and lost their proliferative capacity following six weeks of subculture.

Using *Bixa arellana*, Sha-Valli-Khan *et al.* [21] demonstrated that the combination of NAA and BAP produced white, friable callus with glossy surface, which later developed into white compact callus, and finally regenerated green globular structures. The formation of this green globular structures was the initial sign of somatic embryogenesis as reported by Sudhersan and Abo-El Nil [22] and Zulkarnain [23] on the *in vitro* culture of *Swainsona formosa*. White and compact embryogenic callus were also reported by Tang *et al.* [24] on *Pinus taeda*, and by Fulzele and Satdive [5] on *Nothapodytes foetida*.

4. Conclusion

This experiment demonstrated that a simple protocol for mass propagation of nilam could be developed via somatic embryogenesis using shoot segments as the initial plant material, cultured on solid MS medium supplemented with NAA and BAP. The addition of phytohormones NAA and BAP into culture medium could trigger the proliferation of embryogenic callus, which then regenerated somatic embryos. These somatic embryos were then developed into intact plantlets ready for acclimatization to *in vivo* condition.

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