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Influence of exogenous NAA in *Calophyllum inophyllum* micro-environment seeds germination

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ABSTRACT

Throughout Indonesia, more than a hundred million ha of degraded forest and lands are still to be rehabilitated. High-quality tree seedlings are a key component of landscape restoration. The limited supply of quality seeds has been an obstacle so far. *C. inophyllum* is a tropical tree species that grow well under harsh environmental conditions. It can be used as an alternative tree species for degraded land rehabilitation. We present an efficient and reproducible protocol for improving seed quality with exogenous NAA hormones. The Murashige and Skoog (MS) medium was supplemented with NAA hormones (0 mg/l, 1 mg/l, 2 mg/l, and 3 mg/l of NAA) for observation of the influence of the exogenous NAA on seeds germination in micro-environment controlled. After seven weeks of incubation in axenic culture, the highest concentration of NAA (3 mg/l) showed the fastest average seed-breaking time of *C. inophyllum* (2.0 weeks), After three weeks, the roots were nearly five times longer (4.9 ± 0.1 cm) than the control (1.1± 0.1 cm), and the shoots were nearly three times longer (4.9 ± 0.1 cm) than the control (1.1± 0.1 cm). This research needs to be tested on a larger scale of cultivation.

ABSTRAK

Di seluruh Indonesia, lebih dari seratus juta ha hutan dan lahan terdegradasi masih harus direhabilitasi. Bibit pohon berkualitas tinggi adalah komponen kunci dari restorasi lanskap. Terbatasnya pasokan benih berkualitas menjadi kendala selama ini. *C. inophyllum* adalah spesies pohon tropis yang tumbuh dengan baik di bawah kondisi lingkungan yang keras. Ini dapat digunakan sebagai jenis pohon alternatif untuk rehabilitasi lahan terdegradasi. Kami menyajikan protokol yang efisien dan dapat direproduksi untuk meningkatkan kualitas benih dengan hormon NAA eksogen. Media Murashige dan Skoog (MS) dilengkapi dengan hormon NAA (0 mg/l, 1 mg/l, 2 mg/l, dan 3 mg/l NAA) untuk pengamatan pengaruh NAA eksogen terhadap perkecambahan biji di lingkungan mikro dikendalikan. Setelah tujuh minggu inkubasi dalam kultur axenic, konsentrasi NAA tertinggi (3 mg/l) menunjukkan rata-rata waktu pemecahan benih tercepat *C. inophyllum* (2,0 minggu), Setelah tiga minggu, akar hampir lima kali lebih panjang (4,9 ± 0,1 cm) dari kontrol (1,1± 0,1 cm), dan tunas hampir tiga kali lebih panjang (4,9 ± 0,1 cm) dari kontrol (1,1± 0,1 cm). Penelitian ini perlu diuji pada skala budidaya yang lebih besar.

Keywords: *C. inophyllum, NAA, micro-environment, seeds, rehabilitation*

INTRODUCTION

Calophyllum inophyllum is a species of the family Guttifereae (Clusiaceae), native to East Africa, Australia, India, Southeast Asia, and the South Pacific. Usually, it is called as 'Indian laurel', Alexandrian Laurel, Pannay tree, Beach Calophyllum, Beauty leaf, Sweet Scented Calophyllum (in English), Burmese, Pongnyet, Hawaii, Sultan Champa, Surpan (in Hindi), Kokani, Nagachampa (in Marathi), Nagam, Pinmai, Pinnay, Namere, Punnagam, Punnai, (in Tamil) (Susanto, et al, 2020) *C. inophyllum* has very wide natural distribution in Indonesia, from Sumatera in the west to Papua in the east, and from Java in the south to Kalimantan in the north. It can survive and grow well in a wide range and is also highly tolerant to harsh environmental conditions; therefore, the species is suitable for the conservation and land rehabilitation in several locations in Indonesia (Leksono, et al, 2021).

C. inophyllum is a tropical tree species that grow well under harsh environmental conditions on unproductive land (Friday & Okano, 2006). The species produces flowers and fruit in profusion all year round, and its seeds can be harvested repeatedly from trees aged 4 to 5 up to 50 years old (Leksono, et al, 2017). The trees generally grow in warm temperatures under wet or moderate conditions and tolerate wind, salt spray, drought, and brief periods of waterlogging (Friday & Okano, 2006). Due to its high tolerance to harsh environmental conditions, since more than 50 years ago, the species has been planted for conservation and land rehabilitation purposes in southern regions of the island, Indonesia (Leksono, et al, 2021). Reports on tamanu performance in mineral soils in Indonesia have shown it grows well in coastal areas (Putri, et al, 2019), on marginal land

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(Windyarini, E., & Hasnah, T. M., 2014), rocky soils (Windyarini & Hasnah, 2014) and burnt land (Toungos & Dahiru, 2018).

Several alternatives to tree planting have been implemented in several regions of the world by direct seeding (Bonilla-Moheno & Holl, 2010; Haryjanto et al, 2020). This method can be quickly applied to large areas, costs less than transplanting seedlings, and has better root system development and structure (Bonilla-Moheno & Holl, 2010). However, the lack of information on the initial variability, germination duration, and the effect of hormones that trigger the initiation of optimal *C. inophyllum* seed roots has not been widely reported. On the other hand, seedlings for land rehabilitation projects are strongly influenced by time or planting season (Klavina et al, 2015). Observing the effect of plant hormones on root initiation by utilizing biotechnology in an *in vitro* axenic environment can minimize the influence of abiotic and biotic factors on plant hormone requirements. Exogenous hormone application can also be better controlled. Auxin, such as Naphthalene Acetic acid (NAA), the most extensively used commercial synthetic auxin for promoting root formation, stimulates adventitious root growth (Omar & Ahmed, 2015). Auxin, carbohydrates, and nitrogen in plant material can induce the formation of roots (Djamhari, 2010). Although each plant can produce its hormones, using hormones from the environment can stimulate metabolic processes in plant growth and development (Ramakhrisna & Ravishankar, 2011). Based on this, the purpose of this study was to determine the effect of the biotechnology application of exogenous NAA in *C. inophyllum* micro-environment seed germination. Hormones could improve the percentage of radicals and reduce propagation time (Ramakhrisna & Ravishankar, 2011). Plant hormones are naturally produced by plants that control plant functions and development, such as root growth (Anfang, M., Shani, E., 2021).

METHODS

Sterilization of Seedlings

The *C. inophyllum* seeds were obtained from the Center for Forest Biotechnology and Tree Improvement, Ministry of Forestry, Indonesia. Several modifications of the sterilization method by (Putri et al, 2019; Putri et al., 2020) were used in this study. Sterilization modifications were carried out on the outside and the inside of the laminar airflow (LAF). Sterilization on the outside of the LAF is to immerse the seeds in distilled water overnight. After soaking in distilled water containing detergent for 1 minute and soaking with a fungicide for 3 minutes, rinsing with tap water for 20 minutes, then soaking in hot water (80 ~ 90° C) for 5 minutes to break seed dormancy. Sterilization on the outside of LAF is by immersing the seeds in 15% (v/v) in anti-microbial compound BI (biocide isothiazolone), which contains 5.25% sodium hypochlorite, hydrogen peroxide, sodium perborate, and sodium percarbonate solution. A few drops of Tween™ 80 were added to the solution for 40 minutes, and 70% ethanol for 1 minute. The seeds were rinsed with sterile distilled water for each sterilization. The percentage of axenic culture in each treatment (one seed per tube) was observed.

Seeds Culture

The research was carried out at the tissue culture laboratory at the Center for Forest Biotechnology and Tree Improvement in Yogyakarta, Indonesia (7040'20" S and 110023'30"), 600 meters above sea level, with the highest temperature being 32 degrees Celsius and the lowest temperature being 18 degrees Celsius and a mean humidity of 95%. Researchers observed for six months in the laboratory, from January 2019 to June 2019. The equipment used in this study met tissue culture laboratory standards. The seeds were cultured under micro-environment controlled, 16-hour photoperiod (50~70 µmol/m^2/s) light, with 20 degrees Celsius and a mean humidity of 80%, sub-cultured once a month for three months. Surface-sterilized seeds were cultured on MS (Murashige & Skoog, 1962) medium with no hormones supplemented, and after seven weeks with NAA (1-Naphthaleneacetic acid) (1000 ppm solution) 0 ml/l, 1 ml/l; 2 ml/l and 3 ml/l, 3% sucrose and 0.2% agar, whose pH was adjusted to 5.7 ± 0.1. To examine the effects of NAA hormone, 20 seed replicates for each NAA treatment were prepared from the best growth of the axenic culture. To fulfill the number of axenic cultures for each NAA treatment (20 cultures per treatment consisting of four treatments), the sterilization of seeds was divided into four blocks of 50 seeds each so that a total of 200 seeds were needed to obtain 80 the best growth of the axenic culture. Seed-breaking time in root formation, root length, and height of shoot were used as a parameter of *C. inophyllum* seeds roots regeneration *in vitro*.

RESULT AND DISCUSSION

Axenic Cultures

Micro-environment seeds rooting culture *in vitro* was defined as the axenic culture of any part of one organism under controlled conditions in a culture medium (**Figure 1**). Sterile conditions in axenic cultures were needed so that nutrients are provided only for target seeds, not used for contaminants. *C. inophyllum* seeds sterilization was carried out immediately after about one week of harvesting from the natural environment to the tissue culture laboratory. This sterilization was one of the causes of high contamination and reduced the acquisition of axenic cultures. Prevention of microorganism contamination in plant tissue culture is

essential. Plants growing *in vitro* are under stress and may be predisposed to direct infection, even by non-pathogenic bacteria (Habib et al, 2002).

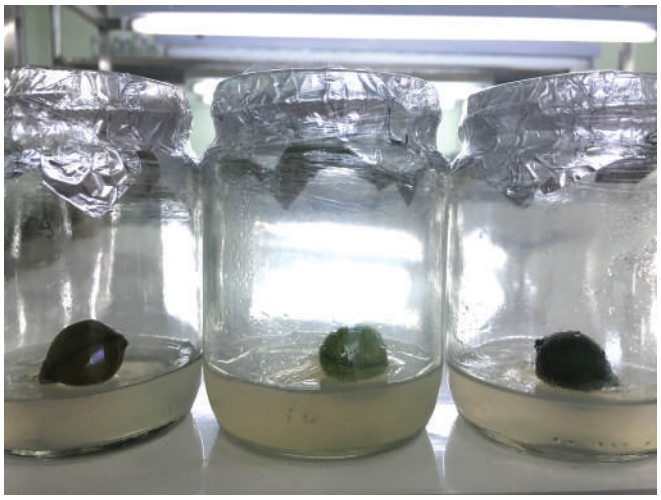

Figure 1. Axenic culture of *C. inophyllum* seeds in micro-environment condition.

The graph in Fig. 2 shows a tendency for the number of contaminated cultures to increase at the beginning of incubation until week 4, but there was no increase in contaminated cultures until week 7. The value still requires better sterilization techniques. However, the source of seeds from the natural environment will cause contamination.

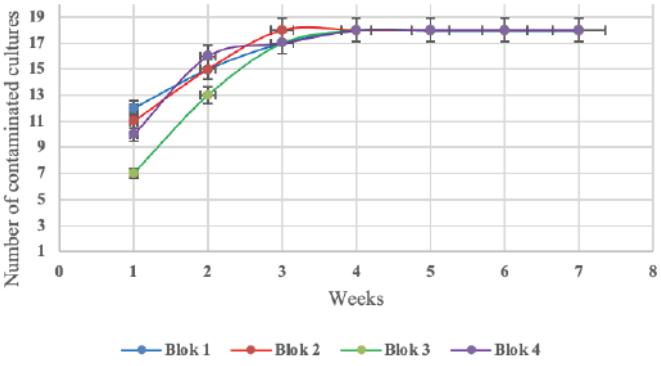

Figure 2. Number of contaminated cultures in 7 weeks incubation (n = 50 cultures/block).

Although the culture medium in which the plant tissue is cultured is a good source of nutrition for contaminant growth, an endogenous contaminant *in vitro* can remain undetected in the micro-environment because the concentration of salts and/or sucrose is not ideal or the pH and/or temperature are not optimal for contaminant growth. When the culture conditions change, as they do during normal plant growth, they can become more favorable to contaminant growth previously undetectable and multiply and even damage the growing plants (Wojtania et al, 2005). The endogenous contaminant can also be difficult to isolate and may only survive or grow well inside the plant. The total contaminated cultures obtained for seven weeks of the observation were 72 cultures from 200 cultures tested (36%), so 128 cultures (64%) were obtained, which could be used for 80 cultures for hormone treatment.

Seed-breaking Time

The NAA treatment with the highest concentration (3 mg/l) showed the fastest average seed-breaking time of *C. inophyllum* (2.0 weeks) compared to the concentration of 2 mg/l (4.6 weeks), concentration 1 (5.1 weeks) and control (6.3 weeks) (**Figure 3**). Seeds of many species do not germinate immediately upon exposure to conditions generally favorable for plant growth, but require a "seed break" of dormancy, which may be related to changes in the seed coat or to the state of the embryo itself. Generally, the embryo has no innate dormancy and will develop after the seed coat is removed or damaged to allow water to enter (Heslop-Harrison & John, 2022). In the observation of bud-breaking, the initial response of *C. inophyllum* seeds showed root growth in all concentrations of NAA treatment (**Figure 4**). Two weeks after seven weeks of root growth, simultaneously shoots grew in all treatments (**Figure 4**). Germination in such cases depends on rotting or abrasion of the seed coat in the soil. Germination inhibitors must be removed with water or the tissue containing them destroyed before germination can occur. Mechanical restriction of embryo growth is only common in species with thick, hard seed coats. Germination then depends on the weakening of the mantle by abrasion or decomposition (Heslop-Harrison & John, 2022).

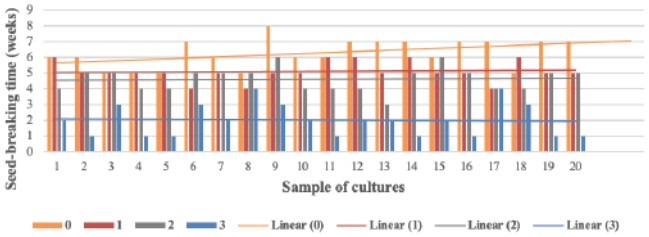

Figure 3. Seed-breaking time of *C. inophyllum* in MS media with 1 mg/l, 2 mg/l, 3 mg/l of NAA added and control at micro-environment.

As in many seeds, the *C. inophyllum* embryo cannot germinate even under suitable conditions for some time. The effect of the micro-environment with conditions of temperature, light, humidity, and light intensity on media containing the NAA hormone, which is measurable, is needed to observe seed development under controlled conditions that are difficult to do directly in nature. This effect can be used as the basis for the required finishing process after "maturation", which is still unclear. Ambient temperature also affects germination, it will fail to grow very late, or growth is not normal. The

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micro-environment study showed the suitability of conditions up to the emergence of rooting and shoots of *C. inophyllum in vitro*. The sensitivity of light and temperature interactions for *C. inophyllum* is not widely known; light needs can be completely lost at a certain temperature.

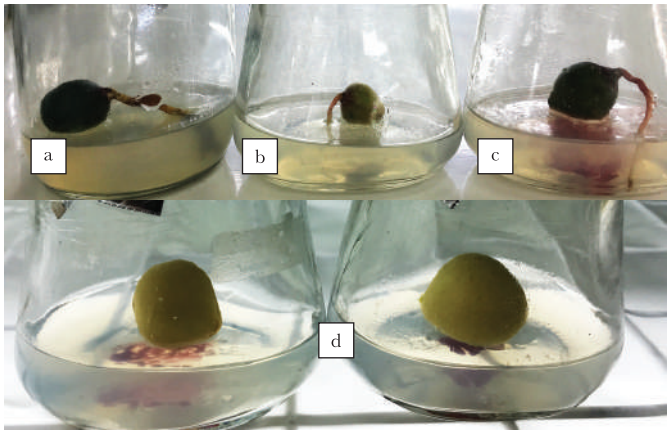


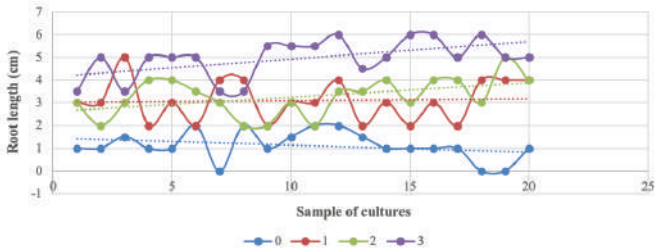
Figure 4. *C. inophyllum* seeds break in 1 mg/l (a), 2 mg/l (b), 3 mg/l (c) of NAA added and control (d) after seven weeks incubation at micro-environment.

C. inophyllum seed appears to have a "critical moisture content", a characteristic feature of a recalcitrant seed. *C. inophyllum* seed can retain viability for an appreciable period (> 8 months) if stored in warmer and slightly humid environments without removing the endocarp, and the seeds appear to be sensitive to low temperatures (Hathurusingha & Ashwath, 2012).

Root and Shoot Length

For three weeks of incubation in the micro-environment at the tissue culture laboratory, the NAA hormone treatment gave a growth response to both root and shoot lengths of *C. inophyllum* seeds. Figure 5, Figure 6, and Figure 7 show the effect of NAA on root and shoot elongation during three weeks of incubation after seven weeks of axenic culture selection and two weeks of seed-breaking maturation. During the 12-week study in this micro-environment, sub-cultures were carried out every month with the same media in each treatment to maintain the availability of *C. inophyllum* nutrients.

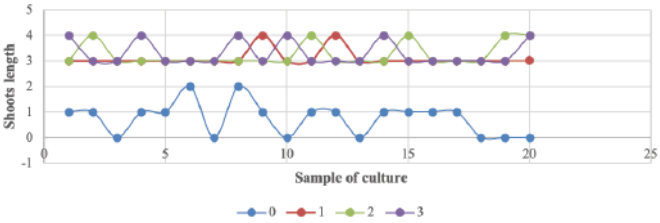
The average addition of root length also followed high exogenous NAA concentrations, the highest at concentrations of 3 mg/l (4.9 ± 0.1 cm), 2 mg/l (3.2 ± 0.1 cm), 1 mg/l (3.1 ± 0.1 cm) and control (1.1± 0.1 cm). In this study, the highest concentration of NAA increased root length almost five times higher than the control. However, Figure 5 shows that all additions of NAA tend to be positively correlated to the increase in the addition of NAA to the culture media. It is also possible that the physical and biochemical microenvironment is suitable for germinating *C. inophyllum* seeds.



Each value is a mean of twenty replicates, significantly different from each other at 0.05 probability level by the Duncan multiple range test. The results of ANOVA showed significant differences (p ± 0.05) between NAA concentration treatment for 200 axenic seeds.

Figure 5. Roots length of *C. inophyllum* in MS media with 1 mg/l, 2 mg/l, 3 mg/l of NAA added and control at micro-environment

In contrast to the exogenous effect of NAA on the root growth of *C. inophyllum* seeds, the response of shoot elongation with all concentration treatments did not show any significant difference. However, NAA still increased shoot elongation three times higher than the control (0.76 ± 0.1 cm). The average elongation of shoots with the addition of 3 mg/l (3.3 ± 0.1) NAA was not significantly different with the addition of 2 mg/l (3.2 ± 0.1 cm) and 1 mg/l (3.1± 0.1 cm).



Each value is a mean of twenty replicates, significantly different from each other at 0.05 probability level by the Duncan multiple range test. The results of ANOVA showed significant differences (p ± 0.05) between NAA concentration treatment for 200 axenic seeds.

Figure 6. Shoots length of *C. inophyllum* in MS media with 1 mg/l, 2 mg/l, 3 mg/l of NAA added and control at micro-environment

Environmental factors play an important part in determining the orientation of the seedling during its establishment as rooting or shooting of a plant and in controlling some aspects of its development. When seeds germinate below the soil surface, plumules may appear and are only visible when light exposure. Correspondingly, the young leaves of the plumules do not expand and turn green unless exposed to light. This adaptive response is regulated by the role of light-sensitive pigment phytochrome reactions. The shoots generally show a strong attraction to light or positive phototropism. Combined with a response to gravity, this positive phototropism maximizes the likelihood that the plant will achieve the most favorable environment for photosynthesis (Hathurusingha & Ashwath, 2012). In a micro-environment with a

completely controlled environment, there is a triggering power for seeds to germinate faster; roots and shoots are expected to be healthier and stronger. These seeds can also be used as a source of material for vegetative propagation explants through *in vitro* multiplication.

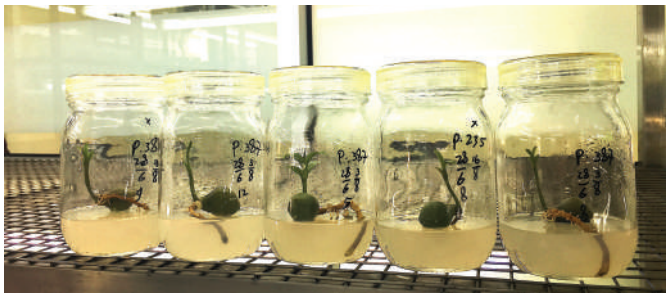


Figure 7. *C. inophyllum* roots and shoots length after 12 weeks incubation in micro-environment.

Inadequate supply and quality of tree seeds and seedlings is a major obstacle to the success of rehabilitation (Sacco et al, 2021; Duguma et al, 2021), especially in terms of the lack of consideration of which species to choose and where and how seeds are obtained. Despite the importance of the genetic quality of planting material for restoration efforts, existing recommendations for seed collection are not consistently integrated. Poor growth and survival of seedlings also increase restoration costs and results (Roshetko et al, 2018). Therefore, increasing the supply of seeds quantity and quality is very important to ensure that it can deliver the desired benefits and contribute to the rehabilitation of degraded lands and the climate crisis. The research is one of the initial efforts to obtain seeds as a source of quality genetic material.

CONCLUSION

As an alternative to the mass production of quality seeds for land rehabilitation, we developed a micro-environment technique by observing the effect of the exogenous hormone NAA on the germination of *C. inophyllum* seeds. The study is a new high-efficiency innovation technique with a mutually reinforcing effect on seedling growth. The highest concentration of NAA in this study increased root length by around five times higher than the control and shoots length by around three times higher than the control. This protocol can be used to spread *C. inophyllum* seeds on a large scale; however, it needs to be tested on a larger scale of cultivation.

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AUTHOR CONTRIBUTIONS

All authors had read and agreed to the published version of the manuscript and had an equal role as main contributors in discussing the conceptual ideas and the outline, providing critical feedback for each section and writing the manuscript.

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