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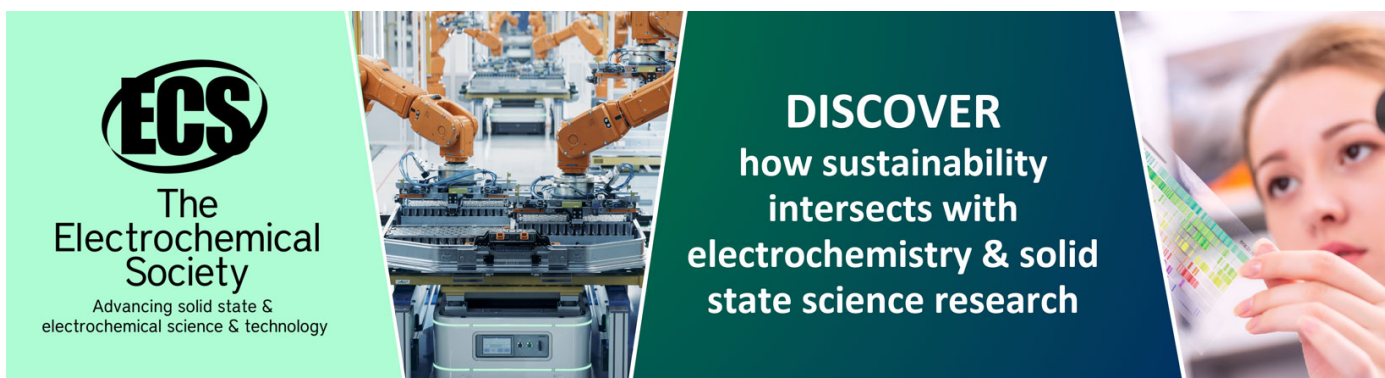
Effect of axenic culture and NAA *in Vitro* on *masoyi* (*Cryptocarya massoy* (Oken) Kosterm) seeds regeneration

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Effect of axenic culture and NAA *in Vitro* on *masoyi* (*Cryptocarya massoy* (Oken) Kosterm) seeds regeneration

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Abstract. The high valuable endemic commodities in Papua, *Masoyi's* (*Cryptocarya massoy*) population facing great threat due to unsustainable harvest system. Generative propagation faces significant challenges due to seed characteristics and habitat conditions. Controlled conditions and the role of hormones have an important effect on generative growth. This study aimed to determine the influence of axenic culture with sterilization treatments Isothiazolone Biocide (IB) and 1-Naphtaleaneacetic Acid (NAA) in Murashige and Skoog (MS) medium on seed regeneration and to observe the development of seedlings at the acclimatization stage. The tissue culture method was used. The highest percentage of axenic cultures (57%) was obtained with 5% of BI. The germination rate of *masoyi* seeds was achieved by 100%. Furthermore, it showed varied responses depending upon concentrations of NAA, the addition of 1 ml l⁻¹ NAA in MS medium is recommended. Acclimatization has been successfully carried out in the greenhouse (67% survival rate) and excellent seedlings growth at nursery (52.35 ± 0.6 cm in height after one year transferred). The impact of the controlled conditions and the addition of NAA to axenic cultures *in vitro* increased the germination of *masoyi* seeds. Axenic culture and hormones were also important requirements for mass propagation of *masoyi* by tissue culture.

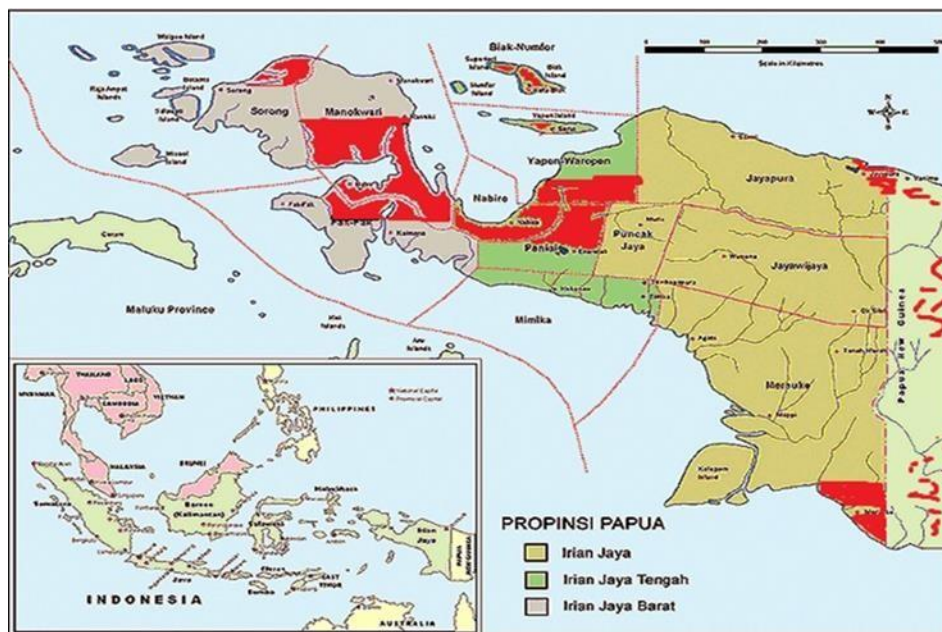
1. Introduction

Masoyi (*Cryptocarya massoy* (Oken) Kosterm)), Lauraceae family is a woody plant endemic in Papua, Indonesia [1, 2]. This plant spreads in several areas in Papua such as Manokwari, Sorong, Nabire, Biak Numfor, Yapen Waropen, Merauke, Jayapura [3]. The *masoyi* distribution in Papua, Indonesia, and Papua New Guinea is shown in Figure 1 [4]. *C. massoy* has a wide distribution in Nabire, Fakfak, Sarimi, Sorong, and Manokwari and produces essential oils with active lactone compounds [5]. Examination of parts of the plants (heartwood, bark, and fruit) showed that C-10 and C-12 massoilactone were primarily contained within the core and bark's oil. At the same time, C-14 massoilactone and δ -decalactone were identified as minor constituents in core wood oil [6]. Fruit oil, in contrast, is found primarily from benzyl benzoate [7]. These compounds were recognized to benefit at a certain level of medical purposes [8]. Moreover, some certain species from its genus are recognized to be utilized traditionally in medical practices by natives [9]. The cytotoxicity value of *masoyi* oil by the distillation method increased in line



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with its concentration. The IC_{50} value of *masoyi* oil against vero cells was 97.4 g ml^{-1} . The IC_{50} is defined as the concentration of an inhibitor where the response (or binding) is reduced by half [10].



Source: [11]

Figure 1. The natural distribution of *masoyi* in Papua, Indonesia (marked in red).

The high world demand for *masoyi* has encouraged an effort to develop *masoyi* outside its natural habitat [3]. Many communities have harvested *masoyi* bark without any replanting, and as a consequence, its population is significantly decreased naturally. On the other hand, seedling germination faces many difficulties, such as natural mother tree reduction, recalcitrant and high seed mortality. A report on the possibility of mass propagation by generative technique was mentioned by [12]. However, the survival rate of the seedlings might be less than expected [13]. Vegetative propagation of *masoyi* by shoot and root cuttings was successful but was limited to cutting media mixture [14]. Further studies on hormones to increase the cutting success rate was still required. Moreover, alternative techniques to enhance the material production to increase the *masoyi* population are urgently required. Therefore, the population's declining status due to an unsustainable harvest system could be avoided.

Micropropagation using an *in vitro* method with tissue culture technique might be a promising alternative to mass production of *masoyi* seedlings. Tissue culture could provide clonal propagation from limited mother trees/genetic sources. In addition, this technique could provide enhanced or superior clonal seedlings through *in vitro* selection [15]. The initial phase of this technique is to obtain an axenic culture as a source of explants material. Axenic culture is a condition without contamination *in vitro* [16]. Providing axenic culture is challenging since the material was taken directly from the forest. Commonly, materials that came directly from the forest tend to bring a great number of contaminants. Therefore, decontaminants and isolation action should be taken as a precaution.

Explant sterilization is a challenging phase since it could accelerate the micropropagation process. A suitable sterilization protocol could produce an optimum number of axenic cultures dedicated as a base of the propagation stage. However, eradicating micro-contaminants hidden below thin layers of cuticula and shoots could be a serious problem. It is also known that certain plants have endophytic organisms that benefit them in *ex vitro* conditions. However, contaminants are undesirable organisms in *in vitro* conditions. Therefore, biocides are usually applied during the sterilization process to eliminate unwanted organisms. The biocides application should also be calculated proportionately since the concentration and types of biocides could deliver various results [17].

Hormones or Plant Growth Regulators (PGR) play a great role in developing cultured tissue plants. The addition of PGR could enhance particular development during *in vitro*. Over studies [18-20], the addition of hormones was reported to affect the growth and division of plant cells. The cells might respond directly or indirectly to the supplementation of the hormones. Amongst studies taken, auxins were the common hormones to be applied due to the wide range of deviations of effectiveness to various plants species [21]. Auxins are also frequently adopted in various studies on plant vegetative propagation. Indole-3-butyric acid (IBA) and 1-naphthalene acetic acid (NAA) are two kinds of hormones that are widely used. IBA was frequently used on woody-plant species, while NAA has less exposure to the typical species. Review by [22] mentioned auxins' essential ability in modifying the growth of plants in their early stage of development. Further, embryogenesis stages, apical meristem development, and root formation were highly affected by the hormone's presence. However, the studies' results involved auxins may vary to the variables of species characteristic, type of auxins, and its concentrations.

This study aimed to obtain the best axenic culture method and optimal concentration of NAA as exogenous hormones *in vitro* for seed germination and to observe the seedling development after acclimatization of *masoyi*.

2. Material and Methods

2.1. Sterilization of seeds

The *masoyi* seeds were obtained from Balai Penelitian dan Pengembangan Lingkungan Hidup dan Kehutanan (BPPLHK) Manokwari, Papua, Indonesia. The sterilization method from seed, as described by [23], was slightly modified. Briefly, sterilizations were carried out on the outside and inside the laminar airflow (LAF). In the outside of the LAF, seeds were immersed in distilled water overnight, followed by soaking in distilled water containing detergent for 1 minute, soaking with fungicide for 3 minutes, rinsing with tap water for 20 minutes, and soaking in hot water (80-90°C) for 5 minutes to break seed dormancy. After sterilization outside, seeds were immersed in 5%, 10%, and 15% (v/v) in an anti-microbial compound of IB (Isothiazolone Biocide) solution inside LAF. A few drops (ca 0.25 ml) of Tween™ 80 were added to the solution for 40 minutes and 70% ethanol for 1 minute. Each seed was rinsed with sterile distilled water and placed in a tube. Percentage of axenic culture was observed every seven days.

2.2. 1-Naphthaleaneacetic Acid (NAA) treatment

Axenic culture of *masoyi* seedlings was selected according to the best sterilization treatment and cultured in Murashige and Skoog (MS) medium containing NAA. The effect of different concentrations of NAA (1 ml l⁻¹, 2 ml l⁻¹ and 3 ml l⁻¹) were observed on seed-break time, shoot length, and root length after eight months.

2.3. Acclimatization

The healthy seedlings, well-developed roots obtained from NAA treatments were chosen. They were washed under running tap water, soaked in fungicide for several minutes and transplanted to the polybag containing soil and compost of 1:1. The seedlings were maintained in the greenhouse under natural light intensity (30%-40%) and sprayed once a day with water. In this experiment, 50 seedlings were transplanted in polybag with plastic cover individually in 4 weeks. The percentage of seedlings survival was recorded 24 weeks after transplanting, and seedlings survival rate was calculated using the following formula:

$$\frac{\text{Number of seedlings survival}}{\text{Number of seedlings transplanted}} \times 100$$

The seedlings were observed for one year in the nursery. The experimental units were designed with a completely randomized design (CRD). Data were analyzed with analysis of variance (ANOVA) and

Duncan's multiple range test (DMRT) with a level of significance at $\alpha = 0.05$. The statistical package (SPSS Ver 24) was used as a tool for analysis.

3. Results and Discussion

3.1. Axenic seeds culture

The axenic culture was the initial tissue culture stage to obtain a source of explant material [24]. Sterile conditions in axenic cultures were needed to avoid contaminants [23]. *Masoyi* seeds were sterilized after three weeks of collection. The delay time between collection and sterilizing seeds has caused the high contamination and reduced the acquisition of axenic cultures. This situation could not be avoided since the seed locations were in remote areas (Fakfak) with transportation limitations, and research activities were done in different locations. Figure 2 shows the percentage of axenic culture on differences of sterilization treatment. The addition of 15% of IB resulted in a better axenic culture percentage compared to the other. The percentage of axenic culture after the 8th week showed a lack of results (57% for 15% of IB). It revealed that sterilization methods for *masoyi* seeds need to be improved.

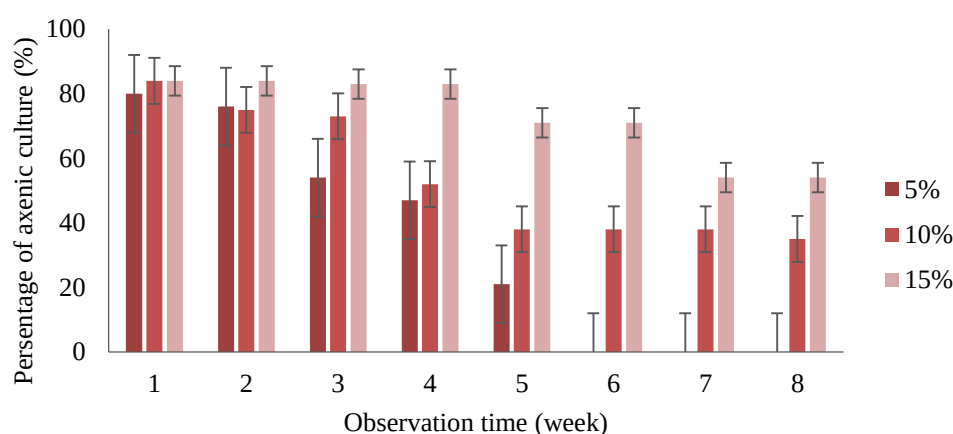
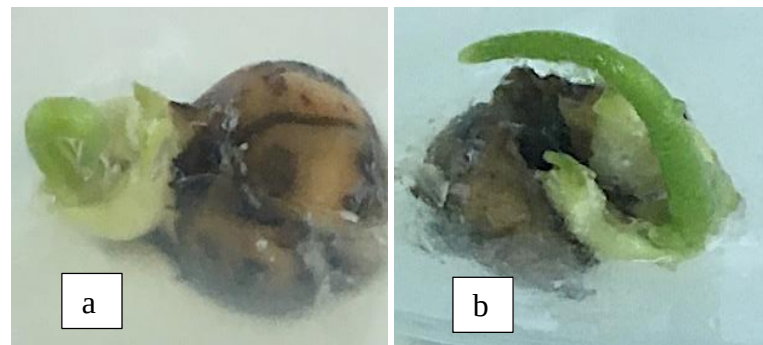


Figure 2. Percentage of axenic culture on the sterilization treatment of 5%, 10%, and 15% anti-microbial compounds (BI) for eight weeks observation.

The percentage of axenic cultures was achieved more than 50% in all treatments until the third week. It indicated that the anti-microbial of IB was effective in avoiding the development of contaminants. In otherwise, the decline in the percentage of axenic culture has been scientifically done after the 5th week. Those indicate that contamination has been done at the highest level. It is believed that after the 5th week, the ability of IB with lower concentration (5% & 10%) to inhibit the contaminant was decreased. Therefore, the contaminant was able to grow in culture media. As shown in figure 3, a higher IB concentration (15%) presented the best performance in inhibiting microbial development in vitro by providing the highest rate of axenic culture percentage. Therefore, a higher concentration of IB in a similar trial might be applied to expect a greater chance of acquiring a higher rate of axenic culture. However, higher IB concentration should be meticulously measured since it could be toxic for the plant tissue itself [17].

3.2. In vitro seed germination

The immature recalcitrant seeds from the axenic culture in the first trial of *masoyi* were used during in vitro germination. Four weeks after sowing, the germination was initiated with the enlargement and rounding of seeds (Figure 3a), followed by shoot formation (Figure 3b). The first seed-break time came three months after the seeds had been transferred to MS media with NAA supplementation.



(Documentation by Putri, 2018).

Figure 3. The *in vitro* initiation of seed breaking begins with the emergence of green shoots after four weeks of incubation (a) and is followed by shoot elongation after the 8th week (b).

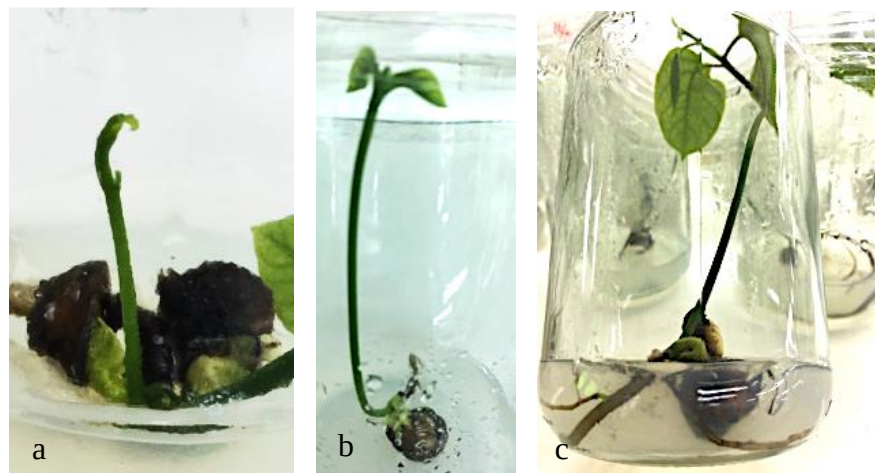
This study showed that there was a 100% of germination rate of *masoyi*'s seeds. However, their responses may vary depending on treatments of NAA concentration. As shown in Table 1, the response of seeds in break-time and shoot length is significantly influenced by NAA concentration supplemented in culture medium. On the other hand, *masoyi*'s root length did not significantly differ over NAA treatment. Therefore, adding 1 ml l⁻¹ in the culture medium would give the best performance in seed germination over the treatments given.

Table 1. Effect of NAA on seed-break time, shoot length, and root length of *masoyi* after eight months incubation *in vitro*.

Concentration of NAA (ml l ⁻¹)	Seed-break time (day)	Shoot length (cm)	Root length (cm)	Rate number of leaves per seed
1	16.15 ± 0.31 ^a	12.45 ± 0.21 ^a	5.02 ± 0.11 ^a	4
2	23.05 ± 0.69 ^b	8.80 ± 0.32 ^b	5.10 ± 0.09 ^a	4
3	54.55 ± 2.92 ^c	6.3750 ± 0.15 ^c	4.85 ± 0.08 ^a	3

Remarks: Each value represents a mean of twenty replicates with standard error (Mean ± S.E). Duncan's multiple range test shows that the means with different letters (a,b,c) are significantly different at a 0.5 probability level. The results of ANOVA showed significant differences ($P \leq 0.05$) between the concentration of NAA tested for seed-break time and shoot length.

The concentration of NAA had a significant effect on shoot initiation and the rooting in *masoyi*. A study by [25] showed that the exposure of seeds cotyledons, even partially, to auxins hormones could trigger germination. The study also indicated that the hormone could intensify the metabolism of nucleic acid and protein synthesis. Further, it was manifested in a progressive germination process that occurred in our study as reflected in seed-break time, shoot length, and root length values of *masoyi*. Overall, adding 1 ml l⁻¹ of NAA to the culture medium showed the optimum treatment of germination media in our study. The optimum values of 1 ml l⁻¹ of NAA might happen since the seeds already contain endogenous auxins within their cotyledons. Therefore, a higher addition of exogenous hormones could drawback the response. As [27] and [20] studied, the oversupply of exogenous hormones to the medium during *in vitro* germination could inhibit cell growth. Therefore, an optimum level of hormones added to the medium should be meticulously considered. The growth of shoots and roots of *masoyi* are shown in Figure 4.



(Documentation by Putri, 2019)

Figure 4. The shoot and root growth of *masoyi*, two months shoot elongation after seed-break (a), four months shoot elongation after seed-break (b), and eight months root elongation after seed-break (c).

3.3. Acclimatization

Masoyi's acclimatization was conducted after explants showed healthy development of roots, as seen in Figure 5c. The explants were taken from the tubes and transferred to polybags containing a mixture of soil and compost. The survival rate of plantlets from *in vitro* germination was considerably high (67%) compared to conventional germination, which has a very high mortality rate, as reported by [12]. The report also mentioned that the conventional *masoyi* germination rate of 82.67% occurred up to 30 days of seedlings marked by the appearance of radicles and followed by the appearance of plumules. However, subsequent germination regeneration did not go well; 90% indicated death [13]. This is due to the seeds' oil content, which is susceptible to pest attack [12]. Acclimatization of *in vitro* germination in the nursery showed that the *masoyi* seedlings grew healthy up to one year after planting and reached 52.35 ± 0.6 cm in the height average. The sterile conditions and the availability of nutrients *in vitro* support for *masoyi* seeds' growth during acclimatization. *Masoyi*'s *ex vitro* acclimatization stage is shown in Figure 5.



(Documentation by Putri, 2020)

Figure 5. Acclimatize the seedlings from *in vitro* germination to the nursery, after one year *ex vitro*: transferred of plantlet (a), covered the seeds as a source of explant material (b).

Transferring explants from *in vitro* to *ex vitro* environment might be a critical phase in plant tissue culture. During *in vitro* conditions, several organs might not yet be formed and function properly [28]. Therefore, gradual acclimatization should be taken precautions to prevent severe damage for plants [29]. A low gradual humidity and light intensity were carried out for *masoyi* explants. As started, high humidity (70%) and daily mean light intensity (2000 Lux) were exposed to the seedlings in a controlled greenhouse. A weekly observation was taken to evaluate the seedlings' condition before a gradual adjustment was taken up to the open environment condition. Further, seedlings from *in vitro* culture propagation are a potential explants source for the next trial. Therefore, proper protection and maintenance from pests and diseases are required. Several attempts were made, including covering the seedlings with plastic and spraying with pesticides. Fertilization, light regulation, and weeding in the nursery are essential to maintaining *masoyi* seedlings' fertility. A healthy source of explant material will affect the future success of *in vitro* regeneration.

4. Conclusion

According to the results of our study, it could be concluded that the concentration of Isothiazolone Biocides (15%) effectively acts as anti-microbial for axenic seed culture. The concentration of 1 ml l⁻¹ of 1-Naphthaleaneacetic Acid was a suitable treatment for enhancing *masoyi* seed germination and seedling growth. Future studies on various biocides applications and concentrations might be a reasonable topic to achieve a greater number of axenic cultures. Therefore, studies on micropropagation and *in vitro* selection to gain superior clones could be conducted. Current study results will also be great information for conservation actors to increase the success rate of seedling germination. Therefore, it could be applied in conservation action by providing a considerable number of healthy seedlings.

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