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Sterilization method of contaminated oil palm plantlets affects the survival rate during acclimatization

Sterilisasi pada planlet kelapa sawit yang telah terkontaminasi mempengaruhi daya hidup selama aklimatisasi

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Abstrak

Kontaminasi pada kultur in vitro merupakan salah satu penyebab kegagalan produksi bibit. Kontaminasi pada fase planlet sangat merugikan mengingat perbanyakan tanaman membutuhkan proses yang sulit dan waktu yang lama. Tujuan dari penelitian ini adalah melihat pengaruh sterilisasi saat aklimatisasi planlet kelapa sawit yang telah terkontaminasi jamur terhadap daya hidup, dan seberapa besar daya hidup yang dapat dicapai dari bahan terkontaminasi. Bahan yang digunakan adalah planlet kelapa sawit yang terkontaminasi fungi yang telah mimiliki akar, 4 daun, dan tinggi rerata 17 cm. Planlet dikeluarkan dari tabung dan dibersihkan dengan air mengalir, kemudian dilakukan perlakuan sterilisasi yaitu P1: perendaman dengan benomil-mankozeb-sodium hipoklorit dan manitol serta pembilasan dengan akuades, P2: perendaman dengan benomil-mankozeb, P3: perendaman dalam mancozeb. Pembersihan dengan air mengalir saja digunakan sebagai kontrol. Hasil menunjukkan bahwa pada umur 10 minggu setelah aklimatisasi, daya hidup planlet pada setiap perlakuan (P1, P2 dan P3) secara signifikan lebih tinggi dibandingkan kontrol. Sterilisasi mempengaruhi waktu kemunculan daun baru, kondisi daun setelah perlakuan sterilisasi dan tinggi tunas. Kontaminasi fungi yang terendah setelah perlakuan ditemukan pada perlakuan P2 diikuti dengan P3. Setelah 3 bulan, daya hidup planlet semakin menurun, dengan daya hidup tertinggi pada perlakuan P3 (32,3%) disusul perlakuan P2 (22,5%). Sebagai kesimpulan, planlet kelapa sawit yang telah terkontaminasi masih dapat hidup dengan perlakuan sterilisasi yang tepat. Sterilisasi mempengaruhi daya hidup dan pertumbuhan planlet kelapa sawit yang telah terkontaminasi di kultur in vitro sebelumnya selama periode aklimatisasi.

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[Kata kunci: Elaeis guineensis*, kultur in vitro, fungisida, sterilan]*

Abstract

Contamination in the *in vitro* culture is a critical problem causing the failure of seed production. Contamination in the oil palm plantlet is detrimental, considering that oil palm propagation is difficult and takes a long time. This research aimed to study the effect of sterilization during acclimatization of the contaminated oil palm plantlets by fungi on viability and to determine the optimum viability achieved from the contaminated materials. The materials used were contaminated plantlets of oil palm with roots, four leaves, and a height of about 17 cm. The plantlets were removed from the tube and cleaned with running tap water, then were sterilized, with treatments P1: soaking in benomyl-mancozeb-sodium hypochlorite and mannitol and rinsing with aquadest, P2: soaking in benomyl-mancozeb, P3: soaking in mancozeb. Cleaning plantlets under running tap water was carried out as a control treatment. The results showed that at 10 weeks after acclimatization, the survival rate of plantlets in each treatment (P1, P2, and P3) was significantly higher than that of the control. Sterilization methods affect the time new leaves emerge, leaf condition after sterilization treatment, and shoot height. The lowest fungal contamination after treatments was found in P2, followed by P3. After 3 months, plantlet survival rate decreased, with the highest survival rate in treatment P3 (32.3%) followed by treatment P2 (22.5%). In conclusion, acclimatization of contaminated oil palm plantlets can be carried out using a suitable sterilization treatment. Sterilization affects the survival rate and growth of in vitro-contaminated oil palm plantlets during acclimatization.

[Key words: *Elaeis guineensis*, *in vitro* culture, fungicide, sterilant]

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Introduction

Oil palm (*Elaeis guineensis* Jacq.) is the main oil-producing crop in Indonesia. The clonal oil palm seedlings can be obtained through *in vitro* culture by somatic embryogenesis technique (Marbun et al., 2015; Sumaryono et al., 2007). This technique has advantages, such as producing true-to-type with pest and disease-free seedlings and allowing germplasm storage by cryopreservation (da Silva & Engelmann, 2017). Oil palm-derived tissue culture can increase potential productivity by up to 20% (Hashim et al., 2018). Nevertheless, there are still many obstacles in supplying tissue culture-derived oil palm seedlings, such as limited sources of explants (ortet), low callus induction rate (Weckx et al., 2019), genetically dependent explant-media response (Sanputawong & Te-chato, 2008; Santos et al., 2018), low formation of somatic embryos, slow *in vitro* regeneration and root formation problem (Sumaryono et al., 2007). It needs two to five years from explant induction to acclimated plantlet (Hashim et al., 2018). Moreover, contamination of *in vitro* culture is a critical problem in clonal propagation of oil palms.

Contamination in oil palm *in vitro* culture occurs in two phases: at the initial culture and later stages (during maintenance). Contamination at initial culture is influenced by the condition of the mother stock (explant), weather conditions during explant collection, sterilization, and culture process (Mng'Omba et al., 2012). Contamination prevention at the initial culture is commonly achieved through surface sterilization using various sterilant agents such as alcohol, NaOCl (Pratiwi et al., 2021), or fungicide (Eziashi et al., 2014). Antibiotics and mercuric chloride can also be used to prevent initial contamination (Eziashi et al., 2014). Contamination during *in vitro* culture maintenance is affected by the aseptic condition during culture (poor handling during subculture, media sterilization failure) and the environment. The contamination percentage generally increases during the rainy season. On the other hand, Indonesia has favored environmental conditions that support microbial growth due to high temperature and humidity.

Plantlets were obtained after a long and complex process of *in vitro* culture. After that, plantlets must adapt to survive in the *ex vitro* environment, which is significantly different from the controlled conditions of *in vitro* culture. In the *in vitro* environment, plantlets absorb nutrients from the culture media, utilize sugar as a carbon source, and grow at controlled temperatures under aseptic conditions (Hazarika, 2003). On the other hand, in the ex vitro environment, plantlets must adapt to absorb nutrients from the soil, obtain carbon from photosynthesis, and cope with fluctuating and non-aseptic environments (Irsyadi, 2021). Therefore, a transitional stage from *in vitro* to ex vitro is needed, called the acclimatization stage.

Acclimatization represents a crucial stage in the final phase of the plant *in vitro* culture production. The success of this stage is paramount, as any failure during acclimatization can lead to the demise of all produced plantlets. The outcome of the acclimatization process is influenced by several factors, including the readiness of the plantlets for acclimatization, the composition of the medium used, and the acclimatization techniques employed (Idris et al., 2015; Sinta et al., 2013; Sinta & Amanah, 2019; Sumaryono & Riyadi, 2011, 2017). Plantlets ready for acclimatization should meet specific criteria, such as possessing vigorous leaves, appropriate height, and a well-developed root system (Sumaryono & Riyadi, 2016), and free from contaminants. Occasionally, the ideal condition for acclimatization cannot be fully achieved. For example, the question lies within contaminated plantlets that meet the physical criteria for acclimatization. Interestingly, no prior research has been conducted on the acclimatization of contaminated plantlets. This leads to poor knowledge of whether the contaminated plantlets can still be acclimatized and how much the survival rate can be achieved during the acclimatization. Therefore, this study aimed to optimize the survival rate of the contaminated oil palm plantlets by implementing a sterilization process during acclimatization. The goal is to explore whether contaminated plantlets can still be successfully acclimatized and what survival rate can be achieved with proper sterilization techniques during this critical stage.

Materials and Methods

Plantlets and sterilization method

The material used was fungi-contaminated oil palm plantlets. The plantlets have an average height of 17 cm, four leaves, and two primary roots, or categorized as class 4 plantlets according to Sumaryono & Riyadi (2011). Fungal contamination is characterized by the appearance of hyphae on the medium's entire surface and the plantlets' lower part. Contaminated plantlets were removed from the tube, washed with running tap water, and cleaned with a brush. Subsequently, sterilization was performed as follows:

P1: Soaked on mancozeb fungicide (1.6 gL^{-1}; 20 min) and benomyl (1 gL^{-1}; 10 min), soaked in sodium hypochlorite/ NaOCl (1.5% ;10 min), rinsed with water three times and immersed in mannitol (1%; 2 min) before planted on the acclimatization medium.

P2: Soaked in mancozeb (1.6 gL^{-1}; 20 min) and benomyl (1 gL^{-1}; 10 min), rinsed with water and then

planted on the acclimatization medium.
P3: Soaked in mancozeb (1.6 gL^{-1}; 20 min), rinsed with water, then planted on acclimatization medium. Washing with water alone was used as a control.

Acclimatization method

The plantlets were planted in a non-sterilized mixture of soil and organic matter (1:3) (v/v) in a small transparent plastic pot (350 mL)), with 5 holes for the water circulation in the bottom part (Sumaryono & Riyadi, 2011). The sterilized plantlets were planted in the medium and covered with a transparent plastic lid of the same size; these lids were sealed to maintain humidity during acclimatization. Before sealing, water was poured onto the medium until it became saturated. The pots were then placed on a plastic tray containing a small amount of water to maintain humidity and located inside a greenhouse under a 90% shade net. After ten weeks of acclimatization, the pot was gradually opened until they were fully open at 12 weeks. The plant maintenance during the acclimatization process includes environmental misting when the air is hot (<30^{o}C); this treatment helps to maintain an appropriate humidity level for the plantlets and assist in their successful acclimatization to the new environment (Sinta et al., 2013; Sumaryono et al., 2012).

Data analysis

The study used a randomized block design, where each treatment and control consisted of 3 blocks containing ten replications, resulting in 30 plantlets as replications for each treatment and control. The parameters observed included plantlet survival rate, leaf color grade, leaf condition during acclimatization, time of appearance of new leaves, shoot height increment, and fungal recontamination. The plantlet survival rate during acclimatization was observed at 10 and 12 weeks, and the calculation is based on the number of surviving plantlets compared to the total number of plantlets. The leaf color grade was measured using a color chart from IRRI (Bulletin, 2013). Data were analyzed using one-way analysis of variance (ANOVA), followed by the Duncan Multiple Range Test (DMRT) at α 5% if significant differences were found.

Results and Discussion

Plantlet growth during acclimatization

a. Leaf color changes and leaf condition

Leaf color grade measurements were performed using a color chart to quantify the level of greenness (Sinta et al., 2013). Leaf with the lowest pigmentation (light green) was categorized as grade two, while those with the highest pigmentation (dark green) were classified as grade five. In this study, the initial plantlet has an average distribution of three leaf color grades: 26% grade two, 56% grade three, and 18% grade four. After sterilization treatment and acclimatization process, the distribution changes in all treatments (Table 1). The decline in leaf color grade started from the 2nd week, and it happened gradually every week until the 10th week when the majority of leaves decreased to grade 2. This gradual reduction in the leaf color grade indicates a change towards lighter green pigmentation in the leaves over the acclimatization period.

The change in leaf color can be influenced by various factors, including nutrient deficiencies (such as Mg and N), pre-senescence conditions, light availability, biotic and abiotic stress. Stress on the plant before the natural aging process (senescence) can impact the leaf color. The alteration in color observed during foliar senescence is closely linked to the regulation of nutrient mobilization and resorption from leaf cells. This process frequently occurs under biotic and abiotic stress (Ougham et al., 2005). The amount and quality of light a plant receives can significantly affect leaf color. Insufficient light or exposure to excessive light levels can cause changes in pigmen-tation. Biotic factors such as pests and diseases caused by fungi or bacteria also lead to changes in leaf color and overall plant health. In this study, the previous fungi contamination on the plantlets was most likely also affect the plant health during acclimatization.

Moreover, this study also observed the leaf condition, including the emergence of new leaves and leaf necrosis. The results showed that new leaves formed most rapidly in treatment P2, where the new leaves appeared after two weeks of acclimatization. Treatment P3 followed with new leaves emerging after four weeks of acclimatization. In contrast, in treatment P1, new leaves appeared after eight weeks of acclimatization, and in the control group, no new leaves were formed even up to 12 weeks of acclimatization. The emergence of new leaves is essential as it indicates growth and vitality during acclimatization. It also shows adaptation to a new environment and recovery time after sterilization.

At the end of acclimatization period, 80% leaves in the control group underwent necrosis. The percentage of necrotic leaves in treatment P1 was 70.6% and 55% in treatment P2. However, the lowest rate of necrosis was observed in treatment P3, where only 35.3% of the leaves were necrosis (Figure 1). Additionally, treatment P3 exhibited the highest percentage of leaves that could remain healthy compared to other treatments. The new leaf percentage during acclimatization was 17.6%, 25%

Table 1. The percentage of leaf color grade distribution
Tabel 1. Persentase distribusi kelas warna daun

Treatment *Perlakuan*	Color grade *Kelas warna daun*	Percentage distribution of leaf color grade by week (%) *Persentase distribusi kelas warna daun pada minggu ke (%)*				
		2	4	6	8	10
Control/ *Kontrol*	4	0.0	0.0	0.0	0.0	0.0
	3	31.3	11.1	28.6	40.0	0.0
	2	68.8	88.9	71.4	60.0	100.0
P1	4	3.3	0.0	0.0	0.0	0.0
	3	63.3	33.3	31.6	16.7	16.7
	2	33.3	66.7	68.4	83.3	83.3
P2	4	9.7	0.0	0.0	0.0	0.0
	3	58.1	21.4	30.4	28.6	9.5
	2	32.3	78.6	69.6	71.4	90.5
P3	4	12.9	0.0	0.0	0.0	0.0
	3	54.8	47.6	61.1	17.6	5.9
	2	32.3	52.4	38.9	82.4	94.1

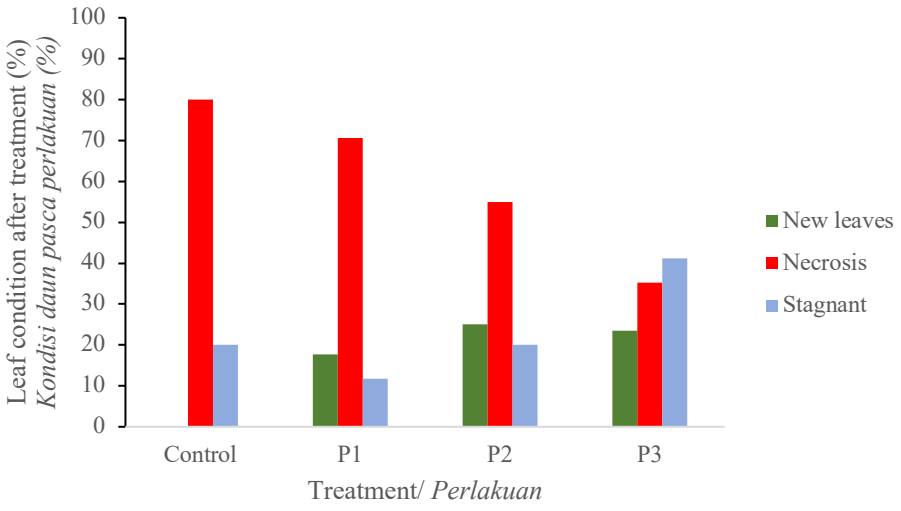

Figure 1. Leaf condition at the end of acclimatization period
Gambar 1. Kondisi daun pada akhir periode aklimatisasi

and 25% in treatment P1, P2, and P3, respectively. In contrast, no new leaves were found in the control group.

b. Shoot height increment

The highest increase in shoot height increment at the end of the acclimatization period was observed in treatment P2, where the shoot height increased by 2.3 cm. The shortest growth was found in the control group, with only a 0.6 cm increase (Figure 2). Meanwhile, treatments P1 and P3 had intermediate growth rates, with shoot heights increasing by 0.9 cm and 1.1 cm, respectively. These updated findings further reinforce that treatment P2 was the most effective in promoting shoot growth during the acclimatization process, resulting in the highest increase in plant height compared to other treatments and the control group.

In the control group, the shoot height did not increase after the 6th week, indicating stagnation in growth. Meanwhile, in treatment P1, the shoot height growth rate slowed after the 8th week of acclimatization. On the other hand, in treatment P3, the shoot height continued to increase, although the growth rate was not as high as in treatment P2.

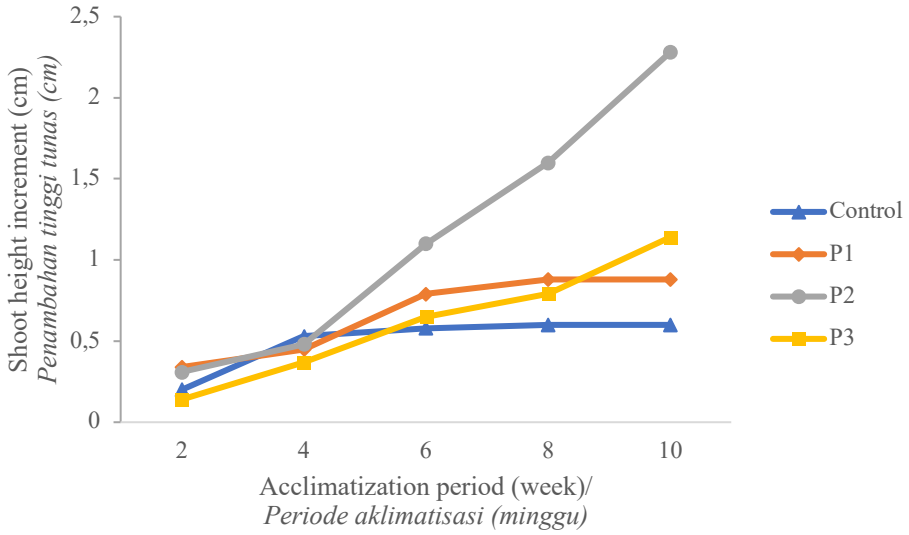

Figure 2. Shoot height increment during acclimatization
Gambar 2. Penambahan tinggi tunas selama aklimatisasi

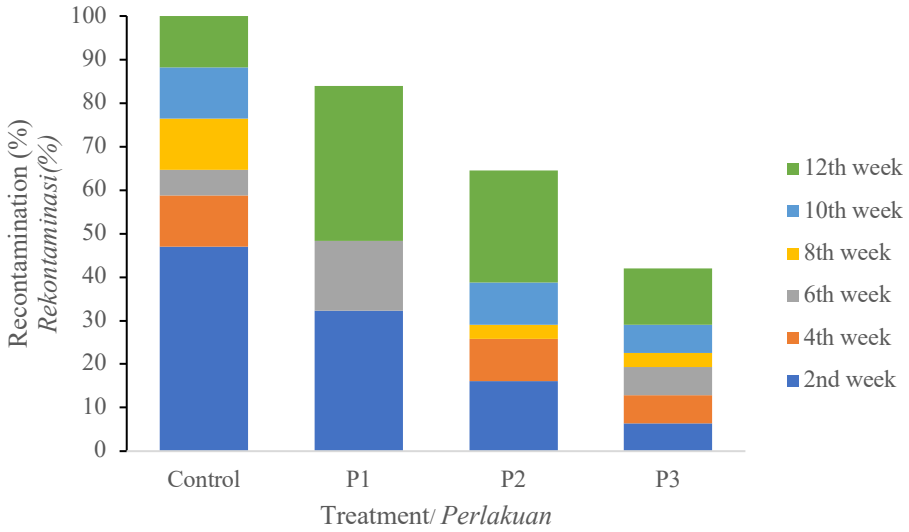

Figure 3. Fungal recontamination percentage during acclimatization
Gambar 3. Persentase rekontaminasi jamur selama periode aklimatisasi

The growth of plantlets during the acclimatization period indicates the successful adaptation of plantlets to the new environment, especially in their usual metabolism, such as the plantlets' ability to absorb and use nutrients from the soil, conduct photosynthesis, and withstand fluctuating environmental conditions. As known, plantlets were previously obtained nutrients heterotrophically, including sugars and minerals, but during the acclimatization plantlets must now be self-sourced through the absorption of fertilizer and water from the soil and photosynthesis (Hazarika, 2003). The growth of these plantlets can be measured based on the increase in plantlet height, the addition of new leaves, stem diameter, and so forth (Idris et al., 2015; Manjusha et al., 2010; Sinta et al., 2013; Sumaryono et al., 2012; Sumaryono & Riyadi, 2011).

Fungal recontamination

This study's materials were already 100% contaminated with fungi. Sterilization treatment was conducted before acclimatization using sterilant containing active ingredients such as NaOCl, mancozeb, and benomyl. Throughout the acclimatization process, it was observed that fungal

recontamination persisted, which was suspected to be one of the contributing factors to plantlet mortality. This recontamination gradually occurred from the 2nd week up to the end of the acclimatization period (Figure 3). The control group observed the highest recontamination, where fungi infected 100% of plantlets. The fungal infestation was characterized by white mycelium on the plantlet's surface. Interestingly, the lowest recontamination was found in treatment P3, with a recontamination rate of 40% in the 12th week.

Figure 3 illustrates that the fungal contamination in P1 was higher compared to P2 and P3. Surprisingly, P1 represents the longest and most complex sterilization process among all treatments. Similarly, in the case of P2, recontamination was higher compared to P3 even though it had shorter sterilization treatment than P2. The intricate sterilization process in this treatment was suspected of potentially diminishing the plantlet's ability to withstand fungal attack, consequently rendering them more susceptible to fungal reinfestation.

Sterilization treatment has been observed to have a weakening effect on plants. The use of sodium hypochlorite, especially at high concentrations, has been reported to reduce germination and induce browning of culture (Hesami et al., 2014; Pratiwi et al., 2021; Varasteh et al., 2015). A study by Algawaiz (2021) showed that injecting sodium hypochlorite into plant roots decreased the mitotic index as the concentration of sodium hypochlorite increased. Similarly, high doses of fungicides have also been found to weaken plants, such as reducing photosynthesis and plant growth (Petit et al., 2012). Although this study employed sterilant doses commonly used in *in vitro* culture, the weakened state of the plantlets due to fungal contamination and the critical acclimatization phase makes them vulnerable to microbial reinfestation.

Plantlet survival rate

Plantlet survival rate was assessed at ten weeks after acclimatization (starting from gradual opening) and continuing until 12 weeks (when 100% covers were removed) (Figure 4). At ten weeks, there was no significant difference in survival rate among treatments, ranging from 57.9% to 64.0 %. The lowest survival rate was observed in the control group (30%). By the 12th week, the highest survival rate was recorded in treatment P3 (32.0%), followed by P2 (22%) and P1 (9.7%). The lowest survival rate was again observed in the control group (5%). These results align with the outcomes of the earlier four parameters: the emergence of new leaves, leaf conditions, plantlet height increment, and fungal recontamination. Plantlets that did not die in this study were categorized as surviving plantlets, whether not infected by fungi or infected. In the 10th week of acclimatization in the control group, there was a 90% recontamination, and then in 12th week recontamination increased to 100%, resulting in the death of plantlets up to 95%.

In this study, plantlets underwent a critical period due to their developmental phase (acclimatization) and condition (contamination). This situation demanded the plantlets confront various challenges, such as adapting to new nutrient sources, performing photosynthesis, enduring fluctuating environmental conditions, and at the same time defending against fungal infections. Hence, appropriate actions are essential to sustain the survival of plantlets.

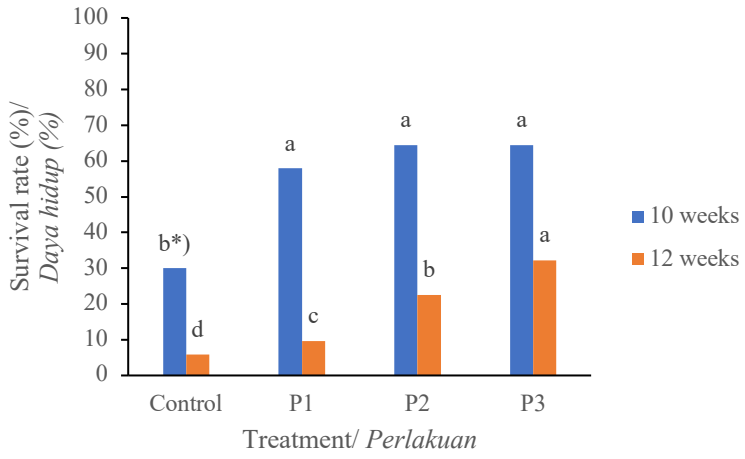

Figure 4. Survival rate of oil palm plantlet during acclimatization
Gambar 4. Daya hidup planlet kelapa sawit selama aklimatisasi

*) Bars with the same letter label are not significantly different according to the Duncan Multiple Range Test (α=5%)
**) Bar dengan label huruf yang sama tidak berbeda nyata menurun uji berganda Duncan (α=5%)*

In this study, one of these actions was to use effective sterilization. Under less-than-ideal conditions (contamination), effective sterilization was achieved through a single level of treatment, specifically immersion in a contact fungicide. P3 treatment resulted in low fungal recontamination, good plantlet vigor, and a high survival rate, as the plantlets managed to withstand the challenges during acclimatization. Dipping plantlets on sterilant before acclimatization is a standard method (Nasution et al., 2020; da Silva et al., 2017). This step is essential to minimizing microbial attack during acclimatization.

Acclimatization techniques vary depending on the plant species and its specific condition. For instance, in *Hevea brasiliensis* plantlet derived *in vitro* microcutting requires an additional hardening phase and appropriate media composition, as well as a closure system and gradual opening during acclimatization (Sinta et al., 2013; Sumaryono et al., 2012). In the case of *Cocos nuficera* var Kopyor, specific plantlet criteria are needed to increase survival rates, such as plantlet height and root and leaf vigor (Sumaryono & Riyadi, 2016). This is in contrast to stevia, where the highest survival rate is achieved when using only one node of plantlet, and the height must not be over-excessive to prevent tipping during acclimatization (Sinta & Amanah, 2019). For oil palm, vigorous criteria is required.

The survival rate of oil palm derived-*in vitro* culture varies depending on the variety, plantlet condition and acclimatization method used, ranging from 22%-97% (Corrêa et al., 2020; Gomes et al., 2015; Sparjanbabu et al., 2020). This research contributes to the development of the protocol for rescuing contaminated plantlets. In this study, it has been shown that even with fungal contamination conditions, plantlets can still be successfully acclimatized with a survival rate of up to 30%. In our view, saving contaminated plantlets is an additional effort that must be made in certain conditions such as the conservation of rare plants, saving important genetic material and in the context of commercial crop production. Further research needs to be carried out to observe differences in plant genetic abilities in efforts to save contaminated plantlets as well as their molecular biology responses.

Conclusion

Fungi-contaminated oil palm plantlets are able to survive during acclimatization, employing an efficient sterilization method involving a single immersion in a contact fungicide solution before acclimatization without being modified by additional sterilant treatments. Sterilization using mancozeb has proven effective in maintaining the plantlet's survival rate of up to 30%. Employing this method has ensured plantlets survival rate and resulted in favorable growth characteristics such as the emergence of new leaves, good leaf condition during acclimatization, plantlet height increment, and notably, the lowest fungi recontamination rate among all treatments.

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