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Callus Induction Of Phalaenopsis Stem (Phalaenopsis sp.) With NAA And TDZ Growth Regulatory In Vitro

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ABSTRACT

Orchid is an ornamental plant that is much favored by the wider community. Orchid flowers have a variety of interesting colors, shapes, and flower patterns. Cultivation is relatively long and requires special treatment. The purpose of this study was to determine the effectiveness of the explant sterilization method using fungicides and fungicides + HgCl2 to determine the response to explants by analyzing data using SPSS version.26 with ANOVA p-value <0.05, followed by Duncan's test to distinguish the average between treatments. o it requires propagation by tissue in vitro. The research design used in this study was RAL by tissue culture or in vitro using a flower stalk sample of the moon orchid (Phalaenopsis sp.) with K0 treatment, TN (TDZ 0.5 ppm, 1 ppm, 3 ppm + NAA 5 mg/L) and T (TDZ 0,5 ppm, 1 ppm, 3 ppm) and with fungicide explant sterilization method 35 replicates and fungicide explant sterilization method + HgCl2 35 replicates. The results of tissue culture research on the response of explants with the fungicide sterilization method (p>0.05), while the results of the fungicide + HgCl2 response did not produce a bubble response because the explants were contaminated and browning. The results of the effectiveness of contamination in sterilization using fungicides (p<0.05), while fungicides + HgCl2 (p>0.05). The results of the effectiveness of browning on sterilization using fungicides and a combination of fungicides + HgCl2 (p>0.05).

Keywords: *Propagation, orchids, tissue culture, hormone*.

INTRODUCTION

Phalaenopsis sp. is one of the ornamental plants that has many species in the world. In addition to natural species, the Phalaenopsis sp. It has many hybrids that make these orchids diverse in color and shape of flowers. The beauty and beauty of its flowers make this plant called the "queen of flowers", so it continues to be in demand, especially by orchid hobbyists. The market demand for orchids tends to continue to increase every year, so that the selling value remains high in the market (Putra et al., 2016).

Phalaenopsis flower stalks grow from stems that come from the axils of leaves and generally carry three to six dormant books and buds, as well as three to twenty-five or more florets

After the first flowers wilt and fall off, the bud eyes on the stalk of the Phalaenopsis flower have the potential to break and grow into keiki or new flower panicles. However, the growth of bud eyes into keiki or flower panicles is only tempted by the environmental temperature and nutritional status of the plant. So orchid stalks must be propagated in facilities that contain a lot of nutrients and the appropriate concentration of ZPT. One alternative that can be done is to propagate orchids together with tissue culture or in vitro methods (Kurniasih et al., 2017).

Figure 2.1 Moon Orchid (Phalaenopsis sp.)
Source : (Miftakhur, 2019)

According to Mubarok (2012) stated that one of the commonly used growth regulators is thidiazuron (TDZ) as a synthetic cytokinin which is the best for the regeneration of several plant species.

Cytokinin is a substance in plants that together with auxins in determining the direction of occurrence is determined by cells. The effectiveness of cytokinins is too varied, among which is determined by the dosage used, age and part of the plant used. The type of cytokinin to multiply calluses, and TDZ is the best compared to other cytokinins (Swandra, 2012).

Usually auxins are used in tissue cultures to stimulate the development of calluses, cell suspensions, and organs. Auxins are useful for the formation of roots and side buds within a certain concentration. The auxin group added in the means to this study is Naphtalene Acetic Acid (NAA) (Mahadi and Imam, 2016).

The development of orchid production in Indonesia is still relatively long. This is because orchid cultivation requires a relatively long time and special treatment, so orchids have high economic value when compared to other ornamental plants. Orchid propagation can be done through various methods, including conventional and in vitro propagation or culture (Kurniasih et al., 2017).

The advantage of the tissue culture method is that it is able to produce plants in large quantities, similar saplings together with their mothers, it takes a shorter time and superior and disease-free results (Zahara, 2017). The obstacle comes from the tissue culture method, namely the occurrence of contamination that can cause damaged treatment facilities and dead plantlets, caused by fungi and bacteria due to the lack of sterilization of planting tools, plantlets, and rooms. In addition, browning occurs in explants (Saepudin et al., 2020).

Based on research by Fauzan (2017), there are several types of sterilization commonly used for explant sterilization. The selection of sterilizers is influenced by the type of plant, the part of the plant to be used and the age of the plant. Sterilants commonly used in tissue culture include 70% alcohol with a soaking time of 0.5-1 minutes, sublimate (HgCl2) with a concentration of 0.01-0.05% and a soaking time of 10-20 minutes.

Fungicides are pesticides that specifically kill or inhibit the growth and development of fungi. In general, fungicides are grouped into the group of dithiocarbabate and belong to selective fungicides (fungicides that kill certain types of fungi) and non-selective fungicides. In addition, fungicides can also be grouped into contact (systemic) fungicides (Surya et al., 2021).

Antracol (Fungicide) is a type of pesticide that contains toxic chemical compounds and can be used to eradicate and prevent fungi/fungi (Sidauruk et al., 2017). Based on the research of Fauzan (2017), there are several types of sterilization that are commonly used for explant sterilization. The selection of sterilizers is influenced by the type of plant, the part of the plant to be used and the age of the plant. Sterilants commonly used in tissue culture include 70% alcohol with a soaking time of 0.5-1 minutes, sublimate (HgCl2) with a concentration of 0.01-0.05% and a soaking time of 10-20 minutes.

Orchid flower stalks can be developed using tissue culture methods with a high success rate. Plants produced through tissue culture are called plantlets (Sandra, 2003). The good growth and development of orchids is greatly influenced by proper sterilization methods, media and ZPT (Wibawati et al., 2010). Therefore, it is necessary to conduct research related to the effectiveness of sterilization methods using fungicides and the combination of fungicides with HgCl2 so as to minimize the occurrence of contamination of the explants and produce a response to the explants of the moon orchid stalk.

RESEARCH METHODS

The treatment design used in this study is a complete random design (RAL) with two factors. The first factor of concentration level was NAA 5mg/L with the addition of each concentration of TDZ1 0.5 ppm, TDZ2 1.0 ppm, TDZ3 3 ppm and without the addition of ZPT NAA with a concentration of TDZ1 0.5 ppm, TDZ2 1.0 ppm, TDZ3 3 ppm concentration using MS media (Murashige and skoog). While the second factor is to find out the comparison of sterilization methods using fungicide and fungicide + HgCl2. And each treatment is repeated 10 times with one type of explant, namely orchid flower stalks planted in each medium as many treatments so that the total number of repetitions is 70 repeats or 70 bottles.

Research Materials

The ingredients used include MS media (Murashige and skoog) explants of moon orchid flower stalks, aquades, 70% alcohol (brataco), jelly (swallow), NAA, TDZ, disinfectant (Lysol 100), liquid detergent (sunlight), bleach (bayclin), fungicide (Anthracol), HgCl2, sugar, coconut water, generic vitamin c, citric acid.

Research Instruments

The instruments used in this study were Laminar airflow, culture bottles, petri dishes, beaker cups, tweezers, scalpels, aluminum foil, ovens, autoclaves, analytical scales, bunsen lamps, erlenmeyers, measuring cups, drip pipettes, spatulas, pots, gas stoves, pH meters, plastic wrap, mortars, books, pencils, rulers, pens, pencils and label paper.

Sterilization Tools

Clean the culture bottles, tweezers, scalpels, scissors and petri dishes with disinfectant and liquid detergent (sunlight) then rinse with clean water and soak for 30 minutes using bleach (bayclin) rinsed using running water in an autoclave at 120 at 15 psi for 3 hours.

Manufacturing Stock Solution
Weigh ZPT NAA and TDZ as much as 0.01 g and dilute ZPT NAA into 100 ml aquades so that the concentration according to the treatment is obtained at 0.5 ppm, 1.0 ppm, 3 ppm, respectively. After that, it is stored in the refrigerator.

Creation of MS Culture Media
This study used MS (Murashige and skoog) media which was added with growth regulators according to the treatment. By adding 20 g/L sugar, 50 ml/L of coconut water, 250 mg of antibiotics (chloramphenicol) 1 capsule/L each for each treatment. After that, pour 1000 ml of aquades into each medium and then stir, then the pH is measured to 5.6-5.8. Next, pour the media into a pot and then put in 7 g/L of gelatin. Make sure the bottle is tightly sealed and then autoclaved for 1 hour at 120 at a pressure of 15 psi. Once finished stored in a room with a temperature of 23-28

Sterilization of Moon Orchid Stalks Explants Using Fungicides
The explant of the moon orchid stalk is washed thoroughly with running water then washed with sunlight and then rinsed with running water 3 times. After that, the explant of the moon orchid stalk is cut 1.5 cm. Wash again with sunlight and then rinse with running water 2 times. Furthermore, the explants of the moon orchid stalks were soaked with a fungicide (Anthracol) 2 g/L for 10 minutes at a dose of 2 g/100 ml aquades and then washed with sterile aquades. After that, soak the explant with a solution of generic vitamin C and citric acid 100 mg/100ml The explant of the stalk of the moon orchid is soaked with 10% bayclin. Soaked with 5% bayclin for 10 minutes at a dose of 5 ml/100 ml of sterile aquades then rinsed with sterile aquades. The explant of the moon orchid stalk is put into a beaker containing 70% alcohol and then put into the laminar airflow and UV irradiated for 30 minutes.

Sterilization of Moon Orchid Stalks Explants Using Fungicide+HgCl2
The explant of the moon orchid stalk is washed thoroughly with running water then washed with sunlight and then rinsed with running water 3 times. After that, the explant of the moon orchid stalk is cut 1.5 cm. Wash again with sunlight and then rinse with running water 2 times. Furthermore, the explants of the moon orchid stalks were soaked with fungicide (Anthracol) 2 g/L + HgCl2 300 mg/L for 5 minutes. After that, soak the explant with a solution of vitamin C and citric acid 100 mg/ 100ml The explant of the stalk of the moon orchid is soaked with 10% bayclin. Soaked with 5% bayclin for 10 minutes at a dose of 5 ml/100 ml of sterile aquades then rinsed with sterile aquades. The explant of the moon orchid stalk is put into a beaker containing 70% alcohol and then put into the laminar airflow and UV irradiated for 30 minutes.

Planting Room Preparation
Before planting, the laminar air flow is sterilized with a UV lamp for 30 minutes and the laminar air flow is sprayed with 70% alcohol.

Preparation of Plants and Planting
The explants of the moon orchid stalks that have been sterilized with 70% alcohol, are taken using sterile tweezers, then rinsed with aquades, then the explants are put in a sterile petri dish. Insert a culture bottle containing the media into the laminar air flow. The mouth of the culture bottle is sterilized with bunsen fire. Next, the stalk is cut which is done aseptically using a scalpel in the laminar air flow until it becomes 4 parts, then planted in the media with the help of tweezers (1 explant 1 bottle) after which the bottle is tightly closed then in plastic wrap, then the bottle containing the explant is stored in the incubation room at a temperature of 28.

Treatment
The treatment in this study was the addition of ZPT with a TDZ concentration of 0.5 ppm, 1 ppm, 3 ppm and for the addition of a combination of TDZ+ NAA, namely TDZ 0.5 ppm, 1 ppm, 3 ppm from each treatment 5 ppm NAA was added (Handini, 2017).
Observation

The observations made in this study were to observe the occurrence of explant response, contamination time and browning time visually once every 1 week, and to calculate the number of contamination and browning in the explant.

RESULTS AND DISCUSSION

Research on the Induction of Lunar Orchid Stalk Explant Callus (*Phalaenopsis sp)* with growth regulators NAA (*Naphthaleneacetic Acid*) and TDZ (*Thidiazuron*) in *vitro* which was carried out from February – May 2021 at the UPT Horticultural Food Plant Seed Center Bali, it was found that each treatment had insignificant results after statistical analysis with the ANOVA test. This observation was made 4 times for 4 weeks or 1 month. The following statistical analysis results are presented in table 1 the average result of moon orchid stalk tissue culture in the sterilization method using fungicide and table 2 the average result of moon orchid stalk tissue culture in the sterilization method using fungicide + HgCl2.

Table 1. Average results of moon orchid stem tissue culture in sterilization method using fungicide

Treat-ment	Respo nse Time	Numb er of Respo nses	Conta minati on Time	Numb er of Conta minati on	Brown ing Time	Brown ing Sum
K0	0a	0a	2from	1 [b]	0 [a]	0 [a]
TN1	1a	1a	1 [a]	1 [a]	1a	1 [a]
TN2	0a	0a	1 [a]	1 [a]	0 [a]	0 [a]
TN3	1a	1a	1 [a]	1 [a]	0 [a]	0 [a]
T1	1a	1a	2 [from]	1 [b]	0a	0 [a]
T2	0a	0a	3b	1 [b]	0 [a]	0 [a]
T3	0a	0a	1 [from]	1b	0a	0 [a]

Description: K0 = Control, TN1 = TDZ+ NAA concentration 0.5 ppm, TN2 = TDZ+ NAA concentration 1 ppm, TN3 = TDZ+ NAA concentration 3 ppm, T1 = TDZ 0.5 ppm, T2 = TDZ 1 ppm, T3 = TDZ 3 ppm, TDZ = Thidiazuron, NAA = Naphtalene Acetic Acid, The numbers in one column followed by the same letter did not differ significantly at the level of 5% according to the Duncan double spacing test.

Table 2. The average result of moon orchid stem tissue culture in the sterilization method using fungicide + HgCl2.

Treat ment	Res ponse Time	Numb er of Respo nses	Conta minati on Time	Number of Contami nation	Brown ing Time	Brow-ning Sum
K0	-	-	2a	1a	1 [a]	1 [a]
TN1	-	-	1 [a]	1 [a]	1 [a]	1 [a]
TN2	-	-	2 [a]	1 [a]	1 [a]	1 [a]
TN3	-	-	2 [a]	1 [a]	1 [a]	1a
T1	-	-	2 [a]	1 [a]	1 [a]	1 [a]
T2	-	-	2 [a]	1 [a]	2 [a]	1 [a]
T3	-	-	1 [a]	1 [a]	1 [a]	1 [a]

Remarks: K0 = Control, TN1 = TDZ+ NAA concentration 0.5 ppm, TN2 = TDZ+ NAA concentration 1 ppm, TN3 = TDZ+ NAA concentration 3 ppm, T1 = TDZ 0.5 ppm, T2 = TDZ 1 ppm, T3 = TDZ 3 ppm, TDZ= Thidiazuron, NAA = Naphtalene Acetic Acid, - = no result. The numbers on a single column followed by the same letter do not differ significantly at the level of 5% according to Duncan's double spacing test. The results showed that the average variable response time of explants to sterilization of explants using fungicides, namely explants in T1 treatment (TDZ 0.5 ppm) experienced a response in the third week in TN1 treatment (TDZ + NAA 0.5 ppm), experienced a response in the fourth week and in TN3 treatment (TDZ + NAA 3 ppm) experienced a response in the fourth week. Treatment of T1 and TN1 balanced cytokinin ratios of 0.5 ppm and the addition of 5 ppm explant auxin can produce a bubble response in the ankle stroke.

According to (Collin and Edward 2004; Lestari, et al. 2020) auxins and cytokinin concentrations from 0 mg/L to 5 mg/L can produce optimal callus growth.

According to Davies (2004) the interaction between auxins and cytokinins in culture *Vitro* able to make the cells in plant tissues undergo a process of division and enlargement, while in the medium without the addition of NAA, auxin at this stage has begun to form a plantletAccording to Ajijah et al., (2010) said that the bubble response in explants is the initial stage of callus formation which indicates the presence of cell activity in the explant. This bubble response occurs at different times where the treatment that gives the fastest response time is the single treatment of 0.5 ppm while the long response in the double treatment is 3 ppm. Compared to the variable response time of the explant in the sterilization method using fungicide + HgCl2, there were no results of the response time because the explant experienced death due to contamination and *browning* on the explant

The next response that occurred during observation was a change in color on the explant to yellowish green or brownish-green. But not all explants undergo such changes. Based on the research of Ariani et al., (2016) the change of stalk explants *in vitro* to yellowish-green and brownish-green shows that the explants are damaged, the green explants slow down the death of the explant. The change in the color of the plant is influenced by the size of the explant used in the study which is not large enough to distinguish the given growth regulator. In addition, the age of the explant also affects the response. The results of table 1 can be seen the treatment of TN1, namely 1a, TN3, 1a, T1, 1a. TDZ+ NAA treatment was better able to keep the explant alive compared to the single and control treatment.

This is because the reaction between the two regulatory substances grows to the interaction of the explant in the medium compared to a single control and treatment. Callus induction in this study was able to provide a bubble response aimed at the formation of callus. The bubble indicates that the state of the cell tissue against the explant is inflated or enlarged. But it does not reach cell division which will later undergo the formation of calluses. After the occurrence of the explant bubble, it will produce calluses (Lizawati et al., 2012). The bubble that occurs is an initial process of nutrient entry from ZPT and media. The next process occurs excessive cell proliferation and development that functions to close the wound (Isra'Aulia and Noriah, 2020).

Table 1 The results of the analysis of contamination time variables in the sterilization method using fungicides show the highest results in T2 treatment. Table 1 shows the amount of contamination using fungicide ingredients in explant sterilization, namely from each treatment having the same effect even though the concentration is different with a significant result, namely 0.00 < p-value.

Table 2 The amount of contamination in the sterilization method using fungicide + HgCl2 had an insignificant result, namely 0.59 > p-value. Sterilization on the immersion of both HgCl2 and Fungicide sterile materials for 5 minutes resulted in a low contamination rate for 1 week. However, the explant completely dies at 2 weeks. Soaking using a single sterilizer of fungicide for 5 minutes showed higher contamination, but with a lower mortality rate. The result is that the addition of HgCl2 300 mg/L has high toxicity to the explant of the moon orchid. The death of the explant that occurs after immersion in a 300 mg/L HgCl2 solution is caused by the bleaching action of two chloride atoms and ions that are strongly bonded to proteins that cause the death of the organism (Admojo, 2016; Himabindu et al., 2012). The results of observations for four weeks after inoculation showed that the contamination that occurred came from explants and culture media. Each contaminant, both bacterial and fungal, consists of several types, some are white, red, and blackish-brown (Handayani et al., 2014).

Contamination that occurs due to bacteria not only in the explant but also in the explant tissue is generally shown 2 times in 24 hours of contamination. Contamination caused by internal infection is the discovery of microorganisms or living bacteria in the explant tissue so that it cannot inhibit the growth of microorganisms by means of external sterilization (Dewi et al., 2016).

The occurrence of contamination in explants *browning* problems in explants is found in this study, where *browning* is a process of physiological deterioration of an explant that is often found in *in vitro* cultures. In *vitro cultures,* browning *events are often found* which ultimately inhibit the development of an explant The use of sterile materials in addition to inhibiting the development of relatively young plant material microorganisms. The *browning* process will inhibit the growth of explants and reduce the physiological function of the explant until the explant dies (Wulandari et al., 2014)

Browning indications arise from the base of the explant exposed to the media, and spread to all parts of the explant. *Browning*) is a symptom of the appearance of brown color on the explant so that it will inhibit the growth of the explant. In the explant, the enzyme polyphenol oxidase is formed which results in the oxidation of phenol compounds into quinones that produce brown pigments when the tissue is injured (Shonhaji et al., 2014).

Table 1 results show the average *browning time* in the sterilization method using fungicides, namely in the TN1 treatment, which is 2 weeks after planting, on the other hand, in the K0, TN2, TN3, T1, T2, T3 treatment there is no *browning.* Based on the results obtained from the fungicide sterilization procedure, the best results from the fungicide sterilization method + HgCl2.Table 5.1 *browning* that occurred in the sterilization method using fungicide materials, namely at the concentration of TN1 (TDZ+ NAA 0.5 ppm) 1 bottle and the treatment of K0, TN2, TN3, T1, T2, T3 did not occur browning and produced a significant result, namely 0, 44 < p- value.

In table 2, the results show that the average *browning* time in the sterilization method using fungicide + HgCl2 is the fastest in the control treatment and the ZPT TDZ+ NAA treatment is 2 or 3 weeks after planting and while in the single treatment is 3 or 4 weeks after planting. Table 2 *Browning* explant in the sterilization method using fungicide + HgCl2 *browning* occurs in each treatment, including K0 there are 2 bottles, TN1 1 bottle, TN2 1 bottle, TN3 2 bottles, T1 2 bottles, T2 3 bottles, T3 1 bottle and does not produce a significant result, namely 0.84 p-value. The use of sterile materials, in addition to inhibiting the development of contaminant microorganisms, can also cause the explants to brown, especially in relatively young plant materials. The *browning* process will inhibit the growth of explants and reduce the physiological function of the explant until the explant dies (Handayani et al., 2014).

Increased concentrations of mercury chloride (HgCl2) did not provide a good response to changes in shoot growth time, bud growth percentage and bud count. High concentrations of mercury chloride cause long-cultivated explants to sprout. This is because the concentration of mercury chloride given has not reached a suitable concentration for sterilization of moon orchid stalks explants. Mercury chloride is able to eliminate the source of contaminants but makes it difficult for the explants to regenerate and form new shoots. The use of HgCl2 in high concentrations affects the damage to plant tissues (Fauzan et al., 2017).

CONCLUSIONS AND SUGGESTIONS

The results of tissue culture research on the variables of explant response time, explant response, contamination time, amount of contamination, and browning time of moon orchid stalks explant can be concluded

1. The results of the study on the explant response of moon orchid stalks can be concluded that the use of fungicides in the sterilization of T1, TN1 and TN3 treatment explants experiences a response to ankle scratches. The use of HgCl2 + fugicide did not experience a response to the explant.
2. The response time of moon orchid explants to fungicide sterilization showed the fastest results in the T1 treatment, which was 3 weeks after planting, while in the TN1 and TN3 treatment, which was 4 weeks after planting. At the time of the response of the explant sterilization

method of fungicide + HgCl2 there were no results due to the contamination of the explant so that the explant did not experience a response.

3. The amount of contamination in fungicide sterilization was more than compared to the explant sterilization method using fungicide +HgCl2 and the contamination time occurred 1 week after planting.
4. The amount of browning in fungicide sterilization was less compared to the explant sterilization method using fungicide + HgCl2 and the browning time occurred 1 week after planting.

SUGGESTION

The suggestion in this study is that at the stage or method of sterilization of explants in the use of HgCl2 + fungicides, soaking is carried out with a period of 3 minutes - 5 minutes so that the explants do not die. Further research needs to be conducted using sterilization methods with other materials other than HgCl2 because it causes no response.

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