PROPAGATION OF PITCHER PLANTS (*Nepenthes gracilis* KORTH. AND *Nepenthes reinwardtiana* MIQ.) THROUGH CALLUS INDUCTION

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ABSTRACT

In vitro propagation of pitcher plants is still limited only using seeds. In contrast, researchers have not widely reported the other *in vitro* methods using leaf explants such as embryogenesis, organogenesis, and callus culture. The research aims to study the growth response of leaf explants in two species of pitcher plants (*Nepenthes gracilis* and *Nepenthes reinwardtiana*), which formed callus in several treatment media combinations. Plant materials used leaf pieces taken from a 4-month pitcher plant culture and grown on the treatment media, namely modified media of Murashige & Skoog with a half concentration ($\frac{1}{2}$ MS) added 2,4-Dichlorophenoxyacetate (2,4-D) with a concentration of 0, 0.5, 1, 1.5, and 2 mg L⁻¹ and kinetin (0.5 mg L⁻¹). The treatment was then stored in a dark environment to induce callus formation. The observation for 12 weeks showed that the time of callus formation in two species of pitcher plant observed was not significantly different. Both species of pitcher plants begin to form callus in the fourth week after treatment. No hormone added in the medium led to no callus formed in leaf explants. The best medium for callus induction in *N. gracilis* is $\frac{1}{2}$ MS medium added with 2 mg L⁻¹ 2,4-D, with callus morphology brownish-white with friable texture. In comparison, this research has not obtained the optimum callus media from leaf explant of *N. reinwardtiana*. Thus further research is still needed.

Keywords: 2,4-D; *in vitro*; kinetin; leaf culture

INTRODUCTION

The pitcher plant belongs to the carnivorous plant family (Nepenthaceae) with the highest number of species, a dioecious plant, a woody or climbing plant, and a terrestrial or epiphytic plant. The plants can live in the lowlands and highlands (Moran and Clarke, 2010; Gaume et al., 2016; Thorogood et al., 2018;

Scharmann et al., 2019; Nainggolan et al., 2020). The pitcher plants have an exotic pitcher with various colors and shapes, which are the evolution of the leaves (Clarke, 1997; Selviana et al., 2018). The pitcher's function in the pitcher plants is a trap for various invertebrates and some vertebrates for nutritional fulfillment (Clarke, 1997; Cheek and Jebb, 2001).

Currently, the pitcher plant is in growing demand by the public, not only hobbyist but also the general public, as an ornamental plant, because of the beauty and exoticism of the shape, pattern, and color of the pitcher and have another potential, namely containing the potential active compound (Chou et al., 2015; Rosli et al., 2018; Goh et al., 2020).

The IUCN red list categorized *Nepenthes gracilis* and *N. reinwardtiana*, which was last estimated in 2014 as low-risk species/ Least Concern (LC) (C. M. Clarke, 2018). However, the Convention on International Trade in Endangered Species of Wild Fauna and Flora classified both species in Appendix II (CITES, 2019). Thus, their trade is regulated and restricted if taken directly from their natural habitat. Therefore, cultivation is needed to anticipate people who encroach on the forest to get the two species.

Nepenthes can be propagated conventionally using seeds and stem cuttings or non-conventional propagation using tissue culture techniques (Devi et al., 2013; Dinarti et al., 2010; Rosmaina and Aryani, 2015; Sukanto, 2011; Nuryadin, 2018). However, *Nepenthes* is a dioecious plant whose insect such as Diptera and Hymenoptera assists the pollination, or sometimes aided by the wind, and many pitchers have offspring from crosses of several species and the resulting impurity seed (Cheek and Jebb, 2001). Therefore, a more efficient vegetative propagation alternative from the same parent is needed. It can be developed for embryogenesis, organogenesis, and callus culture by inducing callus formation.

A callus is an amorphous collection of cells and tissues that divide continuously. Callus culture aims to obtain callus from explants isolated and

grown in a controlled environment. Callus formation from tissue depends on the physiological age of the tissue when isolated, the season at which plant material is isolated, the plant part used, the type and genotype of the plant, as well as the media and growth regulators used (Gunawan, 1992; Sharma and Bora, 2015; Dawa et al., 2017; Jui et al., 2020; Sa'adan and Zainuddin, 2020).

The plant growth regulators (PGR) required from callus induction are usually a combination of auxins and cytokinins (Al-ajlouni et al., 2012; Sharma and Bora, 2015; Wang, 2016). For example, one of the auxins used in callus formation is 2,4-Dichlorophenoxyacetate (2,4-D) (Elaleem et al., 2009; Irvani et al., 2010; Wang, 2016; Huang et al., 2018). The research results by Elaleem et al. (2009) showed that 2,4-D was the most effective PGR for inducing callus in potatoes when applied alone or in combination with cytokinin (BA). 2,4-D was applied to the potato to produce 100% callus with a friable texture and yellow color. Other studies using 2,4-D and using leaf explants to induce callus formation in sugarcane (Rao and Jabeen, 2013), jasmine (Rosyidah et al., 2014), vanilla (Sharma and Bora, 2015), Tongkat Ali (Mahmood et al., 2010), strawberry (Dawa et al., 2017), orchid (Amalia et al., 2017), chrysanthemum (Setiawati et al., 2019), and gerbera (Jui et al., 2020). Researchers have not published much regarding the propagation of pitcher plants using leaf explants through callus induction. However, Novitasari and Isnaini (2020) carried out preliminary research to induce callus in *N. ampullaria* and *N. reinwardtiana*. The optimal medium for callus induction from leaf explants on *N. ampullaria* has been obtained, namely, a combination of 1 mg L⁻¹ 2,4-D and 0.1 mg L⁻¹

kinetin, while the research has not obtained the optimal medium for callus induction on *N. reinwardtiana* (Novitasari and Isnaini, 2020). The purpose of this study was to obtain the response to the growth of leaf explants in two species of *Nepenthes* (*N. gracilis* Korth. and *N. reinwardtiana* Miq.), which formed callus in several treatment media combinations.

MATERIALS AND METHODS

The research was conducted at Tissue Culture Laboratory, Research Center for Plant Conservation and Botanic Gardens – Indonesian Institute of Sciences on November 2019 - February 2020. This study used leaves from two species of pitcher plant culture (*N. gracilis* dan *N. reinwardtiana*) which were cultured for four months in Murashige and Skoog's modified medium with half the concentration (1/2 MS) added 2 mg L⁻¹ Benzylaminopurine (BAP). Young leaves from both species of *Nepenthes* that are four months old after planting were then planted on 1/2 MS medium, added with the treatment of 2.4-D and kinetin. The leaves planted on the treatment media are then incubated in a dark environment with a temperature of 23 ± 2 °C. The experimental design in this study used a completely randomized design with two factors. The first factor is the type of *Nepenthes* (*N. gracilis* and *N. reinwardtiana*), and the second factor is the concentration of 2.4-D with five levels, namely: control (without 2.4-D); 0.5 mg L⁻¹, 1 mg L⁻¹, 1.5 mg L⁻¹, and 2 mg L⁻¹. All treatment media were added with 0.5 mg L⁻¹ kinetin except for control media. Each treatment was repeated three times, and each replication consisted of nine explants. Observations were carried out every week until 12 weeks after treatment. The variables observed were callus

formation time, percentage of callus formation, plant explants' response after treatment, and callus morphology. The callus percentage was calculated using the following formula (Alim et al., 2014):

$$\text{Percentage of callus} = \frac{\text{number of explants that form callus}}{\text{number of all explants}} \times 100\%$$

Observation data were analyzed using Microsoft Excel and SAS program 9.0 to obtain ANOVA, then further tested using Duncan's Multiple Range Test (DMRT) at the 5% level.

RESULTS AND DISCUSSION

Effect of Callus Induction Treatment on Callus Formation Time

The observation of the time variable of callus formation was to see which treatment media could induce the fastest callus formation. The results of observations on the two species of pitcher plants showed no significant difference in the time of callus formation in the treatment of several 2.4-D concentrations given and only different from the control media (Table 1). Compared to the two species of the pitcher plant, *Nepenthes reinwardtiana* was faster to form callus than *N. gracilis*, with the fastest average time to induce callus was 4.93 weeks after treatment on the treatment medium given 0.5 mg L⁻¹ 2.4-D and 0.5 mg L⁻¹ kinetin. Whereas in *N. gracilis*, the fastest callus formation time was 6.42 weeks after treatment in the medium with 0.5 mg L⁻¹ 2.4-D and 0.5 mg L⁻¹ kinetin. The results obtained in Table 1 are slightly different from previous studies, which show that *N. reinwardtiana* forms callus faster than *N. ampullaria*, namely starting in the third week after treatment (Novitasari and

Table 1 Effect of 2.4-D concentration on callus formation time in two species of pitcher plant

The concentration of 2.4-D (mg L ⁻¹)	Average time of callus formation (Week After-Treatment)	
	<i>N. reinwardtiana</i>	<i>N. gracilis</i>
0	0 b	0 b
0,5	4,93 a	6,42 a
1	6,77 a	7,75 a
1,5	5,33 a	9,15 a
2,0	5,17 a	6,88 a

Note: The numbers followed by the same letter in the same treatment and variables were not significantly different in the 5% DMRT test.

Isnaini, 2020). Endogenous hormones in the explants and the combination of exogenous hormone concentrations given, namely 2.4-D and kinetin, probably caused this difference. Moreover, Herawati et al. (2014) stated that a dark environment is the most effective condition to induce callus formation in *Artemisia*.

Effect of Callus Induction Treatment on the Percentage of Callus Formed

The observation of the percentage variable of callus formation was to see the response of leaf explants cultured on ½ MS medium added with 2.4-D hormone in two species of pitcher plant. The results showed that the treatment of 2.0 mg L⁻¹ 2.4-D and *N. gracilis* gave the highest percentage of callus formation compared to other treatments, namely 88.89% (Table 2). Whereas for *N. reinwardtiana*, all the

treatments given had no significant effect on callus formation. However, the percentage of callus formed by *N. reinwardtiana* increased from previous studies (Novitasari and Isnaini, 2020), namely the highest was 16.67% in ½ MS medium with added 2.0 mg L⁻¹ 2.4-D and 0.1 mg L⁻¹ kinetin to 37.03% in the treatment medium 1.0 mg L⁻¹ 2.4-D and 0.5 mg L⁻¹ kinetin (Table 2).

Table 2 shows the cultures of the two species of pitcher plants that did not form a callus without hormones. These results align with previous studies (Novitasari and Isnaini, 2020) and callus induction research conducted by Kumar et al. (2017) on chili plants using hypocotyl as explants to induce callus. Their results stated that the media containing only MS without plant growth regulators did not induce callus formation at all. This study also indicated

Table 2 Effect of 2.4-D concentration on the percentage of callus formation in two species of pitcher plant at eight weeks after treatment

Species	The concentration of 2.4-D (mg L ⁻¹)				
	0	0,5	1,0	1,5	2,0
<i>N. reinwardtiana</i>	0 b	22,22 ab	37,03 a	29,63 a	33,33 a
<i>N. gracilis</i>	0 c	74,08 ab	55,56 ab	40,74 b	88,89 a

Note: The numbers followed by the same letter in the same row were not significantly different in the 5% DMRT

that the addition of auxins or cytokinins or a combination of auxins and cytokinins on MS base media increase callus induction.

Moreover, other studies have also shown the same thing that explants grown on media without growth regulators did not initiate callus formation in barley (Al-ajlouni et al., 2012), *Centaurea zeybekii* (Aydođan and Erdađ, 2015), *Spirodela polyrhiza* (Wang, 2016), *Catharanthus roseus* (Rahman et al., 2019), and rice (Mostafiz and Wagiran, 2018; Khan, 2019). This indicates that exogenous plant growth regulators are crucial in inducing callus formation.

Callus induction will generally increase gradually with an increase in the concentration of 2.4-D given, but it is different from Benzylaminopurine (BAP). The results of callus induction research on *Solanum melongena* L. showed that the best media to induce callus was Murasige and Skoog media which added 3 mg L⁻¹ 2.4-D and 0.05 mg L⁻¹ BAP with a percentage of callus formation 82.67 % with the fastest callus formation time of 7.53 days after treatment (Alim et al., 2014). Increasing the concentration of 2.4-D to 1.0 mg L⁻¹ and 1.5 mg L⁻¹ decreased the percentage of callus formed then increased significantly at a concentration of 2.0 mg L⁻¹ (Table 2). Figure 1a shows a brown (browning) explant. This is thought to be due to the influence of endogenous hormones on the explants and the injury that resulted in browning on cultured leaf explants, so that callus induction was inhibited, and finally, the explants dead and did not form a callus.

Research on callus induction *Centaurea zeybekii* conducted by Aydođan and Erdađ (2015) states that callus is formed when explants are planted on MS media with added cytokinins.

The combination of auxins and cytokinins is required to induce embryonic callus. Different pieces of the same explants and then cultured on the same treatment medium can produce different growth responses. This is thought to be due to the explants' source and the differences in the concentration of endogenous hormones in the explants. In addition, Oxidative stress due to injury to the explants can lead to embryonic callus formation.

The combination of 2.4-D and kinetin in inducing callus *Vinca rosea* was the best combination of media. *Vinca rosea* cultured on MS medium added with 1 mg L⁻¹ 2.4-D and 1 mg L⁻¹ kinetin gave the highest callus percentage response, 95%, 80%, and 60% in leaf, node, and fruit explants. From this study, the combination of auxin and cytokinin proved to be the best medium in producing callus from three types of explants, namely leaves, nodes, and fruit on *Vinca rosea* (Haq et al., 2013). These results align with previous studies that induced callus on ½ MS medium, which added 0, 1, 2, 3, 4, 5 mg L⁻¹ 2.4-D and 0.1 mg L⁻¹ kinetin to *N. reinwardtiana*. In this study, the percentage of callus formed had no significant effect on each treatment (Novitasari and Isnaini, 2020).

Effect of Pitcher Plant Species and 2.4-D Concentration on Callus Morphology

The callus induction media treatment resulted in friable callus and creamy white callus color on *N. reinwardtiana* and brownish white callus on *N. gracilis* (Table 3). Figure 1a and 2a show leaf explants cultured on treatment media without plant growth regulators did not form a callus. The leaf explants of *N. reinwardtiana* experienced browning (Figure 1a), while the leaf explants of *N. gracilis* still looked green but did not show callus formation (Figure 2a).

Table 3 Effect of pitcher plant type and 2,4-D concentration on callus morphology formed at 12 weeks after treatment

Species	The concentration of 2,4-D (mg L ⁻¹)	Callus Color	Callus Texture
<i>N. reinwardtiana</i>	0	-	-
	0,5	Creamy white	Friable
	1,0	Creamy white	Friable
	1,5	Creamy white	Friable
	2,0	Creamy white	Friable
<i>N. gracilis</i>	0	-	-
	0,5	Brownish white	Friable
	1,0	Brownish white	Friable
	1,5	Brownish white	Friable
	2,0	Brownish white	Friable

Figure 1 Callus *N. reinwardtiana* aged eight weeks after treatment on ½ MS medium: a. without hormones b. 0.5 mg L⁻¹ 2,4-D and 0.5 mg L⁻¹ kinetin, c. 1.0 mg L⁻¹ 2,4-D and 0.5 mg L⁻¹ kinetin, d. 1.5 mg

Figure 2 Callus *N. gracilis* aged 12 weeks after treatment on ½ MS medium: a. without hormones, b. 0.5 mg L⁻¹ 2,4-D and 0.5 mg L⁻¹ kinetin, c. 1.0 mg L⁻¹ 2,4-D and 0.5 mg L⁻¹ kinetin, d. 1.5 mg L⁻¹ 2,4-D and 0.5 mg L⁻¹ kinetin, and e. 2.0 mg L⁻¹ 2,4-D and 0.5 mg L⁻¹ kinetin

Figure 1 and 2 show that the callus formed from the pitcher plant leaves with a friable texture. Color change in callus occurred in *N. gracilis* at 12 weeks after treatment from white to brownish-white (Figure 2).

Khan (2019) researched aromatic rice to induce callus from embryo explants using multiple concentrations of 2,4-D (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg L⁻¹). The results showed that the concentration of 2.0 mg L⁻¹ 2,4-D resulted in the highest percentage of callus formation, namely up to 90% compared to other treatments, the resulting callus was creamy yellow and all callus formed had friable texture. Research on callus induction on *Talinum paniculatum* leaf explants conducted by Karuppiyah and Thong (2016) stated that explants grown on MS media added 2.0 mg L⁻¹ 2,4-D and 2.5 mg L⁻¹ BAP was the best percentage that is, 100% and the resulting callus is white and friable.

The research result by Amalia et al. (2017), who induced callus on leaf explants of *Vanda* sp. mentioned that the beginning of callus formation on *Vanda* leaf pieces was marked by the appearance of swelling in the incision or wound area. The wounded leaves form a callus by covering the wound area. The explant cells swelled to cause elongation due to 2,4-D treatment, although not all treatment media had the same effect. Wahyuni et al. (2017) reported that callus formation in the gendarusa was characterized by the appearance of yellowish-green or light green cell clump in the injured explants. Clumps would form a cell mass called a callus. Callus texture from gendarusa leaves grown on MS medium added with a combination of 2,4-D and BAP produces callus with a friable texture. This indicates a change in

the growth phase and strength of cell regeneration.

Wahyuni et al. (2017) also reported that changes in color from yellowish-green to bright green, transparent white, white, yellow, or green to brown, indicating that the cell is still actively dividing or in the division stage, while brown, yellow, or black colors show signs of aging in cells. Amalia et al. (2017) stated that cell wall plasticity and cellulase enzyme formation could be increased by cell division, which can dissolve cell wall cellulases. This event causes oxygen, water, and minerals to pass through the cell walls and then causes cell growth and enlargement. Auxin added to the treatment medium can increase the concentration of intracellular fluid. The auxin spurs the accumulation of structural materials and chemical compounds in the cytoplasm. The presence of cytokinins added to the media causes the absence of embryonic callus in this callus induction.

CONCLUSION

Callus began to form at the fourth week after treatment at *Nepenthes reinwardtiana* and at the sixth week after treatment on *N. gracilis*. The callus formed in the two species of pitcher plants had the texture of friable differed in the color of the callus formed. Callus of *N. reinwardtiana* has creamy white color, while callus *N. gracilis* has a brownish white color. The ½ MS medium added with 2 mg L⁻¹ 2,4-D and 0.5 mg L⁻¹ kinetin was the best medium for inducing *N. gracilis* and *N. reinwardtiana* callus.

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