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In vitro callus initiation from Indigofera (*Indigofera zollingeriana* Miq.)

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Abstract. The aimed of this research was to callus initiation of *I. zollingeriana* Miq using a various concentration of 2,4-D and source of explants. The source of explants was the first factor which consisted of 2 types of explants, leaf (A1) and stem (A2) and concentration of 2,4-D was the second factor with several levels D0 (MS0), D1 (0.0 mg/L), D2 (1.0 mg/L), D3 (2.0 mg/L), D4 (3.0 mg/L), and D5 (4.0 mg/L). The results showed that source of explants and plant growth regulator of 2,4-D afford give a significant effect on the time of callus induction and very significant effect for the weight of callus, but no interaction between source of explants and 2,4-D for a time of callus induction and weight of callus. The fastest of callus initiation on MS media added by 2,4-D with a concentration of 2.0 and 3.0 mg/L (3.55 weeks after planting). The largest callus diameter showed on MS media without 2,4-D. The biggest weight of callus showed on MS media added by 2,4-D with a concentration of 3.0 mg/L. Callus had friable textured and creamy white-colored. Ultimately, the fastest of callus initiation from the source of explants was stem with 3.51 weeks after planting.
Keywords: *Indigofera zollingeriana* Miq, forage, callus initiation, explants

1. Introduction

Indigofera zolingeriana Miq (Fabaceae) that used as forage with high crude protein content. The content of crude protein from *I. zolingeriana* Miq is ranged between 23.80%-28.98% [1-5]. It's also contained saponin that can help the digestion process and reduce methane gas in ruminants [6]. As a forage, this plant is very tolerant to abiotic stress like drought [P1], inundation and high salinity [7].

Abdullah [8] reported that the forage demand in Indonesia is estimated to reach 15.24 million tons of dry matter per year. This demand may not be fulfilled due to the limited supply of forage during the dry season, farmers rely solely on local forage just found around the yards, plantations, forests and fields, and there is competitive use of land for forage production with food crops or horticulture [7]. For these reasons, various efforts are needed to increase the production of forage according to fulfil the demand of forage in Indonesia.

One of the efforts to increase the production of forage is to propagate and cultivate forage intensively, especially for forage with high nutrition. Unfortunately, many of the forage is still difficult to propagate and not many of forages have been cultivated. Propagation of *I. zolingeriana* Miq through seeds is limited because the seeds are small, difficult to germinated and high of dormancy [9,

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10]. One of the alternative techniques to propagate *I. zolingeriana* Miq by using tissue culture. Callus induction and shoots multiplication of *I. zolingeriana* Miq by tissue culture was already reported by [10] using BAP and NAA as plant growth regulator with epigeal and cotyledon node as an explant. This paper was to report the research about callus initiation of *I. zollingeriana* Miq using the various concentration of 2,4-D with leaf and stem as the source of explants to focusing of callus growth and the effect of that two factors for callus induction of *I. zollingeriana* Miq.

2. Plant materials and methods

2.1. Plant materials

Explants used for these experiments were leaves and stems from *I. zolingeriana* that 4-6 weeks on *in vitro* culture germinate in the media of Murashige and Skoog (MS) [11] without plant growth regulator (MS0). The media used for callus induction was MS added by plant growth regulators of 2,4-dichlorophenoxyacetate (2,4-D) and Benzyl Amino Purine (BAP).

2.2. Surface sterilization and germination of seeds

Sterilization of seeds for *in vitro* germination is carried out in laminar air flow using 70% alcohol and cleaning solution containing 0.5% sodium hypochlorite (NaClO) with a concentration of 10-15%. Sterile seeds obtained and planted on solid MS0 media and incubated in the thermostatic chamber at a temperature of 25 ± 2^{o}C to complete germination stage for 4-6 weeks.

2.3. Callus induction

For callus induction, sterile plantlets from *I. zolingeriana* that resulted from *in vitro* germination were taken and placed on sterile petri dishes with sterile tissue at laminar air flow. Sterile explants of leaves and stems then cut into small pieces with a size of 0.5-1.0 cm^2 and each explant was planted in the treatment media.

For induction of callus, media used was MS solid media added by 2.4 D that consist of several levels, i.e. D0 (MS0), D1 (0.0 mg/L), D2 (1.0 mg/L), D3 (2.0 mg/L), D4 (3.0 mg/L), and D5 (4.0 mg/L). Each treatment of media was added with 1.0 mg/L BAP, except in treatment D0 (MS0), it was not added as a control. All treatments were incubated in a thermostatic chamber with a temperature of 25±2^{o}C in dark room. Each treatment was repeated 3 times with each treatment combination consisting of 3 explants.

2.4. Callus observation

Observations of callus were carried out every week with parameters of observation were the time of callus induction (days), structure of callus, the texture of callus, weight of callus (g), color of callus and percentage of callus formation (%). The time of callus induction was observed from the first week after planting where the fastest and late callus of all treatments was initiated to form callus until 8 weeks observation.

Observation for callus texture based on Putri [12] which divides callus texture into three classes, (1) compact callus, (2) crumb callus and (3) mixed callus. Texture callus observation was done by calculating the percentage of callus texture produced. The formula for calculating the percentage of callus texture produced was: number of callus with a compact, crumb or mixed textured which divided with the number of calli totally and multiplied by 100%. Callus color observation was done visually then converted in scoring, with scoring (1) white, (2) white cream, (3) cream, (4) greenish-white, (5) green, and (6) brown.

2.5. Statistical analysis

This research was design by a completely randomized design (CRD) with two factors. Source of explants was first factor which consisted of 2 types of explants, leaves (A1) and stem (A2) explants, while the concentration of 2,4-D that consisting of 5 levels [D1 (0.0 mg/L), D2 (1.0 mg/L), D3 (2.0

mg/L), D4 (3.0 mg/L) and D5 (4.0 mg/L)] as second factor and MS0 as control. The two factors produced 12 treatment combinations and were repeated 3 times with each treatment combination consisting of 3 explants. If the data of variance show significant to very significant, a further test will be carried out with the DMRT or Duncan Multiple Range Test with significant level at $\alpha = 5\%$.

3. Results and discussion

3.1. The effect of 2,4 D and source explants for the interaction of time of callus induction, weight of callus and a diameter of callus

The analysis of variance showed that source of explants and 2,4-D given a significant effect on the time of callus induction and very significantly effect of the weight of callus but no interaction between source of explants and 2,4-D of time from callus induction and weight of callus (table 1). The effect of source of explants and 2,4-D showed the interaction in diameter of callus, with the source of explants showed very significantly and 2,4-D significantly affected. Source of explants capable to absorb the media and induce callus initiation.

Abbas [13] suggested that 2,4-D is plant growth regulator that very effective for induced callus formation and plays a role in continuous cell lengthening and division that cells can grow and develop rapidly [14]. Giving 2,4-D can help accelerate repair of damaged cells in explants due to incision by increasing cell division continuously which can cause enlarged callus cells [15]. Rivai [16] stated that the addition of 2,4-D will increase callus formation and suppress organogenesis.

Using leaves and stem as sources of explants reported more effective for callus induction [17-23] compared with root or another part of plants. Explain by Abbas [13] that each plant species, even parts of plants from different tissues, requires a different composition of media or plant growth regulator to grow properly.

Tabel 1. The effect of 2,4-D and source of explants for the interaction of time of callus induction, diameter of callus and weight of callus

No.	Parameters of observation	Treatments		interaction	Coefficient of difference (%)
		Source of explants	2,4-D		
1.	Time of callus induction	*	*	ns	25.31
2.	Diameter of callus	**	*	*	21.61
3.	Weight of callus	**	**	ns	8.97[a]

Noted: *: significantly different at $p<0.05$, **: very significantly different at $p<0.01$, ns: not significantly different at $p<0.05$, a: Transformation of data $\sqrt{x+0.5}$

Callus began induced at the 3rd and 4th weeks after planting. Induction of callus showed that callus can be initiated with the fastest time of 3.11 weeks on A2D4 media, while the longest time was 6.33 weeks in A1D0 media (table 2). The concentration of 2,4-D with 2.0 mg/L (D3) and 3.0 mg/L (D4) were treatments with the fastest callus time, which was 3.55 week and occurs in all explant sources. Whereas the control (D0) is the longest time for callus, which is 5.58 week after planting and occurs in all sources of explants. Explants that planted in the media without 2,4-D (control treatment) required a

longer time or could not produce callus compared with media added by 2,4-D. Andaryani [24] reported that in *Jatropha curcas* explants also could not induce callus from media without 2,4-D.

Tabel 2. The effect of 2,4-D and source of explants to time for callus induction (weeks).

Source of explants (A)	2,4-D (D) (mg/L)						Average
	MS (D0)	0.0 (D1)	1.0 (D2)	2.0 (D3)	3.0 (D4)	4.0 (D5)	
leaves (A1)	6.33	4.27	4.00	3.89	4.00	4.00	4.42[a]
stems (A2)	4.83	3.33	3.33	3.22	3.11	3.22	3.51[b]
Average	5.58[a]	3.80[b]	3.67[b]	3.55[b]	3.55[b]	3.61[b]	

Note: The numbers followed by different letters in the same row and column showed different significantly from the DMRT at α=5%.

The source of explant given a significant effect from parameter of time of callus induction (table 2). Stem explants were the fastest callus time with 3.51 week and leave explants were the longest callus time with 4.42 week after planting. Stem explants formed more calluses than leaf explants, according to Uddin *et al.* This is due to the presence of wounds on both ends of the stem as a result of being cut with a scalpel so that the stem can initiate callus faster than whole leaves [25]. Bustami [26] added that the presence of wounds cut with a scalpel can facilitate the concentration of 2,4-D contained in diffuse media into explant tissue and help endogenous auxin stimulate cell division, especially cells around the injured area.

3.2. The effect of 2,4 D and source of explants for callus textured, callus color and explant callus formation

Texture of callus used as one of the markers to determine the quality of a callus. According to Putri [12], callus with good quality is characterized by crumbly callus texture. The crumbly callus is callus textured that easy to separate cells into a single cell [27]. The crumbly callus texture is good quality callus because it is related to embryogenic callus [11]. The embryogenic callus is callus that capable to grow to form organogenesis process became shoots, embryo somatic or another part of plants. This was confirmed by [28] that crumbly callus showed cells with embryogenic properties. Young leaves potentially produced callus that can develop into somatic embryos.

The results for callus textured showed that A1D5 and A2D5 media produced the most compact callus with percentage were 37.50% and 50.00% (table 3). Giving 2,4-D with higher concentration showed callus with compact textured, whereas the concentration of 2,4-D below of 4 mg/L will produced crumbly textured calluses. The same study was also reported by Zulkarnain and Lizawati [29] that compact callus was dominated by hypocotyls and cotyledons explants of Jatropha if using high of 2,4-D. Royani *et al.* [15] stated that in peanut plant if 2,4-D was added with high concentration, could increase cell division continuously, that make the callus shape solidify or compact.

Formation of callus with crumbly callus texture could be driven by the presence of endogenous auxin that produced internally by explants cultured [30]. Mardini [31] added that the formation of crumb callus was also influenced by the addition of cytokines (BAP) to media that already contained auxin (2,4-D). The research in *Indigofera suffruticosa* [32] was proved that crumbly callus texture was produced when media was added by two kinds of plant growth regulator, i.e. 2,4-D with concentration of 0.5 mg/L and BAP with concentration of 1.0 mg/L. That result was the same as our result. No mixed textured callus found in this research.

Crumbly callus has the shape of cells with globular small spheres and tenuous structures while the compact callus has the form of cells that fused and a dense arrangement (based on microscope observation). Azizah [33] reported from callus of coffee that crumbly callus was composed of irregular and fragile cells, whereas the compact callus had a nodular shape and was composed of dense cells. Reported by [21] in *Clinacanthus nutans,* friable callus composed by large cells and thin cell wall with distinctive layer form that easily distinguished.

Tabel 3. The effect of 2,4-D and source of explants to the percentage of callus textured, callus color and explant formed a callus

Media	Textured of callus (%)			Colour of callus (%)						Explant callus formation (%)
	(T1)	(T2)	(T3)	(C1)	(C2)	(C3)	(C4)	(C5)	(C6)	
A1D0	-	100	-	-	100	-	-	-	-	22.22
A1D1	33.33	66.67	-	-	33.33	33.33	16.67	16.67	-	66.67
A1D2	-	100.00	-	-	66.67	22.22	-	-	11.11	100.00
A1D3	11.11	88.89	-	11.11	55.56	33.33	-	-	-	100.00
A1D4	16.67	83.33	-	-	66.67	33.33	-	-	-	66.67
A1D5	37.50	62.50	-	-	25.00	62.50	12.50	-	-	88.89
A2D0	12.50	87.50	-	-	37.50	12.50	12.50	-	37.50	88.89
A2D1	-	100	-	-	66.67	22.22	-	-	11.11	100.00
A2D2	-	100	-	-	88.89	-	-	-	11.11	100.00
A2D3	-	100	-	11.11	77.78	11.11	-	-	-	100.00
A2D4	11.11	88.89	-	-	77.78	11.11	-	-	11.11	100.00
A2D5	50.00	50.00	-	-	25.00	37.50	-	-	37.50	88.89

Note: Scoring of textured of callus, (T1) compact callus, (T2) crumb callus and (T3) mixed callus. Scoring of colors of callus, (C1) white, (C2) white cream, (C3) cream, (C4) greenish-white, (C5) green, and (C6) brown.

Callus with creamy white color dominated in this study. A1D0 and A2D2 media produced callus with creamier white with percentage were 100% and 88.89% (table 3). Creamy callus was also produced in almost all treatments, except in the A2D2 and A1D0 media. The callus that produces green color was only found in A1D1 media with percentage was 16.67%. In this study, 2,4-D with higher concentration showed the darker colored of callus. According to Sumaryono and Riyadi [34], the use of 2,4-D can affect the color of callus in quinine plants, the lower of 2,4-D, the lower level of callus browning and vice versa. Arianto *et al*. [30] added that brown callus has a low regeneration because callus cells have decreased cleavage activity.

The color difference of callus showed the level of development of callus. According to Andaryani [24], callus tissue that had been produced from an explant usually gives rise to different color, but good quality callus has a green color. Fitriani [35] added that green color of the callus showed the chlorophyll content in the tissue. Jayanti [36] added that the color change of callus from bright white

to creamy white (dark) cause by the mature age of cells or callus tissue, which indicates an enzymatic reaction that leads to the synthesis of phenol compounds.

3.3. The effect of 2,4 D and source of explants for callus diameter and weight of callus

Observation of diameter of callus showed that the treatment with 2,4-D given the effect significantly for callus diameter and a very significant effect of sources of explants but there was an interaction between 2,4-D and sources of explants on callus diameter. Leaf explants showed the larger callus diameters compared to stem explants. Huda et al. [37] had the same result when using the leaf as a source of explants that showed a larger callus size of leaf than stem as explants in *Stevia rebaudiana* plants.

The largest callus diameter was found in A1D1 media with 1.54 cm (table 4). A1D1 media that only added by BAP has to function for inducing explant development and growth, trigger cell division, widening and increase callus size [31-38]. Many researchers reported that if only BAP added in media also could form the largest callus, like in Gamal [39], melon [40] and cotton [41].

The lowest callus diameter found in A2D5 media with 0.72 cm. Callus in A2D5 media content of 2.4-D with 4 mg/L and stem as a source of explant. As mention above 2.4-D with high concentration could inhibit explants to growth, reduce the capability of cell activity then will stop to callus growth, meanwhile stem as a source of explants showed all callus with a diameter above callus diameter of the leaf (0.72-0.88 cm). Hendaryono and Wijayani [42] added that auxin 2,4-D at high concentrations would be more inhibitory than stimulating callus growth. Puah et al. [21] also reported in *Clinacanthus nutans* that if the concentration of 2,4-D increased, the leaf explants was decreased to form callus. This indicated that 2,4-D having the ability to function as an herbicide. Sumaryono and Riyadi [34] said that 2.4-D with high concentration also affected the color of callus become browning. This will effect of the callus diameter because browning can reduce callus division activity and even grow. Ozel and Maesaroh [10] reported that all callus browning followed by increased necrosis with each increasing concentrations of BAP in *I. zolingeriana*. According to Rahayu and Mardini [43], the difference in callus diameter that had been produced in each treatment was due to different tissue abilities to absorbing water and nutrients.

Tabel 4. The effect of 2,4-D and source of explants to callus diameter (cm)

Source of explants (A)	2,4-D (B) (mg/L)					
	MS (D0)	0.0 (D1)	1.0 (D2)	2.0 (D3)	3.0 (D4)	4.0 (D5)
	(cm)					
leaves (A1)	0.65^{d}	1.54^{a}	1.32^{b}	1.13^{c}	1.05^{c}	1.50^{a}
stems (A2)	0.75^{bc}	0.87^{a}	0.78^{ab}	0.82^{ab}	0.88^{a}	0.72^{bc}

Note: The numbers followed by different lowercase letters in the same row and column or different uppercase letters in the same column show significantly different from the DMRT test at the α=5%.

The effect of 2,4 D and source of explants for the weight of callus showed that A1D4 media was the largest callus weight with 0.72 g, while A1D0 media was lowest callus weight with 0.11 g (table 5). In this study, A1D2 media with 3.0 mg/L concentration of 2,4-D was the best media used to induction of weight of callus on leaves explants (0.72 g) and stem explants (0.40 g), but concentration in 4.0 mg/L decreased callus weight with 0.65 g in leaves explants and 0.37 g in stem explants. Media without growth regulator (MS0), showed the lowest callus weight with 0.11 g (leaf explant) and 0.16 g (stem explant) (figure 1).

The source of explants showed a significant effect on callus weight. Leaf explants were the greatest weight of callus with 0.52 g, while stem explants with 0.31 g. Leaf explants have different structures with stem explants, leaf explants have thinner tissue compared to stem explants that made easier to absorption of nutrients rapidly than stem explants.

Tabel 5. The effect of 2,4-D and source of explants to the weight of callus **(**mg**)**

Source of explants (A)	2,4-D (D) (mg/L)						Average
	MS0 (D0)	0.0 (D1)	1.0 (D2)	2.0 (D3)	3.0 (D4)	4.0 (D5)	
leaves (A1)	0.11	0.67	0.40	0.57	0.72	0.65	0.52^{a}
stems (A2)	0.16	0.31	0.29	0.32	0.40	0.37	0.31^{b}
Average	0.13^{d}	0.49^{ab}	0.35^{c}	0.45^{b}	0.56^{a}	0.51^{ab}	

Note: Data is transformed 1 time and the numbers followed by different letters in the same row and column show significantly different from the DMRT test at α=5%.

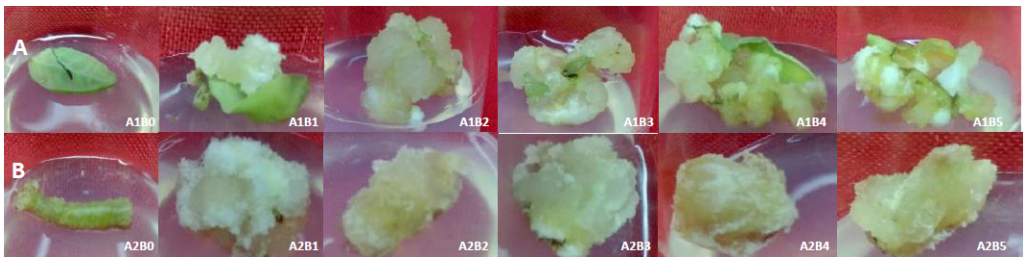


Figure 1. Callus induced from leaves and stem explants of *I. zolingeriana*. A: Leaves explants and development callus from leaves explants, B: Stems explants and development callus from stems explants

2,4-D influences the development of plant cell, which showed indication that 2,4-D can increase osmotic pressure, protein synthesis, cell permeability to water, and soften cell walls [42]. Siahaan and Sumihar [44] added that 2,4-D physiologically plays a role in encouraging enlargement and elongation of cells that made callus cells larger and longer and had been affected by increasing callus weight. The mechanism of biosynthesis of auxin is regulated by tissue-specificity [21].

4. Conclusion

The 2,4-D had a significant effect on the time of callus induction and very significant effect of the weight of callus, but no interaction between source of explants and 2,4-D for the time of callus induction and weight of callus of *I. zolingeriana*. The source of *I. zolingeriana* explant given a significant effect on the time of callus induction with stem was the fastest callus time and leaf was the greatest weight of callus. Media with 3.0 mg/L concentration of 2,4-D was the best media used to induction of weight of *I. zolingeriana* callus both on leaves and stem explants.

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