https://doi.org/10.1051/bioconf/20249101014

Initiation of Red Ginger Callus (*Zingiber officinale* Roxb. var. *rubrum* Rosc.) from Various Explants

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Abstract. The increasing demand for red ginger (*Zingiber officinale* Roxb. var. *rubrum* Rosc.) both at the domestic and international levels has led to the need for gingerol production, a main compound of red ginger, which has various pharmacological activities. The urgency of this research is related to gingerol produced in cultivated red ginger, which often shows variability in quantity due to genetic variation and differences in geographical and environmental conditions where it is grown, so it requires gingerol standardization efforts. Through tissue culture techniques, it is possible to propagate plants in a controlled environment, ensuring genetic uniformity and minimizing variations caused by genetic factors. Red ginger raw materials that can be produced consistently, quickly, and land-efficiently with high gingerol content and pesticide-free have become an essential economic necessity. In this joint study with PT. Bintang Toedjoe, researchers intend to utilize root culture bioprocessing technology to increase gingerol production from red ginger. Root cultures have stabile genetics and growth faster; thus, these techniques imply the formation of organs or structures conducive to enhanced gingerol production. Our research has revealed successful protocols for inducing and multiplying suitable callus for organogenesis. Through the application of hormones, the best callus induction is using a combination of 3 ppm 2,4-D and 0.2 ppm BA with a callus production percentage of 67%. On the other hand, a satisfactory callus multiplication rate was used using 1 ppm 2,4-D with the most significant increase in explant area (79 mm^2) by ruler alignment. Meanwhile, the rooting response was prominent at 1 ppm 2,4-D + 3 ppm BA.

1 Introduction

Red ginger (*Zingiber officinale* Roxb. var. *rubrum* Rosc., Zingiberaceae) is a plant with a more intense aroma and spicier taste than other ginger varieties. Red ginger has been used in Indonesia for healing wounds, as an antibacterial and antioxidant [1]. Red ginger export

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demand is around 4 tons per week [2]. One of the main phenolic contents in ginger is gingerol, a mixture of various compounds with antioxidant, anti-inflammatory and antibacterial activity. Apart from 8-gingerol and 10-gingerol, 6-gingerol is the main compound in ginger fresh, which has multiple pharmacological effects [3]. Gingerol content in rhizomes (104.39 μg/g) is much higher than in stems (0.84 μg/g) and leaves (4.13 μg/g) [4]. Fresh ginger rhizomes contain 6-gingerol highest (75.25 mg/100 g fresh weight), higher than peeled ginger rhizomes (68.15 mg/100 g fresh weight) [5].

Previous research shows variability in gingerol compounds in ginger genotypes [6]. The quality of raw materials based on the gingerol content of red ginger depends on the conditions growing environment [7]. A significant variation was found in the gingerol content and shogaol from ginger samples grown under different conditions [8]. A combination of $ZnSO_4$ and drought stress can also reduce gingerol levels and inhibit plant growth ginger [9]. Variations in gingerol levels in red ginger are one of the main problems in the quality of the raw material for red ginger, so since 2017, PT. Bintang Toedjoe has been forming the red ginger ecosystem by seeking standardization of red ginger cultivation in various Indonesian regions. PT. Bintang Toedjoe has collaborated with the University of Surabaya through red ginger tissue culture techniques to produce consistent and standardized red ginger. The need for red ginger is increasing due to the high export of red ginger in Asia, up to 176% in 2022; even PT Bintang Toedjoe plans to expand the marketing of red ginger products to Japan in 2023.

Based on limited interviews with PT. Bintang Toedjoe, it was found that this was the case in the need for red ginger raw materials that have consistently high levels of gingerol, which is not influenced by genetic variations, growing environment, geographical, environmental conditions, pests-free and pesticide-free. Thus, the production of gingerol in red ginger can be done safely steadily, fast, and relatively does not require large areas of land for cultivation. The novelty of this research is the initiation of red ginger callus culture to induce it to become roots culture. Indirect organogenesis involves the formation of callus tissue, which serves as a crucial intermediate stage. Callus formation is often more efficient through indirect organogenesis, leading to the development of roots [10]. The root culture can grow faster and propagate on a large scale while maintaining genetic and biochemical stability, as we already conducted on *Panax ginseng* [11]. As previous report, roots culture technology can increase the production of vanillin isomers, in *Decalepis salicifolia*, up to 4.9-fold and improve the accumulation of pinostrobin by 3.54 mg/g, in *Boesenbergia rotunda* [12, 13]. This research focuses on the indirect organogenesis of red ginger callus. Our objective is to enhance the efficiency and reduce the cultivation time compared to traditional methods of red ginger cultivation, which utilise rhizomes.

2 Materials and methods

2.1 Media preparation

The media used is Murashige and Skoog (MS) basal medium with vitamins and sucrose at a concentration of 34.43 g/L (according to the manufacture). An appropriate volume of 1000 ppm stock solution of benzyl adenine (BA) and/or 2,4-Dichlorophenoxyacetic acid (2,4-D), is added to the media, and the pH is measured to match the standard (pH 5.80 ± 0.05). If the pH is not suitable, adjustments can be made by adding sufficient 10% H_3PO_4 solution or 0.1 N NaOH. Commercial agar (7 g/L) is then added to the media and is stirred and then heated with a magnetic bar and magnetic stirrer until the media becomes solidified. Sterilization in an autoclave at 121°C (1.5 atm) for 15 minutes. The media was then poured into disposable

petri dishes aseptically. The media sterility was observed for three days before use. In vitro, explants of red ginger were obtained from Kalbe Ubaya Hanbang Bio Laboratory.

2.2 Callus initiation of red ginger

An 8-week-old in vitro seedlings of red ginger (cultured on MS + 1 ppm NAA + 3 ppm BA) are separated into each part including callus (approximately 25 mm^2), shoot bases (approximately 10 mm height) and midrib leaves (approximately 10 mm length), which are used as an explant. The explants are then cultured in various hormone-supplemented media (mentioned in part 2.1). Disposable petri dishes containing explants were then incubated in dark conditions for 43 days. Routine observations are carried out on the morphological appearance of the callus/root, the percentage of callus/rooting, and changes in the size of the explant by ruler alignment. The area measured is the explant area in contact with the media surface. The percentage of callus/rooting (if any) response can be calculated using the following formula.

$$callus\ or\ rooting\ response\ (\%) = \frac{explants\ producing\ callus/roots}{total\ explants} \times 100\% \qquad (1)$$

3 Results and discussion

Initiation of callus formation is carried out so that a callus can reproduce continuously and later be propagated by plants through the construction of organs and embryos, so-called indirect organogenesis. In Table 1, the four media can induce callus formation from shoot base explants. The callus formed is mostly white colour, caused by the incubation process in dark conditions and a balanced proportion of total auxin and cytokinin. All callus was developed either to shoots and/or roots or adventitious roots in different time point. Although callus was able to grow at MS0 (as control), shoot emergence was also found, which corresponds to the prior report in the culture is higher than the auxin concentration [14]. Moreover, it shows roots and roots response before the callus response. The presence of both roots and shoots in the callus might lead to plants with inconsistent morphology. The varied structure and function of roots and shoots could result in plants that are not well-suited for specific applications, such as agriculture or horticulture, where uniformity is often desired [15]. The addition of exogenous hormones to the media is not always directly proportional to the time of callus initiation. In other words, increasing the concentration of hormones may not necessarily result in a faster time it takes for callus initiation to begin. Other factors might be influencing the process, and the relationship is likely more complex. This is because concentrations that are lower or higher than the optimum conditions can reduce the effectiveness of induction and the percentage of callus formed [14]. The efficacy of exogenous hormones also depends on the concentration of endogenous hormones in the explant tissue used. The best medium to induce callus from the base of the shoot is MS medium + 1 ppm 2,4-dichlorophenoxyacetic acid (Fig. 1 A-B), despite it needs 21 days after planting (DAP) to initiate the callus, it has 100% callus response, healthy callus (not much browning in term of callus color), the highest increase in explant area and later regeneration of organ (shoots or roots). Slow regeneration often allows for better control over the growth and development of the regenerated organs. The uniformity and consistency achieved through later regeneration stages are highly desirable for callus multiplication [16].

Table 1. Callus growth from *in vitro* shoot base explants on several types of media for 43 days

Treatment	Parameters					
	Initiation time of callus (DAP)	**Callus color**	**Callus response (%)**	**Description of callus regeneration**	**Increasing explant area (mm^2)**	**Increasing explant height (mm)**
Control	15	White	100	Root and adventitious root (7 DAP) Shoot (10 DAP)	70	5
1 ppm 2,4-D	21	White – light yellowish	100	Root and adventitious root (28 DAP)	79	6
1 ppm 2,4-D + 3 ppm BA	10	Yellowish brown	100	Root and adventitious root (15 DAP)	30	5
1 ppm 2,4-D + 5 ppm BA	21	White – light yellowish	100	Root and adventitious root (28 DAP) Shoot (7 DAP)	55	4

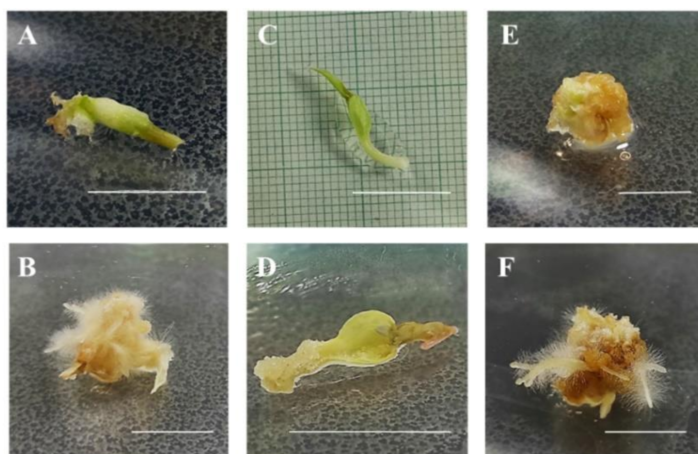

Fig. 1. Callus development from explants: shoot base A) 3 DAP and B) 43 DAP; midrib leaves: C) 3 DAP and D) 43 DAP; and *in vitro* callus: E) 3 DAP and F) 43 DAP [Scale 1:10 mm]

The additional benzyladenine (BA) to the 1 ppm 2,4-D led to a lower increasing explant area, in terms of callus. BA and 2,4-D are both plant growth regulators commonly used in tissue culture. BA is a cytokinin, which promotes cell division and shoot development, while 2,4-D is an auxin that stimulates cell elongation and root formation. The combination of these hormones is often used to induce callus formation and subsequent organ regeneration in tissue culture. If the ratio of these hormones in the culture medium is appropriate, it facilitates the desired cellular responses (callus formation) but sometimes, it also causes undesirable effects (organ formation) [17-18]. Meanwhile, the height of explants (shoot) can serve as an indicator of the response to hormonal treatments. Changes in shoot height may suggest the effectiveness of specific growth regulators or highlight the need for adjustments in hormone

concentrations. This information aids in fine-tuning the culture medium for optimal callus induction and subsequent regeneration [19].

In Table 2, the explants used were midrib leaves, which were subcultured on three media types. Usually, there is already higher indigenous cytokinin than auxin on the shoot culture type. Therefore, the much lower cytokinin on media formulation can be expected to induce the callus culture. Explants grown at MS0 could not induce callus formation, indicating that the leaves and midribs do not have enough levels of endogenous hormones auxin or cytokinin to induce callus. On 3 ppm 2,4-D, the explant was able to form a callus (Table 2). The callus initiation time is relatively long, and the callus formed is also tiny. This indicates that the administration of 3 ppm 2,4-D is starting to affect callus formation. Meanwhile, 3 ppm 2,4-D + 0.2 ppm BA increased the callus formation. Callus initiation time is also relatively faster, and the increase in area is more remarkable than explants on 3 ppm 2,4-D medium. The choice of a higher concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) and a lower concentration of benzyladenine (BA) for callus induction from midrib leaves, compared to the composition used for callus induction from shoots, can be linked to the specific characteristics and requirements of these two types of explants. Midrib leaves and shoot explants have distinct tissue compositions and characteristics. Midrib leaves, being a source of undifferentiated cells, may require a higher concentration of 2,4-D to induce effective callus formation. On the other hand, shoots, with their meristematic regions, may respond well to lower concentrations of 2,4-D [20]. This confirms that these plant growth regulators' concentration influences callus formation. Adding 0.2 ppm BA to a medium containing 3 ppm 2,4-D resulted in a more balance of auxin-cytokinin concentration to initiate callus formation (Fig. 1 C-D).

Table 2. Callus growth from *in vitro* midrib leaves explants on several types of media for 43 days

Treatment	Parameters					
	Initiation time of callus (DAP)	**Callus color**	**Callus response (%)**	**Description of callus regeneration**	**Increasing explant area (mm^2)**	**Increasing explant height (mm)**
Control	-	-	0	Root, adventitious root, and shoot (7 DAP)	73	5
3 ppm 2,4-D	35	Cream	33.33	Adventitious root (43 DAP)	6	1
3 ppm 2,4-D + 0.2 ppm BA	10	White	66.67	-	37	1

The four media listed in Table 3 can increase callus growth from the callus explant. The greater the BA content added, the faster the callus initiation time. The growth regulator 2,4-D acts as a callus inducer, while BA will increase cell division or proliferation in explants [15]. Using 1 ppm 2,4-D + 3 ppm BA medium provided the largest value of increasing explant area (Fig. 1 E-F), indicating that the most callus formation occurred. Therefore, it can be said that this media composition is the best in inducing callus. Using 1 ppm 2,4-D + 5 ppm, BA did not increase the area as large as the other treatments. This indicates that the higher the BA content, the less callus formation occurs. These results correspond to the optimum conditions of callus induction from the shoot explants.

Table 3. Callus growth from *in vitro* callus explants on several types of media for 43 days

Treatment	Parameters					
	Initiation time of callus (DAP)	**Callus color**	**Initiation time of callus (DAP)**	**Description of callus regeneration**	**Initiation time of callus (DAP)**	**Increasing explant height (mm)**
Control	21	White–light cream	100	-	52	2
1 ppm 2,4-D	15	Light cream–red	100	Root and adventitious root (35 DAP)	33	3
1 ppm 2,4-D + 3 ppm BA	10	Faded green–brownish cream	100	Root and adventitious root (28 DAP)	78	2
1 ppm 2,4-D + 5 ppm BA	7	Red–brownish cream	100	Root and adventitious root (43 DAP)	26	0

Regenerating plants with well-developed roots from callus cultures ensures that the production process can be easily scaled up to meet high-demand scenarios. The induction of roots post-callus formation contributes to genetic stability by ensuring that the regenerated plants maintain consistency in their traits [21]. As shown in Table 4, The highest rooting response were obtained with the use of 0.5 ppm 2,4-D (70%), with friable and brownish-white to yellowish-white callus characteristics. A friable callus is loosely arranged and easily crumbles, indicating a more disorganized and loosely aggregated structure. The callus formed with 1 ppm 2,4-D also displayed a friable texture. The friable nature of the callus was consistent between the two treatments, suggesting that the texture of the callus was not significantly affected by the change in 2,4-D concentration. The callus produced with 1 ppm 2,4-D displayed a greenish-white to yellowish-white colour. The shift towards a greenish tone suggests a change in the pigmentation of the callus with the increase in 2,4-D concentration.

Table 4. Adventitious root growth from *in vitro* callus explants on several types of media for 43 days

Treatment	Parameters			
	Callus response (%)	**Rooting response (%)**	**Characteristic of callus**	**Callus color**
0.5 ppm 2,4-D	15	70	Friable	Brownish white – yellowish white
1 ppm 2,4-D	56	15	Friable	Greenish white – yellowish white

On the other hand, when the concentration of 2,4-D was increased to 1 ppm, the rooting response decreased to 15%. As shown in Fig. 2, this suggests that a higher concentration of 2,4-D (1 ppm, Fig. 2 C-D) had a less favourable impact on rooting compared to the lower concentration (0.5 ppm, Fig. 2 A-B) [22]. When transitioning to a liquid medium in a larger scale-up trial, the choice of 2,4-D concentration becomes critical for ensuring efficient rooting. The results suggest that maintaining a lower concentration (0.5 ppm) may be more effective for achieving higher rooting responses. Implementing this lower concentration in the scale-up trial serves as an initial strategy, but continuous monitoring and optimization may be necessary. The findings suggest that it may be a more effective starting point, but adjustments can be made based on the performance and specific requirements of the plant material in the liquid medium.

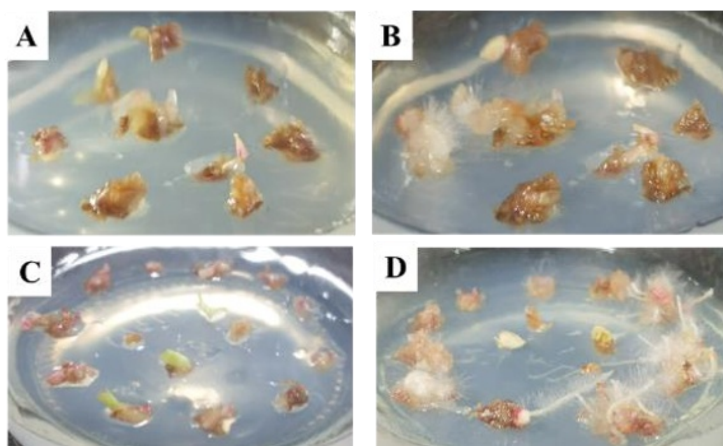

Fig. 2. Adventitious root growth on 0.5 ppm 2,4-D at: A) 28 DAP and B) 43 DAP; 1 ppm 2,4-D at: C) 28 DAP and D) 43 DAP

4 Conclusion

A satisfactory callus induction can be achieved 1 ppm 2,4-D from shoot base explants, and in combination with 3 ppm BA provides a great increase in explant callus area, making it as best option for callus multiplication/ subculture media. The root response was massive when using 0.5 ppm 2,4-D compared to 1 ppm 2,4-D which has a tendency to callus response.

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