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PERSPECTIVE

A perspective on the *in vitro* tissue culture of red ginger and its impact on antioxidant activity

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Abstract

Red ginger (*Zingiber officinale* var. *rubrum*) is one of the ginger varieties grown in Indonesia and Malaysia. Like other varieties of ginger, red ginger has been widely used as a traditional herbal medicine in Indonesia. The antioxidant activity of red ginger is suggested to be higher than other ginger varieties due to anthocyanin, which provide a red -coloured rhizome. The amount of red ginger bioactive compounds is different due to its cultivation place, cultivation method, and processing method. Difficulties of conventional vegetative propagation of red ginger have to be overcome with other appropriate techniques. One of the potential techniques as suitable methods for red ginger cultivation is *in vitro* tissue culture. This technique may produce pathogen-free ginger plantlets with a variation of bioactive compounds. *In vitro* tissue culture also provides higher total phenolic content related to higher antioxidant activity in elicited ginger callus. The best elicitor that yielded the highest antioxidant activity in ginger callus is yeast extract 100 mg/L. With that condition, *in vitro* tissue culture is still promising as an alternative cultivation method for red ginger to promote uniform, sterile and controlled plantlet with targeted bioactive compounds as the researcher urges.

1. Introduction

Most Indonesians often use some herbal medicine for their family medication as first aid before looking for a medical expert. One of the herbal medicines widely used by Indonesians is ginger (*Zingiber officinale*) (Jayanudin *et al.*, 2019; Nurhadi *et al.*, 2020; Lukiati *et al.*, 2020). Indonesia is the 4th out of the top ten ginger-producing countries in the world right after India, China, and Nepal (Shahrajabian *et al.*, 2019a). Based on its morphology, Indonesian ginger is classified into red, elephant or giant, and emprit or small ginger (Daryono *et al.*, 2012). Those three have differences in the rhizome, including shape, odor, color and bioactive compounds (Wibowo *et al.*, 2020). Red ginger (*Z. officinale* var. *rubrum*) is ginger variance cultivated in both Indonesia and Malaysia, which is has a red-coloured rhizome (Nurhadi *et al.*, 2020). In addition to gingerols and shogaols, red ginger is also loaded with anthocyanin and tannin (Shahrajabian *et al.*, 2019b). Anthocyanins have a flavylium cation (AH+) structure which is related to their antioxidant activity (Tena *et al.*, 2020).

Ginger contains many bioactive compounds with different biological and pharmaceutical effects. Ginger contains some antioxidant agents, including terpenoids, polyphenols, and β-carotene. It was reported that the high total phenolic and flavonoid contents correlate with high antioxidant activities (Ghafoor *et al.*, 2020). Some phenolic compounds in ginger are 6-gingerol (Deleanu *et al.*, 2018), 8-gingerol, 10-gingerol, 6-shogaol, 8-shogaol, zingeron (Lukiati *et al.*, 2020), and paradol (Mošovská *et al.*, 2015; Nurhadi *et al.*, 2020). Whereas, some flavonoid compounds in ginger are quercetin, rutin, catechin, and epicatechin (Nurhadi *et al.*, 2020). The most abundant bioactive compounds found in ginger are 6-gingerol and 6-shogaol (Sahardi *et al.*, 2021) (Figure 1).

The red ginger ethanolic extract has 155.784 gallic

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acids equivalents and 609.655% quercetin equivalents of total phenolic and flavonoid contents, respectively (Lukiati *et al.*, 2020). The phenolic and flavonoid compounds in ginger are closely related to its antioxidant activity. Both are natural antioxidants that interact with DPPH (2,2-diphenyl-1-picrylhydrazyl) as free radicals. They transfer electrons to DPPH and then neutralize DPPH radicals. Ethanolic extract of red ginger is categorized as a good antioxidant due to its IC_{50} values which is smaller than 50 µg/mL. The IC_{50} of red ginger ethanolic extract was 44.06 µg/mL (Lukiati *et al.*, 2020). *In vivo*, the antioxidant activity of ginger also affects xenobiotic metabolism by drug-metabolizing enzyme induction. The enzymes including glutathione-S-transferase, glutathione peroxidase, and quinone reductase (Höferl *et al.*, 2015). The ginger extract is known as an antioxidant agent due to its ability to scavenge superoxide anion and hydroxyl radicals. Gingerol inhibited ferrous complex-induced lipid peroxidation in rat liver microsomes *(*AlTahtawy *et al.*, 2011).

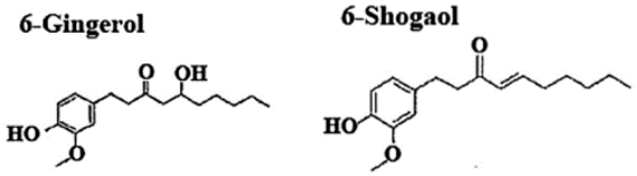

Figure 1. Structure of 6-gingerol and 6-shogaol as the most abundant bioactive compounds in ginger (Shahrajabian *et al.*, 2019b).

The bioactive compounds of ginger depend on many factors, including the cultivation place, cultivation method, and processing method used. Ginger is commonly propagated by rhizome ever since it is cultivated as spices and medicinal properties. Its rhizome propagation is vulnerable because of the low proliferation rate (Ammar *et al.*, 2016) and the presence of some pathogenic bacteria or fungi as rot and wilt diseases causative agents (Mehaboob, Faizal, Raja *et al.*, 2019). Furthermore, ginger has poor flowering and seed set leads to complicacy of breeding (Kambaska and Santilata, 2009; Mehaboob, Faizal, Shamsudheen *et al.*, 2019). To overcome those main difficulties in ginger breeding, an *in vitro* tissue culture was introduced as an alternative cultivation method. *In vitro* tissue culture promotes massive propagation and crop improvement in a shorter time than its common cultivation (Ammar *et al.*, 2016; Pinheir *et al.*, 2020). *In vitro* tissue culture of ginger demonstrated complete elimination of nematodes, creating variation and exploiting crop improvement (Babu *et al.*, 1992). Culture media plays an important role in producing a successful *in vitro* micropropagation. The chemical composition of media will influence plantlet development (Mehaboob, Faizal, Thilip *et al.*, 2019) and its bioactive compounds. This review investigates the effect of *in vitro* tissue culture on red ginger antioxidant activity.

2. Methodology

The databases used during searching works of literature needed for this article were Scopus, PubMed and Google Scholar. The keywords used were *Zingiber officinale* var. *rubrum*, red ginger, antioxidant activity + *Zingiber officinale* var. *rubrum*, *in vitro* culture + *Zingiber officinale* var. *rubrum*, and *in vitro* culture + antioxidant activity.

3. Red ginger *in vitro* tissue culture

In vitro cultivation in a plant is a competitive technique to produce higher bioactive compounds. Some treatments can enhance those production during undifferentiated cell culture (Solanki *et al.*, 2014). Micropropagation, organ culture, somatic embryogenesis, organogenesis, somatic and embryonic suspension culture have been reported as an alternative technique for ginger *in vitro* cultivation (Guo *et al.*, 2007). Many bioactive compounds are known to be synthesized during the plant *in vitro* culture, mostly in higher concentration because *in vitro* culture has a higher rate of metabolism, so it leads to faster proliferation and biosynthetic cycles (Imaneh *et al.*, 2011). Moreover, *in vitro* culture provides disease-free clones with a rapid multiplication rate (Guo and Zhang, 2005) and stable supplies of its bioactive compounds (Ma and Gang, 2006).

Young buds with various sizes (0.5 to more than 4 cm) are commonly used as explants in red ginger *in vitro* tissue culture. The variation of young bud's sizes was targeted to study first bud appearance, survival rates, and number of microshoots per explant. The previous study showed that the smaller-size explants took longer days for first bud appearance, yet they had higher survival rates after 45 days. Furthermore, the bigger size of explants produces a higher amount of ethylene, leading to explant oxidation and death (Zuraida *et al.*, 2016). Ginger buds also can be used for callus induction in tissue culture. Callus plays an important role in the production of higher bioactive compounds in plants, including red ginger. Callus could be induced when the explants (rhizome buds, shoot tips, leaf bases, petals, anthers, or ovaries) of red ginger were placed on a suitable medium (Ma and Gang, 2006). Murashige and Skoog (MS) medium supplemented with indole-3-acetic acid (IAA), naphthaleneacetic acid (NAA), 2.4-dichlorophenoxy acetic acid (2.4-D), Dicamba or benzyl adenine (BA) are commonly used for callus induction (Miri, 2020). The addition of higher benzyl amino purine (BAP) concentration as growth regulators showed a

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higher number of microshoots per explant but lower survival rates after 45 days and longer for the first bud's appearance (Zuraida *et al.*, 2016) (Table 1).

Table 1. Effect of various concentrations of BAP on ginger bud appearance, survival rates, and number of microshoots in 2-4 cm young buds explant (Zuraida *et al*., 2016).

BAP concentration (mg/L)	First bud appearance (days)	Survival rate after 45 days (%)	Number of microoshoots per explants
0	32	35±2.4	0.6±0.01
1	35	35±4.5	2.2±0.31
5	35	25±1.3	3.2±0.06

Propagation technique with *in vitro* tissue culture depends on the aseptic culture establishment, acclimatization, rooting, and shoot regeneration. An effective protocol in tissue culture is needed for successful large-scale *in vitro* micropropagation, including an effective protocol of explant and media sterilization (Tewelde *et al.*, 2020). Rhizome buds as a source of explants have their own challenge because of various soil pathogens exposure. The pathogens must be eliminated from explants by surface sterilization (Zahid *et al.*, 2021). Tap water, distilled water, sterile water, detergent solutions, antifungal and antibacterial agents, $CaOCl_2$, $HgCl_2$, NaOCl, ethanol, and antibiotics are common for explant sterilization and control media contamination (Tewelde *et al.*, 2020). The previous study shows that NaOCl 0.50% (v/v) at 20 mins of treatment yielded the highest number of clean and survived shoot tip explants compared to 10 and 30 mins of treatment (Table 2). On the other hand, ethanol 70% (v/v) required a shorter treatment time for explant sterilization (Table 3). The effectiveness of ethanol sterilization decreased over a longer time of treatment where the number of clean and survived explants decreased with increasing treatment time.

Table 2. Effect of surface sterilization with NaOCl 0.50% (v/v) on the number of clean and survived shoot tip explants (Tewelde *et al*., 2020).

Treatment	Time (mins)	Number of clean explants		Number of survived explants	
		Mean±SD	%	Mean±SD	%
NaOCl 0.50% (v/v)	10	3.67±0.58	37	3.68±0.58	37
	20	8.00±1.73	80	7.33±0.58	73
	30	6.33±0.58	63	7.00±1.00	70

Table 3. Effect of surface sterilization with ethanol 70% (v/v) on number of clean and survived shoot tip explants (Tewelde *et al*., 2020).

Treatment	Time (mins)	Number of clean explants		Number of survived explants	
		Mean±SD	%	Mean±SD	%
Ethanol 70% (v/v)	10	4.66±0.58	47	7.33±0.58	73
	20	3.66±0.58	37	7.00±1.00	70
	30	4.33±1.15	43	6.33±0.58	63

Bacterial or fungal contamination is another problem in plant tissue culture. That contamination causes a significant loss in the quality and quantity of the culture outputs. So, the use of antibiotics or antifungals is highly recommended to minimize contamination. For red ginger *in vitro* tissue culture, the most common source of contamination is field-grown explants because they acquire contamination from soil. Antibiotics are often used to control contamination of explants and growth media, but using single antibiotics may not be effective. Some antibiotics commonly used to overcome microbial contamination are cefotaxime, gentamicin, streptomycin, and kanamycin. The efficacy of those antibiotics increases in combination of two or three pairs of antibiotics (Tewelde *et al.*, 2020).

Protoplast culture is one of the alternatives for ginger *in vitro* cultivation. The most appropriate source of protoplasts for ginger culture is embryonic suspensions because ginger has no seeds. Embryonic suspensions are axenic, rapidly growing, well dispersed, and composed of little cell culture so it is recommended as a source of ginger protoplasts. To establish somatic embryogenic suspensions, healthy emerged buds in size 1-1.5 cm were needed. Isolated protoplasts were cultured in the dark at 25°C in a shallow liquid layer and subcultured every two weeks. The protoplast-derived colonies were then transferred to a solid MS medium for further proliferation in the dark (Guo *et al.*, 2007). In addition to providing ideal sources for red ginger protoplast cultures, embryonic suspension cultures are ideal donors for further investigation of somatic mutation, somatic hybridization, and genetic transformation (Guo and Zhang, 2005).

The production of gingerol and zingiberene in red ginger correlates with callus culture differentiation (Imaneh *et al.*, 2011). The rate of callus induction was varied, depending on the explants. Rhizome buds, shoot tips, and leaf bases were poor for callus induction with only 5% of explants producing callus. On the other hand, petals, anthers, and ovaries were much more efficient at callus induction with 90% of explants producing callus (Ma and Gang, 2006). For callus induction, explants were placed on an MS medium containing some growth regulators, such as indole-3-acetic acid (IAA), naphthaleneacetic acid (NAA), 2.4-dichlorophenoxy acetic acid (2.4-D), or others. Then, for shoot induction, the leaves derived from the callus were placed in a basal medium with benzyl adenine (BA) or Kin. The multiplied shoots were then separated and transferred into ½ MS with sucrose, agar, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), or naphthaleneacetic acid (NAA) for rooting (Miri, 2020).

https://doi.org/10.26656/fr.2017.8(4).324

The production of bioactive compounds in higher plants undergoes a variety of stresses. Those stresses promote the different syntheses of bioactive compounds even in the same species. Likewise, *in vitro* tissue culture has developed as an alternative technique for the production of bioactive compounds with some added values (Narayani and Srivastava, 2017). *In vitro* tissue cultures allow better control of plant development, production of specific tissues, and activation of biochemical routes that trigger the synthesis of specific bioactive compounds (Cardoso *et al.*, 2019). *In vitro* tissue culture has been widely used for the production of disease-free plants, production of plant-derived bioactive compounds, plant genome transformation, and rapid multiplication for plant conservation (Espinosa-Leal *et al.*, 2018).

4. Antioxidant activity of red ginger cultivated by *in vitro* tissue culture

Plants with *in vitro* culture such as callus or cell suspension culture could produce the marketable bioactive compounds in a sterile and controlled environmental condition (Cardoso *et al.*, 2019). *In vitro* tissue culture has been used to produce interesting bioactive compounds in plants (Mohammed *et al.*, 2019). Furthermore, the production of bioactive compounds can be enhanced by the addition of some elicitors in tissue culture (Ali *et al.*, 2018). Elicitors induce stress by activating of several defense related genes or inactivating of non-defense related genes, or expressing of enzymes related to specific biosynthetic pathways of many bioactive compounds (Narayani and Srivastava, 2017). Elicitors like glycine, salicylic acid and yeast extract induce the production of bioactive compounds and enhance the total phenolic content of ginger callus, and consequently the antioxidant activity (Mohammed *et al.*, 2019). The production of bioactive compounds in ginger, including red ginger using *in vitro* tissue culture is an efficient source of pharmaceutical agents and novel bioactive compounds. The success of bioactive compound production in culture conditions is a combination of nutritive and environmental factors, endogenous substances and the addition of plant growth regulators to the culture medium (Rajkumari and Sanatombi, 2020).

The previous study suggested that ginger callus had phenolic content which is related to its antioxidant activity. The use of elicitors, such as yeast extract, glycine, and salicylic acid, influence the production of plant bioactive compounds (Figure 2), including total phenolic content in callus culture and consequently its antioxidant activity, significantly (Ali *et al.*, 2018). Red ginger antioxidant activity, like other ginger species, has been affected by its various phytocompounds such as zingerone, sesquiterpenes, monoterpenes, oxygenated monoterpenes, and polyphenols like gingerols, shogaols, paradols. The spiciness of ginger is because of the existence of gingerol. It is a group of homologous phenols responsible for ginger's antioxidant activity (Mathew and Subramanian, 2014).

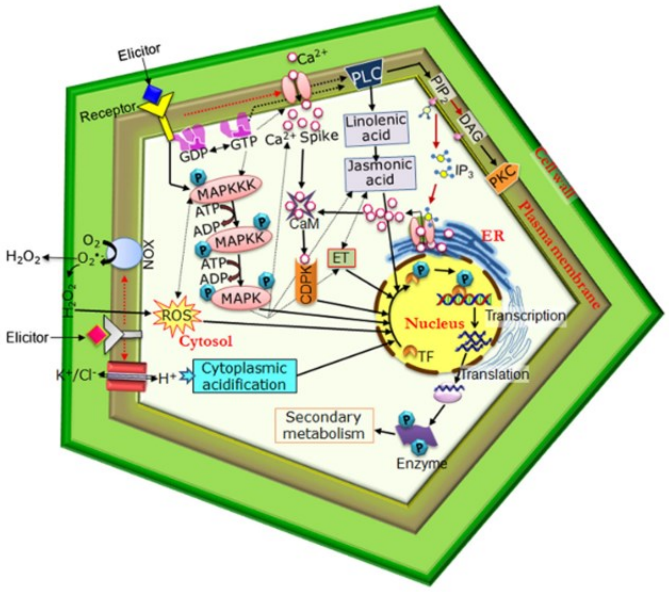

Figure 2. Schematic illustration of elicitor-induced signal transduction pathway for bioactive compounds production (Narayani and Srivastava, 2017).

Within the antioxidant activity assay with free radicals 2,2'-azino-bis (3-ethylbenzenthiazoline-6-sulphonic) acid (ABTS), it showed that the scavenging effect increased significantly with the increase of ginger extract concentration (Bellik, 2014). Ginger cultivated with *in vitro* tissue culture generates different abundance of total phenolic content leading to different antioxidant activity. The total phenolic content of ginger chloroform: methanol (1:1) extract was a bit higher (60.34±0.43 mg gallic acid/g) than petroleum ether extract (52.17±2.41 mg gallic acid/g). Both chloroform:methanol (1:1) and petroleum ether extract of ginger callus showed lower total phenolic content (33.6±0.07 mg gallic acid/g) compared to ginger rhizome extract.

The eliciting process is necessary for enhancement of the total phenolic content in ginger callus extract. The common elicitor used are yeast extract, glycine, and salicylic acid. The production of phenolic compounds in callus is related to mitochondrial activity. When cell dehydrogenase activity and the cytochrome oxidase decline, the concentration of phenolic compounds increases. These processes occur during the stabilization and growth of ginger callus. Yeast extract has been known as the best elicitor to enhance the total phenolic content of ginger callus (45.91±1.8 mg gallic acid/g) compared to salicylic acid (44.89±0.86 mg gallic acid/g)

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and glycine (37.92±0.07 mg gallic acid/g) (Bellik, 2014).

Elicitation of ginger callus with yeast extract 100 mg/L showed higher antioxidant activity in chloroform:methanol (1:1) callus extract than glycine with the same concentration, but still lower than antioxidant activity of ginger rhizome extract. The antioxidant activity of ginger callus extract without elicitor in chloroform:methanol (1:1) is the lowest among others (Table 4). Generally, treating ginger callus with elicitors could effectively enhance the antioxidant activity compared to untreated callus. The ginger extract showed stronger scavenging activity to 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical than 2,2'-azino-bis (3-ethylbenzenthiazoline-6-sulphonic) acid (ATBS) cation radical (Ali *et al.*, 2018).

In vitro tissue culture is an efficient technique for rapid propagation of red ginger and its modification of bioactive compounds. Each plant cell can form a complete plant through *in vitro* tissue culture with the appropriate conditions, including biotic and abiotic factors in the laboratory. Application of *in vitro* tissue culture overcomes the problems of propagation and production of bioactive compounds of ginger, including red ginger. *In vitro* tissue culture could also conserve the genetic variation and population of ecologically and commercially viable genotypes of ginger to prevent the possibility of its destruction (Seran, 2013).

5. Conclusion

Red ginger is a potential source of phenolic related to antioxidant activity and the level could be adjusted by *in vitro* tissue culture technique. *In vitro* tissue culture provides sterile and controlled red ginger plantlets so the growth rate and production of bioactive compounds could be improved. The antioxidant activity of ginger callus was generally lower than its rhizome extract but it can be enhanced by treatment of various elicitors. Elicitation with yeast extract in ginger callus significantly improves the antioxidant activity compared to other elicitors (glycine and salicylic acid). The study of *in vitro* tissue culture of red ginger has to be improved to investigate its potential as an alternative cultivation technique to generate higher bioactive compounds and antioxidant activity to combat free radicals.

Conflict of interest

The authors declare no conflict of interest.

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