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ORIGINAL RESEARCH PAPER

EVALUATION OF GLYCEOLLIN ACCUMULATION AND ANTIOXIDANT PROPERTIES ON SOYBEAN (*GLYCINE MAX* L.) THROUGH COMBINATION OF DIFFERENT BIOTIC ELICITOR AND LIGHT

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Abstract: The dynamic changes of glyceollin contents and concentration depend on various conditions. This study aimed to investigate the role of a pure culture of *Saccharomyces cerevisiae* versus *ragi tape* as a commercial starter combined with light/no light for boosting glyceollin accumulation in soybeans followed by the evaluation of its antioxidant activity. Soybeans were subjected to five different treatments: untreated, *S. cerevisiae* (gS), *ragi tape* (gR), *S. cerevisiae* with light (gSL), and *ragi tape* with light (gRL). The isoflavonoids daidzin, glycitin, genistin, daidzein, glycitein, genistein, glyceollin I, glyceollin II, and glyceollin III were identified and measured by High-Performance Liquid Chromatography (HPLC) assay, and the antioxidant activity was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH). Glyceollin I was increased about 2-fold in gR, 3-fold in gS, 4-fold in gSL and 7-fold in gRL compared to untreated soybeans. The gRL also exhibit a potent antioxidant activity compared to gSL. Taken together, *ragi tape* with light is the most effective elicitor for boosting isoflavonoids contents. Therefore, *ragi tape* with light deserves as a potential elicitor with valuable insights to increase the isoflavonoids contents to optimize the impact of soybeans for health.

Keywords: *glyceollin, isoflavonoids, light, ragi tape, Saccharomyces cerevisiae, soybean*

INTRODUCTION

Soybean (*Glycine max* L.) is one of the important food among the Asian population, and now it is consumed globally due to its positive association with health, predominantly from its isoflavone-rich content [1] Soybean can be directly consumed or well prepared for a variety of food, including non-fermented or fermented soy products. The isoflavonoids-rich content in the whole soybean is commonly found as conjugate glucosides which are poorly absorbed in the digestive tract [2, 3]. In contrast, the beneficial effect of isoflavonoids is majorly due to daidzein, genistein and their aglycones [4]. Furthermore, many researchers are interested in optimizing the beneficial effect of soy on health through enhanced isoflavonoids aglycone. Fermentation is one of the most commonly used techniques for improving isoflavonoids aglycone [5, 6]. However, the cooked fermented soy-products, such as chungkookjang or natto do not contain glyceollin [7].

Glyceollin is a phytoalexin which is synthesized *de novo* from daidzein and accumulated on the soybeans under fungal infection [8]. In recent years, glyceollin gained much attention due to its strong relationship with health, including antioxidant [9], antifungal [10], anti-inflammatory [11, 12], anticancer [13], restore hematopoietic homeostasis [14], and improve insulin sensitivity [15]. The biotic elicitors which have been used to enhanced glyceollin synthesis were *Aspergillus oryzae*, *A. niger*, *A. flavus* [8], *Rhizopus* sp. [11], *R. microsporus* [16], *A. sojae* [7 – 9], and *R. oryzae* [16]. Moreover, the combinations of fungal with light exposure can double the glyceollin accumulation when compared to fungal alone [17]. However, to our knowledge, there is still limited study about the utilization of commercial starters, such as *ragi tape* to enhanced glyceollin accumulation.

Ragi tape is a dry-starter which is commonly used by the local Indonesian people to make traditional fermented food particularly *tape, arak,* and *brem* [18]. *Ragi tape* contains heterogenous microorganism, such as molds (*R. oryzae*, *Amylomyces rouxii* or *Mucor* sp.), yeasts (*Saccharomyces cerevisiae*, *Saccharomycopsis fibuliger*, and *Endomycopsis burtonii*), and lactic acid bacteria [18, 19]. Among the heterogeneous microorganisms, *S. cerevisiae* has a Qualified Presumption of Safety (QPS) status and plays an essential role in many commercially important sectors, including biotechnology for many years [20]. In the food industry, *Saccharomyces cerevisiae* is usually used for the fermentation process [21]. In addition, *ragi tape* could be a potential candidate to be used as biotic elicitor to enhanced glyceollin accumulation due to its low prices and ease of obtaining.

This study was undertaken in order to investigate the impact of both pure culture and commercial starter on glyceollin accumulation under fungal infection to its antioxidant activity. In addition, we also combine the biotic elicitor-treated in soybean with light as an abiotic elicitor to get extensively understanding on isoflavonoid changes during infection.

MATERIALS AND METHODS

Materials

Soybean seeds *var. Anjasmoro* were purchased from Indonesian Legumes and Tuber Crop Research Institute (ILETRI), Malang District, East Java, Indonesia. *S. cerevisiae* was kindly provided by the Animal Physiology, Structure, and Animal Development Laboratory of Brawijaya University, Malang, Indonesia. *Ragi tape* (Harum Manis) was purchased from the local market in Malang, East Java, Indonesia.

Preparation of soybean elicitation

Soybeans modification process was done according to the previous study with slight modification [17]. Soybeans were elicited with *S. cerevisiae* as a pure culture, *ragi tape* as a commercial starter, and light as abiotic stress. Soybeans were divided into five groups, soaking for one day, and elicited for three days (Table 1).

Table 1. *Scheme of soybean treatments*

Treatments	Stage
	Elicitation (3 days)
Untreated	–
gS	dark, with *S. cerevisiae*
gR	dark, with *ragi tape*
gSL	light, with *S. cerevisiae*
gRL	light, with *ragi tape*

Soybean seeds (100 g) were sterilized with 70 % ethanol for 10 minutes. After that, soybeans were washed with sterile distilled water four times [22] and then the sterilized soybeans were soaked for 24 h and placed in the dark room. Subsequently, the soybeans were planted in a sterile plastic box and treated according to Table 1. For treatment with *S. cerevisiae*, the soybeans were planted in a sterile plastic box then inoculated with 1×10^7 *S. cerevisiae* (15 mL suspension/200 g soybeans). For treatment with *ragi tape*, briefly, *ragi tape* was ground until becoming powder then weighed at 10 g. After that, the *ragi tape* powder was mixed into soybeans (10 g/200 g soybeans) until homogenous, then planted in a sterile plastic box. Soybeans were stored at room temperature and exposed with a bulb lamp (only for gSL and gRL) from 05.00 P.M. − 09.00 A.M. for three days consecutively [17].

The elicited soybeans extraction

After three days, soybean was harvested and washed three times with sterilized distilled water. Soybean was then crushed and extracted with 80 % ethanol (50 g/150 mL) and heated at 50 °C in a waterbath for 1 h, cooled at room temperature, then centrifuged at 14000 rpm for 10 minutes [9]. The extract was then filtered through 0.45 µm sterile filter (BD Falcon). Each filtrate was then evaporated using a rotary evaporator and then freeze-dried. The crude extract was stored at −20 °C before use.

High-Performance Liquid Chromatography (HPLC) assay

Each crude extract was analyzed for its isoflavonoids content using HPLC apparatus system (LC-Prominence-20 AT and SPD 20A UV–Vis detector, Shimadzu Co., Tokyo, Japan). Each crude extract was dissolved in bidistilled water and then centrifuged at 8000 rpm for 20 minutes. The supernatant was collected and dissolved again in bidistilled water. Separations were performed using Shim-pack VP ODS C18 (150 x 4.6 mm, 5 µm, Shimadzu Co., Tokyo, Japan) reverse-phase column. The injection volume was 5 µL, and column temperature (CTO 10 ASVP) was set at 25 °C. The wavelength detector (SPD 20A UV–Vis detector, Shimadzu Co., Tokyo, Japan) was set at 280 nm, and the flow rate was 1.0 $mL \cdot min^{-1}$ at the following mobile phase composition: 0.1 % phosphoric acid in water (A), acetonitrile (B) 75/25 v/v, using an isocratic method with total run time of 40 minutes. The analytical standards of daidzin, glycitin, genistin, daidzein, glycitein, genistein, glyceollin I, glyceollin II, and glyceollin III were purchased from Sigma-Aldrich, Merck KGaA (Darmstadt, Germany). The serial dilution of standards (0, 0.1, 0.5, 1, 5, and 10 $\mu g \cdot mL^{-1}$) was prepared in acetonitrile to create calibration curves. Data identification and quantification were recorded using Lab Solution Ver 5.6.1 based on their absorption at 280 nm. All HPLC assay was performed in triplicate.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

DPPH assay was done to measure the ability of the elicited soybean extracts as a radical scavenger. Briefly, the DPPH solution was freshly made according to the previous study [23]. Afterward, 50 µL of the solution of elicited soybean extracts were added to 200 µL of 200 µM DPPH solution. After that, the mixed solution was incubated at room temperature in dark condition for 30 minutes and then measured at 515 nm [9]. The groups of untreated, gSL, and gRL extract were tested for DPPH assay. Ascorbic acid (cat. no.: A92902, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was used as a positive control. All tests were performed in duplicate.

Statistical analysis

Data from the DPPH assay were analyzed descriptively, and data from HPLC were analyzed by one-way ANOVA followed by post-hoc test Duncan's Multiple Range Test (DMRT). P-value < 0.05 was set as significant level. Data were expressed as the mean ± standard deviation (SD) and the statistical analysis was performed using Microsoft Excel for Windows.

RESULTS AND DISCUSSION

Isoflavonoids profile of both *ragi tape* and *S. cerevisiae* treatment in soybeans

Our result showed that both of *ragi tape* and *S. cerevisiae* alters the isoflavonoids content on germinating soybeans (Figure 1).

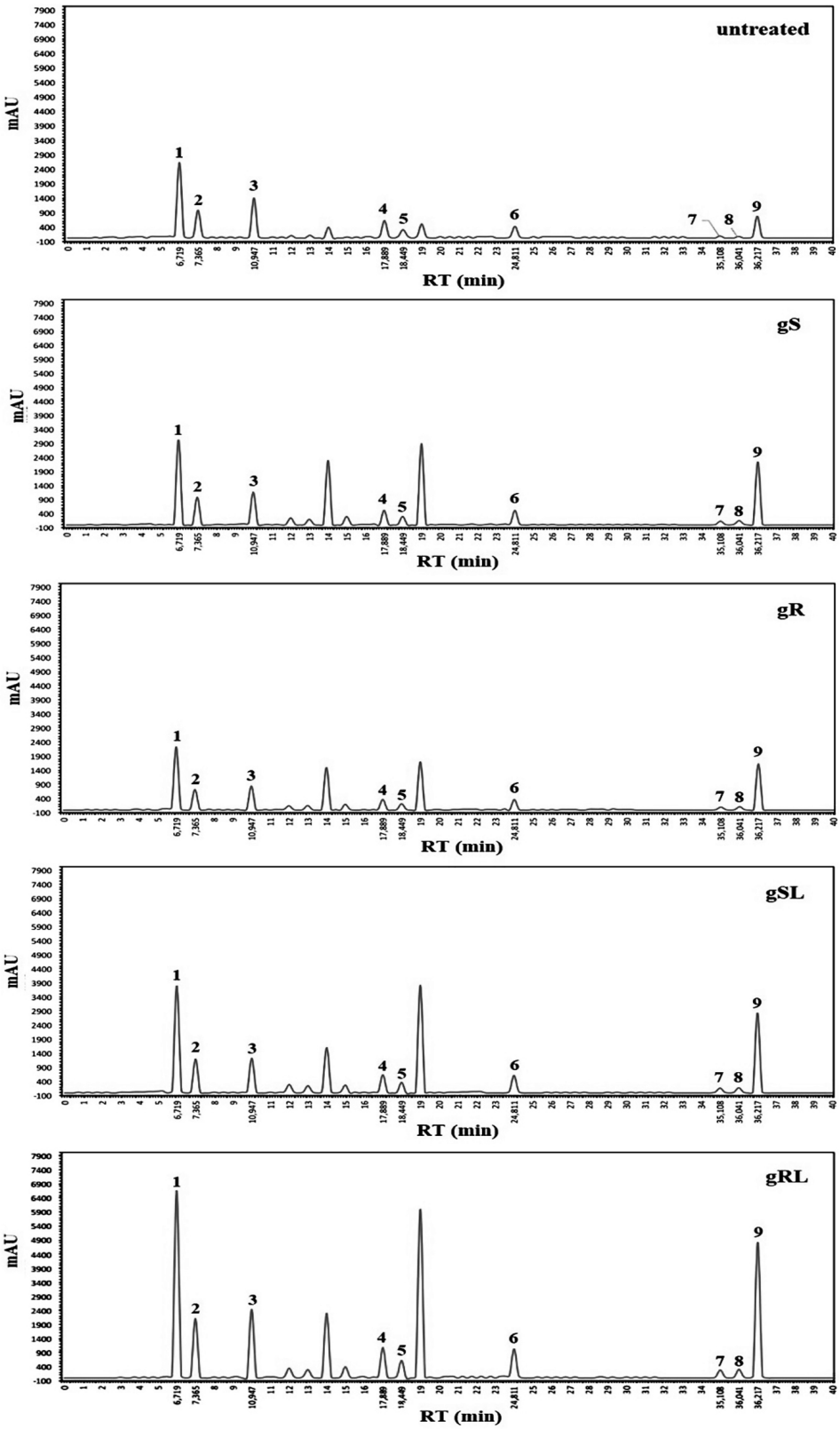

Figure 1. *Comparison of isoflavonoids contents between untreated and treated soybean (peak numbers refer to compounds in Table 2)*

The peaks number 1, 2, 3, 4, 5, and 6 were identified as daidzin, glycitin, genistin, daidzein, glycitein, and genistein respectively, whereas the peaks number 7, 8, and 9 were identified as glyceollin III, II, and I (Table 2). Both treatment *S. cerevisiae* and *ragi tape* lead to accumulation of phytoalexin, glyceollin.

Table 2. *Quantitative analysis of isoflavonoids from untreated and various treated soybeans*

Peak number	RT [min]	Compound	Compound concentration [µg·mL^{-1}]				
			Untreated	gS	gR	gSL	gRL
1	6.719	Daidzin	42.96^{b} ± 4.73	46.37^{b} ± 1.74	34.03^{a} ± 1.75	58.83^{c} ± 3.14	101.04^{d} ± 3.55
2	7.365	Glycitin	16.75^{b} ± 0.55	16.97^{b} ± 0.32	12.68^{a} ± 0.54	21.88^{c} ± 0.75	38.79^{d} ± 0.54
3	10.947	Genistin	21.46^{b} ± 1.47	23.42^{c} ± 0.47	16.99^{a} ± 0.52	30.15^{d} ± 0.13	51.8^{e} ± 0.59
4	17.889	Daidzein	11.7^{b} ± 1.03	11.79^{b} ± 0.61	8.47^{a} ± 0.58	15.29^{c} ± 1.25	25.02^{d} ± 1.15
5	18.449	Glycitein	5.55^{b} ± 0.3	5.84^{b} ± 0.29	4.18^{a} ± 0.27	7.39^{c} ± 0.37	12.89^{d} ± 0.69
6	24.811	Genistein	10.78^{b} ± 0.72	11.78^{b} ±0.57	8.59^{a} ± 0.68	13.78^{c} ± 0.21	25.27^{d} ± 0.55
7	35.108	Glyceollin III	1.88^{a} ± 0.1	4.63^{c} ± 0.08	3.5^{b} ± 0.1	6.66^{d} ± 0.35	11^{e} ± 0.18
8	36.041	Glyceollin II	0.86^{a} ± 0.05	3.73^{c} ± 0.11	2.75^{b} ± 0.06	4.72^{d} ± 0.14	7.97^{e} ± 0.24
9	36.217	Glyceollin I	9.64^{a} ± 0.08	29.48^{c} ± 0.31	21.1^{b} ± 0.12	36.78^{d} ± 0.16	63.02^{e} ± 0.2

RT = Retention Time

The *ragi tape* treatment decreases the content of glucoside conjugates daidzin, glycitin, and genistin, while *S. cerevisiae* treatment increases it. Interestingly, the aglycones daidzein, glycitein, and genistein were decreased significantly in *ragi tape* treatment compared to untreated soybeans. Glyceollin I was increased dramatically about 2-fold in *ragi tape* treatment (20.98 µg·mL^{-1}) and 3-fold in *S. cerevisiae* treatment (29.13 µg·mL^{-1}) compared to untreated soybeans (9.57 µg·mL^{-1}) (Table 2).

Glyceollin II and III also increased in others treatment, although they are not higher as well as glyceollin I. Surprisingly, *ragi tape* alone looks like less effective than treatment with *S. cerevisiae* to alters the contents of glyceollin on exposed soybeans.

Daidzein plays a pivotal role as a precursor of glyceollin, coumestrol, and other flavonoids [24, 25]. Daidzein is an isoflavone aglycone which is low abundant in raw soybeans. The fermentation process increases the daidzein content on soybean, but the fermented soy-product did not contain glyceollin. In contrast, the elicitation process did not increase isoflavonoid aglycones as much as fermented soy-product but followed by glyceollin accumulation [7].

These results are in line with our recent study that there are no changed too much in isoflavonoids aglycones contents, except in soybeans treated with *ragi tape* and light. Interestingly, daidzein contents decreased in soybeans treated with *S. cerevisiae* or *ragi tape*. We assumed that the decline of daidzein contents in *S. cerevisiae* and *ragi tape* treatment was due to the activation of isoflavonoids biosynthetic pathway, resulting in the synthesis of glyceollin as an early defense response for pathogen infection [25].

The *S. cerevisiae* has a fungal cell walls namely β-(1,6)-glucans. The β-(1,6)-glucans act as microbe-associated molecular patterns (MAMPs) which in turn stimulate the plant's defense response [26]. Similar with our result, treated soybeans with *S. cerevisiae* enhanced the glyceollin accumulation and altered isoflavonoids composition. Surprisingly, treated soybean with *ragi tape* did not boost the glyceollin

accumulation better than *S. cerevisiae* treatment in exposed soybeans. These results need further confirmation for understanding the role of heterogenous microorganism in *ragi tape* to alter the isoflavonoids profile.

Isoflavonoids profile of both *ragi tape* and *S. cerevisiae* treatment when combined with light in soybeans

Our result showed that the addition of light in the presence of biotic elicitor on germinate soybeans cause a dynamic changed on the isoflavonoids content (Figure 1). Surprisingly, the combination of *ragi tape* with light has the highest glyceollin content than *S. cerevisiae* or *ragi tape* treatment alone (Figure 1). Both the *ragi tape* and *S. cerevisiae* treatment increase the glucoside conjugates (except genistin in *S. cerevisiae* treatment). Interestingly, the aglycones daidzein, glycitein, and genistein were increased about 2-fold in *ragi tape* with light treatment.

Glyceollin I was increased significantly about 4-fold in *S. cerevisiae* treatment with light (36.6 $\mu g \cdot mL^{-1}$) and 7-fold in (62.82 $\mu g \cdot mL^{-1}$) in *ragi tape* with light treatment compared to untreated soybeans (9.57 $\mu g \cdot mL^{-1}$) (Table 2). Furthermore, light addition together with *ragi tape* increase the glyceollin contents about 2-fold compared to *S. cerevisiae* with light (Table 2). Our result suggests that the combination of *ragi tape* with light can accelerate the alteration of isoflavonoids contents on germinating soybeans.

Light is considered the main factor when combined with biotic elicitor for boosting isoflavonoids and glyceollin content on treated soybeans [17]. Light has a responsibility to increase malonyl-CoA and coumaroyl-CoA production [27], which in turn increase the concentration of daidzein as a precursor of glyceollin. Both treatment *S. cerevisiae* with light and *ragi tape* with the light increase the concentration of daidzein which followed by glyceollin accumulation. Furthermore, the light exposure is known to shift the site of glycinol prenylation through cyclization of a pyran or a furan ring [17].

Biosynthesis of glyceollin I require the glycinol 4-dimethylallyltransferase (G4DT) at the C4 position of pterocarpans. *The stress-induced germinate soybeans has known to produce* G4DT which in turn increase the glyceollin amount. Indeed, glycinol 2-dimethylallyltransferase (G2DT) has also taken the responsibility at biosynthesis glyceollin at the C2 position of pterocarpans [28]. *Ragi tape* contains heterogenous microorganism, including *Rhizopus oryzae*, *Aspergillus rouxii*, *Mucor* sp., *Saccharomyces cerevisiae*, *Saccharomycopsis fibuliger*, and *Endomycopsis burtonii* [19]. Another study was reported *ragi tape* contained lactic acid bacteria, predominantly *Weissella* spp., *Lactobacillus* spp., *Enterococcus* spp., and *Pediococcus pentosaceus* [29]. We assumed that the heterogeneous microorganism in *ragi tape* might work synergistically in the presence of light.

Comparison of radical scavenger activity in untreated and elicited soybean extract

DPPH assay is commonly used for the evaluation of radical scavenging activities. In our experiment, both the treatment of *S. cerevisiae* with light (gSL) and *ragi tape* with light (gRL) has strong DPPH scavenging activities, which indicated their role as a strong antioxidant (Figure 2).

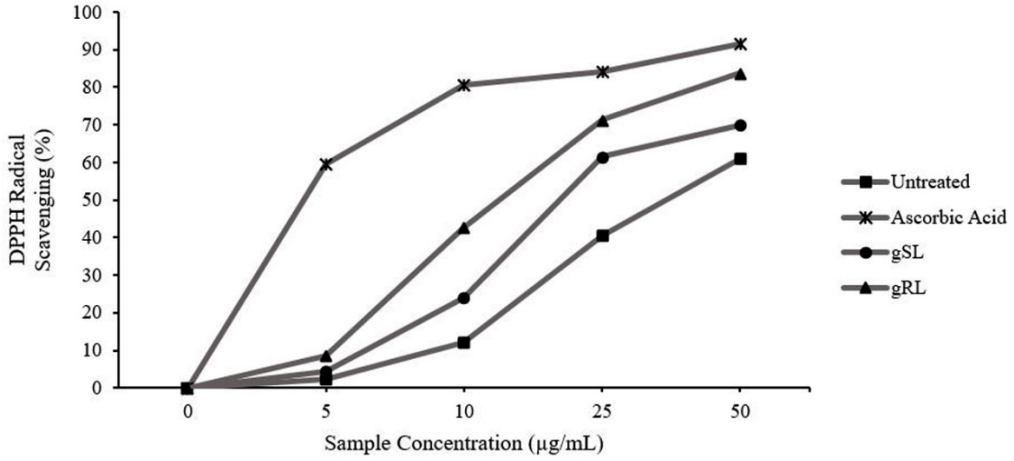

Figure 2. *DPPH radical scavenging activity of elicited soybeans extract (gSL and gRL) vs. untreated and ascorbic acid as a positive control*

Its antioxidant activity follows the high abundance of glyceollin in treated soybeans. As we expected, the increase of glyceollin caused by treatment influence the antioxidant activity of the soybeans. Our result is consistent with a previous study showed that glyceollin has strong radical scavenging activity. Furthermore, glyceollin also showed the inhibition of singlet oxygen, ferric reducing antioxidant power (FRAP), and hydrogen peroxide [9].

Glyceollin I emerge the most crucial factor that influences radical scavenging due to its phenolic hydroxyl bones [9, 30]. The recent study suggested that glyceollin promotes Nuclear Factor Erythroid 2-related Factor-2 (Nrf2) translocation in Hepa1c1c7 and mutant BPRc1 cells, which in turn enhances the expression of several antioxidant enzymes, including heme oxygenase-1 (HO-1), gamma-glutamylcysteine synthase (γ-GCS), and glutathione reductase (GR) [31]. We suggested that commercial starter, such as *ragi tape* combining with light may increase the beneficial effect of soybeans through enhancing the production of glyceollin.

CONCLUSIONS

In conclusion, our results suggest that the dynamic change of glyceollin can optimize by combining the biotic elicitor with light. *Ragi tape* with light may be used as a booster for glyceollin production to maximize its beneficial effect on health and can be suggested as a promising agent with valuable insight in the future to apply as a food supplement or therapy.

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