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Induction of Glyceollins by Fungal Infection in Varieties of Korean Soybean

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Glyceollins, one of the inducible phytoalexins produced by plants, were induced in a number of varieties of Korean soybean through fungal infection. Of the tested soybean varieties, *Tae-Kwang,* though not the most productive, was found to be currently the most suitable for the induction of glyceollins. Amongst the fungal species, *Rhizopus microsporus* var. *oligosporus* was seen to be the most effective elicitor. Halved soybean seeds produced glyceollins upon fungal infection; however, chopped soybeans and homogenized soybeans did not produce significant quantities of glyceollins.

Keywords: Glyceollins, fungal infection, soybean variety, *R. oligosporus*

Glyceollins are a member of a group of inducible phytoalexins produced by plants when they are exposed to a number of stress factors such as microbial infection, chemical, or physical stresses [1–3, 5]. It is thought, due to their antimicrobial activities, that glyceollins are produced in order to protect plants from microbial infection [5]. Recent studies have shown that glyceollins may well have other important biological functions, for instance by serving to increase insulin sensitivity as a result of estrogenic or anti-estrogenic activities, and even preventing cancer [8–11]. It is hence of little surprise that, as a result of these biofunctionalities, efforts have been made towards the utilization of glyceollins as an active ingredient in fermented foods. For example, soy yogurt has been produced from black soybean seeds enriched with glyceollins through fungal inoculation [6]. Glyceollin-enriched soybean seeds have been found to enhance the biofunctionalities of soy milk and fermented soy milk, which already contained bioactive compounds such as daidzein and genistein, aglycones derived from isoflavonoid glycosides during fermentation [2, 4]. The rationale behind the current study is that this same strategy could be utilized to improve the functionality of traditional Korean fermented soy foods such as *cheonggukjang* and *doenjang.* Herein are examined the efficiencies of glyceollin induction in a number of varieties of popular Korean soybean, through the inoculation of fungal species. In this way, it is hoped that suitable combinations of soybean varieties and fungi for the production of fermented Korean soy foods can be determined.

Varieties of Korean soybeans were soaked in distilled water overnight at room temperature and then dehulled, by hand, before fungal inoculation. It was noted that the swollen soybean seeds were very easily halved during the dehulling process. Halved seeds were then placed on top of four layers of filter paper (Hyundai Micro Ltd., Qualitative, Korea), placed in a Petri dish (diameter 150 mm), wetted with 30 ml of sterile water, inoculated with fungal spores (5×10^9 spores/200 g soybean), and then incubated at 25°C for 3 days in the dark. During the incubation period, the soybean seeds were seen to germinate, while fungi grew on the surface of seeds. After incubation, the soybeans were frozen overnight and then freeze-dried. Fungal spores were recovered from mycelia grown on PDA agar (Difco, Becton, Dickinson and Co., MD, U.S.A.) for 1 week at 25°C. Mycelia were collected using a sterile wooden stick, resuspended in a small volume of sterile deionized water, and then filtered using cheese cloth. In this manner, five fungal species were tested for the induction of glyceollins: *Aspergillus awamori* ATCC 14331, *Aspergillus niger* ATCC 4695, *Aspergillus oryzae* ATCC 11493, *Aspergillus sojae* ATCC 9362, and *Rhizopus microspores* var. *oligosporus* ATCC 22959.

For the extraction of glyceollins, freeze-dried soybean samples were mixed with 80% ethanol (7.0 ml/g of dry

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matter) and extracted through continuous shaking in a water bath for 1 h at 50°C After the mixture had cooled, it was centrifuged at 14,000 ×*g* for 15 min. Supernatant was filtered using a 0.45-μm membrane filter and analyzed using a Waters HPLC system (U.S.A.) with a 2996 PDA detector and a wavelength range set at 200~400 nm (285 nm for glyceollins). The HPLC column was a X-bridge (4.6× 150 mm, 3.5 μm; Waters). The mobile phase consisted of water (A) and acetonitrile (B). The gradients used in the sample analysis were 0−1 min, A 100%; 1−17 min, A from 100% to 55%; 17−27 min, A from 55% to 10%; 27−33 min, A 10%; 33−35 min, A from 10% to 100%; and 35−40 min, A 100%. The amount of induced glyceollins was calculated according to a standard calibration curve using glyceollin standards. In soybean, glyceollin exist as three different isomers, type I, II, and III, with type I being the most abundant of them [1, 5]. The glyceollins monitored in this work included all of the three types. The soybean varieties tested were all harvested in 2007, except for *Cheong-Jak* (black soybean, 2003 crop year).

HPLC results showed that *Soon-Chang* was the best variety for glyceollin induction (984±50 μg/g soybean) when challenged by *R. oligosporus* (Fig. 1). *Nam-Poong* (604±2 μg/g soybean) was found to be the second best, followed by *Tae-Kwang* (587±3 μg/g soybean), *Aga* (536 ±2 μg/g soybean), and *Cheong-Jak* (473±12 μg/g soybean). *Dae-Yang* (344±2 μg/g soybean) was found to produce the least glyceollins amongst the species examined. Soybean seeds did not produce glyceollins during incubation if not inoculated with fungal spores (data not shown). Although

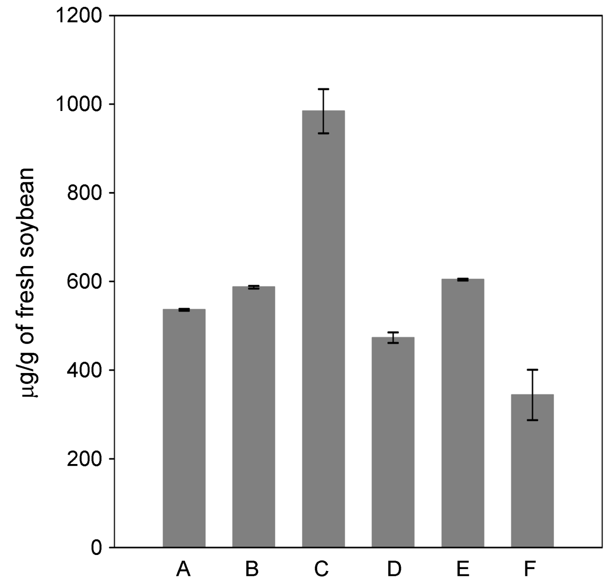

Fig. 1. Induction of glyceollins in different varieties of soybean by infection with *R. oligosporus.*
A, *Aga*; **B**, *Tae-Kwang*; **C**, *Soon-Chang*; **D**, *Cheong-Jak*; **E**, *Nam-Poong*; **F**, *Dae-Yang.*

Soon-Chang seed consistently produced more glyceollins than the other varieties throughout our studies, the homogeneity of *Soon-Chang* seeds was questionable. *Soon-Chang* soybean was cultivated and harvested by local farmers in Soonchang county, Jeollabuk-do, for the production of *cheonggukjang* and *doenjang* through traditional means. However, the genetic homogeneity of the seeds could not be confirmed. Other varieties were cultivated in experimental fields that were supervised by researchers and thus the genetic identity could be confirmed. Since the *Soon-Chang* soybean was found to be superior to the other varieties for yield of glyceollins, future studies on the characteristics and genetic homogeneity of the *Soon-Chang* seeds would appear to be necessary for the optimal production of glyceollin-enriched fermented soy foods. Amongst the other varieties examined, *Tae-Kwang* would appear to be the most suitable for the production of fermented soybean foods fortified with glyceollins because this variety is already cultivated on a large scale, and it is thus less expensive when compared with other varieties such as *Aga* and *Cheong-Jak* (black soybean).

The highest yield for the glyceollin production was observed when the *Soon-Chang* seed was infected with *A. sojae* (1,263±56 μg/g soybean), followed by *R. oligosporus* (984±50 μg/g soybean), *A. oryzae* (645±30 μg/g soybean), *A. awamori* (589±19 μg/g soybean), and *A. niger* (104± 4 μg/g soybean) (Fig. 2A). For *Tae-Kwang*, the best yield was observed with *R. oligosporus* (587±3 μg/g soybean) and followed by *A. awamori* (222±1 μg/g soybean), *A.sojae* (219±15 μg/g soybean), *A. niger* (203±13 μg/g soybean), and *A. oryzae* (200±45 μg/g soybean) (Fig. 2B). For *Aga*, optimal yield was observed with *R. oligosporus* (536± 2 μg/g soybean), followed by *A. sojae* (255±6 μg/g soybean) (Fig. 2C). No glyceollin was detected when *A. oryzae* was used for infection, although this organism is often used for the production of fermented soy foods. These results emphasized the importance of the careful selection of a fungus for a specific soybean variety, if glyceollin-enriched fermented food is the intended product. As a further illustration this has been confirmed in other studies; for example, when Boué infected Pioneer 95B41 seeds with four *Aspergillus* species (*A. sojae*, *A. oryzae*, *A. niger*, and *A. flavus*), *A. sojae* was found to be the best elicitor (955 μg/g soybean), whereas all the other species produced significantly less glyceollins [1].

Among the fungal species, *R. oligosporus* seemed the most suitable elicitor for the induction of glyceollins. Although *A. sojae* was better for the *Soon-Chang* seed, *R. oligosporus* consistently induced higher amounts of glyceollins in all the other varieties. Moreover, soybeans infected with *R. oligosporus* were noted to produce a milder flavor than soybeans infected with other fungi, and hence resulting in a more palatable product (data not shown). Since *R. oligosporus* is well known as the starter for tempeh [7], this organism seems promising not only as an elicitor for

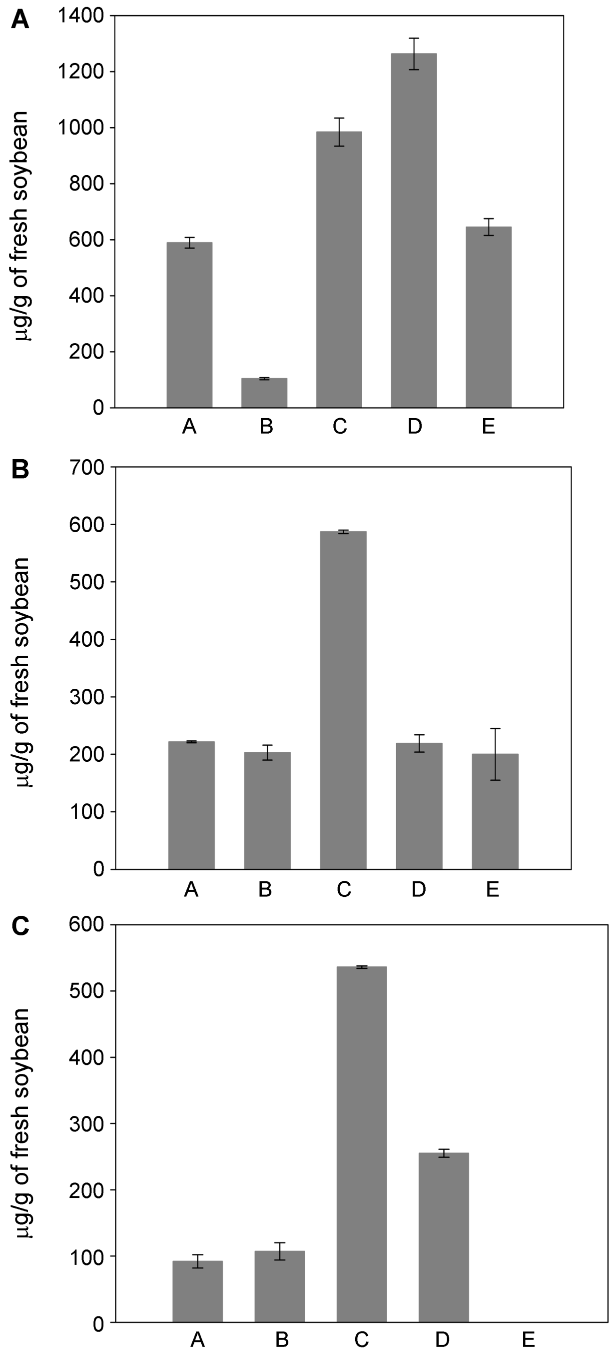

Fig. 2. Induction of glyceollins in *Soon-Chang* (**A**), *Tae-Kwang* (**B**), and *Aga* (**C**), when infected with different fungi.
A, *A. awamori*; B, *A. niger*; C, *R. oligosporus*; D, *A. sojae*; E, *A.oryzae.*

glyceollins but also as a starter for fermented soybean foods fortified with glyceollins.

Halved soybeans produced much more glyceollins than those that had been chopped (cut into several pieces with a razor blade) or homogenized (by a Waring blender) (Fig. 3). This indicates that maintaining the whole structure of a

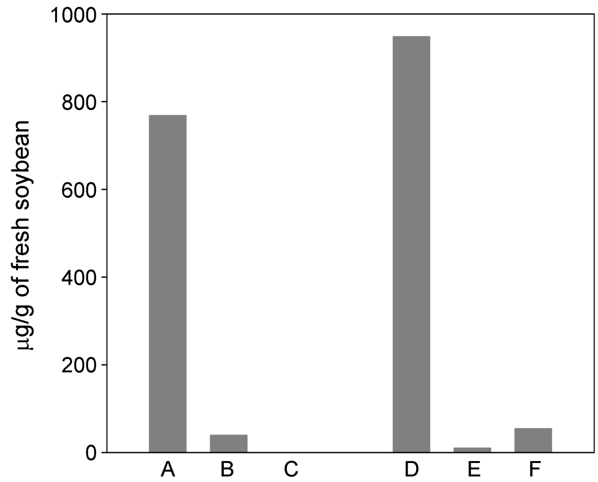

Fig. 3. The amount of glyceollins induced in *Soon-Chang* soybean seeds differently pretreated.
A and D, half-cut; B and E, chopped; C and F, homogenized. A to C, *Rhizopus* infection; D to F, *A. sojae* infection.

seed is important for the synthesis of glyceollins upon fungal infection. Although chopped or homogenized seeds have more surface areas, thus providing seemingly more favorable conditions for mycelia to grow, the biosynthetic capabilities of the seed may be hindered. Depending on the degree of chopping (number and size of pieces), it is conceivable that different results may be obtainable. Hence, more detailed studies are necessary to determine the most suitable physical forms for soybean seeds. Along similar lines of reasoning, different methods of fungal inoculation should be examined.

In summary, of the soyean species examined, and in consideration of factors such as genetic identity, availability, and cost, the *Tae-Kwang* variety would appear to be the most suitable seed in Korea for the massive production of soybean foods fortified with glyceollins. *R. oligosporus* was found to be the best elictor, and halved seeds were better for glyceollins production than chopped or homogenized seeds. The development of new soybean varieties, which induce more glyceollins, is very desirable, while further studies on currently available soybean varieties, including *Soon-Chang*, are also necessary.

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