Role of Raffinose Family Oligosaccharides in Respiratory Metabolism During Soybean Seed Germination

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Role of raffinose family oligosaccharides (RFOs), sucrose and lipid in respiration metabolism during soybean seed germination were investigated to determine appropriate method for producing soybean sprouts containing high amount of RFOs. Soybean seed were soaked into 50 µM 1-deoxygalactonojirimycin (DGJ), which is a specific α-galactose inhibitor, for 90 minutes before and after germination preparation phase (GPP). Soybean seed without DGJ treatment was used as control. The growth rate, respiration rate, respiratory quotient, fatty acids and saccharides contents were measured during cultivation at 20°C. When DGJ was applied before GPP, the degradation of RFOs was significantly reduced, moreover the growth rate and respiration rate were suppressed due to the alternation of respiratory substrate from glucose to lipid in GPP. However, in the DGJ application after GPP, no difference was found from control although RFOs breakdown was functionally inhibited in subsequent radicle elongation phase (REP). These results suggest that RFOs are essential only in GPP as a source of respiratory substrate and lipids play the main role in respiration during REP. Therefore, prevention of RFOs degradation after GPP could be useful to maintain high amount of RFOs in soybean sprouts.

Keywords : germination, raffinose family oligosaccharides, respiration, soybean sprout

INTRODUCTION

The production of soybean sprout containing high amount of oligosaccharides as well as isoflavones can enhance its sales value due to the beneficial effect for human health. Originally, soybean seed contains relatively high amount of raffinose family oligosaccharides (RFOs) such as raffinose stachyose and verbascose, however RFOs are drastically decreased in germination process. Our previous research revealed that RFOs content in soybean sprouts is affected by the cultivation temperature and RFOs remained most at 20°C due to the smallest total respiratory CO₂ production during cultivation among the tested conditions from 10°C to 30°C. In addition, the application of α-galactosidase inhibitor suggested to be a promising method to produce soybean sprouts containing high amount of RFOs (Syukri et al., 2018). However, the optimum cultivation method has not been determined yet, especially the role of RFOs in seed germination process should be clarified for optimization.

Reports are available on the necessity of RFOs in germinating seeds that it is an essential compound as a source of respiratory substrate in germinating legume seeds such as soybean and pea, since they rapidly disappear after imbibition (Reinhold et al., 1981; Gangl and Tenhaken, 2016). Moreover, delay of germination was observed in pea seed when RFOs breakdown was suppressed from the beginning of germination by 1-deoxygalactonojirimycin (DGJ) which is a specific α-galactose inhibitor (Blöchl et al., 2007). On contrary, Dierking and Bilyeu (2009) suggested that oligosaccharides are not essential substrate for respiration in efficient soybean seed germination, because no significant difference was found in growth rate between soybean seeds containing low (16–25 mg g⁻¹) and normal (58–62 mg g⁻¹) level of RFOs. From these previous reports, we hypothesize that there might be a minimum requirement of RFOs for normal germination.

According to Bewley and Black (1978), there are three critical phases on seed germination process (i.e. imbibition phase (IP), germination preparation phase (GPP) and radicle elongation phase (REP)). IP is a period of water absorption by the dry seed resulting in swelling of the seed then rupturing the seed coats and enabling the radicle to come out in the form of primary root. After IP, several metabolic activities were reactivated to begin a complex germination process which is indicated by the emergence of small radicle, and this phase is classified as GPP. After existence of small radicle, cell expansion occurs to elicit radicle elongation in producing hypocotyl of sprouts where it is marked as REP. Through the developing process of seed germination from IP to GPP and subsequent REP, the metabolic process is drastically changed, so that, the role of RFOs might be also differed in each germination phase.

In fact, lipid and sucrose are abundant in soybean seed (Young and Mebrahtu, 1998; Obendorf et al., 2011), and decrease during germination as well (Pazur et al.,...
1962; Brown et al., 1962; Joshi et al., 1973; Kuo et al., 1990). However, to our knowledge, the contribution of each above compound in respiratory metabolism during soybean seed germination has not been clarified yet. In this study, we compared RFOs, sucrose and lipid contents during germination among soybean seeds treated by DGJ before and after GPP for inhibiting RFOs breakdown and non-treated one. The respiration rate and respiratory quotient (RQ) were also measured. Focusing on the germination phases, the contribution of each compound to respiration process of soybean seeds was discussed.

MATERIALS AND METHODS

Plant materials

Soybean (Glycine max, cv. BS5012) were used for producing soybean sprouts and subsequent measurements in this study.

Germination process

Germinations were performed under three different conditions (i.e. DGJ treatment before and after GPP, and control). About 200 g of soybean seeds was sterilized by dipping into 70°C of hot water for 10 seconds. Then the seeds were transferred to 20°C of water and left for 4.5 hours for water imbibition process. After soaking, the seeds were divided into two groups. One group of seeds were moved to 250 mL plastic cup and kept in the incubator (MIR-154-PJ, Panasonic, Gunma, Japan) at 20°C with 70–80 % RH in dark condition for germination. This condition was marked as control. Another group of seeds were soaked into 50 µM DGJ for 1.5 hours subsequently, then moved to 250 mL plastic cup and kept in same incubator for germination. Since, based on our preliminary experiments, the absorption of DGJ by the seeds for an optimum inhibition of RFOs requires at least 1.5 hours (data not shown). This condition was marked as DGJ treatment before GPP. DGJ was purchased from Cayman Chemical (Ann Arbor, MI, USA). Furthermore, when the radicle germs of control become about 0.5–1 cm in length around 24 hours since the beginning of germination, the germs were then divided into two groups, then one group was soaked into 50 µM DGJ for 1.5 hours. Subsequently, the treated germs were put back to 250 mL plastic cup and kept inside the incubator for continuing the germination. This condition was marked as DGJ treatment after GPP. All germinating seeds were watered by 100 mL of water, twice a day (10 am and 4 pm).

Measurement of respiration rate by a flow-through method

During germination, the rate of respiratory carbon dioxide (CO2) production was measured by a flow-through method using gas chromatography (GC) as described in Syukri et al. (2018). Briefly, approx. 40 g of seeds from each treatment were kept in two plastic cups and placed into an acrylic chamber (2 L) equipped with gas inlet and outlet tubes. The chambers were closed and connected with an air compressor to produce air flow through the inlet tubes. The chambers were placed in incubators set at 20°C. The relative humidity inside the chamber was maintained around 70–80 %. Inlet and outlet gas samples were injected automatically into a GC (GC-14A, Shimadzu, Kyoto, Japan) alternately via a 0.5-mL sampling loop attached to a rotating stepping valve. CO2 were separated by a Porapak Q column and measured by a thermal conductivity detector. Helium gas was used as a carrier gas. The gas chromatogram was analyzed with an integrator (C-R7A plus, Shimadzu, Kyoto, Japan) based on a CO2 standard curve. The results were expressed as percentage of total gas volume. For giving water to sprouts, the lid of each sample chamber was temporarily opened and then it was immediately closed and left for 3 hours to equilibrate the gas inside the chamber before continuing for the gas measurement. The rate of CO2 production was calculated from the absolute differences in gas concentration between the inlet and outlet following the equation (Fonseca et al., 2002):

\[
R_{CO2} = \left( \frac{\text{Y}^\text{out}_{CO2} - \text{Y}^\text{in}_{CO2}}{100} \right) \times \frac{F}{W} \times \frac{P}{RT} \times 1000
\]

where \( R_{CO2} \) is the respiration rate for CO2 production of the product (mmol kg\(^{-1}\) h\(^{-1}\)), \( Y_{CO2} \) is a volumetric concentration CO2 in the chamber (in= inlet and out= outlet) (%), \( W \) is the weight of the sample (kg), \( F \) is flow rate (mL h\(^{-1}\)), \( P \) is the atmospheric pressure (=101.3 kPa), \( R \) is the universal gas constant (=8.314 L kPa K\(^{-1}\) mol\(^{-1}\)) and \( T \) is the absolute temperature (K).

Determination of the respiratory quotient

The RQ during germination was also determined by measuring the changes on CO2 and oxygen (O2) concentration in closed system method using CO2 and O2 sensor as described in Limmimitr et al. (2018) with some modifications. Appro. 40 g of soaked seed from each treatment were put into two plastic cups and subsequently placed into an acrylic chamber (2 L). The chamber was then set inside an incubator with temperature at 20°C. This chamber has both inlet and outlet ports that were linked individually to the plastic tube. The tube coupled to the outlet port of the chamber was connected to a semiconductor-type CO2 sensor (GM70, Vaisala, Vantaa, Finland) equipped with an aspiration pump and further along linked to a zirconia-type O2 sensor (MC-8G, Iijima Electronics Co., Aichi, Japan). Changes in both the O2 and CO2 concentrations inside the chamber were then recorded at 10-minute intervals by a data recorder (TR-V550, Keyence, Osaka, Japan). Data on the changes of gas concentration were collected for 12 hours and were used for estimating the respiratory quotient (RQ) value. The RQ value of the germinated soybean sprouts was calculated from the slope of the regression line relating the increase of the CO2 concentration with the decrease of the O2 concentration inside the chamber, as shown in the following equation.

\[
RQ = \frac{\Delta C_{CO2}}{\Delta C_{O2}}
\]

Where \( RQ \) is the respiratory quotient and \( \Delta C \) is the slope of the gas concentrations in the chamber (O2 and CO2) (% h\(^{-1}\)).

For determination of the slope of the gas concentrations in the chamber, sequential sets of 5 data points for each O2 and CO2 concentration were selected, and these were subjected to regression analysis to estimate \( \Delta C_{O2} \) and
RESULTS AND DISCUSSION

Figure 1 demonstrates the comparison of radicle increment of germinating soybean seed for all tested conditions. The change of the radicle length of soybean seed treated by DGJ after GPP was same as control, and it took 96 hours until the hypocotyl grew by 10 cm in length. While in the case of the soybean seed treated by DGJ before GPP, the growth rate was significantly suppressed compared to control, and it took 156 hours to grow. More specifically, the duration of GPP, in which small radicle just starts forming and grows until around 0.5–1 cm in length, prolonged from 24 hours to 48 hours, and that of REP, which is the subse-

ΔC\textsubscript{CO}_2, respectively. The calculation continued, by shifting down one step and computing the slope of the next data set, as the method for obtaining a moving average. After the calculation of RQ values as mentioned above, the RQ values were then plotted against the moving average of 5 data points of the O\textsubscript{2} concentration to observe the changing pattern of RQ.

**Determination of sucrose and RFOs**

Sample for sucrose and RFOs measurements were took periodically until the hypocotyl of the sprouts reach at 10 cm in length. At the same time, each radicle length of 20 soybean sprouts was measured by a ruler to determine the growth rate. The sprouts were divided into 2 groups; cotyledon and hypocotyl. Thereafter, only cotyledon was freeze-dried by bench top freeze-dry system (FDU-1200, Eyela, Tokyo, Japan) and stored at −50°C for further analysis.

A 25 mg of sample was placed in 2 mL self-standing screw cap micro tubes (Watson, Kobe, Japan), and 1 mL of 70 % (v/v) ethanol was added, then vortexed for 30 seconds. Extraction process was subsequently refined by ultrasonic wave at 180 Hz for 45 minutes. The extraction solutions were centrifuged at 14,000 rpm at 10°C for 10 minutes, then supernatant was filtered through a 0.2 μm membrane (RC15 Minisart, Sartorius, Göttingen, Germany) for further high performance liquid chromatograph (HPLC) analysis.

Sucrose and RFOs including raffinose, stachyose and verbascose were analyzed using a HPLC system (Ultimate 3000, Thermo Fisher Scientific, Massachusetts, USA) equipped with a charged aerosol detector (Syukri et al., 2018). The mobile phase was 0.5 % acetic acid in water (solvent A) and acetonitrile (solvent B). The programmed elution was performed in isocratic of 75 % B for 8 minutes and linear gradient for 22 minutes which decreasing of B from 75 % to 60 %. The flow rate of mobile phase was 1 mL min\(^{-1}\). A 30 μL of sample was loaded onto an amino bond column, (Asahipak NH2P-50 4E (5 μm, 250 mm× 4.6 mm i.d.), Shodex, Tokyo, Japan) through an autosampler. Column temperature was maintained at 35°C. Sucrose and RFOs were identified and quantified by comparing to the standard curves of them. We confirmed our data by repeating the whole experiment at least three times.

**Determination of fatty acids**

The residual lipid in samples was extracted according to Folch’s method. A 20 mg of dry sample was weighted into 2 mL self-standing screw cap micro tubes and 0.9 mL of extraction solvent (hexane and isopropanol, 3:2) was added, and homogenized using a bead crusher (Shake Master Neo, BMS, Tokyo, Japan) at 1,500 rpm for 10 minutes, afterward 0.15 mL of water was added and mixtures were then centrifuged at 16,000 rpm for 15 minutes at room temperature. The organic solvent was collected and was then removed from the extract solution through evaporation using a centrifugal evaporator (CV-2100, Eyela, Tokyo, Japan). Methyl-esterification of the fatty acids and purification of the methylated fatty acids were conducted using the fatty acid methylation kit (P/N:06482-04, Nacalai Tesque, Kyoto, Japan) and the methylated fatty acid purification kit (P/N:06483-94, Nacalai Tesque, Kyoto, Japan). Methyl ester fractions obtained by the above procedure were injected into a gas chromatograph (GC 2010, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a capillary column (DB-23 (30 m× 0.25 mm i.d., 0.25 μm film thickness), Agilent, Santa Clara, USA) using helium as carrier gas at a flow rate at 45.4 mL/min. The injector and detector temperatures were both 220°C and 230°C, respectively. The analysis was performed using a temperature program as follows; the temperature was start at 150°C and raised up to 200°C at a ramp rate of 2°C min\(^{-1}\), then it further increased to 230°C at a ramp rate of 4°C min\(^{-1}\). The final column temperature was maintained at 230°C for 5 minutes. A 1 μL of sample was injected for each sample. A standard solution containing a mixture of authentic samples of fatty acid methyl esters was run under the same experimental conditions prior to running the samples. The retention times of the unknown samples of methyl esters were compared with the standards for identification purposes. We confirmed our data by repeating the whole experiment at least three times.

**Changes in radicle length of soybean sprouts germinated under normal and DJG treated conditions (Vertical lines represent standard deviation, n=20).**
The suppression of CO$_2$ production during GPP and another substrate would be from RFOs breakdown suggested to be used as a respiratory substrate during germination period, where glucose (sugar) that derived during GPP and kept constant during REP until the end of experiment. In our previous report, in normal germination period, CO$_2$ production rapidly increased during GPP and kept constant during REP until the end of germination period where glucose (sugar) that derived from RFOs breakdown suggested to be used as a respiratory substrate during GPP and another substrate would be used during REP (Syukri et al., 2018). The suppression of CO$_2$ production during GPP in germinating soybean seed treated by DGJ before GPP might induce the shifting of the respiratory substrate from sugar to lipid due to sugar starvation (Toole and Toole, 2004).

Next, we measured the RQ value in order to speculate a respiratory substrate in soybean seeds during germination. The RQ is calculated as the ratio of the CO$_2$ production rate to the O$_2$ consumption rate, and has been used for discussing the qualitative change of respiration. When the RQ value is 1.0, it is considered logically that sugar is used as a respiratory substrate, whereas an RQ of about 0.7 indicates that lipid is the main source of energy utilization. As shown in Table 1, the RQ values in the DGJ treatment after GPP and control were 0.9 for the first 24 hours which corresponded to GPP, then gradually decreased from 0.8 to 0.6 during REP. From this observation, it can be predicted that the main substrate for respiration was sugar during GPP, after that it changed gradually to lipid in REP. In the case of soybean seed treated by DGJ before GPP, since the RQ value was approximately constant in the range from 0.7 to 0.6 during all germination period, lipid is considered to be used as a main substrate whole through the germination process.

Although soybean seed contain a higher amount of protein compare to lipid, however, as in one of oil seed species, the utilization of lipid as energy source more predominant compared to protein (Graham, 2008). Therefore, to confirm the results mentioned above, changes of fatty acids in germinating soybean seed were compared in Fig. 3. Prior to germination, soybean seed ‘BS5012’ contained five major fatty acids, namely, palmitic, stearic, oleic, linoleic and linolenic acid, especially, linoleic acid was the most abundant (data not shown). In germinating soybean seed treated by DGJ before GPP, the total lipids decreased markedly from 184 µg mg$^{-1}$ to 165 µg mg$^{-1}$ for the first 24 hours, subsequently, it slightly decreased to 159 µg mg$^{-1}$ until the end of germination period. Meanwhile, the

![Fig. 2](image-url) Changes in CO$_2$ production rate of soybean sprouts during germination under normal and DGJ treatment conditions (Vertical lines represent standard deviation, n=4).

![Fig. 3](image-url) Changes of total fatty acids during germination under normal and DGJ treatment before GPP (Vertical lines represent standard deviation, n=3).

Table 1

| Germination period (h) | Control | DGJ treatment after GPP | DGJ treatment before GPP |
|------------------------|---------|-------------------------|--------------------------|
| 24                     | 0.9     | 0.9                     | 0.7                      |
| 48                     | 0.8     | 0.8                     | 0.8                      |
| 72                     | 0.7     | 0.7                     | 0.7                      |
| 96                     | 0.6     | 0.7                     | 0.7                      |
| 120                    |         | 0.6                     |                          |
| 144                    |         | 0.6                     |                          |
| 156                    |         | 0.6                     |                          |
changes of total lipids were observed in similar pattern both in control and in germinating soybean seed treated by DGJ after GPP, the total lipid was relatively stable ranging from 184 μg mg⁻¹ to 179 μg mg⁻¹ for the first 48 hours of germination period, then it slightly decreased to 169 μg mg⁻¹ until the end of germination period. These results clearly indicated that in case of sugar deprivation due to DGJ treatment before GPP, lipid degradation induced to fulfill the energy requirement for starting germination process. Lipid is degraded in the series reaction called β-oxidation for respiration which is more complex compared to sugar catabolism (Bewley and Black, 1994). So that, the suppression of respiration and growth rates during GPP observed in DGJ treatment before GPP could be caused by the alternation of the respiratory substrate from sugar to lipid.

To clarify the necessity of RFOs breakdown for soybean seed germination, we quantified RFOs and the data were shown in Fig. 4. The RFOs (raffinose, stachyose, and verbascose) in control condition rapidly degraded from 43.4 mg g⁻¹ DW to 8.6 mg g⁻¹ DW for the first 48 hours and then gradually decreased to 4.0 mg g⁻¹ DW until the end of germination. In DGJ treatment before GPP, RFOs degraded similarly as control for first 24 hours and after that it was slowly reduced until 48 hours of germination period. After 48 hours, the amount of RFOs was remained unchanged unlike control. On the other hand, when DGJ was applied before GPP, RFOs degradation was inhibited since the beginning of germination and only a slightly reduction of RFOs was observed during whole germination period. Comparing the RFOs degradation pattern with the growth rate (Fig. 1) and CO₂ production rates (Fig. 2) of germinating soybean seed, it can be suggested that RFOs is required only during GPP (the first 24 hours of germination). Because when RFOs breakdown was inhibited during GPP, the trend of growth and CO₂ production rates were similar as control. On the other hand, when RFOs degradation was inhibited during GPP, CO₂ production was suppressed and germination was delayed. This result is in contrast with the suggestion by Dierking and Bilyeu (2009). They compared the growth rate between soybean species containing normal (58-62 mg g⁻¹ DW) and low level of RFOs (16-25 mg g⁻¹ DW) obtained by a genetic modification, and mentioned that RFOs did not play an important role for germinating soybean seed because no difference was found between them. However, in our observation, lack of glucose provision by the inhibition of RFOs breakdown delayed the growth rate and that means RFOs is essential especially in GPP.

Additionally, to know the necessary amount of RFOs for normal germination of soybean seed, we subtracted the amount of RFOs when the GPP was ended (after 24 hours) from that at the point of germination starts. From our data, soybean seeds require approximately 16 mg g⁻¹ DW of RFOs (about 38 % of initial RFOs in un-germinated seed) for normal germination. The higher initial RFOs in un-germinated soybean seeds, the higher of RFOs could be maintained in soybean sprouts by the application of α-galactosidase inhibitor after GPP, because the soybean seed needs low amount of RFOs for normal germination.

During GPP, RFOs break to produce glucose for respiration. As mentioned in introduction, soybean seeds also contain high amount of sucrose that is another source of glucose. Figure 5 demonstrates the changes of sucrose in germinating soybean seed for all tested conditions. Data shows that soybean seeds contain 31.4 mg g⁻¹ DW of sucrose before germination. Sucrose content was increased to 34.3 mg g⁻¹ DW for the first 24 hours of germination period both in soybean seeds treated by DGJ after GPP and in control. The accumulation of additional sucrose is the result of RFOs breakdown during GPP. On the other hand, when RFOs breakdown was inhibited before GPP, sucrose content was remarkably reduced. It indicates that the initial sucrose of soybean seed was solely responsible for respiration during GPP in absence of RFOs breakdown. Afterward, sucrose was degraded rapidly from 34.3 mg g⁻¹ DW to 10.9 mg g⁻¹ DW and 8.7 mg g⁻¹ DW at the end of germination in case of DGJ treatment after GPP and control.

![Fig. 4](image1.png) Changes of total RFOs during germination under normal and DGJ treatment conditions (Vertical lines represent standard deviation, n=4).

![Fig. 5](image2.png) Changes of sucrose during germination under normal and DGJ treatment conditions (Vertical lines represent standard deviation, n=4).
respectively. Many studies indicated that sucrose is the primary organic carbon that plays a role in development of non-photosynthetic tissues (sink) such as seed, fruit, and root in the most higher plants (Amor et al., 1995; Salnikov et al., 2001; Ruan, 2012). Therefore, sucrose here is suggested to be reduced due to other physiological developments of sprouts like cellulose synthesis rather than respiration because lipid is the main source for respiration after GPP (Table 1). In control situation, both the initial seed sucrose and sucrose accumulated by RFOs breakdown works together but if RFOs breakdown is inhibited after GPP, sucrose solely acts for those physiological developments that causes more reduction of sucrose after GPP. From Fig. 5, it could be suggested that the initial sucrose of soybean seeds could contribute to the germination process regardless of whether the RFOs breakdown is inhibited or no after GPP. Therefore, inhibition of RFOs breakdown after GPP could be useful for production of soybean sprout containing high amount of RFOs.

CONCLUSION

In this paper, we described the difference of RFOs, lipid and sucrose contribution in respiratory metabolism during germination process of soybean seed. For a normal germination process of soybean seed, a little amount of RFOs breakdown is indeed required for respiration during GPP. However, RFOs and sucrose are not essential for respiration during REP due to the change of respiratory substrate form sugar to lipid. Sucrose hypothesized to contribute to cellulose synthesis for hypocotyl prolongation mainly. Since RFOs are naturally present in mature soybean seed and only little amount is used for respiration during GPP and does not play an important role during subsequent REP, the prevention of RFOs breakdown during REP becomes a potential way for production of soybean sprouts with high amount of RFOs. However, DGJ, which is a synthetic α-galactosidase inhibitor, is available for preventing RFOs breakdown and it is very costly in practical use, therefore, further research on discovery of other potential α-galactosidase inhibitors which is inexpensive and natural-derived needs to be considered.

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