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Endophytic infection of human pathogen to *Arabidopsis thaliana* and prevention effect using Makgeolli yeast

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Abstract

One of the changes in the diet of westernized Asians due to the globalization of culture is the increase in the consumption of raw vegetables. However, while eating raw vegetables, people can get diseases from harmful bacteria remaining in the vegetables because they were not exposed to heat during cooking. In particular, since these bacteria exist inside plant, they cannot be removed by disinfection and washing the surface of the plant. Therefore, it was investigated whether adding Makgeolli yeast to the soil effectively prevents pathogen infection inside the plant and affects the growth of the soil bacteria. First, *Arabidopsis* roots were exposed to two types of Endophyte bacteria or *E. coli* (DH5α) expressing green fluorescent protein, and then it was confirmed whether the bacteria move to the stem or leaves by observing with a fluorescence microscope. Yeast isolated from rice wine or yeast filtrate inhibited the proliferation of harmful bacteria, *E. coli*. However, they did not affect *Arabidopsis* growth or inhibit the proliferation of beneficial bacteria among the eight bacteria isolated from soil. Finally, after exposing *E. coli* or yeast to *Arabidopsis* subjected to dry stress, the expression of the stress gene *AtRD20* was compared to demonstrate that yeast inhibits the proliferation of *E. coli*. Besides, adding yeast present in fermented foods to the soil will be an effective solution to prevent infection by pathogens caused by the consumption of primitive vegetables in countries that use compost instead of chemical fertilizers in agriculture.

Keywords: Yeast, Endophytic infection, Fermented food, E. coli, AtRD20

Introduction

As the Asian diet, including the Chinese diet, become westernized, the proportion of meat and vegetable increased in the traditional grain-oriented diet. Consumption of western-style dishes such as salads is rising due to western culture, which led to a rise in raw vegetable consumption. However, conventional farming methods involving the use of compost that has been adopted in many Asian countries increase the possibility that crops will be infected by harmful bacteria that cause disease in humans. In a guide document published in February 2008, the US FDA mentioned the dangers of foodborne microbial pathogens such as *Cyclospora cayetanensis*, *Escherichia coli* O157:H7,

Hepatitis A virus, *Listeria monocytogenes*, Norovirus, *Salmonella spp*., *Shigella spp*. which have the potential to infect humans during the consumption of raw crops and also noted the fact that all of these pathogens can cause diseases (Center for Food Safety and Applied Nutrition, 2008). Besides, in 2011, raw vegetables were pointed out as the cause of "hemolytic uremic syndrome," in which more than 1,000 people were infected in Europe (Deadly E. coli found on bean sprouts, 2011).

As the Asian diet, including that of China, became westernized, the proportion of meat and vegetables increased in the traditional grain-oriented diet. Western-style dishes such as salads are increasing, which led to a rise in raw vegetable consumption. However, as many Asian countries adopt traditional farming methods using compost, crops are more likely to be exposed to harmful bacteria that can cause disease in humans. In a guide document published in February 2008, the US FDA mentioned the dangers of foodborne microbial pathogens such as *Cyclospora cayetanensis*, *Escherichia coli* O157:H7, *Hepatitis A virus*, *Listeria monocytogenes*, Norovirus, *Salmonella spp.*, *Shigella spp.* which have the potential to infect humans in the consumption of raw crops, and also noted the fact that these pathogens can cause diseases. (Center for Food Safety and Applied Nutrition, 2008) Besides, in 2011, raw vegetables were pointed out as the cause of "hemolytic uremic syndrome," and more than 1,000 people were infected in Europe. (Deadly E. coli found on bean sprouts, 2011)

Microbial endophytes, which form symbiotic relationships without harming the plant, within plants have been confirmed through past studies (G. C. M. Latch M. J. Christensen, 1985), and studies on this topic have been actively conducted. The route of infection is known to be the root of plants (Rees et al., 2000). Pathogens, such as *E. coli*, present in compost used in traditional farming, can survive continuously (Chen et al., 2018). Since endophytes and plants are in a symbiotic relationship, there is a risk of infection by consuming (organic) vegetables if pathogens present in the compost enter the plant. Research on the symbiotic relationship between plants and fungal endophytes has been actively conducted, but less research has been done on pathogenic endophytes. In particular, few studies have been undertaken on diseases caused by pathogenic bacteria in humans who consume plants and measures to prevent such infections.

It was first discovered by Hayduck, F. that yeast, which is mainly used in fermented foods, has a microbial inhibitory effect. (Hatoum et al., 2012). Subsequently, it was found that yeasts of several strains such as *C. intermedia*, *C. tropicalis*, *C. lusitaniae*, and *S. cerevisiae* inhibit bacteria such as *S. aureus*, *E. coli*, and *P. aeruginosa* (Younis et al., 2017), and *S. cerevisiae* and *P. fermentans* is known to have antifungal effects (Menolli Junior et al., 2020). Efforts to utilize yeast or fermented foods in agriculture have also been carried out for a long time. In 2005, the Yeosu Agricultural Technology Center announced that makgeolli treated with organic acid and sprayed on peppers effectively prevented diseases and pests ("전국일반 : 전국 : 뉴스 : 한겨레," 2005), and in 2014, a study found that yeast extracted from sugar cane helped plant growth (Nutaratat et al., 2014, p. 690).

Aiming to confirm yeast's inhibitory effect on pathogenic endophytes, this study attempted to determine whether pathogens in contaminated soil can penetrate plants and whether the penetration of pathogens can be inhibited when soil is treated with yeast. *Arabidopsis thaliana* was used as the model organism because it does not require much space to grow and has a short generation time ("Analysis of Chloroplast Mutants of Arabidopsis Induced by Diepoxybutane," 2002, p. 400). The DNA sequence of *Arabidopsis thaliana* has been fully identified and is already widely recognized for its suitability in studying the interaction between plant pathogens and *Arabidopsis* (Junker et al., 2012, p. 658), making it suitable as a model organism for this study.

Methodology

Arabidopsis Cultivation

Sterilized *Arabidopsis* seeds were planted on MS agar and germinated under white LED lights (16 hours light/8 hours dark). Containers were filled with Topsoil mixed with distilled water. 2 *Arabidopsis* seedlings were transplanted each and placed in a tray containing water under the LED light.

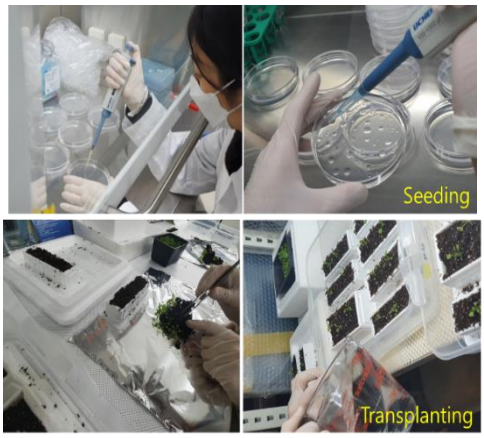

FIGURE 1. Seeding and transplanting *Arabidopsis thaliana*.

Preparation of Bacteria

Endophyte-GFP (Green Fluorescent Protein) bacteria and E. coli-GFP (DH5α) appeared green fluorescence under the blue light of a fluorescence microscope. For isolating soil bacteria, 1g topsoil and 4g distilled water was mixed and inoculated on NA medium and cultured for 48 hours at 28℃. After classifying the bacterial colonies, each colony was re-incubated in 5mL NB medium.

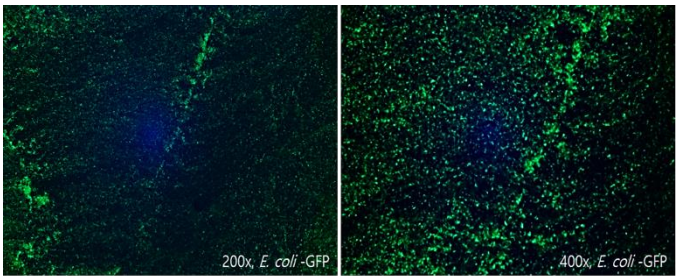

FIGURE 2. *E. coli*-GFP (DH5α) observed at 200x and 400x magnification under Fluorescence Microscope.

Inoculation of Soil Bacteria to Arabidopsis

Roots of *Arabidopsis* were damaged by a knife. 1mL of 0.01A soil bacteria was injected into the soil through a syringe needle. The number and size of leaves of the *Arabidopsis* in each pot were recorded after 96 hours. The size of leaves was measured, as shown in Figure 3.

FIGURE 3. Damaging *Arabidopsis* roots and measuring of *Arabidopsis* leaves using a ruler.

Effect of Yeast on Different Bacteria

Soil bacteria and Makgeolli yeast solution were mixed, as shown in Table 1 and incubated at 28° C for 48 hours. 10μL of the cultured solution was spread on NA and YPD medium and re-cultured. 0.1A yeast filtrate was added to NB medium of concentrations 0%~50%, 0.01A *E. coli*-GFP (DH5α) inoculated and cultured at 37°C for 48 hours.

Inoculation of GFP to Arabidopsis

Ten *Arabidopsis* seedlings each were transplanted to a new MS agar media using tweezers. The roots of five seedlings were damaged with a micropipette, and the pathogen was inoculated on all roots. The seedlings were observed under a fluorescence microscope after growing at room temperature for 48 hours.

Immunity RNA Analysis of Arabidopsis Cultivated under Stress

25 *Arabidopsis* seedlings were left in a mixture of MS broth media, distilled water, sea salt, yeast filtrate, and 15uL *E. coli*-GFP (DH5α). After 48 hours, RNA of *Arabidopsis* was extracted, and cDNA synthesized. RD20-For, RD20-Rev, Actin-For, and Actin-Rev primers were each diluted to 100 pmol/μL. 5μl cDNA, 2μl primer, and 13μl

TABLE 1. Absorbance and concentration of Soil bacteria solutions in 600nm Wavelength.

Soil bacteria	ABS	%T	Bacteria sol. (μL)	Yeast sol. (μL)	NB (μL)
S1	0.198	63.37	25	14	4,961
S2	0.194	63.87	26	14	4,960
S3	0.232	58.53	22	14	4,966
S4	0.302	49.81	17	14	4,969
S5	0.317	48.16	16	14	4,970
S6	0.406	39.18	12	14	4,974
S7	0.349	44.68	14	14	4,972
S8	0.341	45.52	15	14	4,971
Yeast	0.361	43.47	-	14	-

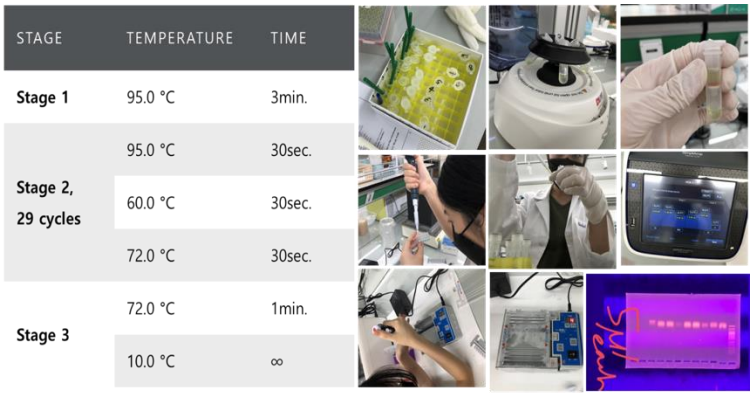

FIGURE 4. 2 Specific Temperature and Duration of 3 stages during PCR and RT-PCR process.

sterile distilled water were added to freeze-dried PCR Premix, PCR of 29 cycles and electrophoresis performed, and the gel was observed on a UV illuminator and gel doc.

Results

Invasion of E. coli-GFP Inside Plants

To examine whether *E. coli* or endophyte penetrate inside of the plant, roots of *Arabidopsis* seedlings grown in MS agar media were inoculated with *E. coli*-GFP or endophyte-GFP bacteria. After 48 hours, the seedlings were pulled out and placed on a glass slide to be observed with a fluorescence microscope.

Although there was no significant difference between plants with damaged roots and plants with intact roots, more bacteria were identified in the stems of plants with damaged roots than that with intact roots. In general, when a plant specimen is observed with a fluorescence microscope, the plant tissue would be destructed by the prolonged exposure of a light source, and bacteria expressing GFP from the destroyed tissue escape. Therefore, non-specific signals from the destructed plant tissues or bacteria expressing GFP that have escaped from the outside would be detected. To prevent the detection of non-specific signals, special care has been taken to speed up the experiment.

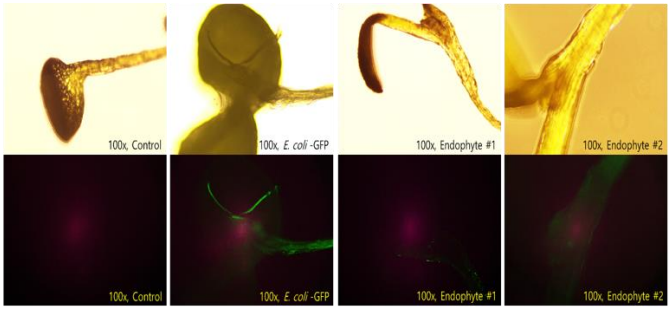

FIGURE 5. *Arabidopsis* infected by *E. coli*-GFP observed under Fluorescence microscope.

No GFP signal was observed in leaves and stems of *Arabidopsis* seedlings that were given distilled water to their damaged roots. Contrarily, *E. coli*-GFP clearly showed green bacteria in *Arabidopsis* leaf veins, and green fluorescence was also clearly observed in *Arabidopsis* stems and leaves exposed to two other types of

endophyte-GFP bacteria. Moreover, GFP signals were detected in the intact roots exposed to *E. coli*-GFP or two other types of endophyte-GFP (Fig. 5). These results mean that bacteria penetrated the plant and moved to the leaves through the vascular system when the roots of plants absorbed water. Therefore, it was found that the infection of bacteria in plants through the roots is possible.

Invasion of Yeast Inside Plants

Generally, the size of yeast is 5-8 μm, whereas the size of rod-shaped bacteria is 2-3 μm and that of coccus-shaped bacteria is 1 μm (Junker et al., 2012, p. 658). The vascular tissue of plant roots is 10-50 μm in size, and bacteria can penetrate the plant along with water, but yeast, being two to three times larger than bacteria, is less likely to do so. An experiment was conducted using *Arabidopsis* in this study to confirm the invasion of yeast into the plant body through the roots, but no evidence of penetration could be found even when the roots were wounded.

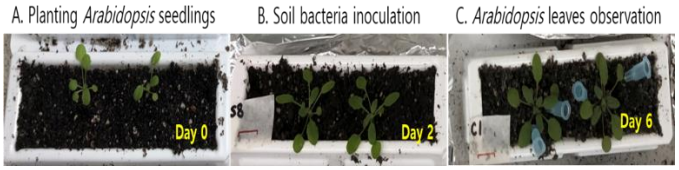

FIGURE 6: Arabidopsis development before and after soil bacteria inoculation.

The Selective Effect of Makgeolli Yeast on Soil Bacteria

To determine the effect of 8 strains of soil bacteria on plants, bacterial solutions were diluted to a concentration of 0.01A with distilled water. 500μL of each strain was injected through a syringe needle into the soil, which *Arabidopsis* was planted on (Fig. 7 & 8). After growing the *Arabidopsis* under identical conditions for 96 hours using a low-surface irrigation method, the size and number of leaves were measured. The development of a flower stalk was also recorded after 1 week to analyze the differences in plants after bacterial injection.

It has been known that plants increase their number of leaves as a response to external stress. Thus, higher number of leaves can be interpreted as the *Arabidopsis* being subjected to stronger stress. An increase in the size of leaves of a plant is a positive (normal) growth pattern. Hence, plants that only showed an increase in their number of leaves while leaf size was relatively unchanged were subject to more stress than plants that did not. The greater $\frac{No.\ of\ leaves}{Length\ of\ leaves}$ is than 1, the more stressed the plant is. Likewise, plants with developed stems promote stem growth for faster reproduction when exposed to stressful environments. When *Arabidopsis* stem growth was promoted, it means that it is under more stress. $\frac{No.\ of\ leaves}{Length\ of\ leaves}$ was greater than 1 on Day 0 in S2, S4, DW, and Control plants, but it seems that the stress was not influenced by soil bacteria. $\frac{No.\ of\ leaves}{Length\ of\ leaves}$ was greater than 1 in S2, S3, S4, S7, S8, DW, and Control after 96 hours (Fig. 9). S3, S7, and S8 appear to have been newly stressed by the soil bacteria. Moreover, since flower stalks were also generated in S3, S4, and DW (Table 2), considering all factors, plants injected with soil bacteria S3, S4, and S8 received the highest stress. Therefore, these data suggest that soil bacteria S3, S4, S7, or S8 harms plant growth, S1, S5, or S6 does not affect plant growth (neutral), and S2 enhances plant growth.

Effect of Yeast on the Growth of Soil Bacteria

To discover the effect of yeast on each isolated soil bacteria, each soil bacteria and yeast were co-cultured and spread on an agar medium to determine the difference in proliferation. Soil bacteria singly cultured were cultured in NA medium, and soil bacteria co-cultured with yeast were cultured in both NA medium and YPD.

When each strain of soil bacteria was co-cultured with yeast, the proliferation of S1 was promoted. The proliferation of S2, S3, S4, S5, S6, or S8 was unaffected while S7 was inhibited. This means that yeast isolated from rice wine has neutral effects on bacteria that affects plant growth. Furthermore, some soil bacteria regulated yeast. Yeast growth was inhibited by S2 bacteria, which enhances plant growth. Other soil bacteria did not affect yeast growth (Fig. 10).

TABLE 2: Development of Arabidopsis flower stalk according to strain of soil bacteria.

	S1	S2	S3	S4	S5	S6	S7	DW	C
Flower stalk	No development	No development	developed	developed	No development	No development	No development	No development	developed

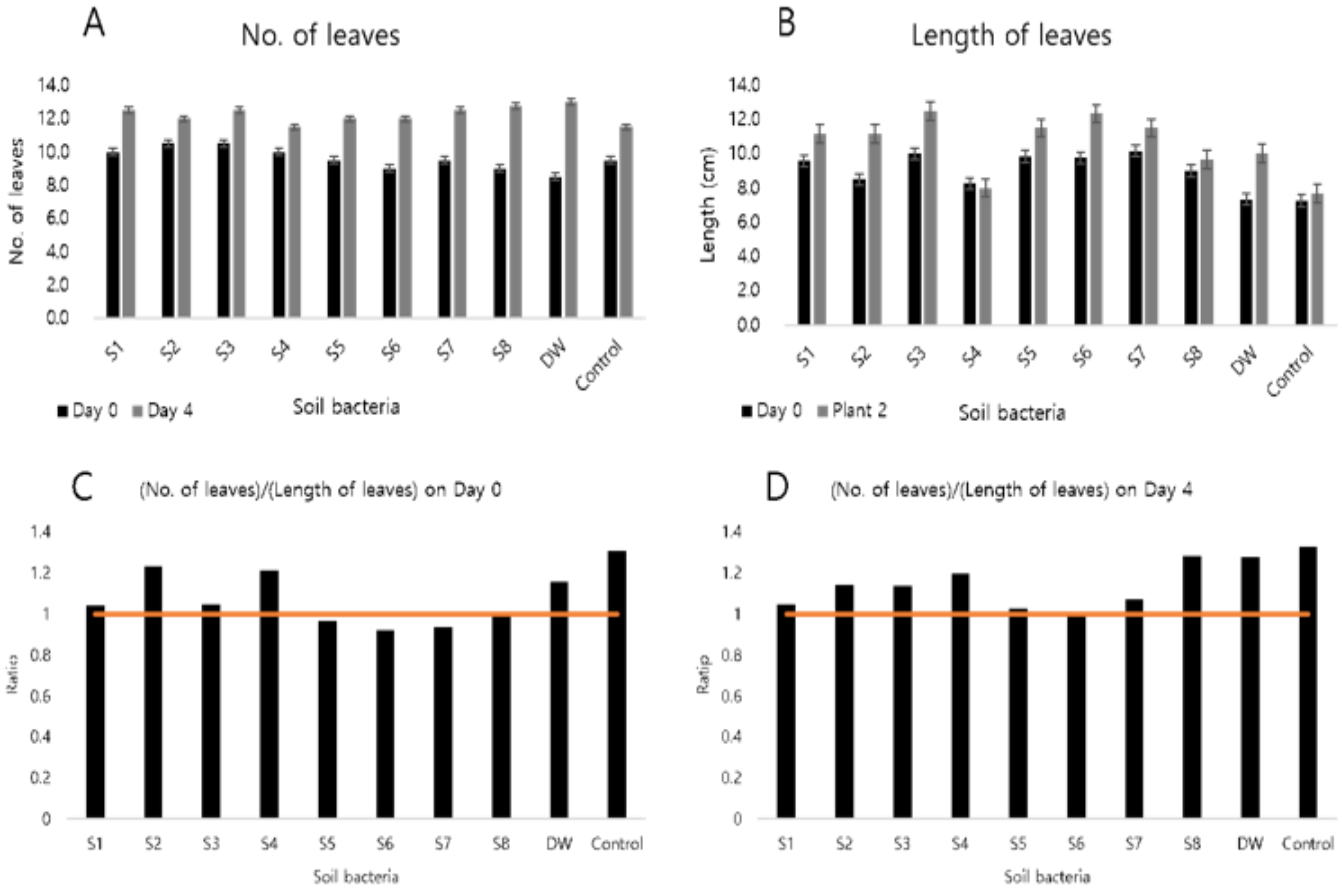

FIGURE 8: The number of Arabidopsis leaves according to the type of Soil bacteria (A), Length of Leaves (B), The ratio of number of leaves to the length of leaves on Day 0 (C), The ratio of number of leaves to the length of leaves on Day 4 (D).

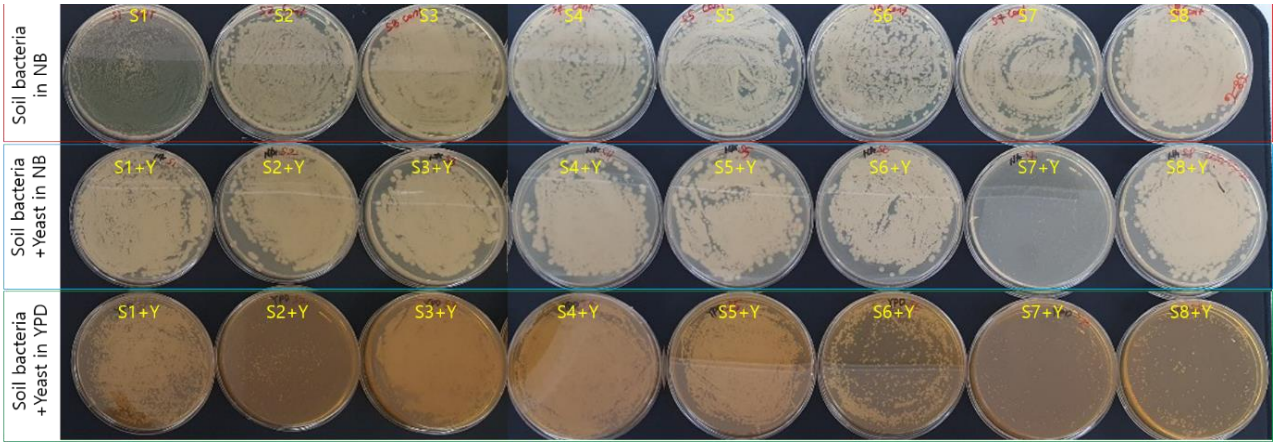

FIGURE 7: Yeast and soil bacteria S1 ~ S8 mutually affecting each other.

Inhibitory Effect of Yeast Extract on E. coli-GFP

To determine whether yeast metabolite inhibits the proliferation of *E. coli*-GFP, yeast filtrate containing yeast metabolites was added to the NB medium at a concentration of 0 to 50 %, and *E. coli*-GFP was cultured in it. The proliferation of *E. coli*-GFP was determined by measuring the absorbance. For 0 % of yeast filtrate, 50 % YPD broth media or 50 % NB solution was used to consider the maximum amount of added yeast filtrate, and the absorbance of *E. coli*-GFP was reduced by 46.7 % from 0.182 of Control to 0.097. When the concentration of yeast filtrate was reached 30 %, the absorbance of *E. coli*-GFP decreased to -0.001. When the absorbance value was negative, it was reflected in the graph as 0. The absorbance of *E. coli*-GFP decreased sharply from 10 % yeast filtrate onwards. Eventually, the absorbance of *E. coli*-GFP decreases as the concentration of yeast filtrate increases. It shows an inverse relationship between the absorbance of *E. coli*-GFP and the concentration of yeast filtrate, and it was concluded that yeast metabolites contain a strong substance that can inhibit bacteria.

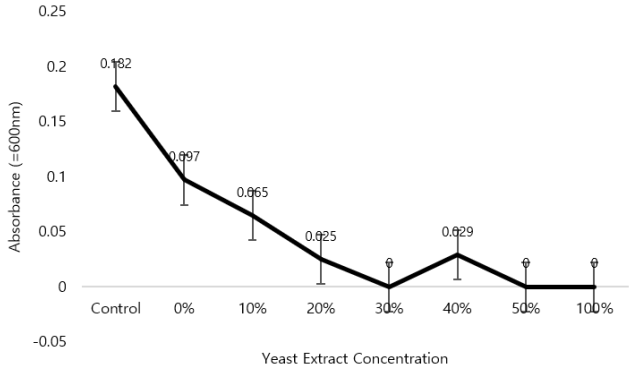

FIGURE 11: Changes in absorbance of *E. coli*-GFP (DH5α) according to the concentration of yeast filtrate. Control is 100% NB media, 0% is a mixed medium of 50% NB media and 50% YPD broth media.

Inhibitory Effect of Yeast Treatment in Arabidopsis under Dry Stress on E. coli-GFP

Although yeast did not affect the growth of soil bacteria, it inhibited the growth of *E. coli*-GFP. Therefore, the inhibition of the proliferation of *E. coli*-GFP by yeast in the soil would decrease the invasion rate of *E. coli*-GFP inside the plant. To investigate the effect of *E. coli* and yeast on *Arabidopsis* under salt stress, *Arabidopsis* was put in a liquid medium added with salt, and the expression of the stress gene, *AtRD20*, was monitored through RT-PCR. *AtActin1* gene was used for the reference gene to confirm the use of the same amount of cDNA.

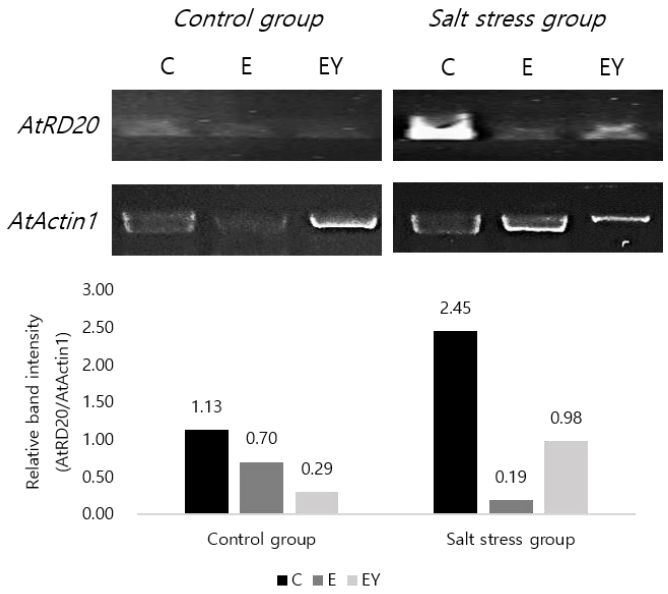

FIGURE 12: Actual and Relative AtRD20 and AtActin1band intensity of control group and salt stress group *Arabidopsis* samples

In plants not under stress, the expression of *AtRD20* was weakly or strongly decreased by treatment with *E. coli* or treatment with *E. coli* and yeast, respectively. Contrarily, expression of *AtRD20* was higher in salt stress-induced plants, and the expression was strongly reduced by infection with *E. coli*. *AtRD20* expression induced by salt stress was reduced to 1/4 due to yeast infection (Fig. 12). It means that *Arabidopsis* not exposed to both *E. coli* and yeast received the most salt stress and that *Arabidopsis* exposed to only *E. coli* and *Arabidopsis* exposed to both *E. coli* and yeast received significantly less salt-stress than the control plant. There was also a difference in the degree of stress between the two *Arabidopsis.* It was observed that *Arabidopsis* exposed only to *E. coli* received the lowest stress. *Arabidopsis* exposed to *E. coli* and yeast had slightly higher stress than the *Arabidopsis*

exposed to *E. coli* alone. Through this, it can be deduced that *E. coli* plays a role in relieving the salt stress that *Arabidopsis* receives, and the reason stress increased when yeast was added because yeast inhibited *E. coli*. Therefore, it suggests that yeast reduces the action and effect of *E. coli* on plant growth.

Discussion and Conclusion

This study was conducted to investigate the possibility of preventing infectious diseases caused by human consumption of raw vegetables by inhibiting the penetration of pathogens into plants by S. cerevisiae, a yeast isolated from rice wine.

First, to determine the effect of *S. cerevisiae* metabolite filtrate on the growth of harmful bacteria, *E. coli* was cultured in yeast filtrate, and the difference in absorbance was analyzed. As a result, it was observed that yeast filtrate significantly inhibits the growth of *E. coli*.

To determine whether harmful bacteria invade plants through the roots, sterilized *Arabidopsis* seeds were germinated and grown in a plant medium. After transferring to another medium, the roots were exposed with two types of endophyte or *E. coli* expressing GFP (endophyte-GFP or *E.coli*-GFP, respectively) for 48 hours, and root samples were observed with a fluorescence microscope. As a result, fluorescence was observed in the stems and leaves of *Arabidopsis*, and it was confirmed that bacterial infection through the roots of the plant is possible.

Next, eight strains isolated from soil were injected into the soil of *Arabidopsis* pots to investigate the effect of yeast on soil bacteria. After 96 hours, the number and size of leaves were measured, and the effect of yeast on bacterial proliferation was analyzed by co-cultivation with yeast and bacteria. As a result, the yeast isolated from rice wine enhanced or did not affect the growth of beneficial or neutral soil bacteria. Moreover, yeast had inhibitory or neutral effects on the proliferation harmful of soil bacteria. Therefore, it suggests that injecting yeast into soil did not negatively affect beneficial soil bacteria and only suppressed some bacteria that interfere with plants' growth and harmful bacteria such as *E. coli*.

Finally, the presence of *E. coli* and yeast, affecting the expression of genes related to drought stress in *Arabidopsis*, was discovered. *Arabidopsis* exposed to both *E. coli* and yeast received the most stress, whereas *Arabidopsis* exposed to only *E. coli* or both showed significantly lower expression of the stress gene AtRD20 than controlled plants. *Arabidopsis* exposed only to *E. coli* received the lowest stress, and when yeast was exposed together, stress was higher than when only *E. coli* was present. These results mean that yeast inhibits the growth of *E. coli*.

Hence, the yeast isolated from rice wine inhibits pathogenic bacterial growth, such as *E. coli* in soil, and prevents bacteria from being transmitted to humans through infection inside plants and enhances plant growth. However, the inhibitory effect of *S. cerevisiae* on the growth of *E. coli* was higher when *E. coli* was cultured in yeast filtrate than when directly co-cultured with yeast. This may be due to certain ingredients of the filtrate, so the ingredient(s)' specific properties will have to be investigated in future research.

This study suggests using fermented foods that can be easily manufactured to suppress plant-endophytic bacteria that cause diseases in the human body. Although the effectiveness of yeast from rice wine, which is Korea's representative fermented drink, was verified in this study, the findings can be applied to any country where fermented foods using yeast are readily available.

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