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Enhancement of antioxidant activity and biomass by mineral water in rehmannia root (*Rehmannia glutinosa* L. purpurea Makino)

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Abstract

We investigated the functional mechanisms of brine mineral water (BMW) in plants. Antioxidant activities were determined by DPPH free radical-scavenging analysis of crude extracts of *Rehmannia glutinosa* treated with BMW. The DPPH was measured by value for the density of antioxidant required to absorbance equal to 50% that of a control containing no antioxidant. Plants treated with 0.05-0.1% BMW showed weak antioxidant activity. The concentrations of the principal mineral components, Mg^{2+}, K^{+}, and Sr^{2+} were increased in samples treated with 1% BMW. Antimicrobial activities of BMW-treated *R. glutinosa* were determined. The greatest inhibition was against *Salmonella typhimurium* and *Klebsiella pneumoniae*. The effect of BMW treatment on plant growth over four weeks was investigated and determined by addition of distilled water into 100% BMW, which plant growth was enhanced about 8 cm by 0.05-0.1% BMW. The expression of marker genes involved in plant metabolism was monitored in control and BMW-treated plants. The *RgAPX* gene was induced in 5% BMW-treated plants after 48 h, and transcription of the *PAL* gene was significantly higher in 5% BMW–treated plants after both 1 and 48 h. In addition, *rbcL* gene expression was higher in BMW-treated than in control plants. These data suggest that BMW has various effects in plants. This work explains the relationship between biological activity and biomass in terms of the expression of functional genes. BMW affects biological activities, growth, and the secondary metabolism of plants.

Keywords: Antimicrobial activities, Antioxidant activities, BMW, Marker genes
Abbreviations: APX_ascorbate peroxidase; BMW_brine mineral water; DPPH_2,2-diphenyl-1-picrylhydrazyl; DSW_deep sea water; ICP_ inductively coupled plasma; PAL_phenylalanine ammonia-lyase; rbcL_ribulose 1,5-bisphosphate carboxylase large subunit; Rg_*Rehmannia glutinosa*..

Introduction

Rehmannia glutinosa Liboschitz (Scrophulariaceae), a comprehensive traditional herbal medicine, is used widely in Korea. It promotes the production of body fluids and removal of pathogenic heat from blood (Won et al., 2010). There have been many reports of its pharmacological activities in the blood, immune, endocrine, cardiovascular, and nervous systems (Won et al., 2010). Also, *R. glutinosa* Liboschitz is known for its broad clinical applications, such as in hemostasis, antitumor treatment, immune-enhancement, anti-hypertension, and bone metabolism (Oh et al., 2003; Chao et al., 2006). This herb contains iridoids, phenolics, glycosides, and norcarotenoids (Morota et al., 1989). The overall pharmacological activities of *R. glutinosa* are associated with the presence of multiple compounds related to various therapeutic functions (Xue and Roy, 2003; Qian et al., 2007). Brine mineral water (BMW) generally refers to seawater at depths equal to those at which photosynthesis occurs, and to which sunlight does not reach (Kim et al., 2009). BMW contains an abundance of minerals, such as Ca, Sr, Mn, Zn, Fe, Cu, Ni, V, and Se, and is more similar to human body fluids than deep seawater (DSW) (Moon et al., 2004). The abundance of minerals has attracted attention, and studies have demonstrated the usefulness of BMW for a variety of applications (Kim et al., 2008). DSW is also enriched with various minerals and useful for a variety of applications (Hachmuth, 1991). DSW has been shown to both control and reduce serum lipid levels in rabbits (Yoshioka et al., 2003).

Various environmental stresses enhance the production of reactive oxygen species (ROS) (Meloni et al., 2003). ROS interact with a number of cellular macromolecules, such as DNA, proteins, lipids, and pigments, which can result in destructive processes in plant cells (Mittler 2002). It is crucial for plants to balance the generation and elimination of ROS during exposure to environmental stresses (Light et al., 2005). Plants with high antioxidant contents have been reported to

have greater tolerance for oxidative injury (Thakur and Rai, 1981; Upadhyaya et al., 1989). APX is the most important peroxidase in hydrogen peroxide (H_2O_2) detoxification, working both in the cytosol and chloroplasts (Gossett et al., 1994). Expression of the *APX* gene is rapidly induced by various stresses (Garg and Singh, 1971). Phenylalanine ammonia-lyase (PAL) is an enzyme upstream of the phenylpropanoid pathway and is involved in the production of a variety of phenolic compounds with structural and defense-related functions (Romero et al., 2008). Ribulose 1,5-bisphosphate carboxylase (Rubisco) is specific to the Bundle sheath (BS) cells of mature C_4 leaves and provides an excellent model system of regulation of photosynthetic gene expression in these plants (Patel and James, 2008). The *rbcL* gene is localized to chloroplasts and translated on prokaryotic-like plastid ribosomes (Sasanuma, 2001). In the present work, we treated *R. glutinosa* with BMW to investigate its functional mechanisms in plants. We focused on BMW up-take into whole plants at various concentrations. The antioxidant and antimicrobial activities of BMW-treated *R. glutinosa* were determined. The main components of the samples were identified using inductively coupled plasma optical emission spectrometry (ICPOES). This work explains the relationship between biological activity and biomass in terms of the expression of functional genes. Our results indicate that BMW can induce an increment of plant biomass by enhancing various biological activities.

Results and Discussion

Antioxidant activity

The primary method of determining free radical-scavenging activity is DPPH analysis (Kil et al., 2009). The DPPH free radical-scavenging activity of extracts of *R. glutinosa* treated with BMW was determined after addition of □-tocopherol and ascorbic acid (Table 2). Crude extracts (1.0 mg mL^{-1}) were subjected to DPPH analysis, and all showed antioxidant activity. Extract activity was BMW concentration-dependent, and ranged from 254 to 318 mg mL^{-1} (Table 2). Most samples treated with 0.05-0.1% BMW showed weak antioxidant activity (Table 2). With regard to RC_{50} values (the concentration of antioxidant required to achieve absorbance equal to 50% of that of a control containing no antioxidants), samples treated with greater than 0.5% BMW had lower radical-scavenging activity (Table 2). Exposure to proton radical scavengers is known to significantly decrease the level of DPPH (Yamaguchi et al., 1998). Thus, free radical-scavenging activity has a marked impact on the phenolic composition of the sample. Mineral water is known to exhibit electron-donating antioxidant activity, as determined by DPPH analysis in a study that aimed to determine its composition (Ham et al., 2005). The antioxidant activities of DSW (deep sea water) and Danasoo were also determined using DPPH analysis, and were shown to increase in a dose-dependent manner (Kim et al., 2008).

Antimicrobial activity of BMW-treated samples

The antimicrobial activity of crude extracts of *R. glutinosa* was determined with a serial two-fold dilution assay. BMW-treatment inhibited the antimicrobial activity of the crude extracts (Table 3). The greatest antimicrobial activity was detected in the extract of plants treated with 0.1% BMW, which had an MIC of 500 μg $Mℓ^{-1}$. The strongest inhibition was against *S. typhimurium* and *K. pneumoniae*. No antimicrobial activity against other strains was detected. *S. typhi* was also used as a bacterial target (Perez and Anesini, 1994). Methanol extracts of some medicinal plants exhibited marked antibacterial activity against *S. typhi* (Rani and Khullar, 2004). *P. vulgaris*, followed by *S. typhimurium* were the least sensitive to medicinal plant extracts (Parekh et al., 2005). Extracts of *Terminalia chebula* and *Ocimum sanctum*, both of which are traditional Indian medicinal plants, exhibited antibacterial activity against *K. pneumonia* (Sharma et al., 2009). Crude *Acacia nilotica* L. extract showed maximum antibacterial activity against *K. pneumoniae* (Mahmood et al., 2012).

Analysis of main components

The effect of BMW on the mineral composition of *R. glutinosa* was investigated (Table 4). The Mg, K, and Sr concentrations were higher in samples treated with 1% BMW. The Mg concentration of samples treated with 1% BMW increased to 16.336 μg ml^{-1} compared to 10.620 μg ml^{-1} in the control. K was also higher (512.921 μg ml^{-1}) than in the control (330.550 μg ml^{-1}). The concentration of Sr was induced significantly in the sample treated with 1% BMW (0.217 μg ml^{-1}), which was 5.7 times that in the control (0.038 μg ml^{-1}).

Inductively coupled plasma (ICP) is used to determine the trace element composition of plant materials (Kos et al., 1996; Barnes, 1998; Amarasiriwardena et al., 1998; Rodushkin et al., 1999). Diluted 70% HNO_3 was used for dissolution of Al, Ca, Cd, Cu, Fe, Mg, Pb, and Zn from plant materials (Wieteska et al., 1996). DSW was shown to contain a high Cl concentration (17.9 μg ml^{-1}) in an analysis of Ca, Mg, K, Na, Cl, and SO_4 content (Shon et al., 2008). This work is the first to describe ICP analysis of BMW-treated plants.

Effect of BMW on plant growth

We investigated the effect of on plant growth BMW treatment for four weeks (Fig. 1). Treatment with 0.1% BMW resulted in medium-to-high plant growth. In addition, root length was the longest (6.5 cm) after treatment with 0.1% BMW. The pattern of general growth increased after treatment with 0.05-0.1% BMW, but was reduced by higher concentrations. Thus the effect of BMW on plant height and root length was similar. Fruit growth and weight are both DSW concentration-dependent (Woo and Kang, 2006). Growth in terms of height, fresh and dry weight, stem diameter, and leaf area was inhibited by DSW with a higher NaCl content (Hong et al., 2006).

Effect of BMW on R. glutinosa antioxidant gene expression

We next investigated the effect of BMW treatment on the expression of marker genes involved in plant antioxidant activity by RT-PCR analysis of cDNA synthesized from samples (Fig. 2). The APX protein functions in the first step of the ascorbate-glutathione cycle and is the most important peroxidase in H_2O_2 detoxification, acting both in the cytosol and chloroplasts (Michalak, 2006). *APX*-related gene expression is rapidly induced by various stresses (Michalak, 2006). In this study, the *RgAPX* gene was induced after 48 h in 5% BMW-treated compared to control plants (Fig. 2). PAL catalyzes the first step in the general phenylpropanoid pathway and supplies substrates for the biosynthesis of various phenolic

Table 1. The primers used for reverse transcriptase-PCR

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
RgActin	aacgtgccagccatgtatgt	gcttctccttcacgtcacga
RgAPX	gaactgtgctccgatcatgc	atggaacttcaggtcctccg
RgPAL1	ttagaacgtcgcctcaatgg	ctcggaaaattgagcgaaca
RgrbcL	ggaagatctgcgaatccctc	gaaacgatctctccaacgca

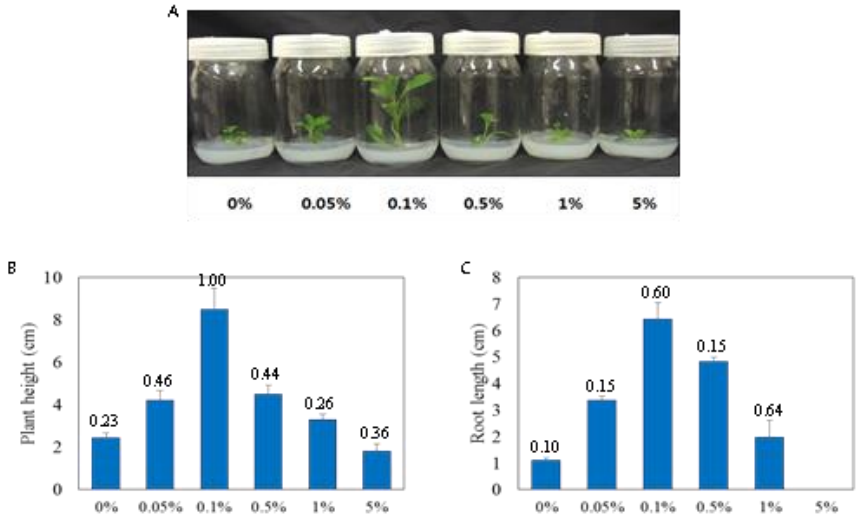

Fig 1. Increasing effect for plant growth in *Rehmannia glutinosa* treated by Brine Mineral Water. Variation on (A) plant growth, (B) plant height, (C) root length. The differences of B and C were represented as a standard deviation (SD).

Table 2. free radical-scavenging activity DPPH[1)] by treatment with various concentrations of Brine Mineral Water (BMW) in *Rehmannia glutinosa*.

Concentrations of BMW (%)	RC_{50} (μg ml^{-1}) [(2)]
0	342.52±4.3
0.05	290.76±5.2
0.1	254.34±4.5
0.5	296.84±6.0
1	318.05±2.5
5	297.41±2.0
α-tocopherol	12.0±0.2
L-ascorbic acid	< 2

1) DPPH: 2, 2-diphenyl-2-picryl-hydrazyl. 2) RC_{50} (μg ml^{-1}) = Amount required for 50% reduction of DPPH after 30min. Each value is mean ± standard deviation of three replicate tests.

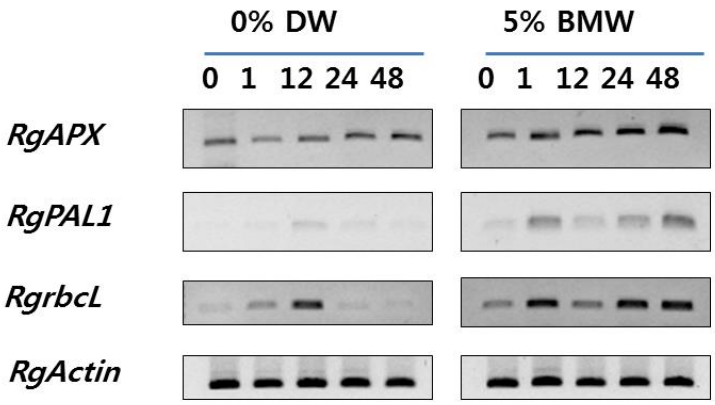

Fig 2. Patterns of gene expression following on different concentration and treat-time (0, 1, 12, 24, 48 hours) in *Rehmannia glutinosa* treated by Brine Mineral Water. The experiment was repeated at least three times and a representative result is shown.

Table 3. Antimicrobial activity in *Rehmannia glutinosa* treated with Brine Mineral Water (ppm).

Microorganisms	MIC[1)] (μg ml^{-1})					
	Brine Mineral Water (BMW)					
	0%	0.0.5%	0.1%	0.5%	1%	5%
Bacillus sublitis	>1000	1000	1000	>1000	>1000	>1000
Staphylococcus aureus	>1000	>1000	>1000	>1000	>1000	>1000
Salmonella typhimurium	1000	1000	500	1000	1000	1000
Klebsiella pheumonia	1000	1000	500	1000	1000	1000
Eschrichia coli	>1000	>1000	>1000	>1000	>1000	>1000
Canolida albicans	>1000	>1000	>1000	>1000	>1000	>1000
Pichia jadinii	>1000	>1000	>1000	>1000	>1000	>1000
Tetracycline	8	8	8	8	8	8

[1)] The MIC value against bacteria were determined by the serial 2-fold dilution method. The values of 500 and 1000 were represented a dilution concentration (microbial culture broth added nutrient medium)

Table 4. Components of mineral deposition determined by ICP/MS measurement from root samples of *Rehmannia glutinosa* treated with Brine Mineral Water (BMW).

Components (μg ml^{-1})	Brine Mineral Water (BMW)				
	0%	0.1%	0.5%	1%	5%
Mg	10.620	5.226	6.423	16.336	11.607
Mn	0.948	0.611	0.480	1.210	0.364
Se	0.004	0.007	0.002	0.007	0.007
V	0.007	0.004	0.004	0.008	0.007
K	330.550	237.273	217.406	512.921	361.166
Sr	0.038	0.028	0.070	0.217	0.275

compounds (Romero et al., 2008). PAL mRNA is induced by a wide array of environmental factors (Sanchez-Ballesta et al., 2000). *PAL* gene transcriptional level was increased significantly in BMW–treated plants after 1 and 48 h (Fig. 2). *rbcL* expression is localized to chloroplasts, and its transcripts are translated on prokaryotic-like plastid ribosomes (Sasanuma, 2001). The *rbcL* genes are likely regulated by light in all plant species (Zhou et al., 2001). In addition, *rbcL* expression was higher in BMW-treated than control plants (Fig. 2). Factors that affect *Rubisco* expression are involved in cell development, photosynthetic metabolism, hormones, senescence, and disease (Patel and Berry, 2008).

Materials and Methods

Sample preparation

Subcultures of *R. glutinosa* tissue for 8 weeks were incubated in the presence of BMW (Geumjin Hot Spring Water, TONGYANG Life Science Corp.) and collected. Whole plant samples (~10 g) were collected from each culture condition, homogenized, and extracted with 80% methanol at room temperature for 48 h. Based on concentration, the extract was adjusted as a 100 μg ml^{-1} by addition of 80% methanol to match with powder weight. Each mixture was then passed through Whatman No. 42 filter paper to remove debris, and the extracts were evaporated at 40°C using a rotary evaporator.

Antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

Sample extracts (~1 mL) were added to 1 mL of 0.15 M DPPH solution. The reaction mixture was shaken well and incubated for 30 min at room temperature in the dark. Absorbance at 517 nm of the resulting solution was measured. The percent inhibition of DPPH was calculated according to the method of Kil et al. (2009).

Analysis of ion content by inductively coupled plasma optical emission spectrometry (ICPOES)

Samples (0.5 g) were suspended for 6 h in a mixture of 7 mL HNO_3 plus 1 mL H_2O_2. Solutions were then evaporated by heating (Goh and Lee, 1999). The resultant samples were dissolved in 50 mL dH_2O and filtered. Ion concentrations were determined by ICPOES (model OPTIVA 7300DV; PerkinElmer, Waltham, MA, USA).

Test of antimicrobial activity

Seven bacterial and fungal strains were used: *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Candida albicans*, and *Pichia jadinii*. The bacteria were cultured in liquid medium with shaking for 12 h at 37 or 30°C. The extracts were then diluted to the highest concentration (100 μg ml^{-1}), and serial two-fold dilutions were made in the range 7.8–100 μg ml^{-1}. Diluted inocula (180 μg) were transferred to wells of 96-well plates. Aliquots (20 μg) of stock solution were then added to the wells. Next, the plates were shaken at 300 rpm for 20 s and then incubated at the appropriate temperature for 24 h. The minimum inhibitory concentration (MIC) was determined as the lowest concentration that inhibited the growth of the microorganisms. Tetracycline was used as the standard antibiotic.

Measurement of plant height and root length

The effect of BMW on plant height and root length was determined compared to the control. The plant height and root

length of samples were measured after *in vitro* culture for four weeks. Values are presented as means ± standard deviations (SD).

Analysis of functional gene expression using reverse transcriptase-polymerase chain reaction (RT-PCR)

Four gene-specific primers (Table 1) were designed and used for amplification of the *RgActin* (EU526396), *RgAPX* (AY462246), *RgPAL1* (AF401636), and *RgrbcL* (GQ436719) genes. PCR amplification was carried out in a thermal cycler using 25 cycles of a 5 min predenaturation at 94°C, 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1 min elongation at 72°C; a final 10 min elongation was performed at 72°C. The relative expressions of *APX*, *PAL1*, and *rbcL* in the leaves of *R. glutinosa* at 0, 1, 12, 24, and 48-h post-treatment were determined.

In conclusion, this is the first report of the biological phenomena induced by BMW. The physical properties of DSW are essential to understanding the mechanisms underlying these results. Few studies have focused on the effect of BMW in plants. We found that BMW affects the antioxidant activities and growth in plants. These results establish the mechanism(s) underlying the function of BMW in plants. Furthermore, BMW had a marked influence on plant mineral content.

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