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Effect of Chitosan on the Biological Properties of Sweet Basil (*Ocimum basilicum* L.)

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The effect of the treatment of chitosan at various concentrations (0.01%, 0.05%, 0.1%, 0.5%, and 1%) upon sweet basil (*Ocimum basilicum* L.) before seeding and transplanting was investigated in aspects of the amount of phenolic and terpenic compounds, antioxidant activity, and growth of the basil, as well as the phenylalanine ammonia lyase (PAL) activity. The total amount of the phenolic and terpenic compounds increased after the chitosan treatment. Especially, the amounts of rosmarinic acid (RA) and eugenol increased 2.5 times and 2 times, respectively, by 0.1% and 0.5% chitosan treatment. Due to the significant induction of phenolic compounds, especially RA, the corresponding antioxidant activity assayed by the DPPH* (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging test increased at least 3.5-fold. Also, the activity of PAL, a key regulatory enzyme for the phenylpropanoid pathway, increased 32 times by 0.5% chitosan solution. Moreover, after the elicitor chitosan treatment, the growth in terms of the weight and height of the sweet basil significantly increased about 17% and 12%, respectively. Our study demonstrates that an elicitor such as chitosan can effectively induce phytochemicals in plants, which might be another alternative and effective means instead of genetic modification.

KEYWORDS: ***Ocimum basilicum*; basil; chitosan; natural antioxidants; secondary metabolite; rosmarinic acid; phenylalanine ammonia lyase; DPPH***

INTRODUCTION

Consumption of vegetables, fruits, and other edible plants has been continuously growing in recent years because epidemiological analysis and dietary intervention trials from the consumption of edible plants have provided compelling evidence of potential health benefits in humans (*1*, *2*), which have been to a large extent attributed to phytochemicals (*3*). Therefore, there is a high interest in increasing the amount of functional phytochemicals in food crops. Genetic engineering technique is an ad hoc approach that has been suggested and practiced for improving phytochemical profiles (*4*). However, in the face of public concerns about the safety of the genetically modified crops, alternative methods, such as wounding, elicitors, and UV-light exposure, upon crops to increase phytochemicals have also been suggested (*5*, *6*). Under various biotic or abiotic stresses, plants can produce or induce not only some compounds such as proteinase inhibitors (PI) directly associated with the defense system to protect themselves from stresses, but also secondary metabolites such as phenolic compounds and terpenoids (*7*−*9*) that have attracted a lot of research interest because of their bioactive functional properties (*10*).

One of the commonly known elicitors is chitosan that is a linear β-(1,4)-glucosamine polymer produced by deacetylation of chitin and is an important structural component of several plant fungi cell walls (*11*). It has been reported that chitosan is effective in inducing various plant defense responses such as induction of phytoalexins, PIs, lignification, phenylalnine ammonia lyase (PAL), and peroxidase (POD) (*12*−*14*). Chitosan was also reported to be a strong antimicrobial agent (*15*). However, the effect of chitosan on induction of secondary metabolites has not been actively investigated except for a few reports that chitosan could increase health-beneficial substances of some agricultural products (*16*, *17*).

In this study, the effect of chitosan on the physiochemical and biological properties of sweet basil (*Ocimum basilicum* L.) was investigated. Phenolic and terpenic compounds in sweet basil were separated and identified by high performance liquid chromatography (HPLC) and gas chromatography−mass spectrometry (GC−MS). Also, antioxidant activity against the DPPH* free radical by the separated phenolics from sweet basil was measured. The effects of chitosan on the induction of PAL and on the growth of sweet basil were also investigated.

METERAIALS AND METHODS

Chemicals. Chitosan (30 cP) was generously provided by Dr. Ham at Mokpo National University in South Korea. DPPH* (2,2-diphenyl-1-picrylhydrazyl), gallic acid, L-phenylalanine, Folin-Ciocalteu's reagent, β-mercaptoethanol, cinnamic acid, bovine serum albumin, and Bradford reagent were purchased from Sigma Chemical Co. (St. Louis, MO). Silica gel (70−230 mesh, 60 Å), eugenol, methyl eugenol, L-linalool, 1,8-cineole, and *n*-alkanes (C_8−C_{30}) were purchased from

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Aldrich Chemical Co. (Milwakee, WI). Rosmarinic acid was obtained from Cayman Chemical Co. (Ann Arbor, MI). All HPLC solvents in analytical grade were from Fisher Scientific (Suwanee, GA).

Plant Culture and Chitosan Treatment. Sweet basil seeds (*Ocimum basilicum* L.), purchased from a local supermarket (Clemson, SC), were soaked in various chitosan solutions (0.01%, 0.05%, 0.1%, 0.5%, and 1% dissolved in 0.5% lactic acid solution) for 30 min. Control seeds were soaked in 0.5% lactic acid and water. The seeds were sown into 48-cell trays containing a commercial potting mixture (Fafard 3-B Mix, Fafard Inc., Anderson, SC). The sweet basil was grown under natural light condition in the greenhouse located at the Clemson University main campus in Clemson, SC, and was watered every other day. Plants were fertilized once a week at irrigation with 1 g/L of a 20 N−4.4 P−16.7 K water solution fertilizer (Peters 20-10-20 Peat-Lite Special, Scotts-Sierra Horticultural Products Co., Marysville, OH) during the experiment. Greenhouse cooling/heating set points were 27/25 °C. At the second leaf stage, 15 sweet basil plants for each treatment were transplanted into 0.4 L plastic pots. Before transplanting, the roots of the basils were again dipped into the same treatment solutions for 30 min that had been applied to seeds.

Determination of the Growth of Sweet Basil Plants. The growth was measured in terms of fresh weight and height increase of the sweet basil plants, of which 10 plants (45-day-old) per each treatment were randomly selected. The weight of the stem and leaves of the sweet basil and the length from the top of the root to the end of the stem representing the height of the basil were measured.

Extraction of Phenolic and Terpenic Compounds from Sweet Basil. After harvesting, sweet basil plants (45-day-old) were ground in liquid nitrogen. Two grams of sweet basil power was mixed with 20 mL of 80% aqueous methanol to extract phenolic compounds, or 20 mL of methyl *tert*-butyl ether (MTBE) to extract terpenoids. The mixtures were shaken at room temperature for 12 h and then centrifuged at 2000*g* for 20 min. After centrifugation, the methanol and the MTBE supernatants were used for the determination of the crude phenolic and terpenic compounds in sweet basil.

Determination of the Total Amount of Phenolic Compounds. The total phenolic content in the sweet basil was determined using Folin-Ciocalteu's reagent by the method of Singleton and Rossi (*18*). Fifty microliters of the methanolic extract was mixed with 450 μL of distilled water and 250 μL of 2 N Folin-Ciocalteu reagents. The mixture was incubated with 1.25 mL of 20% Na_2CO_3 at 25 °C for 20 min and then centrifuged at 2000*g* for 10 min. The absorbance of the supernatant was spectrophotometrically measured at 735 nm. The standard curve was prepared using gallic acid.

DPPH* Free Radical Scavenging Activity. The DPPH* method of Yamaguchi et al. (*19*) was slightly modified in our study for the DPPH* free radical scavenging assay of the sweet basil methanolic extract. The reaction mixture containing 0.1 mL of the sweet basil methanolic extract, 0.4 mL of 0.1 M Tris-HCl (pH 7.4), and 0.5 mL of 0.3 mM DPPH* was shaken vigorously and incubated at room temperature for 20 min in the dark. However, due to the influence of TFA in the mobile phase, the DPPH solution was 10-times diluted, and 100 μL of the collected sample from HPLC column exit was mixed with 50 μL of diluted DPPH solution, 100 μL of methanol, and 100 μL of Tris-HCl buffer. Incubation time was also adjusted to 1 h. The free radical scavenging activity was spectrophotometrically measured at 517 nm. The scavenging activity of DPPH* free radical was calculated with the following formula:

$$\text{DPPH * free radical scavenging activity (\%)} = (1 - \text{absorbance of sample/absorbance of control}) \times 100 \quad (1)$$

EC_{50} value herein refers to the effective chitosan concentration at which DPPH* radicals were scavenged by 50%.

Separation and Identification of Phenolic Compounds. To separate major phenolic compounds, reverse phase C_{18}-high performance liquid chromatography (HPLC) was used. The Pinnacle II C_{18} column (150 × 4.6 mm, 5 μm; Restek, PA) was connected to the Shimadzu LC-10AT HPLC system (Kyoto, Japan) and equilibrated with HPLC water containing 0.05% trifluoroacetic acid (TFA). Fifty microliters of methanolic extract was injected and eluted with HPLC water containing 0.05% TFA and acetonitrile at a flow rate of 1 mL/min. Each fraction of the eluant collected at the detector exit was 1 mL. The absorbance of the eluant was scanned from 200 to 500 nm by a Shimadzu SPD-M10V photodiode array detector (Kyoto, Japan). Authentic standards were used to identify major phenolic compounds of sweet basil.

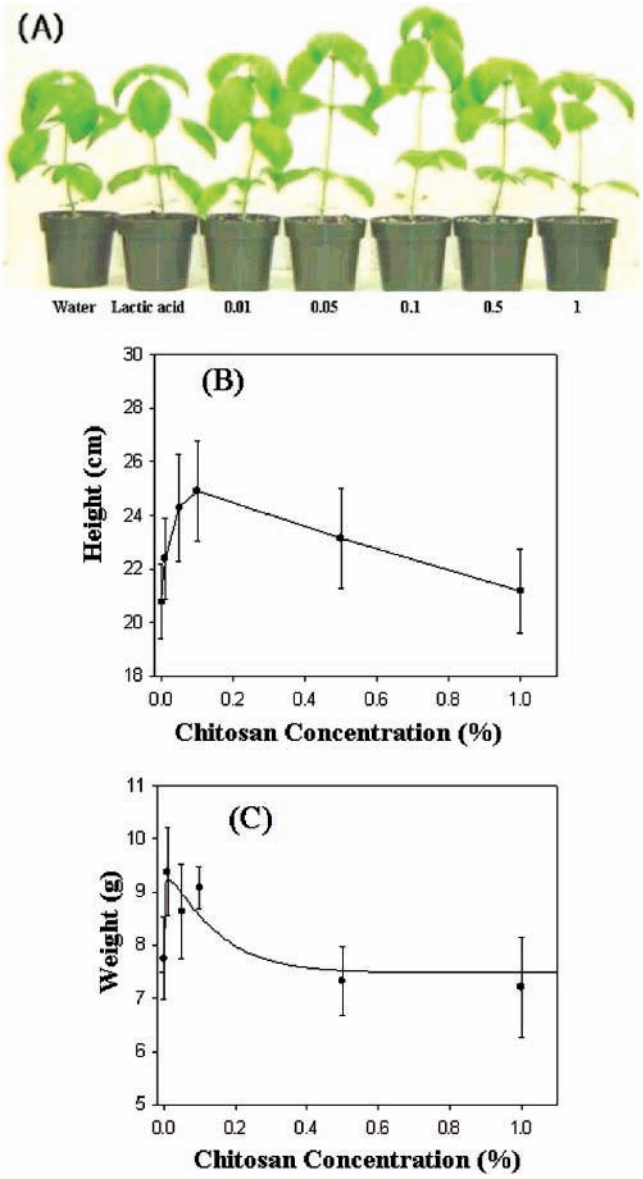

Figure 1. Effect of chitosan on the growth of sweet basil. (**A**) Appearance of 45-day-old sweet basil treated with various concentrations of chitosan, (**B**) height represented by the length from the top of the root to the end of the stem, and (**C**) fresh weight of the leaves and stem of the sweet basils measured.

Identification of the Terpenic Compounds. A DB-5 capillary column (60 m × 0.25 mm, thickness 0.25 μm; J&W Scientific, Folsom, CA) was installed in a Shimadzu GC-17A instrument that was also connected to a GCMS-QP 5050 mass spectrometer (MS) detector (Kyoto, Japan). The GC oven temperature was programmed from 60 to 240 °C at the rate of 3 °C /min and held at 240 °C for 10 min. The injector and ion source temperatures were 200 and 250 °C, respectively. The detector voltage was set at 70 eV, and the MS spectra were obtained in the mass range of *m*/*z* 43−350. Helium was used as the carrier gas at a flow rate of 1.1 mL/min. Three microliters of MTBE extract was injected in a split mode at 1:10. Identification of compounds was based on comparison with mass spectra and retention index (RI) of the authentic standards. The mass spectrum of each compound was also compared to those of the Wiley and NIST mass spectral databases.

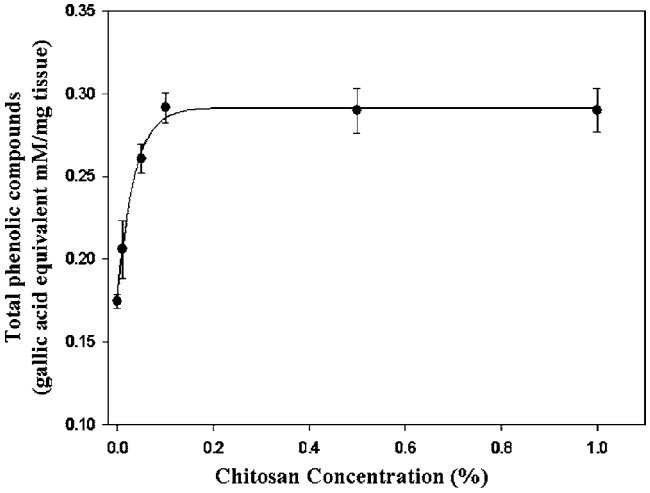

Figure 2. Total phenolic contents of sweet basil treated with various concentrations of chitosan. The amount of total phenolic compounds extracted by 80% methanol from sweet basil was spectrophotometrically determined at 735 nm. Gallic acid was used as a standard compound. Error bars in the figure were standard deviations of triplicate experiments.

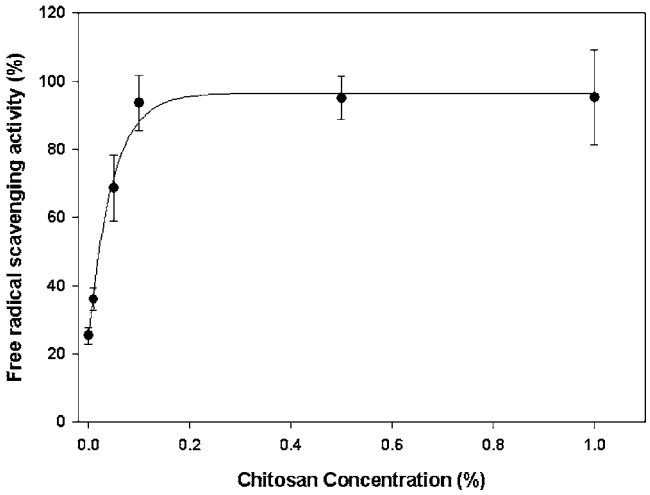

Figure 3. Free radical scavenging activity of phenolic compounds extracted from sweet basil treated with various concentrations of chitosan. DPPH free radical scavenging activity of sweet basil extract (100 μL/10 mg tissue) was spectrophotometrically measured at 517 nm. Error bars in the figure were standard deviations of triplicate experiments.

Determination of Phenylalanine Ammonia Lyase (PAL) Activity. To determine the activity of PAL that is a key regulatory enzyme of secondary metabolites in plants, the crude enzyme of the sweet basil was extracted by 0.1 N Na-borate buffer (pH 8.8) containing 15 mM β-mercaptoethanol. The extracts were precipitated in 80% ammonium sulfate, and the precipitate was dissolved in 0.1 N Na-borate buffer (pH 8.8). After centrifugation at 15 000*g* for 10 min, 500 μL of supernatant liquid was added into 1.5 mL of 0.1 N Na-borate buffer (pH 8.8) containing 10 mM L-phenylalanine. The reactant was incubated at 37 °C for 7 h, and then cinnamic acid produced by the reaction was extracted using toluene. The amount of cinnamic acid dissolved in toluene was measured at 290 nm by a spectrophotometer (*20*), and the PAL activity was expressed in ρkat. One katal was defined as the enzyme activity producing one mole of cinnamic acid equivalents per second. The amount of protein in the crude enzyme was measured by the Bradford assay (*21*) using bovine serum albumin as a standard.

Experimental Design and Data Analysis. To investigate the effect of chitosan on sweet basil, five chitosan concentrations and two controls as treatments were adopted under a randomized complete block design. Fifteen sweet basil plants were used in each treatment for three replicates. Five plants were randomly selected and placed at three randomly selected places in the greenhouse for each replicate. All experiments were in triplicate. Data were subjected to analysis of variance and were analyzed with nonlinear regressions (SAS 9.1, SAS Inst. Inc., Cary, NC).

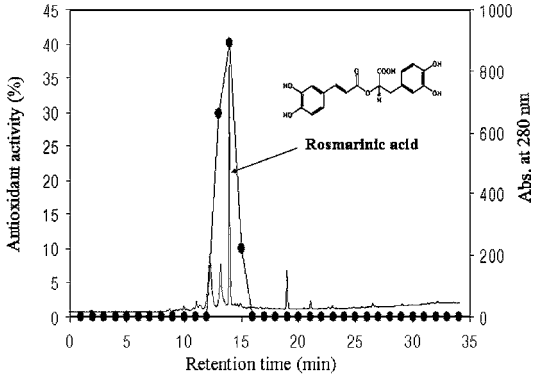

Figure 4. Separation of phenolic compounds from sweet basil using C_{18}-HPLC and the corresponding antioxidant activities of all collected fractions. The Pinnacle II C_{18} column (150 × 4.6 mm, 5 μm) was used with mobile phase of water containing 0.05% TFA and acetonitrile at a flow rate of 1 mL/min. Absorbance of the eluant was scanned from 200 to 500 nm by PDA. Antioxidant activities of all collected fractions were determined using DPPH assay.

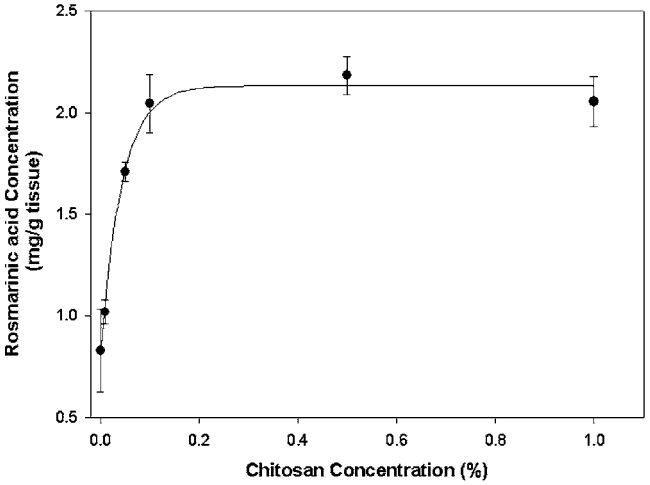

Figure 5. Rosmarinic acid in sweet basil treated with various concentrations of chitosan. Rosmarinic acid was separated by C_{18}-HPLC and determined at 280 nm, and its concentration was measured using the standard curve of rosmarinic acid. Error bars in the figure were standard deviations of triplicate experiments.

RESULTS AND DISCUSSION

Effect of Chitosan on the Growth of Sweet Basil. The growth of sweet basil (**Figure 1A**) increased with the increasing chitosan concentration up to 0.1%, at which the height (**Figure 1B**) and fresh weight (**Figure 1C**) significantly increased about 17% and 12%, respectively. There was no significant difference in height or fresh weight between two control treatments, for example, the 0.5% lactic acid solution and water, on the growth of sweet basil. However, growth of the sweet basil smoothly decreased after the treatment at higher chitosan concentrations (0.5% and 1%). Such observation was similar to those in the previous reports on growth of soybean sprout, *vitis vinifera* L., and laver (*22*−*24*) using chitosan treatment. It was attributed to the higher concentration of chitosan coated outside the seed and root of the plants during the seeding and transplanting, which might have caused an absorptive obstruction of water

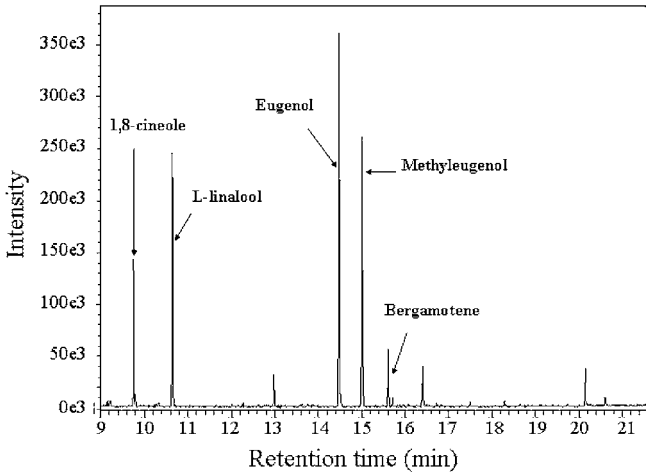

Figure 6. Identification of terpenic compounds from sweet basil using GC–MS. Shimadzu GC-17A GC system was equipped with a DB-5 capillary column (60 m × 0.25 mm, thickness 0.25 μm) and GCMS-QP 5050 mass spectrometer (MS) detector.

and other essential minerals from soil due to the sticky chitosan coating (*24*). In addition, some other reports have indirectly ascribed the effect of chitosan on plant growth to its strong antimicrobial capacity (*25*, *26*) that helps the protection of plants against phytopathogens. Nevertheless, so far the mechanism of the expressed stress response, for example, the significant change of the growth of basil and other plants, induced by chitosan is still not well understood.

Effect of Chitosan on Total Phenolic Content and Antioxidant Activity of Sweet Basil. There was no significant difference in total phenolic content (0.18 gallic acid equivalents mg/mg fresh tissue) between two control treatments (water and 0.5% lactic acid). In contrast, the total phenolic content significantly increased by chitosan treatments (**Figure 2**). The total amount of phenolic compounds reached a maximal amount of 0.29 gallic acid equivalents (mg/mg fresh tissue) at 0.1% chitosan treatment. No further increase in total phenolic content was observed in 0.5% or 1% treatment. This is in agreement with previous reports that chitosan at low concentrations could induce phenolic compounds in soybean cells and tomato leaves (*27*, *28*).

The extract of chitosan treated plants had significantly greater DPPH* free radical scavenging activities than that of the control plants (**Figure 3**). When the chitosan treatment (concentration) was taken into consideration for the same volume of basil extracts against DPPH* free radicals, the EC_{50} value of chitosan concentration was 0.02% and the scavenging activity was saturated (94%) in 0.1% chitosan treatment, which was at least 3.5-fold greater than the activity of control plants (27%). No further increase in free radical scavenging activity occurred when the chitosan concentration increased to 0.5% or 1%.

Moreover, to investigate the antioxidant capacity of the phenolic compounds that were extracted from sweet basil, those showing strong free radical scavenging activity were separated by chromatographic columns, and a major compound was identified.

Among the various phenolic compounds, rosmarinic acid (RA) was the major one in sweet basil (**Figure 4**). Rosmarinic acid has been reported to have various bioactive properties such as antioxidant, antimicrobial, and antiinflammatory activities (*29*, *30*). The DPPH* free radical scavenging activity of HPLC fractions showed that the RA fraction was the major contributor to the antioxidant activity in the methanolic extract of sweet basil (**Figure 4**). The amount of RA in the sweet basil increased up to 0.1% chitosan treatment, and the highest level (2.0 mg/g fresh tissue; **Figure 5**) was close to the accumulated amount of RA in *Coleus blumei* treated by methyl jasmonate and *Pythium* elicitor (*31*). In an interesting coincidence, the general profile of the antioxidant activity of the sweet basil extracts (**Figure 3**) was very similar to that of the induced total amount of phenolic compounds (**Figure 2**) and RA amount in the same basil extracts (**Figure 5**). This implicated that the induced phenolic compounds, especially RA in sweet basil by chitosan

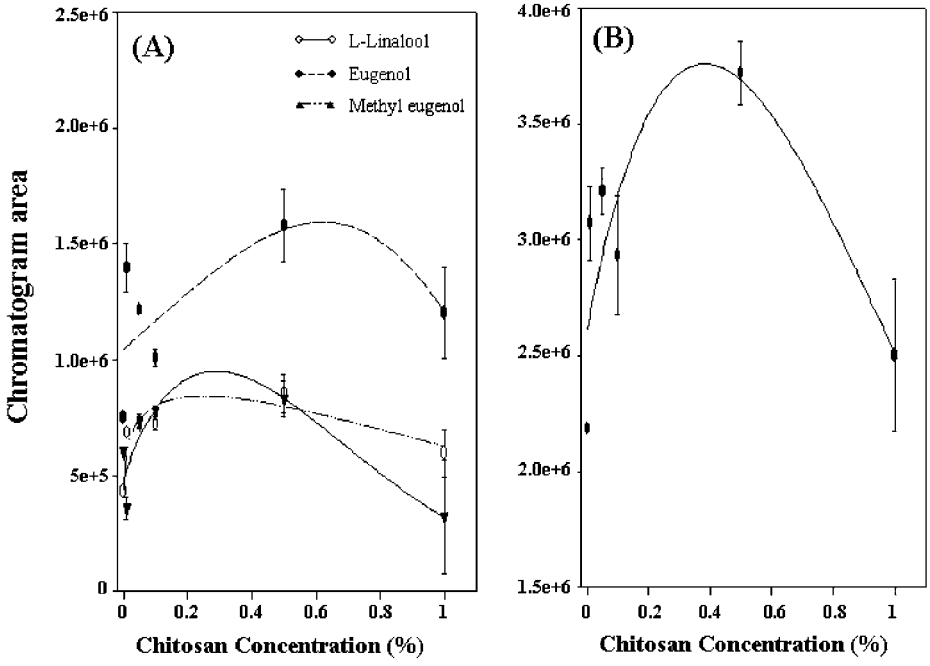

Figure 7. Effect of chitosan on (**A**) terpenic compounds and (**B**) total amount of sweet basil essential oil extracted by MTBE. Major essential oil compounds extracted by MTBE were identified by GC–MS. The total amount of sweet basil essential oil was expressed by the total chromatographic area of five identified compounds (1,8-cineole, L-linalool, eugenol, methyl eugenol, and bergamotene) and three unknown compounds. Error bars in the figure were standard deviations of triplicate experiments.

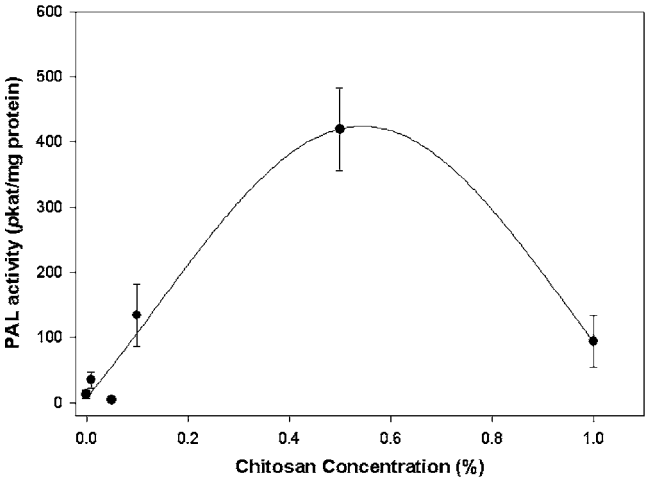

Figure 8. Phenylalanine ammonia lyase (PAL) activity of sweet basil treated with various chitosan solutions. The crude enzyme was extracted by 0.1 N Na-borate (pH 8.8) containing 15 mM β-mercaptoethanol and precipitated in 80% ammonium sulfate. The amount of cinnamic acid produced from phenylalanine by PAL was measured at 290 nm by a spectrophotometer, and the amount of protein in the crude enzyme was measured by the Bradford assay using bovine serum albumin as a standard. Error bars in the figure were standard deviations of triplicate experiments.

treatment, might be responsible for the DPPH* free radical scavenging activity of the basil extract.

Effect of Chitosan on Terpenoid Content of Sweet Basil. Five major terpenic compounds (1,8-cineole, L-linalool, eugenol, methyl eugenol, and bergamotene) were identified in the MTBE extract by GC/MS (**Figure 6**). Eugenol was the major compound in terpenoid fraction and accounted for 33% of the total terpenoids. The contents of methyl eugenol, L-linalool, 1,8-cineole, and bergamotene were in a descending order of 25%, 19%, 11%, and 5%, respectively. However, methylchavicol and methyl cinnamate that were reported as major aromas in European and réunion type and tropical type, respectively, were not present in our treated sweet basil used for this research, which suggested the composition of basil volatile compounds depended on the chemotypes and genotypes of basils (*32*).

The amounts of eugenol and L-linalool, which are produced via the phenylpropanoid and mevalonic acid pathways, respectively, and have been reported to possess various bioactivities (*33*, *34*), increased by chitosan treatment as compared to those in the control (**Figure 7A**). At 0.5% chitosan treatment, the induced amounts of both eugenol and L-linalool in sweet basil were twice as much as those in the control. The amount of methyl eugenol that was toxicologically concerned because of its structural resemblance to known carcinogenic compounds such as estragole and safrole (*35*) also increased by chitosan treatments, but the methyl eugenol content decreased at the high concentration (1%). The total amount of the above-mentioned five terpenoids in the sweet basil increased after the chitosan treatment, and a similar downward bell-shaped response curve with an optimum concentration at about 0.4% was observed (**Figure 7B**). At the chitosan optimum concentration, the total amount of terpenoids was about 45% higher than that of the control. These data were similar to the previously reported results that UV-B treatment or the color mulching increased essential oil content in basil (*36*, *37*). However, there were reports that mechanical wounding did not induce essential oil content except for linalool (*38*).

Determination of Phenylalanine Ammonia Lyase (PAL) Activity. To investigate the relationship between induction of the secondary metabolites and the chitosan treatment, the activity of PAL, a key regulatory enzyme that produces many phenolic compounds in plants through the phenylpropanoid pathway, was determined. As shown in **Figure 8**, chitosan treatment significantly increased the PAL activity. The highest PAL activity (419 ρkat/mg protein) was observed at 0.5% chitosan treatment with an activity that was 32 times greater than that of the control (13 ρkat/mg protein), while the sweet basil treated with the highest chitosan concentration (1%) had only 7 times higher PAL activity than that of control. This result indicated that chitosan, like other elicitors, such as β-glucan and yeast extract (*14*, *39*), could induce secondary metabolites through the phenylpropanoid pathway in plants without any stresses. Moreover, most importantly our study demonstrates that chitosan can possibly and effectively induce phytochemicals in edible and medicinal plants without any genetic modification.

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