J. Microbiol. Biotechnol. (2011), **21**(2), 192–196
doi: 10.4014/jmb.1008.08010
First published online 24 November 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Enzymes Hydrolyzing Structural Components and Ferrous Ion Cause Rusty-root Symptom on Ginseng (*Panax ginseng*)

Lee, Chanyong[1], Kwang Yup Kim[2], Jo-Eun Lee[3], Sunghan Kim[3], Dongkul Ryu[3], Jae-Eul Choi[4], and Gilhwan An[3]*

[1]*Department of Microbiology and Biotechnology, Daejeon University, Daejeon 300-716, Korea*
[2]*Department of Food Science and Technology and Research Center for Bioresource and Health, Chungbuk National University, Cheongju 361-763, Korea*
[3]*Department of Food Science and Technology, Chungnam National University, Daejon 305-764, Korea*
[4]*Department of Crop Science, Chungnam National University, Daejon 305-764, Korea*

Received: August 10, 2010 / Revised: November 5, 2010 / Accepted: November 11, 2010

Microbial induction of rusty-root was proved in this study. The enzymes hydrolyzing plant structural materials, including pectinase, pectolyase, ligninase, and cellulase, caused the rusty-root in ginseng. Pectinase and pectolyase produced the highest rusty-color formation. Ferrous ion (Fe^{+++}) caused the synergistic effect on rusty-root formation in ginseng when it was used with pectinase. The effect of ferric ion (Fe^{++}) on rusty-root formation was slow, compared with Fe^{+++}, probably due to gradual oxidation to Fe^{+++}. Other metal ions including the ferric ion (Fe^{++}) did not affect rusty-root formation. The endophytic bacteria *Agrobacterium tumefaciens*, *Lysobacter gummosus*, *Pseudomonas veronii*, *Pseudomonas marginalis*, *Rhodococcus erythropolis*, and *Rhodococcus globerulus*, and the rotten-root forming phytophathogenic fungus *Cylindrocarpon destructans*, caused rusty-root. The polyphenol formation (rusty color) was not significantly different between microorganisms. The rotten-root-forming *C. destructans* produced large quantities of external cellulase activity (≈2.3 U[μM/min/mg protein]), which indicated the pathogenecity of the fungus, whereas the bacteria produced 0.1–0.7 U. The fungal external pectinase activities (0.05 U) and rusty-root formation activity were similar to those of the bacteria. In this report, we proved that microbial hydrolyzing enzymes caused rusty-root (Hue value 15°) of ginseng, and ferrous ion worsened the symptom.

Keywords: Rusty-root, *Cylindrocarpon destructans*, Fe(III), pectinase, ginseng

Ginseng (*Panax ginseng*) is one of the most important crops in Korea for herbal medicine. About $500 million of ginseng was produced in Korea in 2006 [12]. Ginseng has become popular in North America: the annual average production of ginseng during 1999–2003 was 971 tons in Ontario [22], 741 tons in British Columbia, and 296 tons in Wisconsin [1, 11]. However, the major diseases of ginseng block its production. The most important diseases of ginseng are rotten-root and rusty-root [2, 19, 20]. Rusty-root contains brown spots on the surface of ginseng and decreases its value. Rusty-root formation is slow, and thus if it is detected, farmers will harvest. Rotten-root is caused by the fungus *Cylindrocarpon destructans*. The symptom starts with rusty-root, but the development to rotten-root is fast, taking 2 weeks. Once the disease is found, farmers give up on harvesting because the ginseng structure is no longer rigid [2].

Many studies have reported the cause of rusty-root. The severe damages in ginseng cultivations are due to *Pseudomonas marginalis*, *Microbacterium oxydans*, *Lysobacter gummosus*, *Rhizobium Leguminosarum*, *Pseudomonas veronii*, and *Agrobacterium tumefaciens* [5]. However, the results are controversial. Certain reports indicated that rusty-root symptom was caused by microorganisms. The Gram-negative bacterium *Pseudomonas panacis*, was detected in rusty-root [13], possibly a cause of rusty-root of ginseng. Reeleder *et al*. [16] reported that a species of the fungus, *Rhexocercosporidium*, was only detected in rusty-root and proposed the fungus was a cause of rusty-root. Furthermore, Choi *et al*. [5] isolated *Agrobacterium tumefaciens*, *Pseudomonas marginalis*, *Rhodococcus erythropolis*, *R. globerulus*, *Lysobacter gummosus*, and *P. veronii* in the rusty part of ginseng as endophytes. They confirmed the formation of rusty-root after inoculation of the bacteria on the surface of ginseng. Although the fungus *Cylindrocarpon destructans* is a phytopathogen causing rotten-root of ginseng, it also causes rusty-root symptom at the beginning

*Corresponding author
Phone: +82-42-821-7592; Fax: +82-42-823-4835;
E-mail: ghahn@cnu.kr

of the disease [17, 18]. On the surface of rusty parts of ginseng, a large number of microorganisms were usually detected [4] and the treatment of antibiotics inhibited the formation of rusty-root [14].

Many reports have mentioned the cause of rusty-root to be by chemical reasons. Yang *et al*. [21] reported that the production of phenolic compounds and its transportation to the outside skin of ginseng was the cause of rusty-root. The inoculation of *Agrobacterium tumefaciens, Lysobacter gummosus*, and *P. veronii* caused an increase in the production of polyphenols [7]. In the rusty part of ginseng, large quantities of Fe were present [4, 6, 21].

In this study, we aimed to find the cause of rusty-root. Since polyphenols usually inhibit the growth of microorganisms, we hypothesized that ginseng produced polyphenols against intruding microorganisms. We also hypothesized that to penetrate the ginseng, microorganisms excreted hydrolyzing enzymes, and ginseng responded to these enzymes. Furthermore, since Fe accumulated on the surface of rusty-root, we tested whether Fe ions increased the rusty-root formation. Since Fe is a metal, other metal ions were also tested for their influence on rusty-root. In this paper, we propose that hydrolyzing enzymes for structural components and the ferrous ion are causes of rusty-root.

Materials and Methods

Materials

The enzymes cellulase (Sigma), pectinase (Sigma), pectolyase (Sigma), pectinesterase (Sigma), and lignin peroxidase (Fluka) were used for detection of rusty-root formation activity and enzyme standards. The metal ions from MnO (Alfa Aesar), Mn_3O_4 (Daejung, Korea), Cu_2O (Daejung), CuO (Daejung), $FeCl_2{\cdot}4H_2O$ (Kanto), and $FeCl_3{\cdot}6H_2O$ (Kanto, Japan) were tested for synergistic effect on rusty-root formation. The 11 bacterial strains used here were isolated from a previous study [5]. The degrees of rusty-color formation were severe by *Pseudomonas veronii* (CG123, CG124); mild by *Agrobacterium tumefaciens* (CG101, CG102, CG129), *Rhodococcus erythropolis* (CG114), *Rhodococcus globerulus* (CG125), *Pseudomonas marginalis* (CG104), and *Pseudomonas veronii* (CG125); and slight by *Lysobacter gummosus* (CG116, CG117). The degree of rusty-color formation by *Cylindrocarpon destructans* (CD001) was not determined previously. These bacteria were grown in King's medium (peptone 20 g/l; K_2HPO_4 1.5 g/l; $MgSO_4{\cdot}7H_2O$ 1.5 g/l; and glycerol 15 ml/l) for 24 h with orbital shaking. *Cylindrocarpon destructans* (KACC 410077) was obtained from KACC (Korea Agricultural Culture Collection, Suwon, Korea) and grown in PDB medium (potato dextrose broth; Difco, USA) for 2 weeks. the ginseng (3–4 years old) used in this study was purchased from the Kumsan Ginseng Center (Kumsan, Korea) and kept in a refrigerator.

Rusty-Root Formation and Measurement

Ginseng was cut with sterilized knifes to fit in petri dishes. It was prepared in two ways: wound and raw. For wound ginseng, its surface was scraped with sand paper (# 220). The ginseng was treated with enzymes, microorganisms ($\approx 10^{6-7}$ CFU/ml), and metal ions by covering with filter paper discs (Toyo Roshi Kaisha, Ltd. lot no. 60421693; Tokyo, Japan). The bacteria used here were found as the endophytic bacteria on the rusty-root spots [4]. In addition, the rotten-root forming phytopathogenic fungus *Cylindrocarpon destructans* was used.

The solution for the treatment contained a hydrolyzing enzyme at 1 unit (1 μM substrate/min/mg protein) and metal ions at 3% (w/v). One ml each of the treating solution was applied on the paper discs. The sample ginseng was laid on sterilized cotton balls containing phthalate-sodium hydroxide buffer (pH4.6) and kept at 20°C. The degree of rusty-root symptom was determined by the Color Difference Meter (Model CR-300; Minolta, Tokyo, Japan). The obtained Hunter values (L, a, b) were transformed to Hue value by the method of McGuire [8]: Hue value (°)=$[\tan^{-1}(b/a\times 2\pi)]\times 360$.

Pectinase and Cellulase Activities

Pectinase and cellulase activities were determined by measuring the produced reducing sugar after reaction with pectin (Fulka, catalog no. 76280) and cellulose (Sigma, catalog no. C6288), respectively. The reducing sugar was measured by the DNS method [9, 10]. The endophytic microorganisms grew in YNB medium (Yeast Nitrogen Base without amino acids; Difco, Detroit, MI, USA) containing 1% (w/v) of substrate (pectin or cellulose) for 2 days with orbital shaking. Extracellular enzyme activity was measured with growth culture supernatant obtained after centrifugation in a table-top centrifuge at 14,000 rpm for 5 min. Intracellular enzyme activity was measured with supernatant of cell homogenates obtained by Fastprep FP120 (Thermo Fisher Scientific Inc., Waltham, MA, USA). Citrate buffer (50 mM, pH 4) containing 1% of substrate (pectin or cellulose) and an equal volume of supernatant of cell homogenate were mixed and incubated at 50°C for 60 min. After reaction, the same volume of DNS solution was mixed with the sample solution and incubated at 100°C for 10 min. After cooling, absorbances at 560 nm and 540 nm were measured.

Galacturonic acid and glucose were used as standards. The Bradford method [22] was used for protein quantification and bovine serum albumin was used as the standard (Bio-Rad Protein Assay Dye Reagent Concentrate; Bio-Rad Laboratories, Inc., CA, USA). All samples were prepared in triplicate.

Results and Discussion

Since the brown spots on rusty-root were caused by phenolics [21] and inoculation of microorganisms on the surface of ginseng caused brown spots, it might be caused by invasion of microorganisms through hydrolyzing enzymes. Therefore, the enzymes hydrolyzing the ginseng structure components were tested for the cause of rusty-root. All the hydrolyzing enzymes used (pectinase, pectolyase, ligninase and cellulase) caused rusty-root (Fig. 1). However, the non-hydrolyzing enzyme pectin esterase did not affect the rusty-root formation (Fig. 1). We also tested with unwounded ginseng and the rusty color was significantly formed compared with the control, which was not treated with

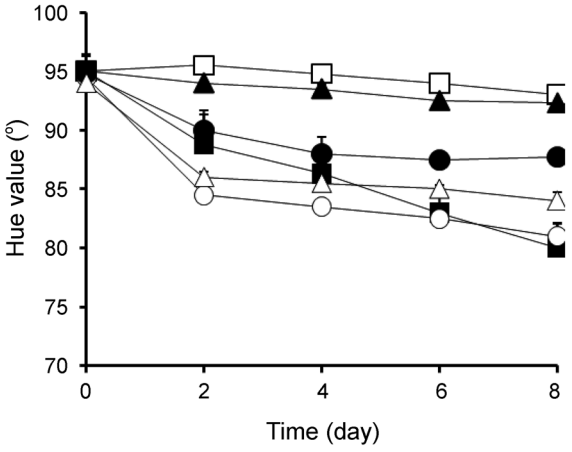

Fig. 1. Effects of hydrolytic enzymes on rusty-root formation.
Symbols: □, control, not treated; ■, pectinase; ▲, pectin esterase; ○, pectolyase; △, ligninase; and ●, cellulase.

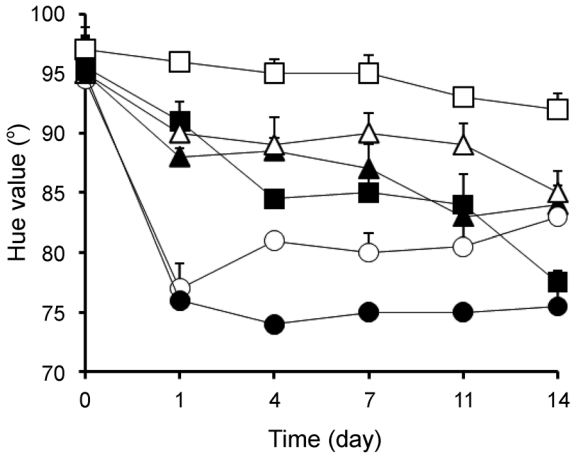

Fig. 2. Effect of Fe ions on rusty-root formation.
Symbols: □, control, not treated; ■, pectinase; ▲, $FeCl_2$; ○, $FeCl_3$; △, pectinase + $FeCl_2$; and ●, pectinase + $FeCl_3$.

enzymes (data not shown). We detected pectinase activity in rusty-root, but the level was too low and thus it was hard to determine if it came from endophytic bacteria or excreted enzyme (data not shown). Probably, pectin hydrolysis was mild and the detection was very limited. However, cellulase in rotten-root was detected, indicating a direct involvement of cellulase (unpublished data).

The rusty-root contained an increased amount of Fe [4] and thus the effect of Fe on rusty-root formation was determined. Ions of Fe, Mn, Mg, Ca, Na, K, and Cd were tested, but only Fe^{+++} affected the formation of rusty-root (data not shown). Therefore, the effects of Fe ions and pectinase on rusty-root formation were further tested.

Fe^{+++} increased rusty-color formation on ginseng at the early stage of treatment. Probably, Fe^{+++} might bind to ginseng and the rusty color of Fe^{+++} caused the rusty-root. However, Fe^{++} gradually did the same during 2 weeks of experimental period (Fig. 2). Iron in the Fe^{++}–polyphenol complex autooxidizes to Fe^{+++} in the presence of O_2 [15]. This result suggested that Fe^{++} oxidized to Fe^{+++} during incubation, but it might be a slow process because of limited O_2 under soil in natural rusty-root formation or aqueous condition in the laboratory. Pectinase (Fig. 2) and other hydrolyzing enzymes (Fig. 1) also increased the rusty color during the experimental period. By hydrolyzing cell structural compounds, polyphenols might be exposed to oxidation conditions and then polyphenols oxidation caused rusty-color formation [7]. Therefore, the rusty-color formation seemed to be due to two sources: Fe^{+++} and oxidized polyphenols. The effect of pectinase + $FeCl_2$ on the rusty-color formation was less severe than the effect of the $FeCl_2$ or pectinase alone (Fig. 2). Pectinase from *Acrophialophora nainiana* was inhibited by Fe^{++}, Cu^{++}, Zn^{++}, Mn^{++}, Al^{+++}, and Ca^{++} [3]. Therefore, pectinase + Fe^{++} was less effective than pectinase alone. Probably, the pectinase–Fe^{++} interaction [3] or exposed polyphenol–Fe^{++} complex formation [15] inhibited the oxidation of Fe^{++} to Fe^{+++}, resulting in less effect of Fe^{++} + pectinase than Fe^{++} alone on rusty-color formation. Fe^{+++} and pectinase synergistically increased rusty-root formation (Fig. 2). Polyphenols might stabilize the rusty color by forming a Fe^{+++}–polyphenol complex [15].

Microbial Effect

Since endophytic microorganisms caused rusty-color formation on ginseng [5], the bacterial activity of the rusty-color formation was tested. The rotten-root forming phytopathogenic fungus *Cylindrocarpon destructans* was also tested because it caused rusty-root [17]. All endophytic microorganisms and the fungus increased rusty-color

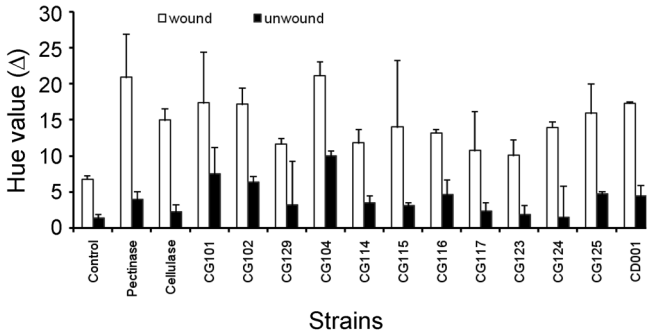

Fig. 3. Effects of microorganisms on rusty-root formation on wound and raw surfaces.
Error bars indicate the standard deviation. Strains: CG101, *Agrobacterium tumefaciens*; CG102, *Agrobacterium tumefaciens*; CG129, *Agrobacterium tumefaciens*; CG104, *Pseudomonas marginalis*; CG114, *Rhodococcus erythropolis*; CG115, *Rhodococcus globerulus*; CG116, *Lysobacter gummosus*; CG117, *Lysobacter gummosus*; CG123, *Pseudomonas veronii*; CG124, *Pseudomonas veronii*; CG125, *Pseudomonas veronii*; CD001, *Cylindrocarpon destructans.*

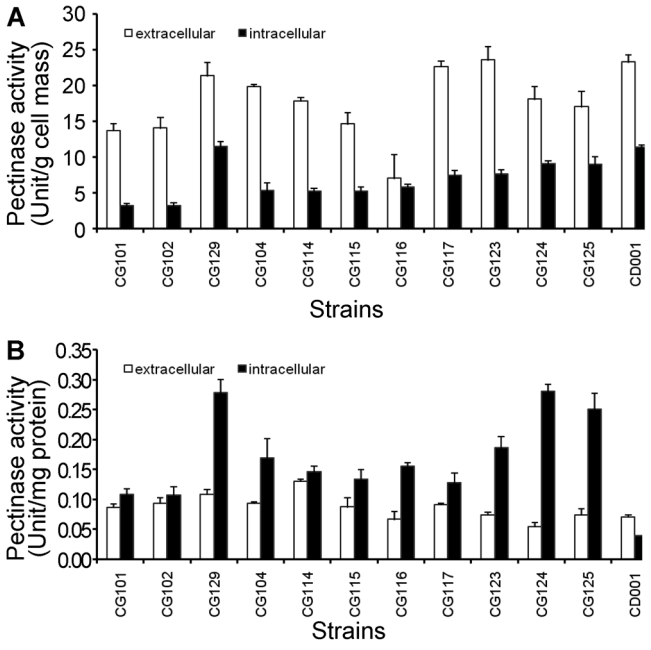

Fig. 4. Pectinase activity of microorganisms causing rusty-root. (**A**) Pectinase (Unit/g cell mass). (**B**) Pectinase (Unit/mg protein). Unit indicates μmol/min. Error bars indicate the standard deviation. Strains: CG101, *Agrobacterium tumefaciens*; CG102, *Agrobacterium tumefaciens*; CG129, *Agrobacterium tumefaciens*; CG104, *Pseudomonas marginalis*; CG114, *Rhodococcus erythropolis*; CG115, *Rhodococcus globerulus*; CG116, *Lysobacter gummosus*; CG117, *Lysobacter gummosus*; CG123, *Pseudomonas veronii*; CG124, *Pseudomonas veronii*; CG125, *Pseudomonas veronii*; CD001, *Cylindrocarpon destructans.*

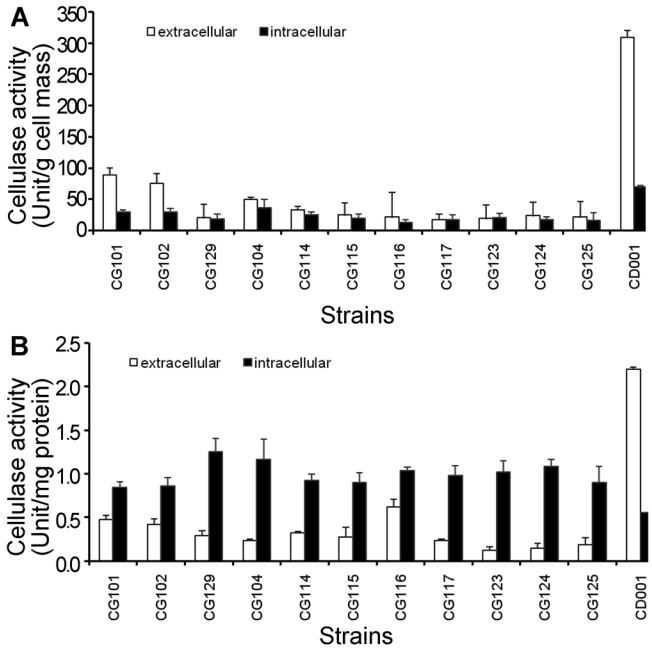

Fig. 5. Cellulase activity of microorganisms causing rusty-root. (**A**) Cellulase (Unit/g cell mass). (**B**) Cellulase (Unit/mg protein). Unit indicates μmol/min. Error bars indicate the standard deviation. Strains: CG101, *Agrobacterium tumefaciens*; CG102 *Agrobacterium tumefaciens*; CG129, *Agrobacterium tumefaciens*; CG104, *Pseudomonas marginalis*; CG114, *Rhodococcus erythropolis*; CG115, *Rhodococcus globerulus*; CG116, *Lysobacter gummosus*; CG117, *Lysobacter gummosus*; CG123, *Pseudomonas veronii*; CG124, *Pseudomonas veronii*; CG125, *Pseudomonas veronii*; CD001, *Cylindrocarpon destructans.*

formation compared with the control (Fig. 3). The degree of rusty-color formation in this study (Fig. 3) was different from previous results [5]. It was probably due to the differences in inoculated bacterial cell mass. In this study (Fig. 3), the same amount of bacteria was applied after measuring the cell mass by the turbidity at 600 nm.

The degree of rusty-color formation and the pectinase activity did not correlate (Fig. 3 and 4). Since pectinase, pectolyase, cellulase, and ligninase caused the rusty color, it could be produced by a complicated work of those hydrolyzing enzymes. Therefore, the pectinase activity might not correlate with rusty-color formation. Moreover, the enzyme activity could be expressed by four ways as shown in Fig. 4, and thus it might be hard to match with the rusty color. Another hydrolyzing enzyme, cellulase, was also tested to explain the discrepancy in pectinase activity. Interestingly, the extra- and intracellular cellulase activities (unit/g) showed meaningful Pearson correlation coefficients with the Hue values of wound ginseng at 0.43 and 0.55, with mild significance at p=0.16 and p=0.07, respectively (data not shown). Interestingly, the cellulase activity of bacteria was higher than the pectinase activity by about 4-fold (Fig. 4 and 5). These results suggested that the major hydrolyzing enzyme activity mainly affected the rusty-color formation.

The cellulase activity of the bacteria was significantly lower than that of the fungus (Fig. 5). *C. destructans* is an important rotten-root forming fungus. The cellulase activity of the fungus, especially secreting enzyme level, was significantly higher than the other bacteria. When cellulase was treated on the surface of ginseng, 3-day treatment (at 1 unit/cm surface of ginseng) was enough to break the physical shape of ginseng. Therefore, the significant toxicity of *C. destructans* was at least partly due to the strong cellulase activity.

Conclusively, the enzymes tested in this study (pectinase, cellulase and ligninase) caused rusty-root color formation and Fe^{+++} synergistically worsened the symptom.

Acknowledgments

This study was supported by the Research and Development Project on Area-based Development in Agriculture, Rural Development Administration, Republic of Korea (Project No. 2007030107000102) and the Technology Development

Program (Project No. 607003-05-2-SB120 and 308023-03-1-HD-110) of the Ministry of Food, Agriculture, Forestry and Fisheries, Republic of Korea.

REFERENCES

1. Anonymous. British Columbia Ministry of Agriculture, Food, and Fisheries. 2003. *Annual BC Horticultural Statistics–2003*. Statistics and Economics Unit, Policy and Economics Branch, Victoria, BC.
2. Brammall, R. A. 1994. Ginseng, pp. 294–299. *In* R. J. Howard, A. J. Garland, and W. L. Seaman (eds.). *Diseases and Pests of Vegetable Crops in Canada*. Phytopathological Society and Entomological Society of Canada, Ottawa, Ontario, Canada.
3. Celestino, S. M. C., S. Maria de Freitas, F. Javier Medrano, M. Valle de Sousa, and E. X. F. Filho. 2006. Purification and characterization of a novel pectinase from *Acrophialophora nainiana* with emphasis on its physicochemical properties. *J. Biotechnol*. **123:** 33–42.
4. Choi, J. E., J. S. Lee, S. M. Yoon, and S. K. Cha. 2002. Comparison of inorganic elements and epidermis structures in healthy and rusty ginseng. *Korean J. Crop Sci*. **47:** 161–166.
5. Choi, J. E., J. A. Ryuk, J. H. Kim, C. H. Choi, J. Chun, Y. J. Kim, and H. B. Lee. 2005. Identification of endophytic bacteria isolated from rusty-colored root of Korean ginseng (*Panax ginseng*) and its induction. *Korean J. Med. Crop Sci.* **13:** 1–5.
6. Lee, S. S., M. G. Lee, and K. T. Choi. 1999. Rusty-root tolerance and chemical components in 4-year-old ginseng superior lines. *J. Ginseng Res*. **23:** 61–66.
7. Lim, T. K., H. W. Park, Y. S. Hwang, and J. E. Choi. 2007. Potential role of polyphenolics and polyphenol oxidase on the induction of browning in ginseng roots. *Korean J. Crop Sci*. **52:** 289–295.
8. McGuire, R. G. 1992. Reporting of objective color measurements. *HortScience* **27:** 1254–1255.
9. Miller, G. L., R. Blum, N. E. Gennon, and A. L. Burton. 1960. Measurement of carboxymethyl-cellulase activity. *Anal. Biochem*. **1:** 127–132.
10. Minjares-Cassanco, A., B. A. Trejor-Aguillas, and G. Aguilar. 1997. Physiological comparison between pectinase producing mutants of *Aspergillus niger* adopted either to solid state fermentation or submerged fermentation. *Enzyme Microb. Technol*. **21:** 26–27.
11. Nicholson, P., D. R. Simpson, G. Weston, H. N. Rezanoor, A. K. Lees, D. W. Parry, and D. Joyce. 1998. Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assays. *Physiol. Mol. Plant Pathol*. **53:** 17–37.
12. Park, C. K., Y. S. Kwak, M. S. Hwang, S. C. Kim, and J. H. Do. 2007. Current status of ginseng (red ginseng) products in market of health functional food. *the Korean Ginseng Res. Ind.* **1:** 9–16.
13. Park, Y. D., H. B. Lee, H. Yi, Y. Kim, K. S. Bae, J. E. Choi, H. S. Jung, and J. Chun. 2005. *Pseudomonas panacis* sp. nov., isolated from the surface of rusty-root of Korean ginseng. *Int. J. Syst. Evol. Microbiol*. **55:** 1721–1724.
14. Park, H. W., E. J. Lee, and J. E. Choi. 2006. Inhibitory effect of disinfectants and antibiotics on rusty-root symptoms in *Panax ginseng* C. A. Meyer. *Korean J. Med. Crop Sci.* **14:** 336–341.
15. Perron, N. R., H. C. Wang, S. N. Deguire, M. Jenkins, M. Lawson, and J. L. Brumaghim. 2010. Kinetics of iron oxidation upon polyphenol binding. *Dalton Trans*. **39:** 9982–9987
16. Reeleder, R. D., S. M. Hoke, and Y. Zhang. 2006. Rusted root of ginseng (*Panax quinquefolius*) is caused by a species of *Rhexocercosporidium*. *Phytopathology* **96:** 1243–1254.
17. Rahman, M. and Z. K. Punja. 2005. Factors influencing development of rotten-root on ginseng caused by *Cylindrocarpon destructans. Phytopathology* **95:** 1381–1390.
18. Rahman, M. and Z. K. Punja. 2006. Influence of iron on *Cylindrocarpon* root rot development on ginseng. *Phytopathology* **96:** 1179–1187.
19. Reeleder, R. D., R. Roy, and B. Capell. 2002. Seed and rotten-roots of ginseng (*Panax quinquefolius*) caused by *Cylindrocarpon destructans* and *Fusarium* spp. *J. Ginseng Res*. **26:** 151–158.
20. Seifert, K. A., C. R. McMullen, D. Yee, R. D. Reeleder, and K. F. Dobinson. 2003. Molecular differentiation and detection of ginseng-adapted isolates of the rotten-root fungus *Cylindrocarpon destructans*. *Phytopathology* **93:** 1533–1542.
21. Yang, D. C., Y. H. Kim, K. Y. Yun, S. S. Lee, J. N. Kwon, and H. M. Kang. 1997. Red-colored phenomena of ginseng (*Panax ginseng* C. A. Meyer) root and soil environment. *Korean J. Ginseng Sci.* **21:** 91–97.
22. Zor, T. and Z. Selinger. 1996. Linearization of the Bradford protein assay increases its sensitivity: Theoretical and experimental studies. *Anal. Biochem*. **236:** 302–308.