



Research article

Changes in element accumulation, phenolic metabolism, and antioxidative enzyme activities in the red-skin roots of *Panax ginseng*



Ying Zhou, Zhenming Yang, Lingling Gao, Wen Liu, Rongkun Liu, Junting Zhao, Jiangfeng You*

Laboratory of Soil and Plant Molecular Genetics, College of Plant Science, Jilin University, Changchun, China

ARTICLE INFO

Article history:

Received 16 January 2016

Received in Revised form

14 June 2016

Accepted 15 June 2016

Available online 25 June 2016

Keywords:

ascorbate

Panax ginseng

phenolic compounds

red-skin ginseng disease

ABSTRACT

Background: Red-skin root disease has seriously decreased the quality and production of *Panax ginseng* (ginseng).

Methods: To explore the disease's origin, comparative analysis was performed in different parts of the plant, particularly the epidermis, cortex, and/or fibrous roots of 5-yr-old healthy and diseased red-skin ginseng. The inorganic element composition, phenolic compound concentration, reactive oxidation system, antioxidant concentrations such as ascorbate and glutathione, activities of enzymes related to phenolic metabolism and oxidation, and antioxidative system particularly the ascorbate–glutathione cycle were examined using conventional methods.

Results: Aluminum (Al), iron (Fe), magnesium, and phosphorus were increased, whereas manganese was unchanged and calcium was decreased in the epidermis and fibrous root of red-skin ginseng, which also contained higher levels of phenolic compounds, higher activities of the phenolic compound-synthesizing enzyme phenylalanine ammonia-lyase and the phenolic compound oxidation-related enzymes guaiacol peroxidase and polyphenoloxidase. As the substrate of guaiacol peroxidase, higher levels of H₂O₂ and correspondingly higher activities of superoxide dismutase and catalase were found in red-skin ginseng. Increased levels of ascorbate and glutathione; increased activities of L-galactose 1-dehydrogenase, ascorbate peroxidase, ascorbic acid oxidase, and glutathione reductase; and lower activities of dehydroascorbate reductase, monodehydroascorbate reductase, and glutathione peroxidase were found in red-skin ginseng. Glutathione-S-transferase activity remained constant.

Conclusion: Hence, higher element accumulation, particularly Al and Fe, activated multiple enzymes related to accumulation of phenolic compounds and their oxidation. This might contribute to red-skin symptoms in ginseng. It is proposed that antioxidant and antioxidative enzymes, especially those involved in ascorbate–glutathione cycles, are activated to protect against phenolic compound oxidation.

© 2016 The Korean Society of Ginseng, Published by Elsevier Korea LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Panax ginseng (ginseng) is a perennial aromatic herb from the Araliaceae family. It has been suggested to stimulate metabolism, and hence maintain and improve health [1]. Its use has lasted for more than 4,000 yrs and spread as an alternative to chemical agents

in Western medicine [2]. Ginseng cultivation requires shade conditions and long period (5–6 yrs). The major production regions of China are in the northeast between the latitudes of 39° and 47° [3], where 50% or more of China's total ginseng crop is produced each year. The quality and price of ginseng depend on its size, shape, and overall appearance.

Abbreviations: AAO, ascorbic acid oxidase; Al, aluminum; APX, ascorbate peroxidase; Asc, ascorbate; CAT, catalase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; ginseng, *Panax ginseng*; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GST, glutathione-S-transferase; GuPX, guaiacol peroxidase; L-GalDH, L-galactose 1-dehydrogenase; MDA, malondialdehyde; MDHAR, monodehydroascorbate reductase; PAL, phenylalanine ammonia-lyase; PPO, polyphenoloxidase; SOD, superoxide dismutase.

* Corresponding author. College of Plant Science, Jilin University, 5333 Xi'an Road, Changchun 130062, China.

E-mail address: youjf@jlu.edu.cn (J. You).

<http://dx.doi.org/10.1016/j.jgr.2016.06.001>

p1226-8453 e2093-4947/\$ – see front matter © 2016 The Korean Society of Ginseng, Published by Elsevier Korea LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Red-skin root disease is frequently characterized by less fibrous roots and red to brown speckles on the superficial skin of the ginseng root. It seriously decreases both the quality and production of ginseng in the Changbai mountain area. Chinese scholars tend to identify the red-skin root disease as a non-infectious physiological disorder and differentiate it from root rusty disease, which is caused by pathogens [4–8]. Soil conditions such as higher moisture, nitrate concentrations, and active Al species were suggested to be closely related to the incidence of red-skin root in ginseng [9]. However, the corresponding changes that occur in the physio-biochemical level in ginseng remain unclear.

In American ginseng (*Panax quinquefolius* L.), rusty root symptoms infected by cylindrocarron include raised, reddish-brown lesions [10], and physiologically caused rust has orange-brown sunken lesions [11]. The physiological basis of either pathogen-induced rusty roots or physiologically caused rust spots in American ginseng appears to relate to the accumulation and oxidation of phenolic compounds in roots [10,12,13]. Phenolic compounds are widely distributed in plants to protect them from adverse environments, diseases, reactive oxygen species (ROS), wounds, and UV light [14]. For example, the accumulation of phenolic compounds in roots and root exudates has been suggested to be related to Al resistance in several plant species [15–17].

The role of the ascorbate (ASC)–glutathione (GSH) cycle in stress resistance, growth, and cell signaling has been well documented by several reviews [18,19]. For example, pumpkin roots were suggested to express higher levels of ASC–GSH system in response to the Al toxicity enhanced production of ROS [20].

Building on our previous results showing that Al toxic species in albic ginseng bed soil contributed to the incidence of red-skin root disease [9], in the present study we explored the origin of red-skin root in ginseng by comparing the phenolic metabolism, reactive oxidation system, and antioxidative system in relation to inorganic mineral element accumulation between healthy and red-skin ginseng tissues.

2. Materials and methods

2.1. Root sampling

Five-year-old ginseng roots were harvested from the first ginseng farm (41°N, 128°E) in the Malugou town of Jilin province, China. The root tissues were stored at –4°C for transport. In the laboratory, ginseng roots were rinsed five times with distilled water to remove the attached soil. After the fibrous roots were cut, the red-skin areas in main roots were scraped with a plastic ruler to collect the first three to four cell layers as epidermis, then the adjacent seven to eight cell layers as the cortex. The corresponding epidermis, cortex, and fibrous roots were sampled from healthy root tissues. Then, 1.0-g sample aliquots were stored at –80°C.

2.2. Elemental analysis

The red skin disease grade was classified according to Li et al [8]. Fibrous roots, epidermis, cortex, and central cylinder portions in main roots of different disease grades ginseng were separated using a plastic ruler. All parts were dried, weighed, and ground to a fine powder and digested with an acid mixture (HNO₃:HClO₄, 4:1). The element concentrations were analyzed by inductively coupled plasma optical emission spectroscopy.

2.3. Determination of the total phenolic compounds

According to the description of Rahman and Punja [13], phenolic compounds were extracted in 80% methanol containing 8% methanoic acid, and reacted with Folin–Denis solution. The spectroscopic absorbance was monitored at 740 nm with gallic acid as analogue for the total phenolic compounds.

2.4. Determination of phenylalanine ammonia-lyase and polyphenoloxidase activities

According to Koukol and Conn [21], phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) was extracted in 0.1M boric acid buffer (pH 8.8) containing 5 mM mercaptoethanol, 1mM EDTA, and 5% glycol (v/v). The enzyme extract was mixed with the boric acid buffer solution (pH 8.8) containing 15 mM L-phenylalanine. After 30 min of incubation at 37°C, HCl was added to stop the reaction. The absorbance was monitored at 290 nm, and a change of 0.01 value within 1 min was defined as 1 unit of enzyme activity.

The polyphenoloxidase (PPO, EC 1.14.16.1) activity was assayed according to Anderson and Morris [22]. PPO was extracted in 50 mM phosphate buffer solution (pH 5.6). The enzyme extract was mixed with 50 mM phosphate buffer (pH 5.6) containing 10 mM o-dihydroxybenzene. The absorbance was monitored at 420 nm, and an increase of 0.01 in the absorbance value within 1 min was defined as 1 unit of enzyme activity.

2.5. Determination of lipid peroxidation and O₂^{•-} and H₂O₂ concentrations

Lipid peroxidation was evaluated in terms of the malondialdehyde (MDA) concentration. Thiobarbituric acid reactive substance was measured as the final product of lipid peroxidation at 600 nm and 535 nm [23].

According to Patterson et al [24], H₂O₂ was extracted with ice-cold acetone and reacted with 10% TiCl₄ dissolved in concentrated HCl and ammonia. The absorbance was monitored at 412 nm.

The O₂^{•-} produced was extracted in 25 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer (pH 6.8) and reacted with *p*-aminobenzene sulfonic acid and *a*-naphthylamine. The absorbance was monitored at 530 nm [25].

2.6. Determination of antioxidative enzyme activities

Frozen samples (1.0 g) were homogenized with 10 mL ice-cold extraction buffer [25mM HEPES containing 2% (w/w) polyvinylpyrrolidone and 0.2mM EDTA, pH 7.8]. After centrifugation, the supernatant was collected for enzyme activity assays.

Guaiacol peroxidase (GuPX, EC 1.11.1.7) activity was assayed based on the oxidation of guaiacol by hydrogen peroxide [26]. The reaction mixture contained 2.9 mL of 50 mM potassium phosphate buffer (pH 7.0, containing 20 mM guaiacol and 10 mM H₂O₂), and 0.1 mL enzyme extract. An increase of 0.01 in the absorbance value within 1 min at 420 nm was defined as 1 unit of enzyme activity. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed at 290 nm [27]. The reaction system consisted of a mixture of 2.9 mL of 50 mM phosphate buffer (pH 7.6) containing 0.25 mM ascorbate and 0.1 mM H₂O₂, and 0.1 mL enzyme extract. One unit of enzyme activity was equivalent to the oxidation of 1 μM ascorbate within 1 min.

The activities of catalase (CAT, EC 1.11.3.6) and superoxide dismutase (SOD, EC 1.15.1.1) were determined according to Dhindsa et al [28]. H₂O₂ was monitored at 240 nm, and a decrease of 0.01 in the absorbance value within 1 min was defined as 1 unit of catalase

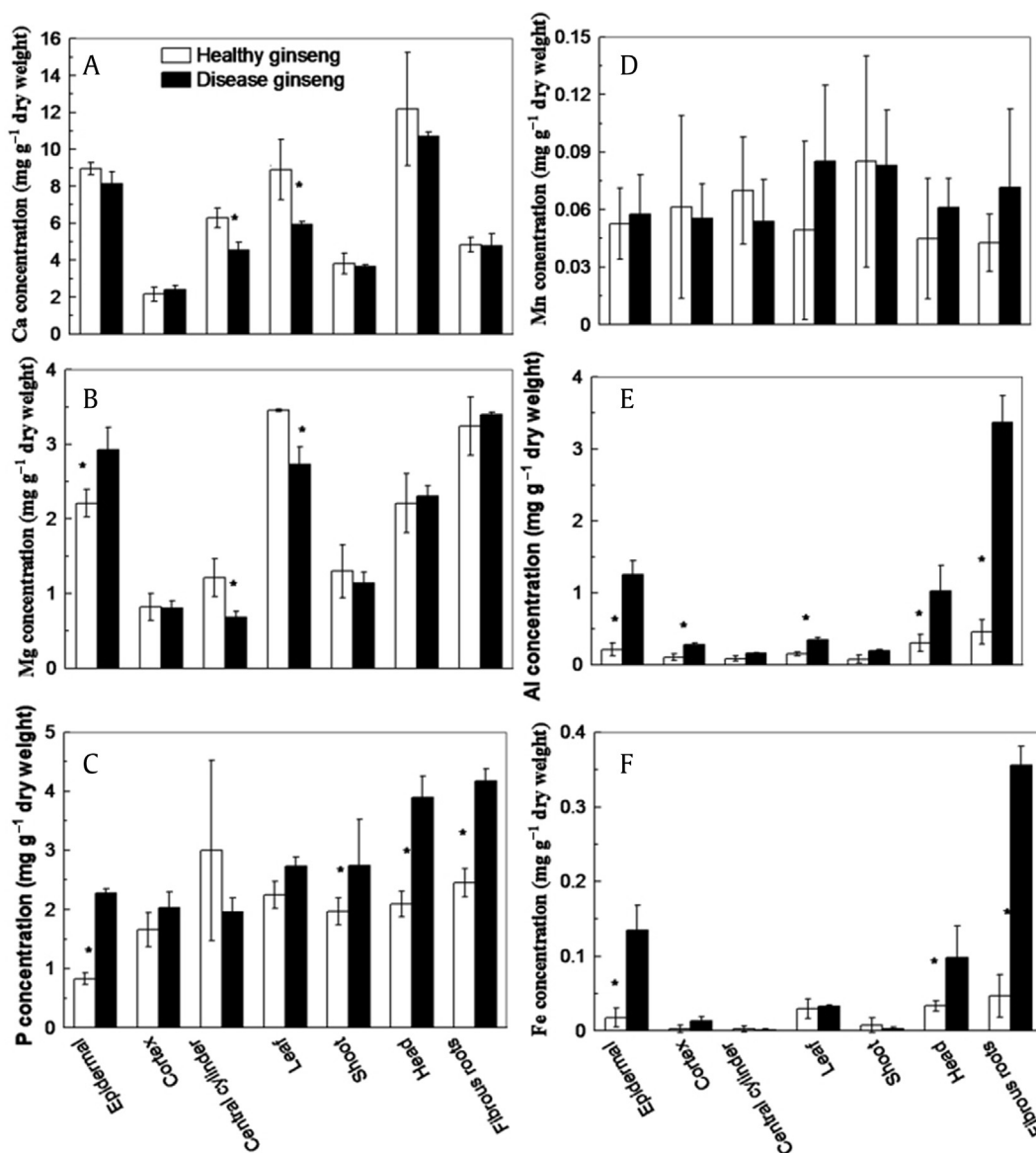


Fig. 1. Comparison of concentrations of elements in different parts of red-skin and healthy ginseng. (A) Calcium (Ca). (B) Magnesium (Mg). (C) Phosphorus (P). (D) Manganese (Mn). (E) Aluminum (Al). (F) Iron (Fe). The epidermis, cortex, and central cylinder were separated from the main root of ginseng. Values are denoted as the mean \pm standard deviation; $n = 3$, $p < 0.05$. * between the black and white columns indicates a significant difference between healthy and disease ginseng.

enzyme activity. NBT was monitored at 560 nm. One unit of SOD activity was defined as 50% inhibition of nitrotetrazolium blue chloride photoreduction.

Oxidation of nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH) was monitored at 340 nm to evaluate monodehydroascorbate reductase (MDHAR) activity with the coefficient of 6.2 mM/L/cm [29]. The formation of ASC was monitored at 265 nm to assay the activity of dehydroascorbate reductase (DHAR) with a coefficient of 14mM/L/cm [29]. According to Foyer and Halliwell [30], the activity of glutathione reductase (GR, EC.1.6.4.2) was determined by monitoring the oxidation of NADPH at 340 nm. The glutathione-S-transferase (GST) activity was monitored at 340 nm with a coefficient of 9.6 mM/L/cm. The ascorbic acid oxidase (AAO, EC 1.10.3.3) activity was assayed by monitoring at 265 nm with a coefficient of 14.3 mM/L/cm. Glutathione peroxidase (GPX, EC 1.11.1.9) activity was measured according to Griffith [31] with monitoring at 412 nm.

L-Galactose 1-dehydrogenase (L-GalDH, EC 1.1.1.316) was extracted and measured according to Gatzek et al [32]. The

absorbance was monitored at 340 nM, and the coefficient was 6.2 mM/L/cm.

2.7. Determination of nonenzymatic antioxidant concentrations

ASC and dehydroascorbate (DHA) were measured spectrophotometrically at 265 nm according to the method of Hodges et al [33]. The reduced form of glutathione (GSH), glutathione disulfide (GSSH), was measured according to Griffith [31], and the absorbance was monitored at 412 nm.

3. Results

3.1. Inorganic element accumulation

A large variation of element composition existed between healthy and red-skin ginsengs, particularly in the fibrous roots and epidermis of main root (Figs. 1A–1F). In comparison with healthy controls, lower concentration of calcium (Fig. 1A), higher

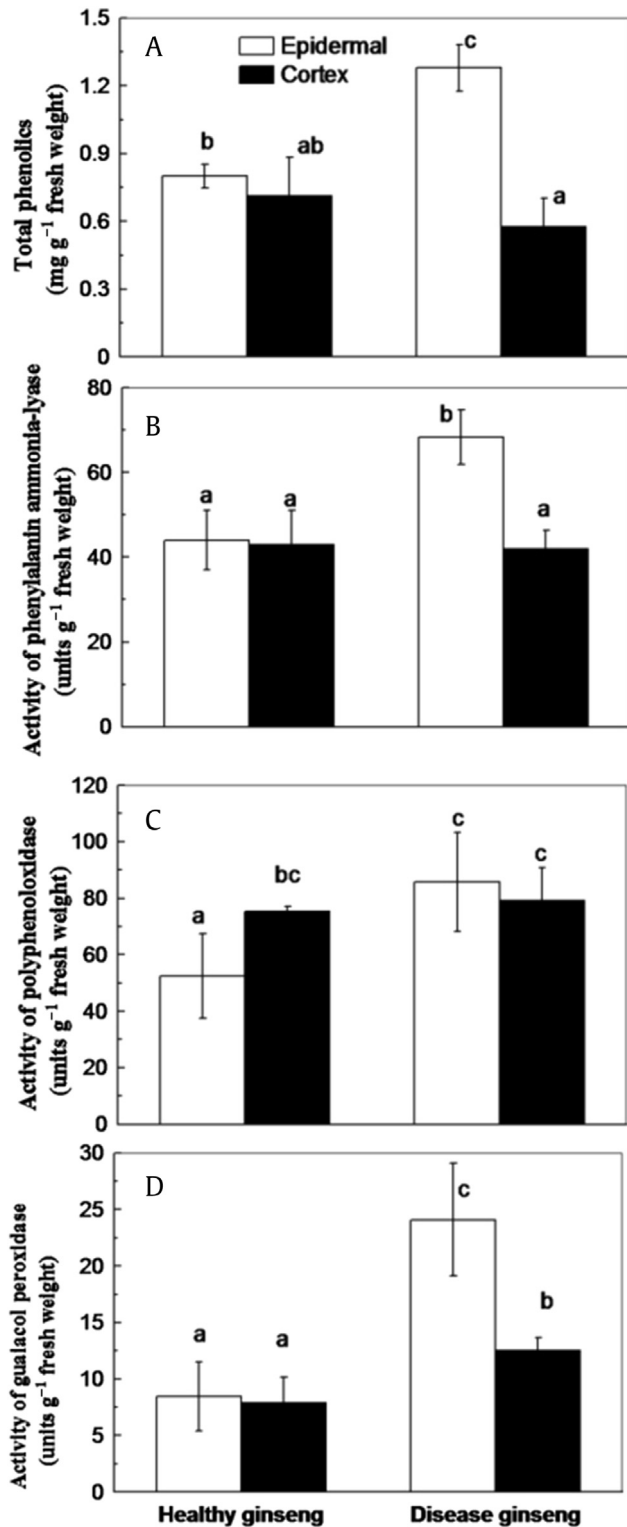


Fig. 2. Comparison between red-skin ginseng and healthy ginseng. (A) Total phenolic compound concentrations. (B) Activities of phenylalanine ammonia-lyase. (C) Polyphenoloxidase activities. (D) Guaiacol peroxidase (GuPX) activities. Values are denoted as the mean \pm standard deviation; $n = 3$. The different letters above column represented statistically different at $p = 0.05$.

concentrations of magnesium (Fig. 1B), phosphorus (Fig. 1C), Al (Fig. 1E), iron (Fig. 1F), and unchanged manganese (Mn; Fig. 1D) were found in the epidermis of red-skin ginseng. Increased Al was also observed in the head, cortex, shoot, and leaves of red-skin

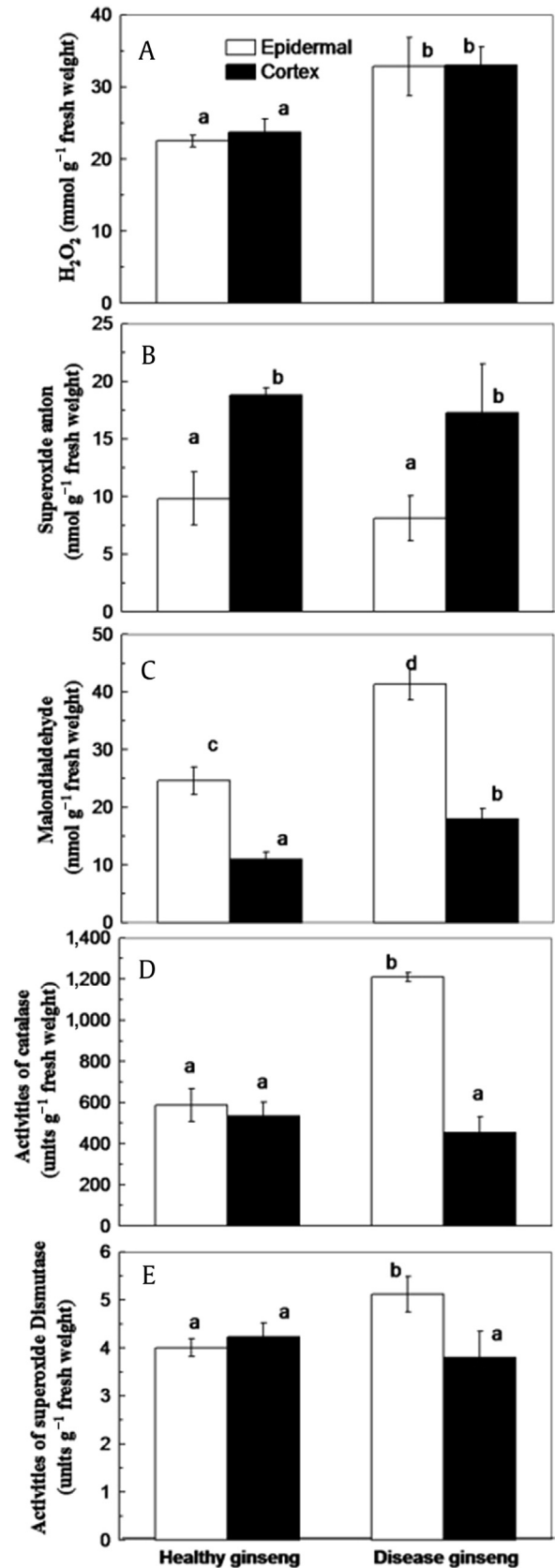


Fig. 3. Comparison between red-skin ginseng and healthy ginseng. (A) Concentration of H₂O₂. (B) Concentration of superoxide anion. (C) Concentration of malondialdehyde. (D) Activities of catalase. (E) Activities of superoxide dismutase. The values are denoted as the mean \pm standard deviation; $n = 3$. The different letters above columns represent statistically different at $p = 0.05$.

ginseng (Fig. 1E). In particular, the amount of AI accumulated in the epidermis of the main root and the fibrous roots of red-skin ginseng were six- and seven-fold higher, respectively, than those in the corresponding parts of healthy ginseng (Fig. 1E).

3.2. Phenolic compound accumulation and their metabolism

PAL is the key enzyme in the phenylpropanoid biosynthesis pathway in plants, which controls the synthesis of phenolic compounds [34]. The total phenolic compounds in the epidermis of red-skin roots were almost double those in healthy roots (Fig. 2A). Accordingly, a significant increase in PAL activity was observed in the epidermis of red-skin roots compared with the healthy roots (Fig. 2B).

GuPX and PPO contribute to the catalysis of phenolic compound oxidation in plants through the reduction of H_2O_2 and molecular oxygen, respectively [35,36]. Higher activities of both PPO (Fig. 2C) and GuPX (Fig. 2D) were observed in the epidermis of red-skin ginseng roots. However, no significant difference was found in the concentrations of phenolic compound and the activities of PPO,

PAL, and POD in the cortex between healthy and diseased ginseng (Figs. 2A–2D). The increase in PAL activity (Fig. 2B), the total phenolic compounds concentrations (Fig. 2A), and the activities of PPO (Fig. 2C) and GuPX (Fig. 2D) are consistent with the assumption that phenolics and their oxidation are related to the reddish-brown speckle symptoms in red-skin ginseng roots.

Higher concentrations of H_2O_2 were detected in both epidermis and cortex of main root of red skin ginseng compared with the corresponding part of healthy ginseng (Fig. 3A). There was no significant difference in $O_2^{\cdot-}$ concentrations between healthy and red-skin ginseng (Fig. 3B). The MDA concentration, which indicates lipid peroxidation, was increased in the epidermis and cortex of red-skin ginseng roots (Fig. 3C). Thus, the presence of more ROS and higher levels of lipid peroxidation were found in the epidermis of red-skin ginseng roots (Figs. 3A–3C). Increased H_2O_2 can provide substrates for GuPX to involve in phenolic compound oxidation.

The activities of H_2O_2 -related antioxidative enzymes were analyzed. Higher activities of CAT (Fig. 3D) and SOD (Fig. 3E) were found in the epidermis of red-skin ginseng roots. The activities of

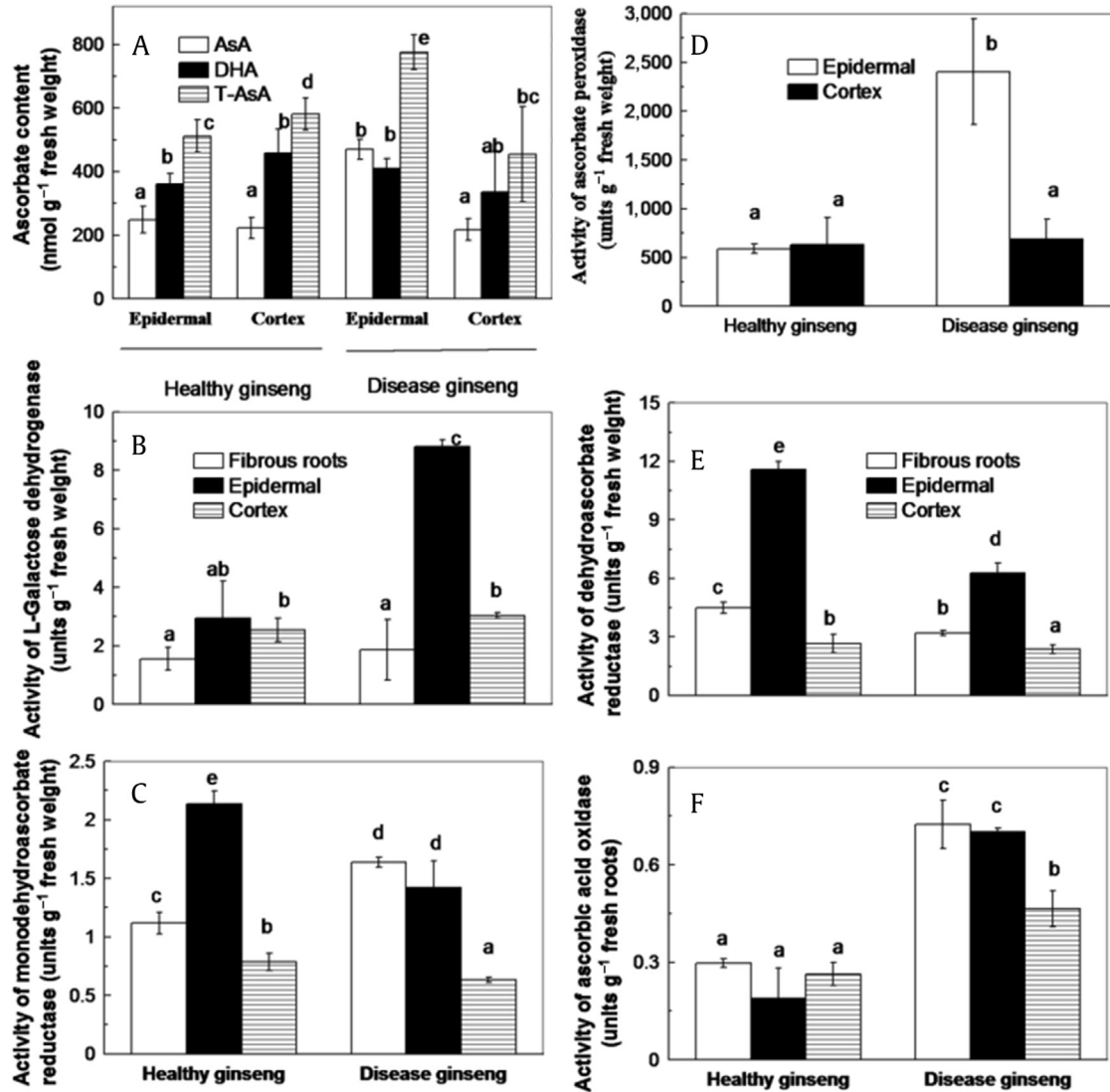


Fig. 4. Comparison between red-skin ginseng and healthy ginseng. (A) Ascorbate concentration. (B) Activity of L-galactose dehydrogenase. (C) Activity of monodehydroascorbate reductase. (D) Activity of ascorbate peroxidase. (E) Dehydroascorbate reductase. (F) Activity of ascorbic acid oxidase. The values are denoted as the mean \pm standard deviation, $n = 3$. The different letters above columns represent statistically different at $p = 0.05$.

CAT in the red-skin root epidermis were two-fold higher than those in healthy ginseng (Fig. 3D).

3.3. ASC metabolism and activities of enzymes in the ASC–GSH cycle

The ASC–GSH cycle plays an important role in the detoxification of ROS by successive oxidation and reduction reactions involving ASC and GSH. Their main cellular redox buffers including ASC and GSH with their respective oxidized forms MDHA, DHA, and GSSH. A higher accumulation of ASC and DHA was observed in the epidermis of red-skin ginseng (Fig. 4A).

The activities of related enzymes in the ASC–GSH cycle were measured (Figs. 4B–4F). L-GalDH and AAO are the key enzymes responsible for ASC synthesis and degradation, respectively. Comparing the healthy ginseng control, activities of both L-GalDH and AAO were significantly higher in the epidermis of red-skin roots (Figs. 4B and 4F). In particular, the activity of L-GalDH is almost three-fold higher in the epidermis of red-skin ginseng than that in healthy ginseng (Fig. 4B). APX can remove H_2O_2 with ASC as the electron donor. MDHAR and DHAR involved in the regeneration of ASC [37] substantially increased APX activity (Fig. 4D), whereas decreased activities of DHAR and MDHAR were found in the epidermis of red-skin ginseng (Figs. 4C and 4E). These findings were consistent with the higher ASC + DHA and DHA

concentrations in the epidermis of red-skin ginseng (Fig. 4A). No significant differences between healthy and red-skin ginseng were noted regarding the activities of the antioxidative enzymes and the antioxidant concentrations in the cortex (Figs. 4A–4F).

GSH is critical for the regeneration of ASC in the ASC–GSH cycle. GPX reduces H_2O_2 to water with GSH as reductant [38]. The conversion of GSH into oxidized GSSH by GPX can be reversed by GR. GST was suggested to detoxify lipid peroxidation and release $O_2^{\cdot-}$ in conjunction with cytosolic peroxidases [39,40]. Increased concentrations of GSH + GSSH (Fig. 5A), GSH (Fig. 5B), and GSSH (Fig. 5C) were found in the fibrous roots and epidermis of red-skin ginseng. Unchanged activities of GST (Fig. 5D) and GPX (Fig. 5E), and higher GR (Fig. 5F) activity were found in red-skin ginseng.

4. Discussion

In this work, the origin of the ginseng red-skin root disease was studied in relation to inorganic element composition and the accumulation and oxidation of phenolic compounds. Particular attention was paid to antioxidative enzymes, especially those involved in the ASC–GSH cycles, to explore the process of protecting against phenolic oxidation.

Phenolics in plants were significantly increased under a variety of stresses, such as Al toxicity [16,17]. The phenylpropanoid pathway is responsible for the synthesis of various secondary

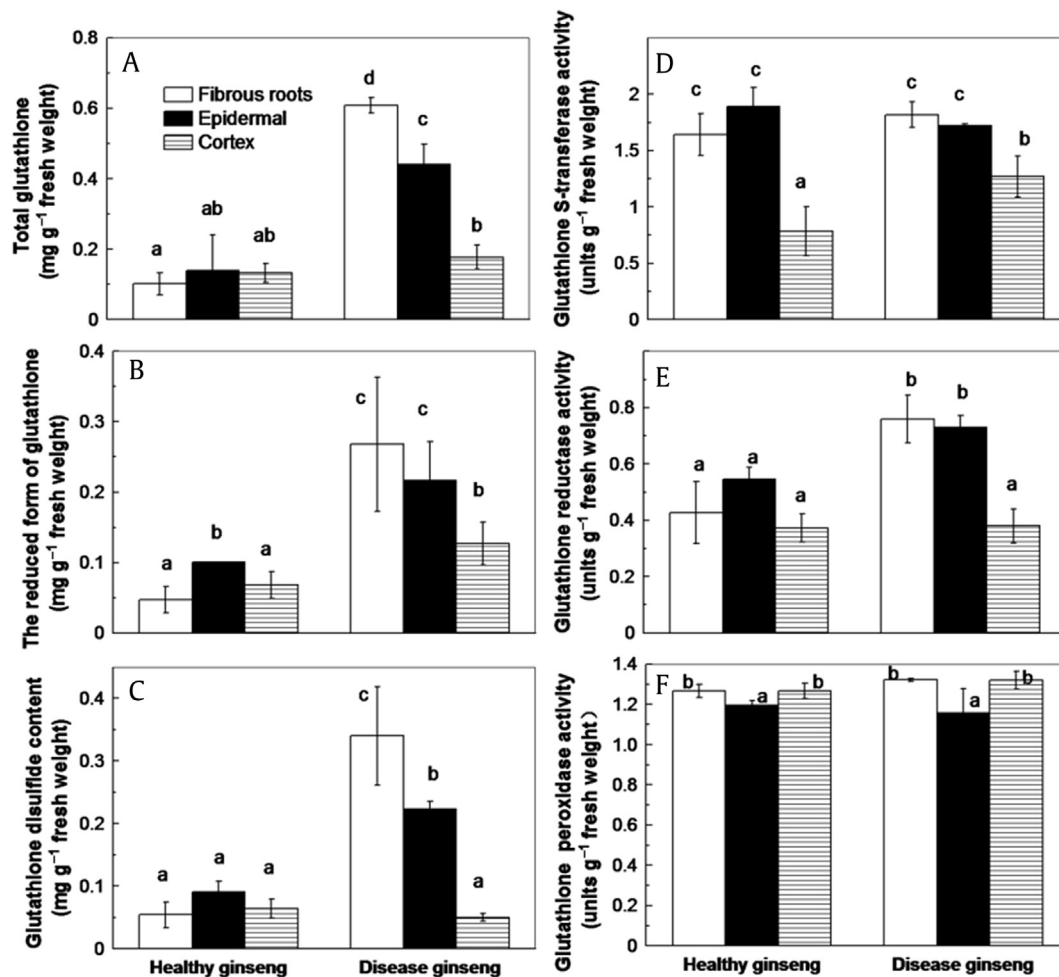


Fig. 5. Comparison between red-skin ginseng and healthy ginseng. (A) Concentration of total glutathione. (B) Concentration of reduced form of glutathione. (C) Concentration of glutathione disulfide. (D) Activity of glutathione S-transferase. (E) Activity of glutathione reductase. (F) Activity of glutathione peroxidase (GPX). The values are denoted as the mean \pm standard deviation; $n = 3$. The different letters above columns represented statistically different at $p = 0.05$.

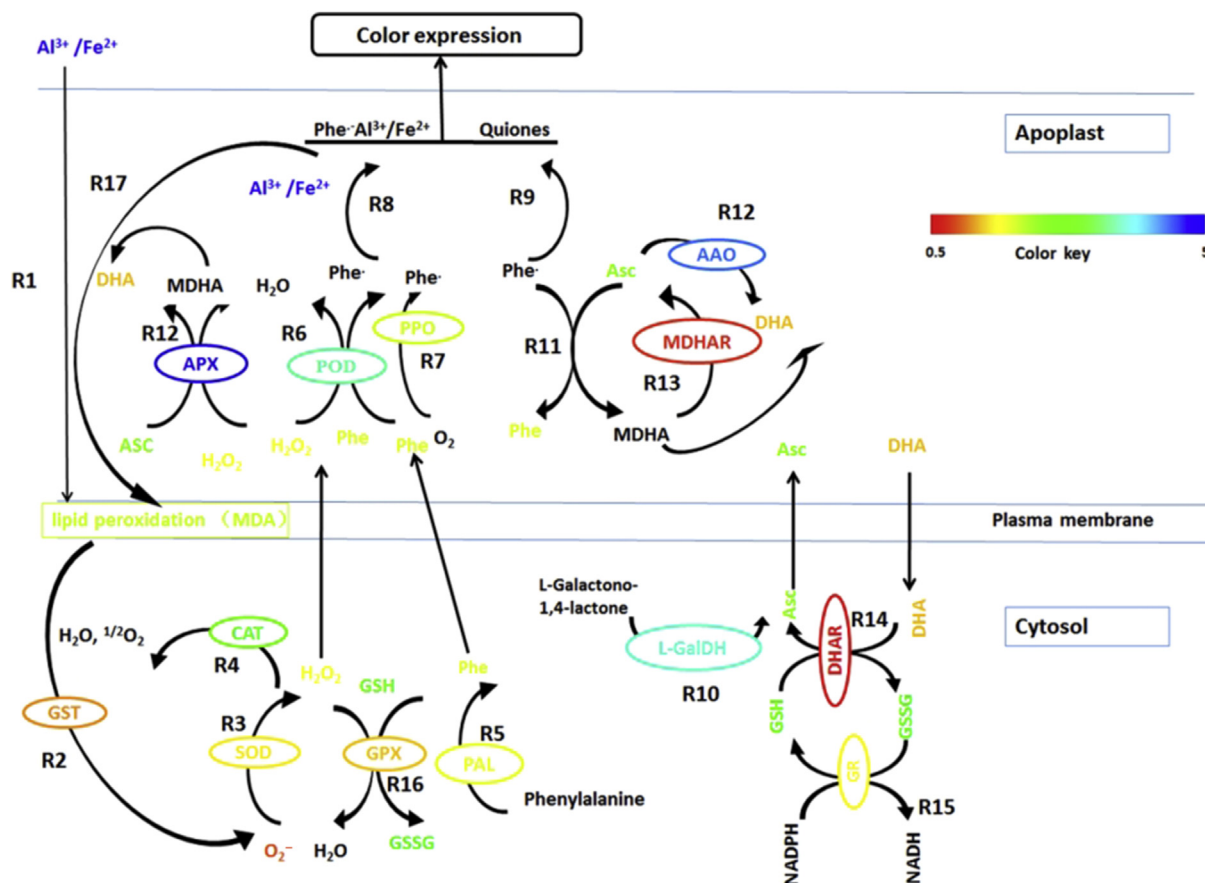


Fig. 6. The proposed phenolics oxidation process and the protection afforded by ascorbate (ASC)–glutathione (GSH) cycles in red-skin roots in *Panax ginseng*. Al and/or Fe stress caused lipid peroxidation (R1). Cytosolic peroxidases (cPX) conjugated with glutathione-S-transferase (GST) to detoxify the lipid peroxidation and release O_2^- (R2). The production of the reactive oxygen system and the higher activities of superoxide dismutase (SOD) and catalase (CAT) were induced to produce and scavenge H_2O_2 , respectively (R3, R4). Simultaneously, higher PAL activity was induced to increase the production of phenolics (R5). The phenol was oxidized to phenol radicals in a reaction catalyzed by guaiacol peroxidase (GuPX; R6) and polyphenoloxidase (PPO; R7) with H_2O_2 and O_2 as the substrate, respectively. The phenol radicals combined with Al or Fe ions (R8) or dismutated to quinone (R9), resulting in the expression of red or brown color. More ASC was produced via a reaction catalyzed by L-GalDH (R10). ASC can reduce phenoxy radicals to their original phenol forms (R11) and can also remove H_2O_2 by APX by undergoing oxidation to malondialdehyde (MDA) and dehydroascorbate (DHA). ASC can also be oxidized to DHA by ascorbic acid oxidase (AAO) (R12). ASC can be regenerated from MDA or DHA by MDAR (R13) and DHAR (R14), whereas GSH can be oxidized to glutathione disulfide (GSSH). The reduction of GSSH to GSG can be realized by GR, which is involved in the ASC regeneration system (R15). GPX reduces H_2O_2 to water by consuming GSH and converting it to GSSH (R16). Lipid peroxidation might also result from the complexation of phenol radicals with Al and/or Fe ions (R17). The color keys represent the ratio of activities or concentrations in epidermal between red skin and healthy ginseng. The black letters represent untested factors.

metabolites, including phenolic esters, coumarins, flavonoids, and lignin [34]. In the present study, higher phenolic compound concentrations and PAL activities were observed in the epidermis of red-skin ginseng (Figs. 2A and 2B). The increased accumulation of phenolic compounds might result from increased PAL activity and could be related to Al toxicity.

Metals, including Al, Fe, and Mn, form complexes with phenolic compounds, which usually contribute to their colorful appearance [41]. For example, Ayala-Silva and Al-Hamadani [42] reported that *Azolla caroliniana* incubated with $Al_2(SO_4)_3$ accumulated nine times more anthocyanins than control plants. Earlier reports demonstrated the presence of elevated phenolics and cations, especially Fe, in rusty tissues compared to healthy tissues of *P. ginseng* Meyer and *P. quinquefolius* L. [12,43,44]. Yang et al [43] reported two- to three-fold higher Fe, Al, and Si levels among the inorganic elements in the epidermis of red-colored ginseng compared to healthy roots.

Our previous report revealed that a large amount of exchangeable Al existed in the albic ginseng bed soils [9]. Furthermore, the occurrence of red-skin root disease was closely related to multiple factors including Al toxicity [45]. Consistently, more Al and Fe were observed in the epidermis, cortex, head, and

fibrous roots of red-skin ginseng (Figs. 1E and 1F). The amount of Fe was rather low in both healthy and red-skin ginseng compared with Al (Figs. 1E and 1F). Al and Fe are suggested to play important roles in contributing to the occurrence of the red-skin symptom in ginseng. The large variation in Ca, Mg, and P concentrations in the epidermis, central cylinder, or leaf between healthy and red-skin ginseng might be a secondary effect of Al or Fe toxicity (Figs. 1A–1C). Higher phosphorus concentrations might immobilize the extra metal cations. No significant difference was observed in the Mn concentration between the healthy and diseased ginseng (Fig. 1D).

Oxidation of phenolic compounds contributes to browning and leads to formation of brown pigments in plant tissues [35]. Wissemeyer and Horst [46] have suggested that accumulations of oxidized Mn and oxidized phenolic compounds in the cell wall contributed to the local brown spots in cowpea leaves. Generally, the oxidation of phenolic compounds involved in two enzymes. On the one hand, with molecular oxygen as the electron acceptor, PPO catalyzes phenolic compounds oxidizing into quinones with toxic oxygen species formation [47]. On the other hand, the GuPX-catalyzed phenol oxidation with H_2O_2 as the electron acceptor results in the formation of phenoxy radicals, which can form

polyphenols to express browning, or combine with metal ions to cause lipid peroxidation [48,49]. For example, Al, Ca, Mg, and Cd have been found to stimulate phenoxy radical-induced lipid peroxidation [50]. Al was shown to have a spin-stabilizing effect on the phenoxy radical [51]. In the present study, higher activities of GuPX and PPO were found in the red-skin root of ginseng (Figs. 2C and 2D), indicating their coinvolvement in phenolic compound oxidation with the expression of red to brown speckles. Consistently, the important oxidative species H_2O_2 was also observed to be enhanced in the red-skin root (Fig. 3A) and could act as a substrate for GuPX. Increased MDA concentration in red-skin epidermis reflected an increase in membrane lipid peroxidation (Fig. 3C), which might be caused by Al or Fe toxic ions or by the combination of phenoxy radical and Al ions.

Considering the importance of phenoxy radicals in polyphenol formation, the redox environment was studied here. Antioxidative enzymes that are involved in scavenging for phenoxy radicals and clearing the H_2O_2 production pathway were selected to investigate how the process of phenolic compound oxidation is controlled.

The combined actions of SOD and CAT are crucial for mitigating the effects of oxidative stress, as the former involves the dismutation of O_2^- into H_2O_2 and the latter decomposes H_2O_2 to water and O_2 . Both CAT and SOD showed increased activity in the red-skin ginseng epidermis layers (Figs. 3D and 3E), indicating their important role in controlling H_2O_2 accumulation.

The ASC–GSH cycle plays an important role in ROS detoxification by successive oxidation and reduction reactions involving ASC and GSH. With ASC as the electron donor, APX effectively removes H_2O_2 and scavenges phenoxy radicals to inhibit phenol oxidation [48,52]. When ASC is also oxidized to DHA, quinoic compounds can be reduced back to their phenol form [53]. MDHAR and DHAR catalyze the reduction of monodehydroascorbate (MHDA) radical or DHA to regenerate the parent compound ASC [37]. Red-skin ginseng exhibited higher activities of the ASC synthetic enzyme γ -GalDH (Fig. 4B) and the degradation enzymes, APX (Fig. 4E) and AAO (Fig. 4F), indicating the important role of ASC in controlling phenol radicals and H_2O_2 . Lower activities of the regenerating enzymes DHAR (Fig. 4D) and MDHAR (Fig. 4C) suggested that less DHA was reduced to ASC, which is consistent with the higher total ASC concentrations and lower ASC/DHA ratio in red-skin ginseng (Fig. 4A).

GSH is also critical for the regeneration of ASC in the ASC–GSH cycle. The high GSH/GSSH ratio in plants could help maintain an appropriate redox environment. In the present study, the higher concentrations of total GSH and oxidized GSSH observed in the epidermis and fibrous roots of red-skin ginseng (Figs. 5A–5C) suggested that GSH might play an important role in protecting against phenolics oxidation.

GPX reduces H_2O_2 to water by converting GSH to GSSH [38]. The GSSH can be reduced to GSH by GR. GST combines with cytosolic peroxidases to detoxify lipid peroxidation products and release O_2^- [39,40]. The activities of GPX and GST remained unchanged in healthy and red-skin ginseng (Figs. 5D and 5F), reflecting their lower contribution of scavenging H_2O_2 and O_2^- production. Higher GR activity was found in the red-skin ginseng, which is consistent with its higher reduced GSH concentration (Fig. 5E).

In conclusion, a model was proposed to explain the occurrence of red-skin symptoms in ginseng (Fig. 6). The Al and/or Fe stress that caused phenolic compound accumulation and oxidation was suggested to contribute to the formation of red-skin disease. The ASC–GSH system was suggested to play an important role in controlling the phenolic oxidation process and thus the occurrence of red-skin symptoms. The regulation of redox environment in ginseng might be useful to alleviate or control the development of red skin ginseng disease.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgments

National Natural Science Foundation of China (No. 40903029), International Foundation for Science (C4711-1) and Jilin Natural Science Foundation of China (20130101084Jc) provided financial support for this research.

References

- [1] Hu SY. A contribution to our knowledge of ginseng. *Am J Chin Med* 1977;5:1–23.
- [2] Baranov A. Recent advances in our knowledge of the morphology, cultivation and uses of ginseng (*Panax ginseng* C.A. Meyer). *Econ Bot* 1966;20:403–6.
- [3] Evans BL. Ginseng: root of Chinese–Canadian relations. *Can Hist Rev* 1985;66:1–26.
- [4] Wang YQ. A preliminary study on cause of ginseng red skin disease in the second ginseng farm of Jinyu county. *Spec Wild Econ Anim Plant Res* 1963;2:9–15 [in Chinese].
- [5] Zhang YC, Wang ZS, Li JF, Gao KJ, Sun SJ, Wang YL. An investigation of cause and protective method of ginseng red skin disease. *Spec Wild Econ Anim Plant Res* 1984;1:21–4 [in Chinese].
- [6] Wu KY. The relationship between ginseng red skin disease and inorganic ions. *Chin Trad Med* 1989;12:13–4 [in Chinese].
- [7] Zhao YF. The progress of diagnosis and comprehensive control of ginseng red skin disease. *Spec Wild Econ Anim Plant Res* 1998;1:41–6 [in Chinese].
- [8] Li ZH, Tian SZ, Sun YJ, Guo SY, Liu ZR. The relationship between ginseng red skin root disease and soil ecology conditions. *Ecol J* 1999;19:864–9 [in Chinese].
- [9] Liu X, Yang ZM, Gao LL, Xiang WY, Zhang B, Xie ZL, You JF. Comparison of the characteristics of artificial ginseng bed soils in relation to the incidence of ginseng red skin disease. *Exp Agric* 2014;50:59–71.
- [10] Parke JL, Shotwell KM. Diseases of cultivated ginseng. *Univ Wisconsin Madison Coll Agric Life Sci Res Bull* 1989;3465:16.
- [11] Campeau CA. Effects of ethephon on floral abscission and root quality of North American ginseng (*Panax quinquefolius* L.). MS dissertation. Ontario, Canada: University of Guelph; 2002.
- [12] Campeau CA, Proctor JTA, Jackson CJC, Rupasinghe HPV. Rust-spotted north American ginseng roots: phenolic, antioxidant, ginsenoside and mineral nutrient. *Hort Sci* 2003;38:179–82.
- [13] Rahman M, Punja ZK. Biochemistry of ginseng root tissues affected by rusty root symptoms. *Plant Physiol Biochem* 2005;43:1103–14.
- [14] Dixon RA, Paiva NL. Stress-induced phenyl propanoid metabolism. *Plant Cell* 1995;7:1805–97.
- [15] Heidarabadi MD, Ghanati F, Fujiwara T. Interaction between boron and aluminum and their effects on phenolic metabolism of *Linum usitatissimum* L. roots. *Plant Physiol Biochem* 2011;49:1377–83.
- [16] Ofei-Manu P, Wagatsuma T, Ishikawa S, Tawarayama K. The plasma membrane strength of the root-tip cells and root phenolic compounds are correlated with Al tolerance in several common woody plants. *Soil Sci Plant Nutr* 2001;47:359–76.
- [17] Kidd PS, Lugany M, Poschenreder C, Gunse B, Barcelo J. The role of root exudates in aluminium resistance and silicon-induced amelioration of aluminium toxicity in three varieties of maize (*Zea mays* L.). *J Exp Bot* 2001;52:1339–52.
- [18] Horemans N, Foyer CH, Asard H. Transport and action of ascorbate at the plasma membrane. *Trends in Plant Sci* 2000;5:263–7.
- [19] Pignocchi C, Foyer CH. Apoplastic ascorbate metabolism and its role in the regulation of cell signaling. *Curr Opin Plant Biol* 2003;6:379–89.
- [20] Dipierro N, Mondelli D, Paciolla C, Brunetti G, Dipierro S. Changes in the ascorbate system in the response of pumpkin (*Cucurbita pepo* L.) roots to aluminum stress. *J Plant Physiol* 2005;162:529–36.
- [21] Koukol J, Conn EE. The metabolism of aromatic compounds in higher plants, purification and properties of the phenylalanine deaminase of *Herdeum vulgare*. *J Biol Chem* 1961;236:2690–8.
- [22] Anderson JV, Morris CF. An improved whole seed assay for screening wheat germplasm for polyphenol oxidase activity. *Crop Sci* 2001;41:1697–705.
- [23] Hodges DM, Delong JM, Forney CF. Improving the thiobarbituric acid reactive substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta* 1999;207:604–11.
- [24] Patterson BD, MacRae EA, Ferguson IB. Estimation of hydrogen peroxide in plant extracts using titanium (IV). *Anal Biochem* 1984;139:487–92.
- [25] Able AJ, Guest DI, Sutherland MW. Use of a new tetrazolium-based assay to study the production of superoxide radicals by tobacco cell cultures challenged with avirulent zoospores of *Phytophthora parasitica* var *nicotinae*. *Plant Physiol* 1998;117:491–9.

- [26] Castillo FI, Penel I, Greppin H. Peroxidase release induced by ozone in *Sw dum album* leaves. *Plant Physiol* 1984;74:846–51.
- [27] Asada K. Chloroplasts: formation of active oxygen and its scavenging. *Methods Enzymol* 1984;105:422–9.
- [28] Dhindsa RS, Plumb-Dhindsa P, Throne TA. Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase. *J Exp Bot* 1981;32:93–101.
- [29] Hossain MA, Nakano Y, Asada K. Monodehydroascorbate reductase in spinach chloroplasts and its participation in the regeneration of ascorbate for scavenging hydrogen peroxide. *Plant Cell Physiol* 1984;25:385–95.
- [30] Foyer CH, Halliwell B. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta* 1976;133:21–5.
- [31] Griffith OW. Determination of glutathione and glutathione disulphide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* 1980;106:207–12.
- [32] Gatzek S, Wheeler GL, Smirnov N. Antisense suppression of l-galactose dehydrogenase in *Arabidopsis thaliana* provides evidence for its role in ascorbate synthesis and reveals light modulated l-galactose synthesis. *Plant J* 2002;30:541–53.
- [33] Hodges D, Andrews C, Johnson D, Hamilton R. Antioxidant compound response to chilling stress in differentially sensitive inbred maize 23 lines. *Physiol Plant* 1996;98:685–92.
- [34] Zhang XB, Liu CJ. Multifaceted regulations of gateway enzyme phenylalanine ammonia-lyase in the biosynthesis of phenylpropanoids. *Mol Plant* 2015;8:17–27.
- [35] Pourcel L, Routaboul JM, Cheynier V, Lepiniec L, Debeaujon I. Flavonoid oxidation in plants: from biochemical properties to physiological functions. *Trends Plant Sci* 2006;12:29–36.
- [36] Marusck CM, Trobough NM, Flurkey WH, Inlow JK. Comparative analysis of polyphenol oxidase from plant and fungal species. *J Inorg Chem* 2006;100:108–23.
- [37] Asada K. The role of ascorbate peroxidase and monodehydroascorbate reductases in H₂O₂ scavenging in plants. In: Scandalions JG, editor. *Oxidative stress and the molecular biology of antioxidant defenses*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1987. p. 715–35.
- [38] Noctor G, Foyer CH. Ascorbic and glutathione: keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol* 1998;49:249–79.
- [39] Richards KD, Schott EJ, Sharma YK, Davis KR, Gardner RC. Aluminum induces oxidative stress genes in *Arabidopsis thaliana*. *Plant Physiol* 1998;116:409–18.
- [40] Sakihama Y, Yamasaki H. Lipid peroxidation induced by phenolics in conjunction with aluminum ions. *Biol Plant* 2002;45:249–54.
- [41] Vermerris W, Nicholson R. Phenolic compound biochemistry (Chapter 2). *Chemical properties of phenolic compounds*. e-ISBN: 978-1-4020-5164-7. 2009p.43. Springer.com.
- [42] Ayala-Silva T, Al-Hamadani S. Interactive effects of polyactic acid with different aluminum concentrations on growth, pigment concentrations, and carbohydrate accumulation of *Azolla*. *Am Fern J* 1997;87:120–6.
- [43] Yang DC, Kim YH, Yun KY, Lee SS, Kwon JN, Kang HM. Red-colored phenomena of ginseng (*Panax ginseng* C. A. Meyer): root and soil environment. *J Ginseng Sci* 1997;21:91–7.
- [44] Wang YP, Li ZH, Sun YJ, Guo SW, Tian SZ, Liu ZR. Studies on the genesis of ginseng rust spots. *Korean J Ginseng Sci* 1997;21:69–77.
- [45] You JF, Liu X, Zhang B, Xie ZL, Hou ZG, Yang ZM. Seasonal changes in soil acidity and related properties in ginseng artificial bed soils under a plastic shade. *J Ginseng Res* 2015;39:81–8.
- [46] Wissemeier AH, Horst WJ. Effect of light intensity on manganese toxicity symptoms and callose formation in cowpea (*Vigna unguiculata* (L.) Walp.). *Plant Soil* 1992;143:299–309.
- [47] Appel HM. Phenolics in ecological interactions: the importance of oxidation. *J Chem Ecol* 1993;9:1521–52.
- [48] Takahama U. Redox state of ascorbic acid in the apoplast of stems of *Kalanchoe daigremontiana*. *Physiol Plant* 1993;89:791–8.
- [49] Takahama U, Oniki T. Regulation of peroxidase-dependent oxidation of phenolics in the apoplast of spinach leaves by ascorbate. *Plant Cell Physiol* 1992;33:379–87.
- [50] Sakihama Y, Cohen MF, Grace SC. Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants. *Toxicology* 2002;177:67–80.
- [51] Kalyanaraman B. Characterization of O-semiquinone radicals in biological systems. *Methods Enzymol* 1990;186:333–43.
- [52] Sanchez M, Queijeiro E, Revilla G, Zarra I. Changes in ascorbate levels in apoplastic fluid during growth of pine hypocotyls. Effect on peroxidase activities associated with cell walls. *Physiol Plant* 1997;101:815–20.
- [53] Isaacs NS, van Eldik R. A mechanistic study of the reduction of quinines by ascorbic acid. *J Chem Soc Dalton Trans* 1997;2:1465–8.