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SHORT COMMUNICATION

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Exogenous ethanol treatment promotes glycyrrhizin accumulation in aseptically grown *Glycyrrhiza uralensis* seedlings

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ABSTRACT

Licorice, the dried roots and rhizomes of *Glycyrrhiza uralensis* Fisch., is one of the most popular herbal medicines used globally. Glycyrrhizin is the primary bioactive component of licorice, exhibiting various pharmacological activities. Herein, we grew *G. uralensis* seedlings aseptically on a medium in the presence of 0–1% ethanol for 10 weeks, elucidating the effect of exogenous ethanol treatment on plant morphological features and glycyrrhizin accumulation. Treatment with 0.1% exogenous ethanol significantly increased the root fresh weight of *G. uralensis* seedlings, whereas treatments exceeding 0.5% exogenous ethanol exhibited phytotoxicity. In addition, the application of 0.1% exogenous ethanol significantly promoted glycyrrhizin accumulation in plant roots relative to the control. Overall, these results indicate that dilute exogenous ethanol treatment positively affects root yield and glycyrrhizin accumulation in the roots of aseptically cultured *G. uralensis* seedlings. The findings of this study may contribute to improving the quality of cultivated *G. uralensis*.

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Introduction

Licorice is one of the most popular herbal medicines used worldwide.[1] In China, licorice is derived from the dried roots and rhizomes of *Glycyrrhiza uralensis* Fisch., *G. glabra* L., and *G. inflata* Batlin, whereas most of the licorice production is derived from *G. uralensis*.[2]

The demand for licorice has increased globally because of its medicinal value and economic benefits.[3] However, available sources of wild *G. uralensis* in China have recently been subjected to reductions due to long-term overharvesting and environmental degradation.[4–6] Moreover, cultivated *G. uralensis* are increasingly replacing wild *G. uralensis*, whereas the quality of cultivated *G. uralensis* is not as good as that of wild plants.[3–5] For example, Wei et al.[7] reported that the contents of glycyrrhizin, the primary bioactive component of licorice, was lower in cultivated *G. uralensis* (1.51% ± 0.49%) than in wild plants (4.43% ± 1.32%).

Abiotic stress influences the production of secondary metabolites in higher plants and may contribute to address this issue.[8] For example, Nasrollahi et al.[9] found that the glycyrrhizin content of *G. glabra* plants collected 28 days after drought imposition was 1.7-fold greater than that of control plants. Furthermore, Wang et al.[10] reported that the accumulation of glycyrrhizin in *G. uralensis* increased in response to 50 mM salinity stress.

Recently, Sako et al.[11] introduced a strategy for plant cultivation called "chemical priming." According to this strategy, plants treated with chemical priming agents before exposure to stress have improved stress tolerance compared to untreated plants. Representative chemical priming agents include reactive chemical species, metabolites and phytohormones, epigenetic regulation-related compounds, and nanoparticles.[11] In particular, ethanol was identified as a chemical priming agent capable of enhancing drought and high-salinity stress tolerance in *Arabidopsis thaliana* and *Oryza sativa* L. cv. Nipponbare.[12,13]

Based on these findings, we hypothesized that exogenous ethanol treatment could promote glycyrrhizin accumulation in *G. uralensis* as part of the response to enhanced salt and drought stress tolerance. Therefore, we aseptically grew *G. uralensis* seedlings in the presence of ethanol for 10 weeks, then elucidated the effect of exogenous ethanol treatment on plant morphological features and glycyrrhizin accumulation. The findings reported by the present study may contribute to quality improvement of cultivated *G. uralensis*.

Materials and methods

Plant material

Glycyrrhiza uralensis Fisch. seeds (Fabaceae) were supplied by Sumitomo Chemical Co., Ltd. (Tokyo, Japan) and were stored at 4°C until the start of the experiments. A voucher specimen was deposited at the Museum of Materia Medica, College of Pharmaceutical Sciences, Ritsumeikan University, Shiga, Japan.

Chemicals and reagents

Glycyrrhizin was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Other chemicals and liquid chromatography/mass spectrometry (LC/MS) grade chromatographic solvents were purchased from either

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FUJIFILM Wako Pure Chemical Corporation or from Nacalai Tesque, Inc. (Kyoto, Japan).

Plant growth conditions

G. uralensis seeds were first washed using the following multi-step protocol: 70% ethanol solution for 1 min, 0.7% hypochlorous acid solution for 5 min, and sterile water three times. Sterilized seeds were then germinated on Murashige – Skoog (MS) medium in Petri dishes. MS medium contains 0.8% (w/v) agar, 3% (w/v) sucrose, and vitamins (i.e., 2 mg/L glycine, 100 mg/L *myo*-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine, and 0.1 mg/L thiamine). The medium was adjusted to pH 5.7 before autoclaving. After 1 week, germinated seeds were transplanted into new MS medium in plant boxes (Bio Medical Science Inc., Tokyo, Japan). New MS medium contains 0.8% (w/v) agar, 3% (w/v) sucrose, and vitamins, and its pH was adjusted to 5.7 before autoclaving. Different concentrations of ethanol (i.e., 0%, 0.05%, 0.1%, 0.5%, and 1% v/v) were added after autoclaving. Plants were then grown for 10 weeks in a plant incubator at 25°C under 18 h/6 h light/dark photoperiod conditions.

Analytical sample preparation

Metabolite extraction from the roots of *G. uralensis* seedlings was carried out as per a previously reported protocol with minor modification.[14] Briefly, raw samples were first freeze-dried using an FDU-2200 vacuum freeze dryer (Tokyo Rikakikai Co., Ltd, Tokyo, Japan) before being pulverized using a PS-SMN06 beaded grinding machine (Bio Medical Science Inc.). Dried and pulverized samples were then extracted with 2 mL of a solvent mixture (i.e., methanol/chloroform/water at a ratio of 2.5:1:1, v/v/v) per 40 mg of dried weight tissue in an ultrasound bath at 35°C for 10 min. After centrifugation, the supernatant was isolated, and the remaining pellet was resuspended in 1 mL of a solvent mixture (i.e., methanol/chloroform at a 1:1 ratio, v/v) per 20 mg of dried weight tissue in an ultrasound bath at 35°C for 10 min. After centrifugation, the supernatant was combined, and 500 µL of water per 10 mg of dried tissue was added to separate the chloroform and water/methanol layers. The water/methanol layer was then filtered through a 0.45 µm Millipore filter (Advantec, Tokyo, Japan) so that 10 µL of the resulting filtrate could be subjected to LC/MS.

Analytical conditions

LC/MS was performed using a LCMS-IT-TOF platform (Shimadzu Corporation, Kyoto, Japan) equipped with an electrospray ionization interface.[15] High-performance liquid chromatography parameters were as follows: column, Waters Atlantis T3 (2.1 mm i.d. × 150 mm, 5 µm, Waters, Milford, MA); column temperature, 40°C; flow rate, 0.2 mL/min; mobile phase, (A) ammonium acetate solution and (B) acetonitrile; gradient conditions, 0–30 min was linear gradient from 10% to 100% B, and 30–40 min was isocratic at 100% B. MS parameters were as follows: ionization mode, negative; source voltage, – 3.5 kV; capillary temperature, 200°C; flow rate of nebulizer gas, 1.5 L/min; ion accumulation time, 100 ms; scan-range; *m/z* 700–1,000. Shimadzu LCMS solution version 3.80 was used for data acquisition and processing.

Statistical analysis

All data are reported as mean ± standard error of the mean (SEM). Statistical analyses were performed using one-way analysis of variance with Tukey's HSD to assess the statistical significance of differences between groups. IBM SPSS Statistics 29.0. was used for the statistical analyses, and $p < 0.05$ was used as thresholds of statistical significance.

Results

To investigate the effect of exogenous ethanol treatment on morphological changes in *G. uralensis*, we first grew plants on a medium in the presence of 0–1% ethanol for 10 weeks (Figure 1a). We then assessed plant morphological parameters, including height, fresh weight, and the number of leaves present (Figure 1b–d). 0.05% Exogenous ethanol treatment showed no significant effects on any of the morphological parameters of *G. uralensis* seedlings. 0.1% Exogenous ethanol treatment positively affected several morphological parameters of *G. uralensis* seedlings, including greater aerial part elongation and root fresh weight relative to the negative control. Most importantly, significant increases were observed in the root fresh weight (232.5 ± 22.4 mg) relative to the control (149.4 ± 20.3 mg) (Figure 1c). Overall, the application of 0.05% and 0.1% exogenous ethanol to *G. uralensis* seedlings did not negatively affect plant morphological parameters. In contrast, the application of 0.5% and 1% exogenous ethanol inhibited seedling growth. Specifically, these treatments resulted in inhibition of root elongation, lower fresh weight of roots and aerial parts, and decreased number of leaves.

Because licorice comprises medicinally valuable root material,[2] and *G. uralensis* seedlings treated with 0.5% and 1% exogenous ethanol showed decreased biomass (Figure 1c), only roots from seedlings treated with 0–0.1% exogenous ethanol were used for further experiments. To avoid the reduced extraction efficiency of metabolites due to variation in moisture content, all raw samples were freeze-dried (Figure 1e). Significant increase of freeze-dried root weight following treatment of 0.1% exogenous ethanol (36.0 ± 3.1 mg) relative to the negative control (20.2 ± 2.0 mg) was observed. Freeze-dried root weight of plants treated with 0%, 0.05%, and 0.1% ethanol represented 14.2%, 13.8%, and 16.0% of fresh root weight, respectively. These results show that the moisture content of roots was approximately 85% regardless of whether roots were treated with low-concentration ethanol or not.

Next, we quantitatively analyzed glycyrrhizin content using LC/MS. Figure 2 shows that the application of exogenous ethanol enhanced the accumulation of glycyrrhizin in the roots of *G. uralensis* seedlings. In particular, treatment with 0.1% exogenous ethanol significantly increased glycyrrhizin content (83.9 ± 17.4 ng/freeze-dried plant root or 2.4 ± 0.5 µg/g freeze-dried root weight) relative to those treated with water (24.2 ± 10.7 ng/freeze-dried plant root or 1.1 ± 0.4 µg/g freeze-dried root weight).

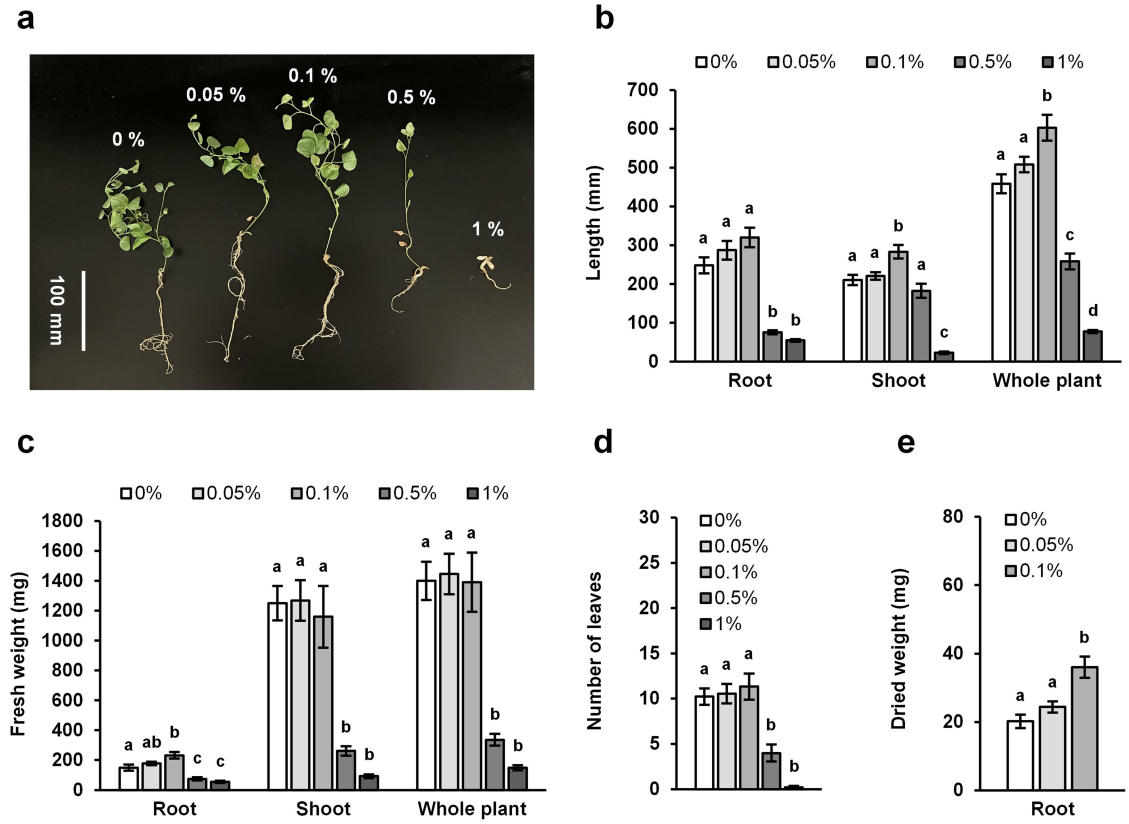

Figure 1. Effect of exogenous ethanol treatment on morphology (a), length (b), fresh weight (c), leaf number (d), and freeze-dried weight (e) of *G. uralensis* seedlings. Values are reported as mean ± SEM ($n = 9$). Different letters indicate significant differences among groups ($p < 0.05$, Tukey's HSD test).

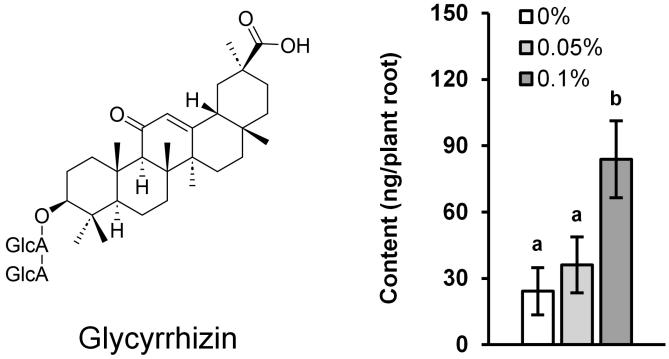

Figure 2. Glycyrrhizin content (ng/freeze-dried plant root) of *G. uralensis* seedlings grown in the presence of different exogenous ethanol concentrations. Ions of 821.40 $[M - H]^-$ were monitored to estimate glycyrrhizin content. Values are reported as mean ± SEM ($n = 9$). Different letters indicate significant differences among groups ($p < 0.05$, Tukey's HSD test). GlcA, glucuronic acid.

Discussion

Application of 0.1% exogenous ethanol was found to stimulate the growth of *G. uralensis* seedlings, whereas treatments in excess of 0.5% exogenous ethanol showed signs of phytotoxicity. Previous studies have reported similar observations. For example, Wu et al.[16] reported that treatment with exogenous ethanol (i.e., in concentrations ranging from 0.00125% to 0.005%) increased the biomass of oilseed rape (*Brassica napus* L.), whereas the application of 0.025% exogenous ethanol significantly inhibited growth in the same species. In soybean (*Glycine max*, Fabaceae), one study found that the application of 20 mM (0.12%, v/v) exogenous ethanol increased both shoot and root biomass relative to an untreated control.[17] However, treatments with concentrations exceeding 5% have been found to inhibit seedling growth.[18] In another study, ethanol treatments of 0.5 mM (0.003%, v/v) and 1 mM (0.006%, v/v) were found to increase shoot dry weight in sweet basil (*Ocimum basilicum*) and sweet wormwood (*Artemisia annua*), respectively.[19] The application of 0.25%, 1.25%, and 5% ethanol treatments significantly inhibited the growth of *Euphorbia heterophylla* L., *Cucumis sativus* L., and *Lycopersicon esculentum* Mill, respectively.[20–22] Taken together, these results indicate that treatment with ethanol can exert positive effects in some cases, whereas at other concentrations it can cause phytotoxicity; this depends on the experimental conditions and the plant species.

The application of exogenous ethanol increased glycyrrhizin accumulation in the roots of *G. uralensis* seedlings. Recent studies investigated the effects of ethanol treatments on the accumulation of secondary metabolites in plants. Das et al.[17] and Rahman et al.[23] showed that 20 mM (0.12%, v/v) ethanol treatment did not affect total phenolic and flavonoid content in soybean. Similarly, Shin et al. [19] reported that significant induction of total flavonoid content was not observed in sweet basil, Korean mint (*Agastache rugosa*), and sweet wormwood treated with ethanol. In contrast, the authors reported that 8 mM (0.05%, v/v) ethanol treatment increased total phenolic content in each of these species.[19] Accordingly, the application of exogenous ethanol may induce the accumulation of other secondary metabolites in plants in addition to glycyrrhizin in *G. uralensis*.

Drought and salt stress can induce the accumulation of secondary metabolites in higher plants and impair the growth of most plants.[24,25] However, in licorice, the moderate drought stress enhanced glycyrrhizin production and root yield without inhibiting plant growth.[26] In licorice exposed to salt stress, promotion of root biomass have not been reported.[27–30] Therefore, our findings are similar to those observed when licorice was exposed to moderate drought stress.

The group of Seki et al.[12,13,31,32] recently clarified the effect of exogenous ethanol as a chemical priming agent for plants. In cassava leaves, exogenous application of 1.0% ethanol was found to induce accumulation of abscisic acid (ABA) and upregulate the expression of ABA signaling-related genes relative to a negative (water) control.[31] No significant differences were observed in jasmonic acid and jasmonic acid-isoleucine contents between cassava leaves treated with 1.0% ethanol and those treated with water.[31] In another study, the leaves of *Arabidopsis thaliana* showed increased leaf temperature by 72 h after 10 mM (0.06%, v/v) ethanol treatment, which indicated stomatal closure and decreased transpiration.[12] In addition, ABA *insensitive 1–1* mutant showed no differences in stomatal aperture and leaf temperature in response to ethanol treatment. These results suggest that exogenous ethanol treatment is related to ABA signaling in plants.[12] Furthermore, other studies have reported the induction of glycyrrhizin accumulation following application of exogenous ABA.[33–35] Therefore, we speculate that the higher accumulation of glycyrrhizin content in response to exogenous ethanol treatment in *G. uralensis* seedlings observed here may occur via altered ABA signaling.

Overall, the findings of the present study support the suggestion by Diot et al. that low concentrations of exogenous ethanol, used at physiological levels, can cause responses similar to hormone signaling, whereas higher concentrations result in phytotoxic effects.[36] These authors also stated that additional studies on the effects of very low doses of ethanol on target plants are required on both the molecular and physiological levels.[36] Further studies on the effects of exogenous ethanol treatment on *Glycyrrhiza* plants are warranted.

Conclusion

In this study, the effects of exogenous ethanol treatment on various morphological features and glycyrrhizin content in *G. uralensis* seedlings were evaluated. 0.1% Exogenous ethanol treatment increased fresh root weight of *G. uralensis* seedlings, whereas treatments in excess of 0.5% ethanol showed phytotoxic effects. In addition, the application of 0.1% exogenous ethanol significantly promoted glycyrrhizin accumulation in the roots relative to the negative control. Taken together, these results show that the application of low-concentration exogenous ethanol can enhance the root biomass production and glycyrrhizin accumulation in aseptically grown *G. uralensis* seedlings. We believe these findings are valuable for improving the quality of cultivated *G. uralensis*.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author' contributions

Y.N.: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualization, writing – original draft preparation, and writing – review and editing. K.T.: Conceptualization, funding acquisition, and resources.

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