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Research Note

Production of astragaloside and flavones from adventitious root cultures of *Astragalus membranaceus* var. mongholicus

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Abstract

Many plants contain various secondary metabolites that have medicinal value. Their content varies across plant parts. We carried out *in vitro* adventitious root induction from leaf explants of Huang-qi (*Astragalus membranaceus*) using different nutrient media supplemented with various plant hormones. The level of astragaloside and flavones were analyzed from the adventitious roots of *A. membranaceus* grown under these different media conditions. Among the different media and plant hormones, Murashige and Skoog medium supplemented with 1.0 mg/L 1-naphthaleneacetic acid resulted in the greatest degree of adventitious root induction. The highest concentration of calycosin (11.7 µg/g dry weight), calycosin-7-glu (13.3 µg/g dry weight), and glucoraphanin (241.9 µg/g dry weight) were observed in roots grown in half-strength Schenk & Hildebrandt mineral solution, half-strength Gamborg's B5 medium, and full-strength Gamborg's B5 media, respectively.

Keywords: adventitious root, astragaloside, *Astragalus membranaceus*, flavones.
Abbreviations: IAA- indole-3-acetic acid; IBA- indole-3-butyric acid; B5- Gamborg's B5; HPLC- high-performance liquid chromatography; MS- Murashige and Skoog; NAA- 1-naphthaleneacetic acid; SH- Schenk & Hildebrandt.

Introduction

Astragalus membranaceus (Fisch.) Bge. or *Astragalus mongholicus* Bge. (Fabaceae) are perennial flowering plants native to the northern, north-eastern, and north-western parts of China, (Ma et al., 2000). *Astragalus membranaceus* is also known as Huang-qi or yellow leader, and the root has been used in traditional Chinese medicine for thousands of years to treat various diseases. The root is considered a tonic to enhance metabolism and digestion, to strengthen the immune system, and promote the healing of wounds and injuries. It is said to prevent cancer, anemia, diabetes, hepatitis, and liver and heart diseases. It is often used as an antiperspirant, immunostimulant, diuretic, and as a supplementary medicine during cancer therapy (Wagner et al., 1997; Zheng, 2005). *A. membranaceus* also has antibacterial, antiviral, and anti-inflammatory properties. Moreover, it contains antioxidants that prevent cell damage caused by free radicals. Extracts of *A. membranaceus* contain many valuable plant constituents, such as triterpenoid saponins (acetylastragalosides, astragalosides, and astragenol), amino acids, flavonoids, isoflavonoids, and polysaccharides. Among these, astragaloside is generally considered to be the primary active ingredient in *A. membranaceus* extract and is well known for its anti-aging properties. Astragaloside IV (Fig. 1-A) is the major active component extracted from *A. membranaceus* and is used in the treatment of many disorders, including cardiovascular diseases. Astragaloside IV has been shown to have protective effects against ischemic injury in the myocardium and central nervous system (Zhang et al., 2006; Luo et al., 2004; Zhou et al., 2000; Qu et al., 2009). Furthermore, it has shown promise as a natural product with both healing and anti-scarring effects for wound treatment. These results provide support for the application of astragaloside IV in the treatment of injuries (Chen et al., 2012). Moreover, calycosin (Fig. 1-B) and calycosin-7-O-β-D-glucoside (calycosin-7-glu; Fig. 1-C) are 2 major isoflavones related to the bioactivity of the herb (Toda and Shirataki, 1998; Wu et al., 2000). Calycosin has been shown to protect endothelial cells from hypoxia-induced barrier impairment (Fan et al., 2003). Calycosin-7- β-D-glucoside appears to be a potential natural anti-inflammatory and anti-osteoarthritic agent and might be used effectively to protect against proteoglycan degradation (Choi et al., 2005) and as a hyaluronidase inhibitory component (Lee et al., 2005). It has been proposed that these two compounds could be used as "marker compounds" for the chemical evaluation or product standardization of *A. membranaceus* (Nakamura et al., 1999). The roots of numerous plant families are the site for biosynthesis or accumulation of major secondary metabolites, including alkaloids, polyacetylene, sesquiterpenes, and napthoquinones. *In vitro* root culture is an alternative method for the production of valuable

secondary metabolites on a commercial scale. Adventitious roots, induced by *in vitro* methods, show a high rate of proliferation and active secondary metabolism (Hahn et al., 2003; Yu et al., 2005). They grow vigorously in phytohormone-supplemented medium and have shown tremendous potential for accumulation of valuable secondary metabolites (Murthy et al., 2008). The development of an efficient method for induction and establishment of adventitious *A. membranaceus* root cultures, as well as identification of conditions for optimal flavonol production in this plant, is warranted for the efficient utilization of this medicinal plant. The objectives of this study were to find out suitable medium with appropriate auxin concentration and analysis of astragaloside and flavones from adventitious root cultures of *A. membranaceus*.

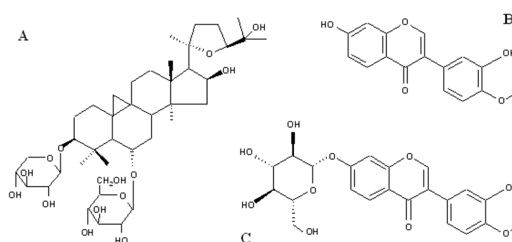

Fig 1. Chemical structure of astragaloside IV (A), calycosin (B), and calycosin-7-O-β-d-glucoside (C).

Results

Adventitious root induction

Leaf explants were cultured on MS medium supplemented with various auxins, such as IAA, IBA, and NAA. After 6 weeks, the adventitious roots, approximately 20%, were produced only on MS medium supplement with 1.0 mg/L NAA (Fig. 2-A). No adventitious roots were observed with other media and hormone combinations (data not shown). Emerging roots were then further cultured in Erlenmeyer flasks containing MS liquid medium supplemented with 1.0 mg/L of NAA (Fig. 2-B).

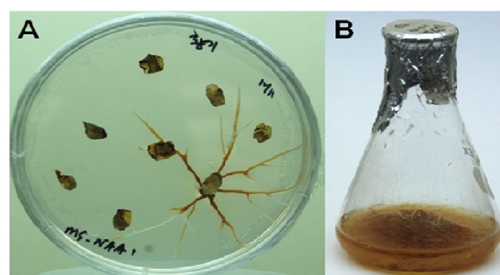

Fig 2. Adventitious root induction and culture of *Astragalus membranaceus* var. mongholicus. A: Adventitious root-induction from leaf explants cultured on MS medium containing 1 mg/L NAA after a 6-week culture. B: Adventitious root culture in MS medium containing 1 mg/L NAA after a 3-week culture.

Effect of different media on adventitious root growth of A. membranaceus

Roots were separated from the solid medium and inoculated into NAA-supplemented MS liquid medium. Culture flasks were maintained under dark conditions and served as sources of inoculums for further experiments. Adventitious roots were cultured by growing the root inoculums in various concentrations of MS, B5, and SH liquid media, fortified with 1.0 mg/L NAA and 3% (w/v) sucrose (Fig. 3). Among the media tested, MS medium sustained better root growth (455.7 mg), followed by ½ MS, ½ SH, ½ B5, B5, and SH media. These results indicated that MS medium was the best for adventitious root induction and that this method can be useful for the large-scale production of *A. membranaceus*.

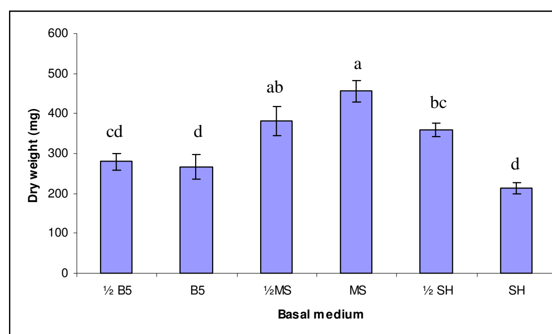

Fig 3. Effect of media on growth of adventitious roots of *Astragalus membranaceus* var. mongholicus after a 3-week culture. Values represent LSD (p=0.05).

Chemical analysis for flavones and astragaloside compound in A. membranaceus

Among the different flavonoids in the roots of *A. membranaceus*, we assessed the content of calycosin and calycosin-7-glu in basal medium (Fig. 4). The calycosin content was found to be the highest in ½ SH medium (11.7 µg/g dry weight), followed by that in ½ B5, ½ MS, SH, and B5 media, while MS medium had the lowest content (3.8 µg/g dry weight). Levels of calycosin-7-glu were the highest in roots grown in ½ B5 medium (13.3 µg/g dry weight), while those of roots grown in SH medium were the lowest (7.0 µg/g dry weight). However, one of the astragaloside compounds, glucoraphanin, was more concentrated in roots grown in B5 medium (241.9 µg/g dry weight), followed by those grown in MS, ½ B5, ½ MS, and SH media, and the lowest (183.6 µg/g dry weight) in roots grown in ½ SH medium (Fig. 5).

Discussion

Leaf explants were cultured on MS basal medium with different concentrations and combinations of auxins. MS basal medium supplemented with 1.0 mg/L NAA was found to be the best for adventitious root induction. This result was concurred with the findings of Kitto and Young (1981), which indicated that among common auxins, NAA is the most effective auxin for inducing root regeneration. Thereafter, the proliferation of adventitious roots was tested using 6 different concentrations of 3 different basal media supplemented with 1.0 mg/L of NAA.

Again, MS medium supplemented with 1 mg/L NAA was observed to be the best for adventitious root growth among the different media tested. This confirmed that MS medium is the most suitable basal medium for adventitious root induction of *A. membranaceus*. The same result was also reported by Khalafalla et al., (2009) in *in vitro* fast-growing normal root culture of *Vernonia amygdalina*. The stimulatory effect of basal medium on adventitious root induction and root quality has already been reported (Baskaran and Jayabalan, 2005). Similarly, in *Bupleurum falcatum* adventitious root cultures, full strength MS medium was found to be sufficient for both root development and saikosaponin production (Kusakari et al., 2000). Amzallag et al. (1992) reported that the promoter effect of mineral concentration of the culture medium on rooting could be attributed to the participation of inorganic ions in processes regulating hormonal balance. Sometimes, high levels of auxin are deleterious to secondary metabolite production in plants (Dornenburg and Knorr, 1995; Chan et al., 2005). Wu et al. (2006) demonstrated that increasing the NAA concentration had a negative effect on biomass, phenol and flavonoid contents in adventitious roots of *Echinacea angustifolia*. However, the response of adventitious roots to different auxins depends on the plant species. For example, treatment with IBA is more effective than NAA in promoting biomass production from root cultures of *Panax ginseng* (Kim et al., 2003). In contrast, NAA is better at inducing the elongation of tomato lateral roots (Taylor and van Staden, 1998). In the present study, roots grown on MS medium had low flavone content, even though this medium produced the greatest growth of adventitious roots when supplemented with 1.0 mg/L of NAA (Fig. 3). Moreover, the flavone and astragaloside contents varied widely, depending on the medium used. In this study, ½ SH, ½ B5, and B5 medium resulted in the highest concentration of calycosin, calycosin-7-glu, and astragaloside, respectively. According to this finding, *Astragalus membrabaceus* requires high nutrient concentrations which are a critical determinant in controlling the growth of adventitious roots. Therefore, in this study, the difference in rooting ability between basal media might be due to their basal salt formulation and the low number of roots obtained on explant cultured on B5 and SH media is probably due to their low ammonium content compared to MS medium. Kim et al. (2009) demonstrated that calycosin from *A. membranaceus* root reduces melanin production by regulating the tyrosinase enzyme. Furthermore, it has been suggested calycosin maybe a potential skin-whitening agent.

Materials and methods

Seed sterilization and germination

Seeds of *A. membranaceus* were surface sterilized with 70% (v/v) ethanol for 30 s and 2% sodium hypochlorite solution for 10 min. Seeds were then rinsed thoroughly with sterilized distilled water and incubated on 25 mL of hormone-free Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) in Petri dishes under appropriate light conditions. The basal medium consisted of a mineral salts-and-vitamins supplement together with 30 g/L of sucrose and 8 g/L of Phytagar as the solidifying agent. The pH of the medium was adjusted to 5.8 before adding the Phytagar; media were sterilized by autoclaving at 121°C for 20 min. The germinated seeds were transferred to a Magenta box containing 50 mL MS basal medium and maintained under controlled environmental conditions until use.

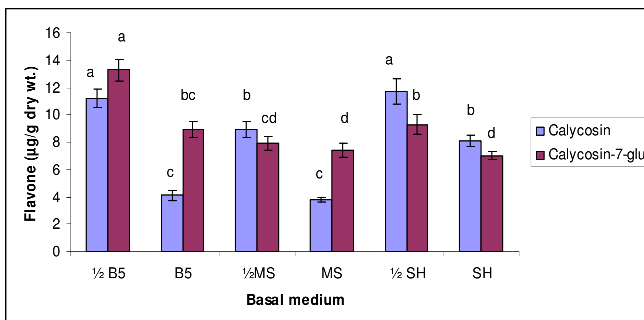

Fig 4. Effect of media on production of flavones in cultures of adventitious roots of *Astragalus membranaceus* var. mongholicus after a 3-week culture. Values represent LSD (p=0.05).

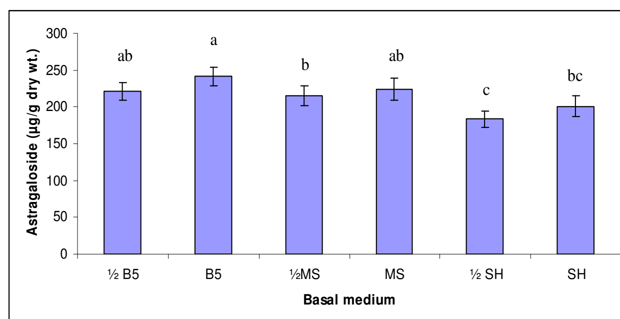

Fig 5. Effect of media on astragaloside production in adventitious root culture of *Astragalus membranaceus* var. mongholicus after a 3-week culture. Values represent LSD (p=0.05).

Adventitious root induction

Leaves (Four weeks old) from *in vitro*-grown plants were excised and cut into small segments (0.5–1.0 cm). The excised leaf segments were cultured on MS medium (Murashige and Skoog, 1962) supplemented with indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) at 0.1, 0.5, and 1.0 mg/L, respectively. The cultures were kept under dark conditions for adventitious root development. After 6 weeks, the emerging roots were transferred to 100-mL Erlenmeyer flasks containing 30 mL MS liquid medium with 1.0 mg/L of NAA for root multiplication. Then, adventitious root culture of *A. membranaceus* was initiated by transferring 2 g of the inoculum from the Erlenmeyer flasks into half-strength (½) MS, full-strength MS, ½ Gamborg's B5 (Gamborg et al., 1968), full-strength B5, ½ Schenk & Hildebrandt (SH) (Schenk and Hildebrandt, 1972), and full-strength SH liquid media, each fortified with 1.0 mg/L NAA and 3% (w/v) sucrose. These flasks were placed on a rotary shaker at 100 rpm, in dark conditions, for 3 weeks. Approximately 20 leaves were used per experiment, and the experiment was repeated thrice in the same environment. The growth rate was measured by taking into account the fresh and dry weight of the roots after 3 weeks of culture. Fresh weight was determined after completely removing the medium by blotting with tissue paper; roots were then placed onto pre-weighed aluminum foil, weighed, and then kept at -80°C for a few hours to freeze-dry the sample for chemical analysis.

Chemical analysis of flavones and astragaloside compounds

Chemical analysis was carried out by high performance liquid chromatography (HPLC) analysis. Freeze-dried samples of A. *membranaceus* adventitious roots were ground into a fine powder using a mortar and pestle. Two hundred milligrams of powdered samples were extracted with 5 mL of 80% (v/v) ethanol at room temperature for 30 minutes. The samples were extracted for three times for the quantification of flavones and astragaloside compounds. Then, the solvent was evaporated and add 1 ml of 80 % methanol. Thereafter, the extracts were centrifuged and the supernatant was filtered with a 0.45-μm Acrodisc syringe filter (Pall Corp.; Port Washington, NY), for HPLC analysis. HPLC analysis was performed with a C18 column (250 × 4.6mm, 5 μm; RStech; Daejeon, Korea). The mobile phase was a gradient prepared from mixtures of acetonitrile and 0.3 % formic acid and the column temperature was maintained at 30°C. The flow rate was maintained at 1.0 mL/min. Injection volume of 20 μL and 280 nm wavelengths were used for detection of flavones and ELSD (Evaporative Light Scattering Detector) was used for astragaloside. The concentrations of flavones and astragaloside compounds were determined by using a standard curve. All samples were analyzed in triplicate.

Statistical analysis

All data are given as the mean and standard deviation of triplicate experiments. The data were analyzed by using the computer software Statistical Analysis System (SAS version 9.2). Treatment mean comparisons were carried out using the Least Significant Difference (LSD).

Conclusion

Adventitious root culturing is an efficient method for producing useful phytomolecules. Here, we developed the most suitable basal medium and auxin concentration for adventitious root induction in *A. membranaceus*. In addition, we could analyze the constitutient of flavone and astragaloside from the adventitious root of *A. membranaceus*. Although previous investigations have identified flavone and astragaloside contents from different plant parts and hairy root cultures of *A. membranaceus*, to date, no other study has yet investigated this in the context of *in vitro* adventitious root culture using different nutrient media. Therefore, our findings provide a useful basis for increasing the production of secondary metabolites in *A. membranaceus*.

Acknowledgements

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References

Amzallag G, Lerner N, Poljakoff-Mayber HRM (1992) Interaction between mineral nutrients, cytokinin and gibberillic acid during growth of sorghum at high NaCl salinity. J Exp Bot 43: 81–87.

Baskaran P, Jayabalan N (2005) Role of basal media, carbon sources and growth regulators in micropropagation of *Eclipta alba* – a valuable medicinal herb. KMITL Sci J 5 (2): 469-482.

Chan LK, Dewi PR, Boey PL (2005) Effect of plant growth regulators on regeneration
of plantlets from bud cultures of *Cymbopogon nardus* L. and the detection of essential oils from the *in vitro* plantlets. J Plant Biol 48:142-145.

Chen X, Peng LH, Li N, Li QM, Li P, Fung, KP, Leung PC, Gao JQ (2012) The healing and anti-scar effects of astragaloside IV on the wound repair *in vitro* and *in vivo*. J Ethnopharmacol 139:721-727.

Choi S, Park SR, Heo TR (2005) Inhibitory effect of astragali radix on matrix degradation in human articular cartilage. J Microbiol. Biotechnol 15: 1258–1266.

Dornenburg H, Knorr D (1995) Strategies for the improvement of secondary metabolite production in plant cell cultures. Enzyme Microbial Technol 17:674-684.

Fan Y, Wu DZ, Gong YQ, Zhou RY, Hu ZB (2003) Effects of calycosin on the impairment of barrier function induced by hypoxia in human umbilical vein endothelial cells. Eur J Pharmacol 481: 33-40.

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirement of suspension cultures of soybean root cells. Exp Cell Res 50: 151-158.

Hahn EJ, Kim YS, Yu KW, Jeong CS, Paek KY (2003) Adventitious root cultures of *Panax ginseng,* Meyer CA and ginsenoside production through large scale bioreactor systems. J Plant Biotechnol 5: 1-6.

Khalafalla MM, Daffalla HM, El-Shemy HA, Abdellatef E (2009) Establishment of *in vitro* fast-growing normal root culture of *Vernonia amygdalina* - a potent African medicinal plant. African J Biotechnol 8 (21):5952-5957.

Kim JH, Kim MR, Lee ES, Lee CH (2009) Effects of Calycosin Isolated from the Root of *Astragalus membranaceus* on Melanin Biosynthesis. Biol Pharmaceut Bull 32(2): 264-268.

Kim YS, Hahn EJ, Yeung EC, Paek KY (2003) Lateral root development and saponin accumulation as affected by IBA or NAA in adventitious root cultures of *Panax ginseng* CA Meyer. *In Vitro* Cell Dev Biol Plant 39: 245-249.

Kitto SL,Young MJ (1981) In vitro *propagation of* Carrizo citrange. Hort Science 16*:* 305-306.

Kusakari K, Yokoyama M, Inomata S (2000) Enhanced Production of saikosaponin in cultured roots of *Bupleurum falcatum* L. using two-step control of sugar concentration Plant Cell Rep. 19: 1115-1120.

Lee YM, Choi SI, Lee JW, Jung SM, Park SM, Heo TR (2005) Isolation of hyaluronidase inhibitory component from the roots of Astraglus membranaceus Bunge Astragali radix. Food Sci Biotechnol 14: 263–267.

Luo Y, Qin Z, Hong Z, Zhang XM, Ding D, Fu JH, Zhang WD, Chen J (2004) Astragaloside IV protects against ischemic brain injury in a murine model of transient focal ischemia. Neurosci Lett 363: 218-223.

Ma XQ, Duan JA, Zhu DY, Dong TTX, Tsim KWK (2000) Species identification of *Radix Astragali* (Huang-qi) by DNA sequence of its 5S-rRNA spacer domain. Phytochem 54: 363-368.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497.

Murthy HN, Hahn EJ, Paek KY (2008) Adventitious roots and secondary metabolism. Chinese J Biotechnol 24: 711-716.

Nakamura T, Hashimoto A, Nishi H, Kokusenya Y (1999) Investigation on the marker substances of crude drugs in formulations. I. Marker substances for the identification of *Astragali Radix* in kampo and drinkable preparations. J Pharmaceut Soc Jap 119:391-400.

Qu YZ, Li M, Zhao YL,Zhao ZW, Wei XY, Liu JP, Gao L, Gao GD (2009) Astragaloside IV attenuates cerebral ischemia–reperfusion-induced increase in permeability of the blood-brain barrier in rats. Eur J Pharmacol 606: 137-141.

Schenk RU, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledons and dicotyledonous plant cell cultures. Can J Bot 50: 199-204.

Taylor JLS, van Staden J (1998) Plant-derived smoke solutions stimulate the growth of *Lycopersicon esculentum* roots *in vitro.* Plant Growth Reg 26: 77-83.

Toda S, Shirataki Y (1998) Inhibitory effects of isoflavones in roots of *Astragalus membranaceus* Bunge (Astragali Radix) on lipid peroxidation by reactive oxygen species. Phytother Res 12:59-61.

Wagner H, Bauer R, Xiao PG, Chen JM, Michler G (1997) Chinese Drug Monographs and Analysis: *Radix Astragali* (Huangqi); Verlag Wald Germany pp 1-17.

Wu CH, Dewir YH, Hanh EJ, Paek KY (2006) Optimization of culturing conditions for the production of biomass and phenolics from adventitious roots of *Echinacea angustifolia*. J Plant Biol 49(3): 193-199.

Wu DZ, Fan Y, Hen ZF, Song CQ, Hu ZB (2000) Effect of flavonoids isolated from *Astragalus membranaceus* on the changes of endothelial permeability induced by low osmolarity. Pharmacol Clin Chin Mater Med 16:16–18.

Yu KW, Hahn EJ, Paek KY (2005) Production of adventitious roots using bioreacters. Korean J Plant Tissue Cult 27: 309-315.

Zhang WD, Chen H, Zhang C, Li HL, Liu RH, Chen HZ (2006) Astragaloside IV from *Astragalus membranaceus* shows cardioprotection during myocardial ischemia *in vivo* and *in vitro*. Planta Med 72: 4-8.

Zheng XY (2005) Pharmacopoeia of the Peoples Republic of China, Vol. 1, Chinese edn. Beijing: Chemical Industry Press 212-213.

Zhou JY, Fan Y, Kong JL, Wu DZ, Hu ZB (2000) Effects of components isolated from *Astragalus membranaceus* Bunge on cardiac function injured by myocardial ischemia reperfusion in rats. Chin J Chin Mater Med 25:300-302.