

Original Research Paper

# Enhancing Astragaloside Production in *Astragalus membranaceus* through Growth Media and Auxins

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**Abstract:** The secondary metabolites of triterpenoid saponins, isoflavonoids and polysaccharides are present in *Astragalus membranaceus* those are biologically active. Astragalosides are a major group of identified compounds that have various pharmacological properties. The present study was considered to enhance astragaloside biosynthesis in hairy root cultures of *A. membranaceus* by using different growth media and concentrations of different auxins. Our findings revealed that Schenk and Hildebrandt (SH) medium responded greatly for the production of the highest dry weight. However, the highest astragaloside production was detected while using SH medium of its half strength. The growth of hairy roots and accumulation of astragaloside in response to different auxinlike Indole-3-Acetic Acid (IAA), Indole-3-Butyric Acid (IBA) and 1-naphthaleneacetic acid (NAA) were different. Hairy root growth rate was increased by the treatment of IBA and NAA, but was unaffected by IAA. Accumulation of Astragaloside was enhanced in response to the treatment of IBA and NAA. In most cases, treatment with the 0.5 mg L<sup>-1</sup> NAA accumulated the highest level of stragaloside in the hairy root of *A. membranaceus*, which might, therefore, be utilized as an ideal mass-scale production system for astragalosides.

**Keywords:** Astragaloside, *Astragalus membranaceus*, Auxin, Media, Hairy Root Culture

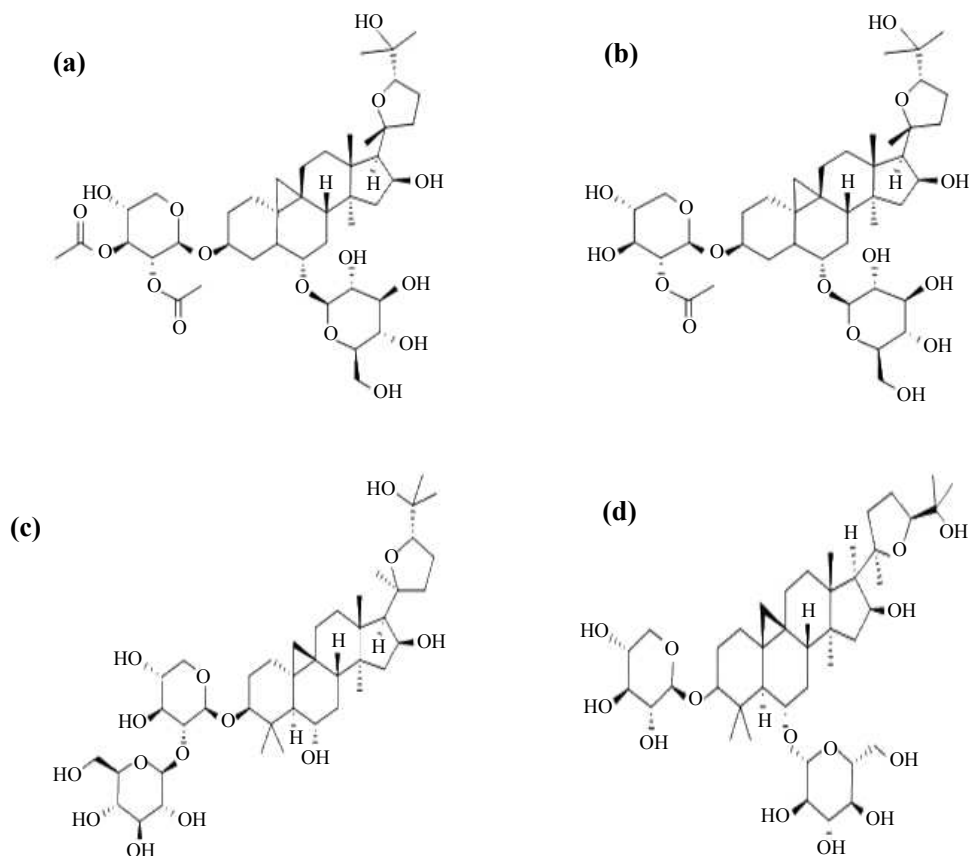
## Introduction

Tissue cultures are generating increasing interesting as potential alternatives for achieving useful and valuable biologically active secondary metabolites (Murthy *et al.*, 2014). For getting higher amount of secondary metabolites and other inevitable biologically active compounds with the shortest possible time and biochemical stability, rapid growth rate, independence from environmental variations and productive capacity of secondary metabolites, hairy root is the very efficient method and can be used in the pharmaceutical and biotechnology industries (Ono and Tian, 2011; Zhou *et al.*, 2011; Talano *et al.*, 2012).

Dried root of *Astragalus membranaceus*, very usually known as Huang Qi, is broadly used in traditional Chinese medicine (Kwon *et al.*, 2013). Its pharmacological properties have also been proven against several important diseases like antidiabetic, analgesic, diuretic, expectorant, hepatoprotective, immunostimulant, sedative and tonic, (Sinclair, 1998).

Several biologically active constituents are present in *A. membranaceus* roots, such as saponins, isoflavonoids, polysaccharides and astragalosides. Astragalosides belong to the class of cycloartane are principal constituents that are also used as quality control markers of the root (Fig. 1) (Kwon *et al.*, 2013). Particularly, the astragaloside IV has the potentiality to use as a neuroprotective, anti-inflammatory and cardio protective agent and has effects on osteogenesis, angiogenesis and metabolic syndrome (Bian *et al.*, 2011; Luo *et al.*, 2004; Xu *et al.*, 2008; Zhang *et al.*, 2011a; 2011b; 2005).

Selection of good cell lines, media, use of proper elicitors, supplementation of nutrient and precursor, biotransformation, immobilization as well as permeabilization have been accounted to enhance the accumulation of biomass and secondary metabolites, which is desirable (Murthy *et al.*, 2014). Therefore, this study was undertaken to enhance hairy root growth and astragaloside production of *A. membranaceus* through different media and growth regulator especially auxins.



**Fig. 1:** Structures of astragalosides showing astragaloside I (a), astragaloside II (b), astragaloside III (c) and astragaloside IV (d)

## Materials and Methods

### Chemicals

The astragalosides I, II, III and IV used for external standards were procured from ChromaDex (Irvine, CA, USA). MS basal medium (M4531), SH basal salt mixture (S4828), B5 basal medium (G4410) and IAA (I5897) were obtained from MB Cell (Los Angeles, CA, USA). IBA (1538) was purchased from Phytotechnology Laboratories (Shawnee Mission, KS, USA) and NAA (1465-25-4) was obtained from Kanto Chemical Co (Japan). HPLC-grade methanol was obtained from J. T. Baker Chemical Co., (Phillipsburg, NJ, USA) and formic acid was purchased from Sam Chun Pure Chemical Co., Ltd. (Gyeonggi, Korea).

### Hairy Root Cultures Establishment

A transformed hairy root was initiated from explants of *A. membranaceus* after being infected with *Agrobacterium rhizogenes* (strain R1000) according to methods described previously (Fig. 2) (Park *et al.*, 2015). They were subcultured every 20 days in half-strength MS liquid medium having 25°C on a shaker (110 rpm) in the dark.



**Fig. 2:** Photograph of 20-day-old hairy root of *A. membranaceus* grown in SH liquid medium

To determine the optimal medium conditions, 2 g fresh hairy root segments was inoculated into 30 mL full- and half-strength MS media (Murashige and Skoog,

1962), Schenk and Hildebrandt (SH) (Schenk and Hildebrandt, 1972) and Gamborg (B5) (Gamborg *et al.*, 1968). The effect of exogenous auxin treatment was determined in 2 g of hairy roots cultured on 30 mL of half-strength MS medium in addition of different concentrations (0.1, 0.5 and 1 mg L<sup>-1</sup>) of IAA, IBA and NAA. After 20 days, all samples were collected and freeze-dried at -80°C for 72 h.

#### Extraction of Astragalosides and HPLC Analysis

Crude extracts from hairy root cultures were obtained by adding 1.5 mL methanol to 100 mg of the dried sample. The supernatant was filtered through a 0.45- $\mu$ m Polytetrafluoroethylene (PTFE) syringe filter (Advantec DISMIC-13HP, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) after centrifugation at 4,000 rpm at 25°C for 10 min. The astragalosides were divided by using C18 column (250 $\times$ 4.6 mm, 5  $\mu$ m; RStech, Daejeon, Korea) through a Futecs model NS-4000 HPLC apparatus (Daejeon, Korea). The drift tube temperature for the Evaporative Light Scattering Detector (ELSD) and nebulizing using a gas flow where rate were set at 70°C and 3 mL min<sup>-1</sup>, respectively. formic acid 0.3% in water (solvent A) and acetonitrile (solvent B) were used as the mobile phase. Samples were eluted with the following gradient: 0 min, 75% A/25% B; 15 min, 65% A/35% B; 3 min, 55% A/45% B; 5 min, 55% A/ 45% B; 25 min, 35% A/65% B; 10 min, 20% A/80% B; 6 min, 20% A/80% B. The flow rate was retained at 0.8

mL min<sup>-1</sup>. The quantification was done by comparing the test sample retention time to that of a reference and standard curve.

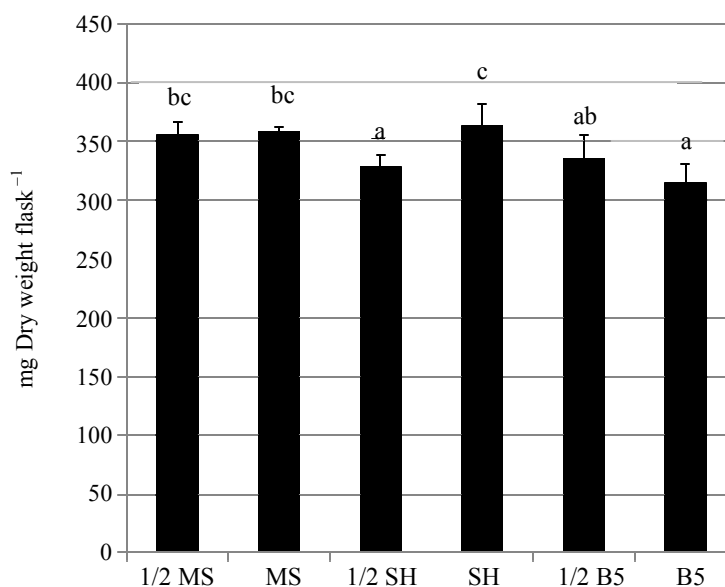
#### Statistical Analysis

All data used in this study are the mean values of three independent biological experiments. The statistical analysis was performed using an Analysis of Variance (ANOVA) with Duncan's multiple range test at a level of significance of  $p < 0.05$  following the statistical package of SPSS program (SPSS Inc., Chicago, IL, USA).

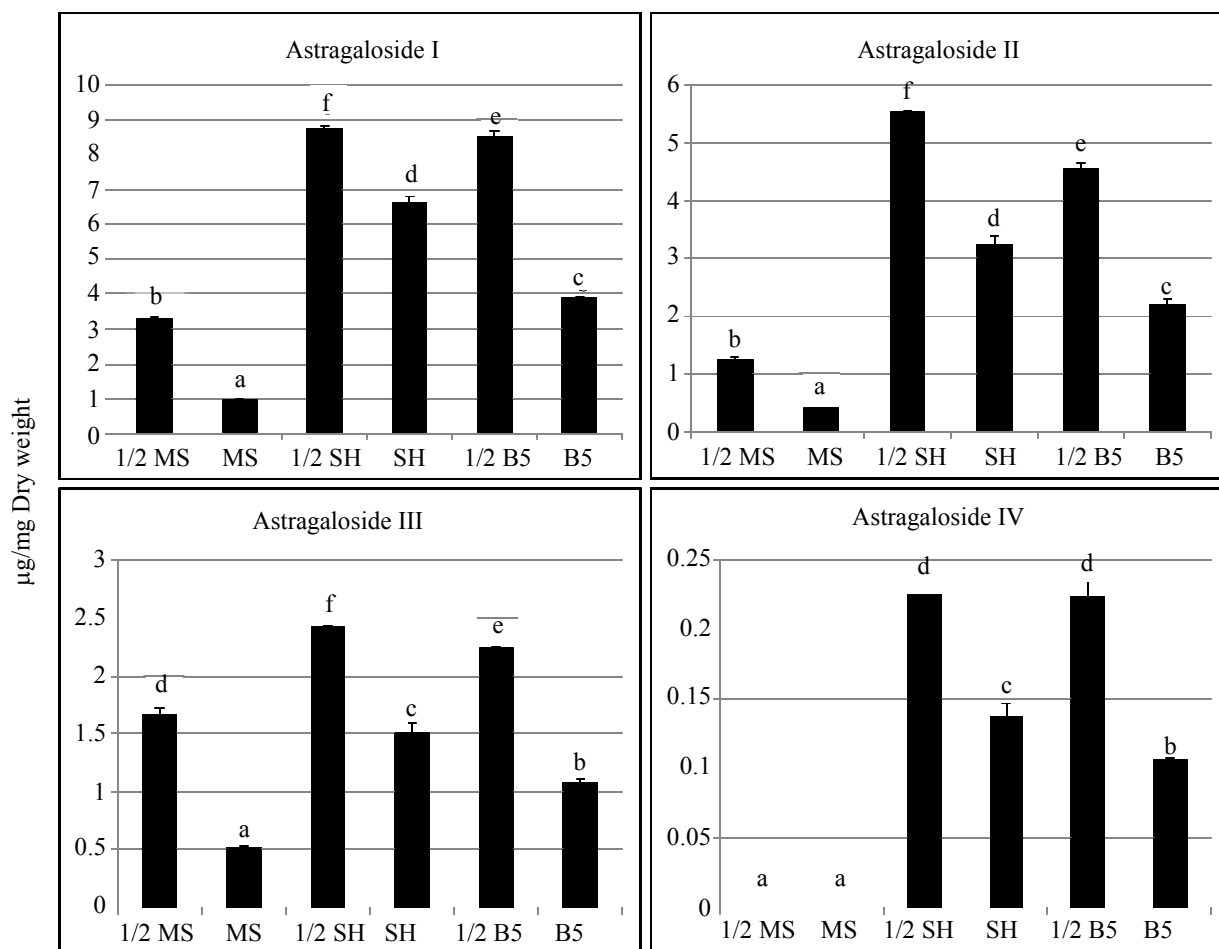
## Results

#### Optimization of Hairy Root Culture

The highest hairy root biomass was produced using SH medium, whereas the lowest was found in B5 medium (Fig. 3). The level of astragalosides was significantly responded by the media type and salt strength (Fig. 4). All types of astragalosides were detected while using SH and B5 media having their full and half strength. However, using MS media with full- and half-strength contained astragaloside I, II and III, but did not detect any astragalosides IV. Moreover, the levels of astragalosides were higher in the half-strength media than they were in the full-strength media. In most cases, the highest levels of astragalosides were observed in half-strength SH medium.



**Fig. 3:** Effects of different growth media on hairy root biomass in *A. membranaceus*. Values are means from three independent replicate results  $\pm$  standard deviation (SD). In a bar, figures with same letters or without letters do not differ significantly whereas figures with dissimilar letter differ significantly at  $p < 0.05$  using DMRT. MS = Murashige and Skoog, SH = Schenk and Hildebrandt, B5 = Gamborg

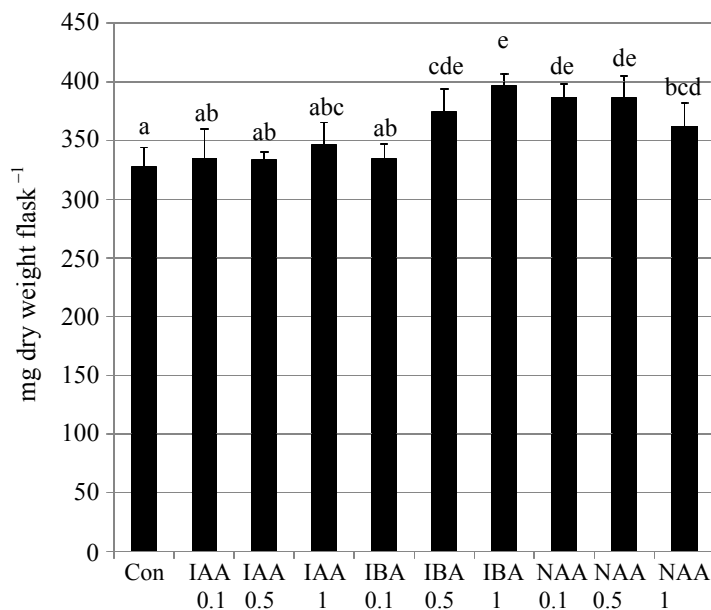


**Fig. 4:** Astragaloside contents in *A. membranaceus* hairy root cultures in different media. Values are means from three independent replicate results  $\pm$  Standard Deviation (SD). In a bar, figures with same letters or without letters do not differ significantly whereas figures with dissimilar letter differ significantly at  $p < 0.05$  using DMRT. MS = Murashige and Skoog, SH = Schenk and Hildebrandt, B5 = Gamborg

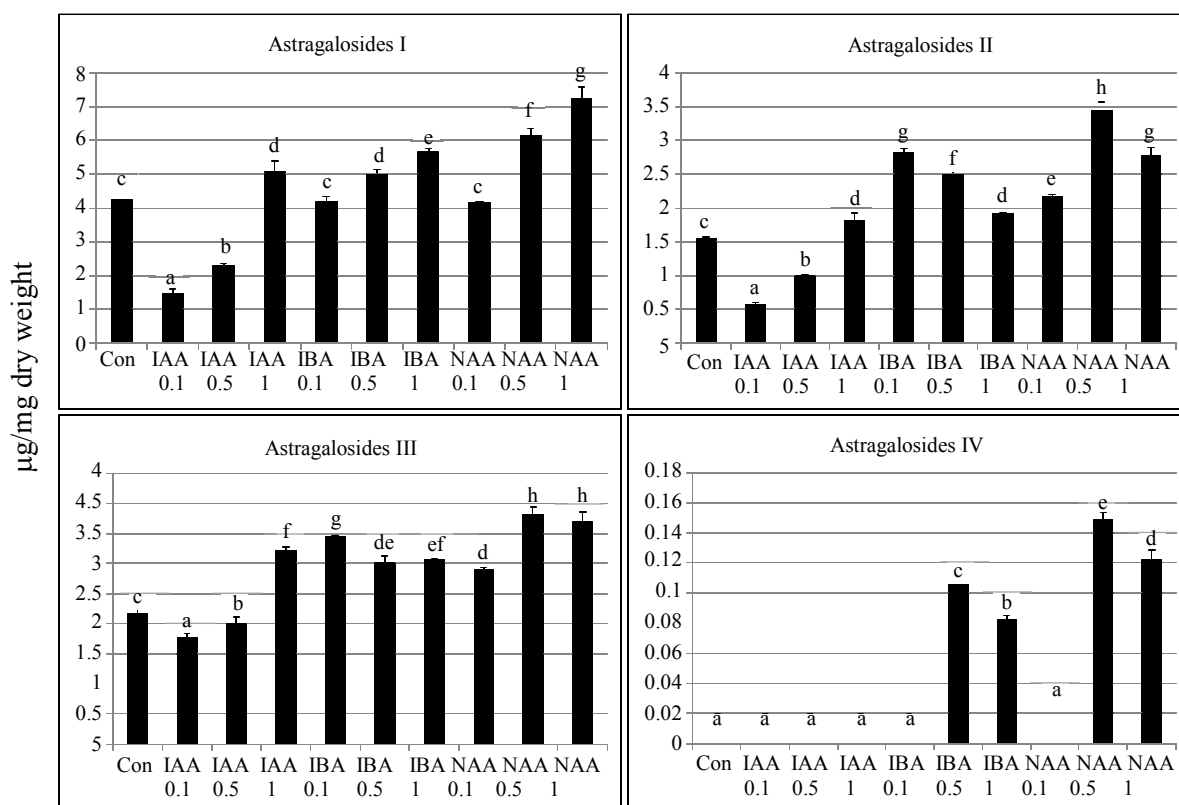
#### Effects of Auxin on Growth of Hairy Root and Accumulation of Astragalosides in *A. membranaceus*

Hairy roots were established using the best growth medium (half-strength MS medium) combined with concentrations (0.1, 0.5 and 1 mg L<sup>-1</sup>) of IAA, IBA and NAA to investigate the combined response of medium and auxins. Different responses to the auxin were detected in hairy root growth (Fig. 5). Furthermore, IAA treatment showed no positive effect on the growth rate of hairy root. Auxin IBA responded positively in a rapid concentration-dependent for hairy root growth. In particular, MS medium of half strength with 1 mg L<sup>-1</sup> IBA produced the maximum biomass productivity. The biomass of the hairy roots was enhanced by NAA at their lower concentrations (0.1 and 0.5 mg L<sup>-1</sup>), but the effect was decreased by high-concentration (1 mg L<sup>-1</sup>) NAA.

Response of exogenous auxins on astragaloside accumulation in hairy roots of *A. membranaceus* is shown in Fig. 6. The levels of astragalosides I, II and III decreased at low concentrations of IAA (0.1 and 0.5 mg L<sup>-1</sup>), whereas the levels of mentioned astragalosides increased when the concentration was 1 mg L<sup>-1</sup> IAA, compared to the control. However, none of the IAA concentrations improved the astragaloside IV production. IBA concentration-dependently increased the content of astragaloside I. In contrast, the synthesis of astragalosides II and III was high in 0.1 mg L<sup>-1</sup> of IBA, but declined with increasing concentrations. The astragaloside IV content was detected in hairy roots treated with IBA of 0.5 and 1 mg L<sup>-1</sup>. Among the treatments NAA at 0.5 mg L<sup>-1</sup> yielded the highest accumulation of astragalosides II, III and IV. In addition, treatment with NAA at 1 mg L<sup>-1</sup> induced the highest formation of astragaloside I.



**Fig. 5:** Effects of auxins on hairy root growth in *A. membranaceus*. Values are means from three independent replicate results  $\pm$  Standard Deviation (SD). In a bar, figures with same letters or without letters do not differ significantly whereas figures with dissimilar letter differ significantly at  $p < 0.05$  using DMRT. IAA = Indole-3-Acetic Acid, IBA = Indole-3-Butyric Acid (IBA) and NAA = 1-Naphthaleneacetic Acid



**Fig. 6:** Effects of exogenous auxins on astragalosides accumulation in hairy root cultures of *A. membranaceus*. Values are means from three independent replicate results  $\pm$  standard deviation. In a bar, figures with same letters or without letters do not differ significantly whereas figures with dissimilar letter differ significantly at  $p < 0.05$  using DMRT. IAA = Indole-3-Acetic Acid, IBA = Indole-3-Butyric Acid (IBA) and NAA = 1-Naphthaleneacetic Acid

## Discussion

Various factors those could lead to enhance growth and accumulation of different secondary metabolite production are an appropriate type and strength of salt of the medium; elicitors, concentrations of carbohydrates, nitrate and phosphate; and levels of growth regulator. Different medium formulations, including MS, SH, B5 and (LS) (Linsmaier and Skoog, 1965), have been developed and widely used for plant cultures (Murthy *et al.*, 2014). Numerous studies have demonstrated that different media with varying salt strengths affect the accumulation of different compounds and secondary metabolites, in different plant or tissue cultures (Murthy *et al.*, 2014). For instance, Nagella and Murthy (2011) reported that a full-strength MS medium had a significant effect on higher production of biomass and higher level of gymnemic acid accumulation in cell suspension cultures of *Gymnema sylvestre*. The highest growth rate in the hairy root cultures of ginseng was observed in MS medium having 0.75-strength, but the level of ginsenoside from a 0.5-strength MS medium was considerably higher than that from other media (Sivakumar *et al.*, 2005). Therefore, optimization of the medium is crucial for establishing optimal plant cultures.

Exogenous growth regulators have been used to investigate the root biomass and accumulation of secondary metabolites in plant cell, tissue and hairy root cultures (Murthy *et al.*, 2014). Among the various growth regulators, auxins, such as IAA, IBA and NAA play important roles in root development and promoting hairy root induction (Cheruvathur and Thomas, 2014). Moreover, the biomass growth and metabolite accumulation in media treated with different types and concentrations of auxins vary based on plant cell cultures (Mantell and Smith, 1983). Our findings strongly support the results of previous studies that determined the response of exogenous auxin on growth of root biomass and accumulation of natural products in variable plant species, such as *Nicotiana tabacum*, *Morinda citrifolia* and *Lippia dulcis* (Sahai and Shuler, 1984; Sauerwein *et al.*, 1991; Zenk *et al.*, 1975)

## Conclusion

Finally, we investigated the optimal conditions for accumulation of astragaloside in hairy root cultures of *A. membranaceus*. The findings of this study confirmed that hairy roots grow better in SH medium containing 3% sucrose and that the half-strength SH medium was superior to other media for astragaloside production. Moreover, treatment with exogenous auxins, especially NAA, significantly enhanced biomass and astragaloside productivity. Therefore, hairy root culture is an ideal ways for the production of large amounts of biomass with increased astragaloside accumulation for *A. membranaceus*.

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## Author Contributions

**Yun Ji Park:** Performed the experiments, analyzed the data and prepare the manuscript.

**Jaе Kwang Kim:** Performed the experiments, analyzed the data and prepare the manuscript.

**Sang Un Park:** Designed the experiments, coordinated the implementation of research work.

## Conflict of Interest

There is no conflict of interest among the authors.

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## Abbreviations

ANOVA, Analysis of Variance; B5, Gamborg; ELSD, Evaporative Light Scattering Detector; HPLC, High-Performance Liquid Chromatography; IAA, Indole-3-Acetic Acid; IBA, Indole-3-Butyric Acid; LS, Linsmaier and Skoog; MeOH, Methanol; MS, Murashige and Skoog; NAA, 1-Naphthaleneacetic Acid; PTFE, Polytetrafluoroethylene; SH, Schenk and Hildebrandt; SPSS, Statistical Package for the Social Science.