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Optimization of flavonoid production in cell cultures of *Astragalus missouriensis* Nutt. (Fabaceae)

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ABSTRACT
The use of flavonoids for prevention and cure of human diseases is already widespread. Quercetin shows anti-proliferative effects against various cancer cell lines. These aspects made flavonoids an interesting object for industrial production. This paper describes a plant-cell-culture production approach that provides increased yields of flavonoids. Cell culture of *Astragalus missouriensis* was established, and flavonoid production was determined and optimized. The chemical investigation of cell extracts led to the isolation of different flavonoids by means of HPLC and TLC. The main aglycon identified was Quercetin in both free and bound forms (as glycosides). Isoquercitrine (quercetin-3-O-glucoside) and Quercitrine (quercetin-3-O-rhamnoside) were the main flavonoid glycosides in all tested cell lines. Rutin (quercetin-3-O-rutinoside), Hyperoside (quercetin-3-O-galactoside), Scopoletin and Phenolcarbonic acids - p-coumaric and chlorogenic have been also detected. In order to increase flavonoid production *in vitro* the effect of plant growth regulators (auxins and cytokinins) and sucrose concentration were examined. Content of flavonoids was severally decreased in higher concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D). Addition of cytokinin stimulated both proliferation and flavonoid production. Kinetin was favorable for flavonoid production in *A. missouriensis* cells. After optimization of production medium maximum total amount of flavonoids 1.34% was achieved. The highest amount of Isoquercitrine (5.3 mg/g DW) and Quercitrine (8.1 mg/g DW) was found in medium with optimal combination of growth regulators naphthaleneacetic acid (NAA) 1 mg/l, Kinetin 2 mg/l and 6% (w/v) sucrose. Rapidly growing cell lines were selected to increase the efficiency of the production of Quercetin derivatives. These results could provide a practical means of in vitro cultivation of this medicinally important plant and for further biotechnological applications.
KEY WORDS: *Astragalus missouriensis, in vitro* cell cultures, flavonoids, Quercetin

INTRODUCTION

The medicinal use of *Astragalus* species dates back over 1000 years. The different species are used in traditional medicine in Bulgaria, Turkey, Rumania, Yugoslavia, Greece, Russia, China, Japan, Mongolia, Korea and other European and Asiatic countries for their hepatoprotective, antioxidative, immune stimulative, antiviral and anticancer properties (1).

Due to the economic and medicinal importance of the *Astragalus* species, they have been investigated for their chemical compounds. The biologically active constituents of *Astragalus* plants represent mainly three classes of chemical compounds: flavonoids, saponines and polysaccharides and have been extensively studied (2-4). A number of flavonoids and their glycosides have been isolated from *Astragalus* species.

Within the *Fabaceae*, production of these compounds has been reported for several genera. However there are several problems connected with this production method (5). Variable quantities and qualities of the plant material, plants that need to grow several years before they are ready for harvesting and over collecting of endangered species (*A.membranaceus*, *A.mongholicus*, *A. missouriensis*, *A. angustifolius*, *A. thracicus*, *A. aitosensis* etc.) are just a few of the problems connected with the production of these natural products. Therefore, cultured cells rather than plants are as a possible alternative production method.

Flavonoids are a large and most important group of polyphenolic compounds synthesized by plants with many potent biological properties. Over the past decade, scientists have become increasingly interested in the potential for various dietary flavonoids to explain some of the health benefits associated with fruit- and vegetable-rich diets. Flavonoids are universally present as constituents of flowering plants, particularly of food plants. The high intake of foods and beverages rich in polyphenols, especially in flavonoids, has been associated with decreased risk of neoplasm. Dietary flavonoids inhibit the proliferation of various cancer cells and tumor growth in animal models (6). Epidemiologic data suggested that flavonoids consumption may protect against cancer induction in several human tissues. Chemoprevention has the potential to be a major component of colon, lung, prostate and bladder cancer control (7). A number of investigators have reported that flavonoids inhibit the tumour growth by interfering with some phases of the cell cycle.

The use of flavonoids for prevention and cure of human diseases is already widespread. These aspects made flavonoids an interesting object for industrial production.

The production of flavonoids via tissue culture techniques have been reported in both callus and cell suspension cultures. The spectrum of the produced compounds and their yields depended on the proper selection of plant species, explant types and culture conditions (1). The studies on culture cells of *Astragalus* began with the report on hairy roots and callus cultures of *A.hamosus* (8-9). Very few plant cell cultures systems have been reported for *Astragalus* ssp. to date - there are only few reports available on flavonoids production in vitro (1). In a previous study, saponin and polysaccharides production in hairy roots and cell cultures of different *Astragalus* species were reported (1, 10).

Northeastern North Dakota is home to the Missouri milk-vetch (*Astragalus missouriensis*). The plant obviously gets its name from the river, not the state, for the species grows on dry plains and river bluffs from Alberta to Texas. However, plants can be found along the Missouri as far east as northeastern Iowa.

This paper describes a plant-cell-culture production approach that provides increased yields of flavonoids. Here, we report a protocol for flavonoid biosynthesis induction in cell culture of endemic *A. missouriensis*. For the first time cell culture of *A. missouriensis* was established, and flavonoid production was determined and optimized. These results could provide a practical means of in vitro cultivation of this medicinally important plant and for further biotechnological applications.

MATERIALS AND METHODS

Plant material

Germination of seeds and callus induction

Seeds from *A. missouriensis* (Chicago Botanic Garden, USA) were surface-sterilized in 80% alcohol for 1 min, then in 10% commercial bleach (Domestos) for 10 min, followed by three rinses with sterile distilled water. Seeds were germinated aseptically in Petri dishes containing 25 ml of growth-regulator-free MS Murashige and Skoog medium (11), supplemented with 3% (w/v) sucrose and solidified with 0.9% agar (w/v). The pH of MS medium was adjusted to 5.6 before sterilization by autoclaving. For callus induction, mesocotyl parts of seedlings were used. Seedlings were grown on MS medium supplemented with 1.1 mg/l 1-naphthaleneacetic acid (NAA), and 1.0 mg/l 6-benzylaminopurine (BAP). Callus proliferation was obtained on MS medium supplemented with 0.1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 mg/l indole-3-acetic acid (IAA) and subculture was performed at three week intervals. Twenty one day-old calli were used to establish cell suspension cultures after the first subculture. Seed germination, callus induction and subculture were carried out in a growth chamber illuminated with fluorescent light.

Cell suspension culture

Twenty one day-old calli were transferred to 300 ml Erlenmeyer flasks (0.5 g fresh mass) containing 50 ml of MS liquid medium enriched with 1.0 mg/l concentrations of 2,4-D and 1.0 mg/l 6-benzylaminopurine (BAP) and agitated on a gyratory shaker (120 rpm, 25 ± 2°C, in light 2000 Lx). Cell suspensions were subcultured at two week intervals. Cultures were maintained in a growth chamber illuminated with fluorescent light and temperature conditions as mentioned above. Two replicates were used for each treatment, and the experiment was repeated two times.

Extraction and isolation of flavonoids

The methanolic extract of the cell mass was dissolved in water. The aqueous remnant was extracted twice with equal volumes of ethylacetate p.a. After the ethylacetate phases were combined and evaporated, the remains were dissolved in methanol p.a. Fractions of the ethylacetate extract over silica gel columns and preparative TLC ($CHCl_3$/$(CH_3)_2CO$/HCOOH 75:16.5:8.5) led to the isolation of flavonoid glycosides. After acid

hydrolysis of the flavonoid mixture one aglycone was isolated by CC and preparative TLC and was identified as a Quercetin. The flavonoids were isolated by preparative and column chromatography to be identified by direct comparison with their respective authentic specimens (TLC, HPLC).

Quantitative analysis

The HPLC with a Thermo Quest HPLC system (Egelsbach, Germany) equipped with a photodiode array detector was used. Separation was performed using a reversed phase Hypersyl C 18 column with guard column (Grom Company, Herrenberg, Germany) and a gradient program with 0.5% orthophosphoric acid p.a. in water (A) and acetonitrile/methanol 400/200 (B) as eluents as follows: 0 to 22 min from 40 to 27% B, from 22 to 24 min to 85% B, and until 30 min back to 27% B. The flow rate increased from 1.0 mL/min at temperature 25 ^{0}C, detection 335 nm, injection volume 20µl. The retention time for Rutin (quercetin - 3-O-rutinoside) is about 9,41 min, for Hyperoside (quercetin-3-O-galactoside) about 10,04 min, for Isoquercitrine (quercetin-3-O-glucoside) about 10,14 min, Quercitrine (quercetin-3-O-rhamnoside) about 11,64 min, for coumarine - Scopoletine about 10.38 min, Quercetin about 15,65 min.

Statistical analysis

To determine flavonoid induction in cell cultures of *A. missouriensis* each experiment was repeated four times. Data were analyzed by one-way analysis of variance. The statistical significance of differences between means was estimated at the 5% level.

RESULTS AND DISCUSSION

Various flavonoids have been found to inhibit the development of chemically-induced cancers in animal models. It was found, that quercetin is most important in this respect (12). Quercetin shows anti-proliferative effects against various cancer cell lines (13). The effect of quercetin on cell cycle progression and growth of human gastric cancer cells, markedly inhibiting the proliferation of gastric and colon cancer cells and arresting the cell cycle at G1 phase.

All cultures of *A. missouriensis* (callus and suspension) produced flavonoids. The main aglycon identified was Quercetin in both free and bound forms (as glycosides). With respect to the potential use of Quercetin as cancer-preventive or chemotherapeutic agents, it is worth mentioning that these aspects made quercetin an interesting object for industrial production (14). Cell-culture–derived flavonoids can be more easily separated in an intact polymeric form than flavonoids within complex plant tissues. Flavonoids produced in uniform plant-cell culture systems offer a novel vehicle for in-depth investigation of these compounds individually. The capacity to uniformly biolabel flavonoids as they are produced and accumulated in cell cultures is a novel tool that facilitates our current efforts to have a rich source of dietary flavonoids.

It was found that darkness suppresses flavonoid biosynthesis and formation of flavonoid glycosides is light-induced (15). That way all cultures were cultivated in continually light (2000 Lx). The chemical investigation of cell extracts led to the isolation of different flavonoids by means of HPLC and TLC. The common structural aspect of produced by cell cultures of *A. missouriensis* flavonoid glycosides is that Quercetin is common aglycon. In general Isoquercitrine (quercetin-3-O-glucoside) and Quercitrine (quercetin-3-O-rhamnoside) were the main flavonoid glycosides in all tested cell lines. Rutin (quercetin-3-O-rutinoside), Hyperoside (quercetin-3-O-galactoside), Scopoletin and phenolcarbonic acids - p-coumaric and chlorogenic have been also detected. Higher formation of flavonoids is correlated to good growth. Response of flavonoid biosynthesis to auxins and cytokinins varies significantly depending on the type of the plant regulators used.

In order to achieve an industrial production one has to obtain a stable high-producing cell line of the *Astragalus* plant. For this - two approaches are being used - (a) screening and selection for high producing cell lines and (b) optimization of growth and production medium and conditions. Rapidly growing cell lines were selected to increase the efficiency of the production of Quercetin derivatives.

Effects of different media components on the flavonoid production

Many factors including the explant source, growth regulators (especially auxins and cytokinins) and other inductive factors (such as bioactive compounds, type of carbon source etc.) are responsible for secondary product biosynthesis in vitro (15-16).

In this study we have examined the effect of plant growth regulators (auxins and cytokinins) and sucrose concentration on the flavonoid production in cell cultures of *A. missouriensis*.

Effects of plant growth regulators

Auxins

Murashige and Skoog medium (MS) was the basal medium. The effect of two kinds of auxins and two of cytokinins were examinated. In various experiments, the basal medium was supplemented with auxins 1-

naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and cytokinins Kinetin (Kin) and 6-benzylaminopurine (BAP) in 10 various combinations, which play an important role in growth rate and biosynthesis of flavonoids. The results are summarized in Table 1. Differences in growth are observes between various media. Regarding growth regulators, it was found that 2,4-D strongly suppress flavonoid biosynthesis in cell cultures of *A. missouriensis* (medium MS10). Content of flavonoids was severally decreased with higher concentrations of 2,4-D. In this respect NAA was weaker than 2,4-D. These results showed that addition of 2,4-D to medium inhibited flavonoid production. Callus cannot be induced in medium containing less than 0.1 mg/l auxin or auxin-free medium.

Cytokinins

Induction of flavonoid production in cell suspension cultures in all tested concentrations of cytokinins (BAP or Kinetin) under the light was achieved - although, Kinetin was the most effective. Flavonoids content differ in medias with varied concentrations of cytokinins. Addition of cytokinin stimulated both proliferation and flavonoid production. When the concentration of Kinetin is doubled (mediums MS2 to MS1), the production of flavonoids increased with 20%. Addition of Kinetin increases flavonoid production and gave the best result in concentration of 2 mg/l (MS1 medium) - 9.3 ± 0.47 mg/g DW (dry weight) total flavonoids (Table 1). This fact indicated that the addition of 2 mg/l Kinetin to MS medium was favorable condition for flavonoid production in *A. missouriensis* cells. The optimal combination of growth regulators is NAA 1 mg/l and Kinetin 2 mg/l (medium MS1).

Effects of carbon concentration

The concentration of carbon source is important factors for biosynthesis induction of many secondary metabolites in plant cultures (15). Sucrose is the common carbon source in most plant tissue cultures. Higher concentrations of sucrose are known to favour growth and saponin production (1). Because the rapidly metabolizing cells cultivated in suspensions are geared toward production of secondary products (rather than diverting energy into differentiation of organs), the carbohydrate and nutrient sources in the cell-culture production broth are used primarily in the synthesis of bioflavonoids.

The effect on flavonoid production of four different concentration of sucrose - 2, 4, 6, 8% (w/v) was examinated in suspension cultures of A. *missouriensis* in light 2000 Lx for 21 days cultivation period. Medium composition MS1 (Table. 1) given 0.93% flavonoids was used as basal cultivation medium in this experiments. At each subculturing stage the fresh weight was noted and the flavonoid production was analyzed. The flavonoid production increase in the percentage of sucrose added to the medium. The maximum total flavonoid production - 1.34% (13.4 ± 0.62 mg/g DW) was found in medium MS1 with sucrose concentration of 6%. Increasing the sucrose concentration to 8% led to negative effect on flavonoid biosynthesis, with decreasing of production to 10.8 ± 0.53 mg/g DW as shown in Fig. 1. These differences in the production of total flavonoids can be attributed to osmotic stress of cells, caused by change of the osmotic balance in high concentration of sucrose. The highest amount of Isoquercitrine (0.53%) and Quercitrine (0.81%) was achieved in medium with optimal combination of growth regulators is NAA 1 mg/l, Kinetin 2 mg/l and 6% (w/v) sucrose.

In order to determinate optimal concentration of sucrose for flavonoid production in suspension cultures of *A. missouriensis*, a regression model by second order approximation was achieved (Fig. 1). The data were calculated on production of main glycosides Isoquercitrine and Quercitrine at two week cultivation period by different concentration of sucrose in MS1 medium. It was found that the optimal sucrose concentration is 5.5%.

CONCLUSION

The ability of manipulating flavonoid biosynthesis in plant species is gaining rapidly in importance as new economically important usesemerged, such as in the areas of food and feed quality and nutraceuticals.

Culture productivity is critical to the practical application of cell suspension culture technology to production of flavonoids. Until now, various strategies have been developed to improve the production of secondary metabolites in *in vitro* cultures, such as the manipulating the parameters of the environment and medium, selecting high yielding cell clones, precursor feeding and elicitation (17).

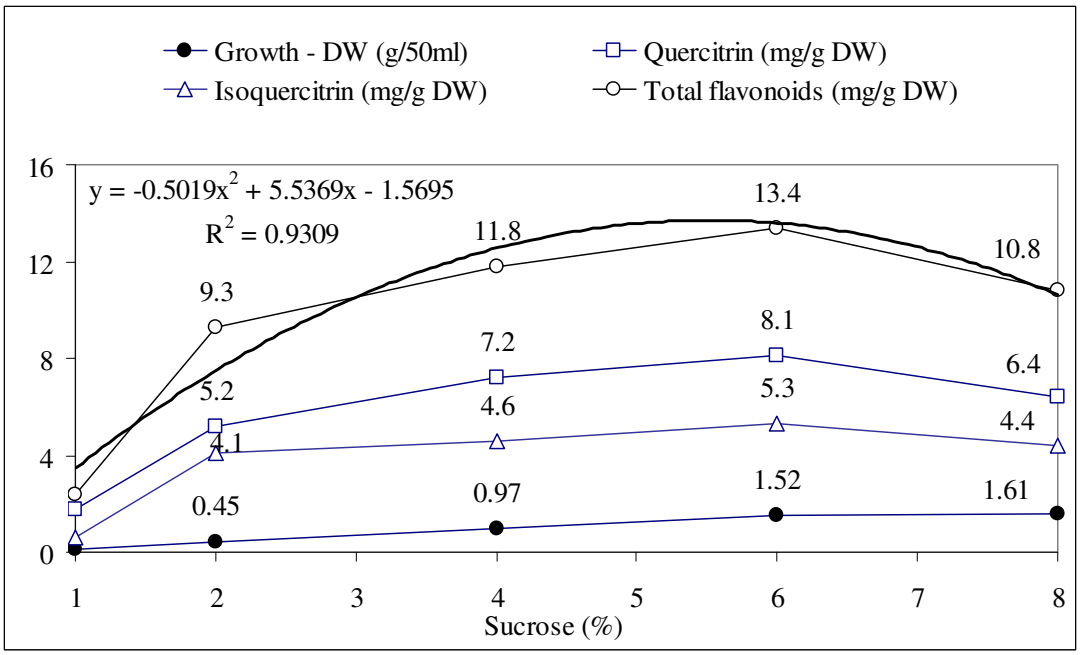
In the study presented, flavonoid produced from cell cultures of *Astragalus missouriensis*, as well as optimization of cultures and production conditions, has been achieved. Flavonoids produced in uniform plant-cell culture systems offer a novel vehicle for in-depth investigation of these compounds individually. Cell-culture–derived flavonoids can be more easily separated in an intact polymeric form than flavonoids within complex plant tissues. The capacity to uniformly biolabel flavonoids as they are produced and

Table 1: Effect of different kinds and concentration of growth regulators on flavonoid production (% DW) in suspension in vitro cultures A. missouriensis

Medium	Growth regulators (mg/l)				Total flavonoids (%)
	2,4-D	NAA	KIN	BAP	
MS1	0	1	2	0	0.93
MS 2	0	1	1	0	0.77
MS 3	0	1	0. 1	0	0.69
MS 4	0	1	0	0.01	0.51
MS 5	0	1	0	0.1	0.49
MS 6	0	1	0	1	0.74
MS 7	0	0.1	0	0.01	0.57
MS 8	0.1	1	0	0.1	0.62
MS 9	1	10	0	0.1	0.23
MS 10	10	0	0	0.1	0.12

(2,4-D): 2,4-dichlorophenoxyacetic acid,
(NAA): 1-naphthaleneacetic acid,
(KIN): Kinetin
(BAP): 6-benzylaminopurine

Figure 1: Influence of sucrose concentration on growth and flavonoid production in suspension in vitro cultures A. missouriensis.



accumulated in cell cultures, and to separate labeled flavonoid mixtures for subsequent bioassay or metabolic tracking, is a novel tool that facilitates our current efforts to discriminate interactions between flavonoid compounds in terms of biological activity (18).

Flavonoid-producing plant-cell cultures from *Astragalus missouriensis* are typically prepared by aseptically germinated seedlings explants from a plant genotype that is a rich source of dietary flavonoids and by inducing rapidly proliferating cells to grow from these tissues on defined medium in vitro. The resulting callus cells can be adapted to liquid suspension cultures, which, when optimized, will rapidly and predictably accumulate a broad range of flavonoid compounds. The cultures are composed of simple uniform cells and do not produce some of the substances (e.g., pectins, fibers, enzymes or excess polysaccharides) that otherwise interfere with flavonoid extraction from complex whole-plant tissues and permit rapid and efficient isolation without many of the interfering compounds that can complicate extraction or

bioassay. The flavonoids correspond to compounds produced in nature by the same plant genotypes, but, through optimization tactics, the levels and the profiles of the flavonoid yield can be enhanced, and isolation from cell cultures is more rapid and streamlined than isolation from in vivo plant tissues.

Recently, the interest of international pharmaceutical industries has been directed more and more to polyphenols. In this case, it has been shown that production of both forms of flavonoids, i.e. aglycons and their glycosylated forms in cell cultures of *A. missouriensis*, is possible. We believe that cell cultures of *Astragalus* plants as source of biologically active flavonoids can play a role in this respect.

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