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CYCLOARTANE SAPONIN PRODUCTION IN HAIRY ROOT CULTURES OF *ASTRAGALUS MONGHOLICUS*

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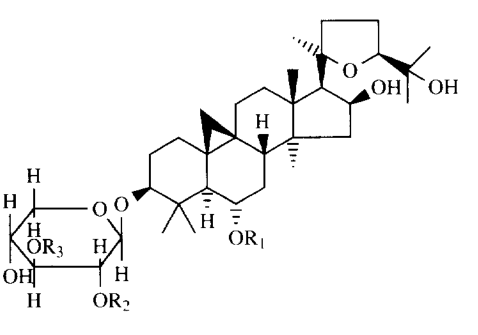
Key Word Index—*Astragalus mongholicus*; Fabaceae; *Agrobacterium rhizogenes*; hairy roots; cycloartane saponins; astragalosides.

Abstract—Transformed root cultures of *Astragalus mongholicus* are induced by inoculation of sterile grown seedlings with *Agrobacterium rhizogenes* strains LBA 9402, ATCC 15834, R 1601 and TR 105. These strains show different abilities to induce hairy roots on this plant. They influence the growth rate, the saponin production, and the ratio of astragalosides. Root growth and saponin production are investigated under various culture conditions. Transformed roots grow better in MS medium without ammonium nitrate and without phytohormone. The saponin production is remarkably increased when sitosterol is added in the culture media. © 1997 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

Astragalus mongholicus is a very old and well-known drug in traditional Chinese medicine. The root of this plant called *Huangqi*, is officially listed in the Chinese Pharmacopoeia, and has been used as a tonic, a diuretic and an anticancer agent [1]. In modern Chinese medicine it is used in Fu zheng therapy as an immunostimulant. Because its properties are somewhat similar to those of the more expensive herb ginseng (*Panax ginseng*) it has been used as a substitute for those species [2, 3]. The limited supply and over-collecting of the plant are two of the problems connected with production of natural compounds, found in *Astragalus mongholicus*. Development of an alternative production method using tissue cultures is a topic of great interest. Transformed root cultures (cooled hairy roots obtained after the insertion of T-DNA from root-inducing (Ri) plasmid of *Agrobacterium rhizogenes* into the plant genome) have the advantages both of fast growth and stable high-level production of secondary metabolites on hormone free medium [4–6].

The chemical nature of the secondary metabolites produced in cell and tissue cultures of *Astragalus* plants have been studied and published in ref. [7]. It was shown that hairy roots of *Astragalus mongholicus* produced the same kinds of saponins as in intact plants [8]. The main saponins were astragaloside I (**1**), astragaloside II (**2**) and astragaloside III (**3**). Their structures have previously been reported and confirmed by NI-FAB, MS/MS, EI-MS, ^{1}H- and ^{13}C-NMR (1D and 2D) [8]. Here we focus our attention on the possibility of increasing the saponin content in these hairy roots. We also examine the influence of bacterial strains, culture conditions, and the addition of sitosterol.



(1) $R_1 = Glc$ $R_2 = CH_3CO$ $R_3 = CH_3CO$

(2) $R_1 = Glc$ $R_2 = CH_3CO$ $R_3 = H$

(3) $R_1 = H$ $R_2 = Glc$ $R_3 = H$

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RESULTS AND DISCUSSION

The hairy root clones generated from seedlings of *Astragalus mongholicus* by infection with *Agrobacterium rhizogenes* were isolated and cultured individually in liquid MS medium without phytohormone. Four of them, transformed through different bacterium, were selected for this study.

The four strains of *Agrobacterium rhizogenes* (LBA 9402, ATCC 15834, R 1601 and TR 105) not only showed significant differences in their transformation ability [9], but also had different effects on the growth process and saponin production in hairy root cultures of *Astragalus mongholicus*. The clones infected by LBA 9402 and ATCC 15834 bacteria grew the fastest, and biomass increased 1–2 times more than the mass of other clones (R 1601 and TR 105) after 28 days of culture. Fluctuation of the rate of biomass production was observed. The initial inoculum of *ca* 0.5 g fresh weight of the transformed roots increased up to about 4 g in 28 days of cultivation. It should be noted that statistically significant correlation between saponin content and final dry mass was observed in hairy root cultures, induced by bacterial strains LBA 9402.

Maximum saponin content was found in hairy root clones, transformed with LBA 9402 bacteria. These results are similar to those, obtained for alkaloid production in some Solanaceous species transformed with the same bacterial strain [10, 11]. The amounts of total saponins in different root clones varied between 3.17 and 5% dry weight. About 16–20% of the total saponins were released into the medium. In order to investigate in detail whether there are differences in the biosynthesis of the various astragalosides, saponin content was measured at different points in the culture cycle. The time course of saponin accumulation by *Astragalus mongholicus* hairy root clones (transformed with different bacterial strains) are shown in Fig. 1. Interestingly, different *Agrobacterium* strains have an effect not only on the total saponin content, but also on the ratio of astragalosides (Fig. 2). In root clones transformed with *Agrobacterium rhizogenes* LBA 9402, R 1601 and TR 105 the main saponin is astragaloside III (**3**), while in root clones, transformed with strain ATCC 15834 the main saponin is astragaloside I (**1**). The structures of astragalosides I, II and III have the same aglycone moiety but different sugar moieties. Astragaloside III (**3**) was the main compound and accounted for 41% of the total cycloartane saponin content in hairy roots transformed with bacterial strain LBA 9402. However, the amount of **3** decreased when hairy roots were induced by *Agrobacterium rhizogenes* strain TR 105 and fell to 35% of the total saponin mixture. The high production of this saponin (**3**), glycosylated only at C-3, suggests that the glucose is linked to C-3 of cycloartenol before the glucose is introduced at C-6. This argument is supported by the fact that no other astragalosides are produced during the first few days of culture. It is interesting to note that the main difference in the amount of various astragalosides can be seen in root clones induced with bacterial strain LBA 9402 (Fig. 1). In hairy roots, transformed with bacterial strain ATCC 15834, the spectrum of produced saponins is dominated by astragaloside I (**1**) (41%). Astragalosides III (**3**) and II (**2**) contribute 34 and 25%, respectively, and the pattern observed with this clone, differs somewhat from that in other root clones.

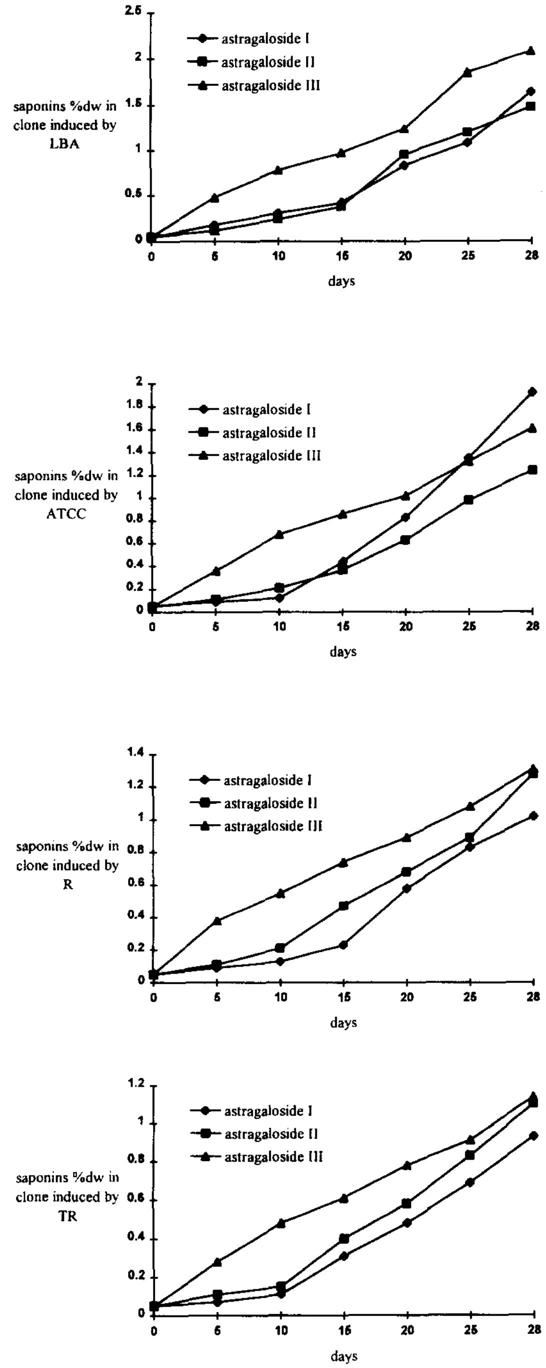

Fig. 1. Time course of saponin accumulation in hairy root culture lines of *Astragalus mongholicus* Bge., induced by *Agrobacterium rhizogenes*, strain LBA 9402, ATCC 15834, R 1601 and TR 105.

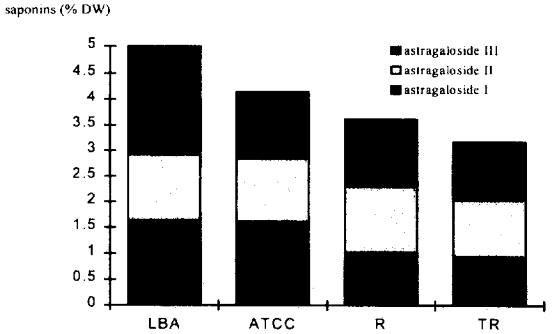

Fig. 2. Influence of *Agrobacterium* strains on production and ratio of cylcoartane saponins in hairy roots of *Astragalus mongholicus*.

This variation in the production of saponins between clones of the same plant species might in part be attributed to the transformation itself, but it is also possible that this difference might be due to a type of adaptation causing changes in the secondary metabolism. Astragaloside II (**2**), which is very interesting because it allows 100% protection of T-lymphocytes *in vitro* against the cytopathic effect of HIV infection and causes 50% inhibition of the growth of tumour cell lines [12], in all culture clones reaches the same maximum at day 28. This result shows that the bacterial strain LBA 9402, which contains the plasmid pRi 1855 [13], is the best strain for inducing highly productive hairy root clones in this plant species.

The recognition of the transformed nature of the hairy roots was based not only on the observation of phenotypic features (active proliferation, lack of geotropism, and abundant lateral branching) but also on the fact that hairy roots of *A. mongholicus* tested positive for opine (agropine and manopine), showing that the roots contained the appropriate enzymes from the plasmids responsible for opine synthesis.

Effect of culture medium

Using the root line, transformed with *Agrobacterium rhizogenes* LBA 9402 we studied the effect of the nitrogen source in the culture medium, containing 2% sucrose, on the growth and saponin production. The main astragalosides were analysed in one run using TLC-densitometry. The growth of hairy roots was investigated for 28 days both with and without ammonium nitrate MS media. From an initial inoculum of *ca* 0.5 g fresh weight a variable rate of biomass production was observed. After a short lag-phase of 4 days, the hairy roots grew rapidly in MS medium without ammonium nitrate, the biomass increased up to 6.36 g fresh weight which was higher than in the presence of ammonium nitrate (4.23 g fresh weight). Ammonium nitrate concentration also affected the morphology of the hairy roots. Since the ammonium nitrate was in the medium, the hairy roots became short and thick with a lower degree of branching, while in medium without ammonium nitrate, the roots became long and thin. However, the resulting cycloartane saponin accumulation in the roots in both media was similar, showing that saponin production in hairy roots of *Astragalus mongholicus* was not impaired by NH_4NO_3. This result is similar to that obtained for saponin production in hairy roots of *Astragalus membranaceus* [7] and in contrast to the results for polysaccharide production in hairy roots of *Althaea officinalis* [14] or diterpene formation in hairy roots of *Salvia miltiorrhiza* [15]. Our results indicated that medium without ammonium nitrate is satisfactory for promoting growth of *Astragalus* hairy root cultures, but the saponin content was not affected significantly.

Effect of sitosterol

Because phytosterols (campesterol, stigmasterol and sitosterol) are present in hairy roots of *Astragalus* species [7], the effect on the total saponin production of these substances was examined. Since the biosynthetic routes to saponins and phytosterols branch at 2,3-epoxysqualene [7] it may be possible to increase the production of saponins by end-product inhibition. In these experiments, the amounts of saponins obtained after the addition of 10 mg of sitosterol in 50 ml MS liquid medium without ammonium nitrate were examined at different stages of growth. The saponin content (total saponins) in the hairy roots induced by LBA 9402 reached 5.25% of dry weight at 28 day of cultivation. When sitosterol was added in the culture media of the hairy roots, astragaloside production was remarkably increased to 7.13% of dry weight and led to an increase of 36% of the total saponin content in comparison with the control. From these data, sitosterol seems to behave as an inhibitor in the biosynthetic route when the amount added is relatively large. The cycloartane production was increased by the addition of sitosterol, similar to that found for ginsenosides in callus cultures of *Panax ginseng* [16]. This seems to be due to the fact that some of the added sitosterol, or of the endogenous phytosterols, could be metabolized in the hairy root tissues when in an abnormally excessive quantity.

In conclusion, these results show that the use of hairy root cultures of *Astragalus mongholicus* can be a valuable alternative approach for the production of cycloartane saponins. Using a selected high productive clone, inducing by *Agrobacterium rhizogenes* LBA 9402, optimized culture medium (MS without ammonium nitrate) and end-product inhibition, a relatively high saponin production can be achieved.

EXPERIMENTAL

Bacterial strains. Agrobacterium rhizogenes strains: LBA 9402, ATCC 15834, R 1601, TR 105 were grown on liquid YMB medium [17], containing yeast extract 1 g/l^{-1}.

Plant material and culture method. Leaf segments from sterile grown *Astragalus mongholicus* Bg plants, were wounded with a sterile needle and the bacteria from the media were spread onto the leaves. After 4 days, the explants were transferred to MS medium without phytohormone, containing 500 mg l^{-1} sodium cefotaxim (Claforan, Hoechst AG, Frankfurt) to remove the excess of bacteria. The cultures were kept in the dark, at 25°. Roots developed after 3–4 weeks of incubation. Single roots (*ca* 20–30 mm long) were transferred to liquid MS medium without phytohormone. For the time course of growth and saponin production in various conditions, 2–3 cm (0.5 g fresh weight) root tips were inoculated in 25 ml medium in 100 ml flasks or 50 ml in 300 ml flasks and cultivated on a gyratory shaker at 120 rpm in darkness at 25°.

Opine assay. Opines in the hairy roots were extracted as described in ref. [18]. The opines were detected on TLC [19] with the reagent of Travelyan *et al.* [20]. Agropine (K.Doerk) and mannopine (Sigma) were used as standard.

Extraction and quantification of astragalosides. Three flasks were used for each experiment. Hairy roots were lyophilized and extracted as described earlier [8]. Silica gel 60 F_{254} plates (20 × 20 cm) were used and developed with $CHCl_3$–MeOH-CH_3CHOCH_3–H_2O (80:50:10:10). After development and drying, the plates were sprayed with *p*-dimethylaminobenzaldehyde reagent, followed by heating at 105° for 10 min. The spots were measured with a TLC scanner (Shimadzu CS-9000) at 483 nm. Calibrations graphs were prepared with standard MeOH solns of astragalosides I, (**1**), II (**2**) and III (**3**). The amounts of the saponins were calculated from the integration of spots corresponding to three different concentrations of astragalosides and three spots of solns of unknown concs.

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