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# Induction of Hairy-Root from *Astragalus* Plant, and Mucilage Production by the Hairy-Root

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A hairy root (HR) culture of *Astragalus gummifer* was established by transforming cultured shoots with *Agrobacterium rhizogenes* (ATCC 15834). These HR produced water-soluble mucilages, containing neutral-mono-saccharides (NMS) such as galactose, glucose, arabinose, fucose and rhamnose. However, neither simultaneously obtained callus nor cultured root produced these water-soluble mucilages. And in some cases, liquid cultured HR secreted mucilage containing galactose and glucose into the medium. But the NMS compositions of these mucilages were neither like those of the gum produced from mother plant nor the glucan of *Agrobacterium rhizogenes*.

*Astragalus gummifer*[1] produce polysaccharide so-called "gum," widely used for icecream and dressing production, as well as in oriental medicine. But because of its high-cost and unstable supply, presently it is replaced by other gum. So we have tried to obtain this gum by plant tissue culture (PTC).

Attempts to produce polysaccharides by PTC have been done since former days.[2,3] One of the oldest study of secondary metabolites by PTC was the attempts obtaining Guar-gum.[4] But in many cases, dedifferentiated callus can only produce little polysaccharide or otherwise, it can not produce any of it. On the other hand, in the study of producing secondary metabolites by PTC, the number of attempts to obtain target materials from HR induced with *Agrobacterium rhizogenes* has increased.[5-7] Transformed root cultures, so-called HR obtained after the insertion of T-DNA from root-inducing (Ri) plasmid of *Agrobacterium rhizogenes* into the plant genome, have both advantages of fast growth and organ differentiation. We report here for the first time the induction of HR from *Astragalus gummifer*, and comparison of the NMS compositions of mucilages of these HR.

## Materials and Methods

*Plant, bacteria, and chemicals.* The *Astragalus* plant (*Astragalus gummifer*) was kindly supplied by Dr. K. Shimomura, Tsukuba Medicinal Plant Research Station, Japan. The bacteria (*Agrobacterium rhizogenes* ATCC 15834) was purchased from American Type Culture Collection, and the bacteria (*Agrobacterium rhizogenes* NIAES 1724)[8] was kindly supplied by Dr. H. Kamada, University of Tsukuba, Japan. The bacteria were subcultured on YEB agar medium (Vervliet *et al.*, 1975)[9] at 25℃ under dark at 3-week intervals. All chemicals were special grade article mostly purchased from Wako Pure Chemicals, Osaka, and from Nakarai Chemicals, Kyoto.

*Establishment of callus, root and HR cultures.* Sterilized *Astragalus* seeds were germinated on a gel solidified with 0.9% agar containing 0.5% sucrose under 18 hr light (2,000 lux) and 6 hr dark at 25℃. The stem (1 cm long) of this plant is subjected to the following methods. The *Astragalus* callus was induced on Murashige and Skoog (MS) medium[10] supplemented with 6-benzylaminopurine, kinetin, zeatin, indole-3-acetic acid (IAA), 1-naphtaleneacetic acid, and 2, 4-dichloro-phenoxyacetic (2, 4-

D), in each appropriate combination and at the concentration, respectively. Obtained callus was subcultured on the MS medium solidified with 0.2% Gelrite, Sanei Kagaku Kogyo Co., Osaka, supplemented with 3 mg/*l* of 2, 4-D and 1 mg/*l* of zeatin. Callus was subcultured at 1-month intervals under dark at 25℃.

Cultured roots were obtained by the same way as callus, and subcultured on the MS medium supplemented with 0.1 mg/*l* of IAA and 0.1 mg/*l* of zeatin. These roots were subcultured at 1-month intervals under dark at 25℃.

Germinated *Astragalus* plants were transplanted to the MS basal media to obtain plantlets. And *Agrobacterium rhizogenes* grown on the YEB agar medium were inoculated onto cutting stem of the *in vitro* plantlet. Two to 3 weeks after the inoculation, HR appeared at the inoculated sites. HR tip segments (1 cm long) were cut off and cultured on a hormone-free MS medium solidified with 0.2% Gelrite containing 500 mg/*l* of cefotaxim-antibiotic Claforan, Hoechst Japan Limited, Tokyo, under dark at 25℃. After several transfers to the fresh MS medium containing antibiotic, the HR tips were transferred and maintained on the hormone free MS medium without antibiotic. The 8 to 10 segments of HR were transferred to 30 ml of hormone free medium in a 100 ml Erlenmeyer flask and cultured under dark at 25℃ with rotary shaking at 60 rpm.

*Detection of opines.* Plant cells transformed by *Agrobacterium rhizogenes* generally produce new compounds called opines, so we detected opines according to the methods of Tate *et al.* (1982).[11]

*Analysis of NMS composition of mucilage from tissues.* About 5 g of each callus, cultured root and HR were weighed and twice of deionized water was added, and homogenized by Physcotron, NITION-I Rhika Kikai Seisakusho Co., Chiba. Homogenized fluid was filtered and this filtrate was poured into 50 ml of 99.5% ethylalcohol, and the produced sediment was dissolved in 15 ml of deionized water and re-poured into 50 ml of 99.5% ethylalcohol. The sediment was then dried and dissolved in 2 ml of deionized water. And after the addition of 0.2 ml of trifluoroacetic acid (TFA), hydrolysed by heating at 100℃ for 2 hr in a nitrogen atmosphere. The hydrolysate was then concentrated to dryness after adding methylalcohol to TFA. The dried material was then reacted by heating at 100℃ for 15 min with 5% hydroxylammonium chloride in pyridine (0.2 ml/mg of aldose). After cooling, acetic anhydride (0.2 ml/mg of aldose) was added and again heated for further 1 hr to dehydrate oxime to nitrile and to acetylate the free hydroxyl groups on the nitrile and the alditol.

The solution was cooled and directly injected into the gas-chromatograph for analysis. Glass columns were used with the following packing materials, column sizes and operational temperature: 3% ECNSS-M on 100–120 mesh Gas-chrom Q (2.4 m) at 190℃.[12,13]

## Results and Discussion

We successfully obtained many HR by *Agrobacterium rhizogenes* (ATCC 15834), but could not obtain any HR by *Agrobacterium rhizogenes* (NIAES 1724). It is conceivable that the latter had a slight effect on *Astragalus gummifer* under the experimental conditions.[14] Three HRs were assayed to detect opines. One of three HRs showed clear spots of both agropine and mannopine (**Fig. 1-4**), but one HR showed only one spot of mannopine (**Fig. 1-3**), and one HR showed neither of them (**Fig. 1-5**). The induced root showed high growth rate such as 1 cm/day even after transferred to the fresh medium. These results suggest that the genes involving opine synthesis would be washed off in short term,[15] or the introduction of only HR inducing gene could be introduced into plant.[14]

Considerably viscous fluid extracted from a squeezed HR, and water soluble mucilage were peculiar to the HR, which have never been detected from callus or cultured root by our described methods (**Table 1**). And some of arbitrarily chosen HR secreted viscous mucilage into the liquid medium (**Table 1**). **Table 1** shows that HR-3 produced water soluble mucilage containing 5 types of NMS, but HR-4 produced that containing 4 types of NMS. And the secreted mucilage contained only 2 types of NMS. On the other hand, mother plant's "gum" contained 6 types of NMS. Furthermore, the amounts of each NMS were different from each other.

The "gum" of mother plant containing much more compounds other than NMS was inevitably more

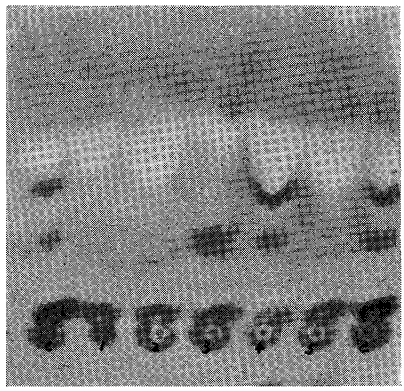


**Fig. 1.** Detection of opines.
C: authentic spot, 1: callus spot, 2: cultured root spot, 3, 4, 5: cultured HR spot.

**Table 1.** Analysis of NMS composition of mucilages from tissue.

| | Rhamnose | Fucose | Arabinose | Xylose | Glucose | Galactose | Total |
|---|---|---|---|---|---|---|---|
| | | | | | weight % (NMS/mucilage) | | |
| Gum | ND | 4.4 | 21.3 | 12.2 | 3.4 | 14.2 | 55.5 |
| HR-4 | 0.3 | ND | 1.2 | ND | 58.2 | 12.5 | 72.2 |
| HR-3 | 0.2 | 0.3 | 11.5 | ND | 54.3 | 11.5 | 77.5 |
| Secreted HR-4 | ND | ND | ND | ND | 66.6 | 14.1 | 80.7 |
| Callus | ND | ND | ND | ND | ND | ND | 0 |
| Cultured Root | ND | ND | ND | ND | ND | ND | 0 |

column: 3% ECNSS-M on 100-120 mesh Gas-chrom Q (2.4 m) at 190℃, ND: not detected.

complex than the mucilage of HR. And the secreted mucilage from HR had considerable viscosity (144 centipoise), but it had less viscosity than the gum-solution (355 centipoise) of the same concentration (0.3%). The composition of NMS in mucilage from HR varied widely and the same HR secreted mucilage contained less number of NMS than the mucilage of squeezed HR. From these results, we deduced that, when the mucilage was secreted into the medium, a part of arabinose and rhamnose were cut, or degraded in the medium.

On the other hand, it has been known that, the bacteria of *Agrobacterium rhizogenes* produce glucan in and out of the cell, and it had considerable viscosity but the composition of NMS was only glucose.[16)]

It is clear from these investigation that, mucilages are composed of several types of NMS and the compositions of NMS in mucilages varied widely, and in some cases, liquid cultured HR secreted mucilage into medium. But these mucilages were not the same as the gum nor the glucan of *Agrobacterium rhizogenes*. Further study over HR and mucilages are currently under examination.

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## アストラガルス属植物からの毛状根誘導と粘質物の生産

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アストラガルス属植物から，*Agrobacterium rhizogenes* (ATCC 15834) 感染によって毛状根を誘導した．これらの毛状根は，中性単糖を含む粘質物を生産し，その中性単糖の組成はガラクトース・グルコース・アラビノース・フコース・ラムノース等であった．しかし，同時に得たカルス・培養根ではこれらの物質の生産は見られなかった．毛状根の中には，液体培地中に生産した粘質物を分泌するものがあり，分泌された中性単糖の種類は減少していた．しかし，これらの中性単糖組成は，母植物の生産する多糖とも，*Agrobacterium rhizogenes* の生産するグルカンとも違っていた．