International Journal of Genetic Engineering and Biotechnology.
ISSN 0974-3073 Volume 1, Number 3 (2010), pp. 149--154
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Generation of Hairy Roots in *Artemisia annua* .Linn. and *A. scoparia*. Waldst et Kit. by using *Agrobacterium rhizogenes*

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Abstract

The increase in content of economically and medicinally important metabolites were attempted in *Artemisia annua* and A. *scoparia*. Sterile leaves of *A. annua* and *A. scoparia* were infected with *Agrobacterium rhizogenes* A4 strain, with the help of a sterilized needle and then inoculated the leaves on a plain MS medium containing ampicillin and sporidex antibiotics and incubated in dark. Formation of hairy roots appeared after 3 weeks of infection in *A. annua* but not observed in the case of *A. scoparia* even after 5- 6 weeks of infection.The so generated hairy roots in *A. annua* were confirmed for the integration of T-DNA by opines analysis. Presence of brown spot coinciding with that of reference compound confirmed the presence of opines and hence the integration of T-DNA into the *A. annua* genome.

Keywords: *Artemisia annua, A.scoparia, Agrobacterium rhizogenes.*

Introduction

Agrobacterium is a Gram-negative soil bacterium, which is also known as Nature's Genetic Engineer because of its capability of causing changes in the genotype of the plant it infects. Plant infections by the soil bacterium *Agrobacterium rhizogenes* result in neoplastic disease with the formation of hairy roots at the site of infection. Expression of a set of oncogenes residing on the stably integrated T-DNA is responsible for the disease symptoms. *Agrobacterium rhizogenes* mediated transformation has been used to obtain transgenic plants in 89 different taxa, reprensenting 79 species from 59 generas and 27 families. A diverse range of

dicotyledonous plant families is represented, including one Gymnosperm.Use of *A .rhizogenes* mediated transformation has tremendous potential for genetic manipulation of plants and has been of particular benefit for improvement of ornamental and woody plants. Weathers *et al. (*1994) reported high level (0.4%) artemisinin in hairy root cultures of *A. annua* transformed with *Agrobacterium rhizogenes.* Genetic transformation of plants using *Agrobacterium rhizogenes,* the causative agent of hairy root disease in several plants has emerged as an important alternative to intact plant as well as cell culture for the production of secondary metabolites (1,3). Hairy roots have been reported to yield higher amounts of secondary metabolites than cell suspension cultures and in some cases intact plant roots. Hairy root is a plant disease caused by *A. rhizogenes* Conn.; a Gram negative soil bacterium. When the bacterium infects the plant, the T-DNA between the TR and TL regions of the Ri plasmid in the bacterium is transferred and integrated into the nuclear genome of the host plant. The transformation process produces hairy roots at or near the site of infection. In addition, opines are produced and serve as specific food for bacteria (2).

In present investigation A_4 strain of *A. rhizogenes* is used for transformation of leaves of *Artemisia annua*. Linn and *A. scoparia* Waldst et Kit.

Material required

For the present investigation sterile leaves of *A. annua* and *A. scoparia* ,A4 strain of *Agrobacterium rhizogenes* were used.

Figure 1: Photograph showing plant of *Artemisia annua*. Linn.

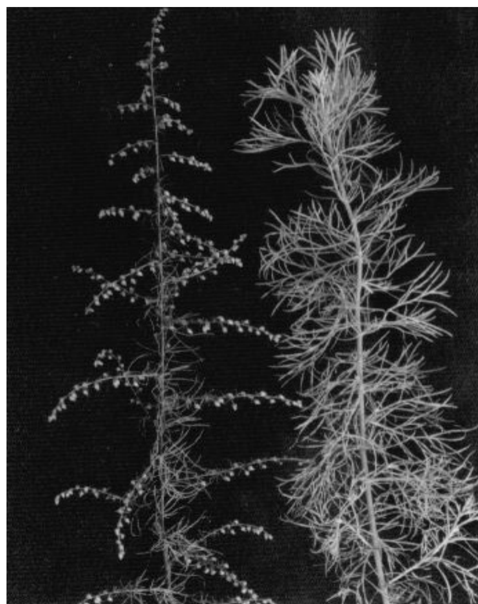

Figure 2: Photograph showing leaf and inflorescence of *Artemisia scoparia* Waldst et Kit.

Culture medium and culture conditions

The infected leaves were inoculated on plain MS medium (without hormones) containing antibiotics (0.5 ml ampicillin 500 ml^{-1}, 0.25 ml sporidex 500 ml^{-1})

Compositon of YEB medium: For 100 ml

YEAST EXTRACT	100 mg
BEEF EXTRACT	500 mg
PEPTONE	500 mg
SUCROSE	500 mg
$MgSO_4$	49 mg
PH	6.8
If solid , agar	1.7 %

Method

A_4 strain of *Agrobacterium rhizogenes* was collected from Department of Botany, Banaras Hindu University, Varanasi in the form of a stab culture. This stab culture was revived on YEB medium. For the purpose of infection a colony of *A. rhizogenes* was picked from the master plate with the help of a sterile toothpick and this toothpick was introduced into a tube containing YEB medium. *A. rhizogenes* was allowed to grow on the medium overnight at 37ºC in a shaker.

Prior to infection the explants were surface sterilized thoroughly. The leaf explants were infected with *A. rhizogenes* A_4 strain with the help of a sterilized needle then the infected leaves were aseptically inoculated on antibiotic containing MS medium and incubated in dark at 25ºC. Hairy root formation observed after 3 weeks of inoculation.

Characterization of hairy roots : The hairy roots so obtained were characterized by opine analysis. Opines are amino acids that provide the bacteria with a source of carbon and nitrogen that is unavailable to the plant. The type of opine produced by the transformed plant cell depends on the specific *Agrobacterium* strain.

The T-DNA has oncogenes (which stimulate tumorigenesis) and opine synthesis genes. All infected cells use plant's energy resources to manufacture opines. Opine catabolism genes in the Ri and Ti-Plasmid can metabolize opines, yielding C and N for the bacteria.

While mannopine strains induce tumors synthesizing mannopine as the only opine, agropine strains induce tumors which synthesize both mannopine and agropine as mannopine is the precursor of agropine.

Agropine strains induced hairy root tumors with higher proliferation and shoot differentiation capacity than mannopine strains.The materials required for the experimentation are as follows:
200 mg of sample ,200 µl of 0.1 M HCl, TLC Plate, Mobile Phase (Formic Acid : Acetic Acid : water :: 5 : 15 : 80),Flat Bed Tank, Reagent A (0.625 gms AgNO3 in 0.25 ml water added to 50 ml acetone ; water added dropwise until the Ag ppt. Dissolves), Reagent B (10 ml of 20 % NaOH added to 90 ml methanol), Reagent C (5 % w/v Sodium Thiosulphate ; 5 gms in 95 ml MQ water),Standard (Mannopine)

Method

200 mg sample was homogenized with 200 µl of 0.1N HCl. The resultant slurry was centrifuged at maximum speed in a microcentrifuge for 5 minutes.10 µl of supernatant was spotted 1.5 cms apart onto a TLC Plate.2 µl of an opine reference solution (mannopine) was spotted onto the same TLC plate. The flat bed tank was filled with mobile phase.The TLC plate was allowed to run into the flat bed tank filled with mobile phase .The plate was removed after the mobile phase reached ¾ distances on the TLC plate and allowed to air dry.Then the TLC was dipped in Reagent A air dried for 20 minutes.Then the TLC plate was dipped in Reagent B and allowed to air dry for 30 minutes.The excess stain was removed by dipping the plate in Reagent C and then washed in running tap water and air dried.

Results and Discussions

Generation of hairy roots was attempted with the objective in mind that these roots serve as biofactories for the synthesis of useful metabolites.

Positive results were obtained in the case of *A. annua* which showed the formation of hairy roots after 2-3 weeks of infection with *Agrobacterium rhizogenes* A4 strain (Figure 3). Very fast growing hairy root lines were obtained.

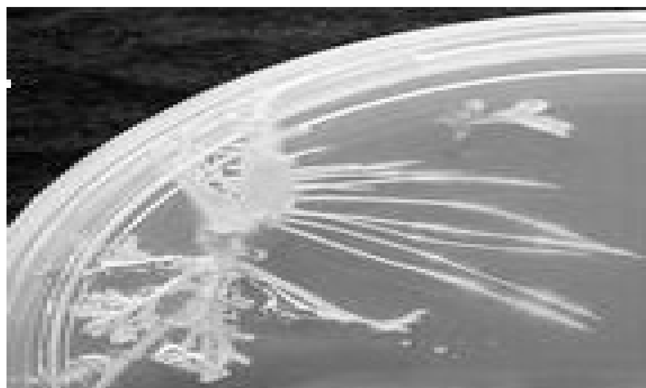

Figure 3: Photograph showing hairy roots generated in *Artemisia annua*. Linn. Using A_4 strain of *Agrobacterium rhizogenes.*

But in the case of *A. scoparia* after a incubation of 5-6 weeks of infection no results were obtained. Hence it could be inferred that for the generation of hairy roots in *A. scoparia* another strain of *A. rhizogenes* should be tried for/or elicitation should be provided by incorporation of acetosyringone, as described in literature.

For the confirmation of integration of T-DNA into the plant genome opine analysis was undertaken. The TLC showing the presence of opines in the hairy root lines of *A. annua* is shown in figure 4.

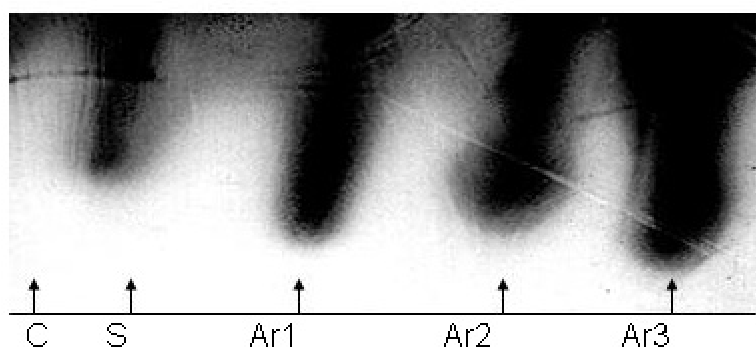

Figure 4: TLC plate showing presence of opine in the hairy root lines of *Artemisia annua* Linn. C = CONTROL (WILD TYPE) S = STANDARD (MANNOPINE).

Three hairy root lines namely Ar1, Ar2 and Ar3 were selected and their spots were applied on a ready made TLC plate.

The TLC plate clearly demonstrates that the hairy root lines Ar1, Ar2 and Ar3 are transformed as they showed presence of brown spots on the TLC plate which confirms the presence of opines in the above lines while no such spot was observed for control.

Since the A4 strain of *A. rhizogenes* is agropine synthesizing strain, the spots were obtained a little below the standard mannopine strain, confirming the presence of agropine in the transformed roots.

The generation of hairy roots was also carried out by keeping in mind that these roots will serve as biofactories for the synthesis and accumulation of these metabolites.

Acknowledgement

The authors are grateful to Dr. Shashi Pandey Rai, Associate Professor,Department of Botany, Banaras Hindu University, Varanasi, India for providing the A_4 strain of *Agrobacterium rhizogenes* and the Head of the Department, Department of Botany, University of Rajasthan, Jaipur, Rajasthan, India for providing the research facilities.

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