In Vitro Culture of Agrobacterium Rhizogenes-Induced Hairy Roots of Salix Alba L.

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Summary

In vitro culture of different types of roots of *Salix alba* was studied. On a variety of low- and high-salt tissue culture media adventitious roots obtained from single-node explants grew very slowly at best. Roots obtained after infection of *in vitro* grown shoots with *Agrobacterium rhizogenes* showed extensive fresh weight increase resulting from elongation growth and ramification.

Key words: Salix alba, root culture, hairy roots, Agrobacterium *rhizogenes*.

Introduction

Many Salicaceae belong to the rapidly growing tree species considered for biomass production (DIMITRI, 1988). The roots of these plants are colonized by several parasites (e. g. nematodes), pathogens (phycomycetes), and favourable fungi of the ectomycorrhizal or VAM type (BUHR, 1964; HARLEY, 1989). These partnerships between a tree root and a second organism are most precisely studied under controlled conditions excluding the interference by additional organisms. Dual cultures between isolated roots and the corresponding partner give optimal opportunity for such studies (MUGNIER and MOSSE, 1987; SAVKA et al., 1990; ZUCKERMAN, 1971). A precondition for such experiments is the availalility of tree root cultures. Here we report the establishment of a culture of hairy roots of *Salix alba* obtained from infection with *Agrobacterium rhizogenes.*

Material and Methods

Growth of plants

Cuttings of *Salix alba* L. were taken from 3 different plants of unknown genetic composition, found on the Neckar river bank in Heidelberg. They were cultivated in the greenhouse in 4-1-pots with a mixture of sand, peat, standard soil (Fruhstorfer P) and Hakaphos green (fertilizer containing N = 20%, P = 2%, K = 15%) of 1 : 4: 4 : 0.01.

The plants were fertilized weekly with Mairol, a liquid fertilizer containing N = 6%, P = 4%, K = 5%. The temperature in the greenhouse was kept above 22 °C throughout the year; the natural light period was extended to 15 hours during spring, autumn and winter using Osram HWL lamps (500 W).

Root sources

a) Normal roots

Young shoots of greenhouse-grown plants were defoliated, surface-sterilized by successive treatments with tap water (10 min), 1% NaOC1 with 0.05% Tween 20 (15 min) and sterile water (3 x 10 min). Single nodes were cultured in Petri dishes (9 cm ⌀) with 20 ml ACM medium (AHUJA, 1983) containing 0 or 0·2 mg/l NAA at 25 °C, 12:12 hours light-dark regime with 3500 Lux fluorescent white light during the light period (BEIDERBECK, unpubl.). Under these conditions the single bud elongated and adventitious roots were formed which served as starting material for root cultures.

b) Hairy roots

24-hour cultures of *Agrobacterium rhizogenes*, strain 15834 (American Type Culture Collection) in NYS medium (LIPPINCOTT and HEBERLEIN, 1965) were harvested by centrifugation (15 min, 6000 rpm), the sediment was resuspended in distilled water and the cell concentration was adjusted to a desired value by absorbance readings (660 nm). Shoots originating from single-node explants (2 cm to 7 cm length) were inoculated with bacterial suspensions (2 x 10^8 colony-forming units per ml) using syringe (⌀ 0.65 mm) and incubated at 25 °C under a light-dark regime of 12:12 with 3500 Lux during the light period.

Hairy roots developing from inoculation sites to a length of 2 cm were used for root cultures.

Root culture

Our standard medium for root culture was a hormone-free, modified ACM medium containing increased concentrations of thiamine (10 mg/l) and sucrose (60 g/l) (ACM_w).

From hairy roots residual bacteria were eliminated by means of an antibiotic. The sensitivity of *A. rhizogenes*, strain 15834, to the antibiotics ampicillin, carbenicillin, chloramphenicol, kanamycin, and rifampicin was tested using the agar diffusion test (HALLMANN and BURKHARDT, 1974). Ampicillin (500 mg/l) and rifampicin (50 mg/l) proved suitable. For the elimination of *A. rhizogenes* from the roots the antibiotics were dissolved in sterile water, filter-sterilized and added to the ACM_w medium at 40 °C to 50 °C.

Liquid cultures were grown in 200-ml baby food jars containing 30 ml to 35 ml medium and agitated on a rotary shaker with 100 rpm. Solid cultures were grown in Petri dishes (9 cm ⌀) containing about 25 ml medium solidified with Difco Bitek(R) agar (8 g/l). Cultures were incubated at 25 °C in permanent darkness. Every 5 days roots were transferred to fresh medium until bacteria-free cultures were obtained. Bacteria-free roots were cultured on the same medium lacking the antibiotic.

Besides the standard medium ACM_w a variety of other media were tried part of which bad been used successfully with root cultures of different species (BUTCHER, 1980 HOOS and BLAICH, 1988; TORREY, 1954), especially the media ACM (AHUJA, 1983), B5 (GAMBORG et al., 1968), MS (MURASHIGE and SKOOG, 1962), and SH (SCHENK and HILDEBRANDT, 1972). These media were modified in different ways by

— reducing the sucrose concentration from 60 g/l to 30 g/l and 15 g/l (ACM, B5, MS) or increasing the sucrose concentration to 80 g/l and 100 g/ (ACM);

— reducing the salt concentration to one half (ACM_w, MS);
— omission of vitamins (ACM_w).
— addition of NAA in concentrations of 0·002 mg/l to 0.2 mg/l (ACM_w, B5), 0.6 mg/l and 2.0 mg/l (ACM_w, SH) and a combination of 2 mg/l NAA and 0.2 mg/l kinetin (ACM_w, SH);

Test for contamination with A. rhizogenes

To test antibiotic-treated hairy root cultures for residual bacteria small root pieces or aliquots of spent culture medium were transfered to solid NYS medium (15 g/l Difco Bitek(R) agar) and incubated at 28 °C for 3 to 4 days.

Evaluations

Fresh weight of root systems was determined in a clean bench. The roots were taken from the medium, blotted dry between two layers of sterilised paper (liquid cultures) or freed of adhering agar traces and weighed. In 5 parallel experiments weight determinations with 6 roots each were performed, mean values were calculated from the 6 measurements and then a common mean value was calculated. In 5 parallel experiments root lengths were determined from the 3 longest secondary roots on each root system, and mean values were calculated as described above.

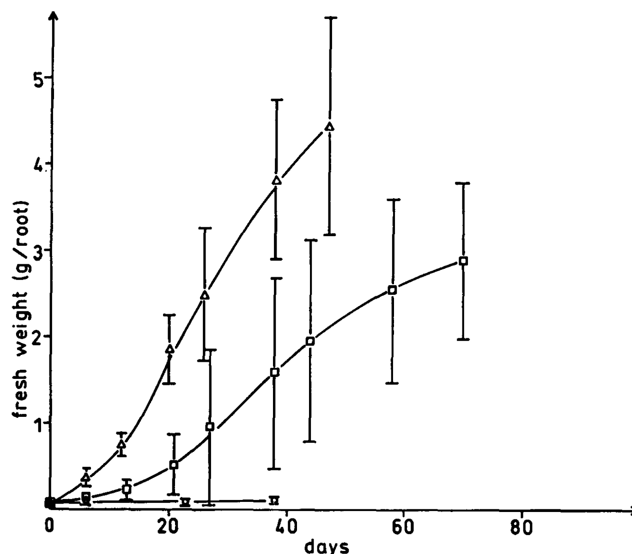

Figure 1. — Fresh weight of normal (o——o) roots on liquid ACM_w ledium and of hairy roots on solid, ampicillin-containing (□——□) or liquid antibiotic-free (Δ——Δ) ACM_w medium (mean values from 5 experiments with 6 roots each; standard deviations.)

Results

Culture of normal roots

Using several different low- and high-salt tissue culture media with different modifications the culture of normal roots of *S. alba* proved very recalcitrant· The most extensive elongation and ramification of the explanted roots was obtained in the medium ACM_w supplemented with 0.2 mg/l NAA, yielding an average increase of root length of 22.4 mm and 3.7 secondary roots during 6 weeks of culture. In all other media root extension and ramification were less abundant or absent. These growth reactions *in vitro* were not considered sufficient for routine work with root cultures.

Culture of hairy roots

Consequently culture of hairy roots (RIKER et al., 1930; MUGNIER, 1988) was tried. After *in vitro* infection of young shoot axes with *A. rhizogenes* 15.4% of the shoots responded with the formation of hairy roots which became visible at the inoculation sites 4 to 5 weeks after inoculation and grew to a length of about 2 cm during another 2 to 3 weeks· These roots differed from normal adventitious roots by an increased root diameter, a high number of secondary roots and a more rapid growth.

They were transferred to liquid or solid medium ACM_w containing an antibiotic and subcultured in the presence of the same antibiotic. After 11 passages in liquid medium 75% of the cultures treated with rifampicin and 20% of the cultures treated with ampicillin became bacteria-free. From solid cultures no bacteria-free roots were isolated after 20 passages on the antibiotics, although a very considerable reduction of bacterial numbers was observed.

On solid medium ACM_w supplemented with ampicillin hairy roots increased their weight 21.7 — fold from 76 mg to 1650 mg during a period of 38 days (for comparison: normal roots showed a 1.3-fold increase). This increase continued up to the 70th day, when 2860 mg were obtained (*Fig. 1*). Up to the 38th day the roots elongated considerably (compare methods of evaluation) and at the same time the root systems became much ramified (*Table 1*).

Solid cultures always were contaminated with few *A. rhizogenes*. Bacteria-free agar cultures were established by transferring bacteria-free roots from liquid medium (see below) to a solidified medium ACM_w lacking antibiotics. After a period of adaptation to the altered conditions these roots grew as rapidly as those with little contamination (*Fig. 1, 2*).

In liquid antibiotic-free medium ACM_w bacteria-free normal and hairy roots were cultured and their growth was compared using fresh weight determinations. Whereas the normal roots did not show a reliable growth reaction (1.4-fold increase during 38 days of culture) the hairy

Table 1. — Elongation and ramification of roots on solid medium ACM_w with ampicillin (550 mg/l) (mean values from 5 experiments with 6 roots each).

Day	root length	side roots of order			
	(mm)	1st	2nd	3rd	4th
normal roots					
0	8.00	0	0	0	0
9	11.02	0.36	2.62	0	0
18	14.43	0.36	2.91	0.33	0
24	16.81	0.36	3.24	0.92	0
38	19.62	0.36	3.24	0.92	0
hairy roots					
0	8.00	0	0	0	0
6	20.24	1.73	6.40	0.40	0
13	33.49	2.16	8.93	10.66	0
21	50.19	2.16	10.85	22.66	3.17
27	62.79	2.16	11.65	37.02	12.93
38	78.77	not countable			

Figure 2. — Hairy roots on medium ACM_w after 28 days of *culture.*

roots increased their fresh weight 45-fold from 84 mg to 3800 mg during the same period with a maximum daily increase of 120 mg/day between the 14th and 38th day (*Fig. 1*). The *in vitro* grown roots ramified considerably (*Table 1*) until a dense network of side roots had formed (*Fig. 2*).

Discussion

Root cultures and especially hairy root cultures have been established for a variety of herbaceous plant species but only rarely for tree roots (INGRAM and HELGESON, 1980; MUGNIER, 1988). No such cultures have been reported for trees considered suitable for rapid biomass production. In accordance with earlier reports on different plant species (QUATTROCHIO, 1985; RHODES et al., 1987) it was not possible to obtain cultures of normal roots of *Salix alba* with growth rates suited for routine experimentation although some optimization was demonstrated. Since hairy roots of herbaceous plants generally have much higher growth rates than normal roots, *Salix* plants were infected with *A. rhizogenes* and roots with high growth rates (elongation, ramification, fresh weight) were obtained. These putatively transformed roots (TEPFER, 1990) will serve as an host source for the study of root symbionts and pathogens.

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Genetic Control of Germination Parameters in Douglas-fir and its Importance for Domestication

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Abstract

The genetic control of germination in Douglas-fir [*Pseudotsuga menziesii* (MIRB.) FRANCO] was studied using wind-pollinated seed collected from 19 seed orchard trees. Seed-donor trees and the seed orchard were carefully selected to minimize any environmental-precondi-