Grapevine root transformation with *Agrobacterium rhizogenes* [1])

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Summary: Grapevine shoots were obtained from tissue cultures of cvs Barbera, Moscato bianco and Nebbiolo; their stems were wounded and inoculated with the following strains of *Agrobacterium rhizogenes*: A4, 8196, NCPP 2659, and 15834. Root production at the inoculation site was obtained in about 25 % of the stems of all cultivars with all the bacterial strains, with the exception of NCPP 2659, which gave a lower root proliferation rate. Cultures of roots obtained after inoculation were established and their growth was enhanced by some media and by addition of cytokinins and auxins to the medium. Root cultures obtained after inoculation with the strain 8196 were genetically transformed, as shown by opine production. In contrast, opines were seldom detected in the root cultures obtained with the other bacterial strains. Light microscope observation showed that the cortex of transformed roots has more cell layers and a larger average cell size than in normal roots.

Key words: Agrobacterium rhizogenes, gene transfer, genetic engineering, genetics, tissue culture, light, growth regulator, root, growth, opine, histology.

Introduction

Grapevine breeding and selection have been undertaken utilizing scientific methods for more than one century, and most of the efforts of breeders have been directed toward the attainment of resistance to damaging pests and diseases or to the improvement of fruit quality and ripening time. Most of these problems, however, have been only partly overcome by traditional breeding methods, because of the difficulty in introducing only a single selected gene into a genome, without introducing other unwanted genetic characters.

The transfer of genetic material between different cells, using various kinds of vectors, is now an established technique for some plants (Armitage *et al.* 1988) and offers interesting perspectives for other less studied plants like grapevine. These methods are particularly interesting for their ability to transfer only the sequences which encode for a particular character. *Agrobacterium rhizogenes* is a commonly employed vector which has been tested on several species; expression of its Ri (root-inducing) T-DNA leads to root proliferation from the inoculation site. Such roots can be grown *in vitro* and whole plants can be regenerated from transformed root cultures (Birot *et al.* 1987; Zambryski *et al.* 1989). However, very little information is available on the possibility of utilizing *A. rhizogenes* on grapevine (Hemstad and Reisch 1985). In this work, we have tested methods to obtain and grow cultures of grapevine roots transformed with *A. rhizogenes*.

Material and methods

Bacterial strains

The *A. rhizogenes* strains used were: 1855, in a preliminary experiment, A4, 15834, 8196, and NCPP 2659. Bacteria were grown at 28 °C in Petri dishes containing the following medium (YMB): K_2HPO_4 0.5 g • l^{-1}; $MgSO_4 \cdot 7H_2O$ 0.2 g • l^{-1}; NaCl 0.1 g • l^{-1}; mannitol 1 %; yeast extract 0.04 %; agar 18 g • l^{-1}.

Cultures were stored at 4 °C for 1-2 months. Grapevine shoots were inoculated with *Agrobacterium* cultures grown for 36-48 h on fresh culture medium.

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Plant material

In vitro rooted stock plants of *Vitis vinifera* cv. Nebbiolo (clone 111), Moscato bianco (clone 4), and Barbera (clone 84) were cultured on a hormone-free JONA and WEBB (1978) medium modified by increasing $FeSO_4$ + NA_2EDTA to 200 μM and thiamine HCl to 15 μM. The pH was adjusted to 5.6 before autoclaving at 120 °C for 10 min.

All the grapevine clones originated from clonal selection performed by this Centre.

Inoculation

5-6 cm long grapevine shoots were obtained by cutting the upper part of the *in vitro* stock plants. The shoots were wounded with a scalpel, partially scraping an internode in the median part of the stem, and inoculated with the bacteria. The shoots were then planted in the hormone-free medium described above. The control shoots were wounded but not inoculated.

In a preliminary experiment, shoots of Nebbiolo were inoculated with *A. rhizogenes* strain 1855. In a subsequent factorial experiment, shoots of Nebbiolo, Moscato and Barbera were inoculated separately with the *A. rhizogenes* strains A4, 8196, NCPP 2659 and 15834. The inoculation was replicated on a minimum of 11 shoots to a maximum of 80 for each treatment. The inoculated plants were kept in a growth chamber at 24 °C, with 250 μmol • s^{-1} • m^{-2} photon flux from fluorescent lamps and 16/8 h photoperiod.

Results were statistically analyzed by using chi square test.

Root cultures

Experiments on root cultures were performed using roots obtained from Nebbiolo shoots inoculated with strain A4.

In an initial experiment, 1 cm root pieces with tips were cultured on three different media, as following:

- medium A: as described by BECARD and FORTIN (1988) (medium MW), with 10 g • l^{-1} agar and 30 g • l^{-1} sucrose;
- medium B: MURASHIGE and SKOOG (1962) medium with half strength salts, 10 g • l^{-1} agar and 15 g • l^{-1} sucrose;
- medium C: MURASHIGE and SKOOG medium modified as suggested by MUGNIER and MOSSE (1987), with 30 g • l^{-1} sucrose bot no agar: a thin layer of medium was poured in each Petri dish.

The pH of all media was adjusted to 5.6 before autoclaving at 120 °C for 15 min. Root cultures were incubated at 24 °C; half of the cultures were kept under continuous dark conditions and another half under a 16/8 h photoperiod as described above. After 2 weeks culture, the growth of each root apex was measured.

In a subsequent experiment, roots were cultured in continuous darkness on medium B containing (GUELLEC, personal communication) 25 mg • l^{-1} of filter-sterilized Claforan (sodium cefotaxime; Roussel Maestretti, Milano); after 1 month, 1-2 cm long root fragments containing root apexes were transferred onto medium B without Claforan and with the following hormones added:

- medium IB: 0.1 μM BAP + 0.15 μM IBA;
- medium IA: 0.1 μM BAP + 0.15 μM IAA;
- medium NA: 0.1 μM BAP + 0.15 μM NAA.

In this case, it was not possible to measure root length because of excessive ramification: thus average root fresh weight was recorded after 4 weeks culture.

Results were statistically analyzed by ANOVA.

Table 1: Percentage of stems with callus and roots and average root number, 18 d after inoculation with *Agrobacterium rhizogenes* strain 1855 on the internode of Nebbiolo grape

	+ 1855	CONTROL	EFFECT
STEMS WITH			
CALLUS (%)	91.3	19.2	* *
ROOTS (%)	69.6	0	* *
AVERAGE ROOT NO.	2.2	0	

Opine analysis by paper electrophoresis

Root extracts were prepared according to PETIT *et al.* (1986) and spotted onto Whatman no. 3MM paper. After electrophoresis (100 V • cm^{-1} for 10 min) in formic acid-acetic acid-water buffer (30/60/910 v/v/v)the dried paper was treated with either silver nitrate reagent or Pauly reagent.

Histological observations

Roots growing out of the inoculation points were collected, embedded in O.C.T. compound (Miles Scient.; Naperville, IL) and sectioned using a cryostatic microtome.

Control roots were taken from the base of non-inoculated shoots, rooted on hormone-free medium; they were treated as above described.

Results

Plant reaction to inoculation

Shoots of Nebbiolo inoculated with *A. rhizogenes* strain 1855 produced slight amounts of callus and roots in the inoculation site, as reported in Table 1.

In the factorial experiment, production of small quantities of callus was more frequent in inoculated plants, independent of grapevine cultivar. No roots were produced in the control plants. Concerning the effects of *Agrobacterium* strain, an average of 21-27 % of the treated shoots produced roots from the inoculation point, with the exception of shoots inoculated with the strain NCPP 2659 (10 %). There was no considerable difference among cultivars, even if some combinations (i.e. Moscato and Barbera + 8196; Nebbiolo + 15834 and Moscato + A4) showed a greater response to inoculation (see Table 2).

Table 2: Comparative results of Barbera, Nebbiolo and Moscato stem inoculations with *Agrobacterium rhizogenes* strains NCPP 2659, A4, 15834, and 8196, 1 month after inoculation. For totals column and rows, values followed by a common letter do not differ significantly at P = 0.05 (small letters) or P = 0.01 (capital letters)

			BARB.	NEBB.	MOSC.	TOTAL
Agrobacterium rhizogenes strain	NCPP 2659	STEMS WITH CALLUS (%)	36	100	35	60 Aab
		ROOTS (%)	9	20	0	10 ABa
		root no.	1	1.7	-	
		root lenght (mm)	20	5.8	-	
	A4	STEMS WITH CALLUS (%)	17	59	50	53 Aa
		ROOTS (%)	0	25	42	24 Aa
		root no.	-	1.6	2.8	
		root lenght (mm)	-	12.4	6.1	
	15834	STEMS WITH CALLUS (%)	72	87	67	75 Ab
		ROOTS (%)	18	40	20	27 Aa
		root no.	2.5	2.2	2	
		root lenght (mm)	50.6	14.2	72.5	
	8196	STEMS WITH CALLUS(%)	36	58	92	59 Aab
		ROOTS (%)	54	8	75	21 Aa
		root no.	3.7	1.5	2	
		root lenght (mm)	7.7	22.7	5.8	
NOT INOCUL.		STEMS WITH CALLUS (%)	27	31	7	23 Bc
		ROOTS (%)	0	0	0	0 Bb
TOTAL		STEMS WITH CALLUS (%)	37 A	60 B	49 AB	54
		ROOTS (%)	16 A	16 A	25 A	18

Table 3: The effect of different media and photoperiods on Nebbiolo root growth (average increase in length as cm). The roots were obtained from stems inoculated with *Agrobacterium rhizogenes* strain A4. Medium A: BECARD and FORTIN (1988); medium B: MURASHIGE and SKOOG (1962), half-strength salts; medium C: liquid full strength MURASHIGE and SKOOG

MEDIUM	24 hrs dark 0 hrs light	8 hrs dark 16 hrs light	
A	15.1 ± 2.8	10.7 ± 2.0	EFFECT
B	6.4 ± 1.4	4.3 ± 7.5	MEDIUM *
C	5.6 ± 0.9	2.5 ± 0.6	
	EFFECT ILLUMINATION *		

Roots usually proliferated where callus was present, but in a few cases the internode formed roots but no callus: this may indicate that the growth of the two tissues is independent.

Rhizogenesis was relatively slow: for instance, after inoculation with the strain A4 only 9.2 % of Nebbiolo shoots produced roots from the inoculated internodes after 18 d, but the percentage increased to 27.3 % after 28 d. If the roots were cut off, the inoculated internodes produced new roots.

Root growth from the wounded and inoculated internode is independent of shoot basal rooting: treated shoots rooted in the basal part of the stem, placed in culture medium, as the control shoots did.

Root cultures

Roots produced by Nebbiolo internodes wounded and inoculated with *A. rhizogenes* strain A4 were cultured on different media. The basal medium described by BECARD and FORTIN (1988) was more effective in promoting root growth than the other media, as reported in Table 3. Roots grew better in continuous darkness.

Addition of BAP (0.1 μM) and auxins (IAA, IBA or NAA 0.15 μM) to the culture medium enhanced root growth, this effect being unrelated to the auxin type (see Table 4).

Opine analysis

Paper electrophoretic analysis revealed that not all the cultured roots synthesized opines. Roots obtained by grapevine internodes inoculated with *A. rhizogenes* strains A4 and 15834 revealed the presence of opines in 20 and 30 % of the samples, respectively, while 83 % of the root

Table 4: Root growth after 1 month of culture on media containing 0.1 mg • l^{-1} BAP and 0.15 mg • l^{-1} of different auxins. Roots were obtained from Nebbiolo shoots inoculated with *Agrobacterium rhizogenes* strain A 4

AUXIN (mg/l)	ROOT FRESH WEIGHT (mg)	EFFECT
IBA 0.15	200 ± 39	
NAA 0.15	189 ± 58	N.S.
IAA 0.15	173 ± 42	

samples obtained from inoculations with strain 8196 had opines. None of the roots grown following inoculation with strain NCPP 2659 produced opines.

It must be noted that among the faster growing root lines (obtained by stem inoculation of strains A4 and 15834) some produced opines and some did not.

Histological observations

Compared to normal roots, roots produced by the inoculated stems showed a strong increase in the cell layer number and in the cell size of the cortical parenchyma. Also, the intercellular spaces appeared larger than in the normal roots. In contrast, no remarkable difference could be noted in the epidermis and the underlying cells.

Discussion

The method described proved to be effective in transforming roots of all the three grapevine cultivars tested. The roots originating from the inoculation site have particular morphological features, and they can be best grown in presence of BAP and auxins, in continuous darkness.

Not all these roots proved to be transformed when the electrophoretic analysis of opines was performed. Fast growing root lines were obtained by stem-inoculation of *A. rhizogenes* strains A4 and 15834; surprisingly, some of those root lines did not produce opines. However, it seems unlikely that in this case roots were not transformed, as preliminary tests showed that non-inoculated roots grew very poorly. The fast growing roots which did not produce opines could be the results of an incomplete transfer of the *Agrobacterium* plasmid. Agropine-type strains like A4 and 15834 have two separated T-regions (T_L and T_R): during transformation plant cells may receive either the T_L-DNA (which induces root proliferation) or the T_R-DNA (coding opine synthesis) or both (Melchers and Hooykaas 1987). More detailed analyses are needed to ascertain the nature of these root lines, and DNA analysis is planned.

Roots originating from inoculations with *A. rhizogenes* strain 8196 are mostly transformed, but they do not grow in the tested culture conditions. They usually darken and die in a short span of time or grow very slowly. It is possible that this problem may be overcome by changing the concentration of growth regulators in the medium.

The production of non-transformed roots from the inoculation point could depend on the wound made before inoculation. If being too deep, the wound could mimic the effects of apical cutting, i. e. root production at the new shoot base.

In conclusion, this method is useful to obtain transformed grapevine roots. Further investigations will be carried out in order to test the response of a larger number of grapevine varieties and to improve root growth in culture.

These investigations will be the first steps toward controlled transformation of grapevine cultivars. However, transgenic plants cannot be obtained unless an effective regenerating system from grapevine roots or callus or cell suspensions is developed. Many efforts are now directed toward this target.

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References

ARMITAGE, P.; WALDEN, R.; DRAPER, J.; 1988: Vectors for the transformation of plant cells using *Agrobacterium*. In: DRAPER, J.; SCOTT, R.; ARMITAGE, P.; WALDEN, R. (Eds.): Plant Genetic Transformation and Gene Expression. Blackwell Sci. Publ.

BECARD, G.; FORTIN, J. A.; 1988: Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol. **108**, 211-218.

BIROT, A. M.; BOUCHEZ, D.; CASSE-DELBART, F.; DURAND-TARDIF, M.; JOUANIN, L.; PAUTOT, V.; ROBAGLIA, C.; TEPFER, D.; TEPFER, M.; TOURNEUR, J.; VILAINE, F.; 1987: Studies and uses of the Ri plasmids of *Agrobacterium rhizogenes*. Plant Physiol. Biochem. **25**, 323-335.

HEMSTAD, P. R.; REISCH, B. I.; 1985: *In vitro* production of galls induced by *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* on *Vitis* and *Rubus*. J. Plant Physiol. **120**, 9-17.

JONA, R.; WEBB, J. K.; 1978: Callus and axillary-bud culture of *Vitis vinifera* 'Sylvaner Riesling'. Sci. Hort. **9**, 55-60.

MELCHERS, L. S.; HOOYKAAS, P. J. J.; 1987: Virulence of *Agrobacterium*. Oxford Surv. Plant Mol. Cell. Biol. **4**, 167-220.

MUGNIER, J.; 1987: Infection by *Polimixa betae* and *Plasmodiophora brassicae* of roots containing root-inducing transferred DNA of *Agrobacterium rhizogenes*. Phytopathology **77**, 539-542.

-- -- ; MOSSE, B.; 1987: Vesicular-arbuscular mycorrhizal infection in transformed root-inducing T-DNA roots grown axenically. Phytopathology **77**, 1045-1050.

MURASHIGE, T.; SKOOG, F.; 1962: A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15**, 473-497.

PETIT, A.; BERKALOFF, A.; TEMPÉ, J.; 1986: Multiple transformation of plant cells by *Agrobacterium* may be responsible for the complex organization of T-DNA in crown gall and hairy root. Mol. Gen. Genet. **202**, 388-393.

ZAMBRYSKI, P.; TEMPÉ, J.; SCHELL, J.; 1989: Transfer and function of T-DNA genes from *Agrobacterium* Ti and Ri plasmids in plants. Cell **56**, 193-201.