Metaldehyd könnten auch illegale Köder (z. B. Hackfleisch) mit Metaldehyd, die von Igeln gern gefressen werden, eine Rolle gespielt haben (GREIG-SMITH et al., 1989).

Literatur

BERTHOUD, G., 1981: Contribution à la biologie du hérisson (*Erinaceus europaeus* L.) et applications à sa protection. Diss. Université de Neuchâtel, Faculté des Sciences.
BOOZE, TH. F. and F. W. OEHME, 1985: Metaldehyde toxicity: A review. Vet. Hum. Toxicol. **27,** 11–19.
DAXEL, R., 1968: Die Abhängigkeit der Wirkung molluskizider Substanzen (Metaldehyd, Isolan, Ioxynil) von endogenen und exogenen Faktoren auf Nacktschnecken. Diss. Technische Univ. Berlin.
ESSER, J., 1984: Untersuchungen zur Frage der Bestandsgefährdung des Igels (*Erinaceus europaeus)* in Bayern. Ber. ANL **8,** 22–62.
FLETCHER, M. R. and K. HUNTER, 1992: Pesticides poisoning of animals 1992: Investigations of Suspected Incidents in the United Kingdom. MAFF Publications, London.
FLETCHER, M. R., K. HUNTER, M. P. QUICK, H. M. THOMPSON, and P. W. GREIG-SMITH, 1990: Pesticide Poisoning of Animals 1990: Investigations of Suspected Incident in Great Britain. Environmental Panel Report, MAFF, London.
GEMMEKE, H., 1991: Untersuchungen zur Abschätzung des Gefährdungspotentials von Schneckenkorn Mesurol für Igel *(Erinaceus europaeus).* Biologische Bundesanstalt für Land- und Forstwirtschaft, unveröffentlichter Bericht.
GREIG-SMITH, P. W., 1988: Wildlife hazards from the use, misuse and abuse of pesticides. Aspects of Applied Biology **17,** 247–256.
GREIG-SMITH, P. W., M. R. FLETCHER, K. HUNTER, M. P. QUICK, A. D. RUTHVEN, and I. C. SHAW, 1988: Pesticide Poisoning of Animals 1988: Investigations of Suspected Incidents in Great Britain. Environmental Panel Report, MAFF, London.
GREIG-SMITH, P. W., M. R. FLETCHER, K. HUNTER, M. P. QUICK, and H. M. THOMPSON, 1989: Pesticide Poisoning of Animals 1989: Investigations of Suspected Incidents in Great Britain. Environmental Panel Report, MAFF, London.
Igel Bulletin: Publikationsorgan des Vereins pro Igel 1991.
KEYMER, I. F., E. A. GIBSON, and D. J. REYNOLDS, 1991: Zoonoses and other findings in Hedgehogs *(Erinaceus europaeus):* a survey of mortality and review of the literature. Vet. Rec. **128,** 245–249.
Plädoyer für den Igel: Rheinisch-Westfälische Igelfreunde (RWI) Perspektiven 1988, ROBOR-GmbH, Hückeswagen.
REEVE, N., 1994: Hedgehogs. T. & A D Poyser, London.
STOCKER, L., 1987: The Complete Hedgehog. Chatto & Windus, London.

Kontaktanschrift: Dr. Hubert Gemmeke, Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Nematologie und Wirbeltierkunde, Toppheideweg 88, D-48161 Münster

Nachrichtenbl. Deut. Pflanzenschutzd., **47** (9), S. 240–244, 1995, ISSN 0027-7479.

Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Biochemie und Pflanzenvirologie, Braunschweig

Experiments to eliminate Agrobacteria persisting in plants

Versuche zur Eliminierung von in Pflanzen persistierenden Agrobakterien

Von J. Landsmann, Elke Graser, Anette Riedel-Preuß und Cornelia van der Hoeven

Abstract

Genetically engineered agrobacteria are routinely used to transform crop plants. These agrobacteria can obviously persist within the transgenic plants although they are not tumorgenic. Our attempts to eliminate the agrobacteria concentrate on the regeneration of secondary shoots from infected tobacco plants. Application of the antibiotics cefotaxim or carbenicillin during the regeneration process have not resulted in sufficient reduction of the bacterial contamination. Shoot tip culture of apical meristems, however, resulted in a large percentage of agrobacteria-free regenerated plants (within the detection limits). The degree of contamination was determined visually and by PCR (polymerase chain reaction).

Key words: *Agrobacterium tumefaciens,* genetic engineering, transgenic plant, meristem culture, antibiotics

Zusammenfassung

In der Gentechnik werden routinemäßig Agrobakterien zur Transformation von Kulturpflanzen eingesetzt. Diese nichttumorgenen Agrobakterien können offensichtlich in den Pflanzen persistieren. Wir versuchen, aus solchen kontaminierten Pflanzen in der Gewebekultur neue, nicht kontaminierte Pflanzen zu regenerieren. Die Verwendung der Antibiotika Cefotaxim (Claforan) bzw. Carbenicillin während der Regeneration hat nicht zu einer ausreichenden Verminderung der persistierenden Agrobakterien geführt. Durch Anwendung der Meristemkultur aus apikalen Sproßspitzen hingegen konnten erfolgreich Agrobakterien-freie Pflanzen (innerhalb der Nachweisgrenzen) erzeugt werden. Zur Bestimmung des Kontaminationsgrades wurden die visuelle Bonitur und die PCR (Polymerase-Kettenreaktion) verwendet.

Stichwörter: *Agrobacterium tumefaciens,* Gentechnik, transgene Pflanze, Meristemkultur, Antibiotika

Introduction

For the transformation of plants by genetic engineering *Agrobacterium tumefaciens* is now a routine tool. The agrobacteria infect wounded plant tissue and transfer parts of their Ti-plasmid, the T-DNA, into the plant chromosomes. The T-DNAs of oncogenic agrobacteria contain genes for plant hormone synthesis leading to gall formation and opine or agropine synthase genes to nourish the infecting agrobacteria. Disarmed agrobacteria used in genetic engineering neither induce tumors nor can they draw on tailor made substrates. It was generally taken for granted that the endobiotic re-

lationship of the disarmed agrobacteria with the plant is a transient one and that no disarmed agrobacteria survive and persist after the early counterselection steps. However, occasionally bacteria can be isolated from transformed plants many months after infection (VAN DER HOEVEN et al., 1991, VAN DER HOEVEN, 1992). We have characterized the reisolated agrobacteria and tried to develop methods for their elimination from the infected plants. Implications for release into the environment of genetically engineered plants will be discussed.

Transformation of tobacco leaf disks and regeneration of plants

Leaves from sterile grown *Nicotiana tabacum* var. W38 were cut into strips vertical to the middle vein and soaked for 10 minutes in 2.5×10^8 *Agrobacterium tumefaciens* LBA4404 per ml MS-medium (MURASHIGE and SKOOG, 1962; HORSCH et al., 1985). The agrobacteria contained the binary vector pPARGUSH (VAN DER HOEVEN et al., 1994a) resp. PARBASTA (VAN DER HOEVEN et al., 1994b) resp. PARPET (LANDSMANN et al., 1988). The leaf pieces were placed on sterile paper cloth to rid them from excess water, then put on MS9-medium agar petri dishes at 26 °C in the dark. After 48 hours of cocultivation the leaves, now visibly surrounded by agrobacteria, were gently washed in MS-medium and placed onto MS9-agar medium containing 100 µg/ml kanamycin and 250 µg/ml Claforan (Hoechst). They were incubated at 24 °C at a 16 h light/8 h dark period and transferred to fresh plates once a week. No agrobacteria grew on this selection/counterselection medium. After four weeks the emerging shoots were transferred to MS-agar medium containing Claforan and kanamycin. Another four weeks later the rooted plants were isolated into MS-agar medium jars containing just the selecting antibiotic kanamycin. Shoot tips comprising at least five leaves were propagated in MS-agar medium in 2–3 weeks intervals. After six passages the antibiotic was omitted.

Those regenerating shoots which rooted on hormone free MS-medium containing 100 µg/ml kanamycin were classified as being transformed. NPT activity (due to the introduced kanamycin resistance gene nptII), GUS activity (due to the introduced ß-glucuronidase gene in PARGUSH plants), PAT activity (due to the introduced phosphinothricin acetyl transferase gene in PARBASTA plants) and CAT activity (due to the introduced chloramphenicol acetyl transferase gene in PARPET plants) were determined in leaves and roots.

For separate analyses with increased sensitivity fake transformations were done with the specially constructed T-DNA deficient binary vector pLX222delta15 (Landsmann, unpublished). This vector does not result in transfer of any genes to the plant chromosomes, nevertheless results in comparable contamination of the regenerated plants with agrobacteria. The resulting plants are kanamycin sensitive.

Reisolation of bacteria

During shoot tip propagation, i.e. more than five weeks after release of Claforan counterselection and more than nine weeks after transformation, occasionally bacteria could be detected growing out of the cut stem site of the majority of 140 transgenic PARGUSH plants. We especially made sure that accidental contamination of the cutting site could be excluded.

Six months after the plant transformation PARGUSH bacteria were isolated from 6 individual transformants. From two different transformations two out of 26 plants (No. H1 and X7) were heavily contaminated with agrobacteria more then two years after cocultivation. From one transformation with PARBASTA seven out of 30 transgenic plants released agrobacteria into the culture medium 3 years after transformation and from two transformations with PARPET three out of 20 transgenic plants regularly released agrobacteria.

Analysis of reisolated bacteria

All isolates were positive in a ketolactose test (BERNAERTS and DE LEY, 1963), indicating they were *A. tumefaciens*. Two PARGUSH and the 3 PARPET isolates were tested in a BIOLOG test (Biolog Inc., Hayward, CA, USA) where they showed slightly different fermentation capabilities. They all were clearly identified as *Agrobacterium* ssp. (SAWADA et al., 1993).

All PARGUSH and PARBASTA isolates grew well on LB-agar medium plates containing 100 µg/ml rifampicin, 5 µg/ml tetracyclin resp. 100 µg/ml kanamycin. Two of the 3 PARPET isolates had lost the tet-resistance marker.

Plasmid preparations from all isolates resulted in plasmid DNA of the expected size, although the vectors were generally not completely stable in *A. tumefaciens* LBA4404 (3 out of 60 HindIII digested preparations from individual PARGUSH colonies showed deviating fragment lenghts). Restriction with EcoRI or HindIII endonucleases showed the restriction pattern of the original binary vectors. Southern hybridization with a PARGUSH probe detected the correct fragments although additional rearrangements might have taken place (VAN DER HOEVEN, 1992).

In order to check the integrity of the constructs two of the reisolated *Agrobacterium* strains were used to transform tobacco with the leaf disk method. Six regenerated plants of each transformation experiment were grown to the rooting stage. The level and variability of GUS activity of the regenerated plants proved to be comparable to the first set of transformants (VAN DER HOEVEN et al. 1994a) indicating the originality of the *Agrobacterium* strains.

Antibiotic treatment to reduce agrobacterial contaminations

Pieces of leaves from transgenic *in vitro* tobacco plants, PARGUSX7, PARPET72 and LX222DELTA15, still harbouring recombinant agrobacteria, were incubated on MS-medium to regenerate secondary shoots. During the regeneration process 0 resp. 250 resp. 500 µg/ml Claforan (Cefotaxim) or carbenicillin (for LX222DELTA15) were applied. Approximately 50 shoots of each experiment were analysed.

From the 2–3 year old transgenic lines only one of the secondary shoots – regenerated on antibiotic containing medium – developed agrobacteria when transferred onto antibiotic-free medium. From the freshly fake transformed LX222DELTA15 plants, however, up to 40 % of the regenerated secondary shoots were still visibly contaminated. This did not depend on the kind or dosage of the antibiotic used.

Primers deduced from the tet gene of the binary vector could detect visible amplification products in 8–21 % of PCR analyses of individual secondary PARPET72 shoots. All of the secondary PARGUSX7 shoots were negative with the tet primers. For both lines there was no correlation to the level or the absence of antibiotic treatment. The tet gene is located outside the T-DNA region and should not be transferred to the plant chromosome upon plant transformation.

Tet-PCR with the secondary shoots of antibiotic treated LX222DELTA15 showed 5–40 % contamination in the first test, uncorrelated to the kind or dosage of the antibiotic used. When tested again four weeks later, the contamination rate in the plant apex had dropped from an average of 20 % to an average of 15 %.

In the absence of any antibiotic the plants regenerated very poorly because of heavy bacterial contamination (Fig. 1). Two of the 16 re-

Fig. 1. Calli and shoots regenerating from transgenic *Nicotiana tabacum* leaf discs. Agrobacteria leak into the culture medium.

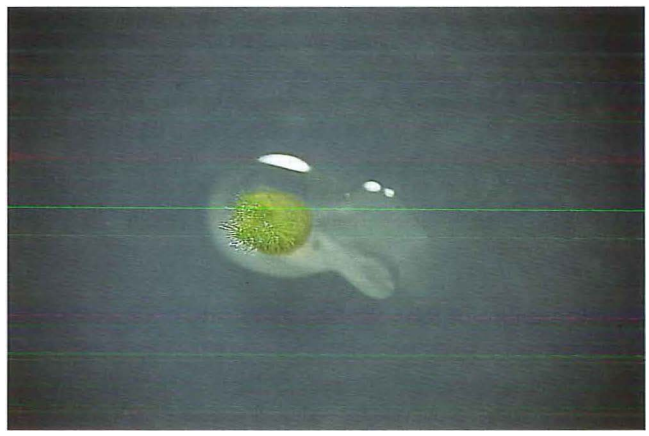


Fig. 2. Meristem preparation from *in vitro* grown transgenic *Nicotiana tabacum*. Agrobacteria leak out of the cutting site into the culture medium.

spective LX222DELTA15 shoots analysed repeatedly showed no tet-PCR amplification products.

These results could be confirmed with PCR primers from the nptII gene as the LX222DELTA15 plants are untransformed.

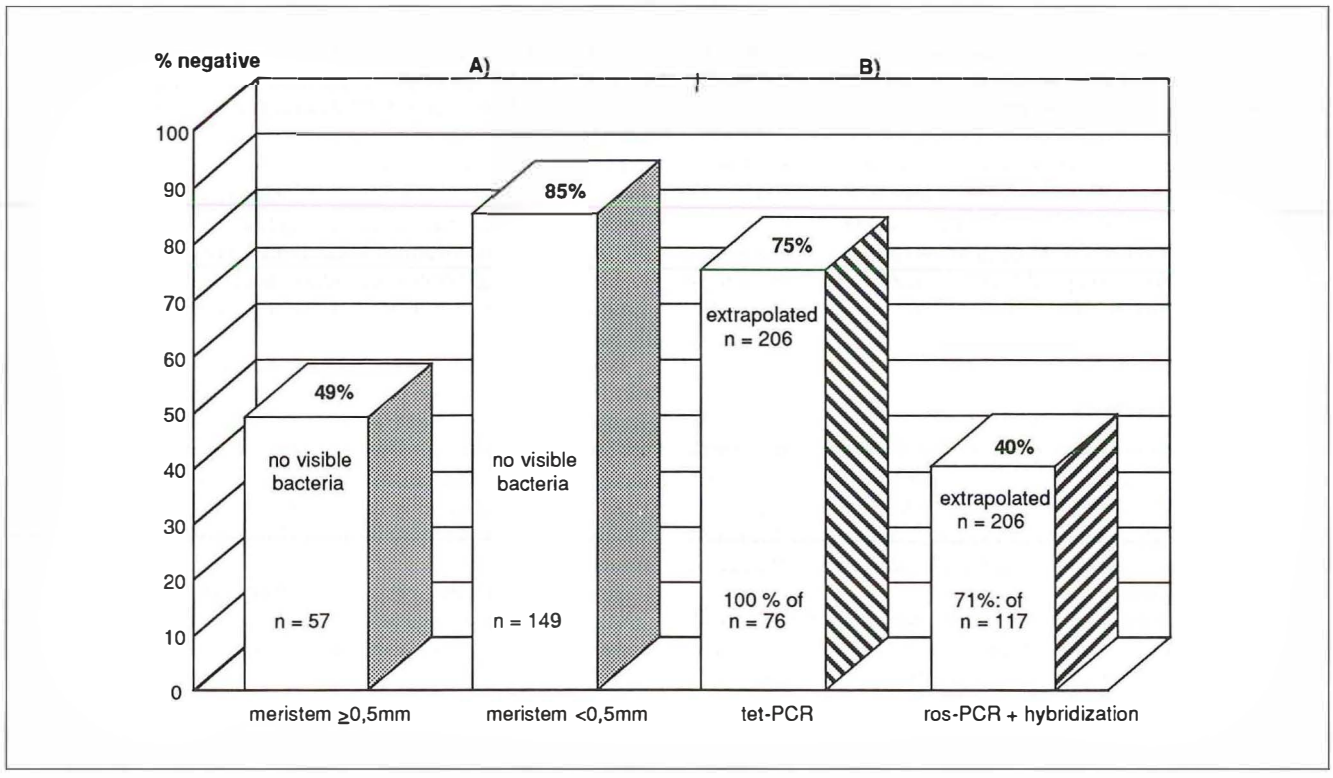
Shoot meristem culture to minimize agrobacterial contaminations

Shoot tips were prepared from transgenic *in vitro* tobacco plants and used to regenerate calli and shoots (GRASER, 1994). 49 % of the regenerated calli from 57 meristem preparations ≥ 0.5 mm in ∅ and 85 % of calli regenerated from 149 meristems < 0.5 mm in ∅ did not develop *Agrobacterium* colonies on the medium (Fig. 2). DNA preparations of these calli were submitted to PCR analyses.

PCR with the tet primers were negative with all 76 tested – visibly bacteria-free – candidates.

PCR with primers from the agrobacterial chromosomal ros gene was done including hybridization with a ros gene probe (COOLEY et al., 1991; DONG et al., 1992; DSOUZAAULT et al., 1993; ZWEIGERDT, 1993). The hybridization was necessary to compensate for the background of amplification products with uninfected plants. 71 % of 117 – visibly bacteria-free – calli tested showed negative results in the ros hybridization of the PCR gels (Fig. 3), indicating a presumably agrobacteria-free state.

Fig. 3. A) Detection of *Agrobacterium* colonies on the medium during plant regeneration from tobacco meristems (percentage of visibly bacteria free preparations).
B) PCR with DNA from visibly bacteria free meristem preparations. Percentage of negative results with the tet primers (deduced from a gene of the transformation vector, not integrated into the plant) on ethidium bromide stained agarose gels, resp. percentage of negative results with the ros primers (deduced from a *Agrobacterium* gene) after hybridization of the gels with a ros gene probe.



Seed production to exclude agrobacteria

525 seeds from 3 individual tobacco transformants were aseptically germinated on MS-medium. 375 of the emerging plantlets were cut into pieces and incubated at 28 °C on LB-medium agar. After 10 days still no agrobacteria were visible. The remaining plantlets were grown for further 4 months, with no bacteria appearing on the MS-medium.

Seedlings from different transgenic tobacco lines were submitted to PCR analyses with the ros and the tet primers. 100 % of 46 tet-PCR analyses of 2 transgenic lines (comprising 2–3 pooled seedlings each) and all 20 tet-PCR analyses of leaves from individual plantlets of another transgenic line were negative.

Discussion

Agrobacteria persisting after the plant transformation appear to be defying even severe attempts of elimination (GOULD and SMITH, 1989).

The general use of the antibiotics carbenicillin or cefotaxim during the plant regeneration step does not kill the agrobacteria. Even high dosage of the antibiotics does not readily eliminate the agrobacteria. Equally important seem to be the washing procedure for the plant leaf discs and the time gap between the plant transformation and the contamination test. Agrobacteria do not seem to substantially multiplicate or actively move within the plant tissue. Although the common antibiotics reduce the contamination (REED et al., 1995) more potent or systemic antibiotics need to be tested.

Shoot tip (meristem) culture is an appropriate method for further minimizing bacterial contamination (THEILER, 1980). Meristem culture in combination with a heat treatment (to slow down virus replication) is commercially used for the elimination of plant viruses (HEDTRICH et al., 1980, HUTH, 1979). There is a dilution effect towards the plant apex. Our experiments with tobacco show that a complete elimination of persisting agrobacteria can probably be achieved through regeneration from uncontaminated tissue. Treatment at 42 °C for several hours had no additional effect.

Proof of the bacteria-free state, however, is not possible. Notwithstanding, tests for contamination depend upon the detection limit (PONSONNET and NESME, 1994; SAWADA et al., 1995) and need to be standardized for general use. The detection limit for visible PCR products on ethidium bromide stained gels lay at < 100 bacteria per PCR reaction tube. Additional hybridization could detect even 1 bacterium. However, when calculating the necessary dilutions (PCR does not work in concentrated extracts) and extrapolating to the plant material assayed it came down to a minimum of 750 agrobacteria which escape detection. In addition, the sample taken may not be representative of the whole plant because of uneven distribution of the contaminants. The additional possibility of persisting free Ti-plasmid DNA in transgenic plants has not yet been investigated.

Technically, special attention has to be given to false positive results due to contaminations in laboratories where the agent to be screened for in trace amounts is prevalent.

As the agrobacteria used for plant transformation obviously can survive within the transgenic plants (ZEIDAN and CZOSNEK, 1994; ESCUDERO et al., 1995), generally applicable methods to eliminate these bacteria need to be developed. *Agrobacterium* is supposed to persist predominantly between plant cells (LEHOCZKKY, 1971; STELLMACH, 1990; MOGILNER et al., 1993). Thus protoplast culture may offer other means of regenerating bacteria-free plants. These, however, will be time consuming and not easily adaptable to recalcitrant crop species.

Finally, our experiments support the notion that seed transmission of agrobacteria can probably be denied, at least for tobacco.

Engineered agrobacteria residing within transgenic plants in the field are capable of gene transfer to other bacteria in the environment. An uncontrolled spread of genes from non-bacterial kingdoms (residing within the T-DNA) to the microflora of natural and agricultural ecosystems is not desired. Thus plants released into the environment should be free from engineered agrobacteria.

It should be noted, however, that in the vast majority of cases no significant impact and thus no risk for the environment would be connected with such a horizontal gene transfer.

Acknowledgements

We would like to thank ROBERT ZWEIGERDT for his help and for some of the ros primers. We also would like to thank UTE KOELLNER for excellent technical assistance.

References

BERNAERTS, M. J. and J. DE LEY, 1963: A biochemical test for crown gall bacteria. Nature **197**, 406–407.

COOLEY, M. B., M. R. D'SOUZA, and C. I. KADO, 1991: The virC and virD operons of the *Agrobacterium* Ti plasmid are regulated by the ros chromosomal gene: Analysis of the cloned ros gene.. J. Bac. **173,** 2608–2616.

DONG, I.-C., C.-W. SUN, K. L. THIES, D. S. LUTHE, and J. C. H. GRAVES, 1992: Use of polymerase chain reaction to detect pathogenic strains of *Agrobacterium*. Phytopathology **82**, 434–439.

DSOUZAAULT, M. R., M. B. COOLEY, and C. I. KADO, 1993: Analysis of the ros repressor of *Agrobacterium* virC and virD operons – molecular intercommunication between plasmid and chromosomal genes. J. Bacteriol. **175**, 3486–3490.

ESCUDERO, J., G. NEUHAUS, and B. HOHN, 1995: Intracellular *Agrobacterium* can transfer DNA to the cell nucleus of the host plant. Proc. Natl. Acad. Sci. USA **92,** 230–234.

GOULD, J. H. and R. H. SMITH,1989: A non-destructive assay for GUS in the media of plant tissue cultures. Plant Mol. Biol. Rep. **7,** 209–216.

GRASER, E., 1994: Eliminierung von Agrobakterien aus gentechnisch veränderten Pflanzen. Diplomarbeit. Universität Göttingen, Germany.

HEDTRICH, C. M., W. FEUCHT, and H. SCHIMMELPFENNIG, 1980: Pathogeneliminierung und Vermehrung von Himbeeren durch Meristemspitzenkulturen. Erwerbsobstbau **22**, 159–160.

HORSCH, R. B., J. E. FRY, N. L. HOFFMANN, D. EICHHOLTZ, S. G. ROGERS, and R. T. FRALEY, 1985: A simple and general method for transferring genes into plants. Science **227**, 1229–1231.

HUTH, W., 1979: Kultur von Himbeerpflanzen aus apikalen Meristemen. Gartenbauwissenschaft **44**, 53–55.

LANDSMANN, J., D. LLEWELLYN, E. S. DENNIS, and W. J. PEACOCK, 1988: Organ regulated expression of the *Parasponia andersonii* haemoglobin gene in transgenic tobacco plants. Mol. Gen. Genet. **214**, 68–73.

LEHOCZKKY, J., 1971: Further evidence concerning the systematic spreading of *Agrobacterium tumefaciens* in the vascular system of the grapevines. Vitis **10**, 215–221.

MOGILNER, N., D. ZUTRA, R. GAFNY, and M. BARJOSEPH, 1993: The persistence of engineered *Agrobacterium tumefaciens* in agroinfected plants. Mol. Plant Microbe. Interaction **6,** 673–675.

MURASHIGE, T. and F. SKOOG, 1962: A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant **15**, 473–497.

PONSONNET, C. and X. NESME, 1994: Identification of Agrobacterium strains by PCR-RFLP analysis of pTi and chromosomal regions. Arch. Microbiol. **161**, 300–309.

REED, B. M., P. M. BUCKLEY, and T. N. DEWILDE, 1995: Detection and eradication of endophytic bacteria from micropropagated mint plants. In Vitro Cell Dev. Biol-Plant. **31**, 53–57.

SAWADA, H., H. LEKI, H. OYAIZU, and S. MATSUMOTO, 1993: Proposal for Rejection of *Agrobacterium-tumefaciens* and Revised Descriptions for the Genus Agrobacterium and for *Agrobacterium-radiobacter* and *Agrobacterium-rhizogenes*. Int. J. Syst. Bact. **43**, 694–702.

SAWADA, H., H. LEKI, and I. MATSUDA, 1995: PCR detection of ti and ri plasmids from phytopathogenic *Agrobacterium* strains. Appl. Environ. Microbiol. **61**, 828–831.

STELLMACH, G., 1990: Experiences with the isolation of latent tumorgenic agrobacteria from grapevines. Nachrichtenbl. Deut. Pflanzenschutzd. **42**, 151–153.

THEILER, R., 1980: Einsatz der Gewebekultur zur Anzucht pathogenfreier Pflanzen; Möglichkeiten und Probleme ihrer Anwendung. Erwerbsobstbau **22**, 226–231.

VAN DER HOEVEN, C., 1992: Untersuchungen zur Variabilität von gentechnisch veränderten Tabakpflanzen mit Resistenz- und Reportergenen. Ph. D. Dissertation. Universität Hannover, Germany.

VAN DER HOEVEN, C., A. DIETZ, J. LANDSMANN, 1991: Agrobacteria shown to