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Eucalyptus occidentalis plantlets are naturally infected by pathogenic *Agrobacterium tumefaciens*

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Abstract

In the Sidi M'djahed nursery (Algeria), over 60,000 eucalyptus (*Eucalyptus occidentalis*) plantlets exhibited tumour-like growths localized at the crown of the plants that resembled crown galls caused by *Agrobacterium tumefaciens*. Bacteria colonizing the galls were isolated and purified. Most (22 out of 24) of the isolates had cultural and biochemical characteristics similar to those of strains of the biovar 1 of *A. tumefaciens*. Twenty out of 22 *Agrobacterium* isolates induced tumour formation on various test plants. In PCR experiments, DNA extracted from these virulent strains yielded an amplification signal corresponding to a 247-bp fragment located within the virulence region of nopaline type Ti plasmid. Consistent with this, the opine nopaline was detected in the tumours induced on test plants – but not on eucalyptus plants. Nopaline was degraded by the 20 pathogenic isolates that were also sensitive to agrocin 84, indicating the presence of a nopaline-type pTi in these strains. The chromosomal region encoding the 16S rRNA was analyzed in a sub-population of the pathogenic agrobacterial isolates. The analyzed strains were found to belong to the ribogroup of the reference strain B6. Interestingly, *Eucalyptus camaldulensis* and *Eucalyptus cladocalyx* grown in the same nursery and in the same soil substrate developed no galls.

Abbreviations: ITS – Intergenic transcribed spacer; MG – Mannitol glutamate; MS – Murashige and Skoog; *tms* – tumour morphology shoot; *vir* – virulence.

Introduction

Agrobacteria are gram-negative soil-borne bacteria that belong to the family *Rhizobiaceae* within the alphaproteobacteria subclass (Woese, 1987). The genus *Agrobacterium* can be divided into three biovars (Kersters and De Ley, 1984). Molecular analyses on genomic DNA and *rrs* (i.e. 16S rRNA) gene support biovar delineation, with several genomic species gathered within biovar 1 (Mougel et al., 2002). The reported study deals with biovar 1 strains belonging to the species *Agrobacterium tumefaciens*.

Agrobacterium tumefaciens is the causative agent of the crown-gall disease that is characterized by the appearance of tumours at the infection site. During the infection process, a piece of the Ti plasmid of *Agrobacterium*, namely the T-DNA, is

transferred from the bacterial genome to the plant nuclear genome where it is integrated and expressed. In the *Agrobacterium* background, the Ti plasmid (pTi) suffices to confer pathogenicity to the host bacterium cell (Hansen and Chilton, 1999; Gelvin, 2000; Zhu et al., 2000; Ziemienowicz, 2001).

The T-DNA contains all the genes transferred to the plant cells. They fall into two categories. The first type of genes, such as *tmr* and *tms*, are responsible for tumour formation, and generally encode enzymes involved in the overproduction of plant growth regulators (e.g. auxin) (Binns and Costantino, 1998). A second group of genes (such as *ocs* or *nos*) encode enzymes responsible for the synthesis of low molecular weight compounds that are characteristic for transformed crown gall cells, the opines (Dessaux et al., 1998). The transfer of the T-DNA to the plant cell is mediated by the products of the non-transferred virulence (*vir*) genes, also located on the Ti plasmid (Hansen and Chilton, 1999; Gelvin, 2000), that encode a translocation apparatus member (Christie, 2004).

As indicated above, the crown gall tumours synthesize opines. As a consequence, the detection of opine compounds in a tumour-like formation indicates *Agrobacterium* infection. Opines in tumours, the nature of which depends upon the Ti plasmids hosted by the infecting agrobacteria, are specifically degraded by the tumour-inducing strain (Dessaux et al., 1992, 1998). Additionally, some opines induce the conjugal transfer of the Ti plasmid (Piper et al., 1999). Opine analogues, such as agrocin 84, naturally occur. Synthesized by 'parasitic' *Agrobacterium* strains (e.g. strain K84), the bacteriocin produced by K84 kills pathogenic agrobacteria degrading the opine that it mimics i.e., agrocinopine (Dessaux et al., 1998; Hayman and Farrand, 1990).

During summer 1999, galled *Eucalyptus occidentalis* seedlings were observed in a forest nursery in western Algeria that also propagated *Eucalyptus camaldulensis* and *Eucalyptus cladocalyx* plantlets. All (100%) the *E. occidentalis* plantlets (i.e. 60,000) were infected irrespective of their location in the nursery, and exhibited galls located at the crown of the plant (Figure 1), reminiscent of *Agrobacterium*-induced galls. Up to date, the host range of biovar 1 agrobacteria includes ornementals (such as rose or chrysanthemum), vegetables (e.g. tomato, *Cucurbita* spp., etc.; De Cleene and De Ley, 1976), fruit trees (e.g. cherry-tree, peach-tree, walnut-tree) and other trees (poplar, weeping fig) (Nesme et al., 1992; Bouzar et al., 1993). Within the *Eucalyptus* genus, only the species *E. camaldulensis* (Ho et al., 1998), *E. globulus* (Azmi et al., 2001), and the hybrid *Eucalyptus grandis* × *E. urophylla* (De Oliveira Machado et al., 1977; Tournier et al., 2003) were sensitive to *Agrobacterium*, albeit under controlled infection conditions in the laboratory. The objectives of this work were therefore to identify and characterize the bacterial strains associated with the galls that appeared on *E. occidentalis* using cultural, biochemical, and molecular approaches.

Figure 1. Galled eucalyptus (*E. occidentalis*) plantlets. In the examined nursery, some 60,000 eucalyptus plantlets harboured overgrowths similar to those shown in this picture. They were located near, or at the crown of the plants. Their size varied from 5 to 25 mm. Six galls were randomly chosen and the bacteria colonizing the overgrowths were isolated on media semi-selective for agrobacteria, as described in materials and methods.

Materials and methods

Collection site and seedling sampling

Galled *E. occidentalis* seedlings (two year-old) were collected during summer 1999 at the Sidi M'djahed forest nursery located near Tlemcen, in Western Algeria. The seedlings were grown in plastic bags containing a mixture of river sand and substrates collected from deep forest horizon, and were irrigated by aspersion. The infected samples were brought to the laboratory and stored in sterile bags at 4 °C until further analysis.

Isolation and cultivation of tumour-colonizing bacteria

Six *E. occidentalis* seedlings, out of 60,000 exhibiting tumour-like formations at the crown, were chosen semi-randomly, by tracing a diagonal line across the *Eucalyptus* propagation zone, and sampling equidistant plantlets that harboured fresh tumours. The selected soil-free tumours were rinsed, cut into pieces and crushed with sterile distilled water to obtain homogeneous suspensions. From this suspension, serial dilutions were plated onto Brisbanne and Kerr's media 1A and 2E (Brisbane and Kerr, 1983), RS (Ophel et al., 1998) and Mannitol Glutamate (MG) agar plates (Keane et al., 1970). These were incubated for 7–10 days at room temperature. After incubation, plates were examined and distinct *Agrobacterium*-like colonies (Moore et al., 1988) were picked off and purified by streaking onto MG agar medium. After purification, ca. four colonies per analyzed galls were retained.

Reference strains and biovar characterization

Agrobacterium strains B6 and C58 (our collection) were used as biovar 1 reference strains. They harbour an octopine- and a nopaline-type Ti plasmid, respectively, and are virulent on a wide range of host plants. The avirulent, Ti plasmid-free derivative C58C1 (our collection) was also used as a negative control in PCR studies. The biovar affiliation of each isolate was determined according to Moore et al. (1988). The tests included oxidation of lactose to 3-ketolactose, tolerance to 2% sodium chloride, growth on tyrosine and ferric ammonium citrate media.

Pathogenicity assays on plant hosts for tumourigenic Agrobacterium

The ability of the isolates to induce tumour formation was determined by inoculating tomato *Lycopersicon esculentum* cv. Marmande seedlings wounded using a scalpel blade at the stem and apex and *Kalanchoe daigremontiana* plant leaves with a loopful of 48 h-old bacterial plate cultures performed on MG agar media (i.e. about 10^8 cells). Following inoculation, plants were checked weekly for tumour formation. Strains which presented no tumourigenic response on these hosts were further assayed on the apex and stem of *Datura stramonium* grown in the greenhouse, and on the hypocotyl axis of *Helianthus annuus* plants grown on MS (Murashigue and Skoog, 1962) medium, containing Cefotaxim (400 mg l^{-1}), as indicated for the tomato and Kalanchoe assays. A control was run which consisted in plants wounded but not inoculated.

Sensitivity to agrocin 84

All isolates were tested for their sensitivity to agrocin 84. The method described by Stonier (1960) and modified by Moore et al. (1988) was used. Strain K84, grown for 24 h on MG broth medium, was spotted onto MG agar plates supplemented with biotin (2 μg ml^{-1}), and incubated at 25 °C for 48 h. Suspensions of the assayed agrobacterial isolates, at ca. 10^8 CFU ml^{-1}, were then sprayed lightly over the medium surface. The plates were re-incubated for 48 h at 25 °C. The diameters of growth inhibition zones visible around the K84 colony were recorded. Bacteria were considered resistant when the diameter of the inhibition zone was smaller than 5 mm.

Opine analysis

The presence of opines in naturally infected *Eucalyptus* seedlings and in induced galls that appeared on test plants was detected by extraction from plant tissues and separation by high voltage paper electrophoresis (HVPE) at pH 1.9, as described by Petit et al. (1983). Opines were revealed using different reagents, exactly as described earlier (Dessaux et al., 1992). Presence of the following opines and opine classes was investigated in the harvested galls: nopaline, octopine, mannityl-opines (mannopine, agropine, mannopinic and agropinic acid), chrysopine-type opines (chrysopine, deoxyfructosyl glutamine, and chrysopinic acid), cucumopine/cucumopine lactam and mikimopine/mikimopine lactam. Opine degradation was investigated by inoculating 200 μl of the strain to assay (at 2×10^8 CFU ml^{-1}) into 800 μl of a degradation mixture that consisted in AT minimal medium (Petit et al., 1978) supplemented with ammonium sulfate (1 g l^{-1}), yeast extract (100 mg l^{-1}) and the appropriate synthetic opines (octopine, nopaline, agropine, mannopine, mannopinic acid, cucumopine and cucumopine

lactame), at 1–5 mM. Assays were incubated at room temperature (ca. 25 °C) for 7 days under shaking. Opine utilization was determined by HVPE as described above (Dessaux et al., 1992).

Molecular analysis of Ti plasmid sequences

Amplifications of Ti plasmid sequences were performed according to Mullis and Falloona (1987) and to Picard et al. (1992) using a genomic DNA extract as a source of template DNA. This DNA was obtained using the DNAeasy kit from Qiagen (Les Ulis, France), according to the manufacturer's instructions. Oligonucleotides used to prime PCR were designed in *vir* and *tms* regions conserved in most pTis, and in the specific *nos* region of nopaline type pTi (Table 1). The PCR reactions were set up in a 25 μl reaction mixture, consisting in 10 mM Tris–HCl, pH 8.3, 50 mM $MgCl_2$ and 0.01% [wt/vol] gelatin. In addition, the medium contained the four dNTPs (20 μM each), 1 μl of DNA prepared in 20% glycerol, the two primers (1 μM of each) and 2 units of Taq DNA polymerase (Gibco BRL, Cergy-Pontoise, France). Cycling parameters were an initial denaturation, 5 min at 95 °C, then 35 cycles of (denaturation, 1 min at 96 °C; annealing for 1 min at 55 °C and extension for 1 min at 72 °C) and final extension phase, 3 min at 72 °C. PCR reactions were performed in a Dry Block Thermal Cycler (Perkin Elmer, Les Ulis, France). Ten μl of each amplification product were loaded on 2% horizontal agarose gels containing 1 μg ml^{-1} ethidium bromide using 1 kb and 123 bp ladders (Gibco/Invitrogen, Cergy-Pontoise, France) as molecular weight standards. Electrophoresis was performed at 6 V/cm for 2 h and resulting electrophoregrams were recorded numerically or on thermal photographic documents. RFLP analysis of PCR amplification products (PCR-RFLP) were performed as described by Ponsonnet and Nesme (1994). Following amplification, ca. 5 μg of the PCR products were digested with 10 units of each restriction enzyme. The DNA of the *tms*(587) region was digested with *Cfo*I and *Dde*II, that of the *vir*(418) region with *Mse*I and *Hpa*II and that of the *vir*(1673) region with *Cfo*I, all according to standard procedures. Restriction fragments obtained after 1 h of digestion were separated by horizontal electrophoresis in TBE buffer on a 2.5% (w/v) Nusieve agarose gel containing 1 μg ml^{-1} ethidium bromide at 2.3 V/cm and then treated as described above for PCR products. Fragments of size lower than 100 bp were not considered for the comparison of restriction patterns.

Table 1. Oligonucleotides used to amplify Ti plasmid (*vir, tms, nos*) or chromosome (*rrs, rrs*+ITS) regions in *Agrobacterium*

Amplified region[a]	Primer pair	Lab code	Primer sequence	Reference[b]
vir(247)	FGP*vir*G15′	F14	GAACGTGTTTCAACGGTTCA	Pic92
	FGP*vir*B_{11} + 21	F44	TGCCGCATGGCGCGTTGTAG	Nes89
vir(1673)	FGP*vir*A2275	F87	TCAAAAGGCAAGCAAGCAGATCTGG	Pon94
	FGP*vir*$B_2$164′	F88	TCAGTGCCACCTGCAGATTG	Pon94
vir(418)	FGP*vir*G15′	F14	GAACGTGTTTCAACGGTTCA	Pic92
	ANTvirB_{11}887	F4626	GGTGAGACAATAGGCGATCT	Pio99
tms(587)	FGP*tms2*-194′	F202	CCTACTCCGGCGTTTCCATG	Nes95
	FGP*tms1*-46′	F203	GTTGGGAGATGATCGCACTG	Nes95
nos(261)	FGPnos975	F89	CATAACGTGCATCATGCATG	Pon94
	FGPnos1236′	F140	CACCATCTCGTCCTTATTGA	Pon94
nos(1234)	FGP*nos*14	F139	GGCAATTACCTTATCCGCAA	Nes95
	FGPnos1236′	F140	CACCATCTCGTCCTTATTGA	Pon94
rrs (1479)	FGPS6	F63	GGAGAGTTAGATCTTGGCTCAG	Nor92
	FGPS1509′	F153	AAGGAGGGGATCCAGCCGCA	Nor92
rrs+ITS (2500–2700)[c]	FGPS6	F63	GGAGAGTTAGATCTTGGCTCAG	Nor92
	FGPL132′	F38	CCGGGTTTCCCCATTCGG	Nor92

[a]The size in bp of the PCR product is given in brackets.
[b]First description, as follows: Nes89, Nesme et al. (1990); Pi92, Picard et al. (1992); Pon94, Ponsonnet and Nesme (1994); Nes95, Nesme et al. (1995); Pio99, Pionnat et al. (1999); Nor92, Normand et al. (1992).
[c]Range of amplified fragment sizes amongst *alpha-proteobacteria.*

Molecular analysis of chromosomal regions

The *rrs* gene determining the 16S rRNA fragment and the vicinal intergenic transcribed spacer (ITS) region of six strains were analyzed by PCR-RFLP as described by Oger et al. (1998). Amplifications were performed using 2 μl of DNA sample extracted using the DNAeasy system from Qiagen (Les Ulis, France), according to the manufacturer's instructions. Primers FGPS6 and FGPS1509′ (Table 1) were used to amplify a 1479 bp-fragment, representing 99.5% of the *rrs* locus. Primers FGPS6 and FGPL132′ were used to obtain the amplification of a larger part of the region determining ribosomal RNA (2500–2700 bp) that included the *rrs* locus and the intergenic spacer (ITS) located in between the *rrs* (16S rRNA) and *rrl* (23S rRNA) loci, plus 132 bp of *rrl* . Preparation of PCR reaction mixtures, conditions for amplifications and PCR-RFLP both for *rrs* and *rrs* +ITS were as described above. Digestion of the DNA encoding *rrs* was performed using the enzymes *Alu*I, *Hae*III and *Hpa*II. The restriction enzymes *Cfo*I (GibcoBRL), *Hae*III and *Mbo*I were used to digest the *rrs* +ITS region, all according to standard procedures.

Results

Isolation and cultivaion of tumour-colonizing bacteria

Six out of the 60,000 contaminated eucalyptus plantlets were chosen as described in materials and methods. The bacteria colonizing the plant overgrowths were isolated with the following pattern: 24 isolates were purified from Brisbane and Kerr media 1A and 2E while none were recovered from the RS medium specific for *Agrobacterium* biovar 3 strains. Out of the 24 isolates, two were fluorescent under UV light and asssumed to be fluorescent pseudomonads. The remaining 22 isolates formed round, smooth, white colonies (2–5 mm diam) on MG medium. These features, combined with biochemical assays (presence of very active urease and betagalactosidase, and ability to hydrolyze esculin) revealed that these strains were likely to be *Agrobacterium* strains (Nesme, unpublished). All isolates produced 3-keto-lactose, and grew in the presence of 2% NaCl on tyrosine and ferric ammonium citrate media, indicating that they belonged to biovar 1 (Moore et al., 1988).

Pathogenicity assays performed on the 22 isolates revealed that 20 isolates induced galls on at least one of the four host plant (*K. daigremontiana*, *D. stramonium*, *L. esculentum, H. annuus*) 3 weeks after inoculation, while the remaining two were not pathogenic at the same time point. To confirm the above results, genetic amplifications were performed by PCR on genomic DNA extracted from the isolates, with a couple of primers targeting sequences of the Ti plasmid located within the *vir* region (*vir*247). DNA extracted from the 20 virulent strains allowed the amplification of a ca. 240 bp fragment (Figure 2), as did DNA from the positive control strain C58, while the two non-pathogenic isolates did not generate any amplification signal (not shown). The 20

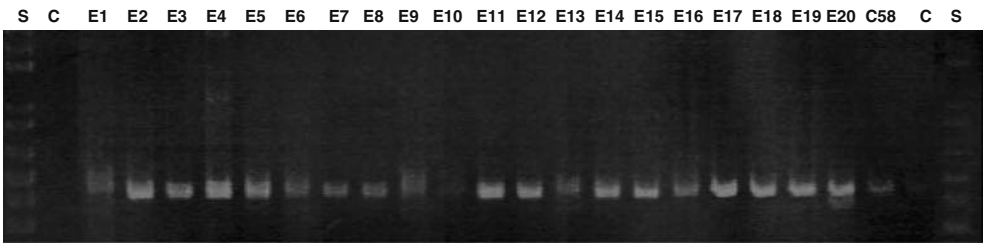

Figure 2. PCR amplification of a 247-bp DNA fragment located within the Ti plasmid *vir* region of pathogenic strains of *Agrobacterium* isolated from eucalyptus galls. PCR amplification was set up using *vir*(247) primers located within the *virB*/*virG* region, and DNA extracted from the 20 pathogenic isolates, numbered E1 to E20. Negative controls (lanes C) are tentative amplification of the DNA extracted from a Ti plasmid-less *Agrobacterium* strain (C58C1). They are presented along with a positive control set up with DNA extracted from the pathogenic reference strains C58. External lanes (S): 123 bp ladder: the three lowest bands have 123, 256 and 369 bp, respectively.

pathogenic isolates were numbered E1 to E20 and retained for further analysis.

Analysis of opine-related functions of Agrobacterium *strains isolated from eucalyptus.*

Presence of the opines and opine classes listed under materials and methods was investigated. None of these opines were detected in the analyzed overgrowths, even using concentrated overgrowth extracts. However, the tumours induced on the test-plants (*K. daigremontiana*, *D. stramonium*, *L. esculentum, H. annuus*) by the 20 pathogenic *Agrobacterium* strains isolated from *Eucalyptus* did produce large amounts of the opine nopaline (Figure 3).

The degradation ability of the 20 isolates was analyzed by monitoring the disappearance of opines from a liquid medium containing these opines as sole carbon sources. The 20 isolates entirely depleted nopaline from the media within 4 days and octopine after 7 days (not shown), but none of the other assayed opines. In addition, these isolates were all sensitive to agrocin 84. All these data are consistent with the presence of a nopaline-type Ti in the assayed strains. In agreement with this, amplification signals were obtained upon PCR involving *nos* primers, *nos*(261) and *nos*(1234), and template DNA from these strains (not shown).

Molecular analysis of both the Ti plasmids and chromosomal backgrounds of the eucalyptus strains

To investigate the diversity of Ti plasmids of the strains and that of their chromosomal backgrounds, defined regions of the Ti plasmid and *Agrobacterium* chromosome were analyzed by PCR-RFLP on a subset of six pathogenic strains obtained from different host plants but randomly chosen (i.e. E1, E9, E11, E15, E17 and E19; Table 2). At the chromosomal level, the examination of the PCR amplification pattern of the *rrs* +ITS region, i.e. the *rrs* gene encoding the 16S rRNA plus the intergenic spacer (*rrs*/*rrl*), revealed that the eucalyptus strains generated a ca. 2700 bp amplification fragment (not shown). The RFLP analysis of the amplified *rrs* locus, performed with three discriminative enzymes, indicated that the isolates yield *rrs* patterns identical to that of B6, the reference strain of the biovar 1 of *Agrobacterium* spp., but differed from C58. In other words, the isolates belong to the same *rrs* cluster as B6. The analysis of the PCR-RFLP of the *rrs* +ITS region revealed that isolates exhibit at least five different backgrounds. In agreement with the above, the background of strains E1 and E19 was not distinguishable from that of strain B6. Others backgrounds such as those of strains E9 or E11 were slightly different (Table 2).

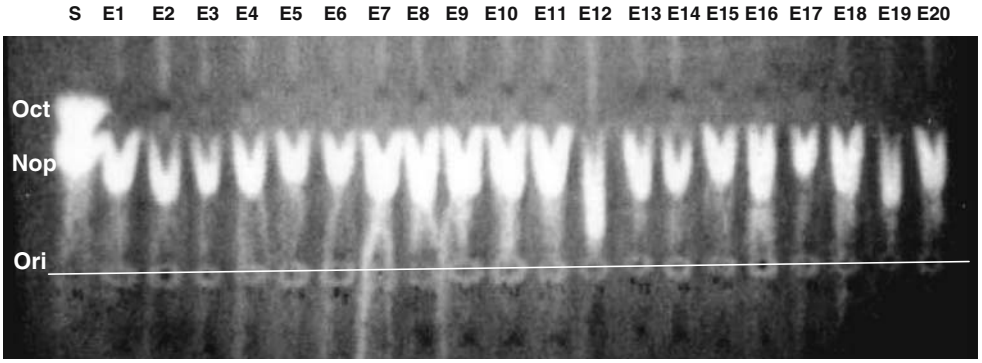

Figure 3. High voltage paper electrophoresis of opines extracted from tumours induced by *Agrobacterium* strains isolated from eucalyptus galls. Tumours were induced by the 20 pathogenic isolates on *Kalanchoe* test plants and opines were extracted 3 weeks post-inoculation. Separation of opines from other plant materials was performed by high voltage paper electrophoresis, at pH 1.9 in acetic/formic acid buffer. The guanidyl-opines octopine and nopaline were revealed using phenanthrene quinone and under UV, all as indicated by Dessaux et al. (1992). Strains that induced the tumours are numbered E1 to E20. Legends are: S, migration standard, that consisted of: Oct, octopine; and Nop, nopaline. Ori, origin of migration.

Table 2. PCR-RFLP analysis of both Ti plasmids and chromosomal backgrounds of selected *Eucalyptus* strains

Strains	Ti plasmid sequences[a]					Chromosomal sequences[a]					
	tms(587)		*vir*(418)		*vir*(1673)	*rrs*			*rrs* + ITS		
	Cfo I	*Dde*I	*Mse*I	*Hpae*II	*Cfo*I	*Alu* I	*Hpa*II	*Hae*III	*Cfo*I	*Mbo*I	*Hae*III
E1	none[b]	none	m1[c]	he1	c1	a1	h1	H1	c1	m1	H1
E9	c1	d1	m1	he1	c1	a1	h1	H1	c1	m3	H1
E11	c2	d1	m1	he1	c1	a1	h1	H1	c1	m2	H1
E15	c2	d2	m1	he1	c1	a1	h1	H1	c1	m2	H2
E17	c1	d3	m1	he1	c1	a1	h1	H1	c1	m4	H3
E19	c1	d1	m1	he1	c1	a1	h1	H1	c1	m1	H1
C58[d]	c3	d4	m2	he1	c2	a2	h2	H1	c1	m2	H4
B6[d]	c1[e]	d4[e]	m3	he2	n.d.[f]	a1	h1	H1	c1	m1	H1

[a]Targeted Ti plasmid regions were the *tms* locus of the T-DNA, and the virulence (*vir*) region. Targeted chromosome regions were the *rrs* locus, and the *rrs* locus plus the intergenic spacer (ITS) located in between the *rrs* and the *rrl* loci.
[b]None, no amplification signal was obtained.
[c]Pattern codes consist of the first letter(s) of the restriction enzyme plus a number characteristic for the pattern. Numbers were given as patterns were identified, hence they are not related one to another, e.g. pattern h4 is not closer to h3 than it is to h1.
[d]C58 and B6 were not isolated from eucalyptus overgrowths but only used as reference strains.
[e]From data obtained by Raio et al. (2004).
[f]n.d., not determined.

Analysis of the Ti plasmid structures showed that the *tms*(587) region varied among the six strains analyzed since four different patterns were observed (Table 2). Interestingly, one strain, E1, did not yield any amplification signal with the *tms* primers. The other amplified regions of the Ti plasmids, i.e. *vir*(418) and *vir*(1673), did not appear to differ from one isolate to the other. Overall, five molecular prints characterized the analyzed Ti plasmids. The most frequently detected Ti plasmid was found twice, associated each time with a different chromosomal background (E9 and E19). Conversely, bacteria with identical chromosomal backgrounds (E1 and E19) harboured different Ti plasmid structures (Table 2).

Discussion

The sub-group of 20 strains obtained from the eucalyptus tumours was originally isolated on the *Agrobacterium* semi-selective media of Brisbane and Kerr (1983). Physiological, biochemical, and genetic data (e.g. a functional Ti plasmids, see below) confirmed that the isolates obtained from the overgrowths were *Agrobacterium* strains, a feature later confirmed by the PCR-RFLP analysis of the *rrs* gene found to be identical or closely related to that of B6, the reference strain of the genus *Agrobacterium*.

The six isolates further analyzed all belonged to the biovar 1 of *Agrobacterium* as judged from both biochemical traits (Moore et al., 1988) and molecular analysis of the chromosomal *rrs* sequence. At a lower phylogenetic level, all six fell within the *rrs* cluster of the reference strain B6 and therefore most likely into one of the genomic species described by Popoff et al. (1984) within biovar 1 *Agrobacterium* spp. However, several biovar 1 genomic species have identical or near identical *rrs* and the information given by the *rrs* gene is not sufficient to identify unambiguously a member of the genus *Agrobacterium* at the species level (Mougel et al., 2002). The more resolute analysis of the *rrs*+ITS locus described by Oger et al. (1998) revealed that isolates are identical or very closely related to B6, and thus are likely to belong to the same genomic species G4. Since B6 is the type strain of *A. tumefaciens* G4, the *bona fide A. tumefaciens* species, agrobacteria isolated from eucalyptus in Algeria are *bona fide A. tumefaciens*. As for other agrobacterial populations isolated from other host plants (Nesme et al., 1987; Burr and Otten, 1999; Pionnat et al., 1999; Raio et al., 2004), agrobacteria isolated from eucalyptus appeared as a population consisting of several strains belonging to the same genomic species. This homogeneity is possibly related to their common nursery origin, or host of isolation, as seen for *A. larrymoorei* isolates obtained from

Ficus benjamina (Bouzar et al., 1995) of for biovar 3 strains (now designed as *A. vitis*) isolated from *Vitis vinifera* (Burr and Otten, 1999).

On the basis of the pathogenicity assay, the sensitivity to agrocin 84 and opine metabolism, the *Agrobacterium* isolates obtained from the eucalyptus tumours carried a nopaline-type Ti plasmid. This conclusion was definitely verified by genetic amplification of Ti plasmid sequences with *nos* primers characteristic for the nopaline synthase gene. Interestingly, this was also verified by the amplification of *vir*(247) since the FGP*vir*$B_{11}+21$ primer is specific for nopaline type pTi (Nesme et al., 1990). Together with the most conserved FGP*vir*G15′ primer, they give rise to amplicons with template DNA from nopaline type pTi (such as pTiC58) but not with DNA template from octopine or agropine-mannopine type Ti plasmids, such as those from B6 or pTiBo542 (Nesme et al., 1990, 1995). The Ti plasmids of the 6 strains were carefully examined using PCR-RFLP analysis of defined pTi regions *tms*(587), *vir*(418) and *vir*(1673). This analysis revealed that the nopaline-type Ti plasmids were closely related and fell into five molecular groups. A possible preferential association of pTi and chromosomal background was investigated, since such an association had previously been found in *Agrobacterium* (Bouzar et al., 1993). In this study, no evidence for a preferential Ti plasmid chromosome background was obtained, as also observed for agrobacterial isolates obtained from *Ficus benjamina* tumours (Raio et al., 2004).

The overgrowths collected from eucalyptus did not appear to contain any opine at the time of the analysis, including nopaline, while this molecule was detected in tumours induced by the pathogenic strains upon inoculation of the test plants. Two explanations may account for this observation. First, the concentration of opines decreases in ageing tumours due to the presence of numerous non-agrobacterial isolates that scavenge the opine(s) (Moore et al., 1997; Nautiyal et al., 1999; Petit and Dessaux, unpublished). Second, opine detection may have been impaired by the presence of large amounts of tanins, phenolics and polyphenolics that the eucalyptus samples contained (Hou et al., 2000). Whether the analyzed overgrowths are crown gall tumours may therefore be debated. The key undisputable outcome of this work, however, is the occurrence of pathogenic agrobacteria on the root system of *E. occidentalis* plants. This result must be examined with the recurring question of reservoirs of pathogenic *Agrobacterium* in mind. The data presented above clearly demonstrate that *E. occidentalis* may host pathogenic agrobacteria, in spite of its high concentrations of natural antiseptic compounds (Harkenthal et al., 1999), and marked resistance to several plant pathogens (Rai et al., 1999). This feature should be taken into account in terms of field management in, or production of soil substrate for tree-nurseries.

Interestingly, in the investigated nursery, the only infected plants belonged to the species *E. occidentalis*. Plants from the two other species cultivated in this nursery (*E. camaldulensis* and *E. cladocalyx*), grown under identical conditions, remained uninfected. *Eucalyptus camaldulensis* plantlets (as well as plantlets from the hybrid *Eucalyptus grandis* × *Eucalyptus urophylla*) are, however, potentially sensitive to agrobacteria, at least under laboratory conditions (Ho et al., 1998; Tournier et al., 2003). These findings therefore invite further stimulating investigation to evaluate whether genetic resistance to pathogenic agrobacteria might exist amongst *Eucalyptus* species in nature.

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