

Persistence of *Agrobacterium tumefaciens* in transformed conifers

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Previous studies have shown that the widely used plant transformation vector *Agrobacterium tumefaciens* can persist in genetically engineered plants *in vitro* and in transgenic greenhouse-grown plants, despite the use of counter-selective antibiotics. However, little is known regarding *Agrobacterium* persistence in tree species. To understand the kinetics of *A. tumefaciens* decline and persistence in transformation experiments, we assayed for the presence of *A. tumefaciens* in spruce and pine embryogenic tissue for up to 10 weeks post-transformation. The *A. tumefaciens* populations declined rapidly in the first five days post-cocultivation but generally declined more slowly in pine, relative to spruce. No bacteria were detected in spruce embryogenic tissue beyond four weeks after cocultivation, however in pine there were ~100 colony forming units per g tissue at 10 weeks post-cocultivation. We present evidence that the detection limit for PCR using *virD2* primers to detect *A. tumefaciens* in a background of pine needle DNA was approximately 10^9 – 10^{10} *A. tumefaciens* cells per g of tissue. We also assayed for *A. tumefaciens* in transgenic pine and spruce embryogenic tissue and from needles, branches, stems and roots of transformed plants, up to four years post-inoculation. Occasionally *A. tumefaciens* was detected in embryogenic tissue up to 12 months post-inoculation. *A. tumefaciens* was never detected in cultured embryogenic tissue more than twelve months after inoculation, nor in developing somatic embryos or germinating plantlets, nor any of the parts of greenhouse-grown plants. From these data we conclude that if *A. tumefaciens* persists in transgenic conifers, it does so beneath our ability to detect it.

Key words: risk analysis / genetic engineering / conifers / transgenics

Abbreviations: *uidA/GUS*: β -glucuronidase gene or protein, respectively; **EDM**: embryo development medium; **mLV**: modified Litvay's medium; **GMO**: genetically modified organism; **CFU**: colony forming unit(s).

INTRODUCTION

In nature, *Agrobacterium* species stably transfer discrete fragments of plasmid-borne DNA (known as T-DNA) into a wide variety of plants (DeCleene and DeLey, 1976). Genes on the T-DNA are integrated into the host plant genome, are transcribed and translated, and these T-DNA-derived proteins cause crown gall disease (reviewed in Nester et al., 1984). This natural ability to transform plants has been exploited in the laboratory to create transgenic plants with specific genes of interest (for recent reviews see Gelvin, 2003; Tzfira and Citovsky, 2003).

In current plant transformation applications, antibiotics are used to counter-select *A. tumefaciens* after trans-

formation of the plant material. However, *A. tumefaciens* is known to persist *in planta* and can be difficult to eliminate using antibiotics (Hammerschlag et al., 1997; Leifert and Cassels, 2001). *Agrobacterium* species are also known for their ability to survive in a number of diverse environments such as water, the rhizosphere, and even in humans (Lehoczky, 1968; Leifert and Cassels, 2001; Marti et al., 1999; Southern, 1996). Thus, if the genetically modified *Agrobacterium* survived antibiotic counter-selection and continued to persist in field-grown transgenic plants, a number of issues of environmental concern could result (Stewart et al., 2000). For example, in theory

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the laboratory strain could be released into the soil *via* the roots where it could subsequently infect other plants or transfer the transgene(s) *via* conjugation to wild type *A. tumefaciens* strains or *via* horizontal gene transfer to other (sometimes distantly-related) microorganisms (Droege et al., 1999; Lilley et al., 1994; Stewart et al., 2000).

Such theoretical risks need to be assessed by the probability of their occurrence and verified by experimental data. Unfortunately, there are disproportionately few publications investigating the survival of residual *A. tumefaciens* in plant tissues after transformation, relative to those on *A. tumefaciens*-mediated transformation *per se*. Of these few studies, Matzk et al. (1996) showed that *A. tumefaciens* was detectable in transgenic tobacco grown *in vitro* for as long as one year after transformation. They also found that *A. tumefaciens* persisted in transgenic tobacco plants that had been in soil for 3–6 months, and this appears to be the only study of *A. tumefaciens* persistence in transgenic plants grown *ex vitro*. Barrett et al. (1997) also found evidence for *A. tumefaciens* persistence *in vitro* in transgenic *Brassica*, *Solanum* and *Rubus* species, although tissue was only examined up to six months post-cocultivation. *A. tumefaciens* was shown to persist in transformed apple cultures, and could only be reduced by infiltrating tissues for one hour with acidified medium followed by an 18 h vacuum infiltration with cefotaxime (5 mg.ml⁻¹) and subsequent incubation on medium containing antibiotics (Hammerschlag et al., 1997). *A. tumefaciens* was also detected in 45–65% of transgenic citrus explants (Cubero and López, 2005) although it was not clear what tissues were examined and at what time post-inoculation they were sampled. In one other related study of non-transgenic plants, engineered *A. tumefaciens* was recovered 1–3 months after agro-inoculation of tomato and avocado plants as well as grapefruit that had been grafted on to Troyer citrange (Mogilner et al., 1993). These experiments indicated that engineered *A. tumefaciens* remained viable in plants for up to three months, which was the longest time period evaluated.

The persistence of *A. tumefaciens* in genetically engineered trees is of significant interest, since they are likely to be in the environment for up to 50 years in plantation forests. While *A. tumefaciens*-mediated transformation methods have been developed for a number of important forest species (e.g. Charity et al., 2005; Klimaszewska et al., 2001; Le et al., 2001; Levée et al., 1999; Trontin et al., 2002), there are currently no published data on the persistence of *A. tumefaciens* in transgenic trees or any data evaluating the persistence of *A. tumefaciens* in any soil-grown plants for longer than six months. However, this sort of information is an important pre-requisite for

field release of transgenic plants from a regulatory standpoint. For example, in New Zealand, the Environmental Risk Management Authority denied planting genetically engineered trees produced *via* *A. tumefaciens*-mediated transformation in the ongoing field trial at Forest Research (now Scion). This was due to the uncertainty of the risk of engineered *A. tumefaciens* strains moving from transgenic trees to the soil surrounding the trees (C. Walter and D. Hannah; personal communication).

To begin to address the issue of *Agrobacterium* persistence in transformed conifers, we examined the effectiveness of counter-selective antibiotics over a period of 10 weeks by determining the rate at which *A. tumefaciens* populations declined in pine and spruce embryogenic tissue immediately after cocultivation. We also used PCR analysis to determine the sensitivity of *virD2* primers to detect known quantities of *A. tumefaciens*, in a background of DNA extracted from pine needles. Finally we evaluated the persistence of *A. tumefaciens* in transgenic pine and spruce embryogenic cultures *in vitro*, in somatic embryos, young plantlets and soil-grown plants from the greenhouse using both an enrichment culture and PCR.

RESULTS AND DISCUSSION

Quantification of *A. tumefaciens* in embryogenic tissue after cocultivation

Quantification 0–7 days post-inoculation

The *A. tumefaciens* population was static or increased slightly during the two-day cocultivation period (Fig. 1A). However, washing the tissue with liquid medium and subsequent culture on a medium with antibiotics drastically reduced the bacterial populations in the remaining five days of culture (Fig. 1A). The *A. tumefaciens* populations declined at different rates for the different species. Of note was that the decrease in numbers of *A. tumefaciens* one day after washing (day 3) appeared to be more dramatic for radiata pine, than for the other species (Fig. 1A). One explanation is that radiata pine is washed through a Buchner funnel with liquid medium (Charity et al., 2005) whereas for the other species, the tissue is resuspended and collected on a Buchner funnel (Klimaszewska et al., 2001). Seven days post-inoculation, all tissue from all species appeared to be bacteria-free by visual inspection but culturing macerated tissue followed by serial dilution showed that *A. tumefaciens* populations were ~10⁴ colony forming units CFU.g⁻¹ tissue for both spruce species, ~10⁵ CFU.g⁻¹ tissue for white and radiata pine and 10⁶ CFU.g⁻¹ tissue for maritime pine (Fig. 1A). Interestingly, all of the pine species were slower to recover

Persistence of *Agrobacterium* in conifers

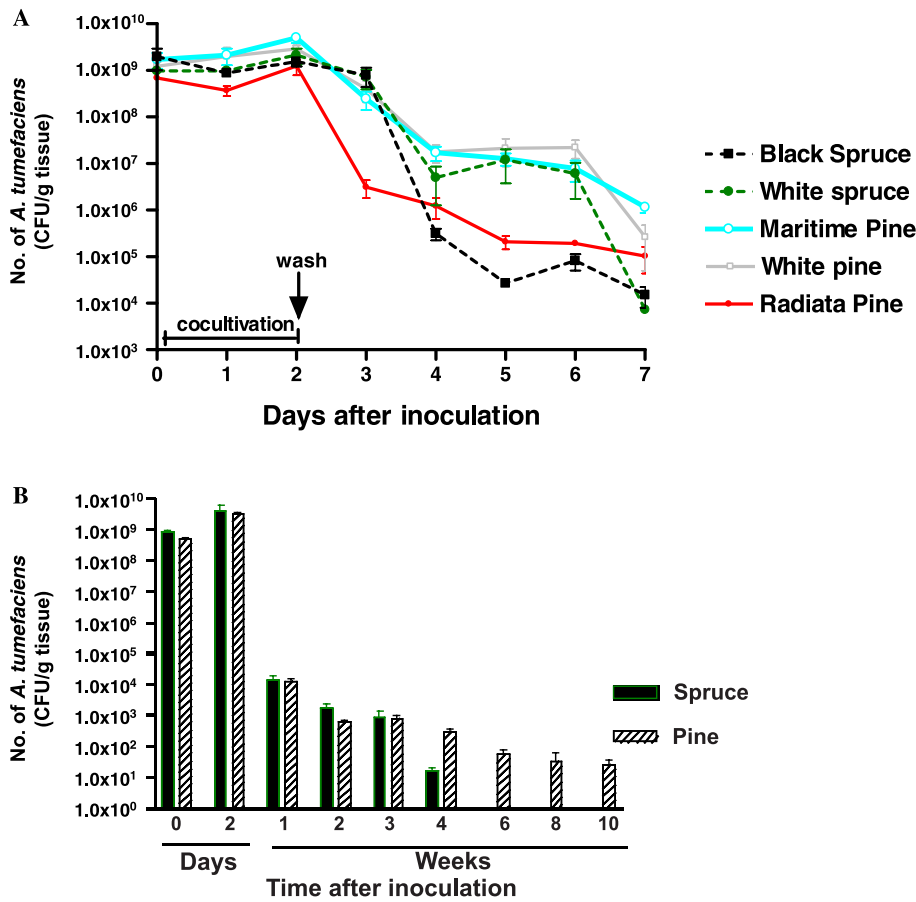


Figure 1. Quantification of *A. tumefaciens* in embryogenic tissue after inoculation. (A) The number of *A. tumefaciens* (CFU.g⁻¹ tissue) isolated from two spruce and three pine species, each day for seven days after inoculation. (B) The number of *A. tumefaciens* (CFU.g⁻¹ tissue) isolated from black spruce and radiata pine up to 10 weeks after inoculation. The data presented in both graphs was the average of two experiments and although there was some variation, the trend for all species and all experiments was similar. Vertical bars represent the standard error.

compared to spruce or control tissue, and the higher residual *A. tumefaciens* may have contributed to the poor vigor of these cultures.

Quantification in embryogenic tissue 1–10 weeks post-inoculation

As observed in the seven-day experiment (Fig. 1A), *A. tumefaciens* increased slightly during the two-day cocultivation and then declined rapidly after washing and transfer to media containing counter-selective antibiotics (Fig. 1B). In these experiments, the concentration of *A. tumefaciens* in black spruce at day 7 was similar to that observed previously in the seven-day experiment (~10⁴ CFU.g⁻¹ tissue). However, the level in radiata pine was lower than in the seven day time-course experiment (~10⁴ CFU.g⁻¹ tissue; compare Fig. 1B to 1A). At 10 weeks post-inoculation approximately 100 CFU.g⁻¹ tissue still persisted in radiata pine but residual *A. tumefaciens* was not detected in black spruce beyond four weeks (Fig. 1B). No *A. tumefaciens* was isolated from any

of the un-inoculated controls in this or the previous experiment (data not shown). *A. tumefaciens* levels decreased more slowly in radiata pine than in black spruce, probably because the black spruce culture medium contained both cefotaxime and Timentin (Klimaszewska et al., 2001), whereas radiata pine was maintained only on Timentin (Charity et al., 2005). Radiata pine could not be transferred to a medium containing cefotaxime because this antibiotic is known to be detrimental to the health of other *P. radiata* explants (such as cotyledons) (Holland et al., 1997). It may be possible to use vancomycin in place of cefotaxime to prevent *A. tumefaciens* growth, since it was not detrimental to radiata pine embryogenic tissue or subsequent somatic embryo development (Holland et al., 2002).

Since pRGR1 does not contain an intron, expression of the *uidA* gene in *A. tumefaciens* was visible as diffuse light blue staining on the tissue and filter paper at day two post-inoculation and also at one week post-inoculation (Fig. 2). This was different from stable expression of the *uidA* gene in embryogenic tissue, distinguished as intense

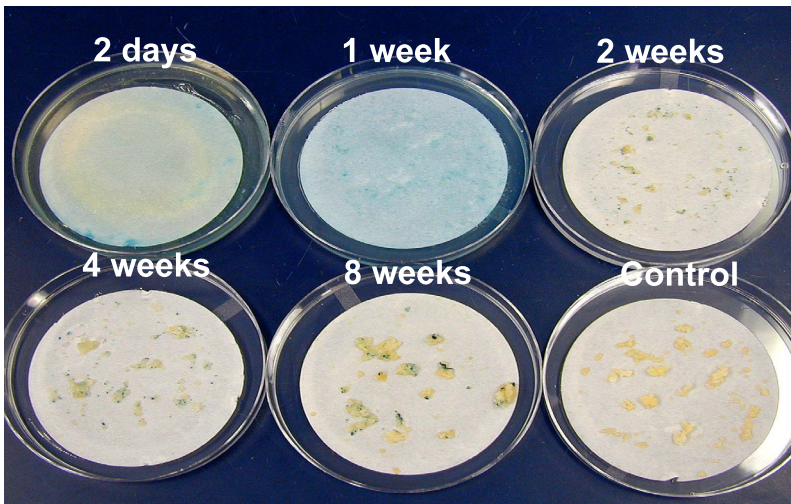


Figure 2. Histochemical staining of embryogenic tissue from 2 days–8 weeks. The photograph shows black spruce embryogenic tissue stained with 5-bromo-4-chloro-3-indolyl β -D-glucuronide to show GUS staining of *A. tumefaciens* itself (diffuse light-blue staining of tissue and filter paper) compared to staining of GUS representing stable transformation of embryogenic tissue (dark blue, discrete areas of staining).

dark blue staining in discrete cells observed as early as two weeks post-inoculation and through to eight weeks (Fig. 2) and beyond (data not shown). Although after one week there appeared to be no *A. tumefaciens* by visual inspection, both the microbiological assay (Fig. 1) and the β -glucuronidase assay (Fig. 2) detected *A. tumefaciens* persistence. Our results are in agreement with the finding of Barrett et al. (1997) who reported that *Brassica* sp. shoots did not appear to be contaminated by visual observation, yet 24% contained *A. tumefaciens* at a range between $\sim 6 \times 10^1$ and $\sim 9 \times 10^8$ CFU.g⁻¹ tissue.

Therefore, at least for conifer embryogenic tissue, the effectiveness of various antibiotics on reducing or eliminating *A. tumefaciens* cannot be solely determined by visual observation, despite the fact that this method had previously been used in the literature (Holland et al., 2002; Hu and Phillips, 2001; Humara et al., 1999; Tang et al., 2000).

Detection of *A. tumefaciens* in transgenic plant material

Determination of the sensitivity of PCR analysis to detect *A. tumefaciens* in a background of pine needle DNA

PCR analysis using primers specific for the *virD2* region of *A. tumefaciens* was used to determine the sensitivity of the technique for detecting known amounts of *A. tumefaciens*. A PCR fragment the same size as observed in the AGL1 positive control was observed in pine needle DNA containing *A. tumefaciens* spiked at $\sim 10^9$ /g and $\sim 10^{10}$ /g tissue (Lanes 9 and 10; Fig. 3A), but not for lesser concentrations.

There were also two bands (~ 1.5 and ~ 1.8 kB, respectively) that amplified in all samples containing pine needle genomic DNA, but these were likely to be nonspecific since they were also amplified in the non-transformed control (Lane 5; Fig. 3A). As expected, the three transgenic lines tested contained only the ~ 1.5 and ~ 1.8 kb non-specific bands and not any fragments amplified by the *virD2* primers (lane 11–13; Fig. 3A). This does not necessarily mean they do not contain residual *A. tumefaciens*, but if it is present, it is below the detection limit of this PCR.

The results are surprising, because at least in our hands, the sensitivity of the PCR reaction could only detect very high numbers of *A. tumefaciens* cells ($\sim 10^9$ – 10^{10} /g). The experiment was repeated with an alternative enzyme (Expand High Fidelity; Roche Diagnostics) but was no more sensitive (data not shown). One explanation is that the method for plant genomic DNA extraction was not equally as efficient for extraction of bacterial DNA. However spiking *Agrobacterium* into needle tissue prior to maceration and DNA extraction was thought to more accurately indicate the true sensitivity of the technique, than adding the *Agrobacterium* DNA afterwards. As a consequence of these results, in our laboratory we no longer use PCR analysis with primers specific for *vir* genes as the only technique to determine if *A. tumefaciens* persists in transgenic plants. Cubero and López (2005) also concluded that enrichment techniques followed by PCR reactions should be used for determining the presence of *A. tumefaciens* that may be present, but not actively growing in transformed tissues.

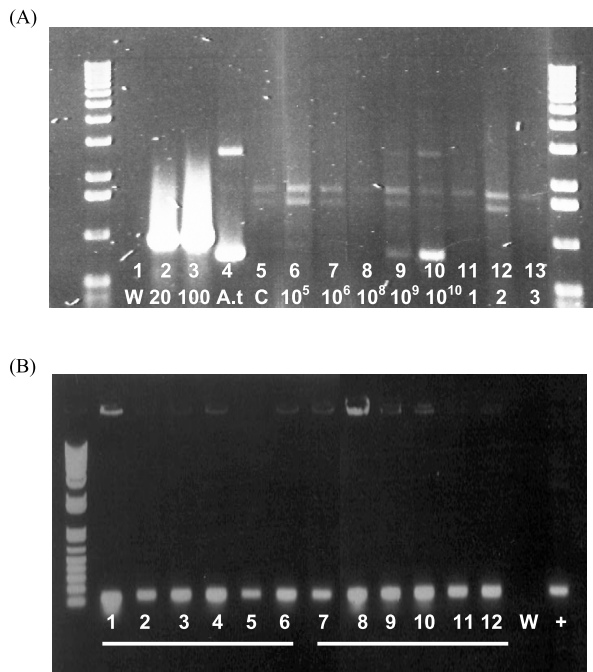


Figure 3. PCR analysis. (A) Determination of the sensitivity of PCR analysis to detect *virD₂* in known quantities of *A. tumefaciens*, spiked into *P. radiata* needles. Lane 1: water control; Lane 2 and 3: 20 pg or 100 pg of *virD₂* plasmid DNA, respectively; Lane 4: *A. tumefaciens* DNA; Lane 5: non-transformed control; Lane 6–10: DNA extracted from *P. radiata* needles spiked with known amounts (per g) of *A. tumefaciens* – 10^5 , 10^6 , 10^8 , 10^9 , 10^{10} , respectively; Lane 11–13: DNA extracted from transgenic *P. radiata* plants. (B) PCR analysis of *A. tumefaciens* DNA isolated from embryogenic tissue using primers specific for the 35S terminator region of pRGR1 or pSAP2C3. Lanes 1–6: *A. tumefaciens* DNA isolated from three black spruce embryogenic tissue, evaluated 3 months after transformation (in duplicate). Lanes 7–12: *A. tumefaciens* DNA isolated from three (out of four) white spruce embryogenic tissue, evaluated 12 months after transformation (in duplicate). W: water control; +: positive plasmid DNA control.

Embryogenic tissue cultured for three months to two years after transformation, developing somatic embryos and in vitro plantlets

The presence of *A. tumefaciens* and other microorganisms was assessed in embryogenic tissue at 3, 12, 16 and 24 months after transformation, in a total of 63 independent transgenic spruce lines (Tab. 1). Although there appeared to be no *A. tumefaciens* growth by visual observation in any of the tissue, using enrichment culturing it was detected in three transgenic lines, three months after inoculation and four transgenic lines, 12 months after

inoculation (Tab. 1). There did not appear to be any other microorganisms in any of the *in vitro* cultures, since aside from the seven colonies described above, there was no other growth on YEP medium without antibiotics. The seven colonies of isolated bacteria were subsequently streaked to single colonies using selective antibiotics. PCR reactions on duplicate samples, using primers specific for the 35S terminator, confirmed that the bacteria harbored the binary vectors used in the original transformation (Fig. 3B and data not shown).

A. tumefaciens was not detected after enrichment culture in any of the 16 or 24-month-old embryogenic tissue or in developing somatic embryos or plantlets (Tab. 1). This is in contrast to the findings of Matzk et al. (1996) and Barrett et al. (1997) who isolated *A. tumefaciens* from 12-month-old transgenic tobacco plants and 24-week-old *Brassica* shoots, respectively. Possible explanations for this difference are that the *Agrobacterium* strains, the concentration of the inoculum and the washing method after co-cultivation for elimination were not the same for any of the species examined. Also, in our work the transgenic plants were regenerated *via* somatic embryogenesis that might have excluded the incorporation of *A. tumefaciens* in the vascular tissue as opposed to organogenesis, used in the latter study. It is also important to note that there are limitations of this experiment, since in most cases, the sample size was small (one replicate) and tissue from only one species was evaluated. Although the sampling method selected 1 g of material representing most of the tissue growing on a single plate, if *A. tumefaciens* was concentrated in a certain area, it may be difficult to detect it using this method.

Transgenic trees grown in the GMO greenhouse for two to four years

Enrichment cultures for *A. tumefaciens* established from needles or branches from transgenic or non-transgenic glasshouse-grown trees frequently contained a variety of fungal, bacterial or yeast-like microorganisms that were able to grow on a selection medium containing kanamycin, gentamycin and carbenicillin (Fig. 4A). Since enrichment cultures could not be assumed to be sensitive enough to detect a single *A. tumefaciens* in transgenic greenhouse-grown trees amongst the background of microorganisms that may have colonized the material, and because microorganism(s) with morphological similarities to *A. tumefaciens* were frequently observed, control experiments with equivalent of 0, 1, 10 or 100 colonies were spiked into non-transgenic plant material. In enrichment culturing experiments with both needle and branch

Table 1. Persistence of *A. tumefaciens* in *in vitro*-grown transgenic plant material.

Species	Construct	Tissue type	Age (months)	No lines tested (REPS ^a)	Positive Result
Black Spruce	pRGR1	Embryogenic	3	4 (2)	3/8 ^b
White Spruce	pSAP2C3	Embryogenic	12	20 (1)	4/20 ^b
White Spruce	pSAP2C3	Embryogenic	16	16 (1)	0/16
Black Spruce	pBIV10	Embryogenic	24	12 (1)	0/12
White Spruce	pBIV10	Embryogenic	24	11 (1)	0/11
Black Spruce	pRGR1	Somatic embryos	3	4 (1)	0/4
White Spruce	pRGR2 pSAP2C3	Somatic embryos	3	2 (1)	0/2
White Spruce	pRGR2 pSAP2C3	Plantlets	4.5	6 (1)	0/6

^a REPS = replicates; ^b positive samples were further analysed by PCR (results for 6/7 of the samples are shown in Fig. 3B).

enrichment cultures, it was possible to detect a single *A. tumefaciens* cell in a background of other microorganisms (Fig. 4A).

The β -3-ketolactose assay using Benedict's reagent was then used to distinguish *A. tumefaciens* from other microorganisms. Negative results were observed for microorganisms from the following categories (a) fungal with obvious hyphae; (b) microorganisms with yellow colonies; (c) microorganisms with orange colonies; (d) microorganisms with pink colonies (data not shown) and (e) microorganisms with creamy-white morphology that were too numerous to form single colonies (Fig. 4A). A positive result confirmed the presence of *A. tumefaciens* in the spiked samples (Fig. 4C) and was used to differentiate the creamy white, microorganism that occurred in high numbers that was frequently isolated from enrichment cultures (Fig. 4C).

The presence of *A. tumefaciens* was assayed in whole plants from the GMO greenhouse. For a total of 226 samples from two conifer species, *A. tumefaciens* was not detected in enrichment cultures from needles, branches, stems or roots of transgenic black spruce or radiata pine over the three-year test period (Tab. 2).

Our results differ from a study of transgenic soil-grown tobacco in which *A. tumefaciens* was found predominantly in the stems and roots after 3–6 months (Matzk et al., 1996). These findings may be a predictor for the location of *A. tumefaciens* in other transgenic plants and are in agreement with evidence for *Agrobacterium* species, which exist as non-pathogenic endophytes, being localized to the roots or root cortex (Hallman et al., 1997; Yang et al., 1999). It is possible that *A. tumefaciens* may

have been present in stems and roots of our transgenic conifers, but subsequently died in the four years after the initial inoculation. Since it was impractical to assay the whole tree, 2–4 replicates of each tissue type were sampled from various parts of the tree, but this may not be sufficient for evidence of lack of contamination, due to the small sampling size. Therefore we can not rule out the possibility that *A. tumefaciens* was present in the needles, stems or roots that we did not sample. Alternatively, since internal migration of *Agrobacterium in planta* has been reported (Cubero and López, 2005; Lehoszky et al., 1968), some bacteria may have already migrated through the stem and passed through into the soil beneath the trees.

Although we did not examine it in this study, if the engineered *A. tumefaciens* did move from transgenic plants through the roots into the environment, the probability of a bacterium establishing itself and maintaining its binary vector in the environment, in the absence of any selective pressure, would be quite low. Furthermore, to be an environmental risk, the engineered *A. tumefaciens* would need to re-infect a suitable host plant, which at worst would result in non-inherited chimeric or transient expression.

Another possible environmental risk is that engineered *A. tumefaciens* strains originating from transgenic plants could conjugate with other bacteria or transfer genetic information *via* non-sexual means to another (possibly distantly-related) organism *via* horizontal gene transfer (HGT). However, since all of the genes in this study were originally derived from microorganisms, it is unlikely that HGT would result in any additional environmental risk

Persistence of *Agrobacterium* in conifers

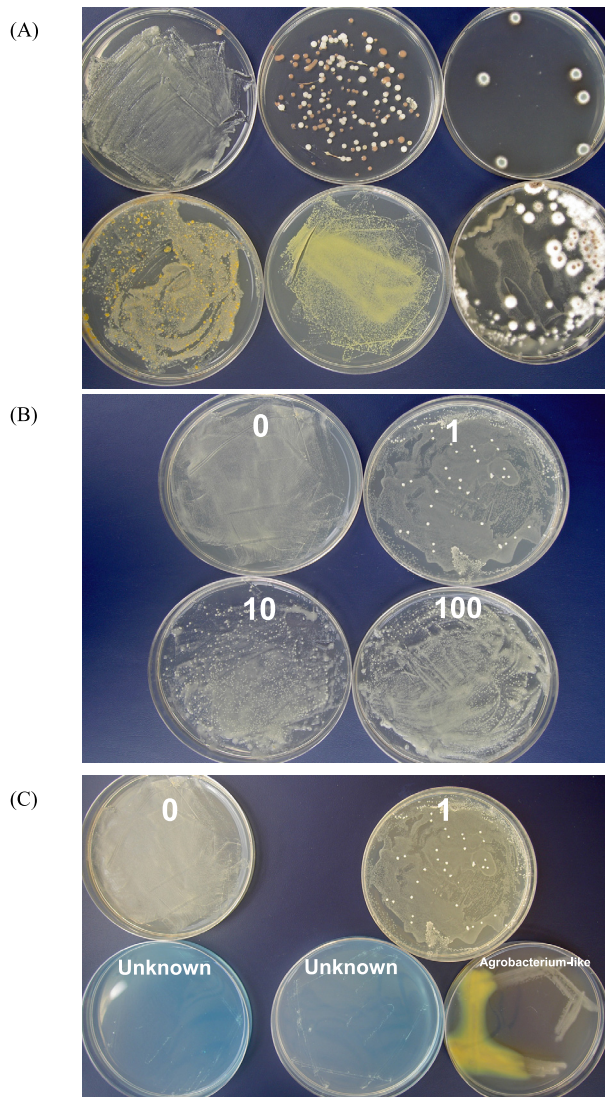


Figure 4. Morphological characterisation of microorganisms and effectiveness of β -3-ketolactose assay. (A) Enrichment cultures derived from non-transgenic and transgenic greenhouse-grown conifers to show the variety of fungi, bacteria and other microorganisms. Microorganisms were categorized on the basis of morphology (as described in the methods) and most are represented in the photograph. (B) The equivalent of 0, 1, 10 or 100 colonies (as indicated) of *A. tumefaciens* were spiked into macerated non-transgenic needles and cultured in liquid bacterial medium to determine the sensitivity of detection amongst the background of other microorganisms. (C) Plating of individual colonies of representative “unknown” or “*Agrobacterium*-like” microorganisms from the control (0) or 1-colony spiked (1) on to lactose containing media to determine if any were positive in the β -keto test. The photograph shows results of staining with Benedict’s reagent to show blue coloration (negative) for “unknown” microorganisms or yellow coloration (positive) for a single *A. tumefaciens*, derived from the “1-colony-spiked” plate.

Table 2. Persistence of *A. tumefaciens* in greenhouse-grown transgenic plants.

Species	Construct	Tissue type	Age (years)	No. ^a of lines tested (REPS ^b)	Positive Result
Spruce	pBIV10	Needles	2	3 (2)	0/6
Radiata pine	pGUL pKEA	Needles	2	10 (4)	0/40
			3	10 (3)	0/30
			4	10 (3)	0/30
Radiata pine	pGUL pKEA	Branches	3	10 (3)	0/30
			4	10 (3)	0/30
Radiata pine	pGUL pKEA	Stems	4	10 (3)	0/30
Radiata pine	pGUL pKEA	Roots	4	10 (3)	0/30

^a No. = number; ^b REPS = replicates.

compared to the presence of those genes already prevalent in nature. In other cases where the genes used were not originally derived from microorganisms, any risk associated with those genes would need to be evaluated on a case-by-case basis. Alternatively, it is possible that the DNA derived from *A. tumefaciens* or from the decaying tissue of the transgenic plants themselves, could remain in the environment. Fragments of genetically modified DNA derived from decomposing transgenic poplar trees have previously been detected in field sites, although for not longer than four months (Hay et al., 2002). It was postulated that any residual DNA would be incapable of transmitting genetic information (Chiter et al., 2000; Hay et al., 2002).

CONCLUSIONS

Although this is a relatively small study of transgenic conifers, our results indicate that long-term *Agrobacterium* persistence in conifers occurs at a very low frequency, and only in undifferentiated material, such as embryogenic tissue. Moreover, if *A. tumefaciens* does persist in differentiated tissue such as whole plants, it does so beneath our ability to detect it. However, since we have demonstrated that a single *Agrobacterium* can be detected in spiked control experiments, any putative undetected bacteria must be in an unculturable state or reside in tissues and organs that we did not sample. Since we did not use methods to detect non-culturable microorganisms and it was impractical to assay entire plants, we cannot categorically rule out these possibilities.

We conclude that the possibility of environmental risk of *A. tumefaciens* persistence in transgenic conifers appears not to be substantiated by the data presented here. Even if *Agrobacteria* were detected at low frequencies, this is a minor issue relative to the risks associated with the production of trees using conventional breeding (Kube and Carson, 2004) or production of transgenic trees, such as the potential for gene flow through the dispersal of wind-borne pollen (Di-Giovanni and Kevan, 1991; Ellstrand, 2001), or undesirable changes in gene expression (Kumar and Fladung, 2001), weediness and invasiveness, impact on non-target organisms and other ecosystem interactions (Mullin and Bertrand, 1998). Furthermore, our results showed that the risk from residual *A. tumefaciens* in transformed conifers can readily be avoided by antibiotic counter-selection for at least ten weeks in combination with enrichment culture testing and subsequent confirmation using PCR analysis to determine the presence of *A. tumefaciens* in *in vitro* material, prior to planting in soil.

MATERIALS AND METHODS

Plant transformation

Embryogenic tissue of two spruce species, *Picea mariana* (black spruce) and *P. glauca* (white spruce) and three pine species, *Pinus pinaster* (maritime pine), *P. strobus* (eastern white pine) (Klimaszewska et al., 2001) and *P. radiata* (radiata pine) (Charity et al., 2005) were inoculated, co-cultivated and washed according to published methods. The supervirulent strain *A. tumefaciens* strain C58 (pMP90) (Koncz and Schell, 1986), containing the binary vector pRGR1 carrying the *uidA* gene (without an intron) driven by $2 \times 35S$ promoter and terminated by the 35S terminator (R. Rutledge, Canadian Forest Service, unpublished) was used. A single colony was grown in liquid YEP medium supplemented with kanamycin (50 mg.L^{-1}), gentamycin (25 mg.L^{-1}) and carbenicillin (100 mg.L^{-1}) (all from Sigma, St. Louis, MO, USA) at 27°C , for ~ 42 h or until the OD_{550} reached 0.6–0.8. A $10 \mu\text{l}$ aliquot of this culture was grown overnight in 10 ml fresh liquid YEP with antibiotics (as above), until the OD_{550} reached 0.6. Differences between the protocols are noted as follows: Cultures were diluted using liquid embryogenesis medium (EM, Smith, 1996) for radiata pine or liquid mLV medium (Klimaszewska et al., 2001) for the spruce species, as well as for eastern white pine and maritime pine. All embryogenic tissue was inoculated with *A. tumefaciens* (strain C58; pMP90) at a concentration of $\sim 10^9$ cells per ml. After cocultivation, radiata pine was washed on a Buchner funnel under gentle vacuum with $\frac{1}{2}$ EMS3 liquid medium to remove excess *A. tumefaciens* (Charity et al., 2005). For the other species, embryogenic tissue from five plates (at a time) were dislodged by manual shaking into an Erlenmeyer flask (250 ml) with 100 ml mLV medium and subsequently collected on new filter papers (Whatman No. 2; Maidstone, England) in a Buchner funnel (Klimaszewska et al., 2001). All tissue was resuspended in liquid EM or mLV at a density of 1 g tissue per 4 ml liquid. Aliquots ($250 \mu\text{l}$) of the cell suspension were pipetted on to filter paper disks or nylon mesh (Madison Filter Media, Auckland, New Zealand) and maintained for the duration of the experiment on semi-solid EDM6 medium containing Timentin (200 mg.L^{-1}) (Duchefa, Haarlem, The Netherlands) for radiata pine or mLV medium containing Timentin (400 mg.L^{-1}) and cefotaxime (300 mg.L^{-1}) (Sigma) for the other species. At each time point there were three Petri dishes (replicates) and one control of 100 mg of un-inoculated tissue.

Quantification of *A. tumefaciens* in embryogenic tissue during and after cocultivation

Quantification 0–7 days

One hundred milligrams of tissue was removed from each of three Petri dishes daily for seven days and macerated in 1 mL of liquid YEP bacterial medium using the FastPrep[®] FP120 apparatus (according to the manufacturer's instructions, BIO 101 Inc., Carlsbad, CA, USA). The samples were serially diluted and plated on YEP medium containing agar (2%), kanamycin (50 mg.L⁻¹), gentamycin (25 mg.L⁻¹) and carbenicillin (100 mg.L⁻¹). The number of colony forming units per g (CFU.g⁻¹) tissue was derived based on counts from Petri dishes where the serial dilutions yielded 0–300 colonies; more colonies resulted in their fusion. On day 0, an aliquot of the *A. tumefaciens* culture was serially diluted to estimate the number of CFU in the inoculum. The entire experiment was repeated.

Quantification 1–10 weeks

In subsequent experiments, only two species (black spruce and radiata pine) were chosen for further work. Embryogenic tissue was inoculated as described above and the numbers of *A. tumefaciens* CFU were estimated from the serial dilutions. Tissue was assayed at two days (at the end of the cocultivation period), then weekly for four weeks, and thereafter every fortnight up to 10 weeks. There were three replicates plus a non-inoculated control at each time point and the experiment was repeated. Because the vector pRGR1 included the *uidA* reporter gene without an intron, GUS was expressed in both bacteria and plant cells. The whole filter paper disc from one dish at each time point was soaked with a solution of 5-bromo-4-chloro-3-indolyl β -D-glucuronide (Sigma) and incubated at 37 °C overnight as per published methods (Jefferson et al., 1987) to detect bacterial GUS expression. This allowed us to follow the effect of antibiotics on the whole population of bacterial cells.

Detection of *A. tumefaciens* in transgenic cultures and plants

Determination of the sensitivity of PCR analysis to detect *A. tumefaciens* in a background of pine needle DNA

Known quantities (from ~ 10 cells– 1×10^7 cells) of *A. tumefaciens* that had been grown from a single cell for two

nights at 27 °C to an OD₅₅₀ of ~ 0.6 were added to 20 mg of young needles harvested from a 2 year old non-transformed *P. radiata* tree. Genomic DNA was extracted in the presence of various quantities of *A. tumefaciens* using the FastPrep DNA[®] Kit H in combination with the FastPrep[®] FP120 apparatus (according to the manufacturer's instructions; Bio 101 Inc.). The primers and PCR conditions for *virD₂* were as previously described (Charity et al., 2002) except that a high fidelity enzymes were used (Pfx DNA polymerase; Invitrogen and Expand; Invitrogen). Approximately 20 and 100 pg of *virD₂* DNA and DNA extracted from *A. tumefaciens* were used as positive controls. Negative controls were water and DNA extracted from non-transformed material. In addition, three two-year-old transgenic *P. radiata* plants that had been produced *via A. tumefaciens*-mediated transformation were tested. The known quantities of *A. tumefaciens* added to 20 mg, were re-calculated to give a concentration equating to the number of *A. tumefaciens* cells per g of tissue.

In vitro-grown embryogenic tissue, developing somatic embryos and plantlets

Detection of residual *A. tumefaciens* and other microorganisms was attempted from embryogenic tissue of 63 black spruce or white spruce lines that had been previously transformed with a range of constructs (Tab. 1; R. Rutledge, Canadian Forest Service, unpublished) but all with *A. tumefaciens* strain C58 (pMP90). Embryogenic tissue of independent transgenic lines was sampled at 3, 12, 16 and 24 months post-inoculation. One replicate of each transgenic line was tested, except for the black spruce cultures that were evaluated after three months, where two replicates (each from separate Petri dishes) were sampled (see Tab. 1). Developing somatic embryos or germinating plantlets, which had been produced *in vitro*, were evaluated after three or four and a half months, respectively. To increase the chance of detecting *A. tumefaciens* that might be present but not actively growing, it was necessary to apply an enrichment culture, derived from Matzk et al. (1996). Briefly, 1 g of transgenic tissue was macerated in 10 ml of YEP liquid medium and grown at 27 °C without antibiotics for seven days. One hundred μ l of the seven-day old culture was grown for two days on semi-solid YEP medium containing agar (2%), kanamycin (50 mg.L⁻¹), gentamycin (25 mg.L⁻¹) and carbenicillin (100 mg.L⁻¹) to specifically select for *A. tumefaciens* containing the binary vector of interest. One hundred μ l was also incubated on medium without antibiotics to select for any other microorganisms. This method only allowed for the

determination of the presence or absence of *A. tumefaciens* or other microorganisms rather than an estimation of colony number. If *A. tumefaciens* was present in embryogenic tissue, DNA was extracted from duplicate bacterial colonies and characterized using PCR and primers designed for the 35S terminator region.

Transgenic trees grown in the GMO greenhouse for up to four years

The sensitivity of the enrichment method for a known concentration of *A. tumefaciens* was first determined in a control experiment. Needles and branches from untransformed trees were surface sterilized in 70% (v/v) ethanol for 2 min, followed by a 10 min wash in 3% (v/v) H₂O₂. Plant material was then rinsed three times in sterile water. One hundred mg of needles or branches were macerated using a Retsch Mixer Mill (Type MM 200; Hann, Germany) in 1 ml liquid YEP medium and spiked with an amount approximately equivalent to 0, 1, 10 or 100 single *A. tumefaciens* cells (based on a serial dilution count). Samples were grown at 27 °C in 10 ml liquid medium with or without antibiotics for three days or until the medium became cloudy. Ten µl was plated on to semi-solid YEP medium with or without antibiotics.

One hundred mg of needles, branches, stems or roots from 10 independent transgenic *P. radiata* lines transformed using the binary vectors pGUL or pKEA (Charity et al., 2005), were surface-sterilized as described above and macerated with 1 ml of YEP and then grown in 10 ml of YEP at 27 °C without antibiotics for seven days or until the medium became cloudy. One hundred µl of each culture was plated on to semi-solid YEP medium containing kanamycin (50 mg.L⁻¹), gentamycin (25 mg.L⁻¹) and carbenicillin (100 mg.L⁻¹) and incubated at 27 °C for three days. Three replicates of each tissue were tested from each of the 10 trees. Three experiments evaluating the presence or absence of *A. tumefaciens* in needles and branches in each tree were conducted at one-year intervals. Due to the destructive nature of the sampling method, there was only one experiment performed on stems and roots in the fourth year of the experiment (also see Tab. 2).

In both the control and transgenic plant material, the plates were scored first using visual observations to categorize the types of microorganisms on each plate. The categories were as follows: (a) fungal with obvious hyphae; (b) microorganisms with yellow colonies; (c) microorganisms with orange colonies; (d) microorganisms with pink colonies; (e) microorganisms that were creamy-white in appearance but too numerous to form single colonies and (f) microorganisms with “*Agrobacterium*”-like morphol-

ogy, that is creamy white, convex and circular. In initial experiments, all types of microorganisms from all plates were streaked to single colonies and grown for two nights at 27 °C on semi-solid lactose medium (Bernaerts and DeLey, 1963). *A. tumefaciens* was distinguishable from other microorganisms because of the formation of yellow coloration after staining with Benedict’s reagent (Bernaerts and DeLey, 1963). Due to the reliability of this assay, only the bacterial colonies with morphological similarity to *A. tumefaciens* (creamy-white, circular, convex and glossy) were screened in subsequent experiments.

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