II.5 Genetic Transformation of Trees in the *Casuarinaceae* Family

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1 Introduction

1.1 Distribution and Importance of the Casuarinaceae

The *Casuarinaceae* family includes about 80 species of shrubs and trees belonging to four genera, *Allocasuarina*, *Casuarina*, *Ceuthostoma*, and *Gymnostoma*. *Casuarinaceae* are primarily native to the Southern Hemisphere, mostly to Australia, where they occur in tropical, subtropical, and temperate coastal regions as well as in arid regions. A few species are native to the Indo-Pacific areas from Malaysia to Polynesia. All members of the family are characterized by highly reduced leaves and photosynthetic deciduous branchlets (Midgley et al. 1983; National Research Council 1984).

Some *Casuarina* trees have the ability to grow well under a range of environmental stresses such as high level of salinity or low water availability (National Research Council 1984; Rockwood 1985). This outstanding ability to grow vigorously on poor soils is due partly to their symbiosis with an actinomycete, *Frankia*, that enables them to fix atmospheric nitrogen. In its native habitat, *Casuarina* root hairs become infected with *Frankia*, and form root nodules which are the sites of nitrogen fixation (Berry and Sunell 1990; Huss-Danell 1990; Baker and Mullin 1992). Many actinorhizal plants are mycorrhizal as well and thus possess the capability of extracting other nutrients from the soil to further enhance their success in poor soils (Rose 1980; National Research Council 1984).

The *Casuarinaceae* are pioneer species, able to colonize severely disturbed sites, and are thought to contribute to the rehabilitation in these sites by stabilizing the soil and building its nitrogen content (National Research Council 1984; Diem and Dommergues 1990). Actinorhizal plants can contribute as much nitrogen per hectare as the most productive legumes.

The ability to grow rapidly on poor soils has led to the widespread use of actinorhizal plants in forestry, landscaping, soil stabilization, and revegetation (Midgley et al. 1983; National Research Council 1984; Dawson 1986; Benoit and Berry 1990; Diem and Dommergues 1990). Various trees of the *Casuarinaceae*

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family are planted from the tropics to temperate zones and are currently used as:
–primary crops for timber and pulpwood;
–interplanted "nurse" plants for other, more valuable species;
–components of multipurpose agroforestry plantations;
–plantations for soil reclamation.
Among the various species, there are *Casuarina* trees capable of stabilizing shifting sand dunes and eroding hillslopes; many of these trees also make useful shade trees, windbreaks, and shelterbelts (National Research Council 1984).

The greatest use of any one actinorhizal genus is probably the production of *Casuarina* for firewood in the tropics. *Casuarina* produces high-quality fuelwood that has a high calorific value (about 5000 cal/g); its wood has a very low ash content and makes excellent charcoal. In Egypt, a country which is poor in forest resources, *Casuarina* wood is used in the form of poles and beams for construction, and is converted into particle boards (El-Osta and Megahed 1990).

1.2 Need for Genetic Transformation

The ability to genetically engineer forest tree species is particularly useful in view of the factors limiting classical genetic improvement such as the large size of mature plants and the long sexual generation time (Hanover and Keathley 1988; Schuerman and Dandekar 1991). Introducing insect resistance genes into *Casuarina* species appears to be a prime target since in tropical regions, where these trees have been extensively planted, a number of wood-destroying insects have been reported. In Taiwan, *Casuarina* trees are attacked by *Lymantria* (Chang and Weng 1985); in Egypt, *Kalotermes flavicollis*, *Stromatium fulvum*, and *Macrotoma palmata* are the most important insect pests of *Casuarina* (Hassan 1990).

Developing gene transfer systems for *Casuarinaceae* would also be of interest in the study of the symbiotic process developed between the actinorhizal trees and their microsymbiont *Frankia*. The broad host range of *Frankia* (the actinomycete has the ability to nodulate plants from nine different families) makes this plant-microbe association a significant one to study in terms of understanding the evolution of nitrogen-fixing symbioses. Nevertheless, in contrast to the *Rhizobium*-legume association, the nitrogen-fixing symbiosis between *Frankia* and woody dicotyledons host plants has received little attention. Studies have been impeded by a number of factors including the bacteria's slow growth and filamentous habit, and by the lack of gene transfer techniques in *Frankia* (Mullin and An 1990). The only actinorhizal nodulin gene which has been characterized is the hemoglobin gene from *Casuarina glauca* (Christensen et al. 1991). Attempts to optimize the symbiosis for a given host plant in a selected environment, to alter host ranges, or to engineer other aspects of the symbiosis will be facilitated by a better understanding of the genetics of the association.

In this chapter, the work concerning the transformation and regeneration of *Allocasuarina verticillata* using *Agrobacterium rhizogenes* is presented (Phelep et al. 1991). Also reported is the stable transformation in callus of *Casuarina*

glauca following transformation with a disarmed strain of *Agrobacterium tumefaciens* containing in a binary vector the *npt*II (neomycin phosphotransferase) gene and the *uidA* (β-glucuronidase) reporter gene.

2 Transformation of *Allocasuarina verticillata* by *Agrobacterium rhizogenes*

Allocasuarina verticillata is a nitrogen-fixing tree about 6–10 m high which has demonstrated good growth on irrigated lands in Egypt and has shown early success in Cyprus, India, Israel, and several countries of southern Africa (National Research Council 1984). The possibility to transform *Allocasuarina verticillata* with *Agrobacterium rhizogenes* was investigated.

2.1 Transformation by the Strains 2659, A4, and 8196

Agrobacterium rhizogenes incites hairy root disease in many plants. The disease, characterized by extensive adventitious root formation at the wound site, results from the expression of *Agrobacterium rhizogenes* genes originating from a large root-inducing (Ri) plasmid which have been integrated into the plant genome (Chilton et al. 1982; White et al. 1982; Birot et al. 1987). Oncogenic strains of *Agrobacterium rhizogenes* can be used to transform a range of plant species, since the induced hairy roots can be regenerated to whole, fertile plants (Tepfer and Casse-Delbart 1987; Weising et al. 1988; Tepfer 1990). The major drawback of this approach is that the regenerated plants exhibit an aberrant phenotype: wrinkled leaves and a reduced apical dominance (Tepfer 1984; Weising et al. 1988).

Three strains of *Agrobacterium rhizogenes* were used in the preliminary experiments of inoculation of *Allocasuarina verticillata*: an agropine-type strain, A4 (Moore et al. 1979), a cucumopine-type strain, 2659 (Davioud et al. 1988), and a mannopine-type strain, 8196 (Koplow et al. 1984). In cucumopine- and mannopine-type Ri (root-inducing) plasmids, the T-DNA consists of a single fragment. In agropine-type plasmids, the T-DNA is divided into two parts which can be integrated independently, TL and TR; genes for opine synthesis are located on the TR-DNA (De Paolis et al. 1985).

Allocasuarina verticillata seeds collected in Australia (Mt. Stromlo) were surface sterilized by a 20-min treatment with 5% calcium hypochlorite, followed by three rinses with sterile distilled water. One to 2 months after germination on water solidified with 8 g/l Difco bacto agar, aseptic plantlets of *Allocasuarina verticillata* were inoculated with *Agrobacterium rhizogenes* by wounding different organs, epicotyls, cotyledons, and hypocotyls with a needle or a scalpel dipped in a culture of agrobacteria; bacterial strains 2659, A4, and 8196 were grown for 1 and 2 days in LB medium (Maniatis et al. 1982). Control plants were wounded in the same way using sterile tools. Inoculated plants were cultured on

BM medium composed of half-strength MS mineral salt solution (Murashige and Skoog 1962), Nitsch and Nitsch (1965) vitamins, 15 g/l sucrose, and solidified by adding 8 g/l bacto agar (Difco). Results of the inoculation are presented in Table 1. With strain A4, cotyledons were found more susceptible to the inoculation than epicotyls and hypocotyls; with the strains 2659 and 8196, respectively, 54 and 45% of the inoculated hypocotyls developed roots showing a typical hairy root phenotype (high growth rate of 1 to 3 mm a day, extensive lateral branching, and lack of geotropism).

Roots induced after wounding by strains 2659 and A4 were analyzed for the presence of specific opines (Fig. 1). Cucumopine was detected in all the extracts of roots induced by the inoculation of strain 2659 (ten extracts were analyzed according to the procedure described by Davioud et al. 1988). With A4-induced roots, 12 extracts were tested for opine synthesis (root extracts were analyzed for the presence of specific opine by high-voltage electrophoresis as reported by Petit et al. 1983). Mannopine and agropine were detected in three extracts, indicating that the TR-DNA carrying the genes for opine synthesis had been transferred to the plant (De Paolis et al. 1985); in nine extracts, no opine was observed, suggesting that only the TL-DNA had been transferred.

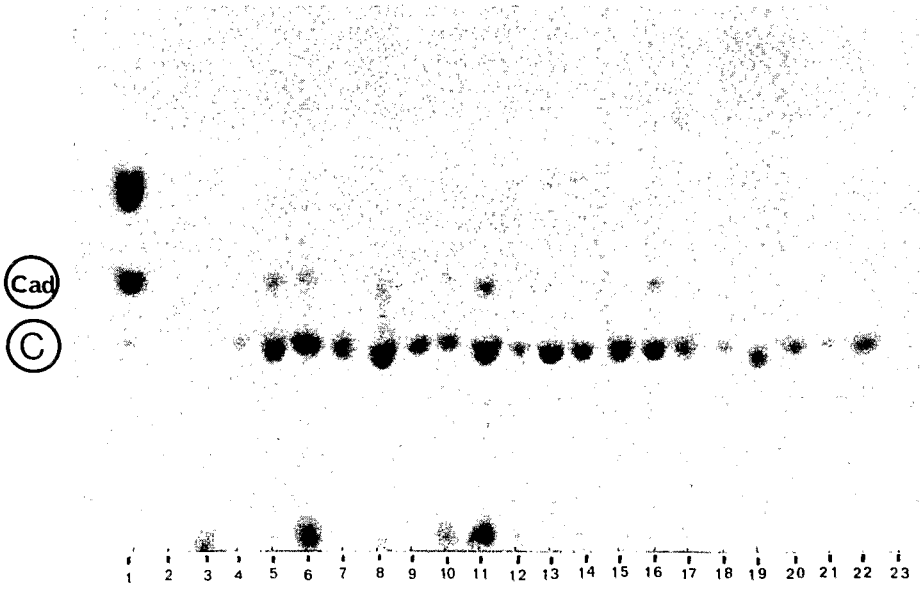


Fig. 1. Electrophoretic analysis of shoot or root extracts for cucumopine. Two μl extract, corresponding to 4 mg tissue was applied in each lane. *Lane 1* Cucumopine standard; *2* control roots; *3* control shoots; *4–9* and *12–22* hairy roots induced by 2659; *4* root induced on a cotyledon; *5–9* roots induced on epicotyls; *12–22* roots induced on hypocotyls; *10* and *11* shoots regenerated from 2659-induced roots. *C* Cucumopine; *Cad* cucumopine acid degradation. (Phelep et al. 1991)

Table 1. Inoculation of *Allocasuarina verticillata* by *Agrobacterium rhizogenes* A4, 2659, and 8196. Aseptic 1-month-old plants were inoculated and grown as described in Section 2.1. Root development was followed for 3 months. No root developed on the control plants wounded by sterile instruments. ND: not determined. (Phelep et al. 1991)

Explants	Bacterial strain	Number of plants tested	Plants with fast-growing roots (%)
Cotyledons	2659	80	10
	A4	42	30
	8196	ND	ND
Epicotyls	2659	86	9.3
	A4	47	19
	8196	ND	ND
Hypocotyls	2659	50	54
	A4	20	20
	8196	50	45

2.2 Regeneration of Transgenic Plants

Roots initiated at the inoculation site were excised and cultivated on solidified BM medium with a 16-h photoperiod (50 μE m^{-2} s^{-1}) at 28°C (Fig. 2A and 2B). Carbenicillin (Sigma, 500 mg/l) was added to the medium for the first two or three subcultures. Shoot regeneration from A4-transformed roots was obtained by adding NAA (0.25 mg/l) and BAP (0.1 mg/l) to the BM medium. Buds developed in 2 months on some of the transformed roots. Shoot regeneration occurred spontaneously on 90% of the *Agrobacterium rhizogenes* 2659-transformed roots after 3 months of culture on hormone-free medium (Fig. 2C). All the shoots rooted when placed in the BM sucrose medium supplemented with 0.1 mg/l IBA.

The phenotype of the transgenic plants was different from those of the control plants (Fig. 2D). The transgenic regenerants had root systems that were more developed, plagiotropic, and branched than the normal nontransformed regenerants. The root dry weight of the transgenic regenerant was five times higher than that of the normal regenerant. The aerial dry weight of the transgenic was two times higher than that of the normal regenerant; the aerial system showed reduced apical dominance with highly branched shoots.

Southern blot analysis was performed on some of the transgenic plants. Total DNA was extracted from four plants regenerated from 2659-induced roots. Integration of the T-DNA was demonstrated following hybridization with two probes, one containing an internal fragment of 2659 T-DNA (a 3.75-kb *Hind*III-*Nhe*I fragment) (data not shown), and the other one containing the right border of the T-DNA and the cucumopine synthase gene (an *Hind*III-*Eco*RI fragment of 2.28 kb) (Fig. 3A).

One plant regenerated from A4-induced roots was analyzed by Southern analysis. This plant did not exhibit any opine synthesis. Nevertheless, the

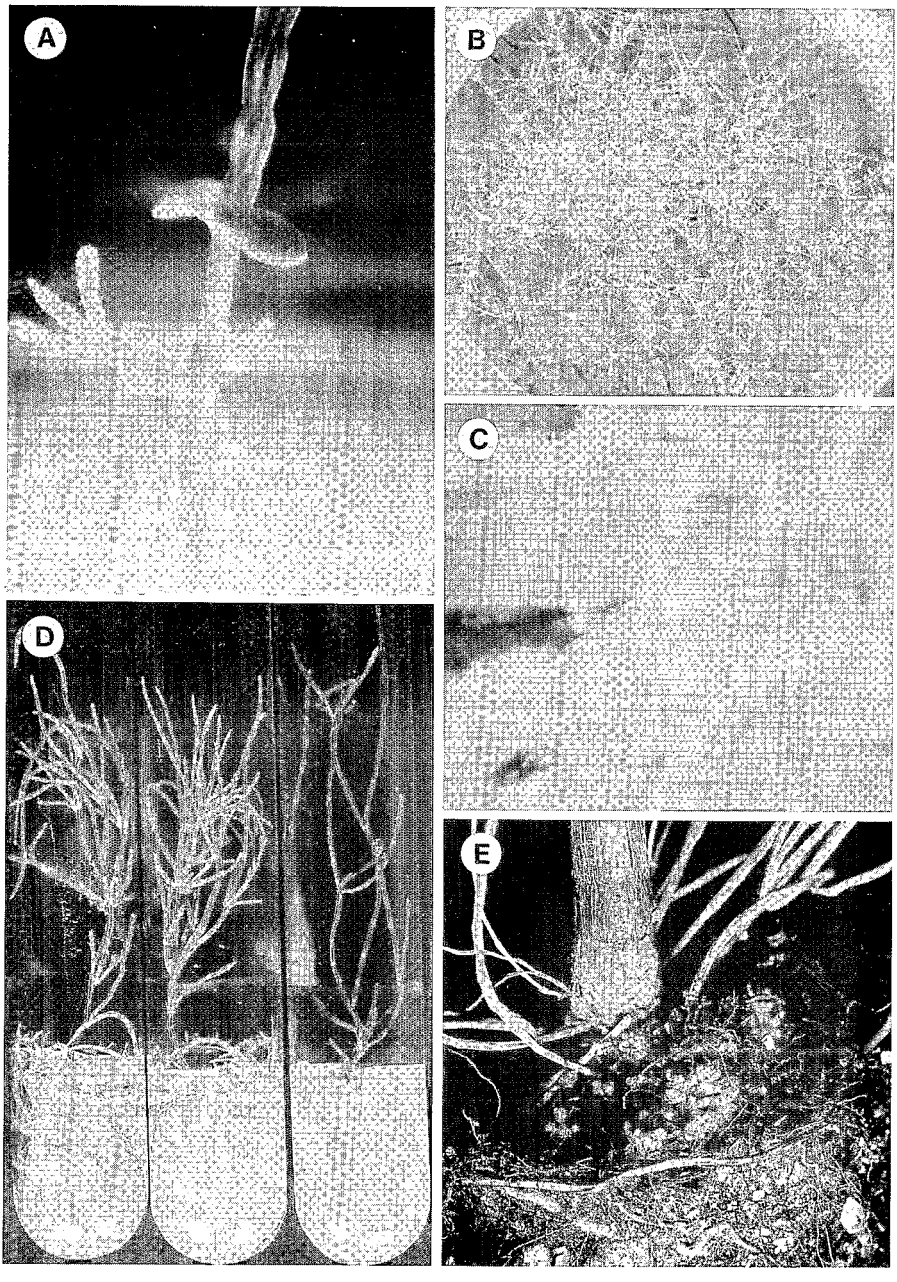


Fig. 2A–E. Transformation of *Allocasuarina verticillata* by *Agrobacterium rhizogenes*. **A** Hairy roots developing on the hypocotyl of a 2-month-old plant inoculated by *Agrobacterium rhizogenes* 2659. **B** Roots transformed by *Agrobacterium rhizogenes* 2659. **C** Regeneration of shoots from A4-transformed roots. **D** Transgenic plants transformed by *Agrobacterium rhizogenes* A4 (*left* and *middle*) and nontransformed control plant (*right*). **E** Nitrogen-fixing nodules on a 2659-transformed plant. (Phelep et al. 1991)

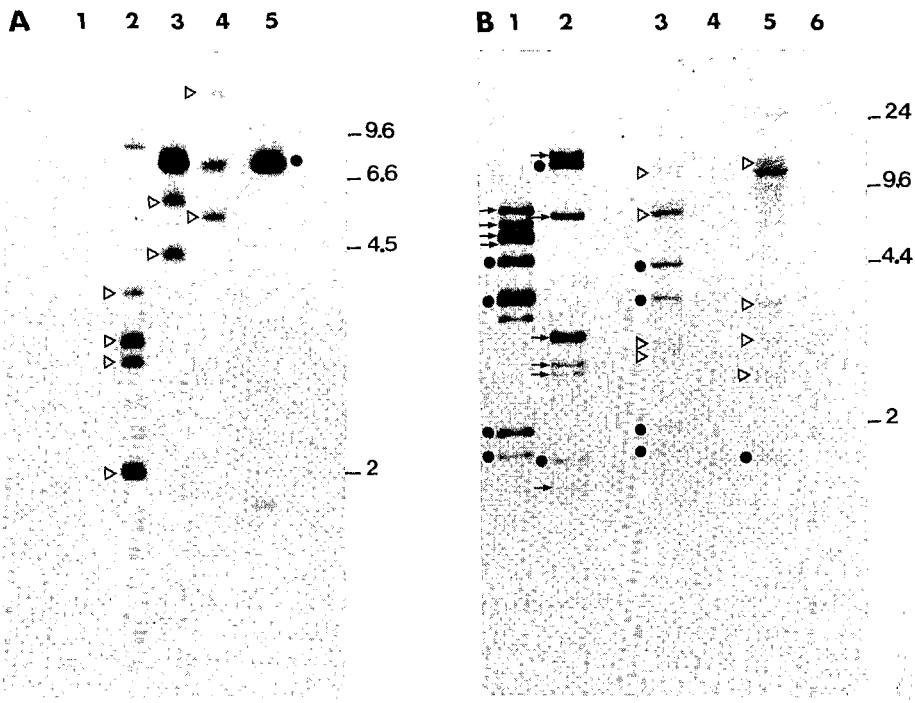


Fig. 3A,B. Southern blot analysis of *Allocasuarina verticillata* plants transformed with *Agrobacterium rhizogenes* 2659 (**A**) or A4 (**B**). **A** DNA was isolated from shoots, digested with PstI, and hybridized with a labeled fragment containing the right border and the cucumopine synthase gene of 2659 T-DNA (Phelep et al. 1991). *Lane 1* control DNA; *2–4* DNA from transgenic *Allocasuarina* plants; *5* control 2659 T-DNA. *Black dots* indicate presumed internal fragments. *Open arrowheads* indicate presumed junction of T-DNA/plant DNA fragments (Phelep et al. 1991). **B** DNA was isolated from shoots, digested with HindIII or BamHI, and hybridized with a probe carrying the entire TL region of A4 (Phelep et al. 1991). *Lanes 1* and *2* Probe DNA digested by HindIII and BamHI, respectively; *3* and *5* DNA from a transgenic plant digested by HindIII and BamHI, respectively; *4* and *6* control *Allocasuarina verticillata* DNA digested by HindIII and BamHI, respectively. *Thin arrows* indicate fragments situated outside the T-DNA. *Open arrowheads* indicate presumed junction of T-DNA/plant DNA fragments. *Black dots* indicate internal fragments. (Phelep et al. 1991)

restriction pattern obtained after hybridization with the probe pLJ1 carrying the TL-DNA of A4 demonstrated that the plant contained at least one TL-DNA insertion (Fig. 3B).

2.3 Nodulation of Transgenic *Allocasuarina verticillata* Plants

Eight transgenic clones of *Allocasuarina verticillata* transformed with *Agrobacterium rhizogenes* 2659 were transferred to a greenhouse when their root system was about 3 cm long; they were planted in plastic pots containing vermiculite

and watered with a Blondon nutrient solution containing 1 mM nitrogen (Blondon 1964). Five clones survived this transfer and have been grown for more than 3 years. Growth of the transgenic plants appeared slower than those of the control plants; after 3 years, transgenic *Allocasuarina* clones were 75 cm high, whereas nontransformed plants were 167 cm high.

Four months after their transfer to the greenhouse, five clones of transgenic *Allocasuarina verticillata* were inoculated by *Frankia* strain DEC, syn. CFN 022901 (Sougoufara 1990). Nodules appeared 2 months after inoculation on only one of the transgenic clones (Fig. 2E). The nitrogenase activity of these nodules was found to be similar to that of the nontransformed control plants (data not shown).

3 Transformation of *Casuarina glauca* Using *Agrobacterium tumefaciens*

Since transgenic *Allocasuarina verticillata* obtained after inoculation with *Agrobacterium rhizogenes* exhibited an abnormal phenotype, alternative methods of transformation using *Agrobacterium tumefaciens* were sought. These experiments of transformation were focused on *Casuarina glauca*, a tall fast-growing tree (typically, 10–14 m high), showing a prolific nodulation, which can survive on difficult sites where other trees fail because of salinity, waterlogging, or shallow water table (National Research Council 1985).

3.1 Transformation by Wild-Type *Agrobacterium tumefaciens* Strains

Effective gene transfer systems require reliable and efficient procedures for plant regeneration from transformed cells. To avoid the difficult step of regeneration, an alternative method for gene transfer in trees has been recently developed with poplar (Brasileiro et al. 1991); the method is based on the coinoculation of stem internodes with two *Agrobacterium* strains: a wild-type *Agrobacterium tumefaciens* strain capable of naturally inducing shoot differentiation on the tumors (Michel et al. 1990), and a disarmed *Agrobacterium tumefaciens* strain containing a selectable marker in a binary vector. The selection and regeneration of shoots on the appropriate selection medium allow the selection of plants containing the T-DNA from the disarmed strain. The advantage over classical explant transformation techniques is that it is not necessary to develop a regeneration procedure; the oncogenic *Agrobacterium* strain provides the optimal balance in growth regulators to allow the shoot regeneration.

This approach has been attempted with *Casuarina glauca*. Two wild-type *Agrobacterium tumefaciens* strains were tested in the coinoculation experiments: the nopaline strain 82139 which has been used by Brasileiro et al. (1991) to transform poplar and Antib 12 (A. Petit, Institut des Sciences Végétales, Gif sur Yvette, France, unpubl.).

Seeds from *Casuarina glauca* were kindly supplied by Dr. El Lakany from the Desert Development Center (Saddat City, Egypt); they were scarified with concentrated H_2SO_4 (95%) for 2 min and rinsed with tap water for 30 min. The scarified seeds were then surface sterilized with 5% Domestos for 20 min, rinsed three times with sterile distilled water, and germinated in Petri dishes on water solidified with 8 g/l Difco bacto agar. The plantlets were maintained on BM medium under the same conditions as those previously described for *Allocasuarina verticillata*. The agrobacterial strains were grown 24 to 48 h at 28 °C on solidified LB medium (Sambrook et al. 1989). For the inoculation, plants were wounded with a needle (Terumo, 0.45 × 12) previously soaked with a fresh colony of the wild-type *Agrobacterium* strain. Inoculations were performed on hypocotyls, epicotyls, or cotyledons. The controls received an identical treatment, without bacteria. Five days after wounding, the plants were transferred to BM solidified medium containing 250 mg/l cefotaxim (Claforan, Roussel).

Results presented in Table 2 and Fig. 4A–C indicate that *Casuarina glauca* was sensitive to both strains 82139 and Antib 12. 83139-Inoculated plants developed tumors only on the hypocotyls. The tumors appeared 4 weeks after inoculation; after 6 weeks, the tumors were green and had an average size of 2 mm. The excised tumors developed on BM medium without any growth regulator. *Casuarina glauca* appeared slightly more sensitive to the inoculation by the strain Antib 12 (Table 2). Tumors developed on both the hypocotyls and epocotyls of the plantlets; tumors were observed 12 days after inoculation; they had an average size of 2 mm 1 month after inoculation. Tiny tumors appeared on the cotyledons, but did not develop further.

The phenotype of the tumors was undifferentiated. Some of the tumors were excised and grown for several months on nutrient medium without any growth regulator; no shoots developed 5 months after inoculation. Thus, the two wild-type *Agrobacterium tumefaciens* strains tested did not appear to be good

Table 2. Inoculation of *Casuarina glauca* by wild-type *Agrobacterium tumefaciens* strains 82139 and Antib12. Aseptic 1- to 2-month-old plants were inoculated by 82139 and Antib12 as described in Section 3.1. Tumor growth was followed for 3 months. No tumor developed on control plants (20 plants for each type of inoculation) wounded with a sterile needle and cultured under the same conditions as the inoculated plants. (Sylla and Franche unpubl. data)

Bacterial strain	Site of inoculation	Number of inoculated plants	Plants developing tumors (%)
82139	Hypocotyls	23	57
	Cotyledons	27	0
	Epicotyls	24	0
Antib 12	Hypocotyls	28	78
	Cotyledons	24	8.3
	Epicotyls	24	57

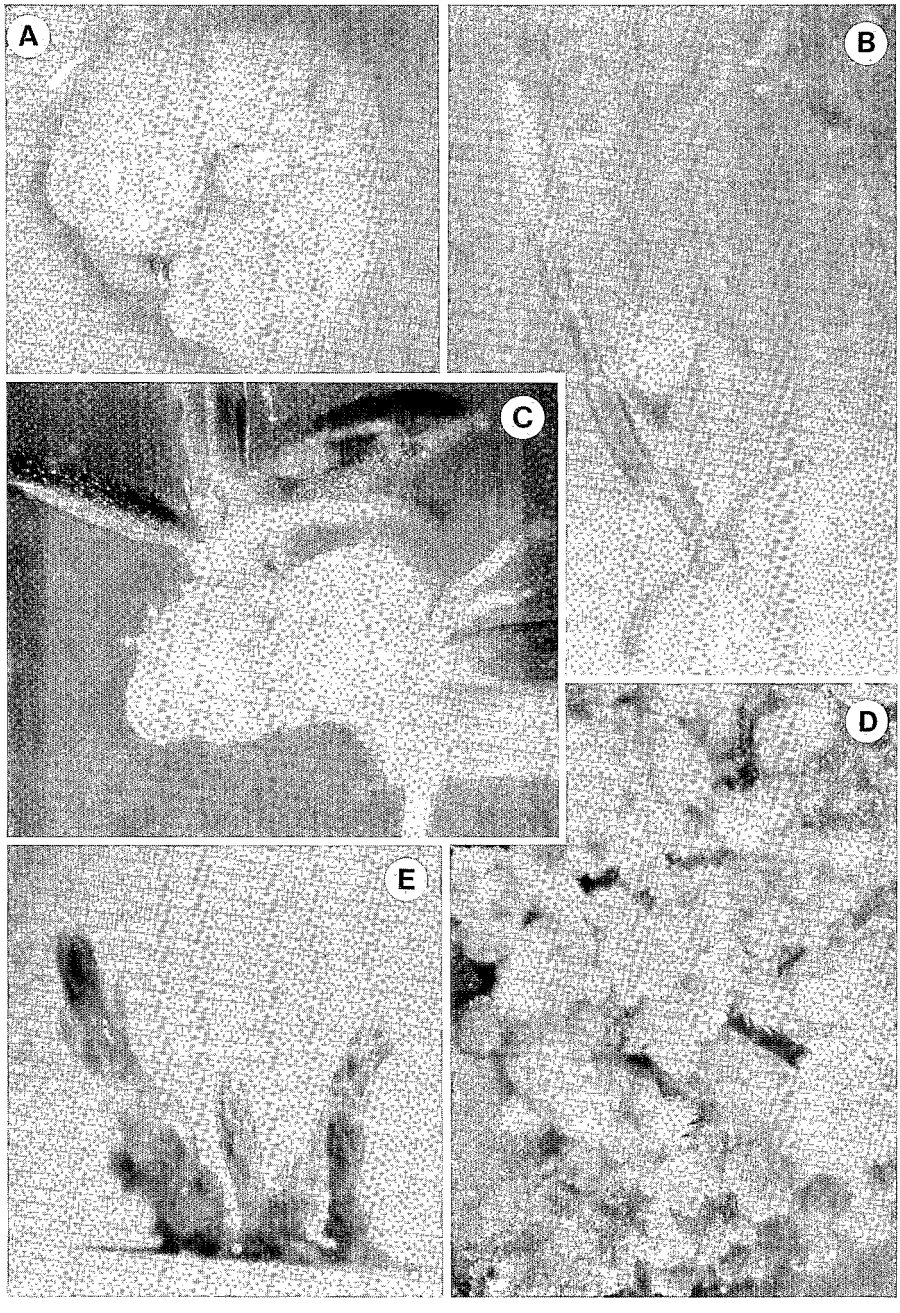


Fig. 4. **A** Three-month-old tumor obtained after inoculation of *Casuarina glauca* by *A. tumefaciens* 82139, and grown on nutrient medium with no growth regulator. **B** Two-month-old tumor induced on the epicotyl of *Casuarina glauca* after inoculation by *A. tumefaciens* Antib12. **C** Two-month-old tumor developing on the hypocotyl of *Casuarina glauca* 2 months after inoculation by *A. tumefaciens* 82139. **D** Bud differentiation on callus cultured for 8 weeks on nutrient medium with 0.1 mg/l ANA and 0.5 mg/l BA. **E** Three-month-old elongated shoots. (Franche and Sylla unpubl. data)

candidates to develop a coinoculation procedure. New oncogenic strains originating from collections or isolated in *Casuarina* plantations will have to be tested.

3.2 Transformation of *Casuarina glauca* with the Disarmed *Agrobacterium tumefaciens* Strain C58C1 (GV2260)

3.2.1 Regeneration of Casuarina glauca

The potential of *Casuarina* for micropropagation by organogenesis has been established by Abo El-Nil (1987) who described the induction of callus followed by bud differentiation from juvenile and mature stem segment explants of *Casuarina glauca*, *C. cunninghamiana*, and *C. equisetifolia* cultures on nutrient medium supplemented with 5 μM 2iP and NAA (0.05 to 0.005 μM). Since we did not succeed in reproducing these experiments of organogenesis in our laboratory, we investigated the possibility to induce shoot differentiation with different concentrations of BA and NAA.

Hypocotyls, cotyledons, and epicotyls were excised from 1- to 2-month-old plants of *C. glauca* propagated from seeds as described previously in Section 3.1; the explants were incubated on BM medium containing different combinations of NAA (0.01, 0.05, and 0.1 mg/l) and BA (0.1, 0.5, and 1 mg/l) and transferred every 3 weeks to fresh nutrient medium. Callus formation was observed 2 weeks after excision of the explants. Root formation occurred in the presence of 0.1 mg/l NAA and 0.1 mg/l BA after 4 weeks of culture. Buds developed after 3 to 10 weeks on some green friable calli growing with 0.1 mg/l NAA and 0.5 mg/l BA (Fig. 4D); bud induction was observed on 17, 14, and 41% of the calli induced from cotyledons, hypocotyls, and epicotyls, respectively. After 2 months of culture, the shoots had an average height of 2 to 4 mm (Fig. 4E). Rooting of the elongated shoots was induced at a high frequency by a 3-day transfer to BM medium containing 10 μM IBA, followed by a culture on BM medium with no growth regulator.

3.2.2 Identification of a Selection Marker

The use of appropriate selectable marker genes in conjunction with efficient regeneration systems is a key to the success of *Agrobacterium* genetic transformation. Selection markers are based on the sensitivity of plant cells to antibiotics and herbicides. The susceptibility of nontransformed *C. glauca* tissues to various concentrations of kanamycin was investigated.

Hypocotyls, epicotyls, and cotyledons excised from 1- to 2-month-old plants of *C. glauca* were cultured on BM medium containing 0.1 mg/l NAA, 0.5 mg/l BA, and kanamycin at a concentration of 10, 25, 50, 100, or 200 mg/l. Callus formation was greatly reduced at 25 mg/l kanamycin and completely inhibited at a concentration of 50 mg/l. These results indicate that *C. glauca* explants exhibit a good sensitivity to kanamycin and that it is an appropriate selectable marker for the transformation experiments.

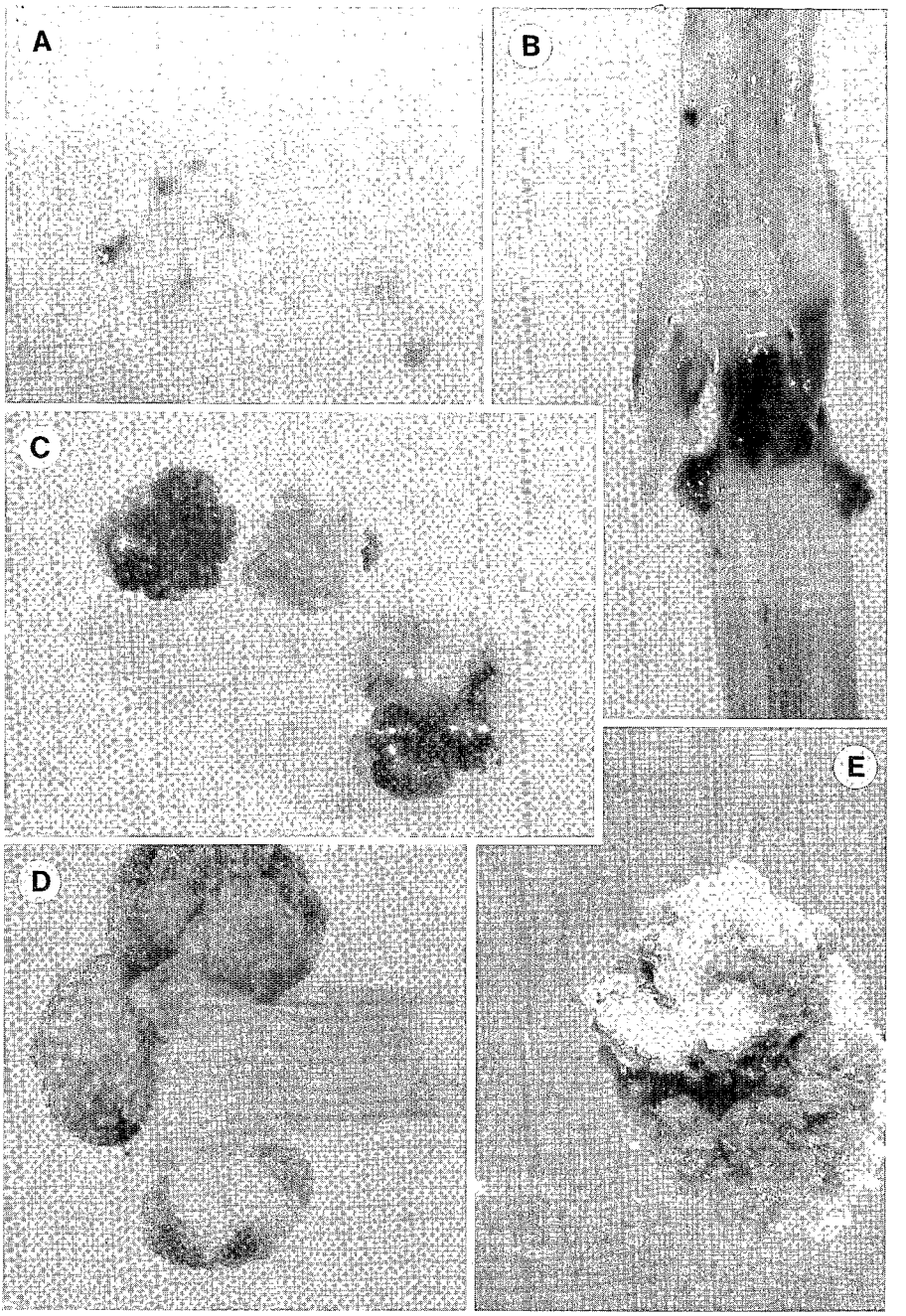
3.2.3 Transformation of Hypocotyls, Cotyledons, and Epicotyls with Agrobacterium tumefaciens

The *Agrobacterium tumefaciens* strain C58C1 (GV2260) (Vancanneyt et al. 1990), carrying a derivative of the binary vector BIN19 (Bevan 1984), was used for the transformation experiments. The plasmid vector contains the *npt*II gene conferring the resistance to kanamycin (Mazodier et al. 1985) and a derivative of the β-glucuronidase (GUS) gene (Jefferson 1987) as a reporter gene; the insertion of a plant intron in the coding sequence of the GUS gene prevents the expression of the reporter gene in *Agrobacterium* and allows one to monitor early events of transformation (Vancanneyt et al. 1990).

Hypocotyls, cotyledons, and epicotyls from 30- to 60-day-old *C. glauca* were dipped for 1 h with an overnight culture of C58C1(pBIN19-GUSINT) diluted five times in BM medium; after blotting, they were placed on nutrient medium containing 0.1 mg/l NAA and 0.5 mg/l BA (the cotyledons were in the adaxial side up position). After 3 days of cocultivation, the explants were rinsed for 1 h in sterile distilled water and transferred onto a selection medium containing 0.1 mg/l NAA, 0.5 mg/l BA, 250 mg/l cefotaxim, and 50 mg/l kanamycin. Nontransformed control plants were incubated under the same conditions. Calli emerging from the wounded edges of the explants were observed 3 weeks after transformation (Fig. 5A–D); within 2 months, among 250 plants tested, 53% of the epicotyls, 37% of the cotyledons, and 17% of the hypocotyls had developed one to five calli growing in the presence of kanamycin. Although *C. glauca* cannot be considered very sensitive to *Agrobacterium* C58C1, these results are promising considering the very small size of the explants, and consequently of the wounded areas where the transfer of the T-DNA occurs: the hypocotyls and the epicotyls are 0.5 to 1 mm in diameter, and the cut area of the cotyledons is approximately 1 to 2 mm wide.

Expression of the β-glucuronidase gene was studied in the kanamycin-resistant calli (Fig. 5C–E). Eighty percent of the calli developed a blue color after incubation in the presence of 5-bromo-4-chloro-3-indolyl β-glucuronic acid (X-glu), the substrate of the β-glucuronidase (Jefferson 1987); light blue to dark blue calli were observed, indicating different levels of expression of the reporter gene. Southern blot analysis of the kanamycin-resistant calli is in progress.

Fig. 5A–E. Histochemical localizations of β-glucuronidase gene expression on explants of *Casuarina glauca* transformed by the disarmed strain of *Agrobacterium tumefaciens* C58C1(pBIN19-GUSINT). Prior observation, the explants were incubated for 24 h in a staining solution containing 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid as described by Jefferson (1987); chlorophyll was removed by incubating the explants for 1 day in 50% ethanol, 10% formaldehyde, 5% glacial acetic acid, followed by several rinses in 70% ethanol. **A** Expression of the β-glucuronidase gene on a cotyledon 2 weeks after transformation by C58C1(pBIN19-GUSINT). **B** Expression of the β-glucuronidase reporter gene on an epicotyl 3 weeks after transformation by C58C1(pBIN19-GUSINT). **C, D** GUS-expressing calli emerging from a cotyledon (**C**) and from an hypocotyl (**D**) transformed by C58C1(pBIN19-GUSINT); the explants were grown for 1 month on nutrient medium supplemented by 50 mg/l kanamycin and 250 mg/l cefotaxim. **E** Callus excised from an epicotyl transformed with C58C1(pBIN19-GUSINT) and cultured for 2 months on selective medium. (Le Van unpubl. data)


A
B
C
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E

Numerous factors are found to be involved to achieve optimal transfer of the T-DNA from *Agrobacterium* to wounded plant cells (Hooykaas and Schilperoort 1992): induction of the virulence genes by acetosyringone and other phenolic compounds (Bolton et al. 1986), pH of the medium during cocultivation, concentration of *Agrobacterium*, strain of *Agrobacterium* and plasmid vector, time of coculture, temperature, light, and sugar content of the medium. The effect of some of these factors has been studied in order to improve the efficiency of the transfer of the T-DNA to *C. glauca*. Figure 6 presents the percentage of explants developing kanamycin-resistant calli according to the duration of coculture with *Agrobacterium tumefaciens*. Optimal transfer of the

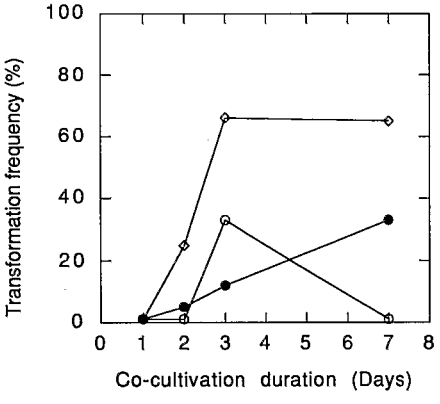

Fig. 6. Effect of cocultivation duration on the frequency of transformation of *Casuarina glauca* by *Agrobacterium tumefaciens* C58C1(pBIN19-GUSINT). Transformation frequency was defined as the percentage of explants developing kanamycin-resistant calli 2 months after transformation. Fifty explants were used for each treatment. No callus was observed on the control nontransformed explants cultured on selective medium. ◇ Epicotyls; ●, cotyledons; ○, hypocotyls. (Le Van unpubl. data)

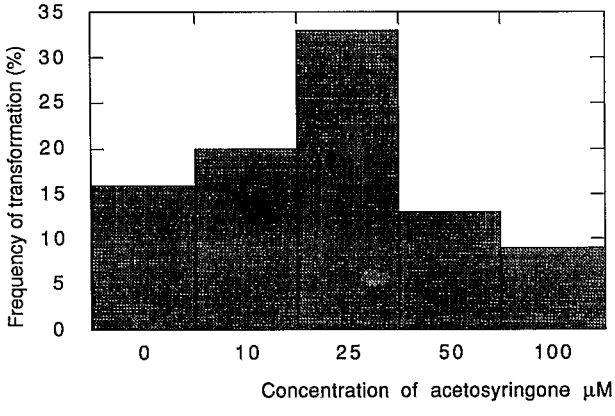

Fig. 7. Transformation frequency after inoculation of *Casuarina glauca* epicotyls with C58C1(pBIN19-GUSINT) in the presence of different concentrations of acetosyringone. Different concentrations of acetosyringone (0 to 100 µM) were added during cocultivation. 150 explants were tested for each treatment. Transformation frequency was defined as the percentage of epicotyls developing kanamycin-resistant calli 2 months after transformation. No callus growth was observed on 50 nontransformed epicotyls cultured for 2 months on selective medium. (Le Van unpubl. data)

T-DNA to wounded *Casuarina* cells occurred within 3 days for the epicotyls and the cotyledons, and after 7 days of coculture for the hypocotyls.

The effect of acetosyringone on the T-DNA transfer into epicotyl cells from *C. glauca* was also investigated. Epicotyls were cut from 2-month-old plants and transformed with the diluted culture of C58C1(pBIN19-GUSINT) as previously described. The explants were cocultivated for 3 days on nutrient medium containing different concentrations of acetosyringone (0, 10, 25, 50, and 100 μM). Then, they were transferred to selective medium and early events of T-DNA transfer were tested after 10 days by incubating the transformed epicotyls in an X-glu solution. Figure 7 presents the percentage of explants exhibiting blue GUS-expressing tissues; the results indicate that 25 μM acetosyringone increases approximately twofold the T-DNA transfer as compared to the nontransformed control plants.

4 Summary and Conclusions

The only actinorhizal plant which has been successfully transformed and regenerated is *Allocasuarina verticillata* (Phelep et al. 1991). The transformation procedure developed in our laboratory is based on *Agrobacterium rhizogenes* and allows one to obtain routinely transgenic plants within 5 months. Hairy root formation was induced by inoculating various organs of *Allocasuarina verticillata* with three strains of *Agrobacterium rhizogenes* (2659, A4, and 8196). The most effective *Agrobacterium rhizogenes* strain among the three tested appears to be 2659; there is a good response of the plants to the inoculation, and regeneration of shoots from transformed hairy roots occurs spontaneously. Newly formed shoots have been multiplied and rooted to produce transgenic plants which are characterized by reduced apical dominance and highly branched aerial and root systems. One of the plants transformed by the T-DNA of *Agrobacterium rhizogenes* 2659 has developed nitrogen-fixing nodules after inoculation with *Frankia*.

Casuarina glauca is sensitive to *Agrobacterium tumefaciens* and undifferentiated tumors developed after inoculation by one of the oncogenic *Agrobacterium tumefaciens* strains 82139 or Antib12. Hypocotyls, epicotyls, and cotyledons of *C. glauca* transformed with the disarmed *Agrobacterium tumefaciens* strain C58C1(pBIN19-GUSINT) carrying the neomycin phosphotransferase gene as a selectable marker have developed calli expressing β-glucuronidase activity. Transformation frequency has been shown to be influenced by explant source, duration of cocultivation, and acetosyringone.

Transformation of actinorhizal plants is still poorly documented. Recently, four wild-type *Agrobacterium rhizogenes* strains have been used to inoculate the actinorhizal tree species *Alnus glutinosa*, *Alnus acuminata*, and *Eleagnus angustifolia* L. (Savka et al. 1992). *Agrobacterium rhizogenes* 8196, a manopine-type strain, K599, a cucumopine-type strain, and two agropine-type strains, A4 and 1855, were tested. *Alnus glutinosa* and *Alnus acuminata* produced hairy roots

containing strain-specific opines when inoculated with any of the four strains of *Agrobacterium rhizogenes*. Nodule-like structures were observed on *Eleagnus* after transformation with K599 and 8196; this host did not respond to the inoculation with the agropine-type strains. The possibility of regeneration of transformed roots of *Alnus* is being currently investigated.

To our knowledge, there is only one report describing the use of two wild-type *Agrobacterium tumefaciens* strains, C58 and Ach5, to transfer DNA into two actinorhizal trees, *Alnus glutinosa* and *Alnus incana*; the agrobacteria were found to induce tumor formation and the transfer of the T-DNA was demonstrated by Southern blot analysis (Mackay et al. 1988). We have established that *Casuarina glauca* develops undifferentiated tumors when inoculated by one of the oncogenic *Agrobacterium tumefaciens* strains 82139 or Antib12.

Since the transgenic *Allocasuarina verticillata* plants have the ability to be nodulated by *Frankia*, they provide a good system to determine the regulation and specific functions of the actinorhizal plant symbiotic genes. Nevertheless, due to the alteration of phenotype exhibited by the transgenic trees, such plants cannot be used for the introduction of agronomically important traits such as insect resistance. The possibility to transfer foreign genes into *Casuarina glauca* with a disarmed strain of *Agrobacterium tumefaciens* carrying a binary vector opens the way to obtain transgenic plants with a normal phenotype.

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