I.5 *Casuarina* and *Allocasuarina* Species

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1 General Account

1.1 Botany, Distribution and Importance of the Tree

Casuarinas are a group of 96 species of trees and shrubs belonging to the family Casuarinaceae (Wilson and Johnson 1989). The family is unique amongst the angiosperms and, having no close relatives, is assigned to an order of its own, the Casuarinales (Beadle 1981). *Casuarinas* are morphologically distinctive with the foliage consisting of long needle-like articulate photosynthetic (assimilatory) branchlets. The branchlets have more or less spaced nodes. At each of these is a whorl of 4–20 leaves reduced to teeth (Fig. 1).

The family Casuarinaceae extends from Australia to the islands of the Pacific and to Southeast Asia. Formerly, all species were included in a single genus, *Casuarina*, but accumulated evidence from morphology, anatomy, cytology, and biogeography resulted in the recognition of four genera: *Allocasuarina*, *Casuarina*, *Ceuthostoma*, and *Gymnostoma*. The phylogeny of the *Casuarinas* is still uncertain, although *Gymnostoma* is probably the least specialized. *Ceuthostoma* and *Casuarina* have a moderate level of specialization, and *Allocasuarina* is the most specialized (Turnbull 1990; Maggia and Bousquet 1994).

Casuarinaceae comprises actinorhizal woody plants that can fix atmospheric N_2 when nodulated by an actinomycete *Frankia*. The family includes important tree species with excellent potential for afforestation in subtropical and tropical areas including arid and semiarid regions provided that water for irrigation is available (El-Lakany 1983a, b; Turnbull 1990). The main significance of Casuarinaceae is that, without any dependence on nitrogen fertilizers, they provide wood and fuel, and sometimes forage, improve soil fertility, are used as shelterbelts, and most often to stabilize desert and coastal dunes in many parts of the world (National Research Council 1984).

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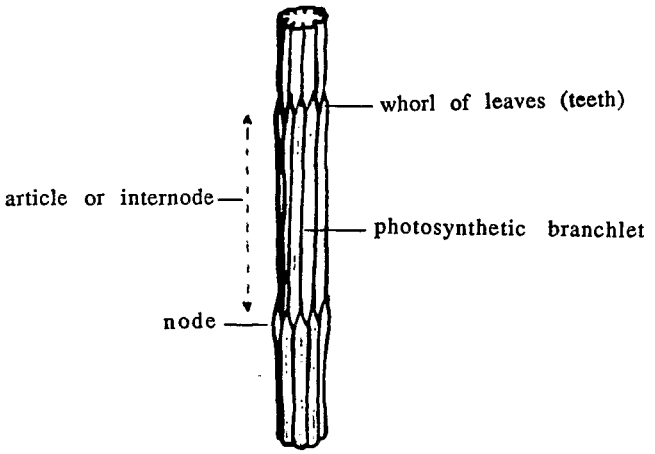

Fig. 1. Photosynthetic branchlet of *Casuarina equisetifolia.* (Wilson and Johnson 1989)

1.2 Conventional Practices for Propagation

Improving the yield and adaptability of Casuarinaceae appears to be an exciting challenge. Two types of approach have been proposed to achieve this goal: the first is to improve the microsymbiont, the second is to develop host plants with superior performance. This second approach comprises the selection of species and provenances, conventional plant breeding by using seedlings, and a number of techniques based on vegetative propagation and tissue culture, the latter strategy being at the core of our presentation. Vegetative propagation of *Casuarina* spp. through cuttings was tested in the 1970s (Somasundaran and Jagadees 1977; Hussain and Ponnuswamy 1980; Torrey 1983). El-Lakany and Shepherd (1984) successfully used stump propagation. Lundquist and Torrey (1984) obtained satisfactory results with mature softwood stem cuttings.

1.3 Need for Unconventional Methods

Our experiments confirmed that cuttings of *C. equisetifolia* were able to form roots, but showed that plants obtained from cuttings collected from mature trees often showed varying degrees of plagiotropic growth. To avoid the rejuvenilization steps which are required when dealing with mature trees, tissue culture techniques allowed the rapid multiplication of *Casuarina* genotypes. This field of research can accelerate tree improvement programs for the rapid multiplication of superior individuals. This was especially the case with clones selected by their high N_2-fixing potential (Sougoufara et al. 1992). Several important species cannot easily be improved through selection of elite trees for seed production owing to low seed viability. Tissue culture may eventually provide the primary

means for the clonal propagation of superior individuals from intra- or interspecific hybrids in *Casuarina glauca* × *cunninghamiana*.

The objective of this chapter is to provide an overview of the different tissue culture techniques that have been recently developed for Casuarinaceae with the goal of improving the yield in the field and to contribute to basic studies on the establishment and functioning of this actinorhizal symbiosis.

2 In Vitro Culture Studies (Table 1)

2.1 Micropropagation

2.1.1 Direct Shoot Regeneration

In our laboratory, direct shoot regeneration has been obtained from two types of explants: juvenile explants excised from young seedlings and immature female inflorescences collected on mature trees.

Juvenile Explants from Young Seedlings. Seeds of *C. equisetifolia* and *A. verticillata* were surface disinfected with 5% calcium hypochlorite for 20 min, then rinsed three times with sterile water. Seeds were germinated in aseptic conditions on medium solidified with agar. One month after germination, epicotyls and roots from plantlets were used as explants.

2.1.2 Axillary Shoot Proliferation from Epicotyl Explants

The original explants were epicotyls excised from *Allocasuarina verticillata* seedlings grown as indicated above. The explants which were, in fact, 3-cm-long typical casuarina branchlets with regularly (5–7) spaced nodes, were placed into MS (Murashige and Skoog 1962) salt medium supplemented with 6-benzylaminopurine (BAP, 0.22 μM) and indole-3-acetic acid (IAA, 0.57 μM). The axillary buds (9–12) located under the leaves at the lowest node grew first and then developed into branchlets (Fig. 2A). Each new branchlet developed other buds at the level of other nodes (Fig. 2B). Addition of maltose instead of sucrose to the medium resulted in a higher rate of shoot multiplication, improved biomass and chlorophyll content, and reduced callogenesis (Cao et al. 1990).

The unusual multiplication rate (40) could be traced to the original anatomic structure of the Casuarinaceae.

2.1.3 Adventitious Shoot Formation on Roots

Root culture of *A. verticillata* can be maintained over several months by successively subculturing excised roots. These cultured roots were used as a source of explants for shoot regeneration.

Table 1. Micropropagation and tissue culture in Casuarinaceae

Species	Explant	Medium and growth regulators (μM)	Results	Reference
Allocasuarina verticillata	Excised roots	MS + maltose 60 g/l NAA 0.25 mg/l BAP 0.1 mg/l	Shoot bud formation and multiplication	Phelep et al. (1991)
A. verticillata	Juvenile shoots	MS + maltose 60 g/l IAA 0.1 mg/l BAP 0.25-0.5 mg/l	Shoot multiplication	Cao et al. (1990)
Casuarina cunninghamiana *C. glauca* *C. equisetifolia*	Epicotyls	MS 2 iP 5 NAA 0.005	Callus formation and shoot formation	Abo El-Nil (1987)
C. equisetifolia	Immature female inflorescence	MS NAA 0.1-0.5 BAP 4–11	Axillary bud multiplication Rhizogenesis	Duhoux et al. (1986)

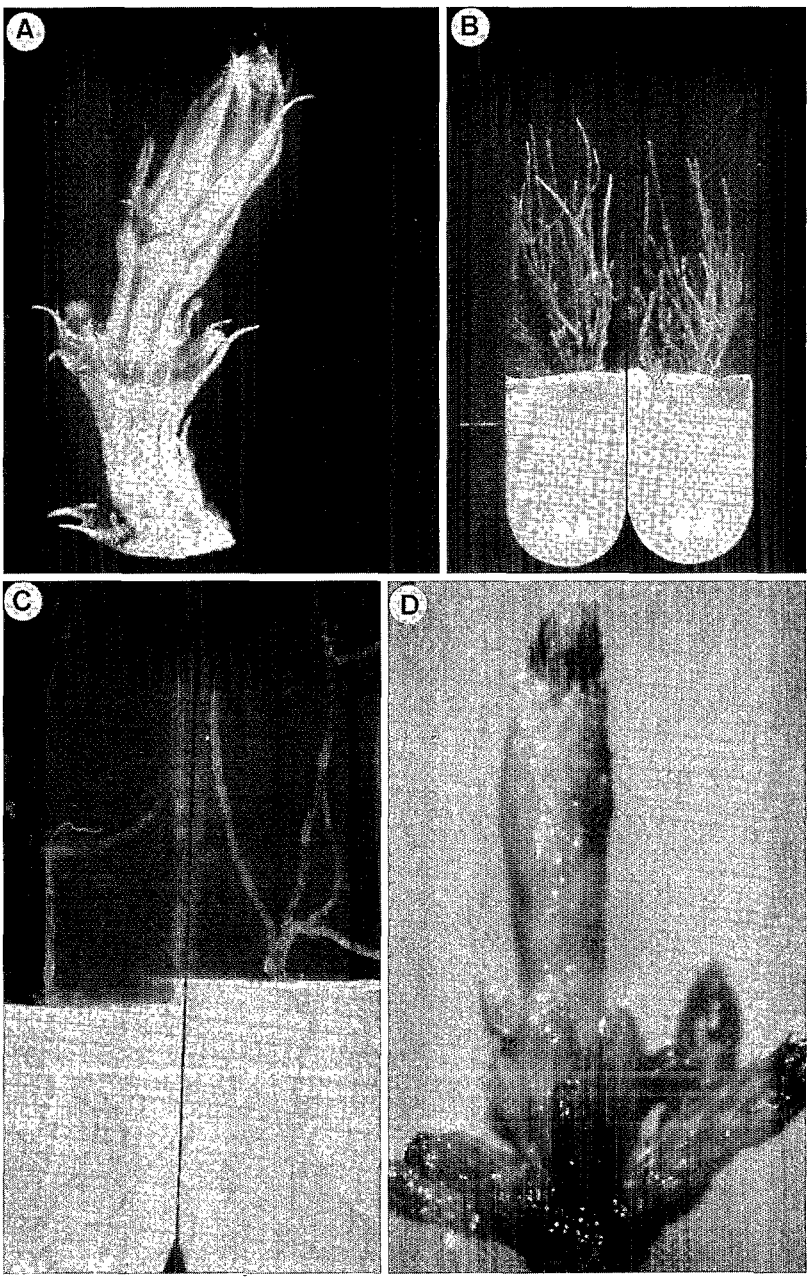

Fig. 2A–D. Micropropagation studies on Casuarinaceae. **A** Numerous axillary buds developing from the axil of leaves (teeth) of *C. equisetifolia*. **B** Clusters of neoformed shoots of *A. verticillata* after 5 weeks of culture. The original explant was an epicotyl of a seedling. **C** Root development on an excised shoot of juvenile *A. verticillata* in response to 0.9 μM IBA. **D** Elongated shoots from the basal part of an immature female inflorescence (IFI) of *C. equisetifolia*. Neoformed shoots originated from axillary buds in the axil of each leaf

Roots from seedlings of *A. verticillata* were excised and cultivated on BDA (Bonner and Devirian 1939) medium modified by Goforth and Torrey (1977) and supplemented with organic compounds (20 g/l sucrose, 100 mg/l mesoinositol, 2 mg/l glycine, 1 mg/l nicotinic acid, 0.5 mg/l thiamine·HCl, 0.5 mg/l pyridoxine·HCl). The explants (2-cm apical root tips) were cultured on solidified medium (4 g/l) in 9-cm diameter Petri dishes. When roots were 2 months old, the 2-cm apical tip of the main axis and secondary roots were excised and then subcultured on a fresh medium. This process was repeated five times and most of the roots still exhibited good elongation. Plant regeneration was obtained from in vitro culture of isolated roots (B. Allioux, Mémoire ENSH, Paris, 1990). Root explants (3 cm) without the root tips were placed flat on the surface of the medium in a Petri dish. BDA medium was tested with three combinations of BAP (0, 0.1, 0.2, and 0.4 μM). Root cultures were placed in continuous darkness or light conditions (16 h light/day, 5000 lx). White light was provided by daylight Sylvania 36 W/GRO fluorescent tubes.

BAP had no effect on shoot formation under light conditions (Fig. 3). On phytohormone-free medium almost 100% of explants produced an average of six shoots per explant. Dark conditions decreased both the frequency of shoot formation and the number of shoots (Fig. 3A, B).

Explants from Mature Trees. It is generally accepted that mature and old trees are difficult to multiply for reasons related to the reactivation of organ growth and the plagiotropic behavior of the explant (Durand-Cresswell et al. 1982). To overcome these obstacles, selected elite individuals are "rejuvenated" or trees are screened to find organs that are still "young". Taking into account the concept that "most trees have zones that retain a degree of juvenility longer than other areas of the tree" (Bonga and Durzan 1982), we used immature female inflorescences (IFI) as explants, hypothesizing that these organs would retain, at least partially, this juvenility character.

This strategy was successfully used in the case of an approximately 30-year-old *C. equisetifolia* tree. Explants made of immature female inflorescences were collected from this tree no more than 3 weeks before flowering (Duhoux et al. 1986). The inflorescences were disinfected and cultivated in a MS mineral medium supplemented with vitamins (5 mg/l; Nitsch and Nitsch 1965), sucrose (30 g/l), auxin (NAA) and a cytokinin (BAP), and solidified with a gelifying agent. The explants were placed in a vertical position with the cut axis of the inflorescence inserted 5 mm deep into the medium.

When placed in the culture medium, IFI exhibited the following modifications. After 2 weeks, the ovoid head formed a vegetative axis while the basal cylindrical peduncle swelled. After 4 weeks, basal vegetative meristems at the axil of IFI leaves evolved into new shoots (Fig. 2D). The most favorable medium contained 11.1 μM BAP and 0.05 μM NAA. In this experiment, we found that all the IFI reacted and gave buds, the mean number of buds being always in the range of five to six per IFI.

In another experiment, a change in the nature of the gel used to solidify the medium induced swelling of the ovoid head of IFIs and reversing of the floret meristems into vegetative meristems (Sougoufara and Duhoux, unpubl.). Under

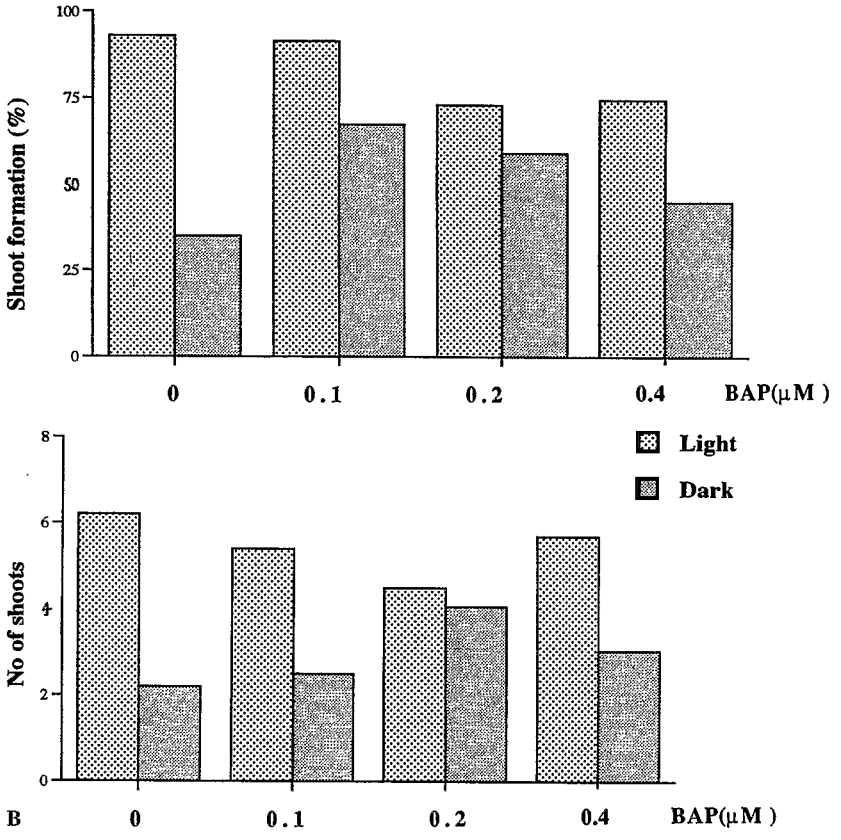

Fig. 3A, B. Shoot formation from in vitro culture of isolated root of *Allocasuarina verticillata*. Effect of exogenously applied BAP on adventitious shoot formation under light and dark conditions. **A** Percentage of shoot formation. **B** Average number of shoots per explant

favorable conditions, after 11 weeks of culture, 30 branchlets could be obtained from both vegetative and floral meristems. To stimulate elongation of the neoformed branchlets, the IFIs were transferred into a new medium to which activated charcoal (10 g/l) had been added. Three weeks later the branchlets were about 2–3 cm longer. They were then cut and transplanted onto a basic medium supplemented with auxin; they developed roots, thus giving vitroplants.

2.1.4 Rooting of the In Vitro-Produced Microshoots

Rooting of the in vitro-produced microshoots of *A. verticillata* was achieved using a half-strength MS basal nutrient medium solidified with 0.8% (w/v) Difco

Table 2. Effect of BAP and NAA concentration in the multiplication phase and effect of IAA and IBA concentration on subsequent rooting of 2–3-month-old *Allocasuarina verticillata* microcuttings

BAP and NAA level in the multiplication phase (μM)		Auxin concentration in rooting medium (μM)		Rooting (%)	Mean length of roots (mm)
BAP	NAA	IBA	IAA		
2.2	5.3	0.9		83	0.5
2.2	5.3		1.7	40	23.2
2.2	5.3		0.5	17	16.7
2.2	2.6	0.9		100	6.2
2.2	2.6		1.7	58	9.8
2.2	2.6		0.5	74	11.4

Bacto Agar (Fig. 2C). Various concentrations of auxin were tested for rhizogenesis and levels of BAP and NAA used during the multiplication phase were taken into account (Table 2). Within 3 weeks, 100% of microshoots rooted with 0.9 μM IBA. The effect of auxin level used in the previous multiplication subcultures was crucial to rooting. The number (data not given) and mean length of roots per explant were increased with IAA or NAA. When IBA was added to the medium the percentage of rooted explants was higher than with addition of IAA and NAA.

The acclimatization of plantlets after their transfer from in vitro to in vivo occurred during the rooting of the microcuttings in the soil within pots placed in a greenhouse under warm and highly humid conditions: 80 to 100% survival was obtained for all clones of species (*C. equisetifolia* and *A. verticillata*) tested.

2.2 Callus Culture and Shoot Regeneration

2.2.1 Callus Induction

The choice of the right explant is critical to achieve plant regeneration. To induce calli, cotyledons and hypocotyls were dissected from 4-5-week-old seedlings. Then cotyledons were transversally sliced in half and hypocotyls cut into 0.5-cm-long segments. These explants were cultured on MS medium with 2% sucrose, supplemented with vitamins (5 mg/l; Nitsch and Nitsch 1965), a cytokinin, BAP, and an auxin, 2,4-D. The medium was gelled with 0.8% (w/v) Difco Bacto agar. The explants were incubated in 10-cm diameter Petri dishes containing the medium (25 explants per treatment) and placed in the same environmental conditions as the juvenile explants (23 °C; under fluorescent light, 5000 lx; photoperiod: 12 h/day).

Calli appeared on the wound surfaces of most explants between 10 and 15 days after transfer onto the culture medium. Although both types of explants produced calli, there were differences between cotyledon and hypocotyl explants,

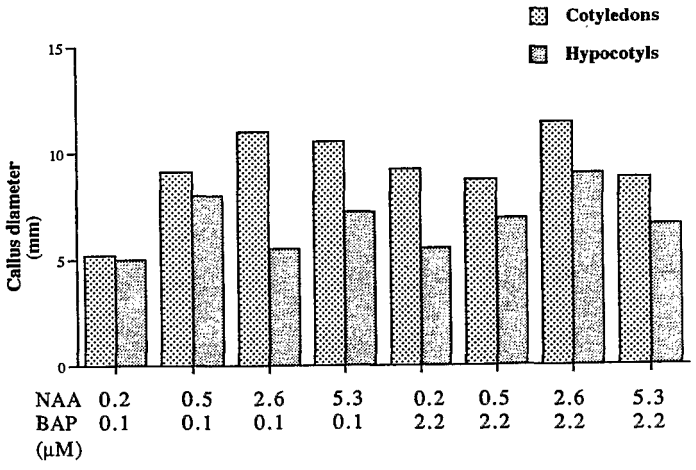

Fig. 4. Mean callus induction rate of explants (cotyledons and hypocotyls) of *Allocasuarina verticillata* after 48 days of culture

as shown in Fig. 4. For all the combinations of NAA and BAP tested, cotyledons induced rapid callus growth.

The morphology (green smooth vs. yellow rough-surfaced) and texture (fragile vs. compact) of the calli varied little and no differences were observed between calli originating from cotyledon and those from hypocotyls.

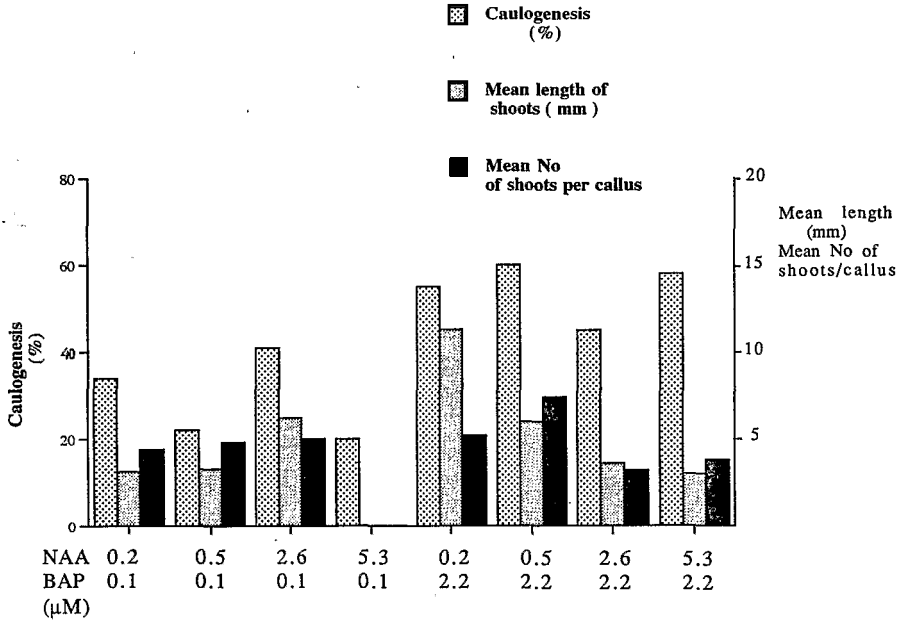

Fig. 5. Effect of growth regulator combinations on percentage of caulogenesis (buds), mean length of shoots, and mean number of shoots per callus in culture of *Allocasuarina verticillata* cotyledons

2.2.2 *Shoot Regeneration*

Figure 5 shows that various combinations of NAA and BAP induced caulogenesis from cotyledon calli. The regeneration of shoots from cotyledon-derived calli is illustrated in Fig. 6A. Only one combination (NAA 5.3 μM, BAP 0.1 μM) did not allow bud formation. Bud organogenesis was most frequent with high levels of BAP (2.2 μM) in combination with NAA (0.2, 0.5, 2.6, 5.3 μM). Half the organogenic calli did not develop, the others developed into shoots, necessitating

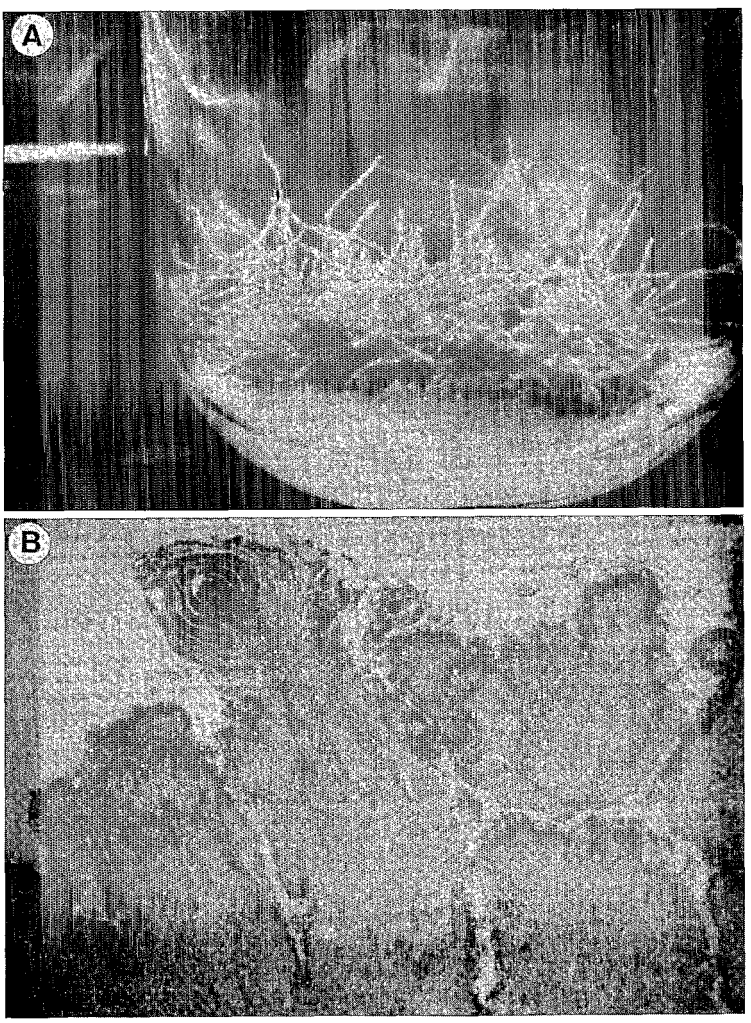

Fig. 6A, B. Callus production and shoot regeneration of *A. verticillata.* **A** Numerous shoots developed from hypocotyl-derived calli in a medium with 2.2 BAP and 0.2 μM NAA. **B** Anatomical aspect of organogenesis. Adventitious buds developed at callus surface

40 days of culture without any transfer to reach the mean length of 11.3 mm with the combination: NAA 0.2 μM, BAP 0.1 μM. Some of the shoots formed with this combination were 40 mm long. The mean number of shoots was between 3.8 and 7.4 per callus.

The regeneration capability of hypocotyl-derived calli and cotyledon-derived calli was similar. The highest number of morphogenetic responses was obtained with media containing 2.2 μM BAP. In these media, the mean number of shoots per hypocotyl-derived callus could reach as high as 16.

Histological examination revealed that in *C. torulosa* adventitious buds from cotyledon-derived calli developed mostly at the callus surface (Fig. 6B).

3 In Vitro Synthesis of Actinorhizal Nodules

In vitro-propagated plantlets and disinfected seedlings of *C. equisetifolia*, *C. glauca*, and *A. verticillata* were routinely tested for in vitro nodulation and nitrogen fixation. Rooted plantlets were inoculated with pure cultures of *Frankia* after their transfer into a nitrogen-free substrate (Hoagland and Arnon 1938). Two types of cultures were used: axenic nodulation using a sterile device described in an earlier study (Galiana et al. 1990), and nonaxenic nodulation using culture tubes Gibson assembly (Vincent 1970).

3.1 Nodulation in Axenic Conditions

The in vitro device for growing and inoculating test plants is described in Fig. 7A. Plantlets (seedlings or rooted microcuttings) were aseptically placed in the sterile device, with their roots inserted between the glass tube and a polypropylene support (Milcap France S. A., Nuaillé 49340 Trémentines France). Culture tubes (25 × 200 mm) were covered with cellulose stoppers (Fig. 8). The medium used was a half-strength N-free nutrient solution (Broughton and Dilworth 1971). The plants were placed in a culture chamber maintained at 28 °C (light 5000 lx, photoperiod: 16 h/day). Plantlets were inoculated by introducing 2 ml of a 3-week-old culture of *Frankia* equivalent to 20 mg of protein (Lowry et al. 1951) into the glass tube along the root system. Four or five months later, the plants were harvested to determine the number and dry weight of nodules, shoot dry weight, and total N content of shoots and roots.

3.2 Nodulation in Semiaxenic Conditions

Tubes containing Hoagland and Arnon (1938) medium solidified with agar were stoppered with an aluminum foil cap, autoclaved, and set in slanted position. Microcuttings were transferred to the tubes with their 0.5-1-cm-long radicles introduced onto the agar slope through a hole in the aluminum foil and the shoot

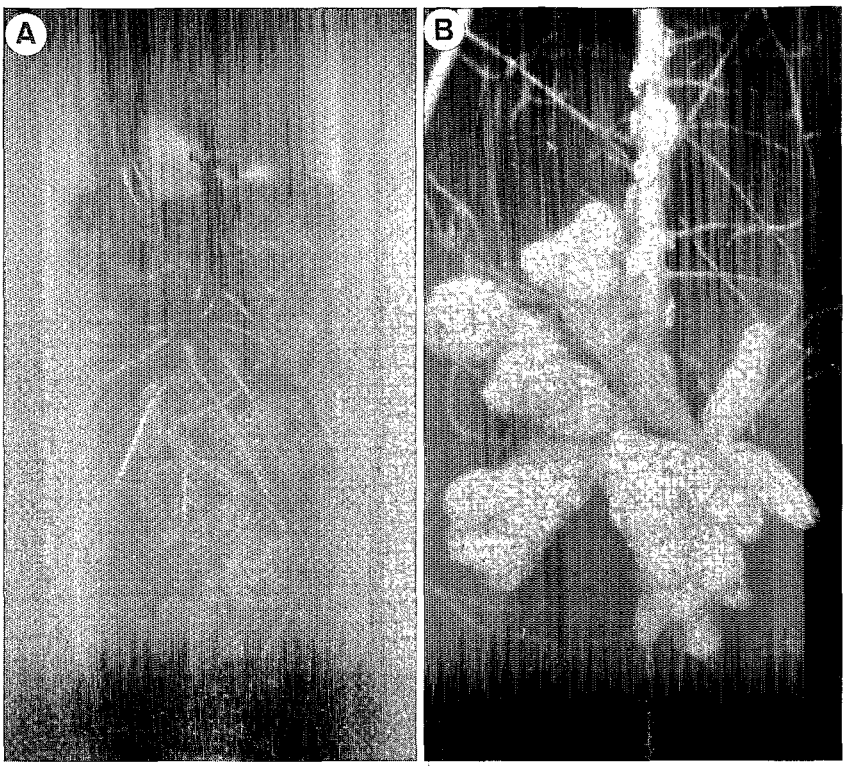

Fig. 7A, B. In vitro nodulation studies on Casuarinaceae. **A** Nodule with numerous lobes and nodule roots of in vitro-propagated *C. glauca* plantlet after 4 months of growth. **B** High magnification of a multilobed nodule of *A. verticillata* initiated in vitro

free outside the tube (Fig. 9). Figure 7B shows a multilobed nodule of *A. verticillata* obtained in this device.

In the first device, nodules were more easily observed than in the second one, but the nodule number was lower. Both devices allowed continuous and nondestructive observations to be made. They appeared to be most convenient (1) to study early steps of nodulation (Fig. 7A, B), (2) to select between the two partners of the symbiosis according to their early performances (which was the case of *Acacia mangium* and other nitrogen-fixing trees, Galiana et al. 1991), and (3) to investigate the specificity of the host plants and that of the associated *Frankia* strains.

A survey of the host specificity of three *Casuarina* spp. and three *Allocasuarina* spp. carried out by Sougoufara (Thèse d'Université, Nancy, 1990) in the devices described above showed that species of *Allocasuarina* failed to be nodulated by *Frankia* strains isolated from *Casuarina*. On the contrary, four *Frankia* strains isolated from *Allocasuarina* nodulated all *Casuarina* and *Al-*

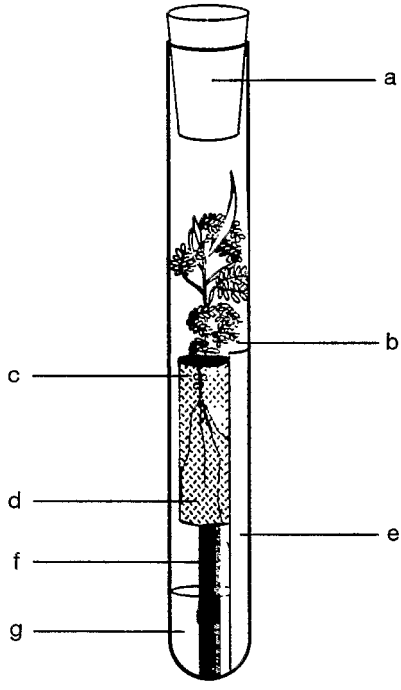

Fig. 8. Device for nodulation in axenic conditions; the plant is *Acacia mangium*. (Galiana et al. 1990). **a** Cellulose stopper; **b** plant placed at the edge of the support; **c** nodules; **d** polypropylene fiber support (Milcap); **e** fiber paper wick; **f** glass tubing; **g** nutrient solution

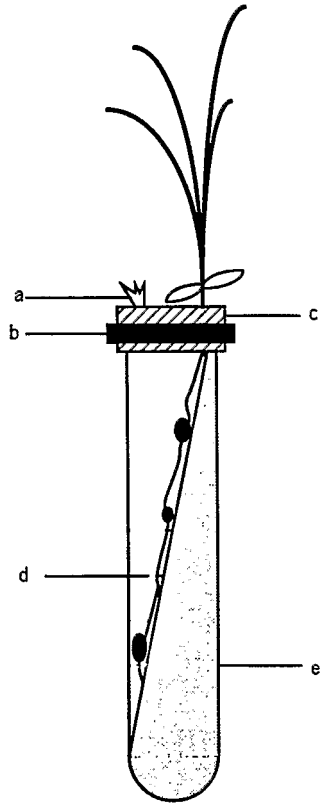

Fig. 9. Device for nodulation under semi-axenic conditions. (Vincent 1970). **a** Cotton wool plug for watering; **b** stronger rubber band; **c** aluminum cap; **d** seedling solution; **e** sloped seedling agar supplemented with charcoal

locasuarina spp. except *A. torulosa*. The survey suggested that species of the genus *Allocasuarina* were more specific in their *Frankia* requirements than species of the *Casuarina* genus, a conclusion already reached by Torrey and Racette (1989), who cultivated their plants in pots. Recently, Maggia and Bousquet (1994) confirmed the strict specificity of *Allocasuarina* species and inferred from their study of the phylogeny of Casuarinaceae that the narrower specificity could be interpreted as a more specialized feature and that evolution had proceeded towards narrower promiscuity and greater specialization.

4 Transformation of *Casuarina* Trees

Gene transfer into trees provides a means of genetic analysis that can bypass sexual barriers and, to some extent, circumvent the limitations of the long breeding cycles of trees. The strategies for tree transformation are based either on the use of *Agrobacterium* strains, or on direct introduction of foreign DNA by microprojectile bombardment (for reviews, see Hanover and Keathley 1988; Schuerman and Dandekar 1991; Jouanin et al. 1993).

Genetic transformation constitutes a valuable technology to accelerate improvement programs of *Casuarina* trees and to study the expression of actinorhizal symbiotic genes in transgenic plants. Both *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens* were used to achieve the transformation of two Casuarinaceae trees, *Allocasuarina verticillata* and *Casuarina glauca*.

4.1 Transformation by *A. rhizogenes*

Three strains of *A. rhizogenes* were used in preliminary experiments of inoculation of *A. verticillata:* an agropine strain, A4 (Moore et al. 1979), a cucumopine strain, 2659 (Davioud et al. 1988), and a mannopine strain, 8196 (Koplow et al. 1984). Two-month-old aseptic seedlings of *A. verticillata* were inoculated with these strains by wounding the hypocotyls with a needle dipped in the agrobacterial culture. Seven to ten days after inoculation, about 50% of the inoculated hypocotyls developed roots, showing a typical hairy root phenotype (high growth rate, extensive lateral branching, and lack of geotropism) due to the expression of the oncogenes of the T-DNA from *A. rhizogenes*. Presence of opines was detected in the transformed root extracts.

The roots transformed by the strain 2659 were then excised and grown on nutritive hormone-free medium. Shoot regeneration occurred spontaneously on 90% of the roots (Fig. 10A) and transgenic rooted plants were obtained within 5 months following plant inoculation (Phelep et al. 1991). The transformation was demonstrated by Southern blot analysis. Transgenic *Allocasuarina* plants have retained the ability to be nodulated by *Frankia* and they still fix nitrogen. Nevertheless, they exhibit an alteration of their phenotype which is characterized by an extensive lateral branching and a lack of geotropism (Fig. 10B). These

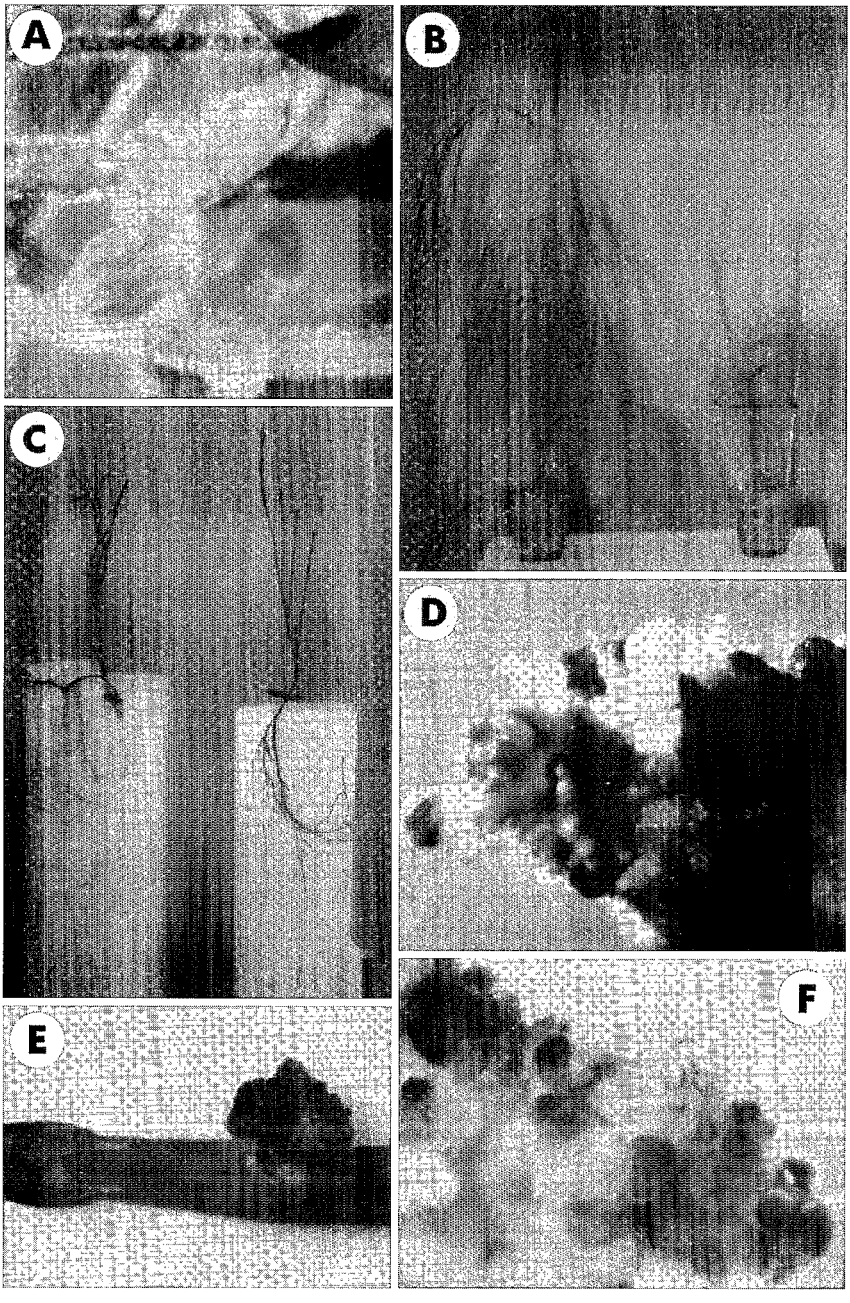

Fig. 10A–F. Transformation of *Allocasuarina verticillata* and *Casuarina glauca* using *Agrobacterium*. **A** Regeneration of shoots from 2659 transformed roots of *Allocasuarina verticillata*. **B** *Allocasuarina verticillata* transformed by 2659 (*right*) and control plant (*left*). **C** Composite plant of *Casuarina glauca* with a root system transformed by A4RS (*left*) and control plant (*right*). **D** Expression of the β-glucuronidase activity in a callus of *Allocasuarina verticillata* transformed by C58C1 (BIN19-GUSINT). **E** Expression of the β-glucuronidase activity in a callus emerging from an epicotyl of *Casuarina glauca* transformed by C58C1 (BIN19-GUSINT). **F** Callus of *Casuarina glauca* transformed by C58C1 (BIN19-GUSINT); few buds expressing the β-glucuronidase activity are visible

transgenic plants are currently used to study the expression of the β-glucuronidase gene under the control of heterologous nodulin promoters.

Using *Casuarina glauca* and the strain of *A. rhizogenes* A4RS (Jouanin et al. 1986), an alternative procedure was developed for rapid production of transgenic nodules. The gene transfer system was based on the work performed by Hansen et al. (1989) with the legume *Lotus corniculatus*. Young seedlings of *C. glauca* were wounded on the hypocotyl and inoculated with an overnight culture of A4RS. After 2 weeks, highly branched roots exhibiting a quick growth were observed at the inoculation site. The normal root system was then removed at the stem basis, while the composite plant was decontaminated by incubation in liquid medium with 500 mg/l of Cefotaxim (Claforan, Roussel). The plants were then cultivated in glass tubes containing nutritive medium (Fig. 10C) prior to inoculation with *Frankia*. Nodules generally appeared after 1 month. Using this procedure, transgenic roots and nodules expressing the β-glucuronidase gene under the control of 35S promoter were obtained (D. Diouf et al. 1995).

The possibility of obtaining transgenic *Casuarina glauca* from transformed roots was also investigated; 30-day-old plants of *C. glauca* were inoculated on the hypocotyl with a needle dipped in an overnight culture of the wild-type *A. rhizogenes* A4. Transgenic roots exhibiting a growth rate of 1 to 2.5 mm per day appeared 7 days after wounding. Three-week-old transformed roots were excised and cultured on nutritive medium without any growth regulator. No further growth was observed on these excised roots (F. Sylla and C. Franche, unpubl.). Additional studies are required to determine if the addition of growth regulators to the roots transformed by A4 could lead to the regeneration of transgenic shoots.

4.2 Use of *Agrobacterium tumefaciens*

Transfer of foreign genes in *Allocasuarina verticillata* and in *Casuarina glauca* using the disarmed strain C58C1(BIN19-GUSINT); (Vancanneyt et al. 1990) was studied. The plasmid vector carries the *nptII* gene conferring resistance to kanamycin and a derivative of the β-glucuronidase reporter gene (Jefferson 1987) expressed only upon transfer to the plant cells and not in *Agrobacterium*.

Mature zygotic embryos of *Allocasuarina verticillata* were surface sterilized by calcium hypochlorite treatment and wounded with a scalpel. After 3 days of cocultivation with an exponential culture of C58C1(BIN19-GUSINT), the embryos were transferred on shoot induction medium containing 100 mg/l kanamycin and 250 mg/l Cefotaxim. Calli emerging from the wounded edges were observed 3 weeks after co-cultivation and buds were differentiated 2 months later. These calli expressed β-glucuronidase activity (Fig. 10D), and the presence of the *uidA* reporter gene was demonstrated by PCR analysis. The possibility of obtaining nodules on transgenic rooted plants is currently being investigated.

The strain of *A. tumefaciens* C58C1(BIN19-GUSINT) was also used to transform *Casuarina glauca*. Hypocotyls, cotyledons, and epicotyls were excised from 1- to 2-month-old seedlings of *C. glauca* and cocultivated with the disarmed strain of *A. tumefaciens*. After 3 days, they were transferred onto callus induction

medium containing antibiotics. Calli growing on kanamycin and expressing the reporter gene activity appeared 3 to 4 weeks after transformation (Fig. 10E) and a few green buds were observed on 20% of them (Fig. 10F; Franche et al. 1994).

Several parameters influencing the transformation efficiency of *Casuarina glauca* were studied such as: addition of acetosyringone (0 to 100 µM) during cocultivation, time of cocultivation (1 to 7 days), pretreatment of the explants with high velocity microprojectiles (1.5 µM beads), age of the explants (15 to 60 days), and pH of cocultivation (5 to 6). Optimal transformation was achieved when the explants were excised from 1-month-old seedlings and cocultivated for 3 days with *A. tumefaciens* in the presence of 25 µM of acetosyringone, at pH 5.6 (Franche et al. 1994). Although a pretreatment of the explants by high velocity microprojectiles created additional wounding sites, it had no effect on the efficiency of transformation (Lappartient and Franche, unpubl.); this result suggests that either the T-DNA could not be transferred into the wounded cells or that the target cells were too damaged to initiate callus growth. A range of hormone conditions are currently being investigated to obtain regeneration of shoots from transgenic calli of *C. glauca*.

Tissue culture techniques can also assist the transfer of DNA to plants via *Agrobacterium tumefaciens* or *A. rhizogenes*. Transgenic casuarinas obtained by this means constitute an ideal material for basic research, especially with the objective of determining the regulation and specific functions of the actinorhizal plant symbiotic genes.

Transformation technology via *A. tumefaciens* depends on the availability of a regeneration procedure such as differentiation of shoots from callus or adventitious caulogenesis. Transformations of *A. verticillata* and *C. glauca* were obtained with this procedure (Le et al. in prep.).

5 Summary and Conclusions

Since Casuarinaceae appear to be an easy plant material to handle in vitro, two approaches are worth developing. The first approach is based on exploiting natural genetic variability. Casuarinaceae species were found to be easily micropropagated and culture of immature female inflorescences was developed for mature trees. Application of rapid in vitro propagation of elite *Casuarina* trees with improved saline or drought tolerance and a high ability to fix atmospheric nitrogen, would have a tremendous impact on the afforestation of semiarid zones and would significantly improve forest productivity.

The second approach is based on the application of gene transfer techniques to casuarinas (Phelep et al. 1991; Franche et al. 1994). Introduction of new traits such as herbicide and disease resistance, as well as modifications of wood quality, could be considered for casuarina trees. Developing gene transfer systems for Casuarinaceae would also be of interest in the exploration of the establishment and functioning of the symbiosis between the actinorhizal trees and *Frankia* (Franche et al. 1994).

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