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Propagation of *Casuarina equisetifolia* through axillary buds of immature female inflorescences cultured in vitro

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Abstract

The study of the actinorhizal symbiosis in *Casuarina equisetifolia* requires an homogenous plant material. Consequently, we devised a method of micropropagation based on the use of immature female inflorescences (IFI) as explants. IFI excised from an adult tree formed multiple buds after 4-week incubation on Murashige and Skoog medium with 0.05 µmol l^{-1} NAA and 11.1 µmol l^{-1} BAP. The axillary buds evolved into 5-6 cm long shoots 5 weeks after the transfer of IFI on a similar medium except for the addition of activated charcoal. Rooting of the shoots was obtained on a third medium, without BAP or charcoal, but with 1 µmol l^{-1} NAA. The plantlets were transferred into soil. Their growth was satisfactory and no plagiotropic tendency was observed.

Abbreviations

BAP : 6-benzylaminopurine ; 2,4-D : 2,4-dichlorophenoxyacetic acid; IAA : 3-indoleacetic acid ; IFI : immature female inflorescences ; NAA : α-naphtaleneacetic acid.

Introduction

Casuarina equisetifolia is widely used in tropical and subtropical countries for dune stabilization, establishment of shelterbelts and production of fuel wood (National Academy of Sciences 1984; Midgley *et al* 1983). The ability of *C. equisetifolia* to grow on nitrogen-poor soils is due to its association with *Frankia*, the symbiotic N_2-fixing actinomycete forming nodules on its roots. We have already shown that the N_2-fixing potential of *C. equisetifolia* exhibited large variations (Gauthier *et al* 1985). Such a variability is undesirable when one needs an homogenous plant material for experimental purposes, such as comparing the effectivity of *Frankia* strains; the solution is then to obtain copies of a given individual, using proper methods of vegetative propagation. On the other hand, one can attempt to exploit the large variability of *C. equisetifolia* populations by selecting fast-growing and actively N_2-fixing specimens and multiplying them using also proper methods of vegetative propagation. Conventional vegetative propagation using cuttings has been used for several years (Somasundaran and Jagadees, 1977; Hussain and Ponnuswamy, 1980). Recently El-Lakany and Shepherd (1984) successfully used stump propagation; Lundquist and Torrey (1984) obtained satisfactory results with mature softwood stem cuttings. Our experiments (not reported here) also indicated that cuttings of *C. equisetifolia* were able to form roots, but we found that cuttings from mature trees often showed varying degrees of plagiotropic growth. 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 Duran 1982), we used immature female inflorescences as explants, hypothesizing that these organs had retained, at least partially, this juvenility character. To date, no other type of explant has been tested.

Material and Methods

Explants were collected on the same tree, 10-15 year-old *Casuarina equisetifolia*, growing at ORSTOM research station Bel Air, Dakar, Senegal. The explants consisted of immature female inflorescences (IFI) at the stage preceding flowering by at least 3 weeks (Fig. 1). An IFI comprises two parts : an ovoid head 1 mm of diameter bearing whorls of bracts with a cymule in the axil of each bract (Flores and Moseley 1982) and a basal cylindrical peduncle bearing whorls of bracts with a vegetative meristem on the axil of each bract.

In the climatic conditions prevailing in the neighborhood of Dakar, flowering of *C. equisetifolia* occurs in February, which should restrict the period when IFI can be sampled to a few weeks in February. However, we found that the flowering stage of *C. equisetifolia* could be extended up to 6-8 months by watering the trees, thus permitting the collection of IFI throughout a long period of the year. However, the development of axillary buds was delayed when IFI were sampled later than February.

To facilitate the transportation of IFI to the lab, segments of branchlets bearing IFI were severed from the trees and the IFI were excised in the lab just before being washed in running tap water and immersed in 0.01 % (V/V) SDS (dodecyl sodium sulfate, SIGMA L 5750) for 30 min. Following surface sterilization in 0.1 % HgCl for 1 min, IFI were rinsed thoroughly in sterile distilled water.

To eliminate the tissues that could have been injured during the sterilization procedure, the lower extremity (1-2 mm for each IFI) was cut off. IFI were then placed vertically onto the nutrient medium, their base being driven into it 2-3 mm deep.

Three culture media were sequentially used.

A medium for caulogenesis : Murashige and Skoog (1962) basal medium with vitamins (Nitsch and Nitsch

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1965) 30 g l^{-1} sucrose, 0,05 µmol l^{-1} NAA, different amounts of BAP ranging from 0 to 13.2 µmol l^{-1} (Table I) and 8 mg l^{-1} Difco Bacto agar.

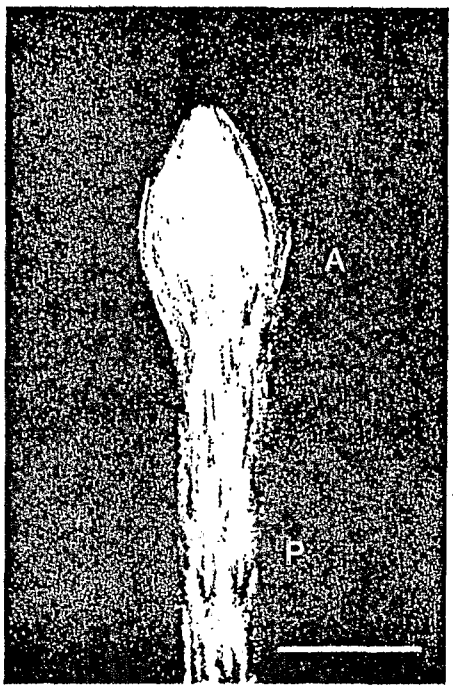

Fig. 1 Immature female inflorescence (IFI) of *Casuarina equisetifolia* used as explant. A, ovoid apex with floret meristems ; P, penduncle (Bar = 1 mm).

B medium for shoot elongation : same as A medium but with 20 g l^{-1} activated charcoal (Merck 2186).

C medium for rhizogenesis : same as A medium but without BAP or activated charcoal; we compared the following concentrations of NAA : 0.05, 0.15, 0.25, 0.5, 1 µmol l^{-1} (Table 2).

All three media were adjusted to pH 5.5 before autoclaving at 110°C for 30 min.

Following sterilization IFI were aseptically placed in 10 cm petri dishes (10 IFI per dish) containing 20 ml of A medium. After 4 weeks IFI were transferred into 23 x 15 mm tubes (one IFI per tube) containing 25 ml of B medium.

Nine weeks later the elongated shoots originating from the axillary buds formed in A medium and developped in B medium were excised thus giving microcuttings. The microcuttings were placed vertically in 23 x 15 mm tubes (one per tube) containing 15 ml of C medium.

All types of cultures were incubated in a growth chamber at 28±1°C with a 17 h photoperiod (4,000 lux).

Results

When placed in A medium, IFI exhibited the following transformations. After 2 weeks the ovoid head formed a vegetative axis while the basal cylindrical peduncle swelled. Later, i.e. after 4 weeks, 5-6 vegetative buds appeared at the base of the IFI (Fig. 2).

Fig 2. Numerous axillary buds formed at the base of the IFI after 4 weeks on A medium (Bar = 1 mm).

Table 1 shows that addition of a cytokinin (BAP) to the medium containing an auxin (NAA, 0.05 µmol l^{-1}) markedly affected bud initiation. The most favorable medium contained 11.1 µmol l^{-1} BAP and 0.05 µmol l^{-1} NAA. In the experiment reported in Table 1 we found that all the IFI reacted, that is, gave buds, the mean number of buds being always in the range of 5-6 per IFI, in the medium with 11.1 µmol l^{-1} BAP.

B - Shoot elongation

Five weeks after being transferred onto B medium axillary buds evolved into green, vigorous-looking 5-6 cm long shoots (Fig. 3). However in the absence of activated charcoal no bud development nor shoot elongation were observed.

C - Root formation

After being excised, shoots obtained in B medium with 11.1 µmol l^{-1} BAP, were used as microcuttings and placed onto C medium (Table 2).

With 0.5 to 1 µmol l^{-1} NAA, a few thick, pink, adventitious roots were formed (Fig. 4). We never observed any callus proliferation at the base of the microcuttings.

D - Transplanting and acclimating

Rooted microcuttings grown in C medium were removed from the test tubes after 4 weeks and transplanted from the agar medium into soil in pots placed in a greenhouse under warm and highly humid conditions. The autotrophic development of the plantlets was satisfactory, and no plagiotropic tendency was observed.

Table 1 Effect of BAP concentration on the mean number of buds originating from each IFI of *Casuarina equisetifolia* cultivated on A medium (0.05 µmol l^{-1} NAA in all media)

Concentration of BAP (µmol l^{-1})	Number of replications	Number of reacting IFI	Mean no of buds per IFI
0	47	47	2.59 ±0.29
4.4	49	49	1.53 ±0.17
8.8	47	47	3.76 ±0.35
11.1	47	47	5.78 ±0.68
13.2	46	46	4.22 ±0.34

Mean values ±SE in last column differ significantly, P = 0.05 (test of Newman and Keuls in Snedecor and Cochran, 1971).

Fig. 3 Elongated shoots originating from axillary buds 5 weeks after transfer to B medium (Bar = 1 mm).

Table 2 Effect of NAA concentration on rooting of microcuttings 3-4 weeks after placement onto C medium (no BAP added).

Concentration of NAA (µmol l^{-1})	Number of replicates	Number of rooted microcuttings	Mean no of roots per microcuttings
0.05	40	0	0
0.15	50	0	0
0.25	47	0	0
0.5	43	14	2
1.0	83	21	2

Fig. 4 Root formation on a microcutting 2 weeks after placement onto C medium (Bar = 1 mm)

Discussion and Conclusion

The ability to vegetatively propagate trees is associated with juvenility (Franclet 1979 ; Franclet *et al* 1980). Since it is recognized that some parts of the trees may be mature or senescent while other portions still display juvenile characteristics (Bonga and Durzan 1982), it can be hypothesized that explants with this desirable characteristic should be more easily propagated. Assuming that IFI of *C. equisetifolia* exhibit this property, we initiated the investigations whose results are reported here. The success of our experiments indicate that IFI of *C. equisetifolia* (and probably also of other Casuarinacea) constitute satisfactory explants when dealing with mature trees. Interestingly enough it has been shown that the period of time during which mature trees bear IFI could be dramatically extended, just by irrigating the plants.

In the case of herbaceous plants, it is known that floral meristems grown *in vitro* can revert back to vegetative meristems; such a reversion has been reported in *Nicotiana tabacum* and *N. glutinosa* (Martin *et al* 1967), *Beta vulgaris* and *Brassica oleracea* (Margara 1982), *Allium porum* (Doré and Schweisguth 1980). The vegetative shoots originating from IFI of *C. equisetifolia* are not a result of such a process, since vegetative buds already exist at a latent stage at the sterile base of the IFI, the development of these buds into shoots being induced by the nutrient medium where IFI are placed. With *C. equisetifolia*, only low amounts of auxins are required to initiate the development of vegetative meristems, whereas much larger additions of IAA (45 µmol l^{-1}) or the use of a strong auxin (2,4-D) are required to get the reversion of floral meristems of herbaceous plants.

Elongation of newly formed shoots occurred only when activated charcoal was added to the nutrient

medium. The beneficial effect of such an addition is not yet clear and several hypotheses to explain it have been proposed (Mission *et al* 1983; Bonga and Durzan 1982).

The method of micropropagation of *C. equisetifolia* based on the use of explants consisting of IFI that is presented here is easy to handle and could probably be adopted on a large scale when the rooting of the shoots is improved. Investigations are under way to improve the shoot branching and the development of the root system of the microcuttings, to find out the most appropriate stage for excising IFI from the trees and to check the nodulating ability of the plantlets when transplanted to pots.

So far, the type of explant used here for micropropagation of *Casuarina equisetifolia* has never been experimented except by Bonga (1984). However, if this author obtained the formation of adventitious shoots in cultures of sections of immature cones of *Larix decidua*, he did not observe rooting in any of the shoots.

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