30. MICROPROPAGATION OF CASUARINA

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1. INTRODUCTION

Casuarina species are members of the family Casuarinaceae, which was considered to be a primitive angiosperm, but lately was found to be closely related to Betulaceae and Fagaceae (10, 20, 25, 30). Casuarinaceae is a small family of four genera, which includes many species of evergreen trees and shrubs with a wide natural distribution in Australia, Southeast Asia, Polynesia and New Caledonia (14, 26). Casuarinas are characterized by scale-like univeined leaves arranged in whorls on green spreading branchlets (3, 25). Flowers are unisexual and plants are monocious in most species (23). The staminate flowers are borne in catkins and the pistillate flowers are spherically arranged on cylindrical inflorescent heads (cones). Seeds are formed inside samara fruits, which are enclosed by two valves (20, 21, 24, 29). Casuarinas are able to fix atmospheric nitrogen through symbiosis with an actinomycete (Frankia) (11, 23).

Casuarinas are important biological components in moist coastal areas or deserts in their natural habitat which spreads from temperate to tropical climate zones (24, 29). Their ability to tolerate inhospitable environments has been greatly enhanced by their high tolerance to salt and drought conditions and by their ability to fix atmospheric nitrogen. Some species are characterized by rapid foliage and root growth, which enable them to capture their sites efficiently and to play a significant ecological pioneering role (9, 12, 14, 16, 23).

Considerable attention has been given to casuarina because of its good potential for afforestation in regions

with poor forest resources (3, 9, 13, 14). In large-scale plantations, casuarinas can be easily established and managed (14). They can be utilized for sand stabilization, shelterbelt, fuelwood, soil rehabilitation and animal fodder (9, 13, 14, 18, 24, 28, 29). Their fast growth and excellent fire wood qualities suggest good potential to overcome fuelwood shortage of many underdeveloped countries (17). Casuarina species that fix atmospheric nitrogen through actinomycete symbiosis have low nutrient requirements and restore soil fertility to agriculturally depleted lands (9, 23).

2. CASUARINA GENETIC TREE IMPROVEMENT

Several casuarina genetic tree improvement programs were established to explore the possibilities of improving and introducing this species into the agroforestry systems of some tropical and subtropical countries (13, 14, 28). Tree improvement starts with provenance trials of seeds from wild or improved populations, followed by cycles of selection and breeding aimed at increasing the frequency of desired genes in subsequent generations (19). Provenance trials showed high inter- and intra-specific genetic variability in a number of important characters, such as; growth rate, stem straightness, and tolerance to adverse environmental conditions (17). Selections were made for several ecotypes. Significant genetic gains were produced and found to be suitable for large-scale plantations (13, 14, 29).

Salt and drought tolerance are among the most desirable traits in breeding of casuarina (9, 14, 17). El-Lakany and Luard (16) found that survival after transplanting, and growth rate of casuarina were affected by soil salinity tolerance. Species were ranked according to their salt tolerance as follows: _C. glauca_, _C. obesa_, _C. equisetifolia_, _C. glauca_ x _C. cunninghamiana_ hybrid, _C. cristata_, _C. cunninghamiana_, _C. stricta_, _C. littoralis_, _C. torulosa_, and _C. decaiseneana_ (16). _Casuarina cunninghamiana_, _C. glauca_ and _C. equisetifolia_ are the most planted species and all are salt tolerant (9, 14, 17). _Casuarina glauca_ was reported to

be also tolerant to water logging, calcareous or heavy clay soils (4, 15, 17).

In a study comparing *C. cunninghamiana* with *C. glauca*, El-Lakany and Shepherd (17) found that height, diameter, branch length and number of cladodes per branch varied significantly within and between the species. Generally, *C. glauca* grew faster than *C. cunninghamiana*. The latter had a thicker stem diameter and less total biomass (14, 17). Variations observed in this study suggested a good potential for genetic gains by selection and breeding for shelterbelt plantation or timber production.

3. CASUARINA VEGETATIVE PROPAGATION

Natural regeneration of casuarina is by seed or coppice (24). It is propagated commercially by sowing seeds in nursery beds. Vegetative propagation by air layering, suckering and rooting of cuttings is possible but the rate of multiplication is slow (14, 24, 34, 35). Application of auxin to girdled air layers stimulated rooting in young trees if treated between April and June, but rooting decreased sharply with aging of ortets (34). Treating lateral tender cuttings of *C. equisetifolia* with a commercial rooting hormone induced rooting in 42% of treated cuttings after 104 days of planting, while untreated cuttings did not root (35).

Techniques for vegetative propagation can be very useful tool in tree improvement program to bring together all superior and desirable clones for clonal testing and breeding. In afforestation and reforestation programs, a clonal plantation of uniform superior clones is a promising method to achieve high levels of genetic gain in the shortest possible time by capturing all additive and dominance gene effects (19). *In vitro* Vegetative propagation may provide better multiplication rates than conventional methods of air layering or rooting of cuttings as with many horticultural plants (32). So far, attempts to culture cells or tissues of casuarina have not been reported (5, 6, 8, 31).

CASUARINA TISSUE CULTURE

Micropropagation was achieved by shoot regeneration m stem segments and callus cultures of juvenile seedlings C. cunninghamiana, C. glauca, their interspecific hybrid C. equisetifolia and of mature flowering male and female es of C. equisetifolia (AboEl-Nil, unpublished data).

. Explants

Two types of explants were used to regenerate shoot and induce callus formation.

4.1.1. Seedling explants. Seeds were germinated in ıt: vermiculite: perlite mixture of 1:1:1, watered by ɔ-irrigation and incubated in 27°C under 16 hours photo-riod. Epicotyls were excised and used as explants when ey were about 3cm long.

4.2.2. Mature trees. Stem tips of about 5cm long re obtained from lateral branches high in the crown of five ar old flowering trees grown in the field.

2. Sterilization and establishment.

Explants were washed in 3% Alconox solution for 10 nutes then sterilized in 1.2% sodium hypochlorite solution r 10 minutes. Explants were then rinsed five times in erile water in a sterile atmosphere. Segments of about 4mm re excised from stems and cultured on the surface of agar lled media. The medium used was that of Murashige and oog (MS) (33), except for the growth regulators which ried from one culture stage to the other as indicated low. All cultures were incubated at 21±1°C under 16-hour hotoperiod at 70 $\mu E/m^2/sec$ from cool white fluorescent amps.

.3. Callus initiation.

Callus was induced from juvenile and mature stem egment explants on MS medium supplemented with 5.0 µM imethylallyl aminopurine (2iP) and naphthaleneacetic acid NAA) (Table 1). Callus growth was induced on media supple-ented with 0.005 µM NAA, while buds and callus were formed t lower concentrations of NAA (Fig. 1 A, B). Juvenile xplants produced more buds than mature explants of C.

equisetifolia. At high NAA concentration of 0.5 μM only green friable callus was formed.

TABLE 1. Morphogenesis of Casuarina equisetifolia, C. glauca, C. cunninghamiana, C. glauca x C. Cunninghamiana hybrid stem segment explants and C. equisetifolia mature stem segments on MS medium supplemented with 5.0 μM 2iP and three concentrations of NAA

Explant	NAA concentrations in μM			
	0.5	0.05	0.005	0.0
Seedling epicotyl segments	Green callus and small roots	Few buds and callus	Buds and callus	Elongation of explants
Mature stem segments	Friable green callus	Few buds and callus	Buds	Elongation of explants

4.4. Organogenesis

Buds were regenerated from callus tissue and stem segment explants on MS media containing 6-benzylaminopurine (BA) at concentrations from 11.0 to 2.2. μM combined with indoleacetic acid (IAA) at 0.5 μM (bud induction medium) (Fig. 1 C, D, F) and (Table 2). In two consecutive subcultures at six-week intervals, buds continue to regenerate and elongate in the absence of exogenous auxin. Bud formation frequency was lower and callus growth was slower in the absence of auxin than when media was supplemented with auxin. Exogenous cytokinin was essential for bud differentiation from callus and stem segments. At the high auxin concentration of 0.5 μM, a dark green friable callus formed on cultured explants of mature trees. This callus did not regenerate buds readily. Dark green friable callus differentiated buds at low frequency when cultured three times on a medium containing 4.4 μM of BA. Callus on explants of juvenile or

ecimens, cultured on media with 5 μM of 2iP combined to 0.005 μM of NAA, was granular in texture, pale slow growing (Fig. 1 E). This callus regenerated ily on a wide range of auxin and cytokinin combina-

ls elongated into shoots upon long incubations on bud ı medium or transfer onto the same basal medium ng 2.2. μM of BA and 3 g/l of activated charcoal G).

Morphogenesis of Casuarina glauca callus on MS medium supplemented with two concentrations of IAA and three concentrations of BA

	BA concentrations in μM			
ra-	11.0	4.4	2.2	0.0
8	Shoots with axillary branching and callus growth	Shoots, callus, no branching	Few shoots pale green callus growth	Roots and callus growth
.0	Shoots with axillary branching	Shoots axillary branching	Shoot with no branch-ing	Poor callus growth

Rhizogenesis

Roots were formed frequently on stem segment explants on callus surface in contact with the medium when cul-d on media with NAA at 0.5 μM or IBA at 6 μM. This rvation indicated that the genus is easy to root in ure and that rooting requirements are broad. Rooting of ıgated shoots was induced at high frequency on MS medium :aining 6 μM of IBA and 3g/l of activated charcoal (Fig. 1

Rooting and root growth was achieved after four-week ubation on rooting medium. Rooted shoots were established ra vitrum by transferring into a soil mixture of peat:

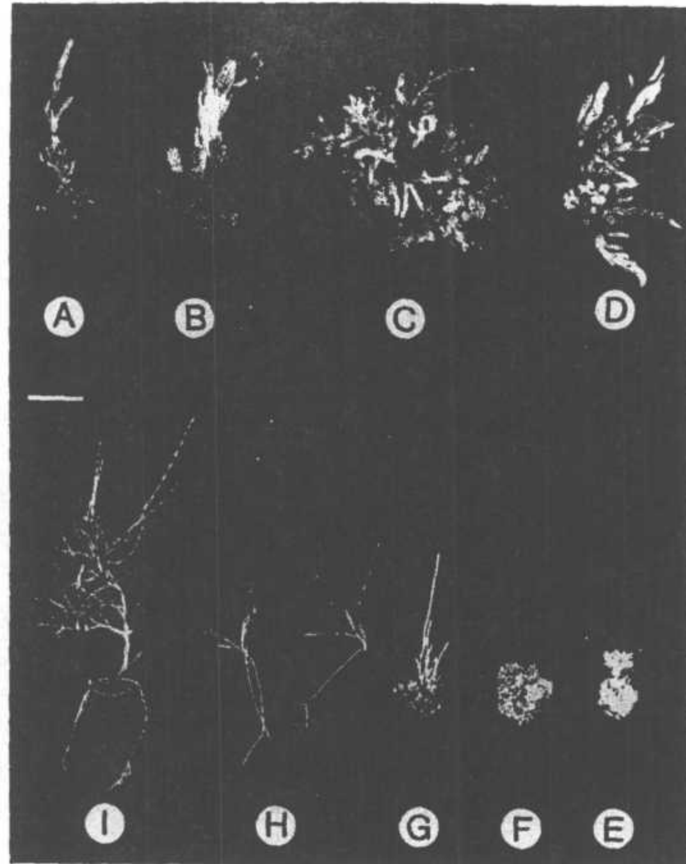

FIGURE 1

In vitro morphogenesis of Casuarina spp. Sieb. A. Callus formation at base of epicotyl explant cultured on MS medium supplemented with 5.0 µM 2iP and 0.05 µM NAA; B. Callus formation at cut ends of a mature stem segment explant cultured on the same medium as in A.; C. Adventitious bud formation from callus growing on medium containing 4.4 µM BA and 2.8 µM IAA; D. Proliferation of axillary buds from epicotyl explant cultured on MS medium containing 5.0 µM 2iP and 0.005 µM NAA; E. Callus culture derived from an epicotyl explant grown on the same medium as in D.; F. Bud differentiation (organogenesis) on callus cultured for 6 weeks on MS medium containing 11.0 µM BA and 2.8 µM IAA; G. Multiple elongated shoots growing on MS medium with 2.2 µM BA and 3 g/l activated charcoal; H. Rooted shoots after 3 weeks incubation on MS medium with 6.0 µM IBA and 3 g/l activated charcoal; I. A plant after extra vitrum establishment. (Scale Bar represents 5 mm for A and C, 2 mm for B, 4 mm for D, and 10 mm for E, F. G. H and I).

perlite: vermiculite at 1:1:1 ratio and incubation in 95% relative humidity for two weeks. Established plantlets were transferred to the greenhouse for further growth before field outplanting. Foliage grown *extra vitrum* was morphologically distinguishable from that formed *in vitro*. The former was much coarser and had less branching than the latter. In the early stage of greenhouse growth, plantlets had from one to four main stems that dominated the rest of the excessively branched crown (Fig. 1 I), while sexually produced seedlings usually only had one main stem with strong apical dominance.

5. CONCLUSIONS

The potential of *Casuarina* spp. for micropropagation by organogenesis has been established. Two approaches were used with juvenile and mature explants alike. One was callus induction followed by bud differentiation, and the other was direct bud induction on stem segment explants. Cultural requirements for regeneration of casuarina were very broad since callus and bud differentiation was achieved on a range of auxin and cytokinin combinations.

It is a prevailing dogma among tissue culture specialists that mature woody and tree species of gymnosperms and angiosperms are difficult to micropropagate (5, 6, 7, 22). A number of mature tree species required special rejuvenation treatments to facilitate their vegetative propagation by tissue culture (2, 22). Casuarina was found to be easy to micropropagating without the special rejuvenation treatments required for many mature tree species.

Application of rapid tissue culture propagation of elite casuarina trees, that are saline and drought tolerant and suitable for economic use as shelterbelt or for wood production, would have a tremendous impact on tree improvement programs and eventually on reforestation (El-Lakany, pers. comm.). Micropropagation of casuarina can be immediately utilized for clonal propagation of unique full-sib hybrids for clonal testing, for multiplication of elite trees that are scarce or endangered, and for germplasm preservation

and exchange. Micropropagated plants had more basal branches than seed propagated plants. This trait may be of significance for using casuarina in shelterbelt plantations in which excessive basal branching and wide crown are highly desirable characters in contrast to timber production in which absence of basal branching with narrow crown is required (13).

Major research problems remain to be solved before large-scale micropropagation and outplanting is possible. Induction of somatic embryogenesis (1) in which production costs may be reduced is of special value to cost-effective agroforestry. Confirmation of physiological uniformity and genetic fidelity of plants produced by micropropagation is a prerequisite for any commercial application of this technology. Lastly, automation of in vitro and extra vitrum manipulations, and outplanting mechanization technologies are critical to achieve large-scale production of plantings for forestry applications.

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