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In Vitro Propagation of Salt-Tolerant Wild Rice Relative, *Porteresia coarctata* Tateoka

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Abstract. Micropropagation of *Porteresia coarctata* Tateoka, a wild relative of rice with useful genetic traits of salinity and submergence tolerance, was achieved through nodal segment culture. Woody Plant (WP) medium supplemented with benzyladenine (5.5 μM) and kinetin (2.3 μM) gave the greatest response to initiation and multiplication. The multiplication rate of 11 shoots/explant with an average shoot length of 3.5 cm was observed after 8 weeks of culture period. The rooting response was observed simultaneously in the multiplication media, but subsequent establishment was poor. When the in vitro raised shoots were transferred to optimal ½ WP and ½ MS media with 10.7 μM α-naphthaleneacetic acid, the rooting response was enhanced. Such rooted plants were hardened successfully under field conditions.

Key Words. Conservation—Mangrove associates—Micropropagation—Nodal explants—*Porteresia coarctata*—Salt tolerance

The genus *Oryza* is comprised of 22 wild species and two cultigens. Some of the wild species have been used extensively as donors of gene(s) controlling resistance to biotic and abiotic stresses in addition to contributing to qualitative characteristics. Several wild species are being researched as possible sources of useful traits with the advent of wide hybridization techniques (Jena 1994, Khush et al. 1977; Stich et al. 1990) and by somatic hybridization experiments (Finch et al. 1990). Research into one of the wild relatives of rice, *Porteresia coarctata*, is receiving attention now because of its inherent capacity to tolerate high levels of salinity and submergence (Flowers et al. 1990) in addition to its perennial character. *P. coarctata* is a tetraploid ($2n = 48$) species producing highly recalcitrant seeds (Probert and Longley 1989) and occurs all over the tropics as a mangrove associate. Because of its vegetative method of propagation, *P. coarctata* helps bind peripheral soils in mangrove forests. Physiological, biochemical, and genetic diversity in *P. coarctata* has been studied (Balakrishna 1995), and its distribution pattern shows a decrease in abundance cover in several mangrove areas because of both natural and anthropogenic pressures.

P. coarctata is a good source of gene(s) for salt and submergence tolerance, and attempts have been made to transfer the salt-tolerant character to cultivated rice, *Oryza sativa*, through wide hybridization procedures (Jena 1994). But the difficulty has been to establish the regenerating material in a suitable medium (Jena, personal communication). Even attempts at anther culture (Zapata, International Rice Research Institute, Philippines, personal communication) have faced problems at the multiplication stage. Another inherent problem with *P. coarctata* is its association with a fungal endophyte, *Acremonium*, species which caused considerable problems in establishing axenic cultures (Ramanan et al. 1996).

We have therefore attempted to develop a clonal protocol with the following objectives: (1) to mass multiply and reintroduce *P. coarctata* into mangrove areas successfully; (2) to use the protocol in wide hybridization experiments, and (3) to establish disease-free material of *P. coarctata* for further studies.

We investigated the effect of Woody Plant medium (WP medium; Lloyd and McCown 1980), so far traditionally used in the multiplication of tree species, for multiplying *P. coarctata*. Combinations of benzyladenine (BA) and kinetin at varying concentrations were studied for shoot initiation and multiplication. Also, the

Abbreviations: BA, benzyladenine, NAA, α-naphthaleneacetic acid, WP, Woody Plant medium, MS, Murashige and Skoog basal medium.
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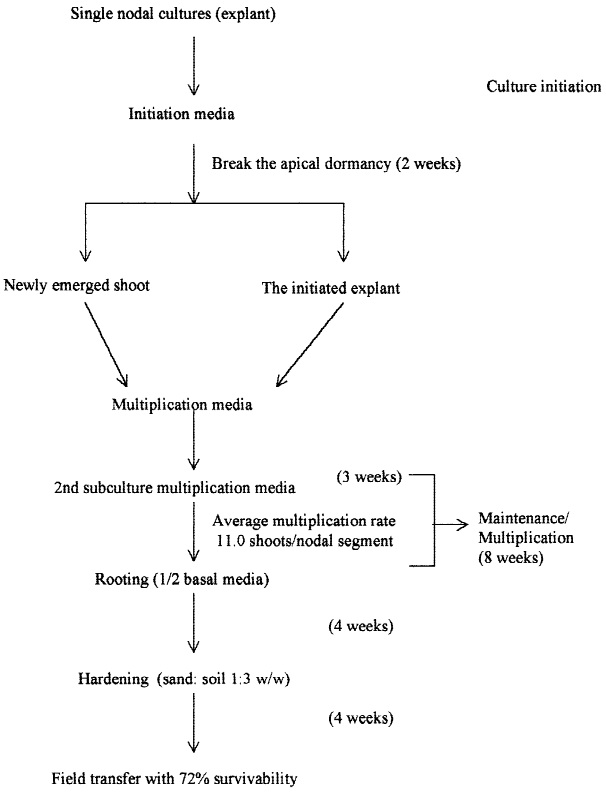

Fig. 1. Flow scheme for the micropropagation of *P. coarctata* (initiation, multiplication, maintenance, rooting, hardening, and field transfer).

effect of different concentrations of α-naphthaleneacetic acid (NAA) for rooting in ½ MS and ½ WP media was investigated. A comparison of initiation, multiplication, rooting, and establishment responses of nodal explants in WP and MS (Murashige and Skoog 1962) media with different hormonal combinations was also attempted in this study.

Materials and Methods

Initiation and Multiplication

P. coarctata collected from the Bhitarkanika, Orissa, mangrove forest was maintained in a glasshouse with 65% relative humidity and 30 ± 2°C day temperature. Profusely growing shoots form mature plants were selected, and nodal segments of 5 cm were placed under running tap water for 1 h. The explants were washed with (1% v/v) Tween 20 and rinsed with water until all traces of the Tween 20 were removed. Explants were pretreated with 70% ethanol for 5 min before surface sterilization with 0.1% (w/v) mercuric chloride for 5 min followed by three to five rinses with sterile distilled water. The surface-sterilized explants were trimmed to 2.5- to 3-cm pieces with single node before placing them in the culture media. WP and MS media with 0.8% agar (Sigma, UK) and 6% sucrose were used as basal media for initiation and multiplication. The pH of the media was adjusted to 5.8 and distributed as 30 mL each in 180-mL glass bottles and autoclaved at 121°C for 15 min. Cultures were incubated at 25 ± 2°C under 16 h/daylight. A light intensity of 75 μmol m^{-2} s^{-1} was provided by cool white fluorescent light. Different concentrations of BA and kinetin were tried singly and in combinations for shoot induction and multiplication. Of several media combinations tried, those inducing the greatest response were recorded. A minimum of 12 replicates was employed for each combination. All experiments were repeated three times. Subculturing was carried out after 3 weeks, and the data were recorded after 8 weeks.

Rooting of Micropropagules

The rooting response of micropropagules was tested on a range of ½ MS and ½ WP media containing 0.8% (w/v) agar, 6% (w/v) sucrose, and different concentrations of NAA (0, 5.4, and 10.7 μM). Single shoots were excised from multiple shoot cultures, produced in optimal micropropagation medium (see the Results section), transferred to 30 mL of each rooting medium (two shoots/180-mL bottle), and incubated as above. Root production was scored at approximately 4-week intervals until the plants were ready for transfer to pots. The above procedures are summarized as a flow diagram (Fig. 1).

Transfer of Plants to the Glasshouse

Rooted micropropagules were washed to remove agar and transferred to pots containing sterilized soil and sand in the ratio of 3:1 (w/w) and were watered daily. The plants were hardened under a 16-h day, 8-h night cycle at 28 ± 2°C, with a relative humidity of 75% in the glasshouse. After 4 weeks the plants were field transferred to the mangrove area for establishment.

Results

Establishment of in vitro cultures of *P. coarctata* posed considerable problems with contamination in primary cultures, which reappeared even after three or four subcultures. This problem was overcome by treating the explants with 0.2% (w/v) Bavistin, a systemic fungicide (BASF, Germany) for 8–10 h and then with 70% ethanol for 5 min.

Production of Shoots

Explants on MS medium and WP medium with either basal salts only or supplemented with BA (2.2 μM) or kinetin (0.4 μM) did not develop new shoots. Nodal segments, when cultured on WP and MS media containing BA (2.2–5.5 μM) and kinetin (0.4–2.3 μM) started to proliferate, producing new shoots after 2 weeks of culture (Fig. 2*A*). The percentage of uninodal shoots producing multiple shoots on combination of BA and kinetin is presented in Table 1. The greatest response for enhanced multiplication was recorded on WP basal medium supplemented with 5.5 μM BA and 2.3 μM kinetin (Table 1). Averages of 11 shots with a 3.5-cm shoot length were observed with the above combination (Fig.

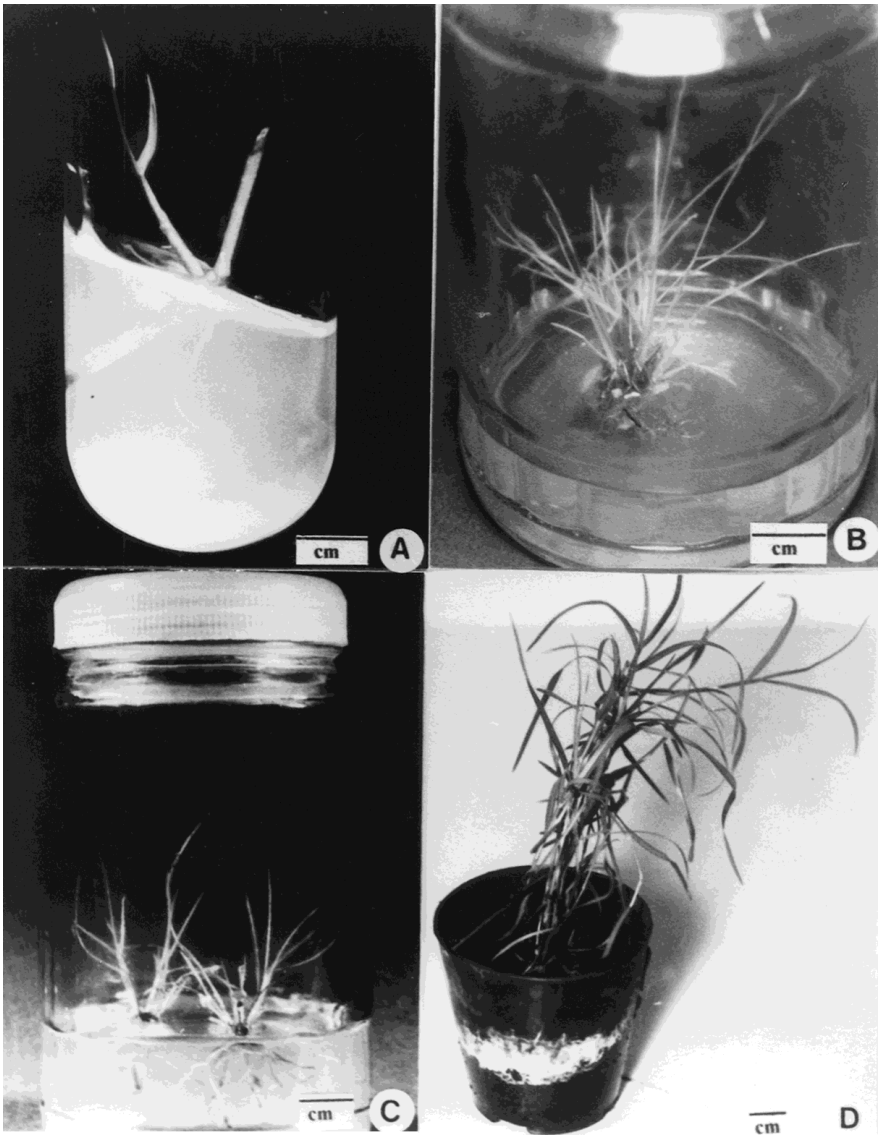

Fig. 2. *Panel A,* shoot initiation on nodal explant in WP medium supplemented with BA and kinetin after 2 weeks. *Panel B,* multiple shoots formed on nodal explant in WP medium (5.5 μM BA + 2.5 μM kinetin) after 6 weeks. *Panel C,* rooting of shoots in ½ WP + NAA (10.7 μM) after 2 weeks. *Panel D,* a 1-month-old plantlet growing in a pot.

2*B*). Subsequent subculturing on the optimal multiplication medium repeatedly for two to three cultures increased the multiplication rate. At the end of the third subculture an average multiplication rate of 20 shoots/explant was achieved. Trimming of shoots after initiation helped to overcome apical dormancy and also enhanced the induction of multiple shoots.

Rooting Response

Multiplied shoots started producing roots in the multiplication medium itself. The number of roots produced in this medium was limited, and the proliferation was also slow. Attempts to harden such rooted plants were not successful. When the multiplied shoots were put on optimal rooting media, the rooting and proliferation re-

Table 1. Effect of cytokinins on multiple shoot induction from uninodal segments of *P. coarctata.* The cultures were scored 6 weeks after initiation, and the data represent the mean of three independent values ± S.E. Results are for 12 replicates. Shoots less than 0.5 cm long were not taken into account. NR, no response. *, $p < 0.05$. Only significant combinations are recorded.

	Cytokinins (μM)		Nodal explants	
Media	Kinetin	BA	No. of Shoots	Average length of shoots (cm)
WP	0.4	5.5	5.8 ± 0.5	7.7 ± 0.2
	1.1	5.5	6.8 ± 0.4	5.4 ± 0.3
	2.3	5.5	11.0 ± 0.7	3.6 ± 0.2
	2.3	2.2	3.6 ± 0.2	9.0 ± 0.3
MS	0.4	5.5	4.7 ± 0.2	8.5 ± 0.7
	1.1	5.5	4.0 ± 0.4	7.3 ± 0.9
	2.3	5.5	7.1 ± 0.5	5.4 ± 0.3
	2.3	2.2	NR	NR

Table 2. Effect of auxin in rooting of shoots produced on media containing 5.5 μM BA and 2.3 μM kinetin after 4 weeks of culture (mean of three values ± S.E.). Results are for 12 replicates, repeated three times. *, $p < 0.05$. Only significant combinations are recorded.

NAA concentration (μM)	½ MS		½ WP	
	No. of roots	Average root length (cm)	No. of roots	Average root length (cm)
0	3.0 ± 0.7	3.2 ± 0.3	4.2 ± 0.6	3.5 ± 0.4
5.3	11.8 ± 0.6	5.8 ± 0.7	14.6 ± 0.6	6.9 ± 0.5
10.7	20.0 ± 0.6	8.0 ± 0.4	4.2 ± 0.6	9.4 ± 0.5

sponse was greater (Fig. 2*C*). An optimal combination of ½ MS or ½ WP medium with NAA (5.4–10.7 μM) was selected to enhance rapid root production (Table 2), thus minimizing the time between inoculation of micropropagated shoots into rooting medium and subsequent transfer to pots. NAA at a high concentration (10.7 μM) showed a significant increase in root number, but roots grew only up to 3 cm in length after a 2-week period. Transfer of such rooted material to auxin-free medium and subsequent transfer to ½ MS and ½ WP media with 10.7 μM NAA after 1 week enhanced root length compared with cultures grown continuously in medium containing 10.7 μM NAA on which the root lengths were shorter (data not presented). Hardening in a controlled environmental chamber and subsequent transfer yielded a good response in establishment, with about a 72% survival rate (Fig. 2*D*). Greater responses in all of the above parameters were observed in the WP-based media.

Discussion

Micropropagation of wild rice species provides an opportunity to maintain a disease-free, clonal population of vegetative material which does not require any attention between subcultures and which can be multiplied and rooted readily for production of mature plants (Finch et al. 1992). Micropropagation has been achieved previously with cultivated rice varieties (Finch et al. 1990, Kumari et al. 1988), and we have now shown the possibility of micropropagating the wild relative of rice, *P. coarctata*.

Micropropagation could aid wide hybridization efforts directly if applied to rice hybrids, recovered from embryo rescue, enabling their ready multiplication (Jena 1994). With the constant association of *P. coarctata* with an endophytic fungus (Ramanan et al. 1996), it has not been possible previously to establish an axenic culture in hybridization experiments. In addition, such an association with an endophytic fungus may not have been conducive to protoplast isolation and fusion studies. With the current micropropagation protocol for *P. coarctata* we have overcome this problem.

As mentioned earlier, *P. coarctata* occurs as a pioneer species in mangrove forests colonizing the fringes of mangroves. With their extensive root system and vegetative propagation method of regeneration they act as good soil binders. Unfortunately this species has disappeared from several mangrove areas in South India, and attempts to reintroduce *P. coarctata* in such areas will also help mangrove area soil conservation. Successful field transfer of our hardened material into the Pitchavaram mangrove forest area has helped this effort as an indirect method of conservation of mangrove associates like *P. coarctata*.

Traditionally, WP medium has been used as a basic medium for propagation of tree species (Mascarenhas et al. 1982). When WP medium was used as basal medium the responses were greater. Our previous attempts to use MS basal medium with a suitable hormonal combination to achieve multiple shoot production, rooting, and establishment did not yield as responses that were as good as in WP-based medium. The possible explanation for this could be that the amount of ammonical nitrogen in WP medium is lower than in MS medium. Also, the major nutrient composition in WP medium corresponds to the mineral composition of the mangrove native environment (Joshi et al. 1975) where *P. coarctata* occurs. Perhaps the habitat of *P. coarctata* being saline and the natural availability of ammonical nitrogen rather low help the plant to respond to a similar situation in culture. This may explain the greater response of *P. coarctata* in WP medium compared with that in MS-based medium.

This protocol can be used as a tool to begin studies on somatic and wide hybridization of *P. coarctata* with *O. sativa*, as this may overcome the possible problems of establishing a good regeneration system.

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