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In vitro shoot culture of wild *Oryzae* and other grass species

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Abstract

A method was developed for the *in vitro* clonal propagation of shoots from a range of wild rice and other grass species that have important genetic traits such as drought resistance and salinity tolerance. The axenic multiple shoot cultures, which were suitable for DNA and protein extraction or direct protoplast isolation, could be maintained without subculture for between 2 and 3 months or rapidly multiplied for the subsequent production of mature plants and seeds. Basal sections of the micropropagated shoots also provided novel explants for the production of highly embryogenic callus, from some species, that could be regenerated into green plants. It is envisaged that this clonal propagation technique could aid the genetic manipulation of cultivated rice by providing a means to vegetatively conserve valuable genetic resources, a technique to rapidly multiply novel hybrid material and a source of embryogenic callus that will allow the application of biotechnological techniques, such as somatic hybridization and genetic transformation, to previously unexploited species.

Abbreviations: BA – N^6-benzyladenine, 2,4-D – 2,4-dichlorophenoxyacetic acid, NAA – α-naphthaleneacetic acid, PAR – photosynthetically-active radiation

Introduction

The genus *Oryza* is comprised of two cultigens and twenty wild species. The wild species represent an important reservoir of genetic diversity and are a source of genes controlling natural resistance to biotic and abiotic stresses, and other characters useful to rice breeders. Interspecific crossing in the genus *Oryza* has rarely been used in the past as a means of improving the cultivated species, *Oryza sativa* L., due to sexual incompatibility barriers. However, future improvement of rice may be achieved by the transfer of useful traits from the wild relatives by modern wide hybridization techniques, utilizing embryo rescue, to produce alien chromosome addition lines (Khush et al. 1977; Jena & Khush 1985, 1990; Sitch et al. 1990) and by somatic hybridization through protoplast fusion (Terada et al. 1987; Hayashi et al. 1988; Finch et al. 1990, 1991). Resources of wild rice germplasm are in some cases scarce, and the advancement of wide hybridization technology and associated techniques, such as the development of species-specific DNA probes and genetic maps of the wild *Oryzae* and related genera, will rely on the ready availability of these species for distribution to laboratories worldwide (D. Vaughan, International Rice Germplasm Centre, IRRI, Philippines. pers. comm.).

Clonal propagation of shoots *in vitro*, or micropropagation, allows the rapid multiplication of a particular plant species in a sterile, disease-free environment whilst minimizing somaclonal

variation, and is thus a particularly useful technique for the multiplication or conservation of male sterile or rare genotypes. We investigated the effects of BA and three different gelling agents on shoot proliferation rates *in vitro* for a range of wild rices and other grass species that possess important traits. The prolific multiple shoot cultures produced have been maintained for many months and can be used as an axenic source of shoot tissue for DNA and protein extractions. In addition, we investigated the effects of NAA on root production and found that all species could be readily rooted and transferred to soil for seed production. During our initial investigations we also observed that basal segments excised from shoot cultures of several species provided novel explants for the initiation of friable callus that regenerated into green plants through somatic embryogenesis.

Our primary aim in this study was thus to develop an efficient micropropagation technique for a range of wild rices, with useful genetic traits, that would provide us with a readily renewable source of axenic plant material and enable us to increase our seed supplies of these rare types. However, we also investigated the use of micropropagated shoots as a source of regenerable embryogenic callus.

Materials and methods

Initiation of micropropagules

Seeds of *O. granulata* (Acc.no. 100879), *O. longistaminata* (Acc.no. 103925), *O. officinalis* (Acc.no. 104619), *O. rufipogon* (Acc.no. 103522) and *Leersia perrieri* (Acc.no. 105164; all supplied by T.T. Chang, IRRI), and *Leptochloa fusca* (supplied by R. Probert, Kew) were after-ripened for 5 days (50°C), dehusked, surface-disinfested [20% v/v Domestos (a commercial bleach preparation containing 9.5% w/v sodium hypochlorite) for 30 mins], washed 5 times in sterile distilled water and germinated on growth regulator-free MS medium (Murashige & Skoog 1962) containing 0.8% w/v Sigma agar and 4% w/v sucrose ('MS0', pH 5.8, autoclaved) in 180 ml glass jars in the light (125 μmol $m^{-2}s^{-1}$ PAR, 16-h daylength) at 25°C. The endosperm and radicle were excised from 7-day-old seedlings. These were inoculated to a depth of 5 mm into 30 ml MS medium (4 explants per 180 ml jar) containing 6% w/v sucrose, BA [8.9 μM (2 mg l^{-1}), 17.8 μM (4 mg l^{-1}) or 26.6 μM (6 mg l^{-1})] and one of 3 gelling agents [0.8% w/v Sigma agar, 0.4% w/v Sigma type I agarose or 0.25% w/v Sigma Phytagel (which gave similar gel strengths)] in all combinations (pH 5.8, autoclaved) and maintained as before. Multiple shoots or 'micropropagules' that developed at the base of the explants (Fig. 1a) were counted at approximately 10-day intervals for one month. Mean shoot production frequencies for each species were calculated from multiple observations in three separate experiments.

Rooting of micropropagules

The rooting response of micropropagules of each species was tested on a range of MS media containing 0.4% w/v Sigma type I agarose, 6% w/v sucrose and either 0, 2.7 μM (0.5 mg l^{-1}), 5.4 μM (1.0 mg l^{-1}), 8.1 μM (1.5 mg l^{-1}), 10.8 μM (2.0 mg l^{-1}) or 16.2 μM (3.0 mg l^{-1}) NAA, ('MSN0', 'MSN0.5' etc.; pH 5.8, autoclaved). Single shoots (Fig. 1b) were excised from the multiple shoot cultures, produced in optimal micropropagation medium ('MSB2A', see results), and transferred to 30 ml of each rooting medium (4 shoots per 180 ml jar) and incubated as above. Root production (Fig. 1c) was scored at approximately 7-day intervals until the plants were ready for transfer to pots.

Transfer of plants to the glasshouse

Rooted micropropagules were washed to remove the gelling agent and transferred to 10 cm plastic pots, containing perlite, in plastic seed-trays with transparent lids, watered twice daily with a 0.22% v/v solution of Maxicrop organic feed (Maxicrop Ltd., Tonbridge, UK) and maintained in a 16-h day:8-h night cycle at 28°C:24°C, respectively, with a relative humidity of 70%. The lids of the seed-trays were removed after 1 week, and the plants transferred to a 3:1 mixture of compost [1:1 mixture of John Innes No. 3 (Fisons plc., Suffolk, UK) and Levington soil-less composts (J. Bentley Ltd., South Hum-

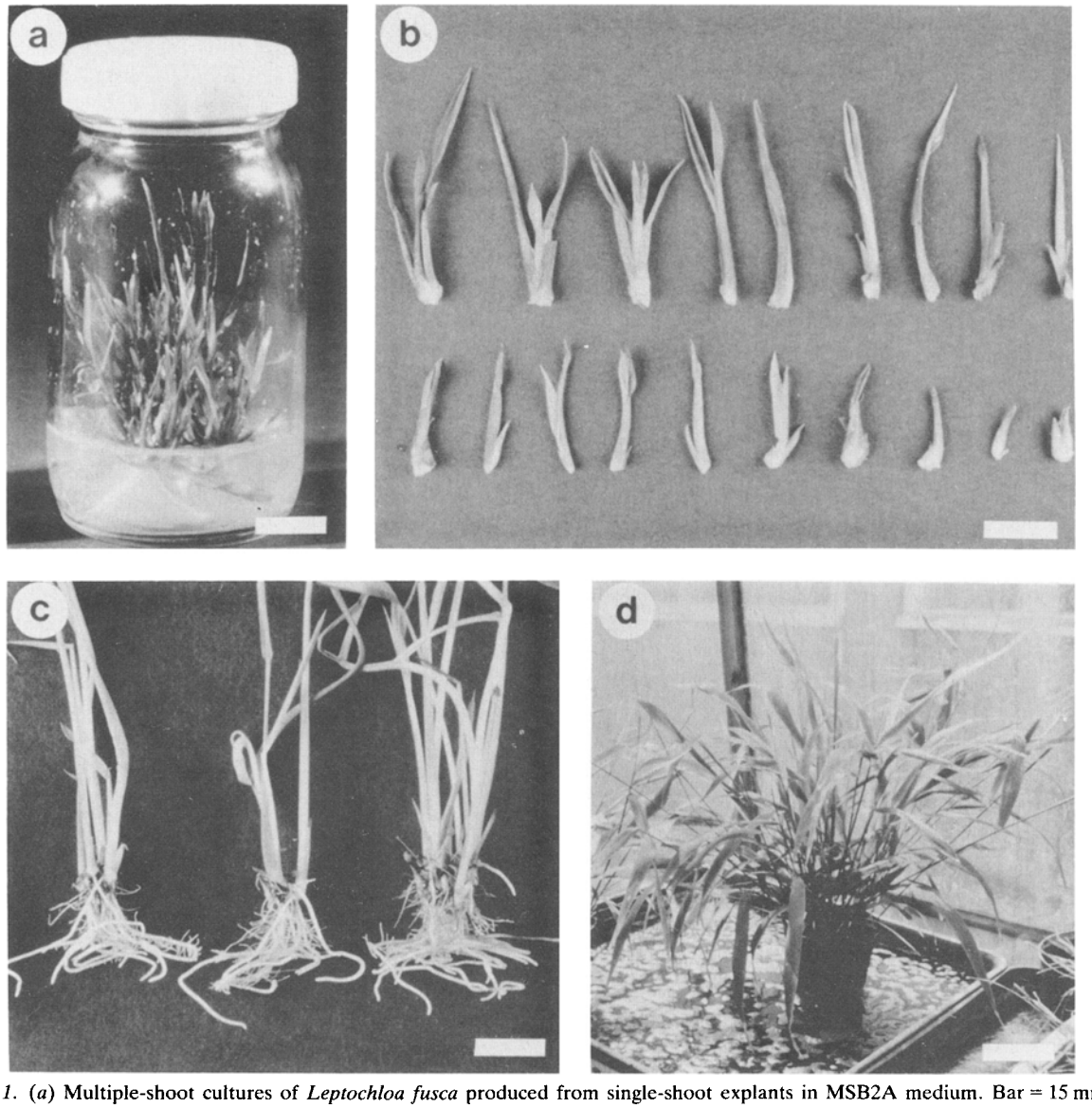

Fig. 1. (*a*) Multiple-shoot cultures of *Leptochloa fusca* produced from single-shoot explants in MSB2A medium. Bar = 15 mm. (*b*) Separated micropropagules from a multiple shoot culture of *O. rufipogon*. Bar = 15 mm. (*c*) Rooted shoots of *O. officinalis* ready for transfer to pots after 14 days. Bar = 10 mm. (*d*) Mature, flowering plants of *O. granulata* derived from micropropagated shoot cultures. Bar = 10 cm.

berside, UK)] and perlite in 20 cm pots after a further 2 weeks. *O. granulata* and *Leersia perrieri* plants flowered and produced seeds under these conditions (Fig. 1d). The other species required a 10-h daylength for flower induction.

Callus induction from micropropagules

Segments (2 mm thick) cut from firm white tissue at the base of micropropagules were inoculated onto 25 ml LS medium (Linsmaier & Skoog 1965) containing 0.4% w/v Sigma agarose and 11.3 μM (2.5 mg l^{-1}) 2,4-D (LS2.5; Thompson et al. 1986) in 9 cm plastic petri-dishes and maintained in the dark at 28°C. Pale yellow calluses with a dry appearance were selectively subcultured onto fresh LS2.5 medium every four weeks. Such calluses from *O. rufipogon*, *Leptochloa fusca* and *O. granulata* resembled embryogenic callus of Japonica rice (Abdullah et al.

Table 1. Compositions of culture media used in this study.

Medium	Use	Salts & vitamins	Sucrose concentration (% w/v)	Concentration of growth regulators ($\mu M/mg\,l^{-1}$)				Agar concentration (% w/v)	Agarose concentration (% w/v)
				BA	kinetin	NAA	2,4-D		
MS0	seed germination	MS	4	0	0	0	0	0.8	0
MSB2A	shoot multiplication	MS	6	8.9/2	0	0	0	0	0.4
MSN0	rooting	MS	6	0	0	0	0	0	0.4
MSN0.5	rooting	MS	6	0	0	2.7/0.5	0	0	0.4
MSN1.0	rooting	MS	6	0	0	5.4/1.0	0	0	0.4
MSN1.5	rooting	MS	6	0	0	8.1/1.5	0	0	0.4
MSN2.0	rooting	MS	6	0	0	10.8/2.0	0	0	0.4
MSN3.0	rooting	MS	6	0	0	16.2/3.0	0	0	0.4
LS2.5	callus induction	LS	3	0	0	0	11.3/2.5	0	0.4
MSKN	plant regeneration	MS	3	0	9.3/2	2.7/0.5	0	0	0.4

MS = salts and vitamins as described by Murashige & Skoog (1962).
LS = salts and vitamins as described by Linsmaier & Skoog (1965).

1986) and produced what appeared to be somatic embryos when transferred to regeneration medium (MS with 0.4% w/v Sigma type I agarose, 3% w/v sucrose, 9.3 μM (2.0 $mg\,l^{-1}$) kinetin and 5.4 μM (0.5 $mg\,l^{-1}$) NAA; 'MSKN'). These structures germinated to concomitantly produce roots and shoots within 2 weeks. Procedures for the initiation of cell suspensions and regenerable protoplasts from micropropagule-derived embryogenic callus of *O. rufipogon* are fully described elsewhere (Baset et al. 1991).

The above procedures are summarized as a flow diagram (Fig. 2). The media used in this study are collectively listed in Table 1.

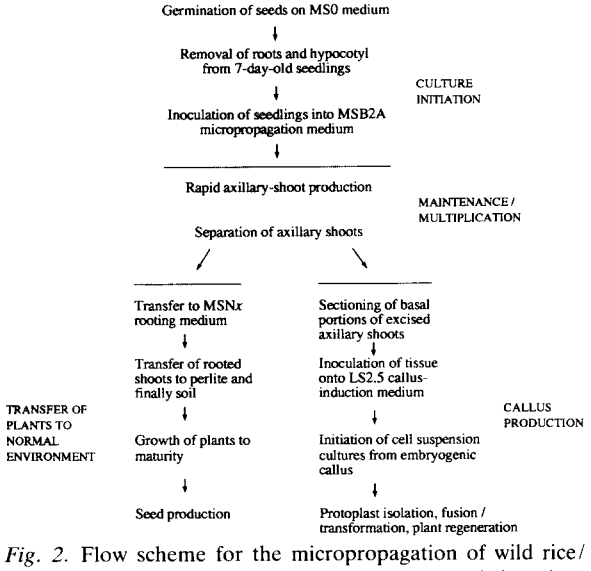

Fig. 2. Flow scheme for the micropropagation of wild rice/grass species. Culture initiation involves seed-disinfestation, seed germination and the preparation of the shoot explants prior to their inoculation into micropropagation medium. The multiple shoot cultures formed are maintained and multiplied by monthly subculture and then rooted for growth to maturity and seed production or dissected and transferred to callus induction medium for the initiation of regenerable, embryogenic callus.

Results

Shoot production

Mean shoot production frequencies from single shoot explants of each species over a period of 31 days are shown (Fig. 3). No differences in shoot production were observed for *O. granulata*, *O. longistaminata* or *Leersia perrieri* for the 9 media examined. For *O. officinalis* with agar as the gelling agent, 26.6 μM BA gave fewer shoots than either 17.8 μM or 8.9 μM BA. When agarose was used, 8.9 μM BA resulted in higher shoot production than 17.8 μM or 26.6 μM BA. For *Leptochloa fusca* with agarose as the gelling agent, 8.9 μM BA gave more shoots than 17.8 μM or 26.6 μM BA. Agarose gave higher shoot production than agar at all BA concentrations. For *O. rufipogon* in media containing 8.9 μM BA, Phytagel gave more shoots than agar or agarose. At 26.6 μM BA, agarose resulted in higher shoot production than agar. Secondary effects of the BA and gelling agents

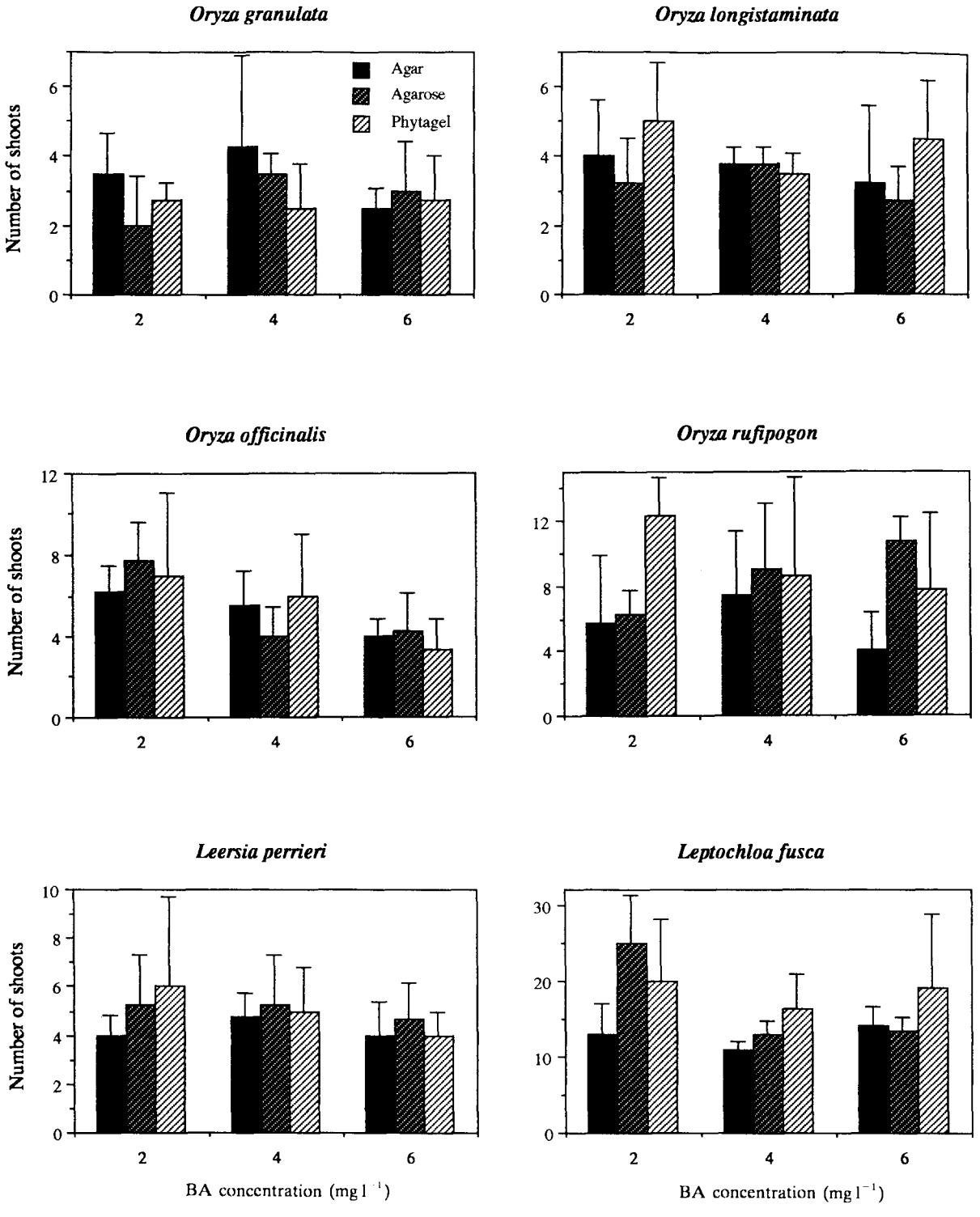

Fig. 3. Mean shoot production from single shoot explants of six wild rice/grass species grown in modified MS basal medium containing a range of different BA concentrations and solidified with three different gelling agents. Bar lines indicate standard errors of the means.

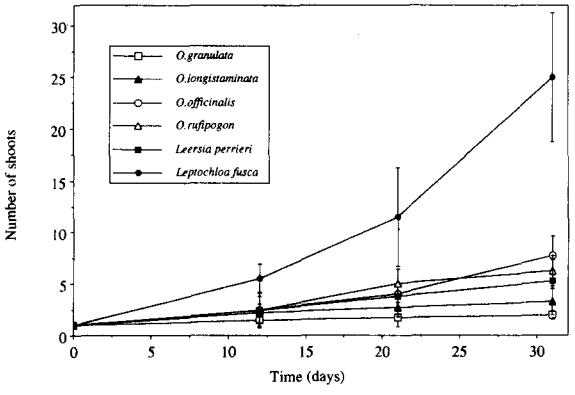

Fig. 4. Mean shoot production over a 31-day period for six wild rice/grass species in MSB2A micropropagation medium. Bar lines indicate standard errors of the means.

on culture development were also observed. Agarose resulted in the least browning and necrosis of the basal region of the shoot cultures, Phytagel the most. Cultures were also more prone to browning at the higher BA concentrations.

The standard multiplication medium subsequently employed for all species contained 8.9 μM BA with agarose as the gelling agent ('MSB2A'). Only *O. rufipogon* produced more shoots at 8.9 μM BA with a different gelling agent, namely Phytagel. However, Phytagel not only resulted in more browning of the cultures but also led to rapid elongation of the shoots produced, such that they outgrew the culture vessels within one month. All the species tested can be maintained in MSB2A in the culture vessels described for between 2 and 3 months without subculture. Figure 4 shows a comparison of the mean rate of shoot production in MSB2A medium for the 6 species examined. *Leptochloa fusca* exhibited a greater shoot production rate than all the other species in this medium.

Root production

Optimal rooting media were those that resulted in the most rapid production of roots thus minimizing the time between inoculation of micropropagated shoots into rooting medium and transfer to pots. As shown in Table 2, root development was markedly slower on transferred shoots of *O. granulata*, *O. officinalis*, *Leersia perrieri* and *O. longistaminata* than on the other species. However, the latter two species produced good root systems in the MSB2A medium and could thus be transferred to pots as rooted multiple-shoot cultures without the need for a rooting phase. Transfer to rooting medium was only necessary if the cultures were separated into single shoots. A separate rooting phase was essential for all other species (Table 2). No phenotypic abnormalities were observed in any of the plants that have so far been transferred to soil despite the fact that *O. granulata*, *O. longistaminata*, *O. rufipogon* and *Leersia perrieri* shoot cultures have been maintained in MSB2A medium for approximately 3 years.

Production of regenerable, embryogenic callus from micropropagules

Basal segments excised from micropropagated shoots of all 6 species produced callus on LS2.5 callus induction medium. *O. rufipogon* and *Leptochloa fusca* exhibited the best callusing response with approximately 5% of the basal shoot segments giving rise to embryogenic callus (Fig. 5a). *O. officinalis* did not produce any embryogenic callus but produced large amounts of rapidly growing non-embryogenic callus. The other species produced small quantities of embryogenic callus at low frequency (Fig. 5b). This

Table 2. Optimal rooting media for micropropagules of 6 wild rice/grass species.

Species	Rooting medium	Days to appearance of first root	Days until ready for transfer to pots
O. granulata	MSN1.5/MSN2	20	35
O. longistaminata	MSN1	9	30
O. officinalis	MSN0/MSN0.5	9	14
O. rufipogon	MSN1/MSN0.5	5	14
Leersia perrieri	MSN0/MSN0.5	11	35
Leptochloa fusca	MSN0/MSN0.5	3	12

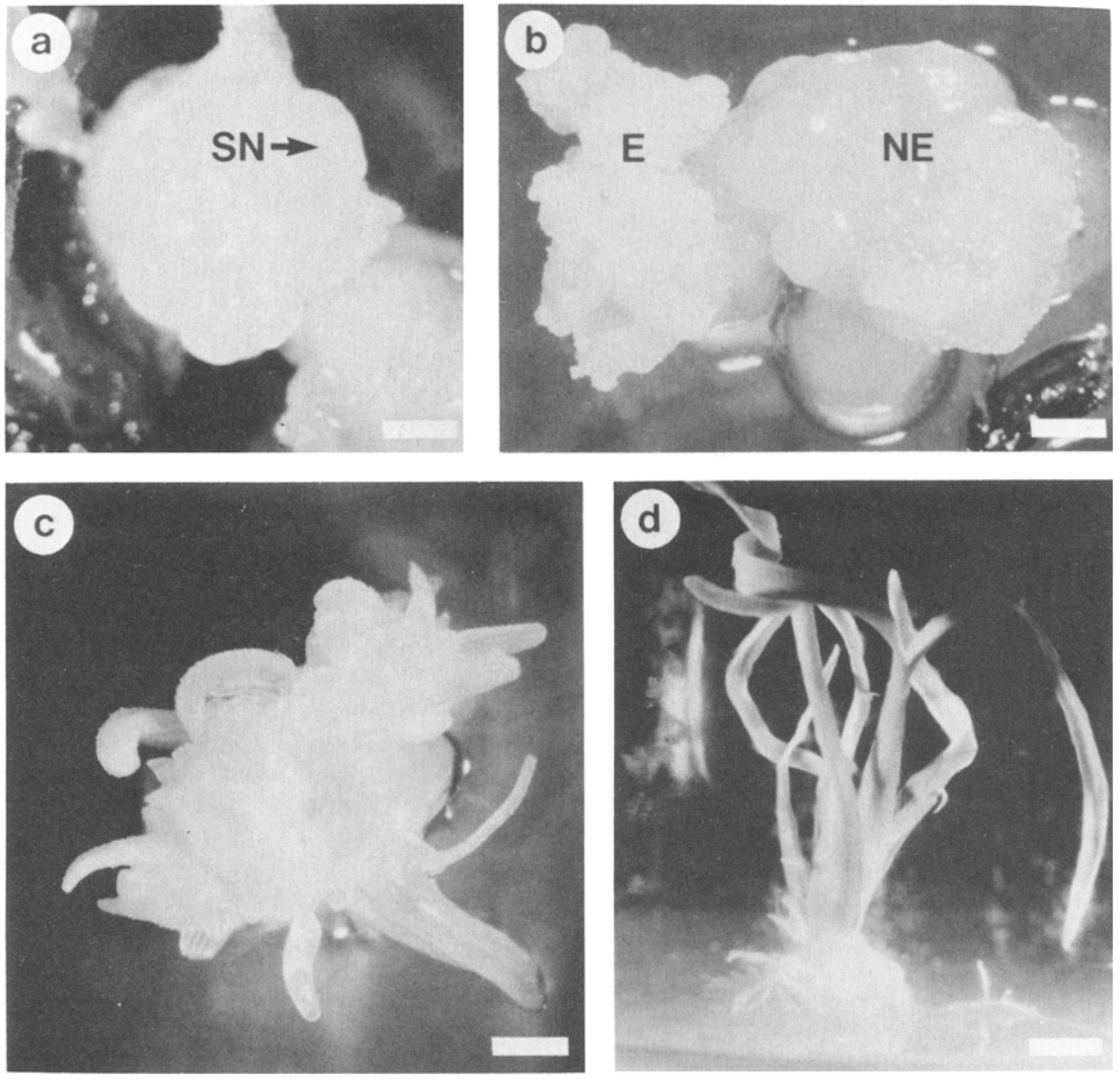

Fig. 5. (*a*) Somatic embryos on the surface of embryogenic callus [note scutellar notch (SN)] produced from a basal segment of a micropropagule of *O. rufipogon*. Bar = 1.5 mm. (*b*) Embryogenic (E) and non-embryogenic (NE) micropropagule-derived callus of *O. granulata* after transfer to fresh LS2.5 medium. Bar = 2.5 mm. (*c*) Shoot production from embryogenic callus of *Leptochloa fusca* on regeneration medium. Bar = 3 mm. (*d*) *O. rufipogon* callus regenerant in MSO medium after transfer to the light. Bar = 5 mm.

was selectively subcultured onto fresh LS2.5 medium after 1 month. Such callus from *O. rufipogon*, *O. granulata* and *Leptochloa fusca* was transferred to plant regeneration medium which resulted in the production of shoots and roots (Fig. 5c,d). Regenerants of *O. rufipogon* and *O. granulata* have been transferred to the glasshouse and have given rise to mature, fertile plants.

Discussion

Micropropagation of wild rice species offers the opportunity of maintaining a disease-free, clonal population of vegetative material that requires no attention between subcultures and can be readily multiplied and rooted for the production of mature plants and large numbers of seeds. The technique may thus prove to be an im-

portant method for conserving rare and genetically important wild rice species. Micropropagation has previously been achieved with cultivated-rice varieties (Kumari et al. 1988; Greco et al. 1990) and we have shown that a suitably modified technique is applicable not only to several wild *Oryzae* but also to other genera. Since micropropagation techniques are now available for both cultivated rice and genetically important wild rices, it is envisaged that micropropagation could directly aid wide hybridization efforts if applied to widecross rice hybrids, recovered from embryo rescue, enabling their clonal multiplication prior to backcrossing. This would improve the chances of obtaining further backcross generations. Rice micropropagation may also be useful in gene mapping projects where a specific mapping population could be clonally maintained for an indefinite period providing sterile plant tissue for DNA isolation. Furthermore, micropropagated shoots provide a ready source of axenic plant material for direct protoplast isolation and we have found that protoplasts isolated directly from plant material of *Porteresia coarctata*, a wild relative of rice, can be used in protoplast fusion studies to produce dividing heterokaryons and somatic hybrid cell colonies (Finch et al. 1990).

Although the use of seedling leaf-base meristems as explants for the production of embryogenic callus of some cereals and grasses is well documented (Conger et al. 1983, 1987; Finch et al. 1991), micropropagated shoots have not been used for embryogenic callus production before. Moreover, this is the first report of plant regeneration from callus of *Leptochloa fusca* (kallar grass), *O. granulata* and an accession of *O. rufipogon*, although regeneration from young inflorescence and seed callus of *O. perennis* has been achieved (Wang et al. 1987). *Leptochloa fusca* is highly salt tolerant and exhibits poor sexual compatibility with rice (Farooq & Mujtaba Naqvi 1987). *O. granulata* is shade tolerant and possibly drought resistant, but is also difficult to cross sexually with rice. Such sexual incompatibility problems could possibly be overcome through the use of somatic hybridization. *O. rufipogon* offers a number of important genetic traits including tolerance to stagnant flood and acid sulphate soils, but a number of accessions also offer new sources of cytoplasmic male sterility (CMS) as recently reported by Sitch et al. (1990). This species can be crossed sexually with cultivated rice, but the CMS trait could be more rapidly transferred through the production of cybrids utilizing protoplast fusion methods previously described for intervarietal cybridization of rice (Yang et al. 1989; Akagi et al. 1989; Kyozuka et al. 1989). We have recently reported that cell suspensions derived from micropropagule-derived callus of *O. rufipogon* yield protoplasts that can be regenerated into plants (Baset et al. 1991). This provides a new opportunity for the production of transgenic wild rice which could provide selection systems for both somatic and widecross-sexual hybridizations.

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