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# Somatic Embryogenesis and Plantlet Regeneration in an Agronomically Important Wild Rice Species *Oryza nivara*

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**Abstract:** Embryogenic callus was initiated from mature seeds on callus induction medium with MS nutrients supplemented with 2, 4-D alone or in combination with BA. The best callus induction period was 4 weeks. The morphology of the embryogenic callus was friable which later turned granular and compact. Somatic embryos germinated to form plantlets after transfer of the callus to regeneration medium (basal MS medium). The total duration of the plant regeneration protocol from inoculation till rooted plantlets were ready for transfer to green house was 20 weeks. Somatic embryos were also encapsulated and the synthetic seeds could be regenerated to produce healthy fertile plantlets.

**Key words:** Calcium alginate beads, embryoids, growth regulators, *Oryza sativa* complex, pre-regeneration medium, synthetic seeds

## INTRODUCTION

The development of new modern rice varieties has depended on the continued availability of genetic diversity. The main sources of this diversity are the traditional varieties and wild species. They are important reservoirs of useful genes for resistance to diseases and insects, tolerance to abiotic stresses and cytoplasmic male sterility sources (Brar and Khush, 2003). In Asia, the population of wild species is becoming rare and threatened due to the destruction of their natural habitat by the extension of cultivated areas or urban pressures and there is considerable genetic erosion in the rice genepool.

*Oryza nivara* Sharma et Shastry, a wild rice specie belonging to the *Oryza sativa* complex with AA genome is the sole donor of a major dominant gene for resistance to grassy stunt virus biotype-1 and possesses resistance to sheath blight (Brar and Khush, 2002). This wild relative of modern cultivated rice also possesses other important agronomic traits such as resistance to blast; stem rot and drought avoidance and Cytoplasmic male sterility (Brar and Khush, 2003). With the application of modern techniques such as protoplast fusion and genetic engineering, such agronomically important traits from wild species can be transgressed to cultivated rice. For the successful application of the aforesaid technologies for crop improvement, the development of efficient plant regeneration system from cultured cells or tissues is required. Plant regeneration has earlier been achieved in *O. nivara* (Oinam and Kothari, 1997) from callus cultures derived from endosperm and radicle using two different basal media for initiation of callus (LS medium) and regeneration of plantlets (MS medium). In this communication, we describe for the first time an improved efficient regeneration system using a single basal medium, based on initiation of embryogenic callus from mature seeds of *O. nivara* which can also be used as the primary source for the successful isolation of protoplast from this specie. We also describe a method for production and germination of synthetic seeds using somatic embryos.

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## MATERIALS AND METHODS

### Plant Material

Dehusked, mature seeds from *Oryza nivara* were surfaced sterilized by immersion in 0.1% (w/v) mercuric chloride solution for 10 min, followed by 3-4 rinses with sterile distilled water. The seeds were dried on sterile filter paper and cultured in culture tubes containing callus induction medium. Ioprata

### Culture Medium

MS Murashige and Skoog (1962) medium supplemented with 3% (w/v) sucrose along with different concentrations of 2,4-D (1.0-6.0 mg $L^{-1}$) with or without BA (0.1-1.0 mg $L^{-1}$) gelled with 0.8% (w/v) agar was used as callus induction medium. The pre-regeneration medium comprised of MS salts with 0.5- 2.0 mg $L^{-1}$ 2, 4-D and 0.1-0.5 mg $L^{-1}$ BA. The effect of MS basal medium devoid of growth regulators was also tested for regeneration. The pH of the medium was adjusted to 5.8 prior to steam sterilization at 121°C for 15 min.

### Culture and Growth Conditions

Seeds were cultured on callus induction medium and incubated in the dark at 25±1°C. After 4 weeks in culture, the embryogenic callus was transferred to fresh culture medium of the same composition for proliferation. The embryogenic calli obtained were subcultured to pre-regeneration medium for 2 weeks and were subsequently transferred to regeneration medium, incubated at 12/12 h photoperiod.

### Acclimatization and Transfer of Plantlets to Soil

Plantlets obtained through somatic embryogenesis were removed from the culture vessel and washed thoroughly in running tap water to remove traces of agar. They were then transferred to plastic cups containing soilrite covered over by polythene bags for hardening. After 2 week in soilrite, the hardened plantlets were subsequently transplanted to potting mixture consisting of sand: soil: cow dung (1:1:1) and maintained in green house. Survival rate of the plantlets was observed.

### Statistical Design and Data Analysis

A set of 20 caryopses per treatment cultured in separate culture tubes was taken as replicate in all experiments except plantlet regeneration and each treatment was replicated five times. The results of callus induction frequency (%), frequency of embryogenic callus and plantlet regeneration frequency were statistically analyzed and the mean separation of statistically significant experiments was made by Duncan's Multiple Range Tests (DMRT) at a probability level of 5%.

### Encapsulation of Somatic Embryos

Somatic embryos were encapsulated by mixing them with 4% (w/v) solution of sodium alginate in MS basal medium and dropping the medium with somatic embryos to a solution of calcium chloride (2.5% w/v). After 30 min of incubation, the beads were recovered by decanting the calcium chloride solution and washing them with sterile distilled water 3-4 times before transferring to petriplates and subsequently sealing with parafilm. The germination capacity of the synseeds was tested by culturing them in basal MS medium.

## RESULTS AND DISCUSSION

Swelling of the mesocotyl and radicle region of the mature embryo was observed 7-8 days after culture on induction medium. This was followed by development of callus. The frequency of callus

Table 1: Data on callus induction and somatic embryogenesis in *Oryza nivara*

| Plant growth regulators (mg $L^{-1}$) | | Callus induction | Embryogenic callus | Plant regeneration |
|---|---|---|---|---|
| 2, 4-D | BA | frequency (%)[A] | frequency (%)[B] | frequency(%)[C] |
| 1.0 | - | 80 (16.0/20)$^{cdef}$ | - | - |
| 1.0 | 0.1 | 82 (16.4/20)$^{efg}$ | 70 (14.0/20)$^{a}$ | - |
| 1.0 | 0.5 | 80 (16.0/20)$^{cdef}$ | 75 (15.0/20)$^{a}$ | - |
| 1.0 | 1.0 | 75 (15.0/20)$^{bcde}$ | - | - |
| 2.0 | - | 100 (20.0/20)$^{g}$ | - | - |
| 2.0 | 0.1 | 100 (20.0/20)$^{g}$ | 95 (19.0/20)$^{b}$ | - |
| 2.0 | 0.5 | 100 (20.0/20)$^{g}$ | 92 (18.4/20)$^{b}$ | - |
| 2.0 | 1.0 | 98 (19.6/20)$^{g}$ | - | - |
| 4.0 | - | 90 (18.0/20)$^{fg}$ | - | - |
| 4.0 | 0.1 | 85 (17.0/20)$^{fg}$ | 80 (16.0/20)$^{a}$ | - |
| 4.0 | 0.5 | 75 (15.0/20)$^{bcde}$ | 68 (13.6/20)$^{a}$ | - |
| 4.0 | 1.0 | 73 (14.6/20)$^{bcd}$ | - | - |
| 6.0 | - | 72 (14.4/20)$^{bcd}$ | - | - |
| 6.0 | 0.1 | 70 (14.0/20)$^{bc}$ | - | - |
| 6.0 | 0.5 | 66 (13.2/20)$^{ab}$ | - | - |
| 6.0 | 1.0 | 60 (12.0/20)$^{a}$ | - | - |
| **Regeneration medium** | | | | |
| No PGR | | - | - | 90 (45/50) |

[A]No. of explants showing callus induction/Total No. of explants cultured, [B]No. of explants producing embryogenic callus/ Total No. of explants cultured, [C]No. of embryogenic callus showing plantlet regeneration/total No. of embryogenic callus cultured. Values followed by different superscripts letter(s) are statistically significant at $\alpha = 0.05$ by DMRT

formation varied considerably with hormonal combinations, but in majority of the experiments, 60-100% of the cultured seeds formed callus at all concentrations of 2, 4-D used (Table 1). Among the different auxin analogues used to induce somatic embryogenesis, 2, 4-D is the most efficient and therefore used in the majority of embryogenic cell and tissue culture systems. 2, 4-D at 2 mg $L^{-1}$ proved to be optimal for callus induction and proliferation. An increase or decrease in 2, 4-D concentration resulted in a reduced response (Table 1). This could be attributed to the dual effect of 2, 4-D above a certain concentration wherein it acts as an auxin and as a stressor (Fehér *et al*., 2002).

As earlier described for rice (Rueb *et al*., 1994), three types of callus tissue with varying morphogenetic potential were distinguished after 3-4 weeks in culture. One was creamy-white friable callus, which later turned granular and compact with embryogenic potential (Fig. 1a), while the other was soft and glutinous callus which remained non-embryogenic (Fig. 1b) and the third was root producing callus with only root primordia. Though friable callus formed on a wide range of 2, 4-D concentrations (1.0-6.0 mg $L^{-1}$), granular compact embryogenic callus was developed only on media containing 2,4-D (1.0-4.0 mg $L^{-1}$) fortified with lower levels of BA (0.1-0.5 mg $L^{-1}$) (Table 1).

The embryogenic calli, when transferred to pre-regeneration medium (MS medium with 0.5-2.0 mg $L^{-1}$ 2, 4-D and 0.1-0.5 mg $L^{-1}$ BA), grew rapidly and developed embryoids within 2-4 weeks (Fig. 1c). A concerted action of 2, 4-D and cytokinins is needed to induce regenerable calli. In *O. nivara*, BA+2, 4-D medium was conducive for the induction of granular- compact embryogenic callus (Table 1). On the contrary, Hirano and Kohno (1990) observed that in American wild rice, *Zizania palustris*, BA did not play a critical role in the formation of embryoids. However, the presence of BA in the regeneration medium did not promote embryo germination and plantlet formation in *O. nivara* (Table 1). Cultures transferred to regeneration medium (MS medium devoid of growth regulators), showed germination of embryoids and developed shoot and roots simultaneously (Fig. 1d), which is indicative of regeneration via somatic embryogenesis (Chaudhury and Qu, 2000). Unlike present findings, Oinam and Kothari (1997) had used MS medium supplemented with BA (2 mg $L^{-1}$) and NAA (0.5 mg $L^{-1}$) in order to regenerate plantlets. They have also subcultured the shoots to growth hormone free medium to initiate rooting before transplantation, while in our study we could achieve both shoot and root formation in the same medium thereby reducing the time period needed


Fig. 1: Morphologies of callus developed from mature seeds of *Oryza nivara*. (a) granular compact embryogenic callus, (b) soft glutinous non-embryogenic callus, (c) different developmental stages of isolated somatic embryos from seed callus, (d) germination of somatic embryos and formation of plantlets in regeneration medium, (e) somatic embryos encapsulated in calcium alginate beads, (f) germination of synthetic seeds in MS basal medium and (g) regenerated plant in pot

to obtain healthy plantlets for transplantation. The regenerants with well-developed roots were removed from culture tubes, washed free of agar to grow in soilrite for hardening. The hardened plants were transferred to the green house for acclimatization prior to transplantation to the field.

Somatic embryos obtained from the above experiments were also encapsulated in calcium alginate beads (Fig. 1e) to produce synthetic seeds. Alginate hydrogel was chosen for artificial seed production because of its moderate viscosity, low toxicity, quick gelation and low cost (Hussain *et al*., 2000). The synthetic seeds produced were cultured in MS medium devoid of growth hormones to initiate germination and retrieval of whole plantlets. After a period of 1 week, the synseeds started to germinate and produced well developed plantlets within another week (Fig. 1f). The plantlets were subsequently transferred to the green house after a week of hardening in soilrite (Fig. 1g). More than 95% of the plants germinated from the synseeds derived from somatic embryos, survived and were fertile.

This simple and improved protocol established for regeneration of the important wild rice species, *Oryza nivara* via somatic embryogenesis using a single basal medium and their encapsulation has immense potential for storage and large-scale propagation of valuable germplasm. Moreover, the somatic embryogenic calli raised through this protocol can be used as a preliminary source for successful isolation of protoplast from this specie which can be utilized in transformation studies as well.

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