into geotechnical behaviour, which is essential for the design of a suitable nodule mining system.


**ACKNOWLEDGEMENTS.** I thank the Director, National Institute of Oceanography, Goa for permission to publish this data and the crew and participants of both the cruises on-board *RV Yachmorgeologia* for collection of part of data. Critical review by the anonymous referee has improved the quality of the paper. The study was financially supported by the Department of Ocean Development, Government of India, New Delhi. This is NIO contribution No. 3708.

Received 23 May 2001; revised accepted 15 October 2001

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**Qualitative assessment of tissue culture parameters useful in transformation of indica rice**

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In vitro response of several elite indica rice cultures was compared with that of the most responding japonica type, Taipei 309, for enumerating critical tissue culture parameters that subscribe to successful genetic transformation. Callus induced from mature seeds of indica rice genotypes was more compact and proliferated slowly. Formation of friable callus, rapid proliferation and sustenance of regeneration capacity for 9–10 weeks, are identified as the key features that favour success of transformation in a variety and recovery of transformant through subsequent selection and regeneration. Differences in regenerating and nonregenerating green regions were presented to differentiate them at the early stages.

**Development** of productive transgenic rice lines requires routine and efficient gene transfer method. The process of genetic transformation entails several steps, the most important being (i) DNA delivery method, (ii) efficient selection for transformants, (iii) regeneration of transformants. While an array of DNA delivery methods have been reported in rice transformation, key to recovery of transformants lies in post-transformation selection and regeneration. Sifting of transformants involves stringent selection for 2–3 cycles on selection medium and thus maintenance of regeneration capacity of transformed tissue assumes significance. Earlier reports on callus induction and regeneration indicate large differences in tissue culturability between japonica and indica varieties. The former were found to be more responsive. Thus, many advances in tissue culture and genetic transformation methods were often demonstrated using japonicas and to a lesser extent with a few responsive indica varieties. Study on the differences between the in vitro response of the two subspecies, japonica and indica, as well as the superiority of japonica types over indica types for parameters that have a direct bearing on transformation, is vital. The present study involves a comparative analysis of tissue culture response of several elite indica genotypes with a japonica genotype, Taipei 309. Critical tissue culture parameters that subscribe towards transformation and recovery of transgenics were identified as aid to genetic transformation of indica genotypes of choice.

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High-yielding rice varieties adapted to different agro-ecological conditions of the Indian subcontinent, such as Nagarjunna, Pranava, Rasi, Pusa Basmati-1 (PB-1), IR 72, Abhaya, Jaya, Ratna, Pothana, Ajaya, Vibhava, Phalgunu, Tellahamsa, Salivahan, Vikas, Swarna, Seshu, Vikramarya, Chandana, Sonasali, Prasanna, Swarnadhan along with japonica type – Taipei 309, were used. Mature de-husked seeds as explants were surface sterilized for 3 min with 70% ethanol followed by 3 min in 0.1% mercuric chloride and by several washes with sterile distilled water. The seeds were blotted on sterilized paper towels and planted on MS medium containing 3 mg l⁻¹ 2,4-D and 0.2 mg l⁻¹ BAP, 50 mg l⁻¹ tryptophan and 300 mg l⁻¹ casein hydrolysate (enzymatic). The seeds were placed with only half of the embryo in contact with the medium. The cultures were raised in dark at 25 ± 2°C for callus induction. After 25–30 days primary callus was dissected aseptically and was subcultured on the same medium at 15–20 days intervals. Regeneration protocol was carried out at 4–5 weeks and 9–10 weeks on MS medium containing 2.5 mg l⁻¹ BAP, 1 mg l⁻¹ K, 0.5 mg l⁻¹ NAA, 1 g l⁻¹ proline and 500 mg l⁻¹ casein hydrolysate (enzymatic). Callus sample (ca. 30 mg) was inoculated for regeneration. Scoring for calli showing green regions was done after 20 days. Sub-culturing on fresh regeneration medium was carried out at 30 days. We describe here the qualitative analysis on stages of in vitro development, viz. callus induction, calli with green regions, calli with only shoots, calli with roots and shoots, rhizogenic calli and plants per unit culture.

Previous investigators working with 60 cultivars, indica, japonica and indica hybrids, including various ecotypes of rice, for regeneration potential, using callus derived from root sections of 5–7-day-old seedlings have indicated variability in plant regeneration. It was a significant observation that while only a few of the indica varieties, japonica x indica hybrids and the large-grained javanica types showed shoot regeneration potential, all the 28 japonica cultivars regenerated plants. Among the japonicas, Taipei 309 was the most responsive genotype to tissue culture and genetic transformation. In an earlier study, quantitative assessment and statistical analysis on sequential growth stages of in vitro revealed large differences among the indica varieties. Genotypes were grouped by canonical analysis, joint score method and nonhierarchical Euclidean methods of statistical clustering, to identify the indica varieties that are proximate to T 309 for their tissue culture response. Vibhava and Seshu, which were clustered along with T 309, were used for genetic transformation. The other varieties required media manipulation for improvement in their tissue culture response.

Plumule and radicle emerged after 72 h on medium containing 2,4-D. Further growth was, however, suppressed and proliferation of scutellum and mesocotyl followed. Elongation of the shoots was the first observed pheno-

Figure 1. Initiation of callus in a, b, T 309 and two indica genotypes c, d, Rasi and Seshu.

menon in all the genotypes. The shoot elongation was rapid in T 309 compared to other indica genotypes (Figure 1a and b). Proliferation of scutellum genotypes at 7–10 days. Apart from the variation in the quantitative response, the genotypes differed in the lag period for callus induction. White to yellow and compact callus was apparent in Rasi, PB-1 and Nagarjunna as early as 7–10 days and in other genotypes at 15–20 days. Primary callus proliferated rapidly in the early responding varieties, a trait of interest for transient expression studies, because the callus grows rapidly till 30 days enabling visualization of gus gene expression. Furthermore, 7–10-day-old calli can also be used for bombardment.

Distinct difference between T 309 (japonica) and others (indica) was observed. Microcalli originated along the entire length of the coleoptile in T 309, a distinct proliferation of only the scutellar tissue leading to a compact callus in all the indica genotypes (Figure 1). Fifteen to twenty-day callus was smooth or undulated (Figure 2a and d). Embryogenic callus, highly organized into globular embryos, associated with unorganized non-embryogenic callus in variable proportions was observed in all the genotypes at 25–30 days. The callus region adjoining the seed attachment was predominantly embryogenic-type. Though no differences between T 309 and other genotypes in the structural organization of the callus at 15 days were present, sub differences were observed at 30 days and were more pronounced at 45 days (Figure 2b, c, e, f). T 309 had creamy white, easily dissociating cell clusters unlike the indica types, which had a compact callus (Figure 2). The compact nature of the callus in indica varieties sustained even after sub-culture, whereas T 309 formed friable callus. Mechanical dissociation of
these compact calli led to necrosis. The extent of friable callus amongst the indica varieties varied. With Rasi, PB-1, IR 72, Nagarjuna and Tellahamsa being more friable, they facilitated cell suspensions and paved the way for protoplast studies and biolistic transformation. In addition, small cell clumps render fewer escapees of untransformed cells after growth on selection media (authors, unpublished results). Further, callus growth in T 309 was also rapid and resulted in larger mass compared to indica varieties. Such behaviour of callus on subculture has been reported in japonica genotypes. Proliferation of secondary callus was rather slow in Vikramarya, Swarnadhan, Prasanna, Chandana and Jaya. Hartke and Lorz noted large differences among genotypes for formation of embryogenic callus, while screening 15 indica rice genotypes. In the present study, such large differences in the formation of embryogenic callus were noted. Embryogenic callus, white to creamy, smooth and knobby was observed in IR 72, PB-1, Vibhava, Seshu, Vikas and Tellahamsa, but not in Swarnadhan, Chandana, Prasanna and Abhaya.

Transfer to regeneration medium led to green regions, not all of which developed shoots. Lustrous green spots in organized calli covered with sickle-shaped trichomes eventually transformed into shoots, while pale green unorganized regions covered with white hairs did not give rise to shoots; both types were observed in the same callus (Figure 3). Development of green region as well as shoots was simultaneous in some of the calli. The non-regenerating green regions gave rise to roots. Shoot-bearing cultures upon transfer produced roots on half strength MS medium, whereas the root-bearing cultures failed to produce shoots even after transfer to the fresh regeneration medium. Besides normal plant regeneration, cultures often showed excessive rooting, dormant green regions without further development and occurrence of necrotic areas.

Development of chlorophyllous regions and emergence of shoots was simultaneous and sustained over 3–4 passages in T 309, whereas in all indica varieties, the stages were in the same sequence and overlapping, but lasted only for two passages. In Vibhava and Seshu, the number of plants regenerated from a single green region were high, while in T 309, the total number of regenerating green regions was high.

Sustenance of regeneration capacity till 9–10 weeks is important to recover plants from transformed sectors.

**Figure 2.** Differences in callus proliferation and morphology between T 309 and indica genotypes. Callus induced in a–c, T 309 and d–f indica variety after 15, 30 and 45 days.

**Figure 3.** Different stages in regenerating and dormant green regions from the callus of rice varieties. a–c, Dormant green regions; and d–f, Stages in regeneration.
Table 1. Regeneration frequency of calli at different ages

<table>
<thead>
<tr>
<th>Genotype</th>
<th>4–5 weeks</th>
<th>9–10 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nagarjuna</td>
<td>29.1 ± 10.9</td>
<td>4.6 ± 4.6</td>
</tr>
<tr>
<td>Pusa Basmati-1</td>
<td>9.3 ± 0.2</td>
<td>0</td>
</tr>
<tr>
<td>Jaya</td>
<td>8.4 ± 8.4</td>
<td>3.6 ± 3.6</td>
</tr>
<tr>
<td>Rasi</td>
<td>25.6 ± 7.8</td>
<td>0</td>
</tr>
<tr>
<td>Seshu</td>
<td>72.8 ± 0.6</td>
<td>44.1 ± 8</td>
</tr>
<tr>
<td>Vikas</td>
<td>38.9 ± 5.6</td>
<td>17.5 ± 7.5</td>
</tr>
<tr>
<td>Salivahana</td>
<td>91.7 ± 8.4</td>
<td>0</td>
</tr>
<tr>
<td>Taipei 309</td>
<td>69.2 ± 10.8</td>
<td>56.7 ± 3.3</td>
</tr>
<tr>
<td>Vihavha</td>
<td>72.2 ± 5.6</td>
<td>59.8 ± 2.7</td>
</tr>
</tbody>
</table>

Table 2. Differences in tissue culture response between T 309 and Indica rice cultivars belonging to indica group

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Indica</th>
</tr>
</thead>
<tbody>
<tr>
<td>T 309</td>
<td></td>
</tr>
<tr>
<td>Induction of callus from the scutellum in seed-derived calli was late and evident at 15–20 days.</td>
<td>Induction of callus was early and evident at 7–10 days.</td>
</tr>
<tr>
<td>Callus was diffused and spread over scutellum and mesocotyl.</td>
<td>Callus was compact and originated from only the scutellum.</td>
</tr>
<tr>
<td>At 20 days, the undulations in the callus were not distinct.</td>
<td>Distinct</td>
</tr>
<tr>
<td>At 40 days, distinct globular organizations were seen.</td>
<td>Compact organization was seen.</td>
</tr>
<tr>
<td>Callus was friable.</td>
<td>Compact</td>
</tr>
<tr>
<td>The proliferation of the callus was rapid on sub-culturing.</td>
<td>Slow</td>
</tr>
<tr>
<td>On transfer to regeneration medium, proliferation and regeneration were simultaneous.</td>
<td>Proliferation preceded by re-differentiation.</td>
</tr>
<tr>
<td>Regeneration capacity was sustained over long period.</td>
<td>There was a rapid decline in regeneration potential.</td>
</tr>
</tbody>
</table>

even after two or three cycles of selection. This was examined at 4–5 weeks and 9–10 weeks (Table 1). T 309 did show 69.5% regeneration at 4–5 weeks and sustained the frequency of regeneration after 9–10 weeks. Vihavha and Seshu showed 72.2% and 72.8% at 4–5 weeks and a decline in regeneration (59.8% and 44%) at 9–10 weeks. Salivahana though showed 91.7% regeneration at 4–5 weeks, quickly lost this potential over time. Similar trend was also noted in other varieties Rasi, PB-1, Nagarjuna, Vikas and Jaya (Table 1). The decline was sharp in Salivahana, Nagarjuna, Rasi and Vikas, but it was not so in Vihavha, Seshu and T 309. Salivahana showed good initial regeneration, but this was not sustained. A sharp fall in the regeneration ability after first passage in two of the three indica genotypes has also been reported. In studies on protoplasts from long-term suspension cultures, plant regeneration seems not a serious impediment as far as japonicas were concerned, but it was difficult with indica. A two-step method consisting of selection of highly regenerable genotypes followed by selection of highly regenerable calli has been reported, to establish japonica rice suspensions retaining a high regeneration potential even after 14 months of culture. Manipulation of hormone contents and supplementation of organic additives is recommended for high-frequency regeneration in long-term cultures. Substantial improvement has been successfully demonstrated through media manipulations or culture practices by Sivamani et al. and Khanna and Raina. There is a dire need to identify simple and dependable criteria to select genotypes, for success in transformation of the genotype of interest. In the present study formation of friable callus, easily dissociating clusters with a few cells, is desirable for transformation. Small cell clumps are preferred for selection of marker gene after transformation, since they increase the permeability, expose more cells to selection agent and thereby improve the selection efficiency and minimize the escapees. Media constituents and supplements such as casein hydrolysate (enzymatic) also determine the quality as well as the quantity of callus proliferation. Table 2 gives the observed differences in the in vitro response between T 309 and indica varieties. Among the indica genotypes, PB-1 and IR 72 could form friable callus, proliferated faster and could be regenerated after 9–10 weeks. Thus initiation and maintenance of suspension cultures was easy and selection on marker substrate was facilitated in these genotypes. These parameters are important for the success of genetic transformation of these two indica genotypes by all the three methods of transformation. The present analysis enabled to focus such in vitro parameters that need to be improved in the indica genotypes of choice.

Though the advent of Agrobacterium-mediated transformation has simplified the delivery of DNA, the transgenic production is limited to japonicas and a few responsive indicas. A study by Jain et al. established distinct positive correlation between embryogenic potential of Basmati rice calli and their competence to be transformed by Agrobacterium. Hence it is the tissue culture maneovurability rather than transformation that limits the transgenic production. Cultural conditions and media manipulations favouring the formation of friable calli, long-term retention of regeneration potential, frequency of calli forming shoots and recovery of greater number of plants per unit culture are prerequisite to select the cells carrying the transferred gene, its manipulation, and subsequent recovery of whole plants with agronomically superior characters.

Vegetation vis-à-vis climate and glacial fluctuations of the Gangotri Glacier since the last 2000 years

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Pollen analysis of a 1.25 m sediment profile from an outwash plain at Bhujhas (3800 m asl) near Gangotri Glacier has revealed the vegetational changes in relation to climatic and glacial fluctuations in the area during the past 2000 years. Around 2000 years BP, open Juniperus–Betula forest occupied the area vacated by the glacier, revealing comparatively cooler and moist climate than the one prevailing at present. Subsequent increase of local arboreal taxa (Juniperus, Betula, Salix) and extra local elements (mainly Pinus) around 1700 years BP, indicates further amelioration of climate, i.e. increase of both precipitation and temperature in this region. Around 850 years BP there is a shift in the vegetational pattern, with sharp increase in Ephedra and other steppe elements notably Artemisia and Asteraceae. This reflects a trend towards drier climatic conditions, which is also evidenced by a decrease in Ferns and Potamogoton. At the upper part of the diagram, i.e. during recent times, climate again reverted to warm and moist, and due to increase in temperature, resulting in the retreat of snout to higher elevations.

The Himalayan glaciers are a storehouse of freshwater and almost all the major river systems in the Indo-Gangetic Plain owe their origin to them. The Gangotri Glacier situated in the Uttarkashi district of Uttarakhand, Western

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