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Micropropagation note

Effect of L-proline and L-tryptophan on somatic embryogenesis and plantlet regeneration of rice (*Oryza sativa* L. cv. Pusa 169)

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Plant cell and tissue culture are likely to continue to be of key importance in the application of molecular biology to crop improvement. Strategies to regenerate plants at high efficiency from tissue culture of cereals have been evolving steadily during the past decades (for a review see Morrish et al. 1987; Lörz et al. 1988). In rice, considerable progress has been made in the last decade and plantlets have been regenerated, via organogenesis as well as somatic embryogenesis, from various explants and also from cells and protoplasts (Maheshwari et al. 1990; see also Raina 1989; Bajaj 1991). The response in terms of plantlets regenerated is, however, poor in comparison to dicots and, in general, *indica* lines have shown a low regeneration potential as compared to the *japonica* lines (Abe & Futsuhara 1984, 1986). Furthermore, a reliable comparison of effectiveness of various promotive factors is complicated due to the fact that very few investigators have given the data in terms of absolute number of plantlets regenerated per unit explant of rice (Heyser et al. 1983; Raghav Ram & Nabors 1984; Abe & Futsuhara 1985; Oard & Rutger 1988; Jones & Rost 1989; Koetje et al. 1989). Search for new promotive factors must continue specially in regard to those capable of promoting regeneration. These could include various organic additives, plant hormones as well as amino acids (Siriwardana & Nabors 1983; Raghav Ram & Nabors 1984; Kavi Kishor & Reddy 1986; Koetje et al. 1989; Ozawa & Komamine 1989; Peterson & Smith 1991; reviews cited above).

The cultivar Pusa 169 used in this investigation is a fine-grained, high protein (9.8%) rice, with many other acceptable features but it has only moderate resistance to bacterial blight, blast, stem borer and brown plant hopper (Siddiq et al. 1989). Development of efficient tissue culture systems, therefore, holds promise to improve it further. Here we report our assessment of the promotive effect of proline and tryptophan on the frequency of callusing and regeneration as also on the absolute number of plantlets regenerated per mature seed used for callus initiation. Finally, scanning electron microscopic evidence for regeneration via somatic embryogenesis in seed cultures is also given.

Seeds of *indica* rice, cv. Pusa 169, were obtained from Indian Agricultural Research Institute, New Delhi. Dehusked seeds were surface-sterilized by immersing in 2.5% sodium hypochlorite for 30 min and subsequently rinsed three times with sterilized distilled water. Seeds were then placed on the surface of callus induction medium consisting of MS medium (Murashige & Skoog 1962) with 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 2% sucrose, and 0.8% agar. The pH of the medium was adjusted to 5.8 before autoclaving. The medium was supplemented with various concentrations of the two amino acids, L-proline (3,6,9,12,15 mM) and L-tryptophan (20,40,90,240,480 μM), added individually to the autoclaved and cooled medium after filter-sterilization. Cultures were kept in diffuse light (8.7 μmol m^{-2} s^{-1}) at 25 ± 2°C. After 30 days, calli formed from scutellar surface were excised from the seed and subcultured on the same medium for another 30 days.

After 60 days of culture, total callus from each seed was separately transferred to culture tubes with cotton plugs containing 20 ml of the regeneration medium [MS + 2.8 μM indoleacetic acid + 23 μM kinetin + 10% coconut water (CW)]. These cultures were kept in 16 h light, provided by fluorescent tubes (Philips TL 40 W/54 or TL 65–80 W/54) at an intensity of 24 μmol m^{-2} s^{-1} and the temperature was maintained at 25 ± 2°C. The cultures showing plantlet regeneration were scored after 30 days, they were subsequently subcultured for another 30 days on the same medium and the frequency of plantlet formation was finally scored. A minimum of 24 cultures was raised for each treatment per experiment and each experiment was repeated at least once. The data shown in Fig. 2 are an average of two experiments and statistical variation is shown in the form of bars.

Samples of thirty-day-old cultures grown on callus induction medium were fixed and processed for scanning electron microscopy according to Vasil & Vasil (1984). After dehydration through an acetone series, the samples were dried at critical point and coated with silver after mounting on stubs. The specimens were observed and photographed with the help of a scanning electron microscope (Philips SEM 501 B).

Within five days after culture on the callus induction medium (MS + 10 μM 2,4-D), the scutellum of the seed showed swelling and started forming callus. After 30 days in culture the calli could be distinguished into two characteristic types, as visualised by light and scanning electron microscopy. One of them was nodular and contained tightly packed cells (Fig. 1A), similar to typical embryogenic calli; the other was of a friable nature, containing long, tubular, loosely arranged cells (Fig. 1B) similar to that in nonembryogenic calli, as described earlier for rice (Nabors et al. 1983). Within the compact and nodular callus, structures similar to bipolar embryos could also be observed (Fig. 1C). Our scanning electron microscopic data, therefore, confirm and supplement the histological evidence for somatic embryogenesis given by Jones & Rost (1989) on seed cultures of rice.

If subcultured on callus induction medium, these calli grew further but did not show any signs of greening or regeneration. Within fifteen days of transfer to regeneration medium, however, several green spots could be observed in the nodular calli (Fig. 1D). The regeneration medium containing 2.8 μM IAA, 23 μM kinetin and 10% CW used for these experiments is comparable to the regeneration medium used by Reddy & Vaidyanath (1985) and Ozawa & Komamine (1989) for *Indica* rice. On further subculture, the calli formed green shoots and roots from their base (Fig. 1E, F). A direct connection of root and shoot axis is indicative of regeneration via somatic embryogenesis, which is a preferred mode of differentiation (Morrish et al. 1987; Lörz et al. 1988; Maheshwari et al. 1990). Somatic embryoids, like their zygotic counterparts, arise from single cells and obviate the problem of possible genetic chimerism arising in other developmental modes. It would be worth mentioning that no albinos were observed in our cultures, which is at variance with the other observations on seed cultures of rice (Raina et al. 1987; Oard & Rutger 1988).

When L-proline and L-tryptophan were added to the callus induction medium, no significant difference in the frequency of callusing itself was observed but a greatly enhanced frequency of embryogenic callus formation was obtained. This is clearly reflected in the percentage of cultures showing regeneration (Fig. 2). It should be noted that cultures without embryogenic calli do not show any regeneration of plantlets. An increase in the proline concentration from 3 to 12 mM resulted in a proportional increase in the percentage of cultures showing plant regeneration (the maximum yield being 63% as compared to 23% without proline); the response declined around 15 mM. Similarly, a four-fold enhancement was observed in terms of number of plantlets produced per culture (Fig. 2). Proline has been reported to promote embryogenesis in somatic tissue cultures of *Zea mays* (Armstrong & Green 1985; Vasil & Vasil 1986). In *Dactylis glomerata* although an enhancement with proline in somatic embryogenesis has been noticed, it is effective only in combination with serine (Trigiano & Conger 1987). Recently, Ozawa & Komamine (1989) have also observed a promotive effect of proline on embryogenesis in suspension cultures of rice. However, the mecha-

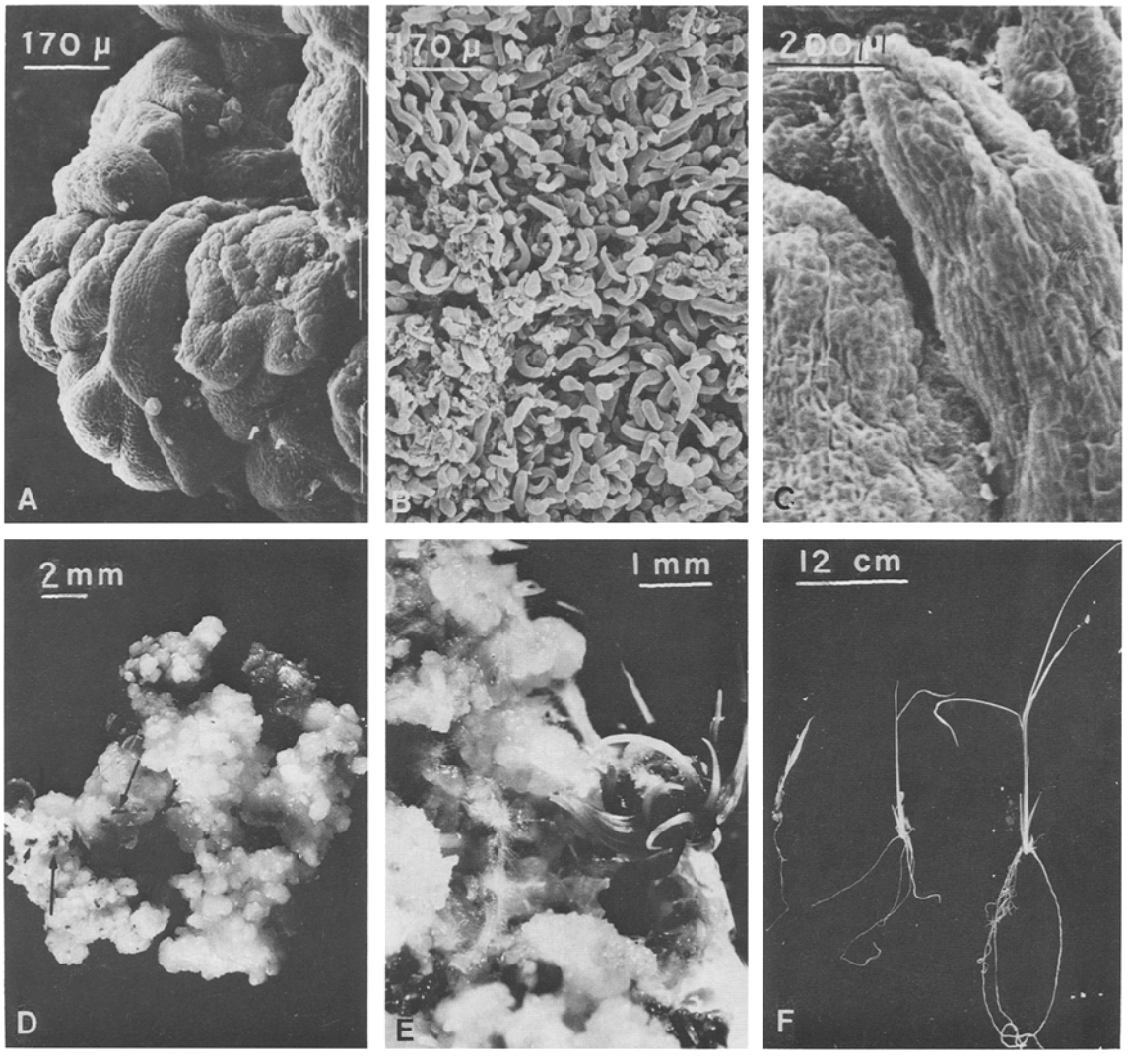

Fig. 1. Regeneration of plantlets in tissue cultures initiated from rice seed. (*A*) Scanning electron microscopic evidence of proliferation of the scutellum leading to the formation of compact, lobed and partially organized structures. (*B*) Friable callus with long and tubular cells. (*C*) Somatic embryoid with defined scutellum and suspensor-like-structure. (*D*) Compact nodular callus produced on callus induction medium with 12 mM proline 15 days after subculture on regeneration medium. Arrows indicate green spots. (*E*) Regeneration of plantlets after 30 days of subculture. (*F*) Plantlets with shoot and attached root in different stages of development at the end of the culture period.

nism of action of proline remains to be elucidated.

Another series of experiments was designed to evaluate the effect of L-tryptophan. Interestingly, tryptophan was found to be effective in a molar range that is about 50 times less than proline. The optimal concentration turned out to be 240 μM and, at this level, both the percentage of cultures showing regeneration as well as the number of plantlets produced per culture were considerably enhanced (250 and 300 per cent respectively over the control; see Fig. 2). In this regard our results are in agreement with the earlier observations of Siriwardana & Nabors (1983) and Raina et al. (1987). Peterson & Smith (1991) also observed increase in regeneration by using tryptophan but only in combination with BAP in the subculture medium. However,

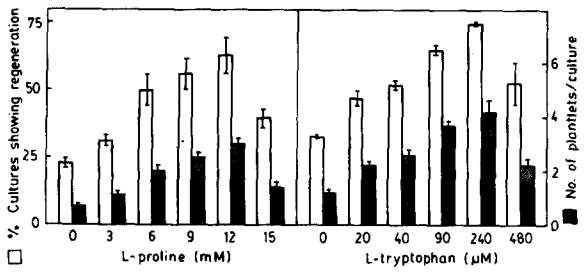

Fig. 2. Effect of L-proline and L-tryptophan on plantlet regeneration from seeds of *indica* rice cv. Pusa 169. Callus cultures were initiated either on MS + 10 μM 2,4-D alone or with varying concentrations of L-proline or L-tryptophan. After sixty days of culture, the callus was transferred to 2.8 μM IAA + 23 μM kinetin + 10% CW for plantlet regeneration. The data given are an average of two experiments. The bar represents variation.

they are at variance with the observations of Koetje et al. (1989) who cultured immature embryos of rice and found that addition of tryptophan increased the amount of callusing but decreased the number of plantlets per explant. It remains to be resolved if the difference in the response is due to the different explants used for induction of callus. The low level of optimal concentration as well as the fact that tryptophan can serve as a precursor of indoleacetic acid has led to the proposal that tryptophan acts by altering endogenous levels of the hormone, IAA (Siriwardana & Nabors 1983). It may, however, be necessary to monitor alterations in endogenous levels of IAA for validating the proposal.

In conclusion, it may be said that we have optimized the concentrations of L-proline and L-tryptophan for obtaining increased regeneration in seed cultures of an *indica* rice cv. Pusa 169 via somatic embryogenesis. Furthermore, the quantitative data for the degree of promotive effect on per cent response as well as absolute number of plantlets produced per explant have been provided, a parameter which should be of value in comparisons involving a search for even more effective additives.

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References

Abe T & Futsuhara Y (1984) Varietal difference of plant regeneration from root callus tissues in rice. Jap. J. Breed. 34: 147–155

Abe T & Futsuhara Y (1985) Efficient plant regeneration by somatic embryogenesis from root callus tissues of rice (*Oryza sativa* L.). J. Plant Physiol. 121: 111–118

Abe T & Futsuhara Y (1986) Genotypic variability for callus formation and plant regeneration in rice (*Oryza sativa* L.). Theor. Appl. Genet. 72: 3–10

Armstrong CL & Green CE (1985) Establishment and maintenance of friable embryogenic maize callus and the involvement of L-proline. Planta 164: 207–214

Bajaj YPS (1991) Biotechnology in Agriculture and Forestry, Vol 12, Rice. Springer-Verlag, Berlin, Heidelberg

Heyser JW, Dykes TA, DeMott KJ & Nabors MW (1983) High frequency long term regeneration of rice from callus culture. Plant Sci. Lett. 29: 175–182

Jones TJ & Rost TL (1989) The developmental anatomy and ultrastructure of somatic embryos from rice (*Oryza sativa* L.) scutellum epithelial cells. Bot. Gaz. 150: 41–49

Kavi Kishor PB & Reddy GM (1986) Regeneration of plants from long-term cultures of *Oryza sativa* L. Plant Cell Rep. 5: 391–393

Koetje DS, Grimes HD, Wang Yi-Chang & Hodges TK (1989) Regeneration of indica rice (*Oryza sativa* L.) from primary callus derived from immature embryos. J. Plant Physiol. 135: 184–190

Lörz H, Gobel E & Brown P (1988) Advances in tissue culture and progress towards genetic transformation of cereals. Plant Breed. 100: 1–25

Maheshwari N, Rajyalakshmi K, Chowdhry CN, Grover A, Tyagi AK & Maheshwari SC (1990) *In vitro* culture of wheat and rice for understanding the molecular basis of somatic embyrogenesis and for transformation. In: Sangwan RS & Sangwan BS (Eds) Impact of Biotechnology on Agriculture (pp 191–213). Kluwer Academic Publishers, Dordrecht

Morrish F, Vasil V & Vasil IK (1987) Developmental morphogenesis and genetic manipulation in tissue and cell culture of the Gramineae. Adv. Genet. 24: 431–499

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Nabors MW, Heyser JW, Dykes TA & DeMott KJ (1983) Long-duration, high-frequency plant regeneration from cereal tissue cultures. Planta 157: 385–391

Oard JH & Rutger JN (1988) Callus induction and plant regeneration in elite U.S. rice lines. Crop Sci. 28: 565–567

Ozawa K & Komamine A (1989) Establishment of a system of high-frequency embryogenesis from long-term cell suspension cultures of rice (*Oryza sativa* L.). Theor. Appl. Genet. 77: 205–211

Peterson G & Smith R (1991) Effect of abscisic acid and callus size on regeneration of American and international rice varieties. Plant Cell Rep. 10: 35–38

Raghav Ram NV & Nabors MW (1984) Cytokinin mediated long-term, high-frequency plant regeneration in rice tissue culture. Z. Pflanzenphysiol. 113: 315–323

Raina SK (1989) Tissue culture in rice improvement: Status and potential. Adv. Agron. 42: 339–397

Raina SK, Sathish P & Sarma KS (1987) Plant regeneration from *in vitro* cultures of anther and mature seeds of rice (*Oryza sativa* L.) cv. Basmati-370. Plant Cell Rep. 6: 43–45

Reddy PJ & Vaidyanath K (1985) *In vitro* selection for salt tolerance in Basmati rice. Ind. J. Plant Physiol. 28: 88–91

Siriwardana S & Nabors MW (1983) Tryptophan enhancement of somatic embryogenesis in rice. Plant Physiol. 73: 143–146

Siddiq EA, Singh VP, Zaman FU, Sadananda AR & Puri RP (1989) 'Pusa 169' an ideal rice variety for wheat-rice rotation. Ind. Farming 38: 23–25

Trigiano RN & Conger BV (1987) Regulation of growth and somatic embryogenesis by proline and serine in suspension cultures of *Dactylis glomerata*. J. Plant Physiol. 130: 49–55

Vasil V & Vasil IK (1984) Preparations of cultured tissues for scanning electron microscopy. In: Vasil IK (Ed) Cell Culture and Somatic Cell Genetics of Plants, Vol 1 (pp 738–743). Laboratory Procedures and Their Applications. Academic Press, Orlando, Florida

Vasil V & Vasil IK (1986) Plant regeneration from friable embryogenic callus and cell suspension cultures of *Zea mays* L. J. Plant Physiol. 124: 399–408