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Plant regeneration from tissue cultures of Pokkali rice is promoted by optimizing callus to medium volume ratio and by a medium-conditioning factor produced by embryogenic callus*

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Abstract. The frequency of plant regeneration from seed-derived Pokkali rice callus has been substantially increased. Four conclusions were drawn from the study: (1) Non-embryogenic callus consisting of elongated, highly-vacuolated cells did not produce regenerated plants. Embryogenic callus consisting of small, non-vacuolated cells produced somatic embryos and regenerated plants. (2) The numbers of plants could be markedly increased by optimizing a medium for embryogenic callus production and a second medium for plant regeneration from embryogenic callus. (3) The optimization of callus to medium volume ratio of 6.5 mg embryogenic callus per 1.0 ml of medium significantly increase plant production on regeneration medium. (4) A further significant increase was obtained by using regeneration medium previously conditioned for one or two weeks by optimal amounts of embryogenic callus. At present, the callus derived from a single seed in six months could theoretically be used in the seventh month to produce 127 500 plants.

Introduction

A major constraint in the application of tissue culture techniques to crop improvement has been lack of sufficiently high-frequency plant regeneration methods for many cereal crops. The use of single medium systems to produce callus and shoots in tobacco [14] and some other dicots has not proven generally applicable to cereals in which the medium which produced callus rarely promotes regeneration. Rather, in cereals (and many dicots [3]), a series of different media may be required for somatic embryogenesis [17] and plant regeneration from morphogenetically capable callus [9, 10]. For example, the shoot axis from mature wheat seeds produces an undifferentiated callus of isodiametric, meristematic-like cells. However, the media promoting formation of this so-called 'smooth embryogenic callus' does not regularly induce the formation of somatic embryoids [6]. Similarly, a medium producing somatic embryos in corn does not induce their continued development into plants [5]. Different species and, within a species, different

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cultivars of cereals, require two or more different media of a reasonably specific chemical composition to complete the sequence from initial explant to regenerated plant.

In rice, the initiation and growth of embryogenic (E) callus with somatic embryos requires one medium, whereas development of the embryos into plants requires a second medium [11, 13]. In general, embryogenic callus forms and continues growth on media containing low levels of 2,4 dichlorophenoxyacetic acid (2,4-D, 0.5–2.0 mg/l) and kinetin (KIN, 0.1–0.6 mg/l) depend[illegible] on cultivar. Addition of high levels (50–100 mg/l) of tryptophan (TRP) enha[illegible] process in some cultivars [13]. Regeneration of plants is promoted by media containing no 2,4-D, low levels of 6-benzylaminopurine (BA, 0.1–0.6 mg/l), and sometimes low levels of indole-3-acetic acid (IAA, 0.5–1.0 mg/l [10]). The effects of proline and abscisic acid on production of embryogenic callus or regeneration of plants from rice tissue cultures has not been investigated. Proline has been reported to promote embryogenesis in other cereals [5] while abscisic acid restores regenerative competence for shoot organogenesis to rice callus ([7], and unpublished observations). Also, the effects of varying nitrate to ammonium ratios, which affect regeneration in some dicots [3, 19], have not been studied.

Initial inoculum size has been studied in relation to callus growth in several dicotyledons [1, 2, 15, 16]. We are not aware that the subject has been investigated in cereals and, in particular, with respect to plant regeneration. The experiments discussed in this paper were conducted to determine whether initial callus inoculum size or the ratio of callus to medium volume affected the rate of plant regeneration from rice embryogenic callus.

Materials and methods

Seeds of rice (*Oryza sativa* L.) cultivar Pokkali, were washed with five drops of liquid detergent in 20 ml of water. Seeds were then washed in running water to remove soap and were sterilized using 95% EtOH for 10 s and 50% Clorox for 30 m. After washing thoroughly five to six times with sterile distilled water, seeds were planted on a medium containing Murashige and Skoog's [8] mineral salts with thiamine, myo-inositol, and 4% sucrose as organic constituents. In addition, 50 mg/l Fe-EDTA was used in place of $FeSO_4$ $7H_2O$. The medium was solidified with 1% agar.

Embryogenic callus was initiated from the scutellum of intact mature seeds in the dark on a medium containing 0.5 mg/l 2,4-D, 0.2 mg/l KIN and 50 mg/l TRP as added growth regulators. It was maintained in the light on a medium containing 0.5 mg/l 2,4-D and 0.4 mg/l KIN. Callus was transferred to fresh medium after four weeks. Plant regeneration occurred in the light on a medium containing 0.5 mg/l BA. Light-grown cultures were placed 25 cm from two Sylvania wide-spectrum fluorescent lamps (F40-GRO-WS) and grown in continuous light. All cultures were maintained at 28 °C.

All culture vials were made of flint glass and have black plastic caps wit cardboard liners. Caps are tightened with no pressure from thumb and for finger. All vials are obtainable from standard catalogs. Four sizes of vials wei used. Size A was (inside dimensions) 36 mm tall and 10 mm wide; size I 60 by 16; size C, 70 by 24; and size D, 80 by 36. The volumes of A–D wei 2.4 mls, 8.7 mls, 30.0 mls and 69.0 mls respectively.

Conditioned medium was prepared by inoculating 65 and 500 mg E cal onto 23 mls of regeneration medium in container D. Cultures were allowed t grow for one or two weeks at which time the medium was partially melted b heating in a 70 °C water bath to allow removal of callus and small plantlet: The medium was then resolidified by cooling and reinoculated with 65 mg c fresh E callus.

Results and discussion

Varying amounts of E callus ranging from 65–800 mg were inoculated int container D with 23.3 ml of regeneration medium. The maximum number o plants per g initial callus after four weeks was produced when the inoculun size was 175 mg (Figure 1). At this point, the ratio of inoculum weight t medium volume was 6.5 mg ml^{-1}. Increasing the inoculum size beyond 17: mg resulted in an abrupt decrease in the number of plants regenerated (Figur 1).

When a standard inoculum of 65 mg callus was used in different container

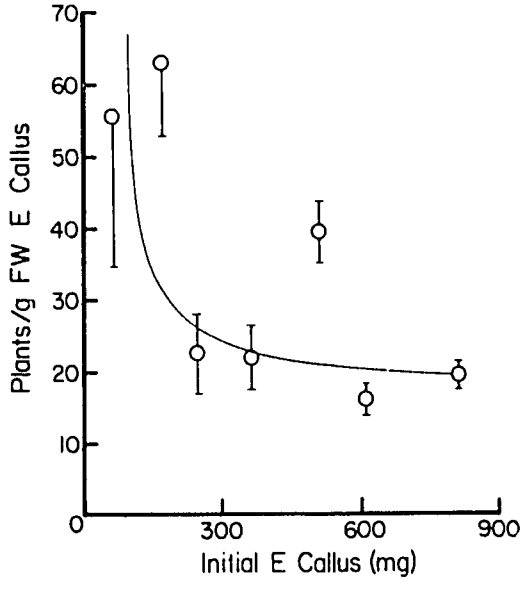

Figure 1. Effect of initial inoculum size on regeneration. Culture vessel D was used. It contained 23.3 ml medium. Standard errors are shown. Sample size was 25–65 containers.

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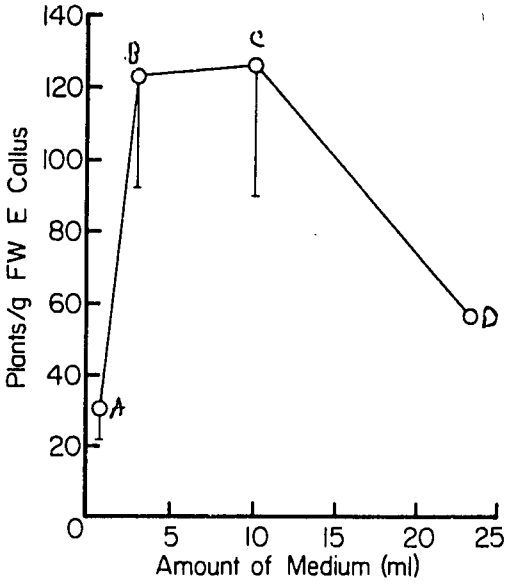

Figure 2. Plant regeneration in four weeks when 65 mg callus was inoculated into container A, containing 0.8 ml medium; B, containing 2.9 ml; C, containing 30.0 ml; and D, containing 23.3 ml. Standard errors are shown. Samples size was 25 containers.

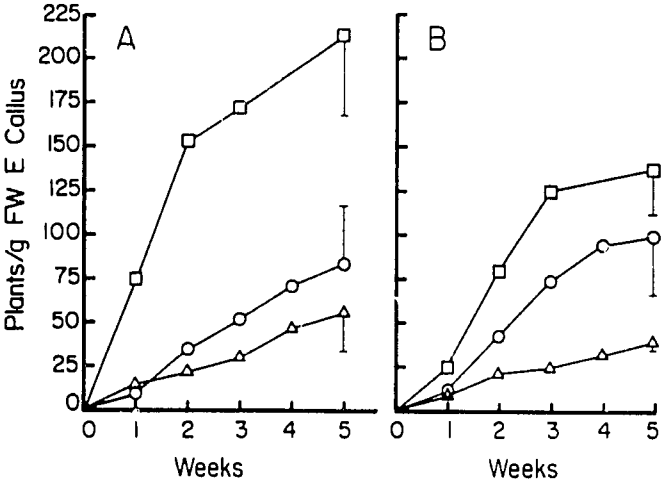

Figure 3. Conditioning of medium. A: initial inoculum size was 65 mg. B: initial inoculum size was 500 mg. △; no conditioning of medium. ○; medium conditioned for 1 week. □; medium conditioned for 2 weeks. In all these cases, after conditioning, the medium was reinoculated with 65 mg E callus. The regeneration from this callus in 5 weeks is evaluated. Standard errors are shown. Sample size was 20 containers. Container C was used with 10 ml medium.

(A–D) containing medium equivalent to one third of their volume, container C produced maximal numbers of plants (Figure 2). In this case, the ratio of inoculum weight to medium volume was 6.5 mg ml^{-1}. An initial inoculum of

Table 1. Regeneration of plants in different culture vessels (amount of medium equivalent to 1/3 volume of the vessel). Sample size was 20 culture vessels.

Container	Amount of medium (ml)	Amount of inoculum (mg)	Inoculum (mg) / Medium (ml)	Plants/g E callus*
A	0.8	23	28.8	50.09 ± 16.57
B	2.9	70	24.1	53.94 ± 12.75
C	10.0	240	24.0	46.18 ± 5.27
D	23.3	513	22.0	44.60 ± 4.72

*Values adjusted to a ratio 25.0

Table 2. Amount of initial E callus for each culture vessel required for maximal regeneration

Container	Medium (ml)	Inoculum size (mg)*
A	0.8	5.2
B	2.9	18.9
C	10.0	65.0
D	23.3	151.4

* Adjusted to nearest decimal

65 mg E callus in container D also produced maximal numbers of plants, in that container, on 10 ml medium (data not shown). With 65 mg callus and 10 ml medium, container C produced twice the number of regenerated plants as container D. This decrease appeared to be related to the more rapid desiccation of medium in container D which had a larger surface area than container C. Experiments to test this hypothesis were not performed.

The above experiments suggested that a critical ratio between the inoculum weight and the medium volume or volume of culture vessel atmosphere might be required for maximal plant regeneration. A ratio of inoculum weight to medium volume between 6.5 to 7.5 $mg\,ml^{-1}$ appeared best from the data. When the ratio of inoculum weight to medium volume was maintained at 25 $mg\,ml^{-1}$, the number of plants regenerated in each container was constant (Table 1). The ratio of 25 was chosen before the optimal ratio had been determined. If 6.5 is taken as the best ratio and containers A–D are filled to one third capacity with regeneration medium, the amount of initial callus required is shown in Table 2.

Other data (not shown) demonstrated many times that, when larger than optimal callus was used as inoculum, plant regeneration was considerably reduced while more callus growth occurred. When inocula smaller than optimal were used, plant regeneration was also reduced and that rate of callus death was increased. Dunwell [4] found similar results for plant regeneration from one hundred tobacco anthers placed on the same amount of medium in

Table 3. Plants which could theoretically be regenerated from one seed under various culture conditions. E callus was initiated from one seed in dark and was maintained for 6 passages (after 1 passage on initiation medium) in light. For the 7th passage, all the E callus was transferred to regeneration medium. Numbers are calculated from experiments in which at least 300 plants were produced. NE callus never produced plants.

Culture Condition	Plants regenerated at the end of 7th passage	Total number of plants regenerated (including those on maintenance medium)
NE Callus	0	0
E Callus unimproved media (2 mg/l 2,4-D)		4 880
E Callus improved media (see Materials and methods)	20 170	25 050
Callus to medium ratio (mg/ml) adjusted to 6.5	64 540	69 420
Regeneration medium conditioned for 2 weeks at optimal conditions	122 620	127 500
Utilizing the conditioning factor directly (proposed)	245 240	250 120

various sized containers. The smallest and largest container gave considerably reduced regeneration of plants. He attributed differences in regeneration frequency to differences in volume of culture vessel atmosphere.

In our experiment, inocula larger than the optimal size soon find medium surface area to be a limiting factor. We investigated the possibility that larger calli deplete the medium of BA, by increasing the concentration above 0.5 mg/l. No increase in regeneration occurred, but the calli may deplete the medium of another hormone factor or required nutrient.

The fate of smaller than optimal calli suggested that the callus may release some factor into the medium which influences regeneration. This 'conditioning factor' would not be produced in sufficient amounts when smaller inocula are utilized. E callus was allowed to condition 10 ml regeneration medium in container C before being replaced with a second E callus. The results (Figure 3a) show that conditioned medium allowed considerably more regeneration than non-conditioned medium. If a 65 mg callus was used as the inoculum and was allowed to condition the medium for two weeks, a subsequent 65 mg callus produced 3.8 times as many regenerated plants as a control placed on non-conditioned medium (Figure 3a). In these experiments, culture vessel volumes were identical for all treatments. The heating of the

medium prior to resolidification makes it unlikely that volatile factors, dissolved in agar, are responsible for the conditions.

Table 3 shows that the overall plant regeneration in Pokkali rice has been increased considerably from initial experiments in which non-embryogenic (NE) callus produced no regenerated plants. Optimizing E callus production and maintenance medium composition by varying cytokinin and auxin concentrations (data not shown) provided the first increase in regeneration simply by increasing the amount of E callus obtained, that is, the number of plants produced per unit callus tissue was unchanged. Subsequently, experiments to optimize the regeneration medium further increased regeneration efficiency. If the best available media were combined with the optimal callus to medium volume ratio and pre-conditioning of the medium, 127 500 plants could be obtained from a single seed in seven months (six months of callus production and one of regeneration). These numbers were calculated from experiments in which several thousand regenerated plants were obtained. Over 100 Pokkali plants were grown to reproductive maturity in the greenhouse.

At present, to obtain this high level of plant regeneration, one half of the E callus must be utilized for medium conditioning. Following heating of the medium and removal of this callus, the callus is unsuitable for subsequent regeneration. If the conditioning factor were isolated and found to be a chemical or chemicals which could be added separately, at least 250 000 plants could be obtained. This procedure could produce large numbers of genetically identical plants for use by plant breeders. In addition, the well-known increase in genetic variation in regenerated plants could be more effective in producing 'mutaclonal' [18] or 'somaclonal' [12] variants using high-frequency regeneration techniques.

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