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HIGH FREQUENCY PLANT REGENERATION FROM MATURE SEED OF ELITE, RECALCITRANT MALAYSIAN INDICA RICE (*ORYZA SATIVA* L.) CV. MR 219

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An efficient *in vitro* plant regeneration system was established for elite, recalcitrant Malaysian indica rice, *Oryza sativa* L. CV. MR 219 using mature seeds as explant on Murashige and Skoog and Chu N6 media containing 2,4-dichlorophenoxy acetic acid and kinetin either alone or in different combinations. L-proline, casein hydrolysate and L-glutamine were added to callus induction media for enhancement of embryogenic callus induction. The highest frequency of friable callus induction (84%) was observed in N6 medium containing 2.5 mg l^{-1} 2,4-dichlorophenoxy acetic acid, 0.2 mg l^{-1} kinetin, 2.5 mg l^{-1} L-proline, 300 mg l^{-1} casein hydrolysate, 20 mg l^{-1} L-glutamine and 30 g l^{-1} sucrose under culture in continuous lighting conditions. The maximum regeneration frequency (71%) was observed, when 30-day-old N6 friable calli were cultured on MS medium supplemented with 3 mg l^{-1} 6-benzyl aminopurine, 1 mg l^{-1} naphthalene acetic acid, 2.5 mg l^{-1} L-proline, 300 mg l^{-1} casein hydrolysate and 3% maltose. Developed shoots were rooted in half strength MS medium supplemented with 2% sucrose and were successfully transplanted to soil with 95% survival. This protocol may be used for other recalcitrant indica rice genotypes and to transfer desirable genes in to Malaysian indica rice cultivar MR219 for crop improvement.

Keywords: Callus induction – *Oryza sativa* ssp. *indica* – mature seeds – plant regeneration – recalcitrant

INTRODUCTION

Over half of the world's population depends on rice as their staple food. The growing human population and resultant increased pressure on arable land and resources have led to the growing acceptance that conventional breeding methods for rice improvement need to be combined with recent achievements in rice biotechnology, including transgenic technology [1, 24]. Rice transformation technology has been applied to address several areas of crop improvement including tolerance to abiotic stress [7, 29]

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetic acid, BAP – 6-benzylamino purine, CH – casein hydrolysate, Gln – L-glutamine, KIN – Kinetin, MS – Murashige and Skoog medium, NAA – Naphthaleneacetic acid, Pro – L-proline.

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tolerance to biotic stress [8, 16] and improvements in quality traits [13]. Most of these achievements were not possible through conventional breeding technologies. In addition to molecular breeding strategies for rice improvement, functional genomic studies in rice, such as RNA interference based knock down of gene expression, also require genetic transformation and plant regeneration protocols to be developed [28]. Whilst there are several regeneration and transformation protocols available in cultivated indica rice genotypes [2, 10, 11, 14, 22, 26], the response of indica, japonica and javonica rice to callus induction and regeneration media is variety specific. Therefore it is necessary to optimize these media individually for the different rice varieties used in different regions [30]. This report describes the establishment and optimization of callus induction and regeneration media for the elite recalcitrant indica rice variety MR219, the major variety used in rice production in Malaysia.

MATERIALS AND METHODS

Manually dehusked seeds of MR219 were surface sterilized with 70% ethanol for three minutes and then with 0.1% mercuric chloride for five minutes, followed by three washes in sterile distilled water. Sterilized seeds were cultured on MS [18] and N6 [5] medium supplemented with 2,4-D (1.5, 2.0, 2.5 and 3.0 mg l^{-1}) and KIN (0.1, 0.20 and 0.3 mg l^{-1}) either alone or in combination with Pro (2.5 mg l^{-1}), CH (300 mg l^{-1}, Gln (20 mg l^{-1}), 3% sucrose and 4 g l^{-1} phytagel at 25 °C in continuous light for 15 days (Table 1). Eight to ten seeds were placed in each plastic Petri dish (100 × 15 mm) containing the various callus induction media. The pH of all media was adjusted to 5.8 before autoclaving at 115 °C for 15 minutes. After 14 days, the proliferated calli were subcultured onto the same medium for another 15 days. Fast growing, cream-colored friable calli (CIM 10) of 5–6 mm diameter were used for regeneration. For regeneration, 30-day-old calli of MS medium (CIM 10) derived calli were transferred to MS and N6 regeneration medium containing BAP (1.5, 2.0, 2.5 and 3.0 mg l^{-1}) and NAA (0.5, 1.0 and 1.5 mg l^{-1}) either alone or in combination with 3% maltose and 4 mg l^{-1} phytagel at 25 °C in the continuous light for 21 days (Table 2). Similarly, N6 medium (CIM 10) derived calli were also transferred to MS and N6 regeneration media containing BAP (1.5, 2.0, 2.5 and 3.0 mg l^{-1}) and NAA (0.5, 1.0 and 1.5 mg l^{-1}) either alone or in combination with 3% maltose and phytagel 4 g l^{-1} at 25 °C in the continuous light for 21 days (Table 3). Developed shoots (3 cm height) were excised and transferred to half strength MS medium containing 2% sucrose for root development. After 15 days, the rooted plants were transferred to pots containing sterile garden soil and coir pith compost (1 : 1).

Callus induction frequency was recorded considering that each callus piece originated from a single seed. The frequency of callus induction and regeneration was calculated as follows: Callus induction frequency (%) = (number of grains producing calli/number of grains plated) × 100 and regeneration frequency (%) = (number of plants recovered/number of calli plated) × 100 [20]. Each experiment was replicated thrice in a completely randomized block design. Data were processed using analysis

of variance (ANOVA) of the IRRISTAT statistical package. Mean values were separated by Duncan's [6] multiple range test (DMRT) at a 5% probability level. Arcsin (p%/100)½ transformation of the variable was performed before analysis and converted back to percentage.

RESULTS AND DISCUSSION

Callus induction was carried out using mature seeds as explants (Fig 1a). Calli were invariably initiated in the scutellum region and were visible within seven days of seed inoculation (Fig. 1b). Reports on choice of explants include immature embryos [4], mature seed [10, 14, 22, 25, 26], meristem discs [3], embryonic suspension cultures [12], shoot meristems [21] and young inflorescence [27]. However, scutellum-derived calli remain the most acceptable explants, since high transformation and regeneration frequencies have been achieved with this material as initial explants [10, 22]. Friable, cream-colored calli were obtained in 14-day-old cultures (Fig. 1c). MS and N6 media were used for callus induction and were supplemented with 2,4-D (1.5, 2.0, 2.5 and 3.0 mg l^{-1}) and KIN (0.1, 0.2 and 0.3 mg l^{-1}) either alone or different combinations (Table 1).

Table 1
Effect of 2,4-D and KIN on callus induction of MR219 in MS and N6 media

Media	Growth regulators (mg l^{-1})		MS Callus (%)[b]	N6 Callus (%)[b]
	2,4-D	KIN		
CIM 1[a]	–	–	20.00±0.67	22.10±0.60
CIM 2	1.5	–	32.00±0.88	35.50±0.40
CIM 3	2.0	–	38.10±0.50	41.30±0.67
CIM 4	2.5	–	52.60±0.35	61.00±0.58
CIM 5	3.0	–	50.00±0.81	58.00±0.87
CIM 6	–	0.10	27.50±0.50	30.70±0.58
CIM 7	–	0.20	28.00±0.40	30.00±0.33
CIM 8	–	0.30	24.00±0.58	27.00±0.71
CIM 9	2.5	0.10	65.00±0.52	72.50±0.40
CIM 10	2.5	0.20	73.00±0.44	84.00±0.30
CIM 11	2.5	0.30	68.10±0.40	79.00±0.33

[a] Callus induction medium (CIM). CIM 1 (MS or N6); CIM 2 to CIM 11 – Media supplemented with 2.5 mg l^{-1} Pro, 300 mg/L CH, 20 mg l^{-1} Gln; All media supplemented with Sucrose 30 g l^{-1}.

[b] Response recorded every 7 d; Total culture period was 21 d; Percentage of callus (mean ± standard error) of three replicates.

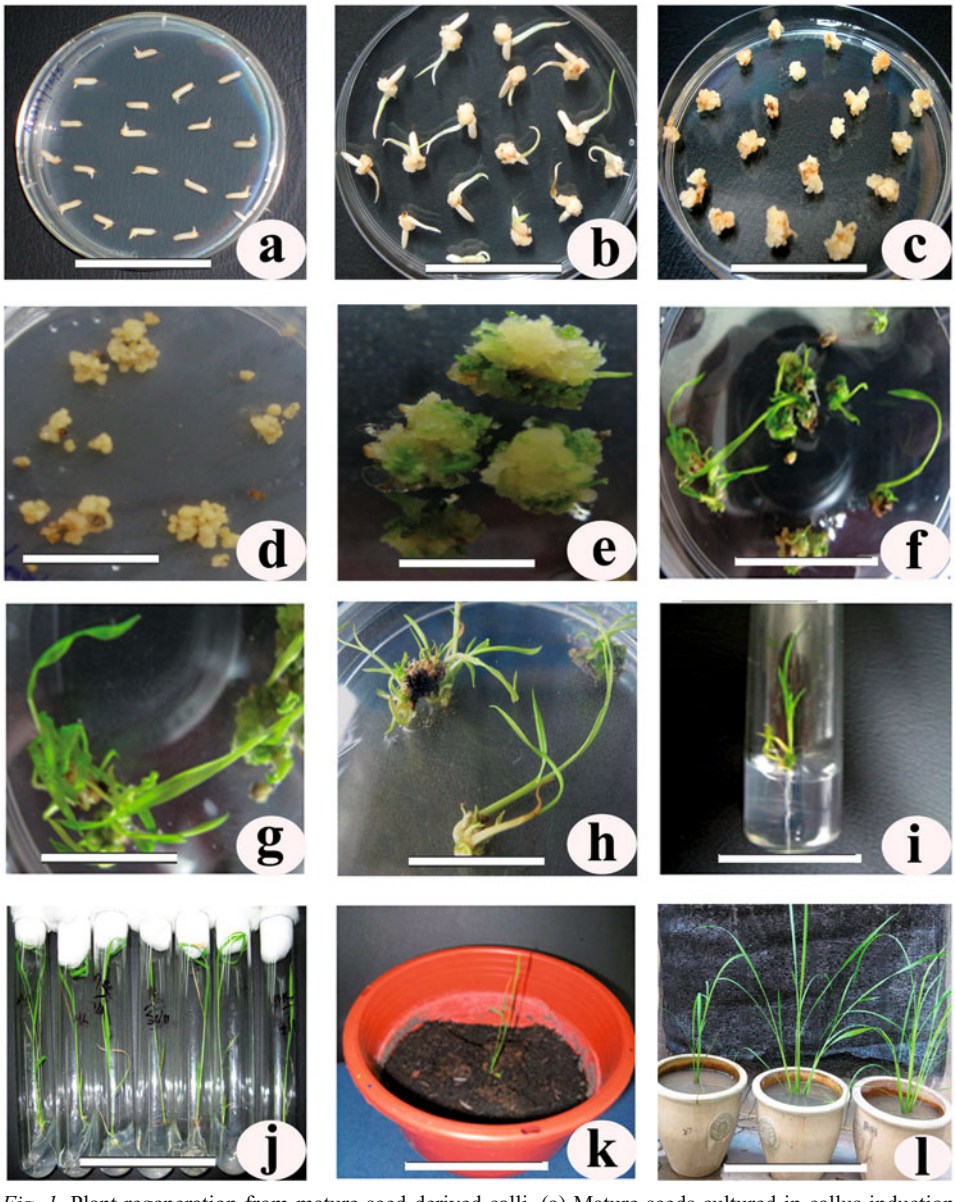

Fig. 1. Plant regeneration from mature seed-derived calli. (a) Mature seeds cultured in callus induction medium (Bar = 50 mm); (b) one-week-old calli induced from the scutellum region of mature seed (Bar = 40 mm); (c) Two-week-old calli (Bar = 40 mm); (d) one-month-old seed derived calli in regeneration medium (Bar = 30 mm); (e) shoot buds initiated from calli cultured in regeneration medium, after six days (Bar = 20 mm); (f and g) shoots developed in regeneration medium, after 15 days (Bar = 30 mm); (h) well-developed elongated shoots in regeneration medium, after three weeks (Bar = 30 mm); (i) excised shoots cultured in half MS medium for rooting (Bar = 30 mm); (j) rooted plantlets *in vitro* (Bar = 70 mm); (k) plantlet grown in a plastic pot containing coir pith compost, five days after transfer (Bar = 100 mm); (l) plantlets grown in *in vivo* condition, one month after transfer (Bar = 400 mm)

Table 2
Effect of BAP and NAA on regeneration of MS medium derived calli (CIM 10) in MS and N6 regeneration media

Media	Growth Regulators (mg l^{-1})		MS Regeneration (%)[b]	N6 Regeneration (%)[b]
	BAP	NAA		
RGM 1[a]	–	–	18.00	15.00
RGM 2	1.5	–	30.00	28.00
RGM 3	2.0	–	32.00	30.00
RGM 4	2.5	–	36.00	32.00
RGM 5	3.0	–	36.00	34.00
RGM 6	–	0.5	20.00	18.00
RGM 7	–	1.0	18.00	15.00
RGM 8	–	1.5	16.00	15.00
RGM 9	3.0	0.5	52.00	48.00
RGM10	**3.0**	**1.0**	**55.00**	**51.00**
RGM 11	3.0	1.5	55.00	51.00

[a] Regeneration medium (RGM), RGM 1 to RGM 11 (MS or N6); All media were supplemented with maltose 30 g l^{-1}.

[b] Response recorded once at 14 d; Total culture period was 21 d; Percentage of regenerants (mean of three replicates).

Additionally, Pro (2.5 mg l^{-1}), CH (300 mg l^{-1}) and Gln (20 mg l^{-1}) were also added to MS and N6 callus induction media (CIM 2 to CIM 11) for further enhancement of callus induction. The effect of 2,4-D and KIN on callus induction of MR219 in MS and N6 medium are shown in Table 1. The callus induction frequency ranged from 20.0% to 73.0% in MS media, whilst in N6 media callus induction frequency ranged from 22.0% to 84.0% (Table 1). The application of 2,4-D (2.5 mg l^{-1}) without KIN induced 61.0% callus induction in N6 medium containing Pro (2.5 mg l^{-1}), CH (300 mg l^{-1}) and Gln (20 mg l^{-1}), compared to 52.6% in MS medium (CIM 4). The present investigation also revealed that the presence of KIN at any concentration (0.1, 0.2 and 0.3 mg l^{-1}) in either MS or N6 media did not enhance callus induction. Among the different combinations of 2,4-D and KIN tested, the combined effect of 2.5 mg l^{-1} (2,4-D) with 0.20 mg l^{-1} (KIN) gave the best response for callus induction on MS (73.0%) or N6 (84.0%) media with 2.5 mg l^{-1} Pro, 300 mg l^{-1} CH, 20 mg l^{-1} Gln, 30 g l^{-1} sucrose and 0.4% phytagel (CIM 10). At this composition, only creamy friable calli were obtained for both MS and N6 medium.

The superiority of 2,4-D and KIN inducing callus induction in indica genotypes was recently reported [22]. The current results also showed that N6 media were better than MS media for callus induction. This may be mainly due to the presence of more

nitrogen in N6 media than MS media, as suggested by Rashid et al. [23]. The present results are similar to those reported by Tariq et al. [25], with indica rice cultivars, where the highest callus induction frequency was produced on N6 medium containing 2,4-D (2.5 and 3.0 mg l^{-1}) alone.

In the present study, 2.5 mg l^{-1} Pro was added to both MS and N6 callus induction media. Proline is normally a stress amino acid that provides protection against stress through various mechanisms [9, 19, 26]. Thus, proline may be helpful for recovery of viable embryogenic calli following various *in vitro* stresses. Toki et al. [26] reported the use of 2.9 mg l^{-1} Pro for callus induction. Similarly, addition of CH to media provides a source of amino acids and has been shown to increase the production of embryogenic calli from rice [17, 22, 30]. Our data also is in agreement with a previous study showing that 300 mg l^{-1} CH enhanced the induction of friable callus in rice [26]. In the present study, 20 mg l^{-1} Gln was added to both MS and N6 media for further enhancement of callus induction. This result is in accordance with Carsono and Yoshida [2] who used 500 mg l^{-1} Gln for embryogenic callus induction in Indonesian rice genotypes.

For plant regeneration, both MS and N6 derived calli were separately transferred to MS and N6 media supplemented with BAP (1.5, 2.0, 2.5 and 3.0 mg l^{-1}) and NAA (0.5, 1.0 and 1.5 mg l^{-1}) either alone or in combinations. Additionally, 2.5 mg l^{-1} Pro

Table 3
Effect of BAP and NAA on regeneration of N6 medium derived calli (CIM 10) in MS and N6 regeneration media

Media	Growth regulators (mg l^{-1})		MS Regeneration (%)[b]	N6 Regeneration (%)[b]
	BAP	NAA		
RGM 1[a]	–	–	20.00	17.00
RGM 2	1.5	–	34.00	31.00
RGM 3	2.0	–	34.00	33.00
RGM 4	2.5	–	37.00	34.00
RGM 5	3.0	–	40.00	36.00
RGM 6	–	0.5	27.00	24.00
RGM 7	–	1.0	23.00	20.00
RGM 8	–	1.5	20.00	18.00
RGM 9	3.0	0.5	65.00	62.00
RGM 10	3.0	1.0	71.00	64.00
RGM 11	3.0	1.5	68.00	60.00

[a] Regeneration medium (RGM), RGM 1 to RGM 11 (MS or N6); All media were supplemented with maltose 30 g l^{-1}.

[b] Response recorded once at 14 d; Total culture period was 21 d; Percentage of regenerants (mean of three replicates).

and 300 mg l^{-1} CH were included in the media as supplements. The regeneration frequencies for MS and for N6 derived embryogenic calli are shown in Tables 2 and 3, respectively. Among the several combinations and experiments tested, the highest regeneration percentage was observed when N6 medium derived calli were cultured in MS or N6 media RGM 10 containing 3.0 mg l^{-1} BAP, 1.0 mg l^{-1} NAA with 2.5 mg l^{-1} Pro, 30 mg l^{-1} CH and 0.4% phytagel (71.0% and 64.0% regeneration, respectively, Table 3 and Fig. 1d) with MS medium giving the higher rate of regeneration. After six days, shoot buds initiated from the calli cultured in the regeneration media RGM 10 containing BAP and NAA (Fig. 1e). After 21 days, the developed shoots (Fig. 1f, g and h) were excised and cultured into half strength MS medium containing 2% sucrose for another 14 days (Fig. 1i). For the present study we included maltose (3%) in the regeneration media to enhance the regeneration frequency, based on previous reports that found maltose to be the optimal carbon source for regeneration [15, 30]. Another study reported sucrose (3%) to be the best carbon source for both callus induction and regeneration [25], however, our frequency of callus induction and shoot regeneration using maltose compare very favourably with those reported by Tariq et al. [25] with sucrose, with 84% callus induction (compared to 73–75% with sucrose) and 71% regeneration (compared to 61–60% with sucrose). After 15 days of culture, the well rooted individual plants (Fig. 1j) from semisolid half strength MS medium were carefully removed and washed free of agar with sterile distilled water before transfer to a sterile coirpith containing plastic pot under *in vitro* conditions (Fig. 1k). After five days, the *in vitro* regenerated plants were transferred to pots containing coir pith compost: garden soil mixture (1 : 1) for further establishment (Fig 1l). The survival percentage of plantlets, as recorded 14 days after transfer to pots containing soil, was over 95%.

In conclusion, an *in vitro* regeneration protocol was successfully standardized for elite indica Malaysian rice cultivar MR219 through proper manipulation of the different medium and its composition. The successful *in vitro* regeneration protocol is highly suitable for other recalcitrant indica rice genotypes. Further, this reliable *in vitro* regeneration protocol will be useful for future genetic transformation studies.

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