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Highly efficient protocol for callogenesis, somagenesis and regeneration of *Indica* rice plants

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ABSTRACT

In the present study, we have reported a simple, fast and efficient regeneration protocol using mature embryos as explants, and discovered its effective applicability to a range of *Indica* rice genotypes. We have considered the response of six varieties in the steps of the regeneration procedure. The results showed that calli were variably developed from the scutellar region of seeds and visible within 6–20 days. The highest and lowest calli induction frequency (70% and 51.66%) and number of induced calli from seeds (14 and 10.33) were observed in MR269 and MRQ74, respectively. The maximum and minimum number (7.66 and 4) and frequency of embryogenic calli (38.33% and 20%) were recorded in MR219 and MRQ74, respectively. However, the highest browning rate was observed in MR84 (87%) and the lowest rate in MRQ50 (46%). The majority of plants regenerated from embryogenic calli were obtained from MRQ50 (54%) and the minimum number of plants from MR84. In this study, the maximum numbers of plantlets were regenerated from the varieties with highest rate of embryogenic calli. Also, various varieties, including MRQ50, MR269, MR276 and MR219, were satisfactorily responding, while MRQ74 and MR84 weakly responded to the procedure. Such a simple, successful and generalized method possesses the potential to become an important tool for crop improvement and functional studies of genes in rice as a model monocot plant.

1. Introduction

Food security has become an extremely important global issue, and spike in prices of important crops such as rice and wheat has occurred in recent years. Rice (*Oryza sativa* L.) is the second widely cultivated crop in the world. It belongs to the genus *Oryza*, family Gramineae (Poaceae) and tribe Oryzeae. It also comprised two subspecies, *Indica* and *Japonica* [1]. The production of rice should be increased up to 40% by 2030 due to the growing global population. Hence, the production of rice varieties with higher potency and stable yield is indispensable to overcome the grain yield reduction and arable land limitation [2]. Plant tissue culture technique covers a wide scope of development, maintenance and usage of genetic variability to improve field, vegetable, crops and aromatic/medicinal plants. In several crops, in order to develop transgenic varieties, tissue culture-based genetic transformation has been commercially utilized [3]. However, the lack of an

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efficient tissue culture method to regenerate the plantlets remains a main barrier for genetic modification of a wide range of plant species. Tissue culture has been verifying some potential, such as producing virus-free plants, improving propagation and developing genetic diversity in the short term [4,5]. One of the most promising goals for plant genetic modification is cultivar improvement, which could be achieved through plant regeneration using somatic embryogenesis [6,7]. The availability of an efficient in vitro regeneration protocol is an essential requisite for doing well transformation and regeneration processes [8]. Furthermore, genetic modification of rice to improve main characteristics needs to have a reliable and well-organized in vitro culture method [9]. The in vitro culture protocol offers material sources that could serve as recipients of introduced exogenous DNA [10]. It also establishes plant propagation and would recognize the genotype that could be used as a recipient of foreign DNA in the following transformation step. Somatic embryogenesis is the common regeneration process in rice. Somatic embryos could be obtained from diverse plant parts, including mature and immature embryos, root, microspores, leaf bases of young seedling, protoplast and young inflorescences and cell suspensions [11–13]. Moreover, the auxin/cytokinin ratio of media resulted in the development of somatic embryos, shoot and root production [14]. We provide here a different regeneration protocol based on initiation of embryogenic calli from mature embryos of *Indica* rice varieties. The objective of this study was to develop a simple yet effective as well as less genotype-dependent plant regeneration method using embryogenesis that would be of the greatest utility in genetic improvement of *Indica* rice varieties.

2. Material and methods

2.1. Plant materials

Mature dry seeds of six elite Malaysian *Indica* rice varieties (MRQ50, MR269, MR276, MR219, MRQ74 and MR84) were used. All seeds were supplied by the Malaysia Agriculture Research Development Institute (MARDI). In addition, the experiment was designed based on Randomized complete block design (RCBD).

2.2. Sterilization procedure

Hull from all mature seeds containing scutellar region of embryo was removed. Dehulled seeds were sterilized with 70% alcohol for 1 min, followed by shaking in a 40% sodium hypochlorite solution containing three drops of polyoxyethylene sorbitan monooleate in an orbital shaker, at 120 rpm for 20 min. Finally, the explants (seeds) were rinsed with sterile distilled water five times.

2.3. Establishment of embryogenic calli from mature embryos

Five sterile seeds were placed in each Petri dish (100 mm × 15 mm) containing 20 mL of MS media supplemented with 2,4-D (MS salts and vitamins, 300 mg/L casamino acid, 2 mg/L 2,4-D, and 2.8 g/L gelrite, pH 5.8). Each four Petri dishes were measured as a replicate and totally 12 Petri dishes, three replicates, were analyzed for every one variety. Then, the cultured seed were incubated in the dark at 25 °C. After three weeks, yellowish white embryogenic calli had developed on the scutellar surface.

2.4. Proliferation of calli

The produced calli were transmitted to the MS media supplemented with 2, 4-D containing MS salts and vitamins, 300 mg/L casamino acid, 2 mg/L 2,4-D, and 2.8 g/L gelrite, pH 5.8 for proliferation of the calli. The calli were kept in the dark at 25 °C for 10 days.

2.5. Shoot production from embryogenic calli

The healthy surviving MR219 embryogenic calli were transferred to the MSKN regeneration medium (MS salts, MS vitamins, 2 mg/L kinetin, 1 mg/L NAA, 300 mg/L casamino acid, 30 g/L sucrose, 0.1 g/L myo-inositol, 2.8 g/L gelrite, pH 5.8). The cultures were kept in the dark for 20 days. The emerging shoots were harvested and then transferred to the fresh regeneration media (MSKN). The cultures were placed in the light at 27 °C with a 16-h photo period (110 $\mu mol/m^2/s$) for 20 days.

2.6. Root production

Finally, the healthy shoots were transferred into the rooting medium (MSO) containing MS salts, MS vitamins, 300 mg/L casamino acid, 30 g/L sucrose, 0.1 g/L myo-inositol, 2.8 g/L gelrite, pH 5.8. The cultures were kept exposed to light at 27 °C with a 16-h photo period (110 $\mu mol/m^2/s$) for 14 days.

2.7. Transfer of regenerated plants to the soil

After 14 days, when a well-developed root system was observed, the culture medium was removed gently from the roots of plantlets using water. After that, the plantlets were transferred individually into the Yoshida culture solution in a greenhouse with a 14-h photo period (160 $\mu mol/m^2/s$), 95% relative humidity, and 29 °C day/light temperature and kept for 21 days. Subsequently, the plants with vigorous root systems were transferred into the pots containing paddy soil, water (1 L per pot and 500 mL each day until maturity, and sufficient initial fertilizer – a mixture of fertilizers, 2.5 g of $(NH_4)SO_2$, 1.25 g of P_2O_5, 0.75 g of K_2O per pot). About 2.5 g of $(NH_4)SO_2$ were added into each pot at the initial stage of flowering. Panicles were harvested when 85% of the color of the grains turned straw gold.

2.8. Characteristics evaluated during the experiment

Various important traits were measured during the experiment to explore the responses of the different varieties to the regeneration procedure. The considered traits included callus induction day, number of contaminated seeds, number of dead seeds, percentage of dead