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SHORT COMMUNICATION

Callusing and regeneration of three genotypes of *Oryza sativa* ssp. *indica* by 2,4 dichlorophenoxy acetic acid (2,4-D)

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Abstract: Consistent callus induction and regeneration of material cultured *in vitro* is a pre-requisite for somatic cell genetic improvement of plants, through methods such as genetic engineering. This study aims to induce rapid *in vitro* callusing and regeneration on three local rice varieties by exposure to different culture media. When mature embryos were exposed to three levels of 2,4- dichlorophenoxyacetic acid (2,4-D) and one level of thidiazuron (TDZ) (with a short hormone free period in between), all varieties showed rapid regeneration in the absence of a visible callus phase. When the seeds were cultured in 2,4-D for three weeks, at a decreasing concentration of 2,4-D every 7 days, and subsequently treated with TDZ, non-embryogenic calli were obtained that failed to regenerate on hormone free medium. When exposed to 2,4-D for 5 days and subcultured in hormone free medium for two weeks, embryogenic calli and greening were observed within that period. The concentration and period of exposure to 2,4-D are critical factors for callus induction and regeneration in the genotypes tested.

Key words: Callusing, *Oryza sativa*, regeneration, thidiazuron (TDZ) 2,4- dichlorophenoxyacetic acid (2,4-D)

INTRODUCTION

Biotechnology provides procedures to develop high yielding and more nutritive crop varieties through gene transformation, for which an efficient plant regeneration system from a cell or callus tissue *in vitro* is a pre-requisite. Of the two ecotypes (*indica* and *japonica*) of the most widely cultivated rice species (*Oryza sativa* L.), *indica* rice cultivars are less amenable to *in vitro* culture. It has been shown that the regeneration percentage in tissue cultured *indica* rice was influenced by genotype, callus induction and regeneration media, and the interaction between the genotype and the two media[1].

Rapid regeneration of plantlets was achieved using 2,4-D and TDZ on mature embryos of rice[2]. The period of *in vitro* culture is important to prevent undesired somaclonal variation, since it has been reported that plants obtained from prolonged *in vitro* culture of rice undergo extensive genetic changes[3]. Hence, rapid *in vitro* regeneration is important. Therefore, the objective of our study was the induction of rapid callusing and regeneration in rice by exposing mature embryos of locally cultivated *indica* rice genotypes to limited durations of 2,4-D and TDZ.

METHODS AND MATERIALS

The study was carried out on three improved Sri Lankan rice varieties, Bg 300, Bg 307 and Bg 357. Seeds of the above varieties were obtained from the Plant Genetic Resources Center, Gannoruwa and multiplied under greenhouse conditions at the Institute of Fundamental Studies, Kandy.

The explants used in the study were dehusked mature seeds. They were thoroughly washed in Teepol solution and surface sterilized using 5% sodium hypochlorite for 20 min followed by 95% ethanol for 1 min and finally rinsed in sterile distilled water. Sterile seeds were cultured for induction of callusing and regeneration by three methods.

Method 1: Seeds were cultured for 3 d in sterile distilled water containing 2,4-D at three concentrations (2.2 mg/L, 4.4 mg/L and 8.8 mg/L). Subsequently, shoots, roots and endosperm were aseptically excised and the embryos cultured in hormone free Murashige and Skoog (MS)[4]

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medium[4] for 5 d and thereafter transferred to sterile distilledwater containing TDZ at 0.44 mg/L for 4 d and finally to hormone free MS medium.

Method 2: Seeds were cultured for 1 wk in sterile distilled water containing 2,4-D at a concentration of 8.8 mg/L. They were subsequently dissected as above and the embryos were cultured in solid MS medium supplemented with 2,4-D at 4.4 mg/L for 1 wk and transferred to solid MS medium containing 2,4-D at 2.2 mg/L for another 1 wk. Thereafter, the seeds were transferred to sterile distilled water containing TDZ at a concentration of 0.44 mg/L for 4 d and then transferred to MS hormone free medium.

Method 3: Seeds were initially cultured for 5 d in liquid 2,4-D medium at 4.4 mg/L. Subsequently, the embryos, dissected as above, were subcultured for 2 wks in solid hormone free MS medium.

All media were supplemented with 2% sucrose, pH adjusted to 5.8, solidified using 0.6% (w/v) agar and autoclaved at a temperature of 121°C and a pressure of 1.2 kg cm^{-2} for a period of 15 min. Liquid media were placed in culture jars of 6 x 5.5 cm and solid media were placed in petri dishes of 9 cm diameter. All cultures were sealed using Parafilm to prevent microbial contamination. Liquid cultures were placed on a rotary shaker at 120 rpm. Solid cultures were placed on culture racks. Culture plates were arranged in a completely randomized design. Each replicate of each variety and treatment contained 20 seeds in four culture vessels. Each culture vessel contained 20 mL of culture medium. Control cultures contained 5 explants per replicate in 20 mL of medium without hormone treatment. All cultures were maintained at 25 ± 2 °C and under constant light. All explants showing multiple shoots from the first method were transferred to solid hormone free MS medium to promote shoot and root growth. The resulting plantlets were acclimatized in hormone free liquid MS medium in test tubes, where the sucrose concentration was reduced from 2% to 0% over 3 wks. Finally, the plants were potted and transferred to the greenhouse. Data were collected on number of responding explants and expressed as a percentage of total explants cultured.

RESULTS AND DISCUSSION

The first method induced regeneration of multiple shoots from single embryos, after five weeks of *in vitro* culture. The response was dependent on the genotype and the concentration of 2,4-D in the callus induction medium (Fig 1). A 2,4-D concentration of 4.4 mg/L[1] gave the best response, showing relatively high regeneration in all varieties. At this concentration the three genotypes responded differently, with Bg 307 producing the highest response at 30% of explants producing multiple shoots. The responses at 2.2 mg/L on Bg 357 and 8.8 mg/L on Bg 300 were weak, with 10% and 3% of the explants producing multiple shoots, respectively (Figure 1). The regenerated plants were acclimatized and seeds obtained from all varieties.

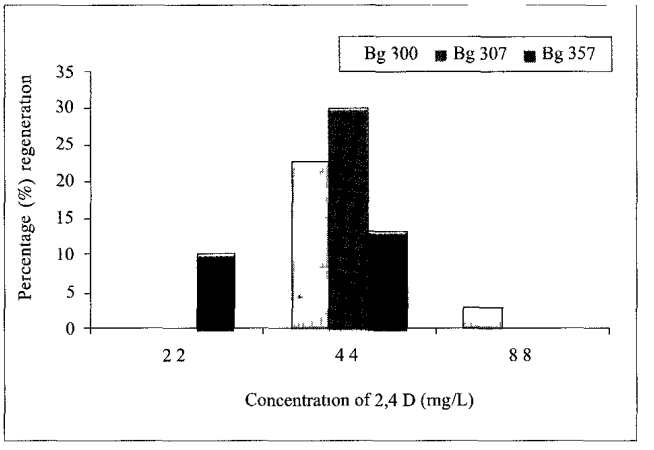

Figure 1: Percentage regeneration of multiple shoots in the varieties Bg 300, Bg 307 and Bg 357. Seeds were cultured in 2,4-D for three days followed by a hormone free period of five days, TDZ for four days and finally in hormone free medium.

The excised shoots and roots in the 2,4-D treated cultures did not regrow, suggesting an inhibition from 3 days of exposure to 2,4-D. The control cultures, where seeds were not subjected to hormone treatment, produced shoots and roots continuously from the excised plant tissues but no callusing or regeneration was observed. The second method produced a dark yellow smooth callus within one week of culture in all varieties. The callus remained unchanged throughout the culture period. Although the concentration of 2,4-D was decreased, the continuous presence of the auxin was not conducive to induce regeneration in the callus. Similar results were obtained previously when a single *indica* rice variety was exposed to 4.4 mg/L 2,4-D for 4 weeks[2]. The control cultures exhibited continuous regrowth of excised shoots and roots, but did not produce a callus phase.

The third method produced embryogenically competent callus from the scutellum, 14-16 days after culture initiation. The callus was large and light yellow with a nodular surface. Callusing and calli greening percentage was genotype dependent. Green shoot buds were given by 30 - 55 % of calli (Figure 2). Calli greening and green shoot buds developed on the calli, one month after culture initiation. The period for callusing and production of green shoot buds was small, as evident above, which is desirable to prevent somaclonal variation

arising in the cultures, as shown previously[3]. The control cultures responded as in the previous procedures.

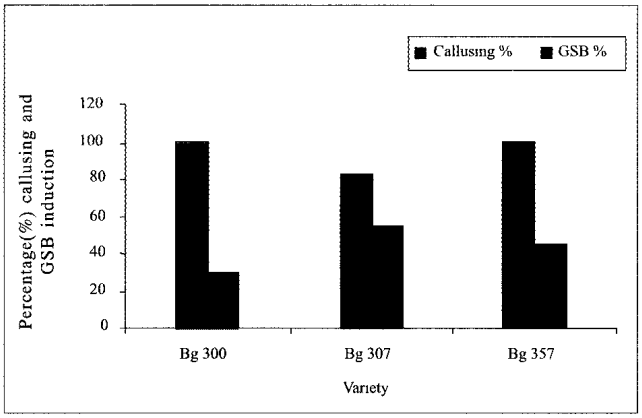

Figure 2: Percentage callusing and green shoot bud (GSB) induction of Bg 300, Bg 307 and Bg 357. Seeds were treated with 4.4 mg/L 2,4-D for 5 days followed by two weeks in hormone free MS medium.

The study shows the genotypic variation of the three rice varieties on multiple shoot regeneration, callusing and green shoot bud induction in response to different concentrations and different methods of exposure to 2,4-D. The three varieties used in the study responded differently to the first and third methods. The variation in response shown by the three genotypes at 4.4 mg/L 2,4-D in the first method was very high. By treating the seeds with 4.4 mg/L 2,4-D for 5 days (Fig 2), all three genotypes produced a high degree of callusing and less variation, while the development of green shoot buds showed more variation.

A study carried out previously[1] has also shown that regeneration percentages of rice varieties in culture are dependent on the culture medium composition and the genotype.

Results of the present study indicates that rice seeds treated with distilled water containing 4.4 mg/L 2, 4 D for 5 days as the effective method for callusing and regeneration of all three methods tested. It is likely that a culture of seeds in distilled water containing 2,4 D subjected to constant shaking may provide an even exposure to the hormone.

Therefore, it can be concluded that for callus induction and regeneration of rice *in vitro*, the concentration and period of exposure to 2,4-D is critical, and depends on the variety under investigation.

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