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Research Article
Enhancing of Drought-tolerant Rice (*Oryza sativa*) Variety MRQ74 Through Gamma Radiation and *in vitro* Pathway

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Abstract

Background and Objective: Aromatic rice MRQ74, known as Maswangi is an important rice variety in Malaysia. However, drought tolerance is essential for the growth of MRQ74. This study was conducted by exposing naked seeds to acute gamma radiation to induce callus embryogenesis. **Methodology:** A dose response experiment was performed at the Nuclear Malaysia Agency to expose the naked MRQ74 seeds to 350 Gy; these seeds were cultured to produce callus on Murashige and Skoog (MS) media supplemented with 2,4-D, (1.0, 2.0 or 3.0 mg L^{-1}) and Kin (0.1 and 0.2 mg L^{-1}) for 4 weeks. Under the optimized conditions in media and regeneration, the callus was introduced to drought stress with 1.5, 3, 4.5, 6 and 7% PEG (MW 6000). **Results:** The most efficient response to callusing was found at 3 mg L^{-1} 2,4-D and 0.1 mg L^{-1} kinetin. Shoot and root formation were respectively optimized in MS+3.0 mg L^{-1} (BAP)+0.1 mg L^{-1} (NAA) and MS+1.0 mg L^{-1} (IBA)+0.1 mg L^{-1} 2,4-D. The highest fresh weight of callus and increase in proline enzyme concentrations were obtained through acute gamma radiation at 7% PEG strength. **Conclusion:** This study successfully establishes a novel potential mutant line as an improved drought-tolerant MRQ74 variant. Further research should be conducted to investigate the nutrient properties and yield of this novel mutant.

Key words: Genotype MRQ74, drought tolerance, gamma radiation, growth regulators, callus

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Rice (*Oryza sativa* L.) as a staple food supports about three billion people and accounts for 50-80% of people's daily calorie intake worldwide[1]. In Asia, low rice productivity is mostly attributed to different abiotic stress conditions, such as drought. Drought stress poses a serious risk to food security in developing countries[2,3]. Low rice yields indicate a constant decrease in drought-prone regions. Thus, effective measures should be implemented to enhance crop productivity in water-scarce areas. Plant tissue culture methods are essential for various academic activities, particularly stress tolerance and various practical features of plant studies. New technological methods have been used to investigate several fields such as, gene guidelines, plant molecular biology, plant biotechnology and molecular breeding. As a significant part in plant tissue culture, plant callus can grow on a solid substrate by employing tissue culture technology[4,5]. Peripheral cells in the callus are rarely spurred into actively dividing cells because of callus diversity. Moreover, distinctions are based on the hormonal balance in the supporting media and physiological conditions of tissues. Mutations change the trait and function of genes involved in plant development as well as generate raw components for genetic development and economic yields[6]. Different mutagenic agents are used to extract desirable mutations at high frequencies. Gamma (γ) irradiation is commonly administered as a changing agent to improve genetic varieties through farming because of its higher saturation capacity in relation to other ionizing radiations[7]. Different doses of γ irradiation can promote plant tolerance to abiotic stress conditions, such as salt and drought[8]. Drought tolerance tests on local cultivars should be conducted to determine critical doses. Many studies documented that the variation in rice genotypes criteria could be utilized as a tool in plant breeding. Meanwhile, data on mechanisms underlying plant responses to drought tolerance for MRQ74 remain insufficient. Thus, rice variety MRQ74 was prepared with tissue culture techniques at 350 Gy dose of cesium-137 to increase the probability of improving the selected mutants as well as reducing the breeding period and time required to develop locally grown and drought-tolerant rice genotype.

MATERIALS AND METHODS

This study was conducted in the Biotechnology Lab of the School of Biosciences and Biotechnology, Faculty of Science and Technology, University Kebangsaan Malaysia. Our experimental material was rice genotype MRQ74. The genotype seeds were subjected to 350 Gy of γ rays (Caesium-137) in the Malaysian Nuclear Agency[9].

Callus induction: Mature embryos were sterilized in accordance with a standard explant sterilization protocol[10]. Explants were washed thrice with distilled water and then with 70% ethanol for 3 min. In a laminar flow cabinet, the explants were treated with 0.1% $HgCl_2$ by adding a few drops of tween-20 for 4-6 min to sterilize the inner surface. Afterwards, the explants were washed with sterile distilled water to eliminate the sterilizing agents. The media was autoclaved. Murashige and Skoog (MS) media were supplemented with various concentrations of growth hormone, 2,4-D at 1, 2 or 3 mg L^{-1} and Kin at 0.1 and 0.2 mg L^{-1}. Before the media was autoclaved, their pH was 5.8±2. After the seeds were inoculated, the irradiated and non-irradiated surface-sterilized seeds of the rice genotype were moved and stored in a controlled growth room in the dark at 25±2°C for callus formation. Petri dishes containing five seeds were utilized for each treatment[10]. After 4 weeks of incubation (Fig. 1), callus induction frequency was calculated as follows:

$$\text{Callus induction frequency (\%)} = \frac{\text{No. of seeds producing callus}}{\text{Total seeds cultured}} \times 100$$

callus fresh weight (mg) was also determined[11,12].

Scanning Electron Microscopy (SEM): Somatic embryos along with embryogenic and non-embryogenic calluses for SEM were prefixed in 5% buffered glutaraldehyde (0.1) (M phosphate buffer, pH 7.2) for 2 h at 30°C. The samples were dehydrated with a graded ethanol series, dried with a CO_2 critical-point drying system (EMITECH K850 critical point dryer), sputtered with gold (SPI SUPPLIES Ion Sputtering System) and monitored with a scanning electron microscope (Fig. 1)[13].

Callus growth with polyethylene glycol 6000 (PEG): A one-month-old well-proliferated callus from the irradiated and non-irradiated seeds was transferred to test tubes containing Kin (0.1 mg L^{-1}), solidified MS media and 2,4-D (3 mg L^{-1}) on the basis of the concentration selected in previous studies. The samples were supplemented with 1.5, 3, 4.5, 6 and 7% PEG (MW 6000) to screen drought tolerance under the described conditions[11]. The medium-lacking PEG

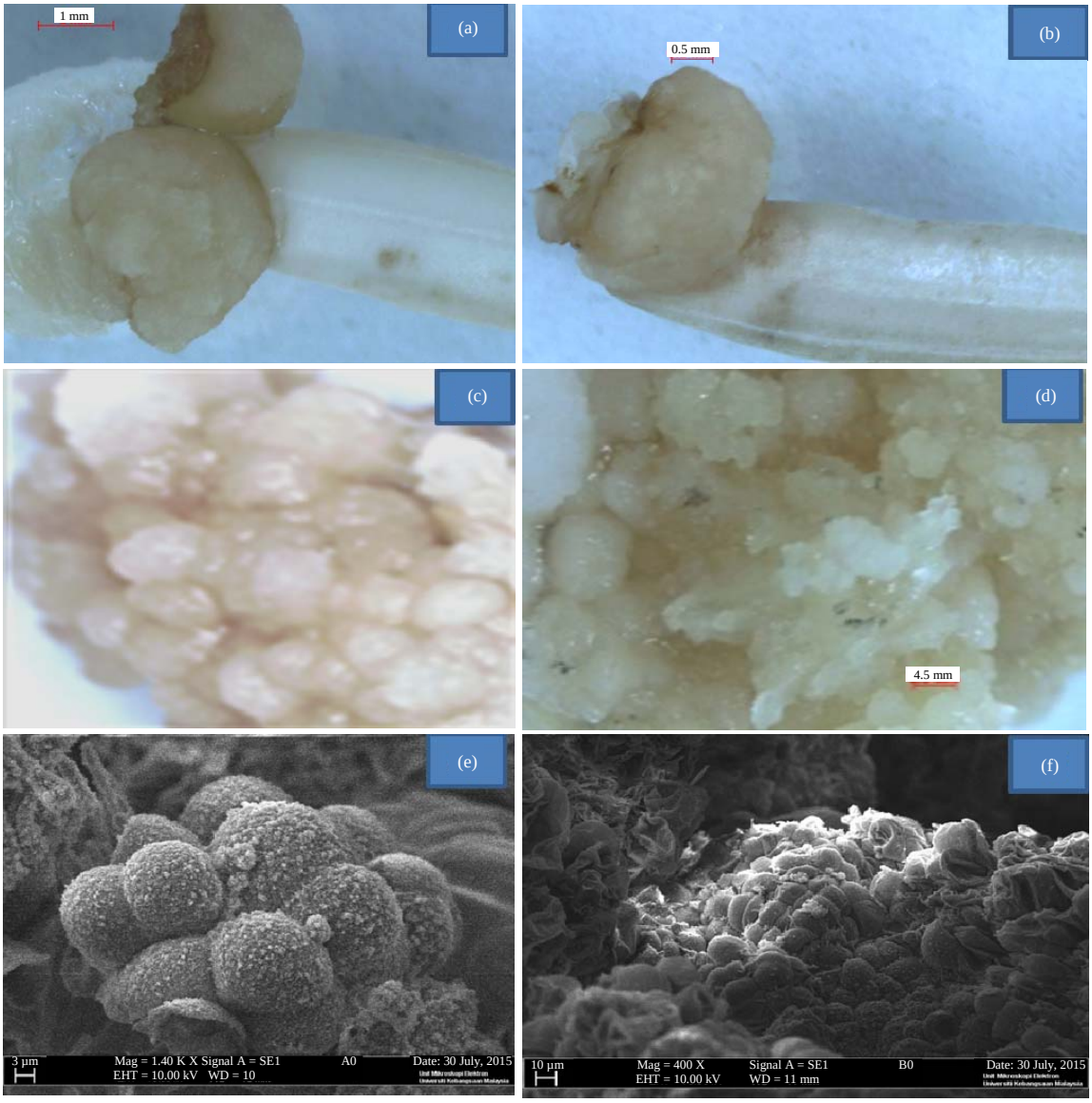

Fig. 1(a-f): Callus formation from mature seeds of rice cv., MRQ74, (a, b) Scutellum was swelling with a callus mass 6-8 days after the seeds were planted on the induction medium, (c, d) After 4 weeks of culture. Scanning of electron micrographs of upper epidermis-like callus surface after placement of callus induction medium and (e, f) Globular embryogenic callus form seed (a, c, e) at 0 (non-irradiated) and (b, d, f) at 350 Gy (irradiated)[13]

was used as a control. After 4 weeks of stress exposure, the fresh weights of the samples and the proline concentrations of the genotype were measured in accordance with the methods described by Bates *et al.*[14] Which are as follows: Proline concentrations were determined from callus with 5 mg fresh weight was homogenized with 3% sulfosalicylic acid. The filtrate was mixed with 2 mL of glacial acetic acid and ninhydrin reagent and incubated at 100°C for 30 min. The samples were rigorously mixed with 4 mL toluene. The light absorption of the toluene phase was estimated at 520 nm through the use of a spectrophotometer. Proline concentration was determined and expressed as µM g^{-1} of fresh weight.

Shoot regeneration: Callus pieces (100 mg) were transferred into a regeneration medium containing an MS medium (10 mL $tube^{-1}$) supplemented with various concentrations of cytokinin BA (1, 2 and 3 mg L^{-1}) and NAA (0.1 mg L^{-1}). All of the cultures were kept at 25±2°C in a 16/8 h (light/dark) photoperiod at a light intensity of 1000 lux. The number of

shoots was determined after 5 weeks of incubation with 10 replicates for each treatment.

Root regeneration: Root-regenerated shoots were initiated on half strength MS medium containing various concentrations of IBA (1.0 2.0 mg L^{-1}) and 2, 4-D (0.1 mg L^{-1}).

Experimental design and statistical analysis: The experiment was designed as a factorial experiment based on a Completely Randomized Design (CRD). The factors included gamma irradiation (2 levels: With and without irradiation) and PEG (6 levels: 0, 1.5, 3, 4.5, 6 and 7). In this study three replications with 10 samples each were applied. The data were subjected to the normality test prior to the analysis of variance using SAS programme (Release 9.1 for windows, SAS Institute Inc., Cary, NC, USA). Significant differences among the mean values of treatments were determined using the Duncan's Multiple Range Test (DMRT) and the Least Significant Difference (LSD) was calculated at the $p \leq 0.05$ level.

RESULTS AND DISCUSSION

In Fig. 2, callus growth was induced to a greater extent from 0.1 mg L^{-1} Kin (57.05%) to 0.2 mg L^{-1} Kin (68.2%). At 3.0 mg L^{-1} 2,4-D, the percentage of callus induction was 59.85%, which was higher than that at 1.0 mg L^{-1} 2,4-D (28.22%). The interaction among 2,4-D, Kin and irradiation indicated that the maximum percentage of callus induction in the irradiated seeds was 77.17% at 0.1 mg L^{-1} Kin and 3 mg L^{-1} 2,4-D; the minimum percentage of callus induction in the irradiated seeds was 20.5 at 0.2 mg L^{-1} Kin and 1 mg L^{-1} 2,4-D.

The mean fresh weight of the callus of the seeds inoculated in the medium with 0.1 mg L^{-1} Kin was 176.23 mg, which was higher than that in the medium with 0.2 mg L^{-1} Kin (170.77 mg). Furthermore, the mean callus fresh weight of the seeds inoculated in the medium with 3.0 mg L^{-1} 2,4-D was 208.74 mg, which was higher than that in the medium with 1.0 mg L^{-1} 2,4-D (145.74 mg; Fig. 3). The interaction between 2,4-D, Kin and irradiation indicated that the maximum callus fresh weight of the non-irradiated seeds was 230.34 mg at 0.1 mg L^{-1} Kin and 3.0 mg L^{-1} 2,4-D; the minimum callus fresh weight of the irradiated seeds treated at 0.2 mg L^{-1} Kin and 1.0 mg L^{-1} 2,4-D was 130.40 mg.

In Fig. 4, the fresh weight of the callus gradually decreased to 221.2, 202.6, 183.7, 167.1 and 164.5 mg as PEG grew to 1.5, 3.0, 4.5, 6.0 and 7.0%, respectively, as stimulated by drought stress. However, the fresh weight of the callus

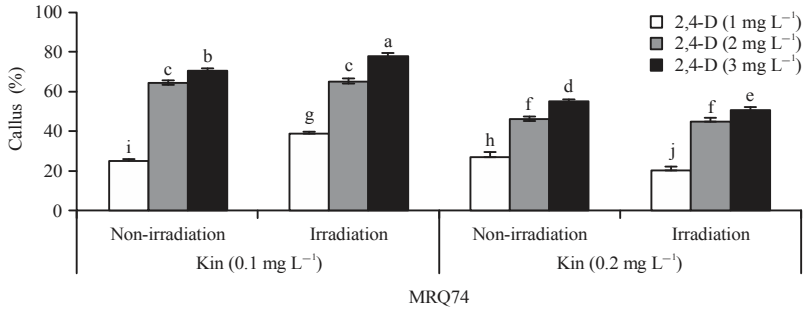

Fig. 2: Effect of 2,4-D and Kin on mean percentage callus induction originated from mature seeds exposed to gamma-ray at 0 (non-irradiated) or 350 Gy (irradiated) after the inoculation of explants onto MS medium for 4 weeks

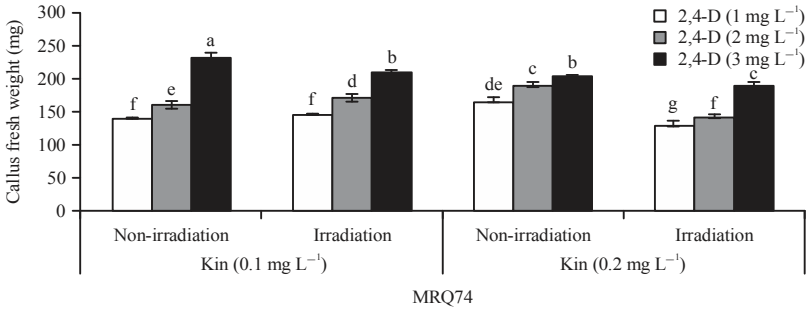

Fig. 3: Impact of 2,4-D and Kin on the mean callus fresh weights (mg) originated from mature seeds exposed to gamma-ray at 0 (non-irradiated) or 350 Gy (irradiated) after inoculating callus pieces onto MS medium for 4 weeks. Initial weight was 50 mg

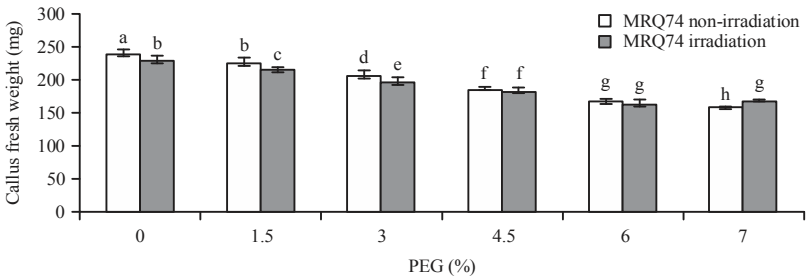

Fig. 4: Impact of PEG and irradiation on callus fresh weight originated from mature seeds exposed to gamma-ray at 0 (non-irradiated) or 350 Gy (irradiated) and PEG treatments at concentrations of 0, 1.5, 3, 4.5, 6 or 7%

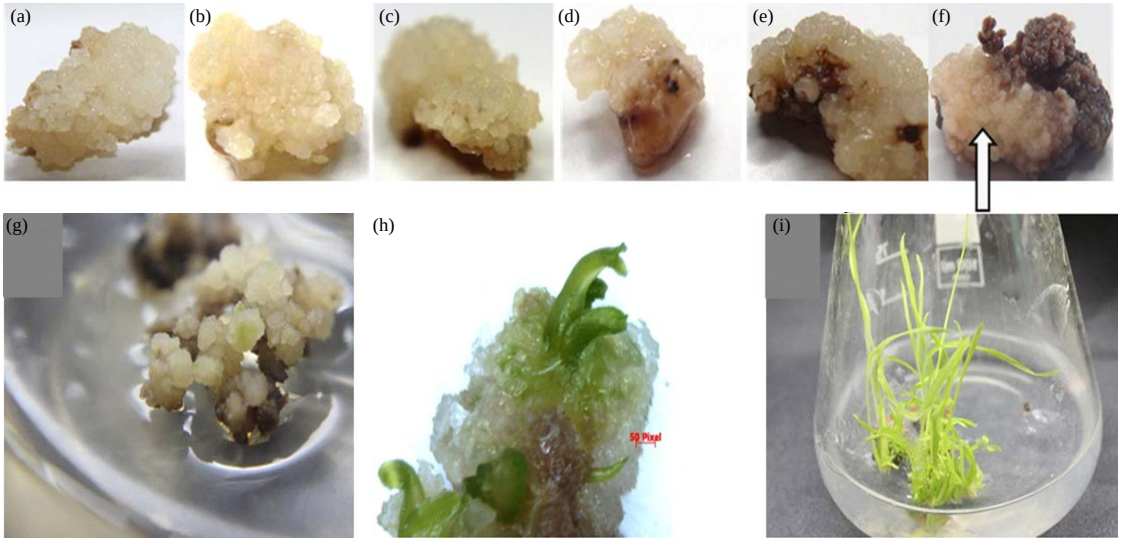

Fig. 5(a-i): Callus produced on MS medium supplemented with 3 mg L^{-1} 2,4-D and 1 mg L^{-1} Kin and challenged with different concentrations of PEG, (a) Callus on medium without PEG, (b-f) Callus growing on 1.5, 3.0, 4.5, 6 and 7% PEG. Progressive reduction in the growth of callus and browning of callus with increased PEG concentration, (g) Surviving callus from mature seeds exposed to gamma-ray at 350 Gy (irradiated) on 7% PEG-supplemented medium, (h) Regenerated plantlets on MS+2 mg L^{-1} Kin+1 mg L^{-1} BAP and (i) Seedling growth

at 0.0% PEG was 237.0 mg. The fresh weight of the non-irradiated seeds callus decreased to 198.8 mg, which was lower than that of the irradiated seeds (193.2 mg). Moreover, the colour of the callus changed from shiny white to dark yellow when the stress level was increased (Fig. 5). The interaction between irradiation and PEG% revealed that the highest fresh weight (243.4 mg) was obtained from the callus of the non-irradiated seeds treated with 0.0% PEG; this value was higher than that of 7.0% PEG-treated seeds (159.3 mg). The highest fresh weight that was obtained from the callus of the irradiated seeds treated with 7.0% PEG was 169.7 mg. This value was also higher than that of the non-irradiated seeds subjected to the same dose of PEG (159.3 mg).

The proline content of the callus increased from 6.059, 8.274, 17.642 and 22.781-29.156 μM g^{-1} as the PEG concentration increased from 1.5, 3.0, 4.5 and 6.0 and 7.0%, respectively, stimulated by drought stress. The proline content was 5.593 μM g^{-1} at 0.0% PEG (Fig. 6). The proline content for irradiated callus was 15.8 μM g^{-1}, which was higher than that of non-irradiated callus (14.0 μM g^{-1}) at 0.7% PEG. The interaction between irradiation and PEG% revealed that the maximum proline concentration was obtained from the irradiated callus treated with 7.0% PEG (32.754 μM g^{-1}). This value was higher than that of the non-irradiated callus treated with 7.0% PEG (25.559 μM g^{-1}). The maximum proline concentration was obtained from the irradiated callus and this value was higher than that from the non-irradiated ones.

Significant differences were found between irradiated and non-irradiated shoot regeneration. In Fig. 7, the percentage of shoot regeneration increased to 31.7% in the

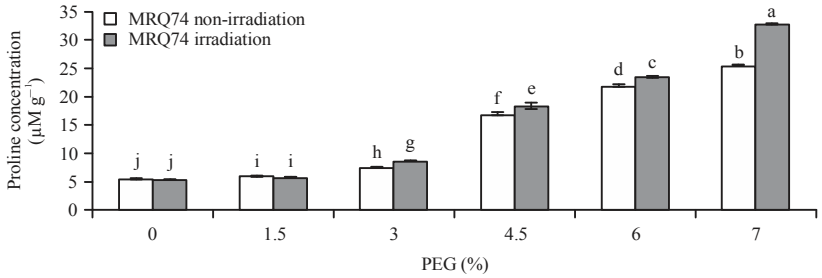

Fig. 6: Impact of irradiation and PEG (%) on the mean of proline concentrations in callus (µM g^{-1}) originated from mature seeds exposed to gamma-ray at 0 (non-irradiated) or 350 Gy (irradiated) and PEG treatments at concentrations of 0, 1.5, 3, 4.5, 6 or 7%

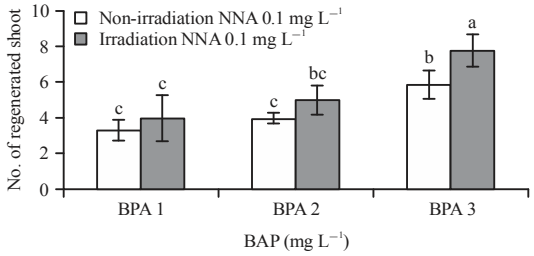

Fig. 7: Impact of BAP mean number of shoot regeneration originated from callus induction originated from mature seeds exposed to gamma-ray at 0 (not irradiated) or 350 Gy (irradiated)

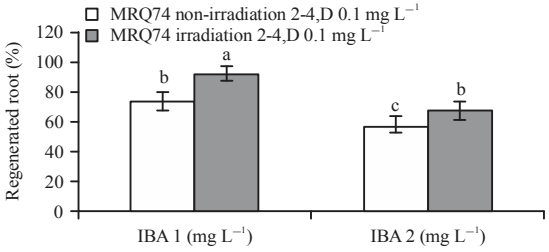

Fig. 8: Impact of IBA mean percentage root regeneration originated from callus induction originated from mature seeds exposed to gamma-ray at 0 (not irradiated) or 350 Gy (irradiated)

irradiated callus and this finding was higher than that in the non-irradiated callus (25.9%). Shoot regeneration was promoted after 3.0 mg L^{-1} BAP (36.7%) was added, which was higher than that after 0.1 mg L^{-1} BAP (24.2%) was added. Interaction between BAP and irradiation indicated that the maximum percentage of shoot regeneration was 40.0% at 3.0 mg L^{-1} BPA with the irradiated callus compared to 3.0 mg L^{-1} BAP, non-irradiated callus that was recorded at 33.3%.

The irradiated and non-irradiated root regeneration significantly differed. In Fig. 8, the percentage of root regeneration increased to 80.0% in the irradiated sample and this finding was higher than that of the non-irradiated sample (65%). Compared with findings at 2.0 mg L^{-1} IBA (61.7%), root regeneration was highly promoted at 1 mg L^{-1} IBA (83.3%). The interaction between IBA and irradiation revealed that the maximum percentage of shoot regeneration in the irradiated sample was 93.3% at 1.0 mg L^{-1} IBA compared with that at 1.0 mg L^{-1} IBA. The maximum percentage of shoot regeneration in the non-irradiated sample was 66.7%.

Callus induction: Callus is extracted from plant tissues and is used for biological studies[2]. Callus production and its next regeneration are initially manipulated through biotechnology[15]. Our results revealed that the percentage of callus induction and the fresh weight of calluses were higher at 3.0 mg L^{-1} 2,4-D and 0.1 mg L^{-1} Kin than at 1.2 mg L^{-1} 2,4-D and 0.2 mg L^{-1} Kin (Fig. 2, 3). Auxin 2,4-D is commonly utilized as a callus-inducing agent for all cereal crop species, including rice[16]. Thus, the initial conclusions of our research considered several elements like hormonal manipulation, which influence callus production in rice. Auxin can stimulate elongation in plant organs and tissues, cell division in meristematic tissues and callus emergence. Differentiations and auxin reactions occur in nucleic acids, plasma membrane and cell wall. Auxin softens and breaks cell walls, as well as return links to new locations under turgor pressure; as a consequence, cell size and breadth increase. The interaction between auxin and cytokinin shows that auxin is essential for DNA synthesis. Based on Fig. 3, the is growth in mean callus fresh weight with growing 2,4-D levels is consistent with that of Wani *et al.*[17] who showed that callus can grow well on MS medium containing 0.5 mg L^{-1} Kin and 2.5 mg L^{-1} 2,4-D and these results are consistent with those observed in sorghum bicolor[18]. This result is consistent with that of Bates *et al.*[14].

The 2,4-D is necessary to produce embryogenic callus[19]. Figure 2 and 3 indicate a growth of the mean percentage of

callus induction and the fresh weight of calluses in the presence of some plant growth regulators mixed with non-irradiation treatments. This observation can be attributed to the effect of endogenous growth regulators, particularly auxin and cytokines, that result in insufficient synthesis and breakdown caused by irradiation[20]. High doses of gamma-rays can cause DNA damage of callus regeneration genes[21]. These results are consistent with those of Dehpour *et al.*[22] who observed a reduction in callus inductions in irradiated rice seeds. Nepal *et al.*[23] reported that the increased inhibition induced by high irradiation doses is due to the cell cycle halting at the G2/M phase during somatic cell division, which damages the whole genome. Bhuiyan *et al.*[24] also observed that the effect of Kin on rice callus propagation is genotype specific. Therefore, depending on the target genotype, Kin can prevent or stimulate callus growth. The weight of the callus highly increased as the concentration of 2,4-D increased. Hence, MS medium supplemented with 2,4-D and Kin (mg L^{-1}) is appropriate for embryogenic callus proliferation[18].

Figure 2 and 3 show an important distinction between non-irradiated and irradiated genotypes. This result agrees with that of Al-Qurainy and Khan[25] who found that mutagenic activity is dependent on medium components, plant genotypes and growth regulators. Radiation by gamma-rays can increase the extent of DNA disruption involving cell inactivation, mutation, chromosomal rearrangement and DNA damage[26]. The gamma-ray utilized in this procedure generates free radicals during interaction with molecules in the cells. These free radicals damage the components of plant cells and affect the anatomy, biochemistry and physiology of plants[27]. Low mutagen doses increases the fresh weight of calluses[28].

Callus growth with PEG 6000: Genotypes exhibit the same reaction under PEG stress in both non-irradiation and irradiation treatments. The fresh weight of callus decreased as PEG concentration increased (Fig. 4, 5). In many plant species, calluses develop as a consequence of the inhibitory effect of PEG. As water content decreases, cell turgor pressure, callus volume and callus growth decrease[29].

Figure 4 agrees with the result of Tsago *et al.*[30] who demonstrated that the weight of rice calluses reduces as PEG 6000 stress increases. Al-Bahrany[31] showed that increased drought stress induced by an increase in PEG concentrations can cause progressive reduction in the rice callus fresh weight. Sakthivelu *et al.*[32] found that callus growth and survival in highly reduced media with higher degrees of PEG 6000 can cause osmotic stress and increase stress duration. The decrease in cell growth may have been caused by the effect of osmotic pressure on the metabolic activity of cells involving enzymatic activity, nucleic acid synthesis, protein synthesis and mitochondrial and plastid activities. These activities may increase the osmotic pressure inside tissue and consequently decrease plant hormone levels essential for cell division and growth under stressful situations[33]. In Fig. 4, an increase in PEG concentrations can decrease the fresh weight of calluses of the irradiated genotype. This result is consistent with that reported by Hasbullah *et al.*[20] who observed that gamma-ray treatment reduces the fresh weights of callus. Interestingly, the fresh weight of callus after irradiation was higher than that of callus before irradiation for the same genotype at 7% PEG. This finding is in line with the results achieved by Oladosu *et al.*[7] who observed that treatment with mutagenic agents can cause a desirable mutation for crop breeders. Mutation breeding is a fast approach for crop improvement. A mutagen is mediated by mutagenic treatments that can disrupt nuclear DNA. Moreover, new mutations are randomized during DNA repair.

In Fig. 6, proline concentrations increased as PEG concentration increased. Crop tolerance can be improved by biochemical markers against osmotic stress. Furthermore, membrane integrity can prevent protein denaturation under drought stress. Proline can interact with enzymes to preserve protein structures and activities. Moreover, high proline concentrations can provide protection against biologically undesirable consequences under stressful conditions[34]. In relation to proline concentrations in callus cultures, proline accumulation initially emerges in response to stress caused by dehydration of plant tissues as a result of drought affect PEG (Fig. 6). The results agree with those observed by Joshi *et al.*[11] who reported that total proline content can increase as PEG concentration increases.

The accumulation of proline is essential for plants exposed to abiotic stresses because of its contribution to osmotic adjustment as other osmolytes grew[3-1]. High proline concentrations unlikely affects macromolecule-solvent interactions. Thus, it is unlikely that proline affects cell membrane functions or antioxidant enzyme (APX, CAT and SOD) activities and mitigates the negative consequences of dehydration[35].

The proline concentrations in the irradiated sample were higher than those in the non-irradiated genotype (Fig. 6). This result reflects that according to Dehpour *et al.*[22] who observed that irradiation proline content increased in rice (*O. sativa* L.) as well as Borzouei *et al.*[36] and Moussa[8] who reported increased proline content in wheat (*Triticuma estivum* L.) and soybean, respectively. Furthermore, the osmolyte synthesis of proline in charge of protective mechanisms is altered by various environmental stresses[37]. Proline is a compatible

osmolyte that interacts with enzymes to preserve enzyme structures and activities. Proline can decrease enzyme denaturation caused by heat, gamma and NaCl stress[38]. In this study, the proline contents of gamma-irradiated callus increased as the PEG concentration increased.

Shoot and root regeneration: An increase in growth regulator type and concentration highly affected the regeneration capacity of mature embryos. The percentages of shoot and root regeneration were higher at 3.0 mg L^{-1} BPA than those at 1, 2 mg L^{-1} BPA (Fig. 7). This result agrees with that of Anand *et al*.[39] who observed that the regeneration efficiency of rice plants is influenced by the compositions of media. This result also agrees with that reported by Satapathy and Thirunavoukkarasu[40] who found that high cytokinin concentrations can promote shoot regeneration. Moreover, these results are consistent with those of Sah *et al*.[41] who observed that high cytokinin concentration and low auxin concentration can promote shoot regeneration.

This indicate that the percentage of root regeneration was higher at 1.0 mg L^{-1} IBA than that at 2 mg L^{-1} IBA (Fig. 8), which agree with those of Amali *et al*.[18] who reported that the addition of IBA to MS enhances the growth of roots. Mouhamad *et al*.[42] reported the same results.

Figure 7 and 8 indicate an increase in the percentage of shoot and root regeneration in the irradiation treatments compared with the non-irradiation treatments. The results agree with those of Kamal *et al*.[43] who found that shoot and root regenerations in mutated rice cultures yield the highest percentages of shoots and roots. Dose irradiation can stimulate growth by changing the hormonal signalling network in plant cells or by increasing the anti-oxidative capacity of cells, thus overcomedaily stress elements under growth conditions easily[35]. The stimulating effects of irradiation on plant growth can be attributed to the stimulation of cell division and alteration of metabolic procedures affecting nucleic acid synthesis[44].

CONCLUSION

In conclusion, optimum medium compositions provide a high volume of callus induction and growth. In this study, the highest percentages of callus induction and growth were obtained by planting mature seeds on MS media supplemented with 3 mg L^{-1} 2,4-D and 0.1 mg L^{-1} Kin at pH 5.8±2. Growth was induced in a dark room at 25±2°C. The fresh weight of the callus decreased as the PEG percentage increased. By contrast, the proline content increased as the PEG percentage increased. High shooting and rooting percentages were observed in MS+3.0 mg L^{-1} (BPA)+0.1 mg L^{-1} (NAA) and MS+1.0 mg L^{-1} (IBA)+0.1 mg L^{-1} 2,4-D, respectively. Compared with that in the non-irradiated treatment at the same PEG concentration, the highest fresh weight of the callus and proline content were detected at 7% PEG in the irradiated treatment. Thus, a positive signal is possibly related to the effect of radiation on these features of drought tolerance.

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