



In Vitro Shoot Regeneration of Rice (*Oryza sativa* L.) Varieties under Artificial Drought Stress

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Abstract

The shoot regeneration efficiency of two rice (*Oryza sativa* L.) varieties (Kasalath and BRRI dhan55) under artificial drought condition created by polyethylene glycol (PEG-6000) was investigated. Calli were induced in Murashige and Skoog (MS) medium supplemented with different concentrations (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 mg/L) of 2, 4-Dichlorophenoxyacetic acid (2, 4-D). Then, *in vitro* shoot regeneration under PEG (6000) was conducted on MS medium supplemented with 1.0 mg/L NAA + 2.0 mg/L BAP + 1.0 mg/L kinetin that gave the highest number of shoots. Shoot regeneration in both varieties reduced with increased levels of PEG (0.5, 1.0, 2.0, 3.0 and 4.0%). Regeneration efficiency of BRRI dhan55 in drought stress was higher compared to Kasalath. Thus, *in vitro* screening of plantlets under PEG can be used to achieve fast and reliable selection for tolerance against drought.

Keywords: Drought, polyethylene glycol, shoot regeneration.

1. Introduction

Drought is one of the major abiotic stresses causing severe yield loss in crop plants worldwide (Akte *et al.*, 2016). Drought stress impairs physiochemical processes of plants such as photosynthesis, respiration, nutrient uptake and metabolism (Piwowarczyk *et al.*, 2014). Plants respond to drought stress through morphological, physiological and metabolic adjustments through different mechanisms (Akte *et al.*, 2016). However, this response often leads to accumulation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide (H₂O₂), hydroxyl radicals and singlet oxygen. These ROS cause peroxidation of lipid

membrane, membrane injuries, protein degradation and enzyme deactivation and lead to the ultimate death of plant (Phung *et al.*, 2011; Akte *et al.*, 2016).

Rice (*Oryza sativa* L.) is the most important cereal crop in the world (Akte *et al.*, 2016). In Bangladesh, it is a staple food and is grown almost all over the country. However, its average yield is low (2.94 t/ha) compared to other rice growing countries such as China (6.8 t/ha) (BBS, 2012; FAOSTAT, 2014). The reasons could be adverse climatic conditions such as flood, salinity and drought (Akte *et al.*, 2016). Rice is highly susceptible to drought stress and drought causes substantial yield reduction at the

reproductive stage. It causes 50% loss of production of rice globally (Wani *et al.*, 2010; Swamy *et al.*, 2012). In the South and South East Asia, drought causes up to 40% yield loss (Pandey and Bhandari, 2006; IRRI, 2009). In Bangladesh, this loss ranges from 37-73% depending on the grain development stage (Mahmood, 2004). Thus, it is imperative to find rice varieties that will be adapted to these conditions.

Screening drought tolerant plants is a way to sustain production under drought environment. However, screening in field is laborious, time consuming and is dependent on resources such as worker, field and power. *In vitro* selection method could be used as it is simple, ideal and effective for screening large set of germplasms (Kulkarni and Deshpande, 2007; Kacem *et al.*, 2017). Artificial drought stress can be achieved *in vitro* by addition of different osmotic agents such as mannitol, polyethylene glycol (PEG), sucrose or sorbitol in the medium for the culture of explants (Kacem *et al.*, 2017). Among these, PEG is the most common and widely used chemical to study plant responses to drought stress (Wani *et al.*, 2010). PEG is non-ionic water-soluble polymer having wider range of molecular weights (Wani *et al.*, 2010; Tripathy, 2015). PEG (6000 MW) has been used in *in vitro* screening of plants such as rice (Wani *et al.*, 2010; Akte *et al.*, 2016; Sabesan and Saravanan, 2016), tomato (Kulkarni and Deshpande, 2007), wheat (Kacem *et al.*, 2017), peanut (Meher *et al.*, 2017) against drought stress. This is because PEG can induce water stress without creating any detrimental effect on the plant. It is also useful in modifying the osmotic potential of culture medium (Wani *et al.*, 2010; Tripathy, 2015; Meher *et al.*, 2017). The present study was conducted to determine the effect of artificial drought stress created by PEG (6000) on callus derived shoot regeneration of two drought tolerant varieties, BRRI dhan55 and Kasalath. The present study aimed at providing a basis for selecting the varieties for further drought tolerance breeding program.

2. Materials and Methods

2.1 Plant material and experimental site

Mature seeds of two drought tolerant rice varieties, BRRI dhan55 and Kasalath were used in the experiment. Seeds of BRRI dhan55 and Kasalath were collected from seed merchant and local farmers respectively. BRRI dhan55 was developed by Bangladesh Rice Research Institute (BRRI) and was released for commercial cultivation in 2011, while Kasalath is a landrace (Parvin *et al.*, 2015). The experiment was conducted in the Plant Genetic Engineering Laboratory of the Department of Genetic Engineering and Biotechnology, Shahjalal University of Science & Technology (SUST), Sylhet.

2.2 Callus induction

Manually dehusked seeds were sterilized by 70% alcohol for 1 minute and 1% sodium hypochlorite (Clotech) for 10 minutes with few drops of tween-20. To induce callus, the seeds were cultured on MS medium in test tubes. The medium was supplemented with different concentrations of 2, 4-D (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 mg/L). In each test tube, one seed was explanted and inoculation was done in a laminar air flow cabinet. The test tubes were incubated in a growth room at 25 ± 3 °C for 16 hours of light ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$) per day. Three weeks after inoculation, the calli were removed from the seeds and were transferred into fresh nutrient medium. Callus induction frequency for the two varieties was recorded three weeks after inoculation. All calli from a single seed were considered as one. The percent frequency of callus induction was calculated as the number of calli obtained/ total number of seeds cultured multiplied by 100.

2.2.3 Shoot regeneration

Shoot regeneration involved two steps. Firstly, there was optimization of the shoot regeneration media. Then, the best shoot regeneration medium was used to investigate the impact of drought stress on regeneration of the two rice varieties under PEG (6000).

To optimize shoot regeneration from calli, one-month old good-quality calli were excised and transferred into MS medium contained in test tubes supplemented with eight different combinations of NAA and two cytokinins (BAP and Kinetin) (Table 1).

To investigate the effect of drought stress on shoot regeneration of the two rice varieties, different concentrations of PEG (0.5, 1.0, 2.0, 3.0 and 4.0%) were supplemented into the medium. All cultures were incubated in growth chambers similar to that mentioned earlier. The frequencies of plantlet regeneration were calculated on the basis of shoot initiation from the calli. The shoot regeneration frequency (number of plantlets/ total number of calli x 100) before and after PEG treatment was also recorded. The medium without PEG was considered as the control.

3. Results and Discussion

3.1 Effect of 2, 4-D on Callus Induction

Callus induction started from the scutellum of the seeds after 3 days of culture in both varieties.

It increased with increased concentration of 2, 4-D then declined (Figure 1). Callus induction in rice variety Kasalath was the highest (73%) in the medium supplemented with 2.0 mg/L of 2, 4-D (Figure 5-A) whereas for BRR1 dhan55 maximum (67%) induction was in 2.5 mg/L of 2, 4-D.

These results are consistent with earlier reports. Shahsavari *et al.* (2010), Haque *et al.* (2013), Pawar *et al.* (2015) and Upadhyaya *et al.* (2015) found higher callus induction with 2.0 mg/L of 2, 4-D while Hussain *et al.* (2010) and Chakraborty *et al.* (2017) showed maximum callus induction with 2-4 mg/L of 2, 4-D. Callus induction frequency might have been influenced by genotypes. Except for 2.5 and 4.0 mg/L of 2, 4-D concentration, Kasalath had the highest callus induction than BRR1 dhan55 in all 2, 4-D concentrations. On average, 47% of inoculated seeds developed callus in Kasalath compared to 39% in BRR1 dhan55. Earlier studies had also revealed that callus induction in rice could be genotype specific (Shahsavari *et al.*, 2010; Hussain *et al.*, 2010; Upadhyaya *et al.*, 2015).

Table 1. Shoot regeneration medium (MS medium containing 1.0 mg/L NAA as constant with different concentration of BAP and Kinetin)

Media Name	Type of combination
K1	BAP 1.0 mg/L + Kinetin 0.5 mg/L
K2	BAP 1.0 mg/L + Kinetin 1.0 mg/L
K3	BAP 1.0 mg/L + Kinetin 1.5 mg/L
K4	BAP 1.0 mg/L + Kinetin 2.0 mg/L
K5	BAP 2.0 mg/L + Kinetin 0.5 mg/L
K6	BAP 2.0 mg/L + Kinetin 1.0 mg/L
K7	BAP 2.0 mg/L + Kinetin 1.5 mg/L
K8	BAP 2.0 mg/L + Kinetin 2.0 mg/L

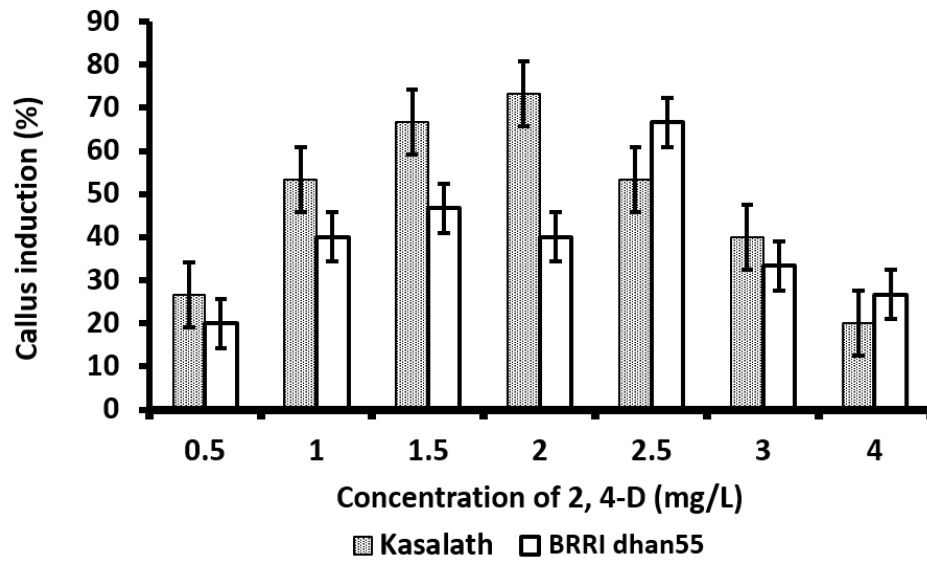


Figure 1. Callus induction of Kasalath and BRR1 dhan55. Bars are standard error.

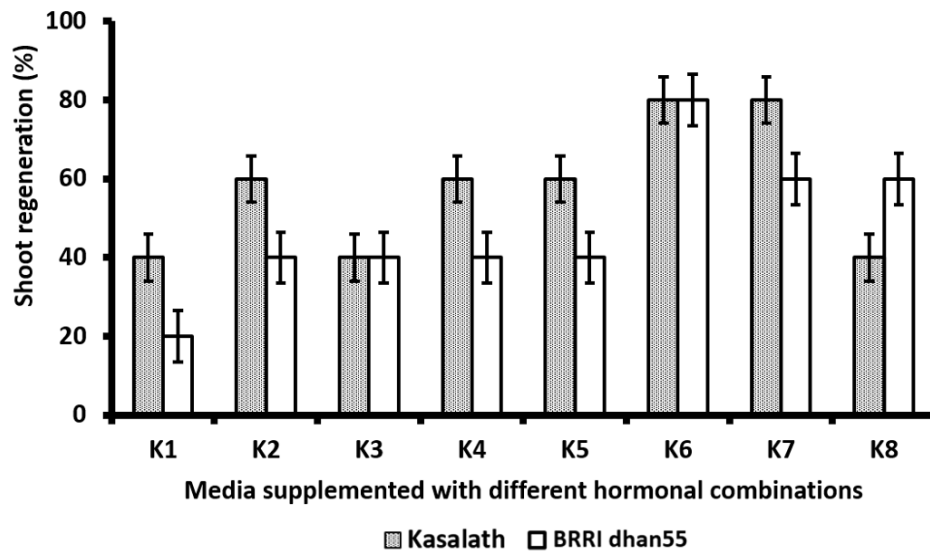


Figure 2. Effect of different hormonal combinations on shoot regeneration from calli. Bars are standard error.

2, 4-D is a synthetic auxin that is commonly used as a plant growth regulator to produce callus. Many studies suggest that MS medium supplemented with just 2, 4-D can produce more calli (Shahsavari *et al.*, 2010). Other studies, however, showed that it is the combination of 2, 4-D and other plant growth regulators that induce more calli. But this is controversial. For instance, Shahsavari *et al.* (2010) did not find any significant effect of a combination of NAA or kinetin with 2, 4-D in callus induction. However, Mohd Din *et al.* (2016) found high level of callus induction in medium supplemented with 2, 4-D and combination of BAP or NAA. The present study also revealed that 2, 4-D inevitably controls callus initiation of rice. MS medium supplemented with 2, 4-D alone produced callus from both varieties (Kasalath and BRR1 dhan55).

Further, callus initiation depended on the concentration of 2, 4-D in the medium. Less number of calli were initiated at concentration above a certain level. This is consistent with reports of Wani *et al.*, (2011); Mohd Din *et al.*, (2016) who also showed reduction of callus initiation with the increasing concentration of 2, 4-D in rice. Suppression of calli initiation at high concentration of 2, 4-D could be due to the residual effect of 2, 4-D on re-differentiation in the mitotic stage (Mohd Din *et al.*, 2016). This means an optimum concentration of 2, 4-D is desirable to get maximum number of rice callus.

3.2 Optimization of auxin and cytokinin for shoot regeneration

MS medium supplemented with different combinations of auxin and cytokinins were optimized for shoot regeneration system that could work for both varieties. About 4-5 week old embryogenic calli were selected and placed on MS medium (K1 to K8) supplemented with NAA (1.0 mg/L), BAP (1.0 or 2.0 mg/L) and kinetin (0.5, 1, 1.5 or 2 mg/L). Results showed a higher regeneration frequency of 80% for both varieties in K6 medium containing MS medium with 1.0 mg/L NAA + 2.0 mg/L BAP + 1.0 mg/L kinetin (Figure 2, Figure 5-B). But the

regeneration capacity of Kasalath was higher than that of BRR1 dhan55 in all media. These results are closely related to those of Shahsavari *et al.* (2010) who showed maximum regeneration frequency of upland rice cultivars on MS medium supplemented with 0.5 mg/L NAA + 2.0 mg/L BAP + 2.0 mg/L kinetin. Furthermore, they found that some rice cultivars had higher regeneration capacity. Hussain *et al.* (2010) also supplemented MS medium with NAA and BAP and found higher plantlet regeneration in MS medium and found higher plantlet regeneration in MS medium supplemented with either 1 mg/L NAA + 2 mg/L BAP or 1 mg/L NAA + 4 mg/L BAP based on varieties.

Hormonal balance between auxin and cytokinin has been showed to significantly alter *in vitro* callus, shoot and root formation. Auxins and cytokinins interact synergistically, antagonistically and additively to influence plant responses. But this is also based on type of tissue and plant species (Coenen and Lomax, 1998).

Thus, the ratio of auxin to cytokinin is vital in *in vitro* shoot formation. Previous studies indicate that plant regeneration in rice from callus has been achieved under different combinations of auxins to cytokinins (Hussain *et al.*, 2010; Wani *et al.*, 2011; Mohd Din *et al.*, 2016). In the present study, the concentration of NAA, BAP and Kinetin in MS medium was crucial for the shoot regeneration and depended on variety too.

3.3 Effects of PEG on shoot regeneration of rice

Polyethylene glycol had a distinctive effect on shoot regeneration from callus. In both varieties, shoot regeneration declined with increased PEG concentration (Figure 3). In the regeneration medium, K6 with PEG concentration of 0.5 and 1.0%, the frequency of shoot regeneration was higher (75 and 70%, respectively) which was similar to the regeneration frequency in unstressed condition (80% in K6 media, without PEG). The lowest percentage of shoot regeneration was obtained in both varieties under severe water stress created by 4% PEG.

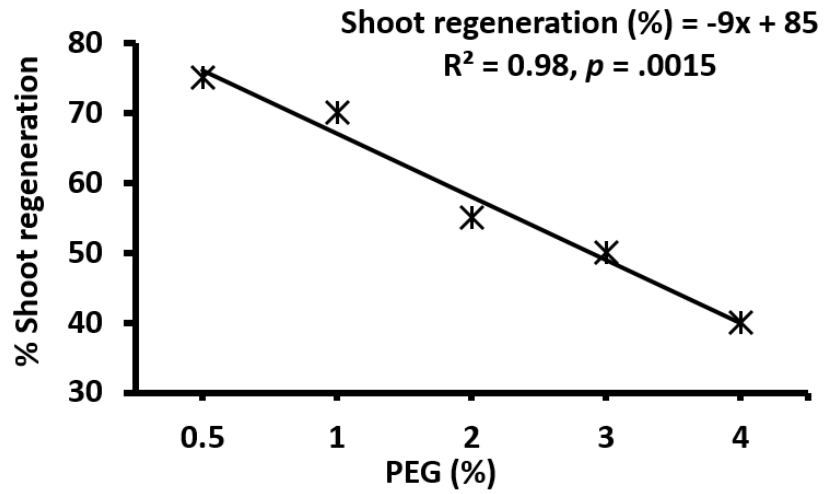


Figure 3. Effect of PEG supplement into MS medium on rice shoot regeneration.

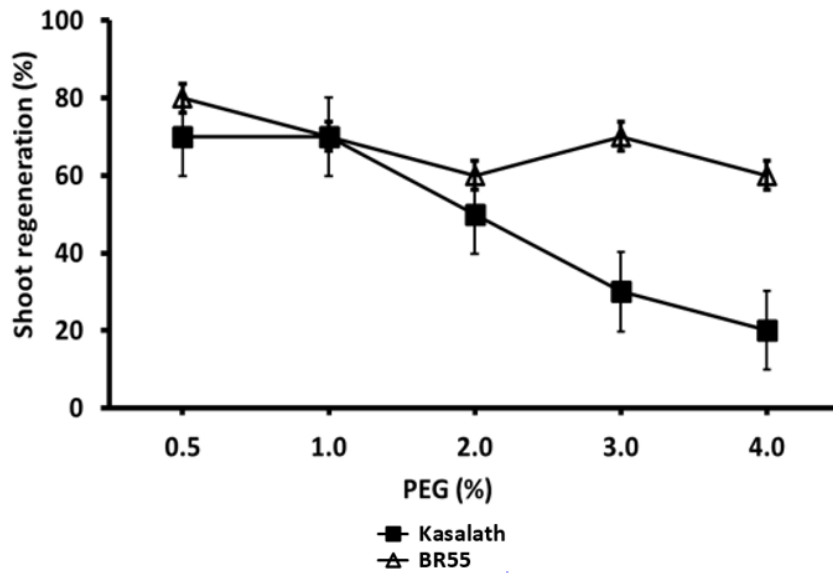


Figure 4. Effect of PEG on Kasalath and BRRI dhan55. The bars are standard errors.

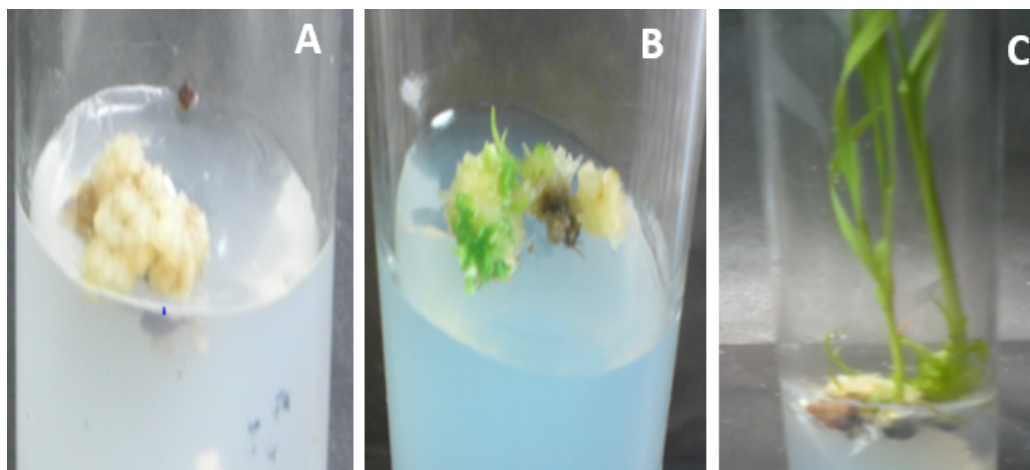


Figure 5. Callus induction and shoot regeneration of rice. A- Callus of Kasalath on callus induction media (MS + 2 mg/L 2, 4-D) after three weeks of inoculation, B- Callus of BRR1 dhan55 on shoot regeneration media (MS + 1 mg/L NAA + 2 mg/L BAP + 1 mg/L Kinetin), C- Shoot regeneration of BRR1 dhan55 after 5 weeks (MS + 1 mg/L NAA + 2 mg/L BAP + 1 mg/L Kinetin + 4% PEG)

However, BRR1 dhan55 had higher shoot regeneration frequency than Kasalath (Figure 4, Figure 5-C). Shoot regeneration frequency for BRR1 dhan55 was 70 and 60% in 3 and 4% concentration of PEG, respectively. Kasalath were 30 and 20% in the same concentration of PEG as those of BRR1 dhan55. Reduction in regeneration frequency with increased PEG concentration has been observed before.

Wani *et al.* (2010) showed both reduced callus induction and shoot regeneration of rice cultivars PAU 201 and PR 116 in medium supplement with different concentrations of PEG. Tripathy (2015) also found similar reduction in regeneration frequency with increased concentration of PEG in upland rice. Further, cultivar depended responses with PEG have also been observed. Akte *et al.* (2016) found that rice cultivar Binadhan-10 performed the best against PEG compared to Binadhan-4, Binadhan-5, Binadhan-6 and Iratom-24. In this study, the

difference in PEG tolerance among the two varieties was expected. BRR1 dhan55 was developed as a variety that could tolerate medium salt and drought stress (Parvin *et al.*, 2015). Kasalath, on the other hand, is a landrace known for several beneficial traits such as early maturity and can tolerate phosphate deficiency and some levels of drought (Kanamori *et al.*, 2013; Sakai, 2014).

Screening plants for drought tolerance is paramount in sustaining production under drought environments. However, screening under field condition is tedious, resource intensive and time consuming. It is easy to use chemicals to induce artificial drought environment. Therefore, a similar method can be used to select for drought tolerance. PEG (6000) can initiate water stress conditions in nutrient cultures and affect plant cells similarly to those under drought conditions. PEG with high molecular weight (more than 4000) is not

permeable to plasma cell membrane. It is also not metabolised and not absorbed by plants. However, it decreases water potential in nutrient solution thus stimulate water stress without other negative effects such as toxicity (Wani *et al.*, 2010; Heringer *et al.*, 2013). Thus, the present study further illustrates the benefit that PEG which can be used to screen plantlets for drought stress.

4. Conclusions

The present study indicated that the concentrations of auxins and cytokinins were an essential factor of rice callus initiation and shoot regeneration. Polyethylene glycol can be used to induce artificial drought condition *in vitro* and assess the responses of rice plantlets in early growth stage to drought stress. Thus, it could be an initial step in screening rice cultivars against drought stress.

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