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In vitro Drought Tolerance of *Oryza sativa* L. 'Tubtim Chumphae' Callus, Valuable Rice of Thailand

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ABSTRACT

Droughts have a serious impact on the environment and negatively influence agricultural productivity. Improved knowledge of plant adaption would help elucidate the mechanisms of their responses to drought stress. Drought tolerance was assessed in the Thai rice cultivar Tubtim Chumphae, using calli as explants. Three-week-old calli were cultured on Murashige and skoog 1962 (MS) medium supplemented with 2 mg/L 2,4-D and 0.5 mg/L BAP for initiation, in combination with 0, 2, 4, 6 or 8% (w/v) PEG 6000. All samples were cultured at 25±2ºC with a 16/8 h light/dark cycle ($40\ \mu mol.m^{-2}.s^{-1}$). Treated calli showed decreased growth performance and survival percentage when cultured at higher PEG 6000 concentrations, except in root formation. Survival percentages of all treatments were significantly different from the control but remained high, indicating drought tolerance of Tubtim Chumphae rice. These findings can be used to improve adaptation mechanisms of drought-tolerant rice cultivars under drought stress and be applied for drought-tolerance-level screening of other crop species. Drought stress information will be useful for rice breeding to increase the resistance of drought-tolerant rice cultivars in conjunction with other agronomic features.

Keywords: Drought stress; PEG 6000; Regeneration; Rice callus; Water deficit

1. Introduction

Rice is a staple food for more than half of the global population [1]. World rice production is around 850 million tons per year, using a rice-growing area of about 256 million hectares, with more than 75% of rice worldwide being consumed by the Asian population [2]. Different forms of environmental stresses, such as nutrient deficiency, high irradiance, toxicity and high temperature all impact rice production, which complicates breeding of drought tolerance species [3]. Droughts are becoming more frequent and severe as a result of the

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current unpredictable and changing environment [4], with a significantly negative impact on agricultural productivity [5]. Most crop plants suffer from drought and wither and die when subjected to a prolonged lack of water [6].

Climate change has affected rainfall patterns and droughts have now become a major concern for rice farmers [7]. Basic knowledge is required to better understand plant responses as adaptation mechanisms to drought stress. Molecular, cellular and morphological adaptations, in the form of physiological and biochemical changes, occur in plants to combat stress exposure at various levels. These changes depend on associated factors such as genotype, stress severity and developmental stage [3, 4, 8].

Drought tolerance is a complicated mechanism controlled by numerous quantitative trait loci (QTL) and is impacted by differing plant phenologies [9]. Drought stress lowers rice seed germination rate by reducing the water content in seeds [10, 11], decreases the strength of seedlings [12] and inhibits leaf expansion, photosynthetic rate and gas exchange properties in the plants [13, 14]. Rice species are more vulnerable to drought situations than other crops, especially during germination and in the early stage of seedling growth [15]. Drought stress during the flowering stage of Yangliangyou 6 (YLY6) and Hanyou 113 (HY113) rice cultivars results in a considerable reduction in grain yield, with a decrease in spikelets per panicle and full grains [16]. Under drought conditions, closed stomata restrict water transfer within the plant leaf. This lowers carbon dioxide intake by the leaves and promotes additional electrons for the production of reactive oxygen species (ROS) [17], causing lipid peroxidation, nucleic acid damage and protein denaturation that all reduce overall metabolism and result in decreased grain production [18].

Breeding programs for drought-tolerant rice varieties are complicated and challenging [15]. Interdisciplinary research has attempted to unravel and comprehend the systems behind plant resilience to drought stress using a range of approaches [3]. This research involves studying the responsiveness of plants to many abiotic and biotic challenges in order to increase yield [19]. However, due to poor trait heritability and the effort necessary for development, conventional breeding techniques to improve drought- tolerant rice varieties under field environments are time-consuming. Plant selection in the field is also difficult due to low heritability and the complex interactions between environmental influences and plant genotypes [2]. Tissue culture techniques aid in the assessment of several stress- tolerance traits due to the simplified control of environmental stresses under *in vitro* situations [6]. Drought-tolerant plant selection can be achieved using *in vitro* cultures, implying a connection between cellular and *in vitro* plant responses [2]. For breeding purposes, the tissue culture approach has been frequently utilized to identify drought-tolerant cultivars [18]. Drought-tolerant rice calli are used to guide and improve drought-tolerant genotype selection efficacy and correctness because callus growth is unaffected by environmental influences [2]. *In vitro* drought tolerance assessment is an important technique to improve selection effectiveness [18], while *in vitro* cell and tissue culturing improve the understanding of physiological, developmental and genetic characteristics of plants in order to enhance diverse rice varieties [20]. Numerous studies have been conducted to assess *in vitro* rice response to abiotic stress [18, 20-22]. Here, *in vitro* drought stress treatment of Tubtim Chumphae rice calli, a valuable Thai rice cultivar, was performed and evaluated.

The Tubtim Chumphae rice cultivar (SRN06008-18-1-5-7-CPA-20) is a hybrid of KDML105 (Mother) and Sangyod Phatthalung (Father). This rice has upright clumps and is a photoperiod insensitive

cultivar. After cooking, the brown rice softens and turns a clear bright ruby-red color. Compared to other commercial Thai rice varieties, the amylose content is low [23]. Seeds of Tubtim Chumphae rice contain abundant antioxidants, phenolics, flavonoids and vitamin E as well as gallic acid, myricetin, luteolin, kaempferol, apigenin and cyanidin-3-glucoside [24], while Tubtim Chumphae rice bran hydrolysates (TCRH) were shown to reduce oxidative stress and improve blood vessel relaxation [25].

2. Materials and Methods

2.1 Callus induction

Tubtim Chumphae rice seeds were kindly provided by Ms. Chanyapat Visaetjirakul, an organic farmer in Bang Pahan District, Ayutthaya Province, Thailand. Dehusked seeds were sterilized for 20 min with shaking in 20% (v/v) sodium hypochlorite (Clorox) mixed with 2-3 drops of Tween 20 and then washed three times with sterile distilled water. The sterilized seeds were then cultivated on MS medium containing 3% (w/v) sucrose and 2 mg/L 2,4-D with 0.5 mg/L BAP for 3 weeks. The cultures were incubated under light conditions as follows: 40 $\mu mol.m^{-2}s^{-1}$ at 25 ± 2ºC (16/8 h light/dark). Calli derived from the seeds were used as explants in all experiments.

2.2 Drought stress treatment

Three-week-old calli were cultured on MS medium supplemented with 2 mg/L 2,4-D and 0.5 mg/L BAP for callus initiation, in combination with 0, 2, 4, 6 or 8% (w/v) PEG 6000. All samples were cultured at 25±2ºC with a 16/8 h light/dark cycle providing 40 $\mu mol.m^{-2}.s^{-1}$. Survival percentage, green spot number per callus and shoot and root number per callus were investigated after 4 weeks of culturing. Live calli were quantified using a 2, 3, 5-triphenyitetrazolium chloride (TTC) assay, with survival percentages calculated using the following equation:

$$\%Survival\ percentage = \frac{Final\ number\ of\ survived\ plants}{Initial\ number\ of\ explants} \times 100.$$

The drought tolerance index (DTI) was used to compare values under drought stress with values under non-stress conditions, as described by Nautiyal et al. [26] (greater than 1 = increase, less than 1 = decrease), as follows: DTI = Data of stress treatment / Data of non-stress treatment.

Each treatment comprised 7 replicates, with 4 calli cultivated in each replicate. There were 28 samples in each treatment, with a total of 140 samples in all five treatments.

2.3 Plantlet regeneration after drought stress treatment

Drought-stress treated calli were cultured on MS medium containing 0.5 mg/L NAA and 4 mg/L BAP. The cultures were incubated at 25±2ºC with a 16/8 h light/dark cycle providing 40 $\mu mol.m^{-2}.s^{-1}$. Regeneration percentage, plant height, root length and green spot number per callus were recorded after 5 weeks of culturing.

2.4 Statistical analysis

The experiments were conducted using completely randomized design (CRD) with five replications. Data were analyzed by one-way analysis of variance (ANOVA) with differences between means separated by Duncan's multiple range test ($p \leq 0.05$). All data are presented as mean ± standard error (SE).

3. Results and Discussion

Callus growth of Tubtim Chumphae rice decreased when cultured at higher PEG 6000 concentrations (Figs. 1-2). Treated calli were small and dried with brown spots compared to the control group (Fig. 2). Survival percentages of treated calli tested by TTC assay were significantly lower than the control (Fig. 3). Only 8% of plants in the PEG 6000 treatment showed a survival percentage lower than 50%, while the other treatments presented a survival percentage

higher than 50% (Fig. 1A). Growth performances including green spot number and shoot number per callus dramatically decreased when treated with higher PEG 6000 concentrations and green spot numbers per callus were significantly different from the control (Fig. 1B). For shoot appearance, adventitious shoot numbers per callus of treated calli differed significantly from the control (Fig. 1C and Fig. 4), while root numbers per callus of treated calli slightly increased but were not significantly different from the control (Fig. 1D). After being cultured on MS medium containing 0.5 mg/L NAA and 4 mg/L BAP for 7 weeks, surviving calli of the control and 2 % PEG 6000 treatment regenerated into plantlets with a 15 and 10% regeneration rate, respectively (Table 1 and Fig. 5).

Table 1. Survival, regeneration percentage, shoot number per callus and shoot length of plantlets regenerated from survived calli after cultured for 7 weeks.

PEG 6000 (%)	% Surv.	% Regen.	Shoot no /callus	Shoot length (cm)
0	45	15	6.00 ± 0.22^{a}	6.32 ± 0.02^{a}
2	25	10	3.50 ± 0.15^{b}	5.48 ± 0.04^{b}

Means ± SE followed by different letters are significantly different in columns by one- way ANOVA and Duncan' s multiple range test (DMRT; $p < 0.05$). Surv = Survival, Regen. = Regeneration.

Two main stresses occur in plants during severe dehydration. These are oxidative stress, generated mostly by photosynthesis at low water content levels, and structural damage that presents as loss of membrane integrity as a result of turgor shift [27]. Our results showed that dried and smaller-sized calli with brown spots occurred after drought- stress treatment in a variety of plant cells. Under drought conditions, many critical nutrients including phosphorous (P) and nitrogen (N) are depleted [11], while drought stress lowers the uptake of critical nutrients like calcium (Ca), copper (Cu), zinc (Zn) and manganese (Mn) in rice; additionally, it ends up altering many biochemical and physiological pathways [28]. Zinc deficiency under drought conditions causes plant cell damage, limiting rice growth and development [29]. Increased osmotic stress causes morphological and physiological changes in calli that affect survival rate, growth rate and regeneration [20, 30]. A high degree of osmotic stress kills tolerant cells, while a low level of osmotic stress allows non-tolerant cell lines to survive. Furthermore, increasing the concentration of PEG in the selection media reduces embryogenic callus induction and plantlet regeneration from selected calli [31].

Different tissue culture media often results in adverse cell development conditions. As a result, most cells die, with only a few continuing to grow. Drought stress causes significant modifications in plants, including generating ROS. This causes oxidation in plant cells and disrupts rice growth [32]. Singlet oxygen and hydroxyl free radicals are among the oxidative species that cause denaturation of rice protein, lipid peroxidation and DNA alterations [3]. Cell tolerance may be impacted by mutation [33] or somaclonal variation [18]. Tolerance at the unstructured cellular level or callus can also act at the whole plant level [34]. *In vitro* screening and selection of calli can be used to determine important criteria for drought tolerance. Selection based on callus is a unique approach that uses grown cells as selection units instead of the entire plant [30]. There are genetic, physiological and biochemical limitations in creating drought-tolerant plants using this technique as it is based on the generation of genetic variation across *in vitro* cells through tissue culture of regenerated plants [21]. This technique allows control of stress uniformity and characterization of cellular activities under stress, independent from regulatory systems at the plant level [6]. Cell wall folding and vacuole fragmentation are two important cellular-level alterations in desiccation-tolerant plants [27]. Cell wall folding has been linked to higher cell wall plasticity

during dehydration as a result of biochemical alterations in the cell wall [35], while central vacuole fragmentation is accompanied by the substitution of water with nonaqueous compounds [27].

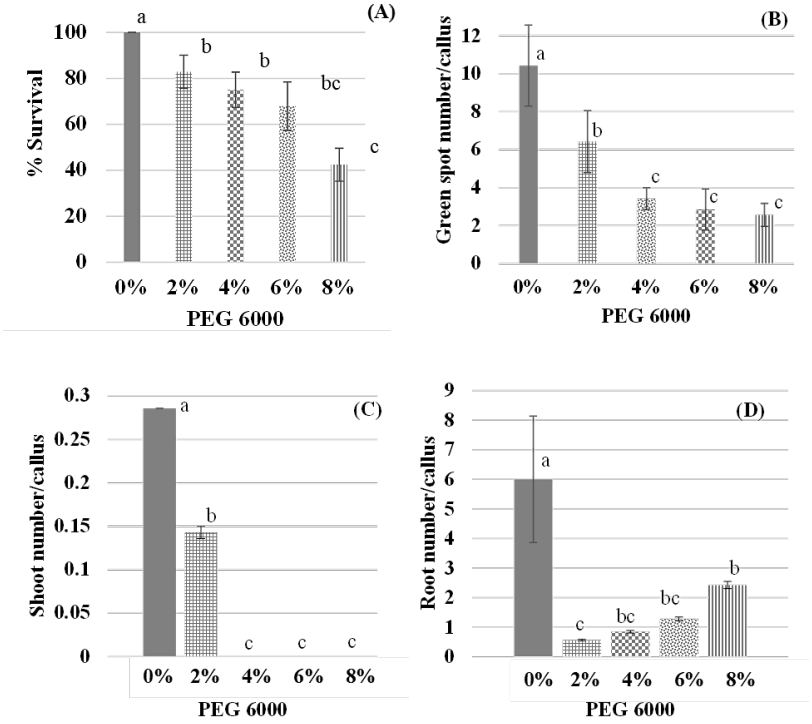

Fig. 1. Growth performance of calli under drought stress: (A) survival percentage, (B) green spot number per callus, (C) shoot number per callus, (D) root number per callus.

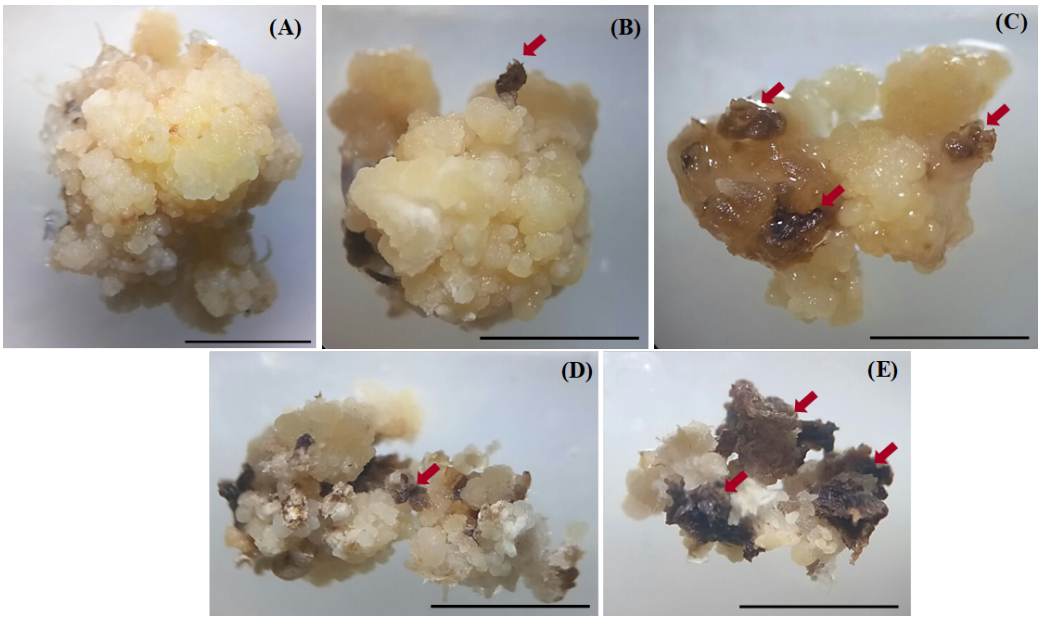

Fig. 2. Appearance of calli after drought stress treatment, brown tissue represents dead areas (arrows): (A) control, (B) 2% PEG, (C) 4% PEG, (D) 6% PEG and (E) 8% PEG. Scale = 5 mm.

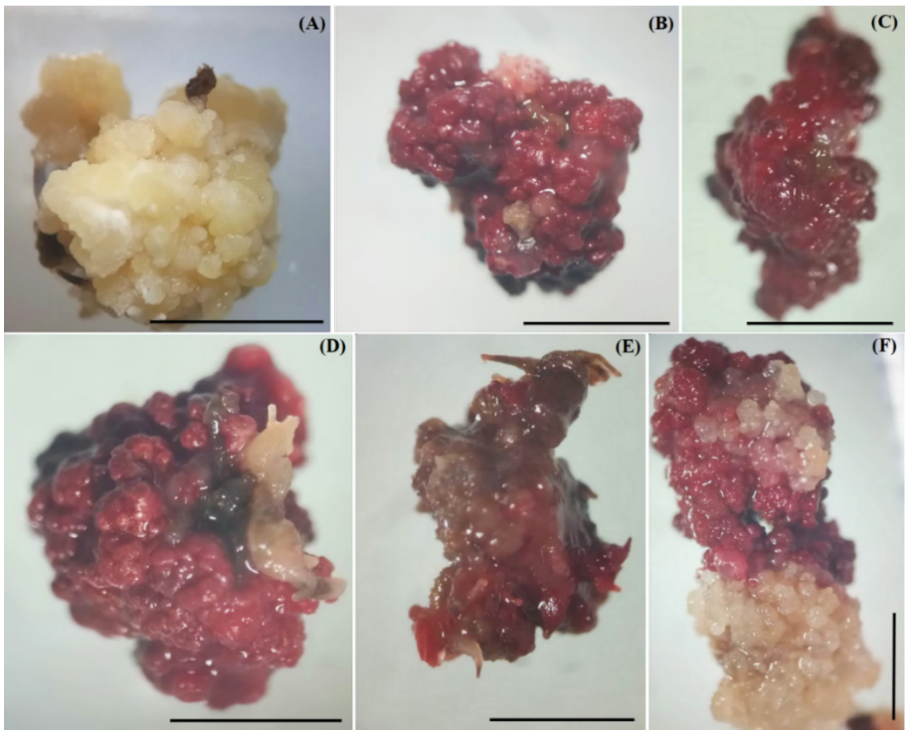

Fig. 3. TTC assays: (A) non treated callus without TTC test, (B) non treated callus after TTC test, (C) 2% PEG, (D) 4% PEG, (E) 6% PEG and (F) 8% PEG. Scale = 3 mm.

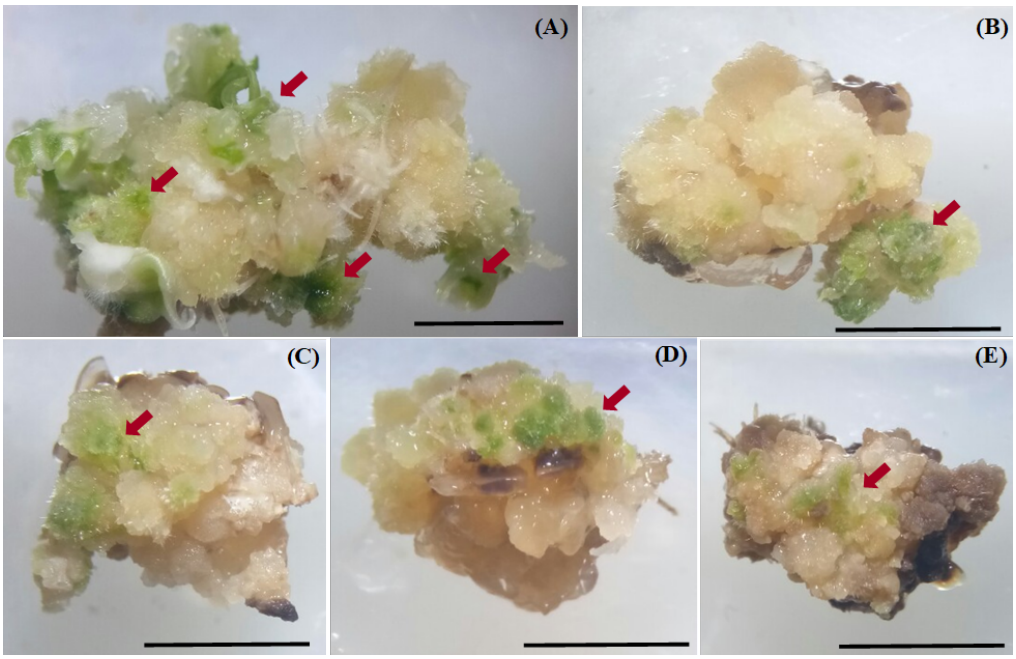

Fig. 4. Green spots and multiple shoots (arrows) of drought stress treated calli: (A) 0% PEG, (B) 2% PEG, (C) 4% PEG, (D) 6% PEG and (E) 8% PEG. Scale = 5 mm.

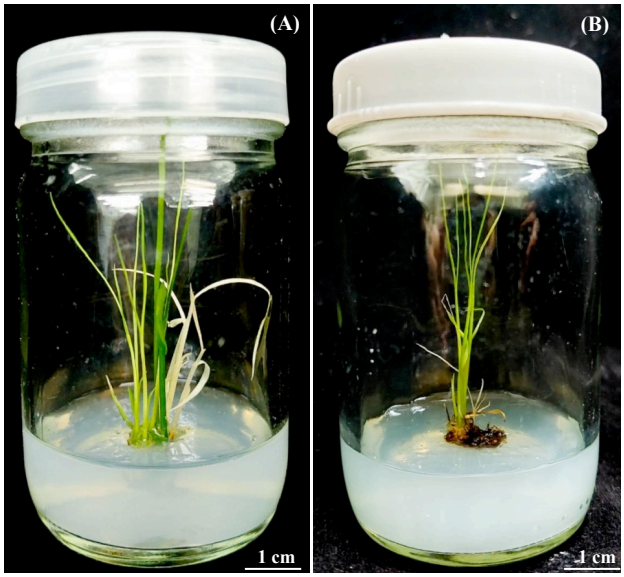

Fig. 5. Regenerated plantlets of the control (A) and 2% PEG treatment (B) after cultured for 7 weeks.

The decreasing trend in calli survival and growth rate with increasing PEG concentrations in this study was consistent with previous findings by others [18, 20, 36]. The surviving calli may be subjected to somaclonal variation that occurs during the *in vitro* regeneration process, producing genetic or epigenetic variants [37]. One appropriate protocol to determine *in vitro* somaclonal variation is the use of molecular markers, while one of the key concerns of tissue culture is maintaining the genetic stability of cells and tissues. Genetic stability assessment by DNA-based molecular markers can be studied using various markers such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphisms (AFLP), Simple Sequence Repeats (SSR), Inter Simple Sequence Repeats (ISSR), Methylation-sensitive Amplified Polymorphisms (MSAP), Sequence-related Amplified Polymorphisms (SRAP), Restriction Fragment Length Polymorphisms (RFLP) as well as phenotypic variation rate [2, 18, 38-40].

Drought tolerance indices of all treated calli were lower than the control (Table 2), while drought tolerance indices of the survival percentage of high PEG 6000 treatment in this study (6% and 8%) showed significant differences from low concentrations (2% and 4%). Three characteristics of survival percentage, being green spot number per callus, shoot number per callus and root number per callus, estimated by the phenotypic correlation coefficient (Table 3), all showed a positive correlation. Survival percentage had a significantly positive correlation with green spot number per callus, while root number per callus exhibited a significantly positive correlation with green spot number per callus and shoot number per callus.

One of the most common methods for inducing drought stress *in vitro* is to utilize high molecular weight osmotic compounds like PEG [41]. PEG is non-ionic, non-toxic and inert with a high molecular weight and is soluble in water [20]. PEG 6000 induces *in vitro* artificial drought stress in callus cultures and has been used to imitate drought stress in cultured plant tissues by lowering the water potential of the culture medium [42]. Water potential has also been shown to cause morphological changes in tissues that have been exposed to osmotic stress,

particularly at the cellular level [43]. Water potential is also crucial for determining *in vitro* regeneration ability after the recovery step with a novel genetic composition achieved through *in vitro* selection [44, 45]. In cultivated cells, PEG does not penetrate or break down in considerable amounts, and it is not absorbed in *in vitro* cultures [40]. Cultured plant cells are stressed by water deficiencies in a similar way that they would be if they were in a real desiccation situation [46]. PEG has been utilized as a selective substance to demonstrate osmotolerance in various calli such as Hassawi rice (*O. sativa* L.) [47], rice (*O. sativa* L.) cultivars PAU 201 and PR 116 [20], wheat (*Triticum aestivum* L.) variety GA-2002 [30], sugarcane (*Saccharum officinarum* L.) [36], *Medicago truncatula* [43], Kurdish rice (*O. sativa* L.) [2], Batutegi and Situpatenggang varieties [22].

Root number per callus of the 8% PEG 6000 treatment exhibited the highest value during the regeneration process, both in number and drought tolerance index (Table 2, Fig. 1D). Other growth performance indicators of this treatment were lower than the control, but root formation was higher than the other treatments. In this study, root formation showed a significantly high positive correlation to green spot number per protocorm (Table 3), as the result of indirect *de novo* root regeneration as adventitious roots derived from calli during *in vitro* culturing with low auxin. However, the formation mechanisms of adventitious roots from calli remain unclear [48]. Under conditions of water shortage, root growth takes precedence over shoot growth. Under drought stress with a reasonably substantial soil water reservoir, several adaptive mechanisms for rice have been established such as osmotic alteration in roots, greater root dissemination into the soil and improved root density with depth [4]. Reducing water potential has also been shown to cause osmotic changes in the root system that help to preserve turgidity and re-establish a water potential gradient for water intake [49].

Table 2. Drought tolerance index of four traits of rice callus under drought stress.

PEG 6000 (%)	Drought tolerance index			
	%Survival	Green spot no / callus	Shoot no / callus	Root no / callus
2	0.83	0.62*	0.50*	0.09**
4	0.75	0.33*	0.00**	0.14**
6	0.68*	0.27**	0.00**	0.21**
8	0.42**	0.25**	0.00**	0.40*

* Significant difference at $p < 0.05$, ** Significant difference at $p < 0.001$

Table 3. Phenotypic correlation coefficients among four characteristics of rice callus under drought.

Characteristic	Survival percentage	Shoot number	Root number
Green spot number	0.358 *	0.479*	0.655**
Survival percentage		0.153	0.283
Shoot number			0.363*

* Significant difference at $p < 0.05$, ** Significant difference at $p < 0.001$

In reaction to abiotic stress, plant organs such as roots and leaves orchestrate defensive responses (internal or external mechanisms) [50, 51]. The first organ affected by water deficit is the root system because water stress is caused by an insufficient or excessive amount of soil water [52, 53]. Plant root properties are critical for increasing productivity under drought conditions [15]. Previous studies have reported that rice varieties with prolific and deep root systems displayed improved adaptability under drought [4, 54]. However, soil microorganisms in plant roots also affect growth and health. In response to drought, the rhizosphere and endosphere microbiome communities suffer significant compositional changes, including decreased taxonomic diversity. Drought-responsive microorganisms, particularly those enriched under water stress situations, may benefit the plant by contributing to drought tolerance and tolerance to other abiotic stresses, as well as providing protection from opportunistic pathogens [55]. The PEG 6000 concentrations used in this study (2, 4, 6 and

8%) were high compared with other rice callus selection studies such as with RD6 cultivar (0, 0.5, 1, 1.5 and 2%) [18] and Kurdish Rice (0, 0.5, 1 and 1.5%) [2]. Our findings suggested that Tubtim Chumphae rice was a drought-tolerant cultivar under *in vitro* drought stress. However, several abiotic stresses such as salinity stress, topography, drought stress, unpredictable weather patterns and disadvantageous soil conditions all contribute to ecosystem complexity [56]. Mechanisms of multiple stresses affecting calli growth and plantlet regeneration require further study to clarify both their individual and combined inputs.

4. Conclusion

An effective technique for plant regeneration and drought tolerance selection was established using Tubtim Chumphae rice calli. Drought stress affected survival rate and callus growth. Drought stress generated by PEG 6000 treatment was suitable for *in vitro* screening of Tubtim Chumphae rice drought- tolerant calli. Drought stress data combined with other agronomic traits can assist rice breeders to improve the resistance of drought- tolerant rice cultivars. Our findings can be utilized to develop adaptation mechanisms for drought- tolerant rice cultivars and screen drought tolerance levels in *in vitro* culture in other crop species.

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