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REGULAR ARTICLE

In vitro screening of rice (*Oryza sativa* L) callus for drought tolerance

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ABSTRACT
While drought resistance is become of increasing importance in rice (*Oryza sativa* L.), selection under actual field conditions is tedious due to low heritability and time required. Selection in tissue culture is thought to be one way to improve selection efficiency, but this requires standardized protocols. Rice cultivars PAU 201 and PR 116 showed significant callus induction, but the capacity for callus induction and regeneration decreased under polyethylene glycol (PEG) (6000) stress in both cultivars. Calli were induced on semisolid Murahige and Skoog (MS) medium supplemented with 2.5 mg l^{-1} 2, 4-dichlorophenoxy acetic acid (2,4-D) + 0.5 mg l^{-1} kinetin (kin) + 560 mg l^{-1} proline + 30 g l^{-1} sucrose + 8 g l $agar^{-1}$. Embryogenic calli showed shoot regeneration on MS medium supplemented with 2.0 mg l^{-1} benzyl aminopurine (BAP) + 0.5 mg l^{-1} kinetin + 0.5 mg l^{-1} napthalene acetic acid (NAA) + 30 g l^{-1} sucrose + 8 g l^{-1} agar. Increased levels of PEG (6000) (0, 0.5, 1.0, 1.5, 2.0 %) were used to create water stress. There was reduction in callus induction ability and plant regeneration efficiency with increasing levels of PEG (6000) stress. These results indicated that PEG (6000) can be used as water stress creating agent under *in vitro* conditions and rice variety PR 116 was relatively tolerant to drought stress as compared to PAU 201. This study will serve as a base line for *in vitro* screening of drought tolerant transgenic rice.

Key Words: *Oryza sativa, PEG, drought stress, callus induction, plant regeneration.*

INTRODUCTION

Of all the cereals, rice (*Oryza sativa*) is most susceptible to damage from water deficit (Lafitte and Bennet, 2003). Drought and salinity are two of the most complex stress tolerances to breed for, as the type (combinations of heat and drought or sodicity and salinity), timing in relation to plant growth stage and intensity of the stress can all vary considerably (Witcombe et al. 2008). According to published statistics, the percentage of drought affected land area in the world more than doubled from the 1970s to the early 2000s (Isendahl and Schmidt, 2006). Drought is a world-wide problem that seriously influences grain production. Increasing human population and global climate change make the situation more serious (Hongbo et al., 2005). Rice, as a paddy field crop, is particularly susceptible to water stress (Tao et al., 2006; Yang et al., 2008). It is estimated that 50% of world rice production is affected by drought (Bouman, et al. 2005). Water deficit is becoming increasingly frequent in irrigated areas due to falling water tables. In total, world rice production uses about 1,578 km^3 of water, which is 30% of the fresh water used worldwide (Trijatmiko, 2005). The international scarcity of water threatens the sustainability of the irrigated rice ecosystem. In Punjab the water table is falling 30 to 90 cm every year depending on the time of sowing/transplanting of the rice crop. A given level of drought, at the vegetative stage, can cause a moderate reduction in yield but drought can entirely eliminate yield if it coincides with pollen meiosis or fertilization (O'Toole, 1982). Genetic improvement of rice for drought tolerance through conventional breeding is slow because of the low heritability of yield under stress, low inherent variation in the field and the limitation that there is usually only one experimentally droughted crop each year (Ribaut et al., 1997).

Plant tissue culture plays an important role in the production of agricultural and ornamental plants and in the manipulation of plants for improved agronomic performance. In vitro culture of plant cells and tissue has attracted considerable interest over recent years because it provides the means to study plant physiological and genetic processes in addition to offering the potential to assist in the breeding of improved cultivars by increasing genetic variability. Regenerated plants are expected to have the same genotype as the donor plant; however, in some cases somaclonal variants have been found among regenerated plants (Karp et al., 1987).

Media composition – mainly the hormonal balance – is an important factor influencing vitro culture initiation and plant regeneration from embryos (Jiang et al., 1998). The auxin 2, 4-dichlorophenoxy acetic acid (2, 4-D) alone or in combination with cytokinins, is widely used to enhance callus induction and maintenance (Castillo et al., 1998). Genetic factors are considered to be a major contributor to the in vitro response of cultured tissues. Differences in the production of embryogenic calli and the regenerated plantlets have been observed, depending on the genotype and source of the explant (Ganeshan et al., 2003). Salinity is the main abiotic stress that has been addressed by in vitro selection, but applications to other stresses such as heat and drought have been reported (Lutts et al., 1996). These techniques are considered to be an important complement to classical plant breeding methods (Zalc et al., 2004). In vitro selection for tolerance to abiotic stress depends on the development of efficient and reliable callus induction and plant regeneration systems. Hence this experiment was carried out to screen the indica varieties PAU 201 and PR 116 for their inherent tolerance against drought stress.

MATERIALS AND METHODS

The study was conducted in the plant tissue culture laboratory, School of Agricultural Biotechnology, Punjab Agricultural University (PAU), Ludhiana, India. Seeds of commercial Indica rice cultivars PAU 201 and PR 116 were provided by the rice section of the Department of Plant Breeding and Genetics, PAU, Ludhiana. Manually dehusked seeds were

treated with bavistin 0.1% for three hours and washed thrice times in sterile distilled water. Seeds were surface sterilized in 0.1% mercuric chloride for 8 -10 minutes followed by three rinses in sterile distilled water in a laminar flow cabinet. Sterilized seeds were cultured in Petri dishes containing semisolid Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 2.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} kinetin + 560 mg l^{-1} proline + 30 g l^{-1} sucrose + 8 g l^{-1} agar. The Petri dishes were sealed with parafilm and placed in a growth chamber in the dark at 25 ± 2ºC. After four weeks incubation, the induced calli were sub-cultured, under the same growth conditions, and in the same MS medium to which various concentrations of polyethylene glycol (PEG) (6000) (0, 0.5, 1.0, 1.5, 2.0 %) were added. The incubation period was two cycles of two weeks each. Resulting calli were excised, transferred, into test tubes containing MS basal salts medium supplemented with 2.0 mg l^{-1} benzylaminopurine + 0.5 mg l kinetin + 0.5 mg l naphthalene acetic acid + 30 g l^{-1} sucrose + 8 g l^{-1} agar 30 g l^{-1} for shoot initiation, for four weeks. Rooting was initiated on half strength MS medium.

The test tubes were placed in a growth chamber under fluorescent light and at an ambient temperature of 25 ± 2ºC. The medium was changed every 15 days and after this period, calli with clearly differentiated shoots were scored as regenerating callus. Each piece of regenerating callus was counted as one regardless of the number of shoots. Regenerating calli, showing shoot and root formations, were transferred on to MS basal medium with no phytohormones and placed in a lit chamber to sustain growth of regenerated plantlets. The pH of all media was adjusted to 5.8 with 0.1 N NaOH prior to autoclaving. The culture medium was autoclaved at 121ºC for 30 min. The data on callus induction efficiency, measured as the number of calli obtained/total number of seeds cultured × 100, and difference in calli fresh weight were recorded after PEG stress. Similarly plant regeneration per cent (number of plantlets/total number of calli) × 100 after PEG treatment was also recoded.

RESULTS AND DISCUSSION

GENOTYPIC CALLUS INDUCTION CAPACITY

The overall aim of the experiment was to screen the rice cultivars PAU 201 and PR 116 for drought tolerance under *in vitro* conditions Callus induction from mature seeds was assessed, further, the response of calli to elevated levels of PEG (6000) was recorded as fresh weight. The response of the regenerating callus to PEG (6000) stress was also observed. Callus induction rates were 44.4 and 53.8%, respectively for PAU 201 and PR 116. This indicated differential genotypic ability for callus induction. The cultivar PAU 201 was less responsive than PR 116, which appears to be best suited for in vitro tissue culture. This agrees with previous reports in which embryogenic and non-embryogenic callus formation, genotypic differences in callus formation as well as plant regeneration were reported to be genetically determined in some cereals including rice (Khanna and Raina, 1998; Hoque and Mansfield, 2004; Khalequzzaman et al., 2005), wheat (*Triticum aestivum* L.) (Özgen et al., 1998) and barley (*Hordeum vulgare* L.) (Lührs and Lörz, 1987). In rice, a significant difference in callus induction was found among different genotypes of Indica rice (Abe and Futsuhara, 1986; Peng and Hodges, 1989; Seraj et al., 1997). Callus, mainly comprising masses of undifferentiated cells, is good starting material for in vitro manipulation. Moreover, calli induced from the scutellar tissue of mature seeds are an excellent source of cells for in vitro regeneration (Hiei et al., 1994; Rashid et al., 1996). In this study the variation in callus induction capacity appeared to be mainly due to genotypic effects.

CALLUS PROLIFERATION AND PLANT REGENERATION RESPONSE TO PEG (6000) STRESS

The main aim of this experiment was to check the inherent capacity of two rice cultivars PAU 201 and PR 116 to PEG stress. Both cultivars showed a similar response under PEG stress. In vitro screening of control calli of rice cultivars PAU 201 and PR 116 for drought

tolerance was carried out by growing embryogenic calli on callus induction medium supplemented with 0, 0.5, 1.0, 1.5, or 2.0% PEG (6000) for two weeks. Approximately 100 mg of one month old embryogenic callus was exposed to each PEG (6000) concentration. Calli on control medium exhibited normal proliferation. As the PEG (6000) concentration in the medium increased, there was a decrease in callus fresh weight (Figure 1, 3). At 1.0% PEG (6000) the fresh weight was 124.4 mg in rice cultivar PAU 201 compared to 198.9 mg at 0% PEG (6000) and 123.7 mg in PR 116 compared to 196.9 mg at 0% PEG (6000).

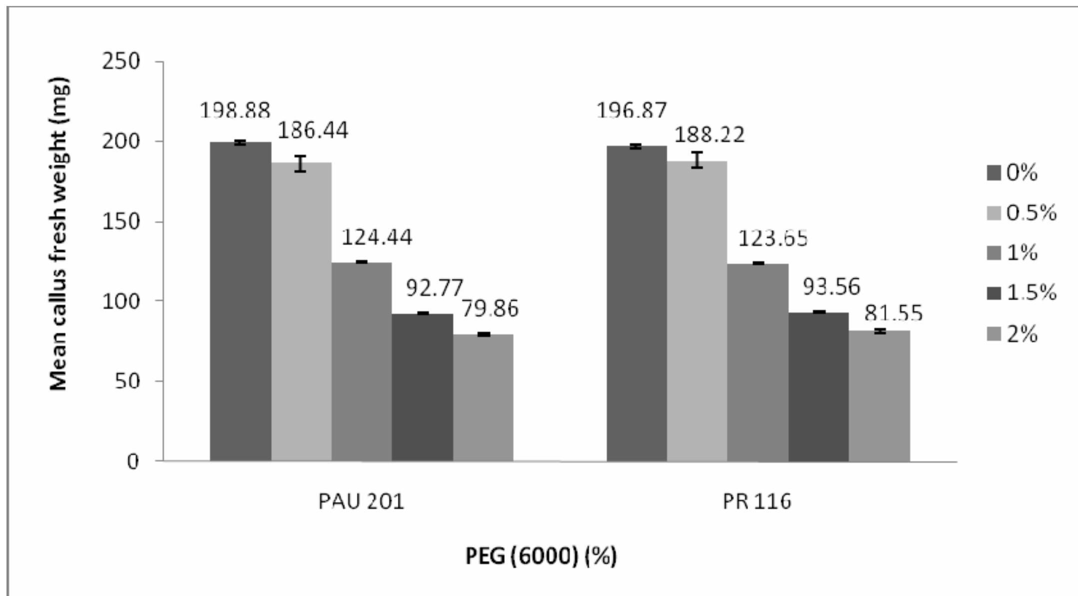

Figure 1. Decrease in mean callus fresh weight with increase in PEG (6000) concentration.

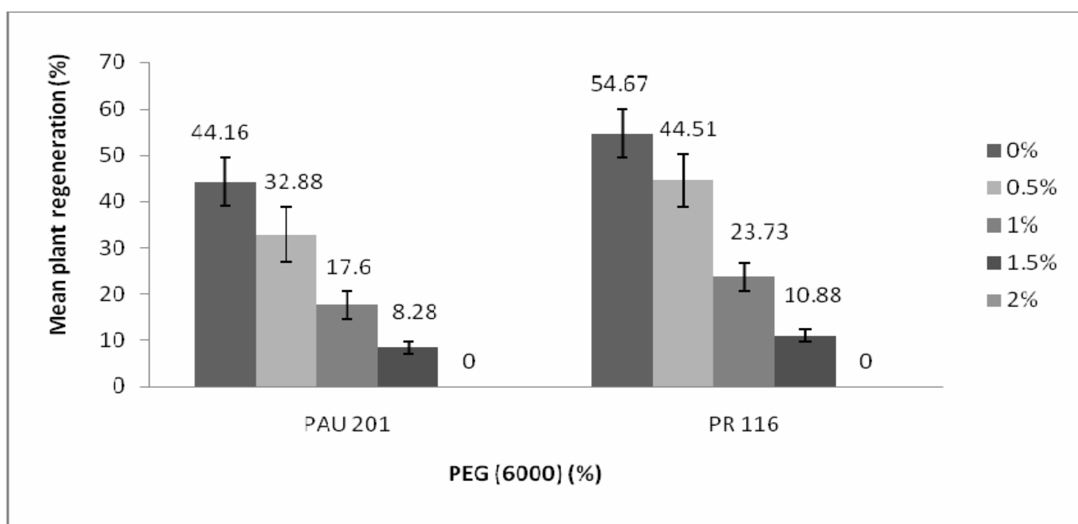

Figure 2. Decrease in mean plant regeneration percent with increase in PEG (6000) concentration.

There was a 74% decrease in callus fresh weight at 1.0% PEG (6000) in rice cultivar PAU 201 and a 73.2% decrease in rice cultivar PR 116.

Dragiiska et al. (1996) developed a system for in vitro selection during somatic embryogenesis in alfalfa (*Medicago sativa* L.). In this work PEG was used as a selective agent for osmotolerance. The procedure involved screening seeds and seedlings until the cotyledonary stage on 10% PEG with further growth of plants on a PEG-free medium.

Initiation of somatic embryogenesis proceeded until the end of the globular stage on PEG. Selection for drought tolerance at early seedling growth stage is frequently carried out by including chemical drought induced molecules such as PEG (6000) in the medium. The role of PEG (6000) in creating chemical drought was demonstrated in wheat by exposing seedling to 20% PEG (6000) for one week (Bayoumi et al., 2008). Lagerwerff et al. (1961) indicated that PEG can be used to modify the osmotic potential of nutrient solution culture and can induce plant water deficit in a relatively controlled manner, appropriate to experimental protocols (Money, 1989; Zhu et al., 1997) Among osmotic agents, PEG is the most widely used osmoticum to study plant status (Handa et al., 1982; Bhaskaran et al., 1985; Newton et al., 1986, 1989). Polyethylen glycol is inert, non-ionic and nontoxic and of high molecular weight. It is very soluble in water, and is available at a wide range of molecular weights (e.g., PEG-4000, PEG-4500, PEG-6000, and PEG-8000) (Lawlor, 1970). It simulates water deficit conditions in cultured cells in a similar manner to that observed in the cells of intact plants subjected to actual drought conditions (Attree et al., 1991; Hohl and Schopfer, 1991). High molecular weight PEG (more than 4,000) induces water stress in plants by decreasing the water potential of the nutrient solution without being taken up and with no evidence of toxicity (Lawlor, 1970). Al-Bahrany (2002) studied the response of Hassawi rice (*Oryza sativa*) callus to varying degrees of (PEG 8000) induced water stress including callus growth, water content and proline accumulation.

Figure 3. *In vitro* screening of rice callus for drought stress using different concentrations of PEG (6000). (a) Control, (b) 0.5%, (c) 1%, (d) 1.5%, (e) 2%.

To characterize callus growth in response to PEG, 2.5 g embryogenic callus was grown in 125 ml flasks containing 50 ml each of liquid MS medium supplemented with PEG (MW 8000) at 0, 50, 100, 150, 200, 250, and 300 g l^{-1}. The results showed that increased water stress was induced by increased concentrations of PEG which caused a progressive reduction in callus fresh weight. Significant reduction in callus fresh weight was observed in response to 50 g l^{-1}, but the inhibitory concentration was 200 g l^{-1}. To check the efficiency of embryogenic calli to regenerate in the presence of drought stress, calli were exposed to elevated levels of PEG (6000) by putting PEG (6000) in the plant regeneration medium. This experiment was performed to check the inherent capacity of calli to regenerate on medium which induced drought stress. Month old embryogenic calli were grown on plant regeneration medium supplemented with 0, 0.5, 1.0, 1.5, 2.0% PEG (6000) for two cycles each of two weeks. There was normal plant regeneration in the no-stress medium, but increased PEG (6000) concentration in medium decreased percent plant regeneration in rice cultivars PAU 201 and PR 116 (Figure2). Per cent plant regeneration was 44.2% at 0% PEG (6000), but decreased to 17.6% at 1.0% PEG (6000). There was 0% plant regeneration at 2.0% PEG (6000) in PAU 201. In PR 116% plant regeneration on the no-stress medium was 54.7%, but increased PEG (6000) concentration in medium decreased of the per cent plant regeneration. At 1.0%t PEG (6000) it decreased to 23.7% and there was no regeneration at 2.0% PEG (6000). In vitro tissue culture could be an important means of improving crop tolerance and yield through genetic transformation as well as by induced somaclonal variation. Therefore it is important to devise an efficient protocol of callus proliferation to start in vitro selection for drought stress tolerance, and to broaden opportunities for genetic manipulation of rice through tissue culture, including trying various explants and media.

The results of this study indicate that the two rice cultivars PAU 201 and PR 116 have good callus induction ability. This experiment provides an indication of the inherent tolerance of rice genotypes to drought. We have introduced the glyoxalase II gene into the commercial rice cultivar PAU 201 (Wani and Gosal, 2011). We will now check the transgenic calli and plantlets for tolerance to drought stress and the above study will be the base line for future screening experiments.

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