A COMPARISON STUDY OF CALLUS AND CELL SUSPENSION CULTURE RESPONSE TO DROUGHT TOLERANCE SCREENING TECHNIQUES IN RICE

S.A. Yousif K.M. Ibrahim A.K. Aurabi

ABSTRACT

Two culture system were used to compare which system is better for producing drought tolerant plants. Sodium Azide (SA) was used as a mutagen to induce genetic variation in two local rice varieties, Amber33 and Amber Baghdad. In vitro screening at the cellular level was performed using PEG (0, 0.5, 1, 1.5 and 2%) as stressing agent to create chemical drought. For shoot formation, the effect of genotypes, PEG and SA were examined on the basis of no. shoot/callus. Proline and carbohydrate concentrations were determined in shoots regenerated from callus or plated cell suspension cultures. Result revealed that cell multiplication increased up to 20 days of culture in almost treatments in 2 culture systems and % growth much higher in cell suspension culture compared with callus culture in all treatments. 1.5 % PEG decreased the growth in Amber 33 and Amber Baghdad only in callus culture, while 2% PEG decreased the growth in both cultivars in callus culture and in Amber33 in cell suspension culture. SA caused an increase in cell growth and division. There was a decrease in plant regeneration in all treatments by increasing PEG levels. 2% PEG in callus culture and 1.5 and 2% PEG in cell suspension culture completely inhibited plant regeneration in both varieties and in both SA treatments. Proline accumulation increased with increasing PEG levels and 1.5 mM SA increased the proline accumulation in both cultivars and in both culture systems. PEG reduced carbohydrate levels significantly in both genotypes and in both culture systems.

Keywords: callus, cell suspension, drought, rice.

INTRODUCTION

Rice is one of the most important crops that provide a half of the world's population with staple diet, yet rice production is affected by environmental stress. Drought is one of the most common environmental stresses affecting rice growth and productivity. In fact, drought can cause severe damage at any stage of rice growth and development, which would lead to yield loss (24). Conventional breeding programs such as hybridization are used to transfer the gene of interest to sensitive plants. In many cases, however, these conventional methods have failed to produce the desired results (19). The use of tissue culture technique has made it possible to improve the tolerance traits through the application of selective pressure in culture conditions (20). In vitro screening on media stressed using different selection agents like polyethylene ethylene glycol (PEG) or manitol was attempted to develop drought-tolerant genotypes. PEG may help in evolving an appropriate selection strategy by acting as a non-penetrating osmotic agent that lowers the water potential of the medium (5). Induced mutations have played an important role in improving various plant characters in different crop species. The characters in which improvement has been achieved include yield, disease resistance, drought tolerance, earliness, adaptability and other morphological characters (2).

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Cell suspension culture is applicable for efficient mass micropropagation (1) and cells in suspension can exhibit in higher frequencies rates of cell division than do cells in callus culture due to the high medium-to-tissue contact. de Touchet *et al.* (7) found that under the best conditions, the initial weight of cells suspension culture increased about 4-fold in a month. Zouine et al. (26) reported that cell suspension resulted in higher somatic embryo number compared to solid medium.

Many difficulties faced the cultivation of rice in Iraq, such as requirement of a continual supply of water, therefore it was important to enhance drought tolerance in sensitive rice genotypes. From other hand, to the best of our knowledge there is no published report so far are available on the comparison between callus and suspension cultures to develop plants with increased drought tolerance. Therefore, this experiment is aimed to compare produce callus and suspension cultures in *in vitro* screening for drought tolerance in two local rice genotypes (Amber33 and Amber Baghdad)

MATERIALS AND METHODS

The experiment was conducted in Agricultural Research Directorate/ Ministry of Science and Technology. For callus and cell suspension cultures MS medium (18) supplemented with 0.5mg/l kin, 2 mg/l 2,4-D and 650mg/l proline (3) were used. Media components were mixed and pH was adjusted to 5.8 before agar was added at 0.8% for semi solid MS medium, then autoclaved (121 ºC, 104 kPa) for 15 min. All cultures were kept in a growth chamber at 25 ± 2ºC in dark.

Callus and cell suspension cultures

Seeds of two local rice genotypes Amber 33 and Amber Baghdad were treated with 0 and 1.5mM Sodium Azide (SA) for 4hrs and washed with tap water 3 times. Seeds were surface sterilized by soaking in 96% ethanol for 2 min and then soaked with 2.5% Sodium hypochlorite for 45 min and washed with sterile distilled water for three times. 3 seeds were cultured in test tube (15x2.5 cm) containing semi solid MS medium to initiate callus from mature embryo. After one month small pieces of calli (150mg) were transferred into semi solid MS medium and callus fresh weights were calculated during a range of culture periods 5, 10, 15, 20, 25 and 30 days for both genotypes. callus growth percentage was calculated by the following formula:

% callus growth= [(Final callus fresh weight – Initial callus fresh weight)/Initial callus fresh weight] x100

Cell suspension cultures were initiated by suspending about 100mg callus pieces into 250ml flasks containing 100ml of MS medium and were placed on a rotary shaker at 100rpm for 30days (14). Growth of cell suspension cultures were determined during a range of culture periods 5, 10, 15, 20, 25 and 30 days for both genotypes, aliquots of 10ml suspension cultures were transferred to 15ml graduated test tubes, centrifuged at 1500rpm for 5min and then the Packed Cell Volume (PCV) was recorded (13).

Cell growth percentage was calculated by using the following formula

[(Final PCV fresh weight – Initial PCV fresh weight)/Initial PCV fresh weight] x100.

Screening for drought tolerance

For callus experiment, 100 mg callus were subcultured into tube containing semi solid MS medium supplemented with the concentrations 0, 0.5, 1.0, 1.5 or 2% of PEG 6000 for both genotypes.

For cell suspension, plating 2.5 ml of suspension cultures into Petri dishes containing semi solid MS medium supplemented with different concentrations of PEG6000 (0.0, 0.5, 1.0, 1.5 or 2.0%). Numbers of colonies were recorded for each treatment.

Plant regeneration

Shoot formation was induced on MS medium supplemented with 4.0 mg/l BA and 0.5mg/l of both NAA and IAA. Shoots were transferred to MS medium supplemented with 1mg/l IBA for root initiation (25). All cultures were maintained at 25 ± 2ºC for 16/8hrs (light/dark) photoperiod at a light intensity of 1000lux. Number of shoots was scored after six weeks of incubation.

Plantlets acclimatization was carried out according to Yousif (25) at which plantlets were left in test tubes containing a rooting medium until the medium was completely dried, then cotton plugs were removed and water was added to the test tubes continuously whenever it required. Plantlets were then transferred and planted in pots inside greenhouse.

Determination of proline and carbohydrate content

Proline concentrations were determined according to Bates *et al*. (4). Total sugar content (carbohydrate concentrations) was determined without the identification of specific sugar components based on the method of phenol sulfuric acid (11).

Experimental design and statistical analysis

All experiments were carried out with 10 replicates for each treatment and subcultured twice at three week intervals.

The experiments were designed as factorial experiments with a completely randomized design (CRD). Analyses were done using the SPSS var. 12 software. Differences between means were determined and compared according to least significant differences at $P \leq 0.05$ (21).

RESULT AND DISCUSSION

Callus and cell suspension cultures

The effect of SA and days of culture periods on growth percentage of rice varieties in callus and cell suspension cultures is presented in (Figure1). Based on the cell growth kinetics, proliferation was evaluated every 5 days in the two culture systems. During incubation, % growth increased due to cell division and enlargement in all genotypes and SA treatments. cell multiplication increased up to 20 days of culture in all treatments except Amber Baghdad with 0 and 1.5 mM SA in callus culture which cell multiplication increased up to 25 days of culture. The stationary phase was characterized by low-capacity cell division at 25 days in Amber33 with 0 and 1.5 mM SA in callus culture. Reducing cell proliferation occurred at 25 or 30 days of culture in all treatments. This continued for a limited period as the growth stopped due to the exhaustion of some factors or the accumulation of certain toxic metabolites in the culture medium (9, 23).

On the other hand the growth percentage was much higher in cell suspension culture compared with callus culture in all treatments. It is considered that the liquid culture system provides an efficient approach for micropropagation and continuous shaking during incubation serves aeration and oxygen and nutrient availability (22). That means cell in suspension culture had better contact and large surface area of the cells directly exposed to the supplied liquid medium whereas only the bottom surface of callus cultures were in contact with the solid medium (8). Based on the results obtained, 20 days old calli or cell

suspension were chosen for the subsequent experiments because the cells at this age are healthy and rapidly dividing.

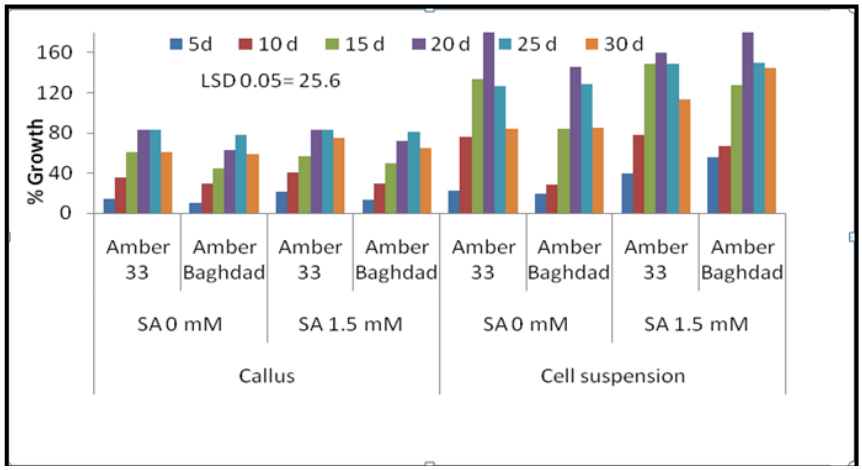

Figure 1: Effect of Genotypes, days and SA on % of growth of callus and cell suspension.

Screening for drought tolerance

Regarding to the effect of PEG on growth of callus and cells in suspension culture, figure (2) revealed the % growth reduction in 0.5, 1, 1.5 and 2% PEG compared with 0 PEG. Although the % growth reduction was lower in both varieties in the cell suspension compared to the callus culture in 0 mM SA treatment, but the pattern of growth was similar in both culture systems as a growth decreased with increases PEG levels. On the other hand, there was an increase in the growth rate at 0.5 and 1% PEG in both cultivars and in both culture systems with the presence of the mutant (1.5 mM SA) and the rate of increase in callus was higher than the suspension culture. 1.5 % PEG decreased the growth in Amber 33 and Amber Baghdad only in callus culture while 2% PEG decreased the growth in both cultivars in callus culture and in Amber33 in cell suspension culture.

More tolerance at the cellular level towards PEG indicates its inbuilt drought tolerance or possible induction of de novo synthesized drought (5). SA caused an increase in cell growth and division, these results was in agreement with those of Hamza (10) who reported that the genetic variations that caused as a result of treatment with SA enhance the activation effect for cell division and enlargement.

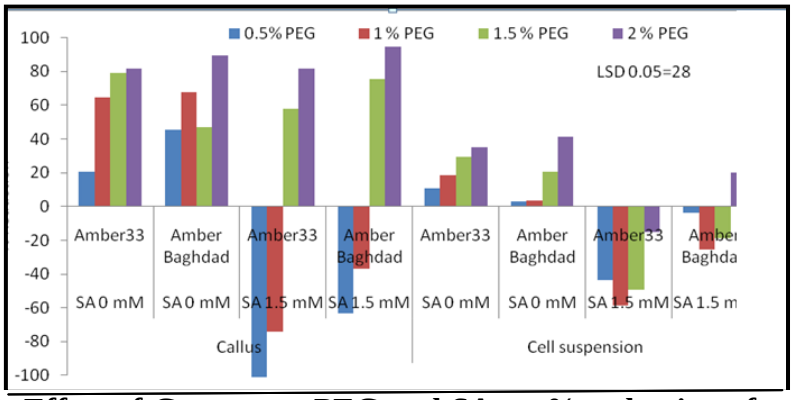

Figure 2: Effect of Genotypes, PEG and SA on % reduction of growth of callus and cell suspension.

In many cases reduction percentage of growth was lower in cell suspension compared with callus culture, this may be elucidate that the liquid medium allows close contact of the tissue with the medium stimulating and facilitating the uptake of nutrients and phytohormones, leading to better growth (17) and also ample oxygen supply to the tissue which ultimately leads to their faster growth (12).

Plant regeneration

There was a decrease in plant regeneration in all treatments by increasing PEG levels. 2% PEG in callus culture and 1.5 and 2% PEG in cell suspension culture completely inhibited plant regeneration in both varieties and in both SA treatments (Figure3). In general no. plant/callus was higher in suspension compared to callus culture in 0.5 and 1%PEG treatments. And it is worth to notice that SA treatment enhanced the plant regeneration in Amber33 and Amber Baghdad in both culture systems. Our finding was consistent with previous findings of Kamal *et al*. (15) who reported that the mutant genotype produced higher percentages of shoots regeneration than non-mutated genotype. The induction of high plant regeneration in 1.5 mM SA genotypes compared with 0.0 mM SA genotypes may due to the SA ability for chromosomal changes as a results of base pair substitution without chromosomal aberration and this result is in agreement with those of Kleinhofs *et al*. (16) who reported that SA creates point mutation in plant genome and the mutant plants are capable to survive under various adverse conditions. Regeneration is not always possible in all plant species, since imposing a selection pressure such as PEG seems to inhibit plant regeneration from undifferentiated cultured cells making it more difficult to identify and isolate variants possessing enhanced drought tolerance. This fact is one of the major limitations that plant breeders face in *in vitro* selection programs, particularly in selection for drought tolerance (10).

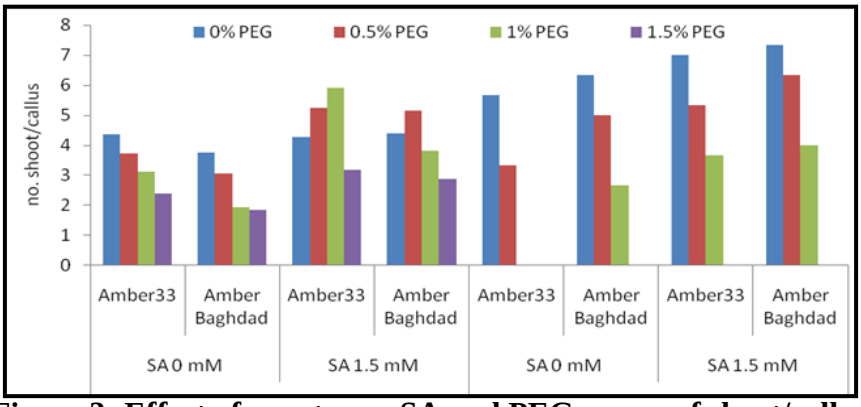

Figure 3: Effect of genotypes, SA and PEG on no. of shoot/callus.

Determination of proline and carbohydrate content

Results of the interaction between genotype, SA and % PEG on proline accumulation in plant induced from callus and suspension culture (Figure 4A and B) revealed a significant increase occurred in proline with increasing PEG levels. SA increased the proline accumulation in both cultivars and in both culture systems. A major role for solute accumulation is osmotic adjustment, which in terms of plant-water relations serves to sustain turgor and cellular hydration and delay wilting. Turgor and cellular hydration dependent functions are better conserved under drought if osmotic adjustment occurs. Some of the accumulated solutes (e.g. proline) are implied to have a role in protecting cellular

organelles or cellular functions (6). It was recognized that proline concentration accumulated in the shoots regenerated from suspension cultures were more than that in callus cultures. It may because those cells grown in suspension cultures are all in full contact with the stress agent (PEG) and no cells escape the stress. In contrast to callus cultures, since only cells grown on the surface of the medium are in full contact, to minimize cell escaper from stress agents, two subcultures at 21 days intervals were carried out in the current experiments and these results are in agreement with those of Ibrahim (13).

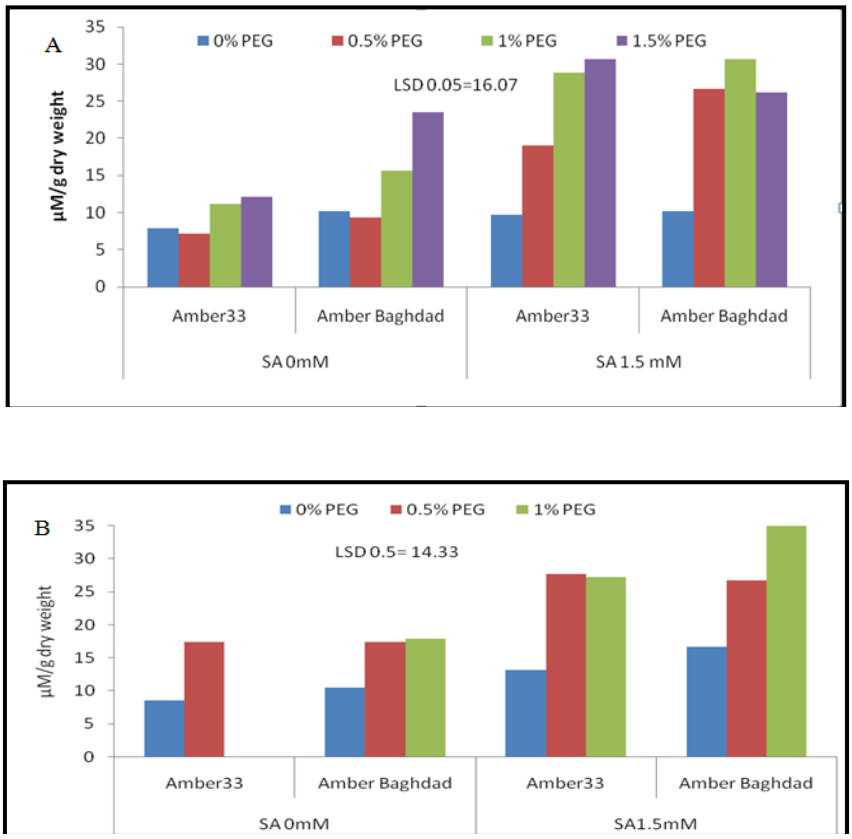

Figure 4: Effect of genotypes, SA and PEG on proline accumulation, A: calluse culture, B: cell suspension culture.

SA treated cultures recorded higher proline concentrations than those of untreated ones; this may be explained that SA develops such mechanism in the cells for drought tolerance expressed as high proline accumulation (10).

PEG reduced carbohydrate levels significantly in both genotypes and in both culture systems (Figure 5A and B) this finding is in agreement with that of Yousif (25) who reported that the reduction in carbohydrate concentrations may because that plant cells tolerate stress by using carbohydrates as an energy source. But these results disagree with those of Ibrahim (13) who reported that carbohydrate concentrations increased in *Coleous blumei* callus cultures after selecting cell lines tolerant to stress agent.

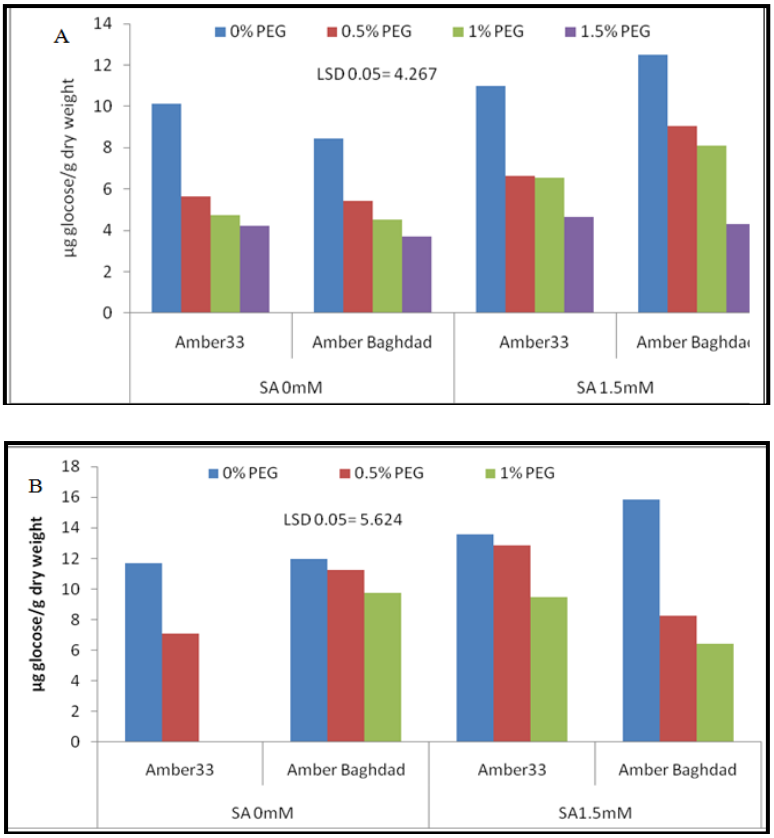

Figure 5: Effect of genotypes, SA and PEG on carbohydrate accumulation, A: callus culture, B: cell suspension culture.

Conclusions

Sodium azide can be utilized for genetic variation and it has a positive effect in callus growth, cell culture biomass, shoots regeneration and proline and carbohydrates accumulations. This protocol (callus and cell suspension cultures) imparts successful technique that provide an efficient approach for drought selection and can be utilized for developing drought tolerant plants. Although growth percentage was much higher in cell suspension culture compared with callus culture in all treatments but from other hands 1.5 and 2% PEG in cell suspension culture completely inhibited plant regeneration in both varieties, while only 2% PEG completely inhibited plant regeneration in callus culture. Therefore we suggest the use of callus culture in in the case of high stress test in the production of drought tolerant plants. The best of our knowledge this is the first report which clearly compared between callus and cell suspension cultures in rice.

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دراسة مقارنة إستجابة الكالس والمعلق الخلوي للانتخاب خارج الجسم الحي لتحمل الجفاف في الرز

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الملخص

تم استخدام زراعة الكالس والمعلق الخلوي لمقارنة أي نظام زراعي هو الأفضل في إنتاج النباتات المتحملة الجفاف. أستخدم الصوديوم ازايد (SA) مطفراً لاستحداث التغييرات الوراثية في صنفين محليين من الرز وهما عنبر 33 وعنبر بغداد. تم اختبار تحمل الجفاف على المستوى الخلوي باستخدام الاثيلين متعدد الكلايكول PEG (صفر، 0.5، 1، 1.5 و 2)% عاملاً لاستحداث الجفاف الكيميائي. أُختبر تأثير المطفر الكيميائي صوديوم ازايد والاثيلين متعدد الكلايكول في إختلاف النباتات من خلال حساب عدد النبيتات المستحدثة/كالس، كما تم اختبار تراكم البرولين والكربوهيدرات في تلك النباتات المستحدثة. أظهرت النتائج حدوث تضاعف للخلايا الى حد 20 يوماً من الزراعة في معظم المعاملات وفي كلا نظامي الزراعة، كما ان نسبة النمو كانت أعلى الخلايا المعلقة مقارنة بالكالس. أما تأثيرPEG في نمو الكالس والخلايا المعلقة، فقد أدى التركيز 1.5% من PEG الى إنخفاض نمو عنبر 33 وعنبر بغداد فقط في معاملة زراعة الكالس بينما التركيز 2% من PEG أدى الى انخفاض في نمو كلا الصنفين في زراعة الكالس وفي الصنف عنبر بغداد المعلق الخلوي. كما لوحظ حدوث زيادة في النمو في معاملات التطفير الكيميائي. كان هناك إنخفاض في اختلاف النباتات في المعاملات جميعها كما أدى وجود PEG بالمستوى 2% في زراعة الكالس و1.5 و2% في الخلايا المعلقة الى تثبيط كلي في إستحداث النباتات. زاد تراكم البرولين مع زيادة مستويات PEG ولوحظ ان معاملة التطفير بالصوديوم ازايد بالتركيز 1.5% أدت الى زيادة تراكم البرولين في كلا الصنفين وفي كلا نظامي الزراعة. في حين حدث إنخفاض في تراكم الكربوهيدرات في كلا الصنفين وفي كلا النظامين.

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