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Abstract

Hassawi rice (*Oryza sativa* L.) is a land race cultivar adapted to eastern Saudi Arabia. A system for in vitro callus induction and plant regeneration was established. The system consisted of two consecutive phases, callus induction and plant regeneration. Callus was established from mature caryopses cultured on MS medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.75, 1, 1.25, 1.5, 2, or 2.5 mg L^{-1} and 6-furfurylaminopurine (kinetin) at 0, 0.5, 1, or 2 mg L^{-1}. Out of the explants, 30% to 80% formed callus depending upon hormonal combination, the remainder either germinated forming whole plants or produced roots only. Highest callus induction percentage occurred on 2.5 mg L^{-1} 2,4-D alone; however, greatest callus weight was obtained on 1.5 mg L^{-1} 2,4-D combined with 2 mg L^{-1} kinetin. To encourage regeneration, callus was transferred to hormone-free MS medium. Plant regeneration was best achieved, over 90%, from callus induced on 1 mg L^{-1} 2,4-D with 0 or 0.5 mg L^{-1} kinetin, albeit these treatments were associated with relatively low callus frequency and small callus weight. Plantlets survived in soil and exhibited normal phenotype.

Sorbitol abstract

Another aspect of the study examined the responses of Hassawi rice callus to varying degrees of polyethylene glycol (PEG)-induced water stress including callus growth, water content, and proline accumulation. 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 cultures were placed on a gyratory shaker set at 150 rpm for 2 weeks. Results revealed that increasing water stress induced by increasing concentration of PEG caused a progressive reduction in callus fresh weight. Significant reduction in callus weight was observed in response to as low as 50 g l^{-1} PEG, but the inhibitory concentration was identified to be 200 g l^{-1}. Increasing PEG concentration was also associated with a progressive reduction in callus water content, which caused increase in proline accumulation reaching significant increase over the control at 100 g l^{-1} PEG. This study serves as a precursor for genetic improvement efforts to enhance tolerance of Hassawi rice to water stress.

1. Introduction

The importance of rice (*Oryza sativa* L.) as a food staple worldwide makes it a prime target for genetic manipulations through biotechnological approaches. An important prerequisite for the application of biotechnology in plant improvement is the availability of a tissue culture system for the crop of interest. Callus induction and plant regeneration of rice has been demonstrated for a number of genotypes. Albeit the success of these regeneration systems for certain cultivars, they are not suitable for all rice genotypes. The genotypic-specificity of the requirements for in vitro culturing different cultivars within the same species is a well-documented phenomenon and has been reported for rice. This phenomenon necessitates the empirical determination of the suitable conditions for the genotype of interest. The suitable requirements can be determined through experimentation with the various tissue culture factors including the medium components (such as basal salts, vitamins, hormones, and sugar), the physical conditions (such as temperature and pliotoperiod), and the plant materials (such as genotype and explant). Based upon the previous work and new experiments involving these factors and combinations of thereof, a tissue culture system can be developed for regenerating the desired plant genotype.

In addition to its use as a micropropagation method, a tissue culture system can be applied for genetic improvement of crops. Somaclonal variation is variation among regenerated plants that occurs as a result of tissue culture. It may arise from pre-existing variation and can be induced with chemical and physical mutation agents. Cell and tissue culture is a rich source of variability that represents a pool upon which a selection pressure can be imposed to isolate unique forms of a clone. Important agronomic traits such as

tolerance to drought, disease agents, and herbicides can be selected for by imposing stress conditions (Skirvin et al., 1994). Selection for these traits can be conducted on the cellular level; thus, providing a large number of cells for selection potentially capable to regenerate plants expressing the desired traits. However, tissue culture applications such as somaclonal variant selection are only possible after the development of a regeneration system for the candidate plant. The proposed study involved the development of regeneration system for "Hassawi" rice, a local cultivar adapted to Al-Hassa area (CATM, 1978), and the selection of somaclonal variants that exhibit tolerance to water stress.

The most common method for plant regeneration of rice consists of two stages, callus induction and subsequent plant regeneration through somatic embryogenesis. In vitro plant regeneration of rice has been the focus of numerous studies resulting in various procedures for rice regeneration that vary in the type and concentration of growth regulators, sugars, solidifying agents, basal salts, and vitamins (Heyser et al., 1983; Raghava and Nabors, 1984; Chen and Luthe, 1987; Kavi Kishor, 1987; Oard and Rutger, 1988; Mirlolii et al., 1989; Abe and Futsuhara, 1989; Ogawa et al., 1992; Tsukahara and Hirosawa, 1992a,b; Kothari et al., 1993; Hiei et al., 1994; Rueb et al., 1994; Guo et al., 1995; Al-Khayri et al., 1995; 1996; Chen et al., 1985). However, none of the previous research has been conducted on rice adapted to Saudi Arabia (e.g. 'Hassawi').

The literature indicates that conditions that are optimal for regenerating one rice cultivar often fail to produce plants in cultures of other cultivars (Abe and Futsubara, 1986; Bhaskaran and Smith, 1990), a phenomenon that is well documented for other species as well. intergenotypic differences in response to tissue culture factors have been observed in rice (Abe and Futsuhara, 1986; Bhattacharya and Sen, 1980; Oard and Rutger, 1988;

Wernicke et al., 198 1). Oard and Rutger (1988) observed that the stimulation of callus growth by the addition of kinetin was genotype-dependent and that different concentrations allowed maximum callus growth of different rice genotypes. Sucrose is generally used as the major carbohydrate source in tissue culture media. However, sorbitol has been reported to enhance plant regeneration of rice when applied to the regeneration medium (Ozawa and Komamine, 1989; Yoshida et al., 1994). Yoshida et al. (1994), in a study with rice cv. 'Kamenoo', observed that increasing the NAA level in the regeneration medium from 0.1 to 1 mg L^{-1} either increased regeneration rate or caused no difference, depending on the other growth regulators present in the regeneration medium. The addition of NAA alone to the regeneration medium was found to inhibit regeneration of the rice cv. 'Sasanishiki', but when kinetin was also included in the regeneration medium, a substantial stimulation of regeneration was obtained (Tsukahara and Hirosawa, 1992a). In previous studies, the concentration of 2,4-D used in the callus induction medium for rice varied among tissue culture systems, ranging from 0.5 mg L^{-1} 2,4-D (Chen and Luthe, 1987), 2 mg L^{-1} (Abe and Futsuhara, 1989- Kavi Kishor, 1987; Rueb et al., 1994), to 4 mg L^{-1} (Tsukahara and Hirowawa, 1992a,b). Oard and Rutger (1988) observed that the optimum concentration of 2,4-D for callus proliferation ranged from 0.2 to 1 mg L^{-1}, depending upon genotype', and that a higher level (2 mg L^{-1}) was inhibitory for all five rice genotypes included in their study.

Like callus proliferation, regenerative capacities vary considerably among rice genotypes (Abe and Futsuhara, 1986; Oard and Rutger, 1988). A sucrose/sorbitol combination was reported to positively affect regenerability of rice callus (Kavi Kishor, 1987; Ozawa and Komamine, 1989; Palit and Reddy, 1990- Tsukahara and Hirosawa,

1992a, 1992b; Yoshida et al., 1994). Most researchers, however, included the sorbitol treatment in the regeneration stage to simulate a water stress situation. Yoshida et al. (1994), in a study with rice cv. 'Kamenoo', observed that increasing the NAA level in the regeneration medium from 0.1 to 1 mg L^{-1} either increased regeneration rate or caused no difference, depending on the other growth regulators present in the regeneration medium. In another study, the addition of NAA alone in the regeneration medium was found to inhibit regeneration of the rice cv. 'Sasanishiki', but when kinetin was also included in the regeneration medium, a substantial stimulation of regeneration was obtained (Tsukahara and Hirosawa, 1992a). Yoshida et al. (1994) found that 0.1 and 0.5 mg L^{-1} kinetin were equally effective in the regeneration of rice cv. 'Kamenoo'. In another study, up to 2 mg L^{-1} stimulated the regeneration of cv. 'Sasanishiki' (Tsukahara and Hirosawa, 1992a). Al-Khayri et al. (1996) observed that inclusion of 2 mg L^{-1} kinetin in regeneration media containing sucrose alone significantly enhanced the regeneration frequency of 'LaGrue' in the presence of NAA and of Ru9l in the absence of NAA but in all genotypes tested. These results further demonstrate the genotype-specific requirements observed throughout these studies. Similarly, Bhattacharya and Sen (1980) indicated that regeneration of rice in response to kinetin was stimulated, inhibited, or unchanged depending upon the genotype and other growth regulators present in the medium.

The genotypic dependency of existing regeneration systems for rice limits their use for other cultivars. Our own observations (Al-Khayri et al., 1995, 1996) emphasized the importance of the genotype and its interactions with the medium components in the development of in vitro systems. The proposed investigation is necessary to satisfy the need for a regeneration system to accomplish genetic improvement of local rice genotypes using

biotechnological approaches. The diversity of the available rice regeneration systems, coupled with the specificity of the requirements dictated by genotypes, necessitate the determination of the culture conditions suitable for the genotype of interest. This study is intended to identify those requirement suitable for callus induction and plant regeneration for "Hassawi" rice, a land race rice adapted to Saudi Arabia growth conditions. Subsequently, the regeneration system can be used for rice improvement using somaclonal variation and possibly for future work in genetic engineering of rice.

Through callus and cell suspension cultures, large number of cells can be routinely manipulated. Mutations which give rise to somaclonal variation can occur spontaneously in cultured cells and can be induced by the application of chemical or physical mutagen (Sala et al. 1990). Since mutant cells can be selected and regenerated into plants, cell selection in tissue culture should give rise to commercially useful mutant crop plants. The selection of cells in vitro has been the focus of numerous research projects (Trolinder and Shang, 1991; Tan et al., 1995). An important characteristic that has been selected for using somaclonal variation is drought tolerance (Kocsy et al., 1991). Our intention is to take advantage of this phenomenon to select 'Hassawi' rice cell lines tolerant to drought and subsequently regenerate rice plants that should exhibit drought tolerance, a trait that is of paramount importance for and regions such as Saudi Arabia.

Cell selection schemes involve the use of either callus cultures or cell suspension induced form callus cultures. The range of variation is influenced by the clone, age of the culture, the use of mutagenic agents, and the selection pressure applied to cell suspension or callus cultures of a clone. The frequency of mutation can be enhanced by prolonging the culture period and further enhanced by exposure to chemical and physical mutagen

(Sala et al., 1990). Selection pressure is imposed after the mutagen is withdrawn. The cultured cells are subsequently transferred to a medium appropriate for plant regeneration. Selection may extend to after plant regeneration, particularly, for those traits that are expressed only in the intact plant (Loh, 1992).

Somaclonal variation appears to result from both pre-existing genetic variation within the explants and variation induced during the tissue culture phase. There appear to be two types of somaclonal variation, heritable and epigenetic. Heritable variation is stable through the sexual cycle or repeated asexual propagation. Epigenetic variation may be unstable even when asexually propagated. Somaclonal variation can involve either single or multiple genes and can be due to alterations in DNA bases, genes, chromosomes, or entire set of chromosomes (Orton, 1984). Commonly, somaclonal variation occurs at rates of 15% to 20% (Evans and Sharp, 1983), in contrast to naturally occurring mutation rates of 1 in 100,000 to 1,000,000 for a given locus. According to Skirvin et al. (1994), a more realistic frequency of 1% to 3% of regenerants are expected to mutate at a particular locus, but it does not mean that 1% to 3% or the regenerants vary from their parents in some physical or biochemical manners.

The value of somaclonal variation for plant improvement is attested by the release of improved named cultivars including ornamental (Griesbach, 1989; Skirvin and Janick, 1976a, b), fruits (Hall et al. 1986a,b) and vegetable crops (Heath-Pagliuso et al., 1989; Moyer and Collins, 1983). Many other somaclones are being tested (Skirvin et al., 1994). In rice the presence of phenotypic variants or mutants among regenerated plants has been documented since the first successful plant regeneration form rice tissue culture, e.g. morphological variants, albino, and polyploids. Useful Mutations that provide a good

source of genetic variations for rice breeding. Researchers have obtained lines apparently tolerant to NaCl, a characteristic that was transmitted to next generation (Wong et al., 1983; Liua and Baob, 1998). The variety exhibited later maturity, increased panicle number, good grain quality, and higher yield than the parent.

As a novel source of variability, somaclonal variation offers a great opportunity for rice improvement. In regions where water sources are limited, somaclonal variation offers a means for the production of drought-tolerant plants. Polyethylene glycol (PEG) has been used as a non- penetrating osmotic agent that lowers the water potential of media and has been used to simulate drought stress in plants and the selection of tolerant cell lines (Sala et al., 1990; Bressan et al., 1981, 1982; Handa et al., 1982; Tschaplinski et al., 1995).

This study was conducted to evaluate the response of rice callus cultures to water stress induced by sucrose and sorbitol combinations or polyethylene glycol (PEG). Responses studied included callus growth, water content, and proline accumulation. This is in an effort to isolate biotypes tolerant to water stress.

2. Materials and Methods

2.1. Plant material preparation

Mature rice seeds of cv. Hassawi were obtained from Hofuf Regional Agricultural Research Center, Ministry of Agriculture and Water, Kingdom of Saudi Arabia. The seeds were dehusked manually and surface sterilized for 1 min in 70 % ethanol followed by 30 min shaking in 2.6 % m/v sodium hypochlorite containing 3 drops Tween 20 per 100 ml solution.

The seeds were rinsed three times in sterile distilled water and cultured on callus induction medium.

2.2. *Culture medium and incubation conditions*

Callus induction and growth medium consisted of MS salts (Murashige and Skoog 1962) supplemented with 1 mg L^{-1} thiamine-HCl, 1 mg L^{-1} pyridoxine-HCl, 1 mg L^{-1} nicotinic acid, 2 mg L^{-1} glycine, 100 mg L^{-1} myo-inositol, 4.52 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.32 μM 6-furfurylaminopurine (kinetin). The medium was adjusted to pH 5.8 with 1*N* KOH and solidified with 8 g L^{-1} agar [Agar-agar/Gum agar] (Sigma Chem Co, St. Louis, MO). Culture medium was dispensed in 150 x 25-mm culture tubes (15 cm^3 medium per tube), and autoclaved at 121 °C and 1 x 10^5 Pa for 15 min.

Seeds were placed horizontally on the surface of the medium (one seed per culture tube) and incubated at 24 ± 2 °C under a 16-h photoperiod of cool-white fluorescent light (40 μmol m^{-2} s^{-1}). After 4 weeks, calli were separated from the seed explants and transferred to fresh identical media to encourage further callus proliferation. Callus cultures were maintained for an additional 4 weeks.

2.3. Effect of 2,4-D and kinetin on callus induction and plant regeneration

To examine the effect of phytohormones on callus formation, the medium was supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) at 0, 0.5, 0.75, 1, 1.25, 1.5, 2, or 2.5 mg L^{-1} and 6-furfurylaminopurine (kinetin) at 0, 0.5, 1, or 2 mg L^{-1}. The medium was adjusted to pH 5.8

with 1*N* KOH, dispensed in 150 x 25-mm culture tubes (15 ml medium per tube), and autoclaved at 121 °C and $1x10^5$ Pa (1.1 kg/cm^2) for 15 min. After 4 weeks, responses of the seeds to each hormonal treatment were determined, including number of seeds that germinated into whole plants, number of seeds that formed roots only, and number of seeds that produced callus. Calli were separated from the seed explants, and individually weighed. Calli were transferred to a fresh callus induction medium and cultured for an additional 4 weeks for further proliferation after which all the resultant calli were transferred to the regeneration medium. The regeneration medium was identical to that used for callus induction except that growth regulators were omitted.

2.4. Effect of sucrose and sorbitol

To examine the response to carbohydrate source and concentration, the medium was augmented with 10, 20, 30, and 40 g L^{-1} sucrose combined with 0, 10, 20, and 30 g L^{-1} sorbitol. In the end of 4 weeks, the number of seeds that induced callus per treatment was noted and calli were separated from the seed explants and transferred to fresh identical media to encourage further proliferation. Callus cultures were maintained for an additional 4 weeks after which individual calli were weighed to determine the effect of osmotic stress on callus growth expressed in fresh callus mass.

2.5. Establishment of cell suspension culture

Callus was inoculated in liquid culture medium (0.5 g per flask) and incubated at 23 ± 3 ^{o}C on a gyratory shaker set at 100 rpm. The medium consisted of MS salts (Murashige and Skoog 1962) supplemented with 1 mg L^{-1} thiamine-HCl, 1 mg L^{-1} pyridoxine-HCl, 1 mg L^{-1} nicotinic acid, 2 mg L^{-1} glycine, 100 mg L^{-1} myo-inositol, and 1 mg L^{-1} 2,4-D. These cultures were maintained to multiply callus for subsequent evaluation for tolerance to PEG-induced drought tolerance.

2.6. Effect of polyethylene glycol

To characterize callus growth in response to polyethylene glycol (PEG), 2.5 g embryogenic callus was grown in 125-ml flasks containing 50 ml each of liquid maintenance medium supplemented with PEG (MW 8000) at 0, 50, 100, 150, 200, 250, and 300 g l^{-1}. The cultures were placed on a gyratory shaker set at 150 rpm for 2 weeks. To determine the effect of PEG concentration on callus growth, callus fresh weight was determined and relative growth rate (RGR) based on fresh weight was calculated according to the following formula: RGR = [ln(final weight)-ln(initial weight)] / weeks. To study the effect of PEG treatments on water content, callus samples of known fresh weight were dried in an oven set at 65 ^{o}C for 48 h after which they were reweighed and the differences in weight were determined. The water content was expressed as a percentage of callus fresh weight. To examine the effect of PEG treatments on proline accumulation in response to PEG, 500-mg fresh callus samples were used for extraction

and estimation of free proline according to Bates *et al.* (1973). To test the regeneration capacity of the callus growing on PEG-containing medium, the callus was transferred to hormone-free medium.

2.7. Determination of free proline content

Extraction and estimation of proline was conducted according to the procedures described by Bates *et al.* (1973). Fresh callus, 500 mg per sample, was homogenized in 10 ml of 3 % (w/v) aqueous sulphosalicylic acid and the homogenate was filtered through Whatman No. 2 filter paper. In a test tube 2 ml of the filtrate was mixed with 2 ml acid ninhydrin and 2 ml glacial acetic acid and incubated in 100 ^{o}C water bath for 1 h. The reaction mixture was terminated by placing in ice bath, extracted with 4 ml toluene, and the chromophore phase was aspirated from the aqueous phase. The absorbance was read at 520 nm using LKB Novaspec Model 4049 spectrophotometer (LKB Biochrom, Cambridge, England).

2.8. Experimental design

The experiment dealing with the effect of 2,4-D and kinetin on callus proliferation and subsequent plant regeneration, the experiment was conducted as a 8 x 4 completely randomized with 36 replications. The main factor were 2,4-D concentrations and kinetin concentrations.

The experiment involving sugar types was setup as a 4 x 4-factorial designed in which the main factors were sucrose and sorbitol concentrations at four levels each. Thirty

seeds were cultured per sucrose and sorbitol combination, and 10 randomly selected calli per treatment were weighed.

The experiment involving polyethylene glycol was setup as a completely randomized single factor design with eight replications. The main factor was PEG concentration at seven levels.

Data were subjected to analysis of variance (ANOVA) and the means were separated, where appropriate, using the least significant difference (LSD) at 5% significance.

3. Results and Discussion

3.1. Effect of 2,4-D and kinetin on callus induction and plant regeneration

Caryopses swelled followed by germination producing small shoots and roots, five days after culture initiation. Some explants ceased the germination process and commenced callus formation. Others continued to grow forming complete plants while others ceased shoot growth but continued root growth. These three responses were dependent upon the hormonal concentration in the culture medium.

Seeds germinated on a medium containing 2,4-D at concentrations lower than 0.75 mg L^{-1} failed to form callus. In response to 2,4-D, a general trend was observed showing that seed germination to form whole plants was inhibited as the concentration of 2,4-D increased, particularly in the absence of kinetin (Fig. 1). The addition of kinetin significantly modified the germination response and either inhibited or enhanced germination depending on the

Fig. 1. Effect of 2,4-D and kinetin concentrations on the percentage of mature rice caryopses that germinated into whole plants.

treatment (Fig. 1). In this study, the inevitable seed germination was not a desirable response since the purpose was callus induction. Therefore, conditions conducive of seed germination are unfavorable and need to be minimized. The most efficient treatment in minimizing seed germination, 10%, consisted of 2 to 2.5 mg L^{-1} 2,4-D combined with 1 mg L^{-1} kinetin.

Another inevitable, undesirable response was root formation rather than callus formation. Root formation was influenced by the interaction between 2,4-D and kinetin concentrations. Similar to germination, rooting was also most inhibited by the two highest 2,4-D concentrations, 2 to 2.5 mg L^{-1}, but in the absence of kinetin. When kinetin was added, the rooting response varied depending upon the hormonal combination. Interestingly, adding 1 mg L^{-1} kinetin to 2 and 2.5 mg L^{-1} 2,4-D caused significant inhibition of seed germination (Fig. 1) whereas root formation was significantly stimulated (Fig. 2).

Within a week of culturing, seeds that completely ceased germination commenced callus induction, which is expressed in the percentage of seed explants that formed callus. Callus formation was observed at the embryo region. Callusing frequency ranged from 35% to 80% depending on the hormonal combination (Fig. 3). In the absence of kinetin, generally the percentage of seeds producing callus increased as the concentration of 2,4-D increased. No significant difference in callus percentage was observed with 2-4,D ranging from 0.75 mg L^{-1} to 1.5 mg L^{-1}. With 2 and 2.5 mg L^{-1} 2,4-D and higher, significant increase in the percentage of callus formation was observed (Fig. 3). The addition of kinetin to the culture medium mostly had no significant effect on the percentage of callus induction when combined with 2,4-D at 0.75 to 1.5 mg L^{-1}, but at higher 2,4-D levels, kinetin significantly inhibited percentage of callus induction. Varying the concentration of kinetin within the range tested, 0.5 to 2 mg L^{-1}, had little or no effect on percentage of callus induction (Fig. 3).

Fig. 2. Effect of 2,4-D and kinetin concentrations on the percentage of mature rice caryopses that developed roots only.

Fig. 3. Effect of 2,4-D and kinetin concentrations on the percentage of mature rice caryopses that formed callus.

The greatest percentage of callus formation was obtained on a medium supplemented with 2.5 mg L^{-1} 2,4-D with no kinetin added.

In addition to influencing the percentage of callus induction, the hormonal treatments also affected the amount of callus proliferation from Hassawi rice seed explants. Callus proliferation, expressed in fresh callus weight, varied according to the concentrations of kinetin and 2,4-D. Callus weight was directly related to the concentration of kinetin. Callus weight increased as kinetin concentration increased with each 2,4-D level, but the amount of increased was dependent upon the 2,4-D concentration. (Fig. 4). This is in contrast to the effect of kinetin on the percentage of callus induction which was either unaffected or reduced depending upon 2,4-D concentration (Fig. 3). The stimulation of Hassawi rice callus proliferation caused by kinetin was consistent with certain rice genotypes, but differs from other rice genotypes that respond oppositely (Al-Khayri et al., 1996; Oard and Rutger, 1988). These observations indicate that the influence of kinetin on rice callus proliferation is genotype-specific, thus either inhibits or stimulates callus growth depending upon the genotype of interest.

In response to increasing the concentration of 2,4-D from 0.75 mg $L^{-1}$to 1.5 mg L^{-1}, callus weight significantly increased, regardless of the kinetin concentration. However, as 2,4-D concentration was increased to 2 and 2.5 mg L^{-1}, callus weight declined (Fig. 4). In other rice tissue culture regeneration systems, the concentration of 2,4-D used for callus induction ranged from 0.5 mg L^{-1} 2,4-D (Chen and Luthe, 1987), 2 mg L^{-1} (Rueb et al., 1994), to 4 mg L^{-1} (Tsukahara and Hirowawa, 1992). Oard and Rutger (1988) observed that the optimum concentration of 2,4-D for callus proliferation ranged from 0.2 to 1 mg L^{-1}, depending upon genotype, and that a higher level (2 mg L^{-1}) was inhibitory. In another

Fig. 4. Callus weight produced from mature rice caryopses as influenced by and 2,4-D and kinetin concentrations.

study, Al-Khayri et al. (1996) testing the effect of 0.5 to 4 mg L^{-1} 2,4-D on callus proliferation, determined that increasing the concentration of 2,4-D above 0.5 was inhibitory but the degree of inhibition varied among genotypes. With Hassawi rice, maximum callus growth was obtained on a medium containing 1.5 mg L^{-1} 2,4-D, particularly when combined with 2 mg L^{-1} kinetin.

A common method to induce morphogenesis and subsequent plant regeneration in callus cultures of monocotyledonous species, including rice, involves either complete elimination of auxin from the culture medium, reducing its concentration to a lower level, or replacing it with a less potent auxin (Al-Khayri et al., 1992; Al-Khayri et al., 1996; Chen et al., 1985; Oard and Rutger, 1988). In the current study, morphogenesis was achieved by transferring the callus to a regeneration medium devoid of 2,4-D as well as kinetin, both hormones were present in the callus induction medium. Hassawi rice callus, initially, continued to proliferate on the hormone-free regeneration medium, but within 6 weeks morphogenesis was observed. Embryogenic callus developed complete plantlets with root and shoot systems, suggesting that Hassawi rice followed somatic embryogenesis pathway rather than organogenesis pathway, which is characterized by adventitious shoot formation. In vitro plant regeneration through somatic embryogenesis is considered the common mode of regeneration in rice and numerous other monocotyledonous species (Al-Khayri et al., 1992; Chen et al.,1985; Chen and Luthe, 1987; Rueb et al., 1994). Somatic embryogenesis is the presumed mode of regeneration in Hassawi rice since whole plantlets were obtained and supported by the in vitro behavior of other rice genotypes; however, histological study is necessary for confirmation.

Plant regeneration capacity of Hassawi rice was significantly influenced by an interaction between kinetin and 2,4-D concentrations for which the cultures were subjected to during the callus induction phase. As the concentration of 2,4-D in the callus induction medium increased from 0.75 to 1 mg L^{-1}, plant regeneration significantly increased; further increase of 2,4-D, however, reduced the regeneration capacity (Fig. 5). In a previous study testing the effect of 0.5 to 4 mg L^{-1} 2,4-D in the callus induction medium, Al-Khayri et al. (1996) observed that increasing the concentration of 2,4-D above 1 mg L^{-1} inhibited the regenerative capacity of callus induced from several U.S. rice genotypes.

The addition of kinetin to the callus induction medium either inhibited, stimulated, or unaffected plant regeneration in Hassawi rice depending upon the 2,4-D concentration. Plant regeneration capacity of callus induced on 0.75 and 1 mg L^{-1} was unchanged when 0.5 mg L^{-1} kinetin was added, but at higher kinetin concentrations the callus exhibited significant reduction in regeneration (Fig. 5). With 2,4-D concentrations higher than 1 mg L^{-1}, kinetin slightly reduced the regeneration capacity when used at 0.5 and 1 mg L^{-1} but enhanced regeneration at 2 mg L^{-1} (Fig. 5). Consistently, Al-Khayri et al. (1996) showed that rice regeneration capacity, in response to kinetin supplemented to the callus medium, was stimulated, inhibited, or unchanged depending upon genotype and 2,4-D concentration.

In the current study, the most effective treatments that resulted in callus with the highest regeneration percentage, 95%, consisted of 1 mg L^{-1} 2,4-D combined with 0 or 0.5 mg L^{-1} kinetin. Coincidentally, these two treatments were not the most conducive of callus induction and proliferation; rather they resulted in a low callusing percentage (Fig. 3) and small callus weight (Fig. 4). This interesting observation demonstrates that culture conditions most favorable for callus proliferation may not necessarily produce highly

Fig. 5. Percentage of callus regenerated plantlets as influenced by 2,4-D and kinetin concentrations supplied to the callus induction medium.

regenerative embryogenic callus and vise a versa. In a study testing the effect of different medium formulations on rice regeneration, Rueb et al. (1994) observed that amount of callus proliferation was influenced by the medium formulation. Relatively small callus produced by one formulation was associated with high regeneration frequency; whereas, larger callus produced by another formulation completely failed to regenerate. This inverse relationship between callus proliferation capability and subsequent plant regeneration capacity was also observed to be genotype-dependent. In a study with several rice genotypes, Al-Khayri et al. (1996) has shown that the genotype with largest callus proliferation exhibited the lowest regeneration frequency while another that produced the least amount of callus had the highest regeneration frequency.

The regenerants survived in the potting soil mix regardless of the in vitro establishment treatment. The survival of the regenerants in soil averaged around 80%. The plants exhibited normal phenotype, grew to maturity, and produced viable seeds under greenhouse conditions.

In summary, an efficient plant regeneration system for Hassawi rice has been developed, presumably, involving somatic embryogenesis. The present system consisted of callus induction phase in which 2,4-D and kinetin were added followed plant regeneration phase in which hormone-free medium was used. The most suitable treatment to produce highly regenerative callus consisted of 1 mg L^{-1} 2,4-D combined with 0.5 mg L^{-1} kinetin. With no kinetin, 1 mg L^{-1} 2,4-D also resulted in a highly regenerative callus but the weight of resultant callus was relatively small. Other treatments augmented with higher concentrations of 2,4-D and kinetin promoted more callus induction and proliferation but the resultant callus was inferior in term of regeneration capacity. Although this newly developed regeneration

system is considered highly efficient, further research on the effect of other medium components and culture conditions would certainly enhance our understanding of the in vitro response of Hassawi rice. The development of a regeneration system for Hassawi rice has met an essential prerequisite for the application of various biotechnological approaches for potential improvement of this important Saudi land race.

3.2. Effect of sucrose and sorbitol

Sucrose is a common culture media additive as the major source of carbon and energy in tissue of rice (*Oryza sativa* L.) (Al-Khayri and Al-Bahrany 2000, Al-Khayri et al. 1996, Duong et al. 2000, Rueb et al. 1994). Other sugars including mannitol, maltose, and sorbitol also have been used, often in combination with sucrose (Kishor and Reddy 1986, Laxmi and Reddy 1997, Okamoto et al. 1996, Swedlund and Locy 1993). The addition of sorbitol has been observed to enhance in vitro culture growth and morphogenesis in certain rice genotypes (Al-Khayri et al. 1996, Huang and Huang 1999, Yoshida et al. 994).

Sorbitol and sucrose act also osmotic agents that may introduce osmotic stress above certain concentrations. Studies on the mechanism of osmotic adjustment in plants are limited by the fact that whole plants contain mostly non-growing cells which makes characterizing biochemical processes in growing cells in response to osmotic changes difficult (Turner and Jones 1980). The use of cultured plant cells provides a means to overcome this difficulty since it allows careful measurements of growth in response to various osmotic changes in the environment (Bressan et al. 1982).

Seed germination proceeded callus formation that emerged from the scutellum

region of the seed. Germinating seeds produced only small shoots and failed to form roots in response to the presence of 2,4-D, which stimulated callus proliferation in about 35 % of the cultured seeds. Although callusing percentage was irrelevant to sugar treatment, maximum callus formation, 47 %, occurred on a combination consisting of 20 g L^{-1} sorbitol and 10 g L^{-1} sucrose, and the minimum, 19 %, was obtained on a medium containing 30 g L^{-1} sorbitol and 20 g L^{-1} sucrose.

In the absence of sorbitol, as the concentration of sucrose increased callus growth improved reaching maximum growth on 30 g L^{-1} sucrose beyond which callus growth was suppressed (Fig. 6). When 10 g L^{-1} sorbitol, the lowest concentration tested, was added callus growth was significantly promoted with each sucrose concentration. The degree of growth enhancement, however, was related to the specified sucrose level in which 30 g L^{-1} sucrose supported highest callus mass (Fig. 6). With 10 g L^{-1} sorbitol, percentage increases in callus mass, compared to the corresponding non-sorbitol treatments, were 37 %, 35 %, 29 %, and 17 %, in response to 10, 20, 30, and 40 g L^{-1} sucrose, respectively. However, additional increase in sorbitol concentration did not further improve callus growth. Sorbitol at 20 g L^{-1} either unchanged, in combination with 40 g L^{-1} sucrose, or suppressed, in combination with 10 to 30 g/L sucrose, callus proliferation. At 30 g L^{-1} sorbitol, further inhibition of callus growth was observed regardless of sucrose level, as compared to 10 g L^{-1} sorbitol. It appears that with high concentrations of sorbitol, above 10 g L^{-1}, the effect of sucrose concentration on callus proliferation gradually diminished.

Osmotic adjustment through the accumulation of cellular solutes, such as proline, has been suggested as one of the possible means for overcoming osmotic stress caused by the loss of water (Al-Bahrany 1994, Shankhadhar et al. 2000). In the present study, generally as

Fig. 6. Effect of sucrose and sorbitol concentrations on callus growth in Hassawi rice.

the concentration of sorbitol increased, proline accumulation in rice callus increased, regardless of the sucrose concentration (Fig. 7). However, the amount of increase varied depending on sucrose concentration. At 10 g L^{-1} sucrose, increasing sorbitol concentration to 10, 20, and 30 g L^{-1} resulted in 1.9, 3.3, and 4 folds increase in proline accumulation compared to no sorbitol. Whereas, with 20 g L^{-1} sucrose, increasing sorbitol concentration to 10, 20, and 30 g L^{-1} resulted in 2, 3.7, and 5.6 folds increase in proline accumulation. At 30 g L^{-1} sucrose, increasing sorbitol concentration to 10, 20, and 30 g L^{-1} resulted in 1.6, 3, and 4.2 folds increase in proline accumulation. Similarly, with 40 g/L sucrose, increasing sorbitol concentration to 10, 20, and 30 g L^{-1} resulted in 1.7, 3.1, and 3.4 folds increase in proline accumulation in comparison to the corresponding sucrose treatments with no sorbitol.

With any given sorbitol concentration, increasing sucrose level further enhanced the accumulation of proline in response for the resultant higher osmotic stress. This suggests that both sorbitol and sucrose contributed to the osmotic stress, and in turns the enhanced accumulation of proline, in an additive manner. The highest proline concentration level, 5.8 μmol g^{-1}(f.m.), was obtained from callus cultured on 30 g L^{-1} sorbitol combined with 40 g L^{-1} sucrose, the highest levels tested. The lowest proline concentration, 0.7 μmol g^{-1} (f.m.), was found in cultures, subjected to the least osmotic stress, grown on 10 g L^{-1} sucrose alone.

3.3. Effect of polyethylene glycol

The present study revealed that increasing water stress induced by PEG caused a progressive reduction in callus fresh weight of Hassawi rice (Fig. 8A). Significant callus

Fig. 7. Effect of sucrose and sorbitol concentrations on proline accumulation in Hassawi rice.

Fig. 8. Response of Hassawi rice to polyethylene glycol concentration in relation to (*a*) callus weight and (*b*) relative growth rate.

growth inhibition was observed in response to as low as 50 g l^{-1} PEG. As PEG concentration was increased to 100 g l^{-1} further significant reduction in callus fresh weight occurred. Increasing PEG to 150 g l^{-1} caused no significant change; however, at 200 g l^{-1} callus fresh weight was significantly further reduced. Beyond this concentration, no significant differences in callus fresh weight were noted. It is worth noting that even at these concentrations callus growth occurred at 20% increase over the initial weight of inoculums. Decreased cell growth must be the most sensitive response of the plant to water stress, since cell growth is quantitatively related to cell turgor which decreases with increased dehydration (Levitt, 1980). Dehydration induced by PEG reduces the availability of water and thus turgidity and growth (Heyser and Nabors, 1981). In a number of plant species, researchers have observed inhibitory effect of PEG on growth and proliferation of callus expressed in fresh weight (Santos-Diaz and Ochoa-Alejo, 1994b; Heyser and Nabors, 1981; Bornman and Huber, 1979). In rice also PEG-induced water stress was shown to inhibit callus growth (Reddy and Vajranabhaiah, 1996). Previous studies have shown that the response of in vitro cultures to water stress is related to genotype (Santos-Diaz and Ochoa-Alejo, 1994b; Cress and Johnson, 1987; Tschaplinski et al., 1995). In rice, genotype differences in response to water stress have been observed (Reddy et al., 1994). Therefore, it was pertinent to determine the response of this particular rice genotype, Hassawi rice, which is adapted to the adverse environmental conditions typical of xeric climate which predominate Saudi Arabia. In the current study, although callus grew on PEG-containing medium, the regeneration capacity was completely lost.

Based on the results from the current study, 200 g l^{-1} PEG is considered the critical inhibitory level which can be used in selection for drought tolerant cell lines of Hassawi rice. The critical inhibitory level may vary depending upon the species. For example, the inhibitory PEG level in *Capsicum annuum* L. and *Larrea tridentate* were 10% and 25%, respectively (Santos-Diaz and Ochoa-Alejo, 1994a), while the inhibitory level for *Populus trichocarpa* Torr. & Gray. and *Populus deltoids* Bartr. was 20% (Tschaplinski et al., 1995). Based on the resultant fresh weight, callus growth expressed in RGR followed the same pattern as callus fresh weight (Fig. 8*B*). Reduction in RGR was observed in response to increasing PEG concentration.

In relation to water content, it has been established that the water potential gradient between the cells and the nutrient medium caused by PEG results in dehydration of the cells (Hasegawa et al., 1984; Heyser and Nabors, 1981). Previous studies have shown that increases in osmotic stress by PEG was accompanied by steep decline in moisture content of tissues Heyser and Nabors, 1981; Bornman and Huber, 1979). In Hassawi rice also increasing PEG concentration was associated with a progressive reduction in callus water content (Fig. 9A).

Regarding proline accumulation, improving crop resistance to osmotic stresses is a major goal of agricultural biotechnology and can be facilitated by the use of biochemical markers, such as proline analysis. This is based on the fact that certain plants have evolved high capacity to synthesis and accumulate non-toxic solutes, predominantly in the cytoplasm as part of an overproduction mechanism to raise osmotic pressure and thereby maintain both turgor and the driving gradient for water uptake under osmotic stresses (Hare et al., 1999). Accumulation of proline in plant tissues exposed to osmotic stress has

Fig. 9. Response of Hassawi rice to polyethylene glycol concentration in relation to (*a*) water content and (*b*) free proline accumulation.

been well established in cell and callus cultures (Hasegawa et al, 1984; Santos-Diaz and Ochoa-Alejo, 1994b; Al-Khayri and Al-Bahrany, 2002; Handa et al., 1986). In the current study, proline content of Hassawi rice callus was shown to increase gradually in response to increasing PEG-simulated water stress (Fig. 9B). Low level of PEG was observed to cause slight increase in proline content; however, significant increase in proline content was seen on 100 g l^{-1}. The level of proline accumulation continued to rise even when increases in growth diminished.

In summary, this investigation has characterized the response of Hassawi rice cell cultures to water stress. Results have shown that increasing PEG concentration caused increased water stress as indicated by the decrease in water content which in turns stimulated proline synthesis and increased its accumulation. This is evident by the highly significant negative correlation (Pearson correlation coefficients = -0.929) between proline and water content of callus exposed to drought stress. The decrease in water content caused a decrease in cell turgor pressure and consequently reduced callus growth as expressed in fresh weight; hence, significant positive correlation between callus weight and water content (Pearson correlation coefficients = 0.779). This understanding of Hassawi rice callus and the information obtained related to physiology and growth is necessary to obtain somaclonal variants.

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Fig. 1. Effect of 2,4-D and kinetin concentrations on the percentage of mature rice caryopses that germinated into whole plants.

Fig. 2. Effect of 2,4-D and kinetin concentrations on the percentage of mature rice caryopses that developed roots only.

Fig. 3. Effect of 2,4-D and kinetin concentrations on the percentage of mature rice caryopses that formed callus.

Fig. 4. Callus weight produced from mature rice caryopses as influenced by and 2,4-D and kinetin concentrations.

Fig. 5. Percentage of callus regenerated plantlets as influenced by 2,4-D and kinetin concentrations supplied to the callus induction medium.

Fig. 6. Effect of sucrose and sorbitol concentrations on callus growth in Hassawi rice.

Fig. 7. Effect of sucrose and sorbitol concentrations on proline accumulation in Hassawi rice.

Fig. 8. Response of Hassawi rice to polyethylene glycol concentration in relation to (*a*) callus weight and (*b*) relative growth rate.

Fig. 9. Response of Hassawi rice to polyethylene glycol concentration in relation to (*a*) water content and (*b*) free proline accumulation.

5. Finance Summary

The project required the purchase of certain supplies as outlined in the proposal. The following table lists the materials purchased, the amount proposed and the amount actually spent. These materials have been purchased during the first phase of the project as dated. Partial salary for research assistance (12 months) was provided. The remaining amount is needed for research assistance and researchers rewards.

Description	**Reference**	**Date**	**Proposed Amount, SR**	**Actual Amount Provided, SR**
Equipments	264/13/A	6/3/1422	12,000	11,974
Materials	286/13/A	17/3/1422	29,500	29,496
Computer	235/13/A	26/2/1422	7,000	7,000
Miscellaneous		4/4/1422	3,700	3,000
Travel			15,000	0
Research assistants salary		25/9/2001 25/3/2002	7,200 7,200 9,600	7200 7200 0
Researcher rewards			52,800	0
Publications			6000	0
Total			**150,000**	**65,870**