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Full Length Article

Scutellum-derived Callus-based Efficient and Reproducible Regeneration System for Elite Varieties of Indica Rice in Pakistan

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Abstract

An expeditious regeneration system was established using scutellum-derived calli from mature seed of six Pakistani rice varieties namely, (Super Basmati, Basmati 385, Basmati 198, Pak Basmati, Basmati 2000 and Basmati 370) using modified MS medium containing various 2,4-D levels ranging from 1.0 to 5.0 mg/L. For regeneration, the embryogenic callus was sub-cultured on MS medium supplemented with 3.0 mg/L of kinetin and 1.0 mg/L of NAA. Increase in 2,4-D concentration enhanced the callus induction and proliferation but, had negative influence on the regeneration potential of calli. Experiments were performed to optimize the responsive age of the cells to regeneration, which was a prime character for efficient regeneration. Furthermore, effects of various gelling agents and carbon sources were also studied. Here, we report an efficient and reproducible regeneration system where responsive age of calli was minimized to 3-5 days with maximum number of shoots produced within a period of three weeks. The regenerants were transferred to soil for acclimatization. Though such research findings have already been published but these results are unique as far as responsive age of cells to regeneration and total time to produce shoots from cells are concerned.

Keywords: Rice; Callus; Regeneration; MS medium; 2,4-D; Kinetin; NAA

Introduction

Rice (*Oryza sativa* L.) feeds more than half of the world population. It provides about 70% of the total food calories consumed on daily basis in Asia. As world population is expected to grow up to 8.9 billion in 2030, we will have to produce 40% more rice to meet the growing demand. Thus, any increase in rice production would contribute towards hunger eradication, poverty alleviation, food security and economic development worldwide. Importance of rice at world level can be assessed by the fact that The United Nations declared the year 2004 "the year of rice" (Datta, 2004).

In Pakistan, rice is the third major crop after wheat and cotton. It is also a high value cash crop (Nawaz *et al.,* 2006) and one of the major export items. Pakistani rice varieties especially 'Basmati' are famous for their aroma and grain length all over the world (Rashid *et al.,* 2001). More than 20 rice varieties have been released for general cultivation (Bashir *et al.,* 2007). Both Japonica and Indica subspecies of rice are grown in Pakistan. Numerous efforts have been undertaken through traditional and mutation breeding, wide hybridization, somaclonal variation and plant transformation, for the development of high yielding varieties with better nutritional quality (Bashir *et al.,* 2007). Despite of all these efforts, yield per unit area of rice in Pakistan is far below the world average and even lower than many neighboring countries (Noor *et al.,* 2005). This prompts the need to improve commercial cultivars in Pakistan.

An efficient and reproducible plant regeneration protocol is a pre-requisite for the successful application of available plant transformation methods. Exploitation of genotypes amenable to callogenesis and subsequent regeneration, source of explant, its developmental stage, manipulation of media composition and culture conditions are thought to be vital steps in establishing a plant regeneration protocol. Rice is considered a model monocot species (Sasaki *et al.,* 2005) and being a member of family Poaceae, it exhibits recalcitrance to *in vitro* manipulations. Yet, the *in vitro* manipulations are inevitable to engineer crop plants. Therefore, we focused to exploit regeneration potential of indigenous cultivars of Indica rice.

Scutellum derived callus induction and subsequent regeneration has been successfully exploited for a number of grasses including wheat (He and Lazzeri, 2001), maize (Abebe *et al.,* 2008, Frame *et al.,* 2011), bentgrass (Lee *et al.,* 2011), sudan grass (Gupta *et al.,* 2004), Bermudagrass (Chaudhury and Qu, 2000), *Brachypodium distachyon* (Bablak *et al.,* 1995) sorghum (Sairam *et al.,* 2000), barley

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(Sharma *et al.,* 2005), oat (Gless *et al.,* 1998), rye (Eapen *et al.,* 1981), Pearl millet (Vasil and Vasil, 1981) and rice (Khan and Maliga, 1999; Saharan *et al.,* 2004; Bano *et al.,* 2005; Toki *et al.,* 2006).

In the present studies, an efficient and reproducible regeneration system for elite Indica rice cultivars of Pakistan was established so that the selection period of upcoming potential genotypes may be shortened and valuable biotechnological applications like plant transformation may be successfully exploited, to harvest its advantages in crop improvement.

Materials and Methods

Plant material

Seeds of six rice cultivars (*Oryza sativa* L. Indica), viz. Super Basmati, Basmati 385, Basmati 198, Pak Basmati, Basmati 2000 and Basmati 370 were obtained from the Rice Research Institute, Kala Shah Kaku, Lahore, Pakistan. Seeds were de-husked manually, dipped in 70% ethanol for 1 min and rinsed thrice with distilled water to remove all the traces of ethanol. Washed seeds were sterilized with 50% commercial bleach and few drops of Tween-20 for thirty minutes on a shaker at 28°C with 180 rpm shaking speed. The seeds were then rinsed with sterile distilled water.

Explant source: Mature seeds were used as an explant source for callus induction from its scutellum. After callogenesis the seed and the elongated plumule were detached using sterile forceps and the whole chunk of each subsequent scutellum-derived callus was used as one explant for *in vitro* regeneration of multiple shoots.

Composition of Media

Explants were cultured in petri plates containing MS salts, growth regulators with varying concentration and vitamins. Different types of solidifying agents [2.66 g/L phytagel (Sigma, USA), 2.66 g/L gelrite (Roth USA), 8.5 g/L phytagar (Phytotech lab, USA)] and sugars (sucrose, maltose and glucose) were also evaluated, in order to see their impact on regeneration, plant growth and multiplication.

Callogenesis: Scutellum-derived calli were induced by culturing surface sterilized mature seeds on a modified Murashige and Skoog's (1962) medium supplemented with 3% sucrose, *myo*-inositol 100 mg/L, glycine 2 mg/L, nicotinic acid 0.5 mg/L, pyridoxin 0.5 mg/L, thymine 0.1 mg/L and solidified with phytagel 3.66 g/L. In addition, this medium contained various concentrations of 2,4-D (1.0-5.0 mg/L), in order to optimize the most suitable concentration of 2,4-D for callogenesis resulting enhanced regeneration.

Regeneration: Scutellum derived calli were shifted to a modified Murashige and Skoog's (1962) medium supplemented with 3% sugar (sucrose, maltose or glucose) *myo*-inositol 100 mg/L, glycine 2 mg/L, nicotinic acid 0.5 mg/L, pyridoxin 0.5 mg/L, thymine 0.1 mg/L and solidified with gelling agent (phytagel, gelrite or agar). These media also contained kinetin (3.0 mg/L) and NAA (1.0 mg/L) as reported by (Joyia and Khan, 2012).

Culture conditions: The cultures were incubated at 26±1°C with 16/8 h light/dark conditions for regeneration and complete dark for callogenesis.

Acclimatization of Regenerated Plants

The regenerated plants were cultured onto MS basal medium (Murashige and Skoog, 1962) in sterilized magenta boxes. The developed plants were then shifted to clay pots having soil and peat moss (1:3) for further growth and development. Plants were washed to remove phytagel entrapped in rooting system to minimize fungal growth. They were well watered and covered with polyethylene bags for 7-8 days, to avoid evapo-transpirational losses of water.

Experimental Layout, Data Collection and Analysis

Data were recorded for callus induction (%) in order to evaluate the callus induction efficiency of different genotypes. Moreover, data were also taken to access the suitable age of calli, effects of various carbon sources and gelling agents for enhanced regeneration under light conditions. The percentage of callus induction and regeneration response of each genotype was calculated using formulae used elsewhere of Hoque and Mansfield, (2004).

All of the experiments were laid out in Completely Randomized Design (CRD) in a factorial arrangement with 3-5 replications per treatment. About 20 seeds were cultured per plate for callus induction and 9 calli per plate for regeneration experiments. Statistical procedures adopted include Analysis of Variance (ANOVA) and Duncan's Multiple Range (DMR) test (Steel and Torrie, 1986).

Results

Optimization of 2,4-D Levels and Varietal Response to Callogenesis using Scutellum as Explant

Six elite Indica genotypes grown in Pakistan were used for callus induction and proliferation on MS medium containing five different levels (1, 2, 3, 4 and 5 mg/L) of 2,4-D. Callus induction was monitored and an inspiring observation was recorded that callus induction started within 8-16 h of incubation on 2,4-D in almost all genotypes. A highly genotype-specific response was observed on various 2,4-D concentrations. A specific callus induction trend was observed for almost all varieties used (Fig. 1a) where increasing amount of calli with increasing concentration of 2,4-D (1.0–5.0 mg/L) was recorded. However, maximum callogenesis was observed at a level of 5.0 mg/L of 2,4-D in Basmati 385 and Basmati 370 (Fig. 1a).

Statistical analysis revealed a significant difference (P<0.01) among genotypes for callus induction; whereas, there was no significant (P>0.05) difference for callus induction in various levels of 2,4-D. Increasing concentration of 2,4-D although improved callus induction and proliferation but with non-significant at (P>0.05) difference. Similarly, the interaction of 2,4-D level to genotype (P>0.05) was also found to be non-significant (P>0.05). Further, two distinct types of calli were observed in all the genotypes used in the experiments. (i) Nodular, whitish in color and compact embryogenic (E) calli. (ii) Soft, mucilaginous, sticky, yellowish and watery in appearance non-embryogenic (NE) calli.

Varietal Response to Regeneration Medium

Scutellum-derived calli of six elite indica genotypes induced on five different levels (1-5 mg/L) of 2,4-D were evaluated for regeneration using regeneration medium (RM). The whole chunk of callus (being very small) induced from scutellum of each seed (Fig. 2) was shifted to regeneration medium; hereafter named as explant. Regeneration of various genotypes was found to be different as compared to callus induction response of the respective genotype. Varieties showing high callogenesis generally showed poor regeneration (compare Fig. 1a and b). Callus induction response of Basmati 385 and Basmati 370 was high compared to other varieties. Similarly Basmati 198 and Super Basmati also showed superior callus induction (Fig. 1b and Fig. 2). Contrary to the callogenesis, regeneration response of only Super Basmati was encouraging (3.272 shoots per explant). On the other hand Basmati 385 and Basmati 198 showed maximum non-embryogenic calli resulting in very low regeneration response as (1.036 shoots per explant) and (0.333 shoots per explant) respectively (Fig. 1a). Other three genotypes (Basmati 370, Basmati 2000 and Basmati Pak) showed less callogenesis but their regeneration response was better than Basmati 385 and Basmati 198. So, the regeneration response of various genotypes was found as: Super Basmati > Basmati Pak > Basmati 2000 > Basmati 370 > Basmati 198 > Basmati 385

The level of 2,4-D initially used for callus induction also affected the regeneration potential. Callogenesis generally increased with increasing level of 2,4-D, although regeneration response was different. The increase in 2,4-D level during callogenesis, showed a negative effect on regeneration consequently reducing the ratio of embryogenic calli and regeneration response. Super-Basmati exhibited highest regeneration (3.272 shoots/explant) for callus induced on 2,4-D (1 mg/L). Similarly, calli of Basmati 2000 and Basmati Pak induced on the same concentration of 2,4-D (1 mg/L) showed fairly good regeneration response as (1.740 and 1.295 shoots/explant respectively) (Fig. 1). On the other hand, Basmati 385, Basmati 198 and Basmati 370, in spite of having high callogenesis, exhibited very low regeneration

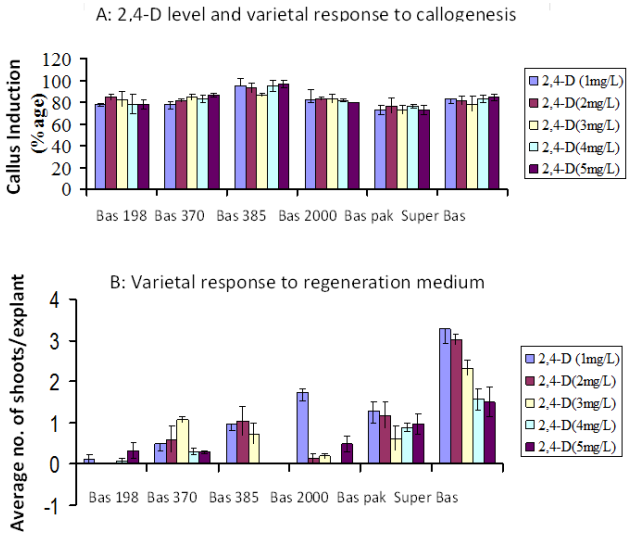

Fig. 1: Comparison of callogenesis and subsequent regeneration of six elite indica rice varieties grown in Pakistan. (A) Comparative callus induction on callus induction medium supplemented with various 2,4-D levels (1-5 mg/L) Best callogenesis response was observed in Basmati 385 at 5 mg/L whereas Basmati 198 and Super Basmati also showed good callogenesis. (B) Regeneration of calli of different genotypes induced on various levels of 2,4-D (1-5 mg/L). Best regeneration response (avg. no. of shoots per explant) was observed in Basmati Super

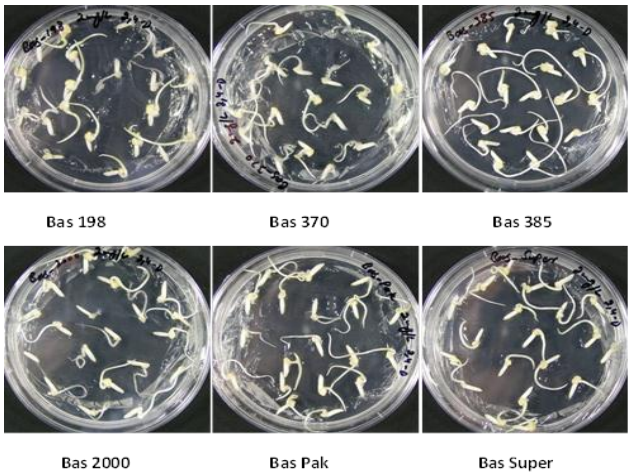

Fig. 2: Comparative plant regeneration response of Basmati Super calli of different age (3-15 days) on regeneration medium. Best regeneration response (avg. no. of shoots per explant) was observed in 5 days old calli. 3 days old calli also exhibited good regeneration response

for the calli induced on all concentrations of 2,4-D (Fig. 1a and b). Statistical data analysis also revealed a highly significant difference (P<0.01) among the regeneration response of various genotypes with respect to 2,4-D level for callogenesis.

Combining the results of callogenesis and

regeneration, it was concluded that calli of Super Basmati induced on callus induction medium containing 2,4-D (1 mg/L) would be most suitable target for transformation because this genotype produced large amount of embryogenic callus producing maximum number of shoots per explant. On the other hand, genotypes Basmati 385 and Basmati 198 though exhibited higher callogenesis but disappointingly low regeneration response was recorded. Basmati Pak and Basmati 2000 having reasonable regeneration potential could not produce large amount of calli as compared to Super Basmati. On the basis of these results, an optimum level of 2,4-D (1 mg/L) responsible for sufficient callogenesis along with good regeneration potential, was selected for further experiments. Basmati Super was used in proceeding experiments because of two reasons; firstly, its good regeneration response in these experiments; secondly, its popularity among the farmers in Pakistan for its good agronomic traits and among the consumers all over the world for its good grain qualities and aroma.

Age Response of Calli to Regeneration

Reliable plant regeneration from a sufficient amount of embryogenic callus enhances efficiency and use of tissue culture techniques in plant molecular biology. The age of callus is a significant factor that affects the totipotency and differentiation of callus. We used calli of different age such as 3, 5, 7, 9, 11, 13 and 15 days. In this experiment we observed that calli older than 7 days became non-embryogenic (soft, mucilaginous, sticky, yellowish and wet in appearance). Such calli kept on proliferating, when sub-cultured on regeneration medium (RM) but never regenerated. Statistical analysis revealed a significant ($P<0.01$) difference among age of calli for regeneration. The maximum regeneration was observed from 5 days old calli whereas minimum from 15 days old calli (Fig. 3). Hence, 5 days old calli were found to be the most suitable for regeneration and hence, was used in further experiments.

Impact of Gelling Agents on Regeneration

The regeneration media solidified with suitable gelling agent(s) permit the cultures to sustain their normal biochemical and physiological processes during growth. Calli of 3, 5 and 7 days were cultured on media solidified with 2.6, 2.6 and 8 g/L of phytagel and gelrite and agar respectively. Plant regeneration response was better in case of phytagel and gelrite than agar. Shoots appearing on phytagel or gelrite containing regeneration media were dry in appearance having better growth than the shoots appearing on agar containing media. Moreover, their use enhanced the ratio of embryogenic calli, which produced greater number of green shoots per explant. Hence, overall regeneration response of calli to the media solidified with phytagel and gelrite was much better as compared to the

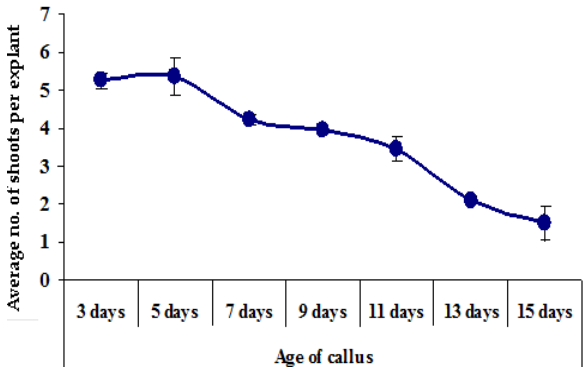

Fig. 3: Comparative plant regeneration response of Basmati Super calli of different age (3-7 days) on regeneration medium. (A) Solidified with different gelling agents. Best regeneration response (avg. no. of shoots per explant) was observed in 5 days old calli on media solidified with Phytagel and Gelrite, (B) supplemented with different carbon sources. Best regeneration response (avg. no. of shoots per explant) was observed in 5 days old calli on media supplemented with maltose

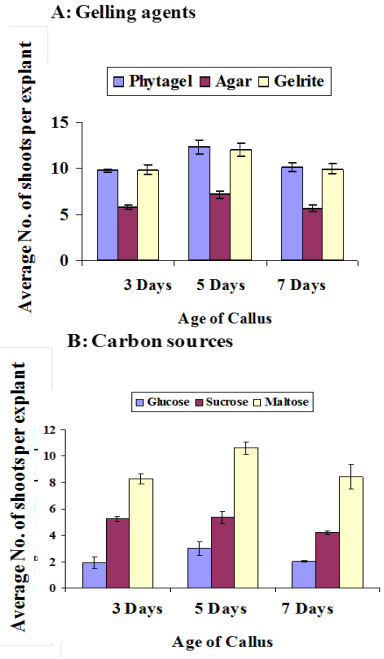

Fig. 4: Callus induction and proliferation from mature seed scutellum on MS medium supplemented with 2,4-D. Maximum callus induction was observed in Basmati 385 at 5 mg/L whereas Basmati 198 and Super Basmati also showed good callus induction.

regeneration of calli on agar solidified media. Among various age of calli, use of 5 days old calli was observed to be the best resulting in higher plant regeneration response (Fig. 4a).

Analysis of variance revealed that there was a significant ($P<0.01$) difference among the effect of gelling

agents on regeneration. Comparison of mean values showed maximum regeneration (12.35 shoots per explant) on media solidified with phytagel. Gelrite exhibited comparable results with regeneration (12.01 shoots per explant), while minimum regeneration (7.174 shoots per explant) was observed in agar solidified media. Hence, the use of phytagel or gelrite was preferred in further experiments.

Effect of Various Carbon Sources on *In Vitro* Regeneration

Three carbon sources namely glucose, sucrose and maltose were evaluated for their effect on *in vitro* regeneration of rice genotype 'Super Basmati'. Calli of 3, 5 and 7 days old calli were cultured on regeneration medium containing glucose, sucrose and maltose (30 g/L each). Plant regeneration response was highest in case of maltose as compared to sucrose and glucose. Comparison of mean values revealed that regeneration was highest (10.629 shoots per explant) when 5 days old calli were cultured on regeneration medium, having maltose as a carbon source. However, number of shoots regenerated on sucrose and glucose-containing media was relatively low (5.351 and 2.99 shoots per explant), respectively. Amongst these calli, 5 days old calli resulted in higher plant regeneration (Fig. 4b and Fig. 5). Statistical analysis also revealed a highly significant ($P<0.01$) difference among the genotypes towards regeneration at various carbon sources.

Discussion

The world's major food crops mostly being monocots are recalcitrant and in spite of enormous efforts, progress made is very limited. Successful transformation of any crop plant is directly related with the development of an effective regeneration system (Joyia and Khan, 2012). Corroborating this concern, the importance of the regeneration step has been stressed. Thus effective utilization of biotechnological approaches, protoplast fusion, somaclonal variations and genetic transformation rely on efficient and reliable regeneration systems. Here we report the development of an efficient regeneration system using mature seed scutellum derived calli of six elite rice genotypes grown in Pakistan. The recalcitrance of monocotyledonous crops has been resolved to great extent by growth hormones particularly auxins and cytokinins (Ho and Vasil, 1983). In the present study 1 mg/L of 2,4-D was used for efficient callogenesis and the said amount of 2,4-D is lower than earlier reports (Sikder *et al*., 2006; Shahsavari, 2011). Kinetin and NAA were found to be suitable for improved regeneration and the results are in line with (Bano *et al.*, 2005). Mature seed scutellum was used as explant for all genotypes under study, producing maximum callus witnin minimum time period of five days only. The reason behind this totipotency is the presence of parenchymatous tissues with thin cell walls and densely packed cytoplasm, full of nutritive compounds in scutella of mature seeds, because scutellar tissue is meant for the absorption of nutrients from endosperm (Campbell *et al.*, 2008).

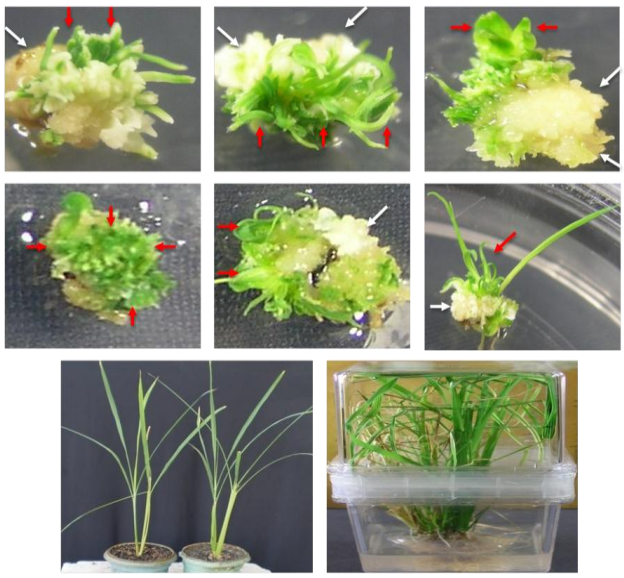

Fig. 5: High frequency multiple shoot regeneration from the embryogenic calli of Basmati Super on regeneration medium supplemented with maltose 30 g/L, Kinetin 3 mg/L, NAA 1 mg/L and solidified with phytagel 2.66 g/L. White arrows show (NE) non-embryogenic part and red arrows show embryogenic part of calli producing multiple shoots on each explant

Endress (1994) suggested that 2,4-D generates DNA hypermethylation, which is responsible for maintaining the cells in a highly active mitotic stage and, therefore, in a pro-embryonic phase. Rueb *et al.* (1994) reported that the use of high 2,4-D concentrations are necessary to induce somatic embryogenesis in rice, but at the same time they exert an inhibitory effect on *in vitro* plant regeneration because residues of 2,4-D remain within the cells and pose negative effects on re-differentiation during regeneration. Therefore, we attempted to evaluate the minimum dose of 2,4-D responsive to induce callogenesis. 2,4-D (1 mg/L) was found to be the best for maximum embryogenic callus production which is lower than earlier reports (Sikder *et al*., 2006; Shahsavari, 2011).

Literature review shows that previously calli of 15 to 20 days (Lee *et al.*, 2006), five to eight weeks (Ge *et al.*, 2006), even six months or more had been used for regeneration. It was time consuming, labor intensive and required a lot of resources. Here we report a trouble-free, very rapid, highly efficient and reproducible regeneration system. We have minimized the responsive age of calli to 3-5 days only. This system has pledged a quick response of such a young calli to regeneration medium producing multiple shoots from each explant. So, we are able to expedite the tissue culture procedure and shorten the time required to regenerate.

Tissue culture media solidified with suitable gelling agent(s) offer appropriate milieu for the response of explants in order to proliferate and regenerate. Gelling agents provide optimum strength in order to support tissue responses hence, well prepared and solidified artificial media provide suitable conditions for the growth of plant cells and tissues. The mechanism governing the effects of gelling agents is complex. However, detailed studies indicate that the differential properties of various gelling agents depend upon the degree of clarity, polymerizability and water retention capacity, which in turn, influence mineral and carbohydrate availability (Spomer and Smith, 1996; Beruto *et al.*, 1999a,b). It is accepted that choice of gelling agents plays a predominant role in determining culture responses *in vitro*. In some systems a simple alteration of gelling agent may improve the culture of recalcitrant genotypes. Literature review illustrates that agarose-solidified media were found to be effective for rice protoplast culture (Thompson *et al.*, 1986); gelrite enhanced somatic embryo initiation (Santarem *et al.*, 1997) and reduced vitrification (Zimmerman and Cobb, 1989) whereas phytagel had been found to promote regeneration (Chevereau *et al.*, 1997) in various plants. In the present studies we found that phytagel and gelrite produce comparable good regeneration in mature seed scutellum derived calli. These results have been found in agreement with the above mentioned studies.

In plant tissues, the first step for sugar's utilization is the cleavage of the glycosidic bond by either synthases or invertases, resulting in the production of hexoses, which are an essential carbon and energy sources for the accumulation of different storage products and tissue growth (Wobus and Weber, 1999) under both *in vitro* and *in planta* conditions. It is a well known fact that the choice of carbon source, its concentration and their interaction, pose a significant influence on *in vitro* growth regulation (Samir, 2005). Thus, in an attempt to evaluate the appropriate sugar for enhanced regeneration, the effect of sucrose, maltose and glucose was evaluated. Sucrose was the best sugar as far as callogenesis is concerned whereas, maltose was appropriate for regeneration step.

As a result of these studies, a protocol has been developed for embryogenic callus formation and its subsequent regeneration into plants that were shifted to the magenta boxes for further growth and ultimately established in soil.

In précis, 2,4-D (1-5 mg/L) was suitable for callus induction but minimum concentration responded best for regeneration. Moreover, the research findings are unique as far as responsive age of callus for regeneration has been minimized to 5 days only. Maltose and phytagel have been found effective to produce multiple shoots per explant.

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