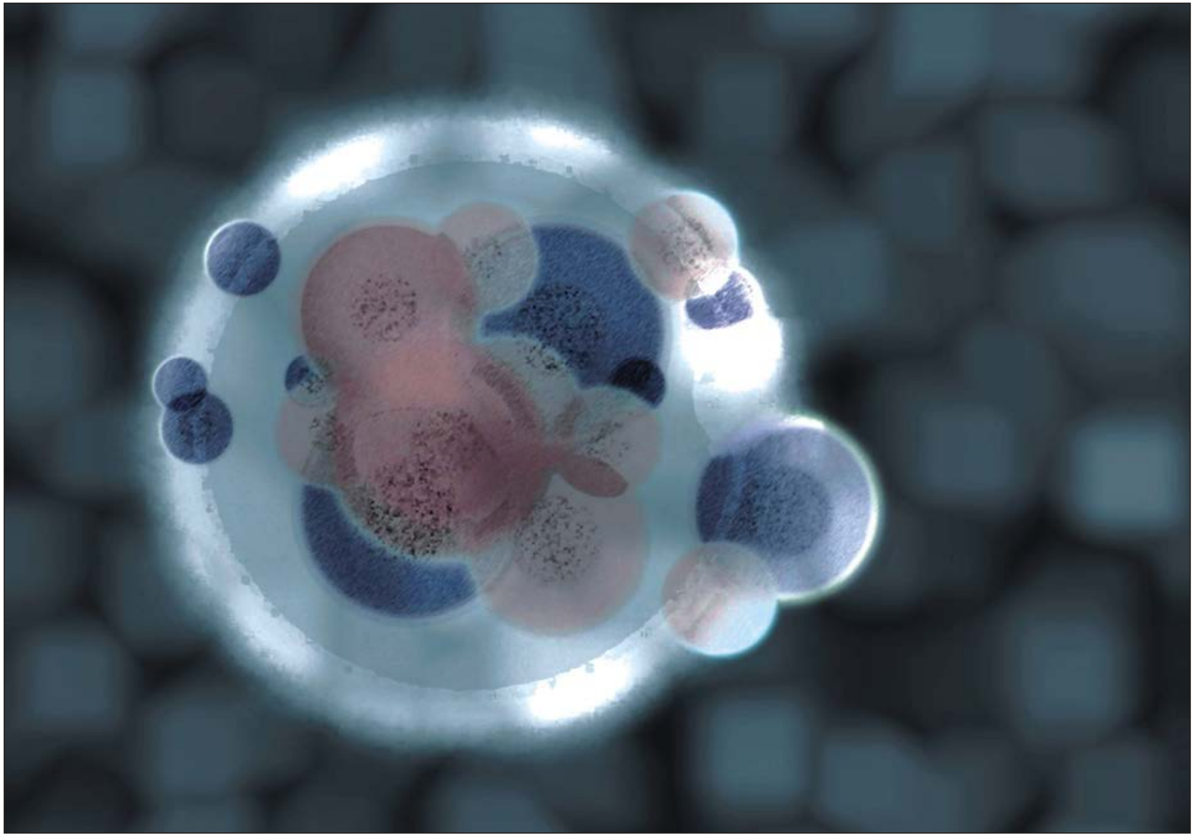

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Factors Enhancing Induction of High Frequency Plant Regeneration from Somatic Embryos of Indica Rice (*Oryza sativa* L.)

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Abstract: This study was aimed to asses the promotive effect of maltose, sucrose, proline, charcoal etc. on frequency of regeneration and embryogenesis. Factors affecting somatic embryogenesis and subsequent plant regeneration *in vitro* in basmati rice cultures were highly genotype specific. The number, mass and morphology of the callus formed on the scutellum were dependent on the medium used. They were affected by the auxins, sugars, amino acids, growth regulators etc. Supplementation of the control medium independently with maltose (30 and 60 g L^{-1}), proline (560 m g L^{-1}), Elevated sucrose (60 g L^{-1}), activated charcoal (2 g L^{-1}), cefotaxime (100 mg L^{-1}) enhanced somatic embryogenesis considerably. The nodular embryogenic calli were further confirmed through histological analysis and their plant regeneration ability. The plant regeneration was also influenced both by the genotype as well as the composition of the medium used for induction of plant regeneration. Higher plant regeneration was achieved on medium containing maltose (30 and 60 g L^{-1}), proline (560 mg L^{-1}), elevated sucrose (60 g L^{-1}) and activated charcoal (2 g L^{-1}). Plants obtained were phenotypically normal and identical to control seed derived plants and exhibited normal fertility. A limited humidity and an optimal aeration of the culture tubes enhanced the frequency of embryogenesis and plant regeneration.

Key words: Embryogenesis, regenerants, basmati, proline, maltose

INTRODUCTION

Rice is a grain belonging to the grass family. It has been cultivated for more than 7000 years as a major crop and currently supports nearly one half of the world population[1]. Basmati rice belongs to Indica group of rice which constitute a small but special group of rices which is considered best in quality and it possesses pleasant and equistic aroma, sweet taste, soft texture, delicate curvature and extra elongation with least breathwise swelling on cooking. Basmati rice which possesses low tillering ability is susceptible and exhibit higher yield losses in the same ecosystem under similar cultural practices. A tissue culture system with efficient degree of somatic embryo production would be useful in genetic manipulation studies aimed at rice improvement. Several potentially useful genes can now be introduced into cultivated rice and prospects for further rice biotechnology are promising[2]. In most, if not all cases, the limiting factors for obtaining transformed plants lies in the regeneration of plant material rather than transformation procedure itself[3]. Compared to *Japonica* rice, Indica rice has been less responsive to *in vitro* culture[4,5] and high frequency somatic embryogenesis with distinct somatic embryo formation, has been limited[6]. Somatic embryogenesis directly from explants could circumvent these problems. It is also being viewed as an important tool for mass cloning of plants through production of synthetic seeds. Somatic embryo production from carrot callus cells, more than 35 years ago has been recognized both as an important pathway for the regeneration of plant from cell culture systems and as a potential model for studying the early regulatory and morphogenetic events in plant embryogenesis. Somatic embryogenesis is the process by which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without the fusion of gametes. In rice, considerable progress has been made in the last decade and plantlets have been regenerated, via organogenesis as well as somatic embryogenesis[7,8]. However, very limited research has been done on basmati rice particularly on Indian basmati varieties. A number of factors (genotype, medium constitution, auxins, sugars, amino acids, growth regulators etc.) influence the process of somatic embryogenesis and subsequent plant regeneration. To obtain a reproducible and efficient

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procedure for regeneration of basmati rice plants through somatic embryogenesis, different factors that result in high frequency regeneration from mature embryo derived calli of the three basmati rice varieties were compared. It is concluded that regeneration was faster and most of the plants obtained were highly fertile. Here we reported our assessment of the promotive effect of proline, cefotaxime, maltose and activated charcoal on the frequency of embryogenesis and regeneration.

MATERIALS AND METHODS

Indica rice cultivars Pusa Basmati 1, Basmati 370 and 386 were screened for somatic embryogenesis at tissue laboratory of Department of Plant Breeding, Punjab Agricultural University, Ludhiana (June to November, 1999). Callus cultures were initiated from mature seed embryos. Dehusked healthy seeds after surface sterilization with $HgCl_2$ (0.1%) containing sodium lauryl sulphate (0.1%) for 10 min were rinsed thrice with sterile water and aseptically placed on Medium Supplemented (MS) with 2, 4-D (2.5 mg L^{-1}) + kin (0.5 mg L^{-1})[9]. Calli thus obtained were maintained and multiplied through periodic subculturing on to different medium compositions (MS-A to MS-F):

Control: MS+2, 4-D (2/4 mg L^{-1}) + kin (0.5 mg L^{-1}) + sucrose (30 g L^{-1}) + agar (8 g L^{-1})
MS-A : MS+2, 4-D (2/4 mg L^{-1}) + kin (0.5 mg L^{-1}) + sucrose (60 g L^{-1}) + agar (8 g L^{-1})
MS-B : MS+2, 4-D (2/4 mg L^{-1}) + kin (0.5 mg L^{-1}) + sucrose (30 g L^{-1}) + proline (560 mg L^{-1}) + agar (8 g L^{-1})
MS-C : MS + 2, 4-D (2/4 mg L^{-1}) + kin (0.5 mg L^{-1}) + sucrose (2 g L^{-1}) + agar (8 g L^{-1})
MS-D : MS + 2, 4-D (2/4 mg L^{-1}) + kin (0.5 mg L^{-1}) + maltose (30 g L^{-1}) + agar (8 g L^{-1})
MS-E : MS + 2, 4-D (2/4 mg L^{-1}) + kin (0.5 mg L^{-1}) + maltose (60 g L^{-1}) + agar (8 g L^{-1})
MS-F : MS + 2, 4-D (2/4 mg L^{-1}) + kin (0.5 mg L^{-1}) + sucrose (100 mg L^{-1}) + agar (8 g L^{-1})

The cultures were incubated at 25±2°C at 70-80% humidity. Primary and secondary calli cultures were kept in total dark.

After 2-3 weeks, calli showing nodular regions were examined and selected under stereo-microscope and were fixed for histology in formalin acetic-alcohol (FAA) for 24 h and passed through dehydration series of ethyl alcohol for 1-2 h. each, followed by xylene clearing series[10] and then embedded in paraffin wax with cerasan (melting point 35°C). Sequential paraffin sections (5-10 μM) were cut using rotary microtome stained with saframin-fast green and mounted with permount. The specimens were observed and photographed with the help of a scanning electron microscope.

The embryogenic calli were transferred to different regeneration medium compositions:

Control : MS + BAP (2.0 mg L^{-1}) + NAA (0.5 mg L^{-1}) + sucrose (30 g L^{-1}) + agar (8 g L^{-1})
MS-1 : MS + BAP (2.0 mg L^{-1}) + NAA (0.5 mg L^{-1}) + maltose (30 g L^{-1}) + agar (8 g L^{-1})
MS-2 : MS + BAP (2.0 mg L^{-1}) + NAA (0.5 mg L^{-1}) + maltose (60 g L^{-1}) + agar (8 g L^{-1})
MS-3 : MS + BAP (2.0 mg L^{-1}) + NAA (0.5 mg L^{-1}) + sucrose (60 g L^{-1}) + agar (8 g L^{-1})
MS-4 : MS + BAP (2.0 mg L^{-1}) + NAA (0.5 mg L^{-1}) + sucrose (560 mg L^{-1}) + agar (8 g L^{-1})
MS-5 : MS + BAP (2.0 mg L^{-1}) + NAA (0.5 mg L^{-1}) + sucrose (2 g L^{-1}) + agar (8 g L^{-1})

(MS-1 to MS-5) to check the effect of different factors on % shoot regeneration and an illumination of 5000 lux was given with 16/8 h (light/dark) regimes.

For induction of roots regenerated shoots were transferred to rooting medium (½ MS). Plantlets with well developed root system were removed from the test tubes and thoroughly washed under running tap water. Plants were then hardened by keeping them on soaked cotton in open test tubes and small plastic modules in MS salts (¼ MS) without sucrose and vitamins and also in tap water, under strong light intensity (5000 lux) for about 15 days. Water was changed daily or ever twice a day to avoid contamination. Hardened plantlets were transferred to normal field soil in polyethylene bags in the glass house.

RESULTS AND DISCUSSION

Callus induction: Swelling of the mesocotyl and radicle region of the seed was observed 4-5 days after culturing on induction medium [MS+2,4-D (2/4 mg L^{-1}) + kin (0.5 mg L^{-1})] (Fig. 1A). This was followed rapidly by the development of the primary callus (Fig. 1B).

Pusa Basmati 1 exhibited early response and rapid callusing within 7 days, whereas, Basmati 370 and Basmati 386 took almost 10 days for callus initiation. Moreover, Pusa Basmati 1 and Basmati 370 exhibited callusing on MS medium supplemented with 2, 4-D (2 mg L^{-1}) and kin (0.5 mg L^{-1}). Whereas, in Basmati 386, it was essential to increase the concentration of 2, 4-D from 2 to 4 mg L^{-1} for obtaining good growing calli. Best callus induction (91.9%) and its subsequent proliferation was observed in

Pusa Basmati 1 followed by 86.0 and 79.56% in Basmati 370 and 386, respectively. Partial browning of the subcultured calli was a common phenomenon in Basmati 370 and Basmati 386 which hampers the uptake of nutrients by the explant, thus results in the decrease in callusing. Browning is due to the phenolic compounds, that are largely located in the vacuoles, get mixed with the contents of plastids and other organelles and consequently the dark pigmentation appears. These are highly reactive compounds that polymerize rapidly and form bonds with proteins and also inhibit enzyme activity and thus may result in lethal browning of explant and medium as well[11]. Addition of activated charcoal (2 g L^{-1}) in the culture medium, checked browning and improved callus growth and subsequently, somatic embryogenesis in Basmati 370 and Basmati 386 (Fig. 1C).

Somatic embryogenesis: After 4 weeks of incubation, the primary calli obtained from the seeds were excised and transferred to the different medium compositions (MS-A to MS-F) to see effect of various factors on somatic embryogenesis and data was recorded 4-5 weeks of incubation. The frequency of callus induction and the phenotype of the callus formed was medium dependent. The secondary calli of the three varieties exhibited varied degree of somatic embryogenesis as was evident from the nodular appearance on the growing calli. After 30 days in culture, the calli displaced three morphological phenotypes. Calli with a dry, compact and nodular appearances formed numerous globular structures and were called embryogenic (E) (Fig. 1D). Translucent and somewhat slimy callus never formed embryos and was called Non Embryogenic (NE) (Fig. 1D), as described earlier Nabors *et al.*[12]. The third type has a pronounced rhizogenic capacity and some of these calli were entirely composed of root primordia. The embryogenic calli were characterized on the basis of texture, colour, histology and regeneration parameters.

Six different medium were tried to study the % somatic embryogenesis in the three basmati rice varieties (Table 1). Statistical analysis showed that variety mean squares and medium mean squares were significant at 5% level with Critical Difference (CD) being 1.45 and 2.21, respectively. Thus, it could be concluded that the three varieties showed a significant difference in their response for somatic embryogenesis (%). The different medium used also differed significantly from each other with highest response in MS-B. Very high frequencies of embryogenic calli were observed, irrespective of the genotype when amino acid, proline (560 mg L^{-1}) was used in the culture medium. No significant difference in the frequency of callusing itself was observed but a greatly enhanced frequency of embryogenic callus formation was obtained[13]. This is clearly reflected in the percentage of cultures showing embryogenesis (Table 1). Proline provides buffering reaction and thus resists any change in the pH of the medium during culture. Addition of activated charcoal (MS-C) has also been found to improve somatic embryogenesis by decreasing the formation of phenolic compounds during culturing specifically in Basmati 370 and 386 (Table 1).

The change in MS medium in carbon source from sucrose (30 g L^{-1}) to maltose (30 and 60 g L^{-1}) resulted in higher percentage of embryogenic calli in all the three varieties. The embryogenic callus on the medium with maltose was creamish yellow, highly embryogenic, compact and increased in mass. Maltose stimulates cell differentiation and metabolism and due to its slow metabolism, it is long duration energy source. Compared to other carbohydrates[14]. Increase in level of maltose from 30 to 60 g L^{-1} (MS-E) lead to enhance the frequency of somatic embryogenesis in all the three varieties (Table 1). When 30 g L^{-1} (MS-D), maltose was used, somatic embryogenesis was reduced but it was better than 60 g L^{-1} (MS-A) sucrose. As the elevated level of sucrose (60 g L^{-1}) caused necrosis of most of the calli and the extent of somatic embryogenesis was reduced. It was interesting to note that the effect of an antibiotic cefotaxime (100 mg L^{-1}) (MS-F) on induction and maintenance of somatic embryogenesis, that played an appreciable role in improving somatic embryogenesis in all the three varieties (Table 1).

Histological analysis: Microtome sections of these calli were observed under scanning electron microscope and somatic embryos at various developmental stages were seen (Fig. 1E). There was a positive relationship with the nodular texture of the calli and the number of somatic embryos seen in the per unit area under the microscope. Nodular calli of Pusa Basmati 1 exhibited greater number of embryos per callus section as compared to Basmati 370

Table 1: Somatic embryogenesis (%) on different MS medium compositions

	Somatic embryogenesis (%)		
Treatments	Pusa Bas 1	Bas 370	Bas 386
Control	63.33	43.33	26.66
MS-A	70.00	56.66	53.33
MS-B	96.66	73.33	60.00
MS-C	73.33	36.66	46.66
MS-D	76.66	63.33	43.33
MS-E	86.66	70.00	56.66
MS-F	93.33	70.00	50.00

CD for variety means at 5% level of significance = 1.45
CD for medium means at 5% level of significance = 2.21
CD for variety x medium interaction at 5% level of significance = 3.83

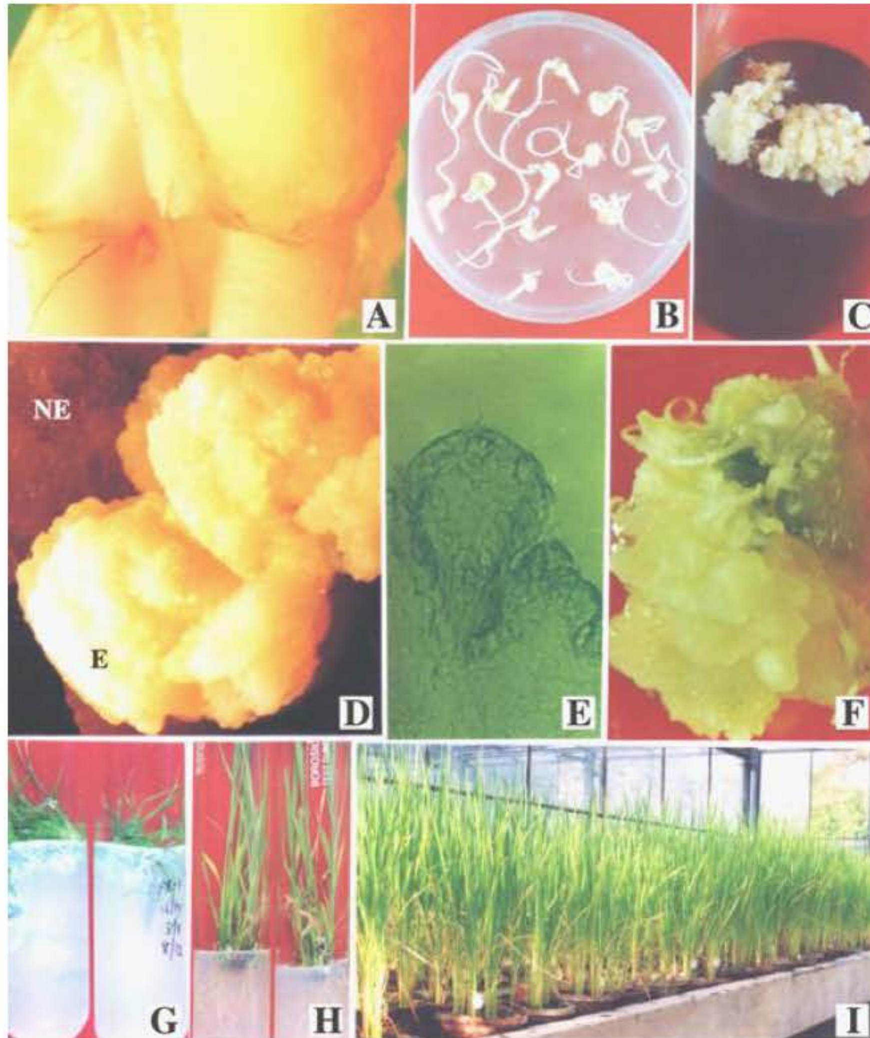

Fig. 1: Induction of embryogenic callus and subsequent plant regeneration in basmati rice

A) Stereomicrograph (16X) showing the origin of the callus from scutellar tissue.
B) Callus proliferation of Pusa Basmati 1 from mature seeds.
C) Subcultured calli of Basmati 386 showing good proliferation on medium containing activated charcoal (2 g L^{-1});
D) Stereomicrograph (16X) showing embryogenic (E) and nonembryogenic (NE) sectors in subcultured callus of Basmati 370;
E) Microtome section of embryogenic calli of Pusa Basmati 1 showing heart shaped somatic embryo;
F) Sterecomicrograph (16X) embryogenic callus of Pusa Basmati 1 exhibiting shoot regeneration;
G) Somatic embryos derived plants of Pusa Basmati 1;
H) Somatic embryos derived plants of Basmati 386;
I) Tissue cultured derived plants in soil.

and 386. Calli initiated on medium MS-B (Table 1) were fast growing highly embryogenic and had only few rhizogenic regions. Besides the higher frequency of embryogenic callus obtained with medium MS-B, the average number of embryo like structure per callus differed considerably for the other medium. Medium MS-A and MS-C induced callus with only few embryo like structures while this number increased upon subculture, but the number of embryo like structures remained far less than the number obtained with medium MS-B.

Shoot regeneration: Embryogenesis was further confirmed by studying the plant regeneration ability of the embryogenic calli. Four weeks after culturing, all

Table 2: Shoot regeneration (%) from embryogenic calli on different MS medium compositions

	Shoot regeneration (%)		
Treatments	Pusa Bas 1	Bas 370	Bas 386
Control	51.02	48.00	44.44
MS-1	50.00	50.00	50.00
MS-2	65.38	53.57	56.52
MS-3	40.00	35.29	28.57
MS-4	66.66	55.00	52.00
MS-5	62.50	45.45	40.00

primary calli, carrying the embryoids, were dissected from seed, coleoptile and roots and transferred to different regeneration medium. Five different medium compositions (MS-1 to MS-5) based on MS salts were tried to see the effect of different factors on per cent shoot regeneration (%) (Table 2). Statistical analysis showed that variety mean squares and medium mean squares were significant at 5% level with CD being 1.29 and 1.83, respectively. Thus, it could be concluded that the two varieties showed a significant difference in their response for shoot regeneration (%). The different medium used also differed significantly from each other. Two responses were observed, small calli upto 10 mm^2 usually became green after 5-7 days and several green spots could be observed within 15 days of transfer to regeneration medium and germination of the embryoids (Fig. 1F). Development of the scutellum and coleoptiles was observed 7 days later. These small calli usually regenerated fully and produced multiple shoots (Fig. 1G). Calli larger than 25 mm^2 did not regenerated but became rhizogenic and necrotic. A direction of root and shoot axis is indicative of regeneration via somatic embryogenesis, which is a preferred mode of differentiation[15]. Somatic embryoides like their zygotic counterparts, arise from single cells and deviate the problem of possible genetic chimerism arising in other developmental modes. It would be worth mentioning that a very few albinos (2-3%) were observed in our cultures, which is at variance with the other observations on seed cultures of rice[16,17]. Regeneration of albino shoots is an undesirable phenomenon, which occurs due to lack of chloroplast caused by the cultural conditions[18].

The origin and physical state of the explant has been described to be important for successful regeneration through somatic embryogenesis. It was observed that immature embryos were found to be most responsive for culturing. Since this explant is restricted to a short period of the growth cycle of the rice, the continuous availability of mature embryos would make them more suitable. The plant regeneration was influenced both by the genotype as well as the composition of the medium used for regeneration. Regeneration medium (MS-4) gave a high percentage of regenerated plantlets. After one month on this medium, hardly any callus tissue was detectable between the shoots. When other regeneration medium were used, a considerable growth of callus occurred. Medium supplemented with maltose (60 g L^{-1}) (MS-2) also induced a fairly good plant regeneration (65.38%) in Pusa Basmati 1. Among the three basmati varieties, highest plant regeneration (66.66%) was observed in Pusa Basmati. It was followed by Basmati 386 (56-62%) and Basmati 370 (55%) (Table 2). Induction (MS-B) and regeneration (MS-4) medium gave a high percentage of regenerated plantlets in basmati cultivars, Pusa Basmati 1 and 370. Whereas, in Basmati 386, induction (MS-B) and regeneration (MS-2) medium resulted in high frequency of regeneration.

Induction of roots and hardening: After induction of roots on rooting medium (½ MS) plants with well developed shoot and root system, were removed from the test tubes and were hardened by placing in the open test tubes containing (1/4 MS) under strong light condition. There was no mortality during the hardening process in any of the variety. Plants growing in the soil earthen pots exhibited normal growth and seed set (Fig. 1I).

In conclusion, the somatic embryogenesis and subsequent plant regeneration was genotype dependent. The concentration of proline, maltose, sucrose and charcoal were optimized for obtaining increased regeneration in seed cultures via somatic embryogenesis, because the potential of the embryogenic callus to produce somatic embryos and their conversion into plant provides the best and accessible target tissue in mutant selection and genetic transformation and these factors could be helpful in shortening the time of *in vitro* culture.

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