The Bioscan 9(3): 1077-1080, 2014
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

PRODUCTION OF HIGH QUALITY EMBRYOGENIC CALLUS OF RICE

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KEYWORDS
Rice
Swana*Sub1*
embryogenic calli
2, 4-D and tissue culture

Received on :
20.12.2013

Accepted on :
04.07.2014

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ABSTRACT
The study was undertaken to standardize an efficient and effective protocol for callus Induction, subsequent growth and regeneration in rice variety Swarna Sub1.MS, B5 and N&N media were used for callus induction.Overall,MS medium was found better for callus induction as compared to N&N medium. Callus induction percentage was highest in SS29 (80.00) followed by SS4 which gave 76.67%, SS32 and SS44 gave 66.67%. The growth Regulator 2, 4-D with varying concentrations were tested for callus induction. Production of embryogenic calli increased as the concentration of 2, 4-D was increased from 0.5 mg/L to 1.5 mg/L.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most versatile cereal food crop and it is the primary source of food and calories for about half of the world population (Vega *et al*., 2009). India is among the largest rice growing countries accounting for about one third of the world acreage under the crop.During past few decades, biotechnological techniques such as somaclonal variation, *in vitro* selection, production of doubled haploid lines from anther culture and genetic transformation are being employed in ricedevelopment for the creation of novel rice varieties. A mature rice seed, compared with immature tissues, which contain a large number of actively dividing cells, is suitable to induce embryogenic calli , it has distinct advantages in practical experiments because it is readily available throughout the year (Carsono and Yoshida, 2006). Maeda *et al*. (2002) reported that white and green patches are often seen on the surface stratum of callus with high regeneration ability. Lee *et al*. (2002) found that the number, colour, size, shape and appearance of the embryogenic calluses varied among the rice genotypes depending upon the basal medium, indicating that induction of high-quality rice callus influenced by genotype, medium, and the kind of explants as well as their interactions. Callus induction and regeneration is still a challenging task in most rice varieties. Keeping the above facts in view, the present study was investigated to develop a reproducible and an efficient procedure for callus induction and plant regeneration of embryogenic calli from mature seeds of Swarna*Sub1* for future genetic transformation studies.

MATERIALS AND METHODS

The nuclear seeds of *Oryza sativa* L. cultivar Swarna *Sub1* were collected from Department of Seed Technology, Narendra Dev University of Agriculture and Technology ,Kumarganj, Faizabad, Uttar Pradesh. The rice husks were removed from the seeds and were washed for 20 minutes in running tap water for removal of dust followed by disinfecting with laboratory detergent (Domex , 15%) solution for 15-20 minutes and also with indophil (0.1% fungicide) for five minutes. The mature seeds were disinfected with 0.1% $HgC1_2$ for five minutes and finally rinsed with sterilized distilled water. Murashige and Skoog (MS, 1962), Gamborg *et al*. (B_5, 1968) and Nitsch and Nitsch (N and N, 1969) media with varying concentrations of plant growth regulator 2,4-D (2,4-Dichlorophenoxyacetic acid) were used for callus induction whereas MS medium supplemented with BAP, NAA and KIN at various concentrations was used for plantlets regeneration.

RESULTS AND DISCUSSION

Callus induction and growth

Media for the present study was initially standardized by inoculating seeds in MS, B_5 and N and N media fortified with 2, 4-D (2 mg/L) for checking their callus induction efficiency (Table 1). As MS media gave better results than B_5 and N and N media, MS media was selected for further studies. The rice callus can be induced and grown on both MS and N and N media (Rashid *et al*., 2004) but the low response in terms of callusing as well as supporting regeneration from explants of N and N and B_5 media may be due to their lower N_2 content (Jubair *et al*., 2008). Pandey *et al*. (1994) reported that the

Table 1: Selection of media for callus induction and growth in Swarna *Sub1*

Media	Hormone 2,4-D (mg/l)	Response Colour	Type	Appearance	Callus induction
MS	2.00	Light Yellow	Highly compact	Smooth, oily	Profuse
B_5	2.00	Yellow	Friable	Rough dry	Scarce
N&N	2.00	Brown	Friable	Rough dry	Scarce

Table 2: Effect of different plant hormones on rice callus induction, formation rate, appearance, colour and type

Media Code	Hormone Concentration	Induction %	Formation rate	Appearance	Callus Colour	Callus Type
SS1	M.S. Basal	0.00 $\pm$0.00^{k}	-	-	-	-
SS2	0.5mg/L 2,4D	43.33 $\pm$4.71^{efg}	+ +	Dry	Light Yellow	Compact
SS3	1.0 mg/L 2,4D	56.67 $\pm$4.71^{cde}	+ + +	Oily	Brownish Yellow	Compact
SS4	1.5 mg/L 2,4D	76.67 $\pm$4.71^{ab}	+ + + +	Oily	Light Yellow	Compact
SS5	2.0 mg/L 2,4D	63.33 $\pm$4.71^{bcd}	+ + + +	Oily	Light Yellow	Compact
SS6	2.5 mg/L 2,4D	56.67 $\pm$4.71^{cde}	+ + +	Oily	Light Yellow	Compact
SS7	3.0 mg/L 2,4D	56.67 $\pm$4.71^{cde}	+ + +	Oily	Dark Yellow	Compact
SS8	3.5 mg/L 2,4D	46.67 $\pm$4.71^{ef}	+ +	Dry	Yellow	Compact
SS9	4.0 mg/L 2,4D	43.33 $\pm$4.71^{efg}	+ +	Dry	Yellow	Compact
SS10	4.5 mg/L 2,4D	43.33 $\pm$4.71^{efg}	+ +	Oily	Blackish Brown	Compact
SS11	5.0 mg/L 2,4D	30.00 $\pm$8.16^{ghi}	+	Oily	Black	Compact
SS12	0.5 mg/L 2,4-D + 0.5mg/L NAA	16.67 $\pm$9.47^{ij}	+	Dry	Light Yellow	Compact
SS13	1.0 mg/L 2,4-D +0.5mg/L NAA	26.67 $\pm$4.71^{hij}	+	Oily	Brownish Yellow	Compact
SS14	1.5 mg/L 2,4-D +0.5mg/L NAA	30.00 $\pm$8.16^{ghi}	+	Dry	Brownish Yellow	Friable
SS15	2.0 mg/L 2,4-D +0.5mg/L NAA	30.00 $\pm$8.16^{ghi}	+	Dry	Brownish Yellow	Friable
SS16	2.5 mg/L 2,4-D +0.5mg/L NAA	26.67 $\pm$4.71^{hij}	+	Dry	Black	Compact
SS17	0.5 mg/L 2,4-D +1.0mg/L NAA	43.33 $\pm$4.71^{efg}	+ +	Dry	Brownish Yellow	Compact
SS18	1.0 mg/L 2,4-D + 1.0mg/L NAA	63.33 $\pm$4.71^{bcd}	+ + + +	Oily	Light Yellow	Compact
SS19	1.5 mg/L 2,4-D + 1.0mg/L NAA	46.67 $\pm$4.71^{ef}	+ + +	Oily	Light Yellow	Compact
SS20	2.0 mg/L 2,4-D + 1.0mg/L NAA	13.33 $\pm$9.43^{jk}	+	Oily	Brownish Yellow	Friable
SS21	2.5 mg/L 2,4-D + 1.0mg/L NAA	56.67 $\pm$4.71^{cde}	+ + +	Oily	Light Yellow	Compact
SS22	0.5 mg/L 2,4-D + 1.5mg/L NAA	30.00 $\pm$8.16^{ghi}	+	Oily	Deadly Brown	Compact
SS23	1.0 mg/L 2,4-D + 1.5mg/L NAA	30.00 $\pm$8.16^{ghi}	+	Oily	Brownish Yellow	Compact
SS24	1.5 mg/L 2,4-D +1.5mg/L NAA	26.67 $\pm$4.71^{hij}	+	Dry	Light Brown	Friable
SS25	2.0 mg/L 2,4-D + 1.5mg/L NAA	16.67 $\pm$9.47^{ij}	+	Dry	Brownish Yellow	Friable
SS26	2.5 mg/L 2,4-D + 1.5mg/L NAA	16.67 $\pm$9.47^{ij}	+	Dry	Brownish Yellow	Friable
SS27	0.5 mg/L 2,4-D + 0.5mg/L BAP	43.33 $\pm$4.71^{efg}	+ +	Oily	Brownish Yellow	Compact
SS28	1.0 mg/L 2,4-D + 0.5mg/L BAP	50.00 $\pm$8.16^{def}	+ +	Oily	Brownish Yellow	Friable
SS29	1.5 mg/L 2,4-D + 0.5mg/L BAP	80.00 $\pm$0.00^{a}	+ + + +	Oily	Light Yellow	Compact
SS30	2.0 mg/L 2,4-D + 0.5mg/L BAP	56.67 $\pm$4.71^{cde}	+ + +	Oily	Brownish Yellow	Friable
SS31	2.5 mg/L 2,4-D, 0.5mg/L BAP	53.33 $\pm$4.71^{cde}	+ + +	Oily	Deadly Brown	Friable
SS32	0.5 mg/L 2,4-D + 1.0mg/L BAP	50.00 $\pm$8.16^{def}	+ +	Dry	Dark Yellow	Friable
SS33	1.0 mg/L 2,4-D + 1.0mg/L BAP	66.67 $\pm$4.71^{abc}	+ + + +	Oily	Light Yellow	Compact
SS34	1.5 mg/L 2,4-D + 1.0mg/L BAP	36.67 $\pm$8.47^{fgh}	+ +	Oily	Deadly Brown	Friable
SS35	2.0 mg/L 2,4-D + 1.0mg/L BAP	26.67 $\pm$4.71^{hij}	+	Oily	Brownish Yellow	Friable
SS36	2.5 mg/L 2,4-D + 1.0mg/L BAP	26.67 $\pm$4.71^{hij}	+	Oily	Brownish Yellow	Friable
SS37	0.5 mg/L 2,4-D + 1.5mg/L BAP	43.33 $\pm$4.71^{efg}	+ +	Oily	Dark Yellow	Compact
SS38	1.0 mg/L 2,4-D + 1.5mg/L BAP	63.33 $\pm$4.71^{bcd}	+ + + +	Oily	Light Yellow	Compact
SS39	1.5 mg/L 2,4-D + 1.5mg/L BAP	46.67 $\pm$4.71^{ef}	+ +	Dry	Deadly Brown	Compact
SS40	2.0 mg/L 2,4-D + 1.5mg/L BAP	43.33 $\pm$4.71^{efg}	+ +	Dry	Light Yellow	Friable
SS41	2.5 mg/L 2,4-D + 1.5mg/L BAP	43.33 $\pm$4.71^{efg}	+ +	Dry	Light Yellow	Friable
SS42	0.5 mg/L 2,4-D + 0.5mg/L Kin	53.33 $\pm$9.47^{cde}	+ + +	Oily	Light Yellow	Compact
SS43	1.0 mg/L 2,4-D + 0.5mg/L Kin	56.67 $\pm$4.71^{cde}	+ + +	Dry	Dark Yellow	Friable
SS44	1.5 mg/L 2,4-D + 0.5mg/L Kin	66.67 $\pm$9.43^{abc}	+ + + +	Oily	Light Yellow	Compact
SS45	2.0 mg/L 2,4-D + 0.5mg/L Kin	53.33 $\pm$4.71^{cde}	+ + +	Oily	Brownish Yellow	Compact
SS46	2.5 mg/L 2,4-D + 0.5mg/L Kin	43.33 $\pm$4.71^{efg}	+ +	Dry	Brownish Yellow	Friable
SS47	0.5 mg/L 2,4-D + 1.0mg/L Kin	13.33 $\pm$9.43^{jk}	+	Oily	Light Brown	Compact
SS48	1.0 mg/L 2,4-D + 1.0mg/L Kin	16.67 $\pm$9.47^{ij}	+	Dry	Light Yellow	Friable
SS49	1.5 mg/L 2,4-D + 1.0mg/L Kin	36.67 $\pm$4.71^{fgh}	+ +	Oily	Brownish Yellow	Compact
SS50	2.0 mg/L 2,4-D + 1.0mg/L Kin	56.00 $\pm$4.71^{cde}	+ + +	Oily	Deadly Brown	Compact
SS51	2.5 mg/L 2,4-D + 1.0mg/L Kin	26.67 $\pm$9.43^{hij}	+	Oily	Brownish Yellow	Compact
SS52	0.5 mg/L 2,4-D + 1.5mg/L Kin	23.33 $\pm$4.71^{hij}	+	Oily	Brownish Yellow	Compact
SS53	1.0 mg/L 2,4-D + 1.5mg/L Kin	46.67 $\pm$4.71^{ef}	+	Oily	Deadly Brown	Compact
SS54	1.5 mg/L 2,4-D + 1.5mg/L Kin	56.00 $\pm$4.71^{cde}	+ + +	Oily	Brownish Yellow	Compact
SS55	2.0 mg/L 2,4-D + 1.5mg/L Kin	50.00 $\pm$8.16^{def}	+ +	Oily	Brownish Yellow	Compact
SS56	2.5 mg/L 2,4-D + 1.5mg/L Kin	63.33 $\pm$4.71^{bcd}	+ + + +	Oily	Light Yellow	Compact

- = no callusing; + = meager callusing; + + = moderate callusing; + + + = high callusing; + + + + = profuse callusing; Values are means of 3 replicates. Mean values followed by the same letters are not significantly different at p ³ 0.05 DMRT.

success of *in vitro* culture largely depends on the nutritional media, growth regulators, genotypes and the interaction of genotype with the medium.

Whereas in MS media, callus induction percentage was highest in SS29 (80.00) followed by SS4 which gave 76.67%, SS32 and SS44 gave 66.67% (Table 2). Production of embryogeniccalli increased as the concentration of 2, 4-D was increased from 0.5 mg/L to 1.5 mg/L. Before now, it was reported that among the auxins, 2,4-D increase the rate of cell division and this attributes to increased amount of callus and to initiate and sustain embryogenic callus growth in rice (Wagiran *et al*., 2008; Revathi and Pillai, 2011), when applied at low concentration (2 mg/L 2,4-D and or 1.5 and 2.5 mg/L 2,4-D) showed high callus induction percentage in all the rice dehulled seeds (Tam and Lang, 2003). However, the combination of 2, 4-D with kinetin was more effective in producing embryogenic and or organogeniccalli (Wang *et al*., 2004), when addition of NAA and or BAP could enhance the quality of the initiated callus (Turhan and Baser, 2004). While cytokinin may increase the growth rate of pre-embryogenic masses (Kommamine *et al*., 1992). Results obtained by Rashid *et al*. (2009) that increase in the concentration of 2, 4-D and kinetin reduced the percentage of embryogenic calli production. Moreover, difference in callus proliferation rate between different auxins may be due to the difference in the physiological activity of the auxins (Wagiran *et al*., 2008) and differences in response of genotypes, especially even when carried out after a short time of callus maintenance (Muhammad *et al*., 2005; Htwe *et al*., 2011). The embryogeniccalli were relatively smooth (oily), compact, nodular in appearance and milky white (yellowish) in colour (Rachmawati and Anzai, 2006; Mahmood *et al*., 2012). Yellow or milky white colour of the 2, 4-D derived callus might be due to the inhibitory effect of 2, 4-D on chlorophyll formation. Our observations are confirmed with Li *et al*. (2009) and Narciso and Hattori, (2010) who reported that embryogenic type of callus in *indica* rice varieties was characterized as yellowish, compact, smooth, big in size and iso-diametric cells termed as embryogenically active. Embryogeniccalli grew about 5 to 10-fold in volume 30 days after transfer. However, the non embryogeniccalli turned brown and died.

In the present study, a combination of BAP along with 2, 4-D was found to be superior to kinetin + 2, 4-D in callus induction. Embryogeniccalli being composed of individual compact and spherical cells that were rather regular in size and tightly held together and appeared to organize globular embryos in small compact clusters. By comparison, non embryogeniccalli showed elongated and loosely held cells on the surface. Fazelienasab *et al*. (2004) reported that the compact type of callus indicates the unorganized cell division of tissues with highly viable cells for further growth of culture. These observations are in close conformation with Haeyoung *et al*. (2007), Singh *et al*. (2009) and Rashid *et al*. (2009).

Callus induction is the first step in rice regeneration to obtain high quality of embryogeniccalli because of the efficiency of regeneration of a plant is highly dependent on the quality of the calli. Rachmawati *et al*. (2004) visually selected the embryogenic rice calli on the basis of their appearance. The embryogenic callus was relatively compact, oily and nodular in appearance. On the basis of performance of callus on the above characters, callus obtained from eleven treatments were selected for further studies, which are SS4, SS5, SS18, SS19, SS21, SS29, SS33, SS38, SS42, SS44 and SS56.

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