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Study of Physiological Potentiality of Callus and Plant Regeneration through Somatic Embryogenesis in Rice Variety Swarna

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ABSTRACT

The regeneration protocol was developed from dehusked and sterilized seeds of rice variety Swarna (MTU 7029) to study callus induction, callus growth rate and regeneration potentiality. Different ranges of 2,4-D (1-4 mg L^{-1}) and combinations of 6-Benzyl Aminopurine (BAP) (2-4 mg L^{-1}) and α-Naphthalene Acetic Acid (NAA) (1 mg L^{-1}) were used for callus induction, somatic embryo formation and plantlets regeneration respectively. Ninty six percent callus induction efficacy was found with 4 mg L^{-1} 2,4-D. Whereas the high frequency of plantlets regenerated from embryogenic calli were observed in the combination of 4 mg L^{-1} BAP and 1 mg L^{-1} NAA; the embryogenic calli obtained from 2 mg L^{-1} 2,4-D concentration performed better in this media (4 mg L^{-1} BAP and 1 mg L^{-1} NAA) than the other three used concentrations of 2,4-D. Physiological potentiality of 45 days old calli in terms of fresh and dry weights and proline content were showed a positive correlation. However, 4 mg L^{-1} 2,4-D in medium showed low regeneration capacity of calli.

Key words: Explant, dehusked seed, *Oryza sativa*, physiological potentiality, proline

INTRODUCTION

Rice (*Oryza sativa* L.) belongs to the family Gramineae is the most important food crop in the world and feeds over half of the global population (Sasaki, 2005). In Asia it covers half of the arable land used for agriculture. During the past few decades plant tissue culture is the most useful technology to improve the agricultural crops (Cantrell and Hettel, 2004; Yoshida *et al*., 1983; Rueb *et al*., 1994; Mandal *et al*., 1999). The use of *in vitro* screening technique to develop plants with increasing abiotic stress tolerance, through screening of millions of a cells in a few flasks and petridishes, instead of a few thousand whole plants on hectares of field plots, offer convenience, ease and confidence (Biswas *et al*., 2002).

Efficient plant regeneration from different explants is an important component for genetic improvement of crop plants through biotechnology (Christou, 1997). Therefore, it is desirable to develop an efficient *in vitro* protocol for high frequency plant regeneration from different tissues of indica rice. Plant regeneration in rice via organogenesis and embryogenesis has been reported from different explants since the 1970's, such as root (Cary *et al*., 2001), mature embryo (Huang and Wei, 2004), leaf (Kopertekh and Stribnaya, 2003), immature embryos (Oduor *et al*.,

2006), anthers (Xiu-hong *et al*., 2005; Roy and Mandal, 2011; Niroula and Bimb, 2009), inflorescence (Kavas *et al*., 2006) etc. Plant regeneration has also obtained from rice protoplasts (Kermanee, 2004). However, despite tremendous effort, regeneration frequency generally remains low in indica rice as compared to japonica rice varieties (Abe and Futsuhara, 1985).

Swarna (Parentage: Vasisa X Mahsuri) is a highest yielding long duration (150 days), mainly semi dwarf (100-130 cm) variety but it has some drawbacks; its water requirement is more due to long duration crop and it is the highest yielding variety under the irrigated transplanted condition but in India maximum area comes under rainfed condition so the results are yield reduction. In late sown crop, much amount of chaffy grains are produced in Northern region mainly, in which flowering synchronized with the low temperature which affects the pollen fertility resulting low seed setting and ultimately drastically reduced yield but late sown are practiced due to rainfed condition (Sharma, 2010). Hence, the genotype Swarna was used in the present piece of work.

Objective of the present study was to determine the most suitable concentration and combination of growth regulators for excellent callus induction and regeneration; further the study was also extended to know the physiological potentiality of the callus for better regeneration of plantlets with higher stress resistance capacity.

MATERIALS AND METHODS

The present study was carried out at Tissue Culture Laboratory at Department of Genetics and Plant Breeding and Department of Plant Physiology, I.Ag.Sc., BHU, India, in the year 2008-09. Dehusked seeds of mature grain from the rice (*Oryza sativa*) variety Swarna were used for callus induction, procured from the Genetics and Plant Breeding Department of the same institute.

The dehusked seeds were surface sterilized with 70% ethanol for 30 sec and rinsed with autoclaved double distilled water for 3 times, after that the seeds were soaked in 0.1% mercuric chloride ($HgCl_2$) for 5 min with intermittent shaking followed by 5-6 rinses in sterile water (Raveendar *et al*., 2008). After surface sterilization the dehusked seeds were kept on autoclaved filter paper in a petridish to remove the excess water. After removing the water from the seed surface, these seeds were inoculated into culture tubes containing MS (Murashige and Skoog, 1962) basal media supplemented with different concentrations (1, 2, 3 and 4 mg L^{-1} and abbreviated as MS1, 2, 3 and 4) of 2,4-D (2,4-dichlorophenoxyacetic acid) for callus induction (Jubair *et al*., 2008) in two lots, one for regeneration purpose and another for the study of physiological potentiality of the callus. Each lot containing 5 replication and each replication consisting of 20 tubes. The pH of the media was adjusted to 5.8 with 1N NaOH and 1N HCl using electronic pH indicator. The media was autoclaved at a temperature of 121°C and pressure of 15 psi for 20 min. Inoculation was carried out under a sterilized environment in a laminar air flow cabinet. After inoculation, culture tubes for callus induction were incubated in dark at 25±1°C for 30 days. After that the 30 days old calli with sufficient size (2-3mm) were transferred to MS based regeneration medium modified with different combinations of BAP and NAA [2:1, 3:1 and 4:1 mg L^{-1} abbreviated as MSR1, 2 and 3 (MS medium for regeneration)]. The tubes were kept at 16 h light/8 h dark at 2000 lux light intensity of cool white fluorescent light at 25±1°C temperature. Somatic embryo formation and the greening of the embryogenic calli start at 10-12 days after transfer. The combinations of these hormones produced the shoots and roots on the same media but sometimes roots were not formed and if formed they were not well developed because in most of the cases shoots were come out in clusters, then the unrooted green shoots were transferred into the rooting medium which contain half strength MS medium without growth regulators. The frequencies of callus induction,

embryogenic calli and regeneration were determined with the first lot and with the second lot; fresh and dry weights and the proline content were measured in the 45 days old calli.

To measure the different parameters of callus formation the following formula were used:

- Callus induction frequency was calculated as follows:

$$\frac{\text{No. of calli} \times 100\%}{\text{No. of inoculated seeds}}$$

- Frequency of developing embryonic calli was calculated as follows:

$$\frac{\text{No. of embryogenic calli} \times 100\%}{\text{No. of inoculated seeds}}$$

- Regeneration frequency was calculated as follows:

$$\frac{\text{No. of regenerated calli} \times 100\%}{\text{No. of calli inoculated}}$$

- Fresh weight of the calli: Three calli was taken for one replication and the fresh weights of the calli were taken in 3 replication by using the electronic balance (Model No. ADAIR DVTT INSTRUMENT PVT. LTD.)
- Dry weight of the calli: To take the dry weights of the calli, samples were kept for an hour in an oven pre-set at 100-110°C. Thereafter it was placed in an oven set as 60±2°C till to get constant weight.
- Proline estimation: Estimation was done in 5 replication each containing 50 mg dried calli, taken from each of the media containing different concentrations of 2,4-D ranging from 1-4 mg L^{-1}. Proline content of the dry calli was measured by using the standard method (Bates *et al*., 1973).

Statistical analysis: The data collected for fresh and dry weights and proline content were analysed by using Randomised Block Design (RBD), the significance of treatment effect was tested with the help of F-test and the differences between treatments by Critical Difference (C.D) at 1 and 5% level of significance were determined. In the other way some parameters like callus induction, embryogenic callus frequency, regeneration percentage and number of green shoot obtained per explant were calculated on the basis of percentage and Standard Deviation respectively.

RESULTS AND DISCUSSION

Table 1 represents the data regarding the callus induction and regeneration percentage of rice variety Swarna in presence of different concentrations of 2,4-D and BAP+NAA, respectively. Among used concentrations 4 mg L^{-1} of 2,4-D showed maximum percentage (96%) of calli formation followed by 3 and 2 mg L^{-1} 2,4-D but the lowest concentration that is 1 mg L^{-1} showed poorest formation of calli. Further, the result represents that the use of 2 mg L^{-1} 2,4-D showed maximum percentage (35%) of embryogenic calli and that was followed by other two higher concentrations of the same chemical however, when MS medium was supplemented with 1 mg L^{-1} 2,4-D failed to show any embryogenic calli.

Table 1: Effects of (a) different concentrations of 2,4-D on the callus induction percentage and embryogenic calli percentage and (b) Different combinations of BAP +NAA on regeneration medium during somatic embryogenesis of the rice var. Swarna.

Concentration of 2,4-D (mg L^{-1}) in MS medium	Callus induction (%)	Embryogenic calli (%)	No.of calli plated on each media	Regeneration percentage			Average No. of shoots per explants		
				2 BAP+1NAA (MSR1)*	3 BAP+1NAA (MSR2)	4 BAP+1NAA (MSR3)	MSR1	MSR 2	MSR 3
							(Mean±SD)		
MS1	10	0	3	0	0	0	-	-	-
MS2	90	35	30	0	23	33	-	9.1±3.0	16.4±4.2
MS3	94	27	31	0	19	22.5	-	4.2±2.2	7.5±2.9
MS4	96	21	32	0	12.5	15.6	-	5.0±2.9	8.2±3.0

*Unit of BAP and NNA is mg L^{-1}

Table 2: Effects of different concentrations of 2,4-D on fresh weight (g), dry weight (g) and proline content (mg g^{-1} dry weight) at 45 days old calli of rice var. Swarna (Statistical design- RBD)

Concentration of 2,4-D (mg L^{-1})	Fresh weight (g)	Dry weight (g)	Proline content (mg g^{-1})
1	0.528[a]	0.062[a]	0.945[a]
2	1.338[b]	0.214[b]	1.276[b]
3	1.648[c]	0.258[c]	1.397[c]
4	1.838[d]	0.334[d]	1.528[d]
SEM (±)	0.020	0.009	0.018
CD at 0.005	0.044	0.020	0.039
CD at 0.001	0.062	0.028	0.055

*unit of BAP and NAA is mg L^{-1} Values with different letter (s) in column are significantly different at p 0.05 and 0.01

It is clear from Table 1 that the no. of calli plated on each regeneration media, comprised with 3 combinations of BAP and NAA. However, maximum of calli has been plated on regeneration medium obtained from MS4 medium. The result represents that the MS1 failed to regenerate any plantlets whereas maximum number of plantlets regenerated in MS2XMSR3 (33%) media followed by MS4XMSR3 (22.5%) and MS3XMSR3 (15.6%). On the contrary some of the plantlets are formed also in MSR2 combination while used along with MS2, MS3 and MS4 but followed the same trend as mentioned above. The average number of shoots per explants resulted from the regeneration medium. The maximum of shoots observed in third regeneration medium (MSR3) (16.4±4.2) followed by second regeneration medium (MSR2) (9.1±3.0) in the callus of 2 mg L^{-1} 2,4-D concentration. The trend of shoot regeneration; in these two medium were same where 4 mg L^{-1} 2,4-D containing sets occupied second position followed by 3 mg L^{-1} 2,4-D sets.

From Fig. 1, it was noted that upon transferring the calli to regeneration medium green tinge was visible on calli within 10-12 days (Fig. 1a, b). After greening of the callus somatic embryos were formed within 3 weeks and different stages of the embryos were observed like globular, torpedo and cotyledonary stage (Fig. 1c-e). Shoot regeneration started within 30-35 days along with the roots, although somatic embryo is a bipolar structure but in some cases the shoots were transferred into rooting media for root formation (Fig. 1f-h) and plantlets were kept for hardening (Fig. 1i).

Table 2 represents the physiological potentiality of the 45 days old calli in terms of fresh and dry weights and proline content. Maximum fresh weight of calli was obtained in the 4 mg L^{-1} 2, 4-D (1.83 g) sets and minimum amount of calli present in the lowest used concentration (1 mg L^{-1}). The second and third positions in this respect achieved by 3 and 2 mg L^{-1} 2, 4-D.

The study of dry weight and proline content of the calli represented the same trend. The result of these parameters showed that the higher concentration of 2,4-D, not only efficiently improves

Fig. 1 (a-i): (a) Formation of callus, (b) greening of the callus, (c) embryo at globular stage, (d) torpedo stage of the embryo, (e) cotyledonary stage and regeneration of the embryo, (f-g) multiple shoot regeneration, (h) complete plantlets and (i) hardening of plantlets

the amount of callus from the dehusked seeds of rice but also improves the proline content (1.528 mg g^{-1}) of it which shows statistically significant results among them.

However, the result of the present study showed that the 4 mg L^{-1} 2,4-D best for callus formation from the dehusked rice seed. The same type of callus induction in rice was observed with different concentrations of 2,4-D (Salam *et al*., 2008), in that case the best concentration of 2,4-D was 2 mg L^{-1} (Katiyar *et al*., 1999; He and Jia, 2008) whereas, in present investigation the best concentration was 4 mg L^{-1} 2,4-D for callus induction (Table 1). Therefore the result suggests that the callus induction of rice depends on various used concentrations of 2,4-D; however the regeneration % and shoot formation from embryogenic calli (obtained from 2, 3 and 4 m gL^{-1} 2,4-D) of rice var. Swarna responded better in regeneration medium third (MSR3) followed by MSR2 but

among all the concentrations of 2,4-D, 2 mg L^{-1} (the callus obtained from this) responded better in regeneration % and shoot formation. The same type of chemical composition in MS medium was used by Jubair *et al.* (2008) for regeneration of rice variety Topa. However, Lee *et al.* (2002) reported that the highest frequency of shoot regeneration were observed for Dong-Jin, Hwa-Chung and Nak-Dong on MS medium containing 2 mg L^{-1} of NAA and 2, 1 and 4 mg L^{-1} of kinetin, respectively. Bejoy *et al.* (2006) also reported more shoot regeneration of *Cucurma haritha* on MS as compared to White's medium.

The highest used concentration of 2,4-D was found to be achieved the maximum proline representing an important compatible osmolyte (Taylor, 1996; Yamada *et al.*, 2005) represented the maximum stress ameliorating characteristics of the formed calli. However, from the observation it can be suggested that although the calli obtained from the medium having 2 mg L^{-1} 2,4-D showed more regeneration capacity (Table 1) but it may be predicted that the plants regenerated from media containing 4 mg L^{-1} 2,4-D may have more resistant characters as compared to the others.

With the extensive use of arable lands the soil may develop salinity in succeeding years and it may be a major limiting factor for rice productivity. Further Swarna, being a very popular rice variety and it is grown in a major part of the country. From the extensive study of the literature it has been realised that the callus of variety Swarna lacks the study regarding stress tolerance. It is well established that proline status of the plant organ and cell culture is an active research area in biotic and abiotic stress physiology (Jain *et al.*, 1991; Sheoran and Nainawatee, 1990; Aghaei *et al.*, 2009; Amirjani, 2010). Increased levels of proline act as compatible solutes, osmosolute and hydrophobic protectant for a number of enzymes and cellular structure. Hence in the present study along with the measurement of fresh and dry mass of callus the proline content was also measured and it was observed that the maximum proline content was found in the callus where the media was provided with the highest used concentration of 2,4-D that is 4 mg L^{-1}; in the same concentration fresh weight which representing the more water holding capacity of the tissue and dry weight both were found maximum; on contrary the regeneration capacity of the callus was less in this concentration and that was found maximum in 2 mg L^{-1} 2,4-D (Table 1). However, it was reported that proline is a potent osmoregulator in rice tissue and indicated that low proline content in the callus representing salt sensitive variety whereas high proline content callus represents salt tolerant variety (Basu *et al.*, 1996, 1999). It is well established that very low concentration of 2,4-D works as phytohormone auxin whereas its high concentration acts as herbicide hence it acts as a stressor in the later case. This explores a view that the callus regenerated from comparatively higher concentration of 2,4-D may have high proline concentration with low regeneration capacity may generate more sturdy and stress tolerant plantlets in the tissue culture system. Sturdy plant which will be easier to handle in the due course of time in fastly originating saline condition for extensive use of arable land.

CONCLUSION

The present study concludes that the use of chemical like 2,4-D can be used in various range during the formation of callus and the consecutive estimation of proline status in it provide an idea of the formation of plantlets having more stress ameliorating characteristics. This study further may open a channel for the selection criteria of regenerating plantlets at an early stage.

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