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CALLUS INDUCTION AND REGENERATION IN SEED EXPLANTS OF RICE (*ORYZA SATIVA* CV. SWAT-II)

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Abstract

Seeds of rice (*Oryza sativa* cv. Swat-II) were inoculated onto MS medium containing various concentrations of auxins and cytokinins. Excellent callus resulted on MS containing 2,4-D and Kn. However, this callus was non-embryogenic. Addition of tryptophan (TPN) @ 50 mg/l induced embryogenic callus. Increase in callus mass was obtained by a change in auxin and cytokinin level. Plantlet regeneration occurred on MS containing BAP at 0.5 mg/l in combination with IAA at a concentration of 0.2 mg/l. The plantlets were transferred to the field after hardening.

Introduction

Rice is the staple food of around 30-40% of the world population. Over 90% of rice is cultivated in Asia. It is an important source of carbohydrate for human consumption and also provides feed and forage. Rice and rice oils are used in cosmetics while starch in textiles.

Global population is expected to reach around 10 billion by 2050. Thus there is a need to improve upon the yield of the local varieties/cultivars, because loss in production could lead to hunger and famine, especially in the developing countries. The improvement can possibly be achieved by genetic manipulation. One possible way is through tissue culture (somaclonal variation) without interfering too much with the genetic make up of the individual. For this an efficient protocol needs to be developed to achieve plant regeneration.

Tissue culture of monocots is difficult compared to dicots (Morel & Wetmore, 1951). Maeda (1965) induced callus in rice seedlings on BM containing 2,4-D. Kawata & Ishihara, (1968) and Nishi *et al*., (1968) obtained callus from rice roots on LS medium fortified with 2,4-D and IAA. Wang *et al.,* (1987) studied regeneration in rice through somatic embryogenesis. It has been observed that rice produces two types of calli viz., embryogenic and non-embryogenic. The former is produced on the surface region, grows faster and gives rise to plants through somatic embryogenesis (Vasil, 1987). Zafar *et al*., (1992) obtained highest callus formation in basmati rice cv.370 on MS with 2 mg/l of 2,4-D. However, somatic embryogenesis was achieved on either N6 or MS medium with 2.0 mg/l each of 2,4-D and Kn. Somatic embryogenesis and regeneration was enhanced when proline or tryptophan was added to the medium alongwith 2,4-D.

Due to a variety of biotic and abiotic interferences, erratic variations in the environment have been noticed. Therefore, the need of the day is to breed resistant and high yielding varieties. Studies has been undertaken to achieve this target e.g., Jan *et al*., (1980) selected some lines of US rice resistant to fungal diseases. Thanh &Tuyen (1990) selected and regenerated rice plants *in vitro* for tolerance against acidic condition.

Th present study was undertaken with the purpose to develop an efficient protocol for regeneration of some local rice cultivers and then study their tolerance against some abiotic stresses viz., drought, salt, temperature etc.

Materials and Methods

The test organism was *Oryza sativa* L. cv. Swat II obtained from National Agricultural Research Centre, Islamabad. The seeds were dehusked and washed with tap water to remove dust and other surfactants. These seeds were then sterilized with 60% alcohol for about 10 minutes and then 1% Mercuric chloride for 2-5 minutes. The sterilized seeds were thoroughly rinsed with distilled water to remove possible traces of Mercuric chloride. These seeds were then soaked for 25 minutes in sterilized distilled water before inoculation.

The basal medium used throughout these studies was that of Murashige & Skoog (1962) salt solution supplemented with various growth hormones as outlined in the result section. Sucrose at 4% was used as the sole carbon source. The pH of the medium was adjusted at 5.8 with either 1.0 N HCl or NaOH and the medium solidified with 0.8% agar. The medium was then autoclaved at 15 psi with a temperature of 121ºC for 15 minutes. Growth hormones were added to the medium before autoclaving. The cultures were then incubated in biotron with 16 hours of light cycle in a 24 hours cycle and the temperature regulated at 25 ± 1ºC. The culture media consisted of (a) callus induction medium, (b) medium for production of embryogenic callus, (c) proliferation of embryogenic callus medium and (d) regeneration medium.

Results

Callus induction: Sterilized caryopsis of *Oryza sativa* cv. Swat II were inoculated on plain MS medium for callus formation and morphogenesis. The seeds germinated after 3 days of inoculation. However, no callus formation took place even after a culture period of 6-10 weeks (Table 1). Therefore, different concentrations of various hormones were added to the BM to observe their callogenic effect. Evans *et al*., (1981) noticed that 2,4-D is effective in callus induction of cereals, while others were able to induce callus in rice with a combination of 2,4-D and a cytokinin (Jabeen & Siriwardana, 1987; Chowdhry *et al*., 1993). Further addition of tryptophan is helpful for the differentiation of embryogenic callus.

a. Callus induction with 2,4-D and kinetin: Sterilized seeds of rice were inoculated on MS medium containing various concentrations of 2,4-D and Kn. Some callus formation occurred at 1.0 mg/l of 2,4-D and 0.5 mg/l of Kn. This callus was soft and friable, translucent and of a yellowish or brownish colour. Almost similar results were obtained with other combinations of 2,4-D and Kn. Further, this callus was mostly non-embryogenic and never regenerated plantlets after several subcultures on regeneration medium.

As evident from the literature that addition of tryptophan enhances formation of embryogenic callus, therefore in another experiment it was added either at 50 mg/l or 100 mg/l to the basal medium.

b. Callus induction with 2,4-D, Kn and TPN: The sterilized caryopsis of rice were inoculated on MS medium containing the following combinations viz.,

i. 2,4-D (1.0 mg/l) + Kn (0.5 mg/l) +TPN (50 mg/l)
ii. 2,4-D (1.0 mg/l) + Kn (1.0 mg/l) +TPN (50 mg/l)
iii. 2,4-D (1.5 mg/l) + Kn (0.5 mg/l) +TPN (50 mg/l)
iv. 2,4-D (1.0 mg/l) + Kn (0.2 mg/l) +TPN (100 mg/l)

Table 1. Callus induction on *Oryza sativa* cv. Swat-II seeds cultured on MS medium containing different combinations of phytohormones.

Nature of explant	Pytohormones mg/l			Culture period (weeks)	Remarks
Seeds	Kn		2,4-D		
= "	0.5		1.0	4	Soft, friable, yellowish-brown callus induction
	2,4-D	Kn	TPN		
= "	1.0	0.5	50	4	Excellent compact callus induction.
= "	1.0	1.0	50	4	Soft, non-embryogenic, yellowish brown callus induction.
= "	1.5	0.5	50	4	Compact, yellowish white callus induction.
= "	1.0	0.2	100	4	Soft, friable, yellowish white callus induction.
	2,4-D	NAA	TPN		
= "	1.0	0.5	50	6	Embryogenic, compact, creamy white callus induction.
	2,4-D	BAP	TPN		
= "	1.0	0.5	50	4	Embryogenic, compact callus induction.

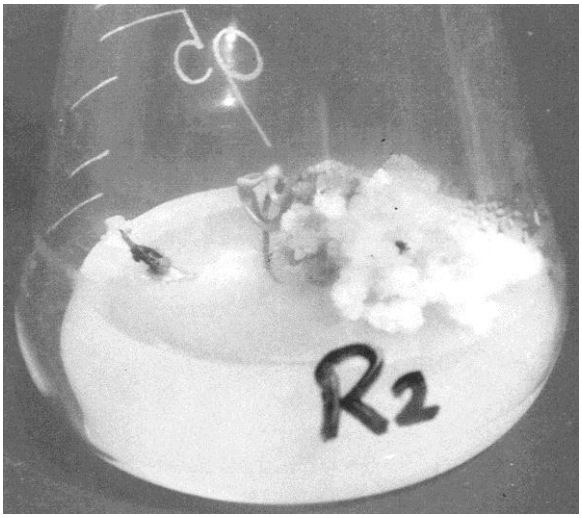

Fig. 1. Callus induction in rice caryopsis when cultured on MS supplemented with 2,4-D (1.5 mg1/ l), Kn (0.5) and TPN (50 mg1/ l). The cultures were raised in cooled incubators (25 ± 1°C) illuminated with white fluorescent light of 16/8 hrs light and dark cycle.

When the seeds were inoculated on the basal medium containing the growth regulators of the first set, seedlings got established within 5-6 days and after about 10 days callus formation occurred at the juncture of the root and plumule. This callus proliferated with the passage of time and an excellent callus growth was obtained after 4 weeks of culture. These calli were hard, compact and creamy white in colour (Fig. 1).

Most of these calli were embryogenic which afterward regenerated plantlets when inoculated on regeneration medium (Table 1).

Very little callus induction took place on MS fortified with 2,4-D (1.0 mg/l), Kn (1.0 mg/l) and TPN (50 mg/l). This callus was soft, friable and of a yellowish brown colour. However, this callus was non-embryogenic. The callus which got initiated on MS containing 2,4-D (1.5 mg/l), Kn (0.5 mg/l) and TPN (50 mg/l) was somewhat compact and yellow to white in colour. Only a small percentage of this callus turned out to be embryogenic in nature. The callus induced on MS containing 1.0 mg/l of 2,4-D, 0.2 mg/l of Kn and 100 mg/l of TPN exhibited slow growth, was soft and friable and of a yellowish or whitish colour. Again this callus was of non-embryogenic type.

c. Callus induction with 2,4-D and NAA: To obtain embryogenic callus, seeds of rice were inoculated on MS containing 1.0 mg/l of 2,4-D, 0.5 mg/l of NAA and 50 mg/l of TPN. The seeds germinated after about 3 days and callus formation occurred after about 10 days. A copious callus resulted within 6 weeks, which was creamy white, compact, usually of embryogenic type and regenerated plantlets when transferred to an appropriate medium.

d. Callus induction with 2,4-D, BAP and TPN: Kn was substituted with BAP to enhance callus induction. A pronounced callus resulted on MS containing 2,4-D (1.0 mg/l), BAP (0.5 mg/l) and TPN (50 mg/l) after about 4 weeks. This callus was compact, hard and creamy white in colour. Most of this callus was embryogenic.

II. Callus proliferation: For study of stress tolerance an efficient and quickly proliferating callus is required which should also have a good regeneration capability. In the previous section, studies with induction of embryogenic callus have been described. In the present section, results with proliferation of the callus are reported.

a. Callus proliferation with NAA and Kn: Calli obtained in the previous section were inoculated on BM supplemented on various combinations of growth hormones. On the basal medium containing Kn (1.0 mg/l) and NAA (0.5 mg/l) an excellent callus developed within 3 weeks (Table 2). These calli were compact in texture and of a yellowish and dark green colour. This greening was due to the production of green projections, which probably were embryoids. These embryoid like structures developed into plantlets after 4 weeks of culture on a fresh medium (Fig. 2). Nonetheless, concurrent with this, browning (darkening) of the callus was noticed in a majority of the cases.

b. Callus proliferation with 2,4-D and BAP: The callus induced on MS + 2,4-D (1.0 mg/l), and Kn (0.5 mg/l) was inoculated on MS containing 2,4-D (1.0 mg/l) and BAP (0.5 mg/l). A moderate callus resulted after about 4 weeks of subculture. This callus was whitish to whitish brown in colour and strangely gave rise to thin roots.

c. Callus proliferation with 2,4-D, BAP and TPN: The mother callus was inoculated on MS containing 2,4-D (1.0 mg/l), BAP (0.5 mg/l) and TPN (20 mg/l). The callus growth was slow which was whitish or yellowish in colour and too compact in texture (Table 2).

Table 2. Callus proliferation and plantlet regeneration in *Oryza sativa* cv. Swat–II cultured on MS medium containing different combinations of phytohormones.

Nature of explant	Pytohormones Mg/l			Culture period (weeks)	Remarks
	Kn		NAA		
Callus	1.0		0.5	3	Excellent, dark green, compact callus developed
	2,4-D		BAP		
= "	1.0		0.5	4	Moderate callus developed with some shoots.
	2,4-D	BAP	TPN		
= "	1.0	0.5	20	4	Too hard whitish-yellow slow growing callus developed
	2,4-D	Kn	TPN		
= "	1.0	0.5	20	4	No callus proliferation.
= "	1.0	0.5	40	4	Slight proliferation of callus occurred.
	BAP		IAA		
= "	0.5		2.0	4	Shoot buds developed on callus.
= "	0.5		2.0	10	Shoot buds developed into plantlets.

Fig. 2. Embryoid-like structures formation and their development into plants when the calli from Fig.1 were cultured on BM + Kn (1.0 mg1/ l) and NAA (0.5 mg1 / l).

d. Callus proliferation with 2,4-D, Kn and TPN: The mother callus was inoculated on MS containing 2,4-D (1.0 mg/l), Kn (0.5 mg/l) and either 20 or 40 mg/l of TPN. Negligible callus proliferation occurred on 20 mg/l of TPN after a culture period of 4 weeks. This callus was loose, friable, translucent and of a yellowish white colour. Further, it did not have much regenerating capacity. A slight improvement in callus proliferation on 40 mg/l of TPN was observed. Some whitish projections were discernable within 2 weeks on this callus. However, no definite bud formation was observed and thus no regeneration.

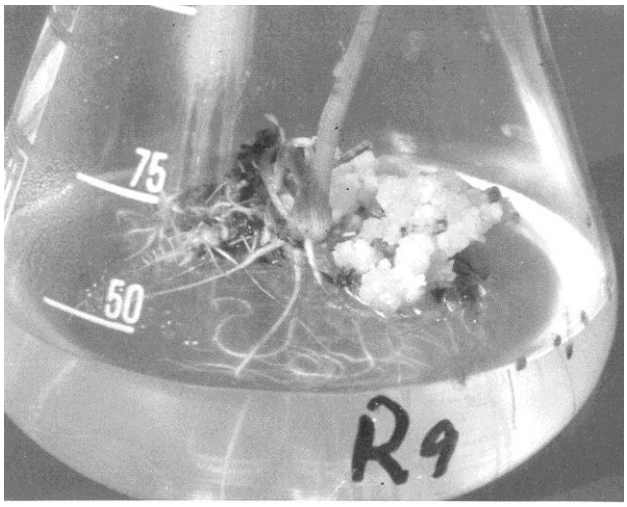

Fig. 3. Plantlet and root formation increased when calli were cultured on BM + BAP + 1AA (see text for explanation).

As a result of these studies, MS containing 2,4-D and Kn was a suitable medium for callus induction. MS fortified with 2,4-D, Kn and TPN was an excellent medium for induction of embryogenic callus. Similarly, a medium containing the above growth regulators was suitable for embryogenic callus proliferation. However, for plantlet regeneration MS fortified with NAA and Kn was the most suitable.

III. Plant regeneration: Plantlet regeneration from somatic embryogenesis is reported in another communication (Ilahi *et al*., 2005). Presently direct plantlet formation is reported briefly. The callus growing on 2,4-D (1.0 mg/l), Kn (0.5 mg/l) and TPN (50 mg/l) was inoculated on MS + BAP (0.5 mg/l) + IAA (0.2 mg/l). Shoot buds appeared on this callus within 2 weeks. However, the callus exhibited some browning. Nonetheless, it exhibited good growth on fresh medium and developed more buds after 4-5 weeks. These buds developed into complete plantlets after a culture of about 10 weeks. Plantlet regeneration capacity increased with an increase of IAA to 0.3 mg/l. Small green projections appeared on the callus, which later developed into shoot buds. These buds developed into plantlets, when the callus, alongwith the shoot buds, was subcultured on a medium containing the same hormonal combination. Rooting frequency was also enhanced by further increase in IAA concentration (Fig. 3; Table 2). The plantlets so regenerated have been transferred to the field after hardening. Thus sufficient material will be available for stress induction of the regenerating callus and plantlets.

Discussion

Totipotency of somatic plant cells is the basis for micropropagation which is being extensively used for obtaining a large number of genetically identical plantlets.These plantlets then would serve as an excellent tool for tolerance to various stresses which is the ultimate aim of the present studies, because an efficient regeneration system is required to obtain plantlets for manipulation. Thus *Oryza sativa* cv. Swat-II seeds were

cultured on MS medium supplemented with various growth regulators. A reasonable amount of callus developed when the seeds were cultured on 2,4-D and Kn especially at 1.0 mg/l and 0.5 mg/l respectively. However, this callus was non- embryogenic and never produced plantlets even after several cultures. Raghawa Ram & Nabors (1984) reported the necessity of a cytokinin and an auxin for the production of callus. Similarly, Siriwardana & Nabors (1983) observed that tryptophan is obligatory in addition to an auxin and a cytokinin for induction of embryogenic callus. Identical results were obtained on rice cv. Swat-II as an efficient callus was obtained on MS containing 2,4-D and Kn but embrgogenic callus was obtained when this medium was fortified with 50 mg/l of tryptophan. Embryogenic callus could also be induced with a change in the growth regulators viz., addition of 2,4-D + NAA or 2,4-D + BAP + TPN.

Nonetheless, excellent callus proliferation was obtained when the basal medium was supplemented with NAA and Kn (1.0 +0.5 mg/l). Similarly, 2,4-D, Kn and BAP + TPN at altered level also induced a reasonably proliferating callus. Collins *et al*., (1978) reported that callus proliferation varies with both genotype, auxin source and concentration. Bano (1997) reported variation in callus proliferation as a result of different combinations and concentrations of growth regulators

Shoot regeneration has been obtained on MS containing BAP and IAA. These hormones supplied at 0.5 and 0.2 mg/l respectively induced formation of shoot buds, which later on developed into complete plantlets. Further, increase of BAP and IAA concentration in the medium increased plantlet regeneration frequency. Our results are in agreement with those of Wang *et al*., (1987) who reported that high concentration of cytokinin and low of auxin promoted plantlet regeneration. Increased concentration of IAA i.e., 0.3 mg/l was sufficient for the induction of roots in the regenerated plantlets. Thus as a result of present studies a protocol for regeneration of plantlets has been developed. These plantlets will be used for stress induction in future studies.

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