Pak. J. Bot., 37(2): 237-242, 2005.

MICROPROPAGATION OF RICE (*ORYZA SATIVA* L. CV SWAT-II) THROUGH SOMATIC EMBRYOGENESIS

IHSAN ILAHI, SHAZIA BANO, MUSARRAT JABEEN AND FAZAL RAHIM

Department of Botany,
University of Peshawar, Peshawar, Pakistan

Abstract

Callus was induced on mature caryopsis of a local variety of rice (*Oryza sativa* L. cv. Swat-II). The frequency of callus induction was studied on modified MS medium using a variety of combinations of 2,4-D and Kn. Addition of tryptophan to different combinations of auxins and cytokinins increased the embryogenic callus mass. Calli have been successfully proliferated on MS supplemented with 1.0 mg/l of Kn and 0.5 mg/l of NAA. For regeneration the embryogenic callus was cultured on MS containing different concentrations of NAA and Kn and BAP and IAA. The somatic embryos developed into complete plantlets on regenerative media. The plantlets were then transferred to natural conditions for acclimatization.

Introduction

Rice is a cereal crop of tremendous economic importance. It is a staple food for a large human population. It is feared that world population would be around 10 billion by 2050. Thus more food will be required to feed the human population. This will be in the backdrop of diminishing cultivated land. Studies are underway to increase yield as well as quality of rice. Introduction of golden rice is a tremendous achievement in this direction.

The available cultivable land is being utilized for non-agricultural purposes (Shazia, 1996). Similarly, salinity is making a great chunk of land uncultivable. Attacks by pests and insects are responsible for decrease in production. Thus there is a constant need to improve crops to overcome all these hazards. Resistant varieties/cultivers have to be developed to cope up with the situation. Induction of tolerance requires an efficient breeding system. These days it can be achieved by *in vitro* studies. However, before actual breeding studies, methods have to be developed to devise a protocol for efficient plant regeneration, which could afterward be used for induction of tolerance.

Somatic embryogenesis in rice have been reported from culture of leaf tissue (Wernicke *et al.*, 1981), root tissue (Abe & Futsuhara, 1985), inflorescence (Chen *et al*., 1985) and protoplast (Yamada *et al*., 1986). Rueb *et al*., (1994) developed a reproducible and efficient procedure for regeneration of rice plants from callus through somatic embryogenesis.

Organogenic capacity of callus tissues depends upon the plant species, type of explant from which the callus was derived, age of callus tissue and composition of the nutritional medium. Another important factor is nature and level of various growth regulators.

Although there has been some success in plant regeneration from rice tissue culture, the protocol so developed is not applicable to all the cultivars of rice. Therefore, suitable method has to be developed for the local rice varieties. Swat- II rice was selected for the present studies because it is a localized variety and no regeneration system has been reported earlier.

Materials and Methods

Callus cultures of rice were initiated on mature caryopsis on a modified Murashige & Skoog's (1962) medium supplemented with 4% sucrose. In addition this medium contained various growth hormones. After dehusking, the seeds were washed with tap water to remove dust and other particles. The cleaned explants were placed in 70 % alcohol for five minutes and surface sterilized with 1% $HgCl_2$ solution for three minutes. The seeds were then rinsed three times with sterile distilled water to remove all the traces of mercuric chloride. The explants were afterwards inoculated onto the medium. The pH of the medium was adjusted to 5.6 and then solidified with 0.9% agar. The phytohormones were added to the medium before pH adjustment. The medium was then autoclaved at 15 psi for 15 minutes at 120^0C. Cultures were kept in 16 hours cycled fluorescent light cooled incubators with temperature regulated at 25 ± 1^0C. Phytohormones used and their respective concentrations are mentioned in the results section.

Results

To achieve embryogenesis and plantlet regeneration the following procedures were adopted.

a. Callus formation: Sterilized caryopsis of rice *(Oryza sativa* L. Swat-II) were inoculated on MS medium supplemented with 4% sucrose as the carbon source. Various growth regulators at 1.0mg/l 2,4-D + 0.5 mg/l Kn + 50 mg/l TPN, 1.0 mg/l 2,4-D + 1.0 mg/l Kn + 50 mg/l TPN, 1.0 mg/l 2,4-D + 0.5 mg/l BAP + 50 mg/l TPN, 1.0 mg/l 2,4-D + 0.3 mg/l Kn were added to the basal medium. Before the actual experimentation, the seeds were inoculated on plain basal medium (i.e. MS). After about three days the caryopsis germinated on MS containing 4% of sucrose. Moreover, a little callus formation was observed at the base of the coleoptile after another 10 days. This callus was isolated and inoculated onto the same fresh medium. However, it did not exhibit a reasonable growth. The callus so formed was then inoculated onto MS containing 2,4-D (1.0 mg/l) and Kn (0.3 mg/l). A reasonable callus formed after about four weeks of culture. This callus was soft, friable, brown coloured and non-embryogenic (Table 1). Further proliferation was observed when this callus was subcultured on the same medium. However, this callus was accompanied by no embryo formation. The callus so obtained was used for regeneration as reported in a later section.

To induce embryogenic callus, when the caryopsis were inoculated onto MS containing 2,4-D (1.0 mg/l) + Kn (0.5 mg/l) + TPN (50 mg/l), a yellowish white, compact and somewhat embryogenic callus got induced after about six weeks (Table 1, Fig. 1). Nonetheless, a soft, friable and non-embryogenic callus resulted on MS containing 1.0 mg/l each of 2,4-D and Kn and 50 mg/l 0f TPN. However, a copious, compact and embryogenic callus also got induced on MS containing 2,4-D (1.0 mg/l) +BAP (0.5 mg/l) +TPN (50 mg/l). All these calli when subcultured on the same fresh media, which subsequently exhibited reasonable proliferation and maintained their embryogenic or non-embryogenic nature for a long duration.

b. Callus proliferation: The callus obtained on MS + 2,4-D (1.0 mg/l)+Kn (0.3 mg/l) was inoculated onto MS with various growth hormones. When the callus was inoculated onto MS + IAA (0.5 mg/l) + BAP (1.5 mg/l), a slight callus proliferation was observed (Table 1, Fig. 2). Green columnar projections also developed in this callus which later on developed into plants. Probably the structures developed were shoot primordia.

Table 1. Callus induction, proliferation, somatic embryogenesis and plantlet formation in rice caryopsis cultured on MS medium under the influence of various phytohormones.

Nature of explant	Pytohormones mg/l			Culture period (weeks)	Remarks
	2,4-D		Kn		
Caryopsis	1.0		0.3	4	Soft, friable, brown coloured non-embryogenic callus induction.
	2,4-D	TPN	Kn		
= "	1.0	50	0.5	6	Yellowish-white, compact callus induction.
= "	1.0	50	1.0	4	Soft, friable, non-embryogenic callus induction.
	2,4-D	TPN	BAP		
= "	1.0	50	0.5	4	Copious, compact, embryogenic callus got induced.
	IAA		BAP		
= "	0.5		1.5	4	Slight callus proliferation along with shoot primordia.
	Kn		NAA		
= "	0.5		0.5	4	Callus proliferation along with meristemoid formation.
= "	1.5		0.5	4	Luxuriant callus proliferation along with embryoid formation.
= "	2.0		0.5	2	Excellent embryoid induction
	BAP		IAA		
= "	1.5		0.5	4	Somatic embryos got developed on the callus, which later on developed into complete plantlets.

The callus obtained on MS + 2,4-D (1.0 mg/l) + Kn (0.3 mg/l) was sub-cultured onto MS containing various concentrations of Kn and NAA. When the callus was inoculated on 0.5 mg/l each of Kn and NAA, callus proliferation alongwith shoot buds (meristemoids) formation was observed. These meristemoids afterward developed into plantlets. Meristemoid formation increased when the concentration of Kn was raised from 0.5 mg/l to either 1.0 or 1.5 mg/l (Table 1).

The callus, which formed on MS + 2,4-D (1.0 mg/l) + Kn (0.5 mg/l), was sub-cultured on MS containing various concentrations of different growth hormones (Table 1). As evident from the result, bud like structures (meristemoids) formed in this callus which exhibited reasonable regenerating capability.

c. Somatic embryogenesis: Embryogenic calli obtained on mature caryopsis (Table 1) were further transferred to different regeneration media i.e., Kn 0.5 mg/l + NAA 0.2 mg/l, Kn 1.5 mg/l + NAA 0.5 mg/l, Kn 2.0 mg/l + NAA 0.5 mg/l and BAP 1.5 mg/l + IAA 0.5 mg/l etc. When the embryogenic callus was sub-cultured on MS medium containing 0.5 mg/l Kn and 0.2 mg/l of NAA, excellent callus growth with moderate regeneration was observed within a culture period of three weeks. This callus was friable, loose textured and creamy- yellow with slight tinge of green colour. This greening was due to the globular structures, probably the embryoids, which later on gave rise to complete plantlets within four weeks (Table 1). Similarly, luxuriant callus proliferation along with abundant embryoid formation was also observed on MS medium supplemented with 1.5 mg/l Kn and 0.5 mg/l NAA. These embryoids gave rise to plantlets after about four weeks (Table 1, Fig. 2).

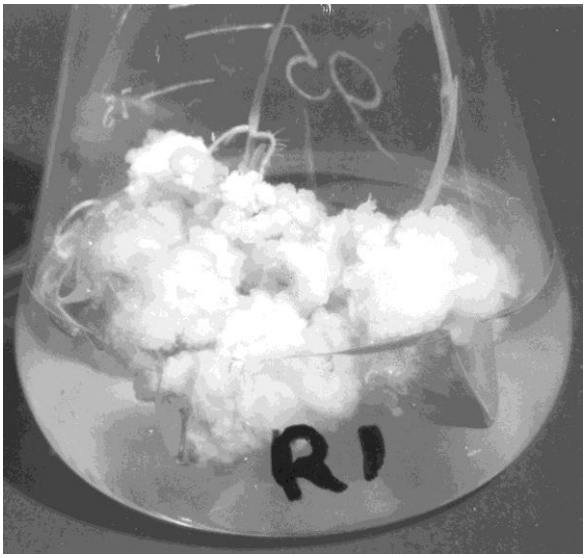

Fig. 1. Callus induction on rice caryopsis cultured on MS medium containing 1.0 mg/l 2,4-D, 0.5 mg/l Kn and 50 mg/l trytophan.

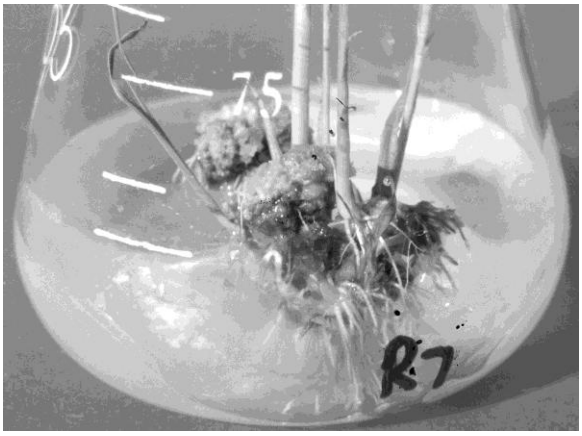

Fig. 2. Embryoid induction and its subsequent regeneration into plantlets on MS containing 1.5 mg/l Kn and 0.5 mg/l NAA.

When the concentration of Kn was further enhanced to a level of 2.0 mg/l, while keeping NAA concentration at 0.5 mg/l, excellent embryoid induction was observed within two weeks. The number of embryoids were about 20 per callus calture in the third week. These embryoids started germination and developed into complete plantlets after four weeks (Table 1).

Luxuriant small green columnar projection formation, probably the somatic embryos, was also noticed on the callus which was cultured on the MS + BAP (1.5 mg/l) + IAA (0.5 mg/l). Within four weeks, these embryos started germination and developed into complete plantlets. These plantlets were healthy and could be easily transferred to field conditions.

The *in vitro* regenerated plantlets after attaining a size of 2-3 inches were then taken out from aseptic containers to the natural conditions. Before transfer to the pot, the plantlets were first cultured in water for 2-3 days. The regenerants were then transferred to small pots containing loamy soil. These plantlets were kept in a green house which got acclimatized to the natural conditions and exhibited normal growth and development.

Discussion

The present studies were undertaken to induce reproducible embryogenic callus and produce plants through somatic embryogenesis. As evident from the results a reasonable callus was induced when MS was supplemented with 2,4-D and Kn. These studies are in line with the reports of Shazia (1996) who obtained good callus on a medium containing an auxin and cytokinin. This combination was excellent for callus proliferation and regeneration. Bud like structures and subsequent plantlets formation has also been reported in wheat (Ilahi, 2000).

Further a soft, friable and non-organogenic callus formation occurred in rice caryopsis when the MS medium contained 1.0 mg/l 2,4-D and 0.3 mg/l Kn. The addition of tryptophan to increase the formation of green and compact regions in the callus also helped in an increase in embryogenic callus mass. Similarly excellent embryogenic callus growth was observed with 1.0 mg/l 2,4-D + 0.5 mg/l Kn + 50 mg/l TPN. These findings are in line with those where TPN was found to promote the growth of embryogenic callus in rice culture (Siriwardana & Nabors, 1983).

Quick success in morphogenesis may be correlated with the natural regenerative potential of plant tissues. In rice somatic embryos got developed on the embryogenic callus under the influence of appropriate balance of exogenous auxins and cytokinins. However, tryptophan is also required for embryogenic callus induction. These studies are in line with those of Chowdhry *et al*., (1993). The embryos then developed into complete plants, which were transferred to pots after proper acclimatization.

The embryogenic callus obtained on MS +2,4-D + Kn + TPN was subcultured on MS + Kn + NAA. The embryoids then developed into plantlets. As reported earlier, the cereals need a callus induction medium followed by the proliferation medium. To induce plants another medium has to be developed (Shazia, 1996). Ling & Yoshida (1987) reported that 1.0 mg/l each of 2,4-D and Kn was necessary for induction of embryogenic callus in the Japonica rice. However, lower concentrations were required for plantlet development. Similar results were obtained by Carmen *et al*., (1987) that higher concentration was necessary for embryogenic callus induction and maintenance, but a lower for plant formation in the embryos. As a result of these studies, a protocol has been developed for embryogenic callus formation and its subsequent regeneration into plantlets.

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(Received for publication 20 January 2005)