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RESEARCH PAPER

In vitro plant regeneration from male flowers of banana

Md Tipu Sultan[1], Mahboob Hossain Khan[1], Md Lokman Hakim[2], ANK Mamun[2], Md Alam Morshed[1, 3 *], Md Rofiqul Islam[2], Md Rokibul Islam[1]

[1]Department of Biotechnology and Genetic Engineering, Faculty of Applied Science and Technology, Islamic University, Kushtia 7003, Bangladesh.

[2]Institute of Food and Radiation Biology (IFRB), Atomic Energy Research Establishment (AERE), Savar, Dhaka, Bangladesh.

[3]Department of Pharmacy, North South University, Bashundhara, Dhaka 1229, Bangladesh.

*Corresponding author: morshedbt@gmail.com

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Abstract

The present study was undertaken with a view to establish a protocol on *in vitro* regeneration of plants by using young male flowers of banana (*Musa* sp. cv. Sabri) as explant. Appropriate developmental stage of immature male flowers for inoculation, medium composition for induction of calli, regeneration of plants, rooting of *in vitro* regenerated shoots, acclimatization of *in vitro* regenerated plantlets and *ex vitro* establishments of plantlets were worked out. Young male flowers obtained by striping away the bracts in between 24 to 26 were found suitable as explants for induction of callus. The isolated male flowers were cultured on MS (Murashige and Skoog) medium supplemented with different concentrations and combinations of three auxins viz. 2, 4-D (2,4-Dichlorophenoxyacetic acid), NAA (Napthaleneacetic acid) and IAA (Indole-3-acetic acid) for induction of callus. Only two of the total twenty

two medium composition yielded calli. The better response (20%) was recorded in MS medium containing 2.0 2, 4-D + 0.5 NAA + 0.5 IAA (Indole-3-acetic acid) (mg/l). The calli were cultured on MS medium fortified with different concentrations of BA (Benzyladenine), NAA, IAA and Glutamine or Caesin hydrolysate (CH) to regenerate shoots. MS medium having the supplementation of 1.0 BA + 0.5 IAA + 500 CH (mg/l) was appeared best for regeneration of shoots. Single isolated regenerated shoots were implanted on MS medium supplemented with three different concentrations (0.5, 1.0 and 2.0 mg/l) of IBA or NAA to induce root. IBA at a concentration of 1.0 mg/l produced best rooting. The plantlets were gradually acclimatized and transferred to the soil. The survival percentage was about 90%.

Key words: *Musa* sp. cv. Sabri, indirect organogenesis, callus, plyploids, somaclonal variation.

Introduction

Banana is one of the most important and remunerative cash crops grown round the year in Bangladesh. The energy and nutritional status of banana are much higher than other common tropical and subtropical fruits. The average yield of banana is 14 t/ha, which is lower compared to other banana-producing countries in the world (Islam and Hoque, 2004). Higher yield of banana can play a pivotal role in the economy of Bangladesh. It is possible to increase the yield of banana by using disease free high yielding variety, modern technology of production as well as post harvest management. Banana (*Musa* spp.) is one of the most important nutritious fruit crops of the world and grown in many tropical areas where they are used both as a staple food and dietary supplements (Assani *et al.*, 2001).

In our country, Sabri (AAB) is the second important commercial cultivar of banana after Amritsagar (AAA) (Islam and Hoque 2004). However, cultivar Sabri is highly susceptible to panama disease (*Fusarium* wilt) caused by *Fusarium oxysporum* ssp. *cubense*. So, there is a scope to improve this variety by the development of somaclonal variant through indirect oraganogenesis. Most of the edible bananas are

sterile polyploids and must be propagated vegetatively. So, genetic improvement of this plant through cross breeding is an insurmountable task. Tissue culture technique using shoot or meristem tips are suitable for large-scale production of uniform and vigorously growing propagules for field establishment. The combination of mutation breeding and *in vitro* culture has been suggested as an alternative approach for banana improvement (Novak *et al.,* 1990). However, the main limitation of this technique is the high degree of chimerism. Repeated vegetative propagation is needed to dissociate chimeras, but the minimum requiring number of cycles is unknown (Roux, 2004). So, somaclonal variation may be an alternative option for the improvement of banana (A.H. Kabir et al., 2008; Nasrin,S. et al., 2003; Denise M. Seliskar et al., 2000; Larkin, P.J. et al., 1981;). Keeping the above significant points in mind, the present research aimed at regenerating plants through indirect organogenesis in banana by using young male flower buds as explants. The specific objectives of the present study are (i) to find out suitable stage of development of male flower bud as explant, (ii) to optimize growth regulators in culture medium for induction of calli and for shoot regeneration from calli, (iii) to optimize auxins in the culture medium for induction of root in regenerated shoot, and (iv) to acclimatize, harden and establishment of the plantlets in the soil.

Materials and methods

Male buds of banana cultivar 'Sabri' (AAB) were collected from the orchard grown plants located in the premise of the Institute of Food and Radiation Biology (IFRB), Atomic Energy Research Establishment (AERE), Savar, Dhaka, in May, 2007. Very young male flower buds, isolated from the male buds, were used as explants. Male flower buds (Fig. A) were collected from the plants grown in field (orchard) and were surface sterilized by cotton swab soaked in 70% ethanol. To collect the young male flowers, 15/16 bracts were removed together with their male flower from the rachis (Fig. B). Then the male flower buds were again cleaned with absolute alcohol by using cotton swabbed with ethanol. Young male flowers were isolated with the help of a pair of sterilized forceps and transferred to the sterilized Petri dish containing moistened filter paper to prevent desiccation of flowers (Fig. C).

Isolated 5-6 young (immature) male flowers were inoculated in one glass jar containing callus / induction agar-gelled (8% w/v) MS based medium (Fig. D, E). MS

medium contained basic macro- and micro-nutrients, and supplemented either with four different concentrations (1.0, 2.0, 3.0 and 4.0 mg/l) of 2,4-D singly or a combination treatment of 2,4-D+IAA, 2,4-D+NAA and 2,4-D+NAA+IAA. In combination treatment, two concentrations of 2, 4-D (2.0 and 4.0 mg/l) were used and against each concentration of 2, 4-D, there were three concentrations viz., 0.1, 0.5 and 1.0 (mg/l) of other auxins like IAA, NAA and NAA+IAA (Table 1). Sucrose at a concentration of 3% was used as carbon source in all of the cases. The pH of the medium was adjusted to 5.7 before autoclaving. Cultures were kept at 25±2°C in darkness and maintained on the same medium without subculture until callus is produced. Calli thus obtained were cut into suitable pieces which ranged 2-4 for each callus depending on size and were transferred to fresh medium composed of MS medium (Table 2) fortified with three different concentrations of BA (0.5, 1.0 and 2.0 mg/l) together with a fixed concentration of NAA (0.2 mg/l) and glutamine (100 mg/l) or with a fixed concentration of IAA (0.5 mg/l) and Caesin hydrolysate (500 mg/l), were employed to regenerate shoots from calli and were kept under fluorescent illumination (at 1500 lux intensity) with 16/8 h light/dark cycle. Shoots thus obtained from calli in regeneration medium were multiplied by subculture to a considerable number to be used to induce root. Two auxins viz., IBA and NAA were used in three different concentrations (0.5, 1.0 and 2.0 mg/l each) in MS medium for root induction (Table 3).

The regenerated plantlets after developing sufficient root system were transferred to small earthen pots containing garden soil and compost in 1:1 ratio. The plantlets containing pots were covered with polythene bags to protect sudden desiccation. The inner sides of this bag were sprayed with water at every 24 h to maintain high humidity around the plantlets. The polythene bags were gradually perforated to expose the plantlets to the outer normal environment and subsequently removed after seven days. By this time the plantlets were established in to the soil. Data were recorded on days required to callus induction, per cent of callus induction, number of shoots and roots, length of shoots and roots and expressed as mean with standard error (SE) whenever applicable.

Fig. 1. Male flower culture in of banana cv. Sabri (AAB). **A**: Male buds harvested for isolation of very young male flower buds. Plate **B**: Male buds after removal of 15 layers of bracts together with male flowers **C**: Isolated young male flowers of cv. Sabri ready for inoculation. **D**: Young male flowers cultured on agar-gelled MS media. **E**: Young male flower buds cultured on phytagel-gelled MS media. **F**: Induction of calli from immature male flowers cultured on to MS medium fortified with 2.0 2, 4-D+0.5 NAA+ 0.5 IAA (mg/l). **G**: Initiation of shoot regeneration from callus cultured on to MS medium supplemented with 1.0 BA+0.5 IAA+500 CH (mg/l). **H**: Rooting of *in vitro* regenerated shoot cultured onto MS medium supplemented with 1.o (mg/l) IBA. **I**: Acclimatized and hardened plantlets in polypags containing garden soil and compost (1:1).

Results and discussion

In the present study, callus induction was carried out with male flowers at different developmental stages. i.e., at bracts number 20 to 30 of banana cultivar Sabri. Among them the male flowers of bracts numbering 24 to 26 gave best response to callus induction, which was almost similar to the result that was obtained by Bakry *et al.* (2008). They investigated through the haploid induction, androgenesis from anther culture of banana *Musa balbisiana* (BB) that, the male flower of bracts number of 21 to 22 was the best developmental stage for callus induction. This explant was cultured onto MS medium supplemented with different concentrations of 2, 4-D singly or in combination with NAA or IAA or NAA+IAA in order to find out the suitable culture media for callus induction. Among all the treatments, maximum (20%) callusing was found in the medium containing 2.0 2, 4-D+0.5 NAA+0.5 IAA (mg/l). Here 2, 4-D singly at the concentration of 1.0, 2.0, 3.0 and 4.0 (mg/l) did not induce any callusing (Table 1). MS medium supplemented with 2.0 2, 4-D+0.5 NAA+0.5 IAA (mg/l) was observed to be the best for induction of callus from young male flowers in the present study (Fig. F). Many workers observed 2, 4-D as the most effective auxin for callus induction as common as in monocot and even in some dicots (Hong and Debergh 1995; Alsadon *et al.* 2004). Kulkarni *et al.* (2006) obtained embryogenic calli from male flowers of banana by culturing them onto MS medium supplemented with 2, 4-D.

The calli thus obtained were further transferred to fresh MS medium containing different concentrations of BA (0.5, 1.0 and 2.0 mg/l) with either the same concentration of NAA (0.2 mg/l) and glutamine (100 mg/l) or (0.5 mg/l) IAA (0.5 mg/l) and caesin hydrolysate (500 mg/l) in order to find out suitable media composition for regeneration of shoots. Out of six treatments, two combinations of BA 1.0 + IAA 0.5 + CH 500 (mg/l) and 2.0 + IAA 0.5 + CH 500(mg/l) were found to regenerate shoots (Table 2). The maximum response (Fig: G) was found in 1.0 BA+0.5 IAA+500 CH (mg/l) followed by 2.0 BA+0.5 IAA+500 CH (mg/l).

Table 1. Effects of different concentrations of 2,4-D alone or in combination with NAA, IAA or NA+IAA in MS medium for callus induction from young (immature) male flowers of banana cv. Sabri.

Growth regulators (mg/l)	No. of male flowers inoculated	No. of male flowers responded to induce callus*	% of male flowers produced callus
2,4-D			
1.0	40	0	0
2.0	40	0	0
3.0	40	0	0
4.0	40	0	0
2,4-D + IAA			
2.0 + 0.1	40	0	0
2.0 + 0.5	40	0	0
2.0 + 1.0	40	0	0
4.0 + 0.1	40	0	0
4.0 + 0.5	40	0	0
4.0 + 1.0	40	0	0
2,4-D + NAA			
2.0 + 0.1	40	0	0
2.0 + 0.5	40	0	0
2.0 + 1.0	40	0	0
4.0 + 0.1	40	0	0
4.0 + 0.5	40	0	0
4.0 + 1.0	40	0	0
2,4-D + NAA + IAA			
2.0 + 0.1 + 0.1	40	3	7.5
2.0 + 0.5 + 0.5	40	8	20.0
2.0 + 1.0 + 1.0	40	0	0
4.0 + 0.1 + 0.1	40	0	0
4.0 + 0.5 + 0.5	40	0	0
4.0 + 1.0 + 1.0	40	0	0

* Calli was induced after 7-9 weeks of culture in the dark. 12 replications were used for each hormonal treatment.

Table 2. Effects of different concentrations and combinations of BA with auxins and additives in MS medium on regeneration of shoots from calli.

Growth regulators/ additives (mg/l)	No. of calli inoculated	No. of calli responded to produce shoot	No. of shoots regenerated from calli*	Length of shoot (cm)
BA + NAA + Glutamine				
0.5 + 0.2 + 100	6	0	0	0
1.0 + 0.2 + 100	6	0	0	0
2.0 + 0.2 + 100	6	0	0	0
BA + IAA + Caesin hydrolysate				
0.5 + 0.5 + 500	6	0	0	0
1.0 + 0.5 + 500	6	4±0.25	11±0.54	3.22±0.55
2.0 + 0.5 + 500	6	2±0.21	5±0.43	5.11±0.96

* Data were collected after 6 weeks of culture. 12 replications were used for each hormonal treatment. Data were expressed as Mean ± SE.

Table 3. Effects of different concentrations of IBA and NAA on root induction in *in vitro* regenerated shoots.

Auxins	Concentrations (mg/l)	Number of roots/shoot*	Root length (cm)*	% of shoots rooted
IBA	0.5	4.3±0.66	2.20±0.76	92
	1.0	5.6±1.13	3.96±0.51	100
	2.0	4.5±1.61	3.65±0.86	83
NAA	0.5	3.8±1.35	1.59±0.45	66
	1.0	4.6±0.64	2.38±0.44	58
	2.0	4.2±0.49	2.79±0.61	66

* Data were collected after 5 weeks of culture. Average values of 12 replicates and standard error.

This significant result is also supported by the result obtained by Hakim *et al.* (2008). They regenerated the plants from anther-derived callus of banana cv. Bichikala by culturing the material onto MS medium supplemented with 0.5 BA + 0.4 IAA+500 CH (mg/l).In case of BA, Hakim *et al.* (2008) got suitable shoots at the concentration of 0.5 (mg/l) but we got suitable shoot at the concentration of 1.0

(mg/l) which is exactly similar to the result reported by Kulkarni *et al*. (2006). They reported germination of somatic embryos derived from calli of male flowers of banana by culturing them on MS medium fortified with 1.0 BA (mg/l). We got almost the same result as that was reported by Hakim *et al*. (2008) in case of IAA, where they used IAA at the concentration of 0.4 (mg/l) whereas we used 0.5 (mg/l) and we got exactly similar result with CH 500(mg/l).

Shoots, regenerated from the calli, were sub-cultured to increase their number for root induction. The individual shoots were placed onto MS medium (Table 3) containing three different concentrations (0.5, 1.0, 2.0 mg/l) of IBA or NAA for root induction. All the treatments followed in root production. Both IBA and NAA at a concentration of 1.0 (mg/l) produced the best result for induction of root in *in vitro* regenerated shoots of banana cv. Sabri. The present findings are in conformity with those of Raut and Lokhande (1989) and Khanam *et al*. (1996) who used MS medium supplemented with different concentrations of IBA for good rooting of banana. On the other hand, Bhaskar *et al*. (1993) observed best rooting on Khudson's medium supplemented with 5 mg/l NAA in case of banana.

Most of the edible bananas are sterile polyploids and must be propagated vegetatively through suckers. So in bananas, there is very little scope to create variability which is the raw material of evolution or to develop improved varieties. Indirect organogenesis via callus from immature male flowers in banana opens up a new way to develop somaclonal variants. Somaclonal variation among plants regenerated through callus culture was reported by Kaeppler *et al*. (2000) and Nasrin *et al*. (2003). Plants propagated *in vitro* are not transferred readily to an open environment. In this present investigation, regenerated plantlets were established in small poly bags containing garden soil and compost (1:1). They were acclimated to outdoor conditions. About 90% plantlets were established successfully in the field conditions (Fig. I).

Findings of the present study elucidate that plant regeneration could be possible from the calli developed from immature male flowers of banana. Although the frequency of callus induction and plant regeneration from immature male flowers in the present study are not so high, there is still a scope to improve organogenesis by further manipulations of the medium composition and cultural conditions.

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