

Recovery of Green Plantlets from Albino Shoot Primordia Derived from Anther Culture of Indica Rice (*Oryza sativa* L.)

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Abstrak: Suatu kaedah mudah dibangunkan yang membenarkan regenerasi tumbuhan albino daripada kultur anter Hobigonj Boro (Hbj B) IV and Hbj B VI, dua *varieti* tempatan padi indica beraromatik dari Bangladesh. Tiga faktor penting telah dikenalpasti untuk tunas/pucuk primordia albino untuk bertukar menjadi pucuk/tumbuhan hijau dalam kultur; komponen-komponen medium induksi M10, saiz kalus (0.2–0.4 cm panjang) dan tinggi tunas/pucuk primordia (2–3 mm). Perpindahan serta-merta tunas/pucuk primordia (2–3 mm) daripada medium M10 ke medium regenerasi diikuti dengan inkubasi berterusan di bawah lampu pendarfluor (100-lux, 25±1°C) telah mencetuskan penukaran tunas/pucuk primordia albino menjadi hijau dalam 2–3 hari. Saiz kalus tidak menunjukkan sebarang perubahan. Pucuk albino yang dihasilkan daripada kalus anter yang dikultur di dalam media KA, KB, KC, KD dan KE tidak pulih dalam kedua-dua varieti. Perpindahan pucuk primordia albino lebih pendek atau panjang dari 2–3 mm daripada kelima-lima kultur tersebut ke medium regenerasi tidak menyebabkan pucuk primordia untuk bertukar menjadi hijau. 100% pucuk primordia albino yang dihasilkan daripada Hbj B VI dan 79% daripada Hbj B IV dalam medium M10 bertukar menjadi hijau selepas dipindahkan ke medium regenerasi. Kultur seterusnya dan subkultur tumbuhan hijau menunjukkan formasi cepat pucuk hijau yang banyak.

Kata kunci: Hobigonj Boro (Hbj B) IV dan VI, Sifat Albino, Padi Indica Beraromatik, Kultur Anther, Tumbuhan Hijau

Abstract: A simple method was developed to permit albino plant regeneration from anther culture of Hobigonj Boro (Hbj B) IV and Hbj B VI, two local varieties of aromatic indica rice from Bangladesh. Three crucial factors were identified for the albino shoot primordia to change into green plantlets in culture; components of M10 induction medium, callus size (range 0.2–0.4 cm long) and height of shoot primordia (range 2–3 mm). Immediate transfer of shoot primordia (2–3 mm) from M10 medium to regeneration medium followed by continuous incubation under fluorescent light (100-lux, 25±1°C) triggered albino shoot primordia to turn green in 2–3 days. Callus size did not show any effect on the change. Albino plantlets derived from anther callus cultured in KA, KB, KC, KD and KE media did not recover in both the varieties. Transfer of albino shoot primordia shorter or longer than 2–3 mm from the above 5 cultures to regeneration medium did not cause the shoot primordia to turn green. 100% albino shoot primordia initiated from Hbj B VI and 79% from Hbj B IV in M10 medium changed to green plantlets upon transfer to regeneration

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medium. Subsequent culture and subculture of green plantlets showed rapid formation of many new green plantlets.

Keywords: Hobigonj Boro IV and VI, Albinism, Aromatic Indica Rice, Anther Culture, Green Plant

INTRODUCTION

Anther culture technique is widely used to shorten the breeding cycle in plant variety development programs. The technique affords rapid generation of a large number of doubled haploid lines from F₁ hybrids, reducing the number of generations required to reach homozygosity in the developing variety. A number of studies have revealed that the composition and physiochemical conditions of culture medium retain and revive the embryogenic capability of rice callus and enhance green plant regeneration (Karim & Zapata 1996, 1990; Shahjahan *et al.* 1992; Mohiuddin *et al.* 2006; Geng *et al.* 2008). However, albino plant regeneration is a common feature from anther culture especially in rice varieties (Babbar *et al.* 2000). Reports show that the frequency of albino regeneration ranges from 10% (Genovesi & Magill 1979) to 100% (Tsay *et al.* 1982) in rice. Similar frequencies are observed in in vitro culture conditions for other crops such as *Triticum aestivum* (Ouyang *et al.* 1973), *Datura metel* (Narayanaswamy & Chandy 1971), *Capsicum annum* (Maheswary & Mak 1993) and *Aesculus carnea* (Radojevic *et al.* 1989).

Regeneration of albino plants from in vitro anther or callus culture from various species, including rice, has become a significant problem (Babbar *et al.* 2000). Research on anther culture found that the production of albino plants from many species was controlled by both genetic and environmental factors (Babbar *et al.* 2000; Tsay *et al.* 1988). Sun *et al.* (1979) reported that the basic cause of albinism in rice is impairment of DNA (probably due to presence of chemicals added to the media) in plastids or nuclei or in both of them. This study also identified the absence of ribosomes in ill-developed plastids as another cause of albinism in rice.

Apart from genetic factors many physical conditions including the chemical composition of growth medium was found crucial for overcoming albinism and induction of green plants in rice (Babbar *et al.* 2000). The increased efficiency of producing doubled haploid green plants in rice is a prerequisite for generating true to type genotypes through anther culture. Keeping this objective in view, an intensive study was carried out to recover green plantlets from albino shoot primordia.

MATERIALS AND METHODS

Anthers from two aromatic indica rice varieties were used in this study; Hobigonj Boro (Hbj B) IV and Hbj B VI. Plants were grown in a net house under optimum agronomic practices during the Boro season (November to April). Shoots with appropriately staged anthers (uninucleate/miduninucleate, reduction division) in

spikelets were collected when the distance between the subtending leaf and the flag leaf was 12–16 cm in length. Before plating the anthers, shoots were incubated at 8°C for 8–10 days (Afza *et al.* 2000; Shahjahan *et al.* 1992). Panicles were separated from shoots, cut into small pieces and surface sterilised by submerging in 70% denatured alcohol (Keru and Co., Bangladesh) for 2–3 minutes before being placed onto sterile filter paper in Petri dishes (90 x 15 mm) to absorb the excess alcohol.

Spikelet tips were cut horizontally using forceps and a pair of scissors. The spikelets were stroked to release the anthers onto the surface of callus induction medium (CIM). The CIM medium was B5 (Gamborg *et al.* 1968) containing 1.0 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), 0.5 mg/l indole-3-acetic acid (IAA) and 0.5 mg/l 6-benzylamino purine (BAP). The medium was solidified with 8.0 g/l agar (Merck, Germany) and was designated as KA or B5 (control in this case). Four additional media designated KB, KC, KD and KE were prepared for this study by supplementing B5 medium (Gamborg *et al.* 1968) with above mentioned hormonal concentrations (KA) and concentrations of sodium chloride (NaCl) and sodium sulphate (Na₂SO₄) described in Table 1 (Karim & Zapata 1996).

Table 1: Hormonal and salt concentrations of defined media.

Type of media	Composition	Concentration of NaCl (g/l)	Concentration of Na ₂ SO ₄ (g/l)
KA	B5 medium containing 1.0 mg/l 2,4-D, 0.5 mg/l IAA and 0.5 mg/l BAP and 8.0g/l agar	0.0	0.0
KB	KA +	0.0	7.5
KC	KA +	5.0	0.0
KD	KA +	0.0	5.0
KE	KA +	2.5	0.0

A new medium known as M10 (Karim & Zapata 1990) was prepared with modified B5 medium (B5 medium with addition of 0.03 mg/l CoCl₂, 6H₂O, 0.2 mg/l glutamine and 0.5 mg/l L-cystiene) (Karim & Zapata 1996) with hormone combinations of 1-naptha-leneacetic acid (NAA) (1.5 mg/l), 6-benzyladenine (BA) (0.5 mg/l), indole-3-butyric acid (IBA) (0.5 mg/l) and Kinetin [N-2-furanylmethyl)-IH-purine-6-omine] (K) (0.75 mg/l).

All media (KA, KB, KC, KD, KE and M10) was adjusted to pH 5.7 with 1N/0.1N NaOH before autoclaving at 121°C for 15 minutes at 1.05 kg/cm⁻² pressures (15–20 psi). 15 ml of autoclaved medium was dispensed on sterile glass Petri dishes (60 x 15 mm, Germany) and cooled. Anthers were plated and the Petri dishes were sealed using semi-transparent and moisture resistant Parafilm^R (USA). Petri dishes were incubated in the dark at 25±1°C to initiate callus growth to the desired size (range 0.2–0.4 cm) and to initiation of shoot primordia.

Appropriately sized shoot primordia (2–3 mm) and calli of optimal size (0.2–0.4 cm) were transferred to regeneration medium consisting of MS medium (Murashige & Skoog 1962) supplemented with 1.0 mg/l each of K and NAA.

Regeneration medium was adjusted to pH 5.7 and autoclaved prior to addition of 8.0 g/l agar. Callus cultures were incubated under cool white fluorescent and incandescent lamps (approximately 100 lux) at $25\pm 1^{\circ}\text{C}$ for regeneration. Regenerated plantlets were transferred to MS medium without hormones in test tubes (20.5 cm/8 in long) for further growth and rooting, and incubated in the conditions described above.

To acclimate plantlets, healthy, green, well-elongated (≥ 16 cm) and well-rooted regenerated plants were transferred to soil in small plastic pots. Pots were covered with transparent plastic bags and maintained in the shade in natural environmental conditions of $27\pm 1^{\circ}\text{C}$ and relative humidity of over 90%. After 1 week, the plastic bags were removed but the pots remained in the shade for another 3–4 days. Surviving plants were replanted in larger pots in a net house to allow for growth, elongation, flowering, fruiting and maturity.

The experiments were done using the Completely Randomised Design (CRD) method. The cultured samples were placed in different places but had the same in vitro culture conditions for callus initiation and shoot regeneration. Data on callusing and callus size were recorded before transfer to regeneration medium. Similarly, data on regeneration frequency and number of green and albino plant production were recorded after root shoot formation. A descriptive analysis was conducted using the recorded data.

RESULTS

Effect of Media on Callus Induction

Anthers of Hbj B IV initiated callus from all media with or without salt. Hbj B VI was observed to initiate callus formation from media KA, KB (with Na_2SO_4 , 7.5 g/l), KC (with NaCl, 5.0 g/l) and M10. The effect of media on callus initiation in Hbj B IV and Hbj B VI is shown in Figure 1. Callus initiation varied with the growth media for both varieties. A high number of anthers was observed for Hbj B IV and Hbj B VI initiated callus when cultured on KA and M10 media. The percentage of callus initiation from Hbj B IV was poor in KB, KC, KD and KE media, and Hbj B VI was not responsive in KD or KE media. Among all the media tested, M10 without salt supplementation showed the highest percent of callus induction with 10.3% and 9.2% in Hbj B IV and Hbj B VI, respectively (Fig. 1).

Lower percentages of callus induction were observed in both varieties in KA medium compared to M10 medium (Fig. 1). Callus mass in M10 medium was lower in comparison with other tested media. Two differently coloured calli, viz. white and light yellowish, was observed to initiate from anthers of both tested varieties. The texture of the white callus was compact while the light yellowish callus was fragile and glistening. M10 medium most often supported initiation of the white and compact callus.

Callus initiation started after 20 days of inoculation from Hbj B IV anthers and after 24 days of culture from Hbj B VI anthers on M10 medium without salt. Callus initiation for both varieties occurred within 29–37 days after inoculation on other tested media. Callus induction took longer with the inclusion of salt (NaCl and Na_2SO_4) in the media. Within 7–10 days calli reached at least 0.2 cm in size in all media except M10. Some calli did not enlarge and became blackish when

left on the same medium for a period of 3–4 weeks. Sub-culture at 7–10 day intervals caused an increase in size and cessation of blackening. Black calli did not expand further.

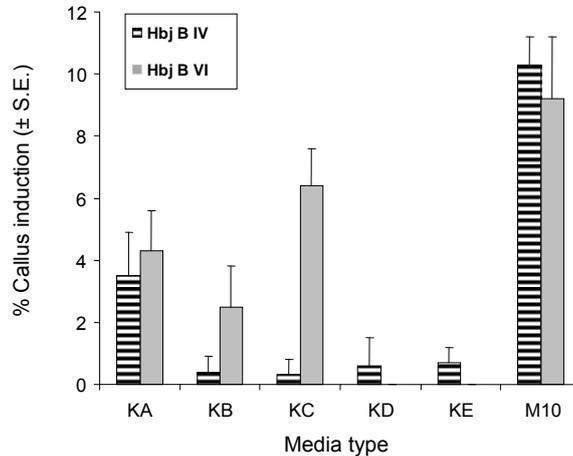


Figure 1: Callus induction from anthers of Hbj B IV and Hbj B VI.

Media Effect on Initiation of Albino Shoot Primordia

Regeneration of albino shoot primordia from callus occurred differently in both varieties and was dependent upon culture media (Fig. 2). Hbj B IV and Hbj B VI developed albino shoot primordia from calli in KA and M10 regeneration media. Hbj B IV anthers that provided poor amounts of callus were observed to develop into albino shoot primordia (100%) in M10 medium [Fig. 2 and 5(a)]. Poor regeneration was observed in this variety (8%) when anthers were cultured in KA medium. Unlike Hbj B VI, no regeneration response was observed on Hbj B IV on KB, KC, KD and KE media (Fig. 2) where poor callus initiation was found from anthers in these media in the presence of salts of NaCl or Na₂SO₄ (Fig. 1).

This study showed that the percentage of albino shoot regeneration varies in Hbj B VI on KA (42%), KB (39%), KC (30%) and M10 (42%) media. Without salt, KA induced a higher rate of regeneration in Hbj B VI in comparison to KB and KC media (Fig. 2). Although callus initiation percentage was quite high from Hbj B VI (6.4%) in KC medium, a lower regeneration (30%) was observed compared to other media tested (Fig. 2). A higher number of days (4–5) were required for observation of shoots from callus initiated in all media other than M10 media.

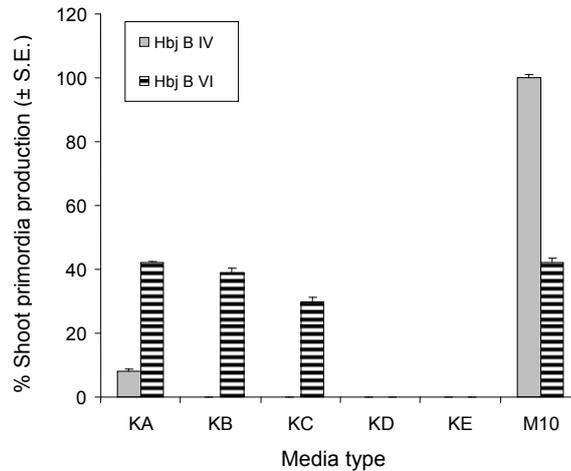


Figure 2: Albino shoot primordia initiation from anther derived callus of Hbj B IV and Hbj B VI.

Effect of M10 Medium on Rapid Albino Shoot Primordia Initiation

Hbj B IV and Hbj B VI responded differently when callus was induced using M10 medium in comparison to other tested media. In 8–12 days time, albino shoot primordia were observed for both varieties grown on M10 medium. A longer time was required for initiation of shoot primordia from callus induced in other media (e.g. KA, KB and KC) when transferred to a regeneration medium. Although higher percentages of callus were induced from Hbj B IV and Hbj B VI (10.3% and 9.2% respectively), the amount of callus initiated from both varieties on M10 medium was poor compared to other media tested. Albino shoot primordia directly initiated from both varieties when cultured in M10 medium. Subculture of callus was not necessary for initiation of shoot primordia from both the varieties cultured in M10 medium. In 4–6 days the initiated albino shoot primordia grew to 2–3 mm in height. At this stage both broad and slender leaves became apparent. A higher number of broad leaved shoots was observed from both varieties when grown in M10 media.

Effect of Callus Size on Albino Shoot Regeneration

Calli 0.2–0.4 cm in size was observed in initiated shoot primordia grown under fluorescent light and incubated in the dark. Calli shorter than 0.2 cm from Hbj B IV and Hbj B VI produced in CIM did not produce albino shoot primordia. On the other hand, calli longer than 0.4 cm turned black on most of the media within 10–12 days time and did not initiate any shoot primordia in both varieties.

Albino shoots were healthy and showed vigorous growth with extensive roots. Most albino shoots initiated rapidly from calli induced from a variety of media. A higher number of albino shoots were observed to initiate from Hbj B VI rather than Hbj B IV except when induced on M10 medium. Calli smaller or bigger than 0.2–0.4 cm initiated on M10 medium occasionally produced albino

shoot primordia. This phenomenon was not observed in calli of both varieties initiated from the other media tested.

Effect of Albino Shoot Primordia Length on Recovery of Green Shoots

All albino shoot primordia (100%) from Hbj B VI within the range of 2–3 mm in length obtained from M10 medium turned green in 2–3 days time (Fig. 3) upon immediate transfer to regeneration medium and incubation under fluorescent and incandescent light (intensity approximately 100 lux) at 25°C. On the other hand, under the same conditions only 79% albino shoot primordia of Hbj B IV gradually turned green [Fig. 5(b)] when incubated under the same culture conditions (Fig. 3). Only 21% of the albino shoot primordia from this variety (Hbj B IV) remained albino and no recovery was found even with incubation for a longer time under the same conditions.

The recovery of green plantlets in this study was observed to occur in three ways: (i) a gradual conversion of albino shoots to green, (ii) direct recovery of albino shoot primordia to green and (iii) direct regeneration of new green shoot primordia from the base of albino shoots. After immediate recovery new shoot primordia were produced from the base of recovered shoots and elongated rapidly [Fig. 5(c)]. Green shoots of Hbj B IV (310 in total) and Hbj B VI (215 in total) showed recovery from albino shoots/shoot primordia (Fig. 4). Green plantlets were obtained from these shoots upon individual culture in MS medium without added hormones. MS medium allowed for multiplication, further elongation and rooting of the plantlets [Fig. (5d)].

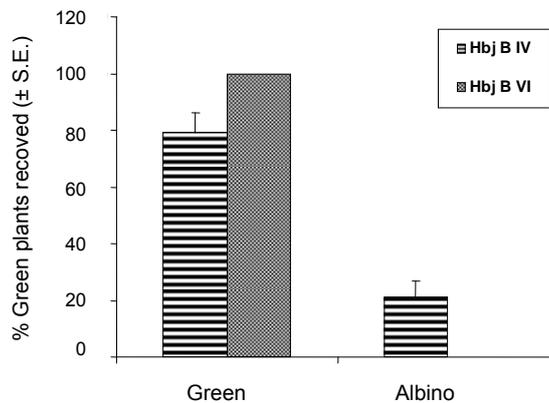


Figure 3: Recovery of green plants from albino shoot primordia of Hbj B IV and Hbj B VI on regeneration medium.

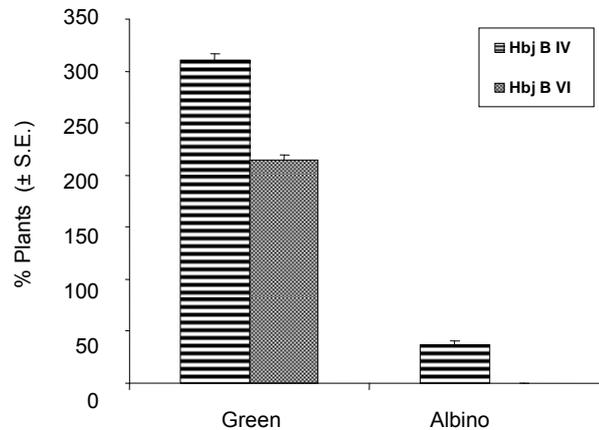


Figure 4: Regeneration of green plants from Hbj B IV and Hbj B VI in regeneration medium.

Albino shoot primordia 2–3 mm in length initiated from both varieties under KA, KB and KC media did not recover in regeneration medium even though they were incubated under the same cultural conditions. Longer incubation under fluorescent and incandescent light did not turn albino shoot primordia green. Albino shoot primordia/plants shorter or longer than 2–3 mm initiated from both varieties under all tested media combinations including M10 never turned green. Delaying the transfer to regeneration medium prior to incubation in the same culture conditions also did not cause the albino shoot primordia to turn green [Fig. (5e)].

Acclimatisation of Green Plants

All healthy, green, well-elongated and well-rooted regenerated plants were acclimatised to natural environmental conditions with a 100% survival rate. Survival was also excellent under net house conditions. Poorly developed plants turned healthy and roots developed well when gradually acclimatised to ambient natural conditions and humidity level (around 65%–70%). Healthy plants were transferred to soil in pots in the net house for subsequent growth, elongation, flowering, fruiting and growth to maturity.

DISCUSSION

Our results show that different types of media including M10 (without salt) induced callus from Hbj B IV and Hbj B VI rice varieties with varying induction percentages. Similar callus induction percentages were observed in other rice varieties when cultured on varied types of media (Mohiuddin *et al.* 2006). Different percentages of regeneration were obtained from anther derived callus of Hbj B VI induced from KA, KB, KC and M10 media similar to results reported by Karim and Zapata (1996) using specific media combinations.

We have observed production of over 400 albino shoot primordia/plantlets from anthers of both Hbj B IV and Hbj B VI varieties when cultured on media of different compositions except M10 (unpublished observation). In this study, we observed albino shoot primordia and plantlet production from these same five media (KA, KB, KC, KD and KE). Albino shoot primordia were observed in callus derived from M10 medium 3–4 days earlier in comparison to other tested media (KA, KB and KC). This suggests that the addition of CoCl_2 , glutamine and L-cystiene to M10 medium played a vital role in early/rapid shoot primordia initiation from aromatic indica rice varieties. Similar effect from the inclusion of these chemicals have been reported by others (Karim & Zapata 1990; Mohiuddin *et al.* 2006, 1995). Our study shows direct shoot primordia initiation from callus of both aromatic rice varieties in M10 medium, establishing an effective one-step regeneration procedure.

Calli 0.2–0.4 cm in size initiated albino shoot primordia both under fluorescent light and dark incubation. This specific calli size is essential for induction of shoot primordia from both aromatic rice varieties. In addition, albino shoot primordia is induced earlier from M10 medium.

These shoot primordia recovered and 100% of Hbj B VI and 79% of Hbj B IV primordia turned green. This suggests that the chemical composition of M10 medium in combination with the length of shoot primordia plays an important role in recovery from albinism. On the other hand, delayed initiation of albino shoot primordia from KA, KB and KC media did not allow recovery, confirming that delayed callus initiation does not allow recovery from albinism. It is probable that calli from KA, KB and KC media have poorly developed plastids that do not allow for chlorophyll synthesis during the initiation phase. 21% of albino shoot primordia from Hbj B IV remained albino, likely due to impaired chlorophyll synthesis. Similar results were observed in cereal anther culture in a study conducted by Vaughn *et al.* in 1980. In 1979, Sun *et al.* observed the absence of ribosomes and thylakoids in albino rice plants containing ill-developed plastids.

Albino shoot primordia 2–3 mm in length initiated from both varieties grown on KA, KB and KC media did not recover in regeneration medium, indicating that the chemical composition of the above media are not suitable for recovery of albinism. Addition of individual salts to the callus induction medium did not allow recovery and displayed lower percentages of regeneration in this study. Earlier studies have shown that addition of salts to media resulted in significant regeneration from rice seed culture (Mohiuddin *et al.* 2006). Albino shoot primordia/plants shorter or longer than 2–3 mm in length initiated from both varieties never turned green under all tested media combinations. Delaying transfer to regeneration medium followed by incubation in culture media also did not cause albino shoot primordia to turn green. This confirms that shoot primordia length of 2–3 mm and initiation from M10 medium are important factors for recovery and colour change of shoot primordia. These results show that recovery from albinism is possible with application of both suitable chemical and physical factors. A study by Maheswary and Mak (1993) showed a similar importance of both chemical and physical factors for green plant regeneration from anthers of *Capsicum*.

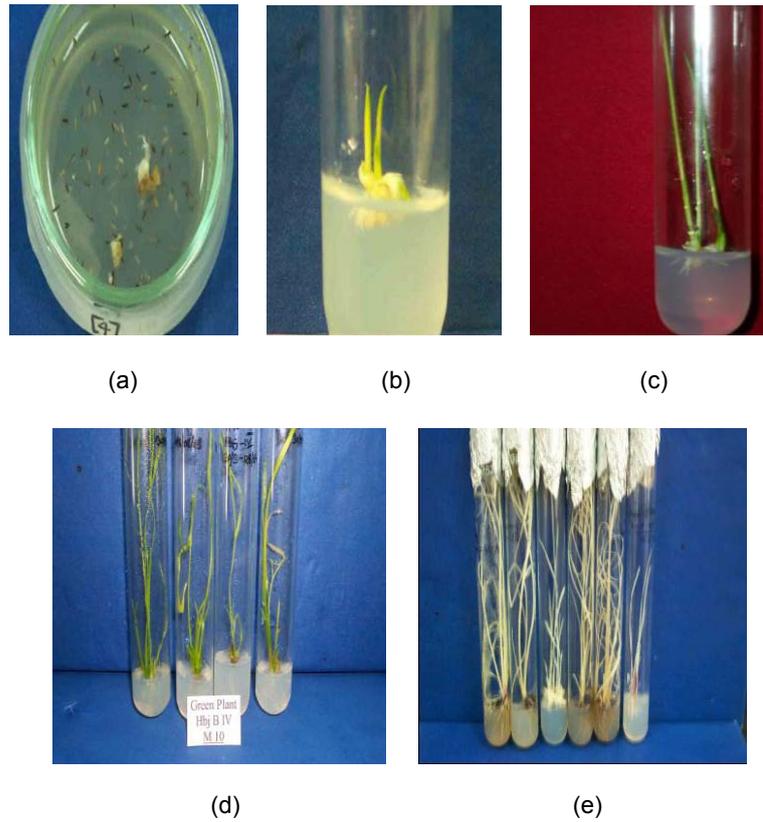


Figure 5: Recovery of green plants derived from anther culture of indica rice (*Oryza sativa* L.) var. Hbj B IV and Hbj B VI: (a) albino shoot primordia initiated from M10 medium (Hbj B IV); (b) gradual recovery of albino plants to green (Hbj B IV); (c) rapid elongation of green plants after immediate recovery (Hbj B VI); (d) multiplication of green plants (Hbj B IV); (e) albino plants regenerated from defined media tested in this study.

CONCLUSION

We have previously observed the regeneration of over 400 albino plantlets from anthers of both Hbj B IV and Hbj B VI varieties when cultured in different media compositions (KA, KB, KC, KD and KE) except M10 (unpublished observations). In this current study we observed excellent recovery of Hbj B IV and Hbj B VI plantlets from albinism when we took into consideration the following chemical and physical factors: (i) callus size between 0.2–0.4 cm, (ii) shoot primordia 2–3 mm long and (iii) use of M10. The routine use of M10 medium and specific callus and primordia size in anther culture should allow for significant restoration of albino plants to green plants.

ACKNOWLEDGEMENT

This research has been possible due to the Biotechnology Division of the Bangladesh Rice Research Institute's PETRRA Project "Development of High Yielding Rice Varieties for the Coastal Wetlands of Bangladesh" funded by Department for International Development (DFID).

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