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Improved, Efficient and Reliable Plant Regeneration Protocol for a Recalcitrant Black Rice (*Oryza sativa* cv. Chakhao amubi)

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Abstract

Rice tissue culture is very well established for the Indica and Japonica varieties in white rice. Black rice is recalcitrant for the regeneration due to their pigment. Since, there is a limited genetic transformation protocol available for the black rice so crop improvement efforts have not made so much progress. Here, we have developed an improved, efficient and reliable regeneration protocol for Chakhao amubi cultivar of black rice using mature seeds as an explant and through somatic embryogenesis pathway. The effects of growth regulators, gelling agent, and photoperiod, and various stages of complete protocol is well established. The regeneration protocol developed in this study will be well suited for introducing the agronomical important genes and functional genomic studies in black rice.

Keywords: Black rice, Chakhao amubi, Embryogenic callus induction, Somatic embryogenesis, Plant regeneration

Introduction

Rice is the major staple food for the people in India and for more than 50% of the world's population. It is the primary source of nutrition for more than half of the world's population, primarily in Asia. It is grown on 43.86 million hectares in India, with a yield of 117.47 million tonnes (DAC & FW, 2019-20). It plays an important role in the nation's economy. Depending on the colour pericarp, there are many types of rice black, brown, purple and red and coloured rice provides several health benefits (1). Rice is a pre-eminent crop in North-East India and is widely grown in lowland, upland, and deep-water situations, accounting for around 72% of the total land area. This region is thought to be home to at least 10000 indigenous cultivars (2). The states of Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim, and Tripura in Northern India have a rich array of regionally adapted non-Basmati aromatic germplasm. It is defined as a huge geographical area with high rainfall, humidity, variable topography, high natural selection pressures, and environmental stresses. Joha, chakhao, and tai cultivars grown in the states of Assam, Manipur, and Mizoram are the most prominent fragrant rice cultivars in this region (3). Chakhao landraces are a Manipur unique rice with a pleasant aroma and high quality. Manipur is one of India's eight North Eastern states and is noted for its numerous traditional rice types, cultivars, and landraces that are valued for their cultural and nutraceutical properties (4). Black rice is also known as Chak hao (meaning delicious rice) in Manipur, Chak means rice and hao means delicious. Recently, Black rice (Chak hao) has bagged GI tag in April 2020, with a certificate number 364. The application was registered by North Eastern Regional Agricultural Marketing Corporation Limited (NERAMAC).

In India, Manipur is the highest producer of Black rice cultivated by Meitei farmers. In Manipur, there are four landraces of black rice: Chakhao amubi, Chakhao angouba, Chakhao poireiton, and Chakhao angangba. This rice is gluten-free, cholesterol-free, and low in sugar, salt and fat. It is a whole grain, exceptionally nutritious rice that is high in fibre, anthocyanin, antioxidants, vitamin B complex, vitamin E, iron, thiamine, magnesium, niacin, and phosphorus. According to Cornell University researchers, antioxidants are nearly six times more abundant in black rice. Black rice is known as a treasure of macro and micro-nutrients as it contains many minerals and vitamins. It is estimated that 50 g of black rice per day offers 35% of the RDA for selenium, copper, zinc, and magnesium. Protein quality and quantity are better than in any other rice variety as it contains 18 amino acids. Being a natural iron-rich food, it is ideal for those who are concerned about receiving enough iron on plant-based diet (5). Calcium (Ca) and iron (Fe) are abundant in black rice (21.38/100 g), but sodium (Na) is low (10.19 mg/100g). It has a high magnesium and potassium content (Potassium 186.54 mg/100 g and Magnesium 107.21 mg/100 g). Among all rice varieties studied, black rice (5.89%) had the highest total saturated fatty acid and unsaturated fatty acid content (6). All rice samples included oleic and linoleic acid, with black rice having the highest concentration. The soluble dietary fibre (SDF) (%) in black rice is 8.17±0.07, while the insoluble dietary fibre (IDF) (%) is 14.49 ±0.07 (78). One half-cup cooked or one-fourth cup uncooked black rice contains (in daily recommended values) 160 kcal energy, 1.5 g fat, 34 g carbohydrate, 2 g fibre, 7.5 g protein, no saturated fat, and no cholesterol (5) and www.blackrice.com.

According to earlier research, black rice has higher antioxidant activity and phenolic content than white rice (7). Several studies have revealed that black rice is an excellent source of phytochemicals**.** The dehulled seeds of Japanese black-purple rice were qualitatively and quantitatively characterised for anthocyanin, flavones, flavonoids glycosides (Quercetin-3-O-glucoside, isorhamnetin-3-O-glucoside, and myricetin-7-O-Glucoside), carotenoids, vitamin E (tocopherols and tocotrienols), and y-oryzanols that gives health benefits, which also ensures the usage of black rice as a nutritious food (8). Chakhao rice takes 108 to 165 days to bloom flower and reaches a height of 130 to 165 cm. Chakhao cultivars have a narrow spikelet fertility range and yield few tillers and panicles per plant (9). Chakhao rice produces a yield of 1.3-5.01 tonnes ha-1, depending on the farming regime (9, 10). In spite of high nutritional and medicinal values, the yields of these cultivars are very poor as they are highly susceptible abiotic and biotic stresses. Efforts should be made to develop high yielding varieties without losing their aroma, cooking quality and grain quality.

Although during green revolution, conventional breeding had played a major significant role in crop production but those methods are not sufficient to feed today's growing population. Standardisation of protocols for effective regeneration and transformation systems for various crops has proven to be a challenging task, and biotechnology interventions for crop breeding have established an advantage over conventional approaches. Although, there are manyplant regeneration protocols for white rice are well established (11-14), but studies on *in vitro* culture and regeneration in black rice are very scarce. Attempts to develop a tissue culture-based methodology for regenerating black rice plants from calluses have so far either failed or produced extremely low regeneration frequencies. Development of extremely efficient and reliable plant regeneration systems have significant potential to assist in the genetic transformation of indica rice cultivars. In the present study, an improved, efficient and reliable regeneration protocol with high regeneration frequency was developed, which will be useful for generating black rice transgenics for its genetic improvement.

Materials and Methods

Plant material and sterilization of seeds

A Manipuri Black rice cultivar Chakhao amubi was selected for the present study. Seeds were collected from Assam Agriculture University, Assam, India. Dehusked healthy mature seeds were initially washed two times with sterile distilled water for removing dust particles. Seeds were sterilized with 70% ethanol (v/v) for 30 sec and then immediately rinsed with autoclaved distilled water. Seeds were surface sterilized using 4% sodium hypochlorite along with few drops of teepol for 20 min with intermittent shaking. The seeds were then washed thoroughly with autoclaved distilled water for removing the detergent. Sterilized seeds were soaked for overnight before they were inoculated on embryogenic callus induction medium.

Induction of embryogenic callus

The over-night-soaked sterilized seeds were dried on sterile Whatman paper before inoculation. For embryogenic callus induction, 12-15 seeds per petri plate were inoculated on Callus Induction Medium (CIM) and kept under dark at 28±2˚C. CIM was supplemented with MS salts Himedia PT021 with different concentration of 2,4-D, 3% Maltose, 2.8 g/L proline, 0.6 g/L casein hydrolysate. Gelling agents used for solidifying the medium were 0.8 % agar and 0.4% phytagel. pH was adjusted to 5.8 before autoclaving. A total of three different combination of 2,4-D and gelling agents used were listed in Table 1. After 15 days, embryogenic calli were selected and cut it into two pieces and sub-cultured on CIM for multiplication. The frequency of callus induction was calculated by the following formula:

$$\text{Callus induction frequency (\%)} = \frac{\text{No. of seeds produced calli}}{\text{No. of seeds inoculated}} \text{ X } 100$$

Data obtained were subjected to two-way anova test analysis, based on 110 seeds per experiment, which was repeated thrice by using 110 seeds in each case for the induction of callus.

Table 1: Composition of various media used in the study

Medium type	Medium	Medium composition	Plant hormones	Gelling agent
Callus Induction Medium	CIMI1	MS Salts Himedia PT021, 3% Maltose, 2.8 g/L Proline, 600 mg/L CEH pH 5.8	2 mg/L 2,4-D	0.8% Agar
	CIMI2	MS Salts Himedia PT021, 3% Maltose, 2.8 g/L Proline, 600 mg/L CEH pH 5.8	2.5 mg/L 2,4-D	
	CIMI3	MS Salts Himedia PT021, 3% Maltose, 2.8 g/L Proline, 600 mg/L CEH pH 5.8	3.0 mg/L 2,4-D	
	CIMII1	MS Salts Himedia PT021, 3% Maltose, 2.8 g/L Proline, 600 mg/L CEH pH 5.8	2 mg/L 2,4-D	0.4% Phytagel
	CIMII2	MS Salts Himedia PT021, 3% Maltose, 2.8 g/L Proline, 600 mg/L CEH pH 5.8	2.5 mg/L 2,4-D	
	CIMII3	MS Salts Himedia PT021, 3% Maltose, 2.8 g/L Proline, 600 mg/L CEH pH 5.8	3.0 mg/L 2,4-D	

Shoot Regeneration Medium	MSRMIa	MS Salts Himedia PT021, 3% Maltose, 600 mg/L Proline, 2 g/L CEH, pH 5.8	2 mg/L BAP + 5 mg/L NAA	0.4% Phytagel
	MSRMIb	MS Salts Himedia PT021, 3% Maltose, 600 mg/L Proline, 2 g/L CEH, pH 5.8	2 mg/L BAP 1 mg/l Kinetin + 0.5 mg/l NAA	
	MSRMIc	MS Salts Himedia PT021, 3% Maltose, 600 mg/L Proline, 2 g/L CEH, pH 5.8	4.5 mg/l Kinetin	
	MSRMId	MS Salts Himedia PT021, 3% Maltose, 600 mg/L Proline, 2 g/L CEH, pH 5.8	2.5 mg/l BAP + 0.5 mg/l NAA	
	MSRMIe	MS Salts Himedia PT021, 3% Maltose, 600 mg/L Proline, 2 g/L CEH, pH 5.8	2 mg/l Kinetin + 1 mg/l BAP + 0.2 mg/L NAA	
	MSRMI-Ia	MS Salts Himedia PT021, 3% Maltose, 600 mg/L Proline, 2 g/L CEH, pH 5.8	2 mg/L BAP + 0.5mg/L NAA	0.8% Agarose
	MS-RMIIb	MS Salts Himedia PT021, 3% Maltose, 600 mg/L Proline, 2 g/L CEH, pH 5.8	2 mg/L BAP + 1 mg/l Kinetin + 0.5 mg/l NAA	
	MS-RMIIc	MS Salts Himedia PT021, 3% Maltose, 600 mg/L Proline, 2 g/L CEH, pH 5.8	4.5 mg/l Kinetin	
	MSRMI-Id	MS Salts Himedia PT021, 3% Maltose, 600 mg/L Proline, 2 g/L CEH, pH 5.8	2.5 mg/l BAP + 0.5 mg/l NAA	
	MS-RMIIe	MS Salts Himedia PT021, 3% Maltose, 600 mg/L Proline, 2 g/L CEH, pH 5.8	2 mg/l Kinetin + 1 mg/l BAP + 0.2 mg/L NAA	
Rooting Medium	RM	Half MS Salts Himedia PT021, 3% Sucrose	-	0.4% Phytagel

Plant regeneration

Calli obtained were cut into two pieces and sub-cultured on the same CIM for multiplication of calli. To get highly efficient regeneration, sub-cultured embryogenic calli were considered. A total of five different combination of media for regeneration tested were listed in Table 1. Different combination of BAP, NAA and along with Kn were used for regeneration in this study. Calli were inoculated on regeneration medium in the petri plates and kept under 16/8 photoperiod with a light intensity of 40 µE mol $m^{-2}s^{-1}$ at 28 ± 2°C. After shoot buds were initiated from calli, they were transferred to shoot elongation medium in jam bottles. Shoot regeneration frequency was calculated on the basis of number of calli cultured on shoot regeneration medium and number of calli with shoot regeneration.

$$\text{Shoot regeneration frequency} = \frac{\text{No. of calli showing shoot regeneration}}{\text{No. of calli inoculated}} \times 100$$

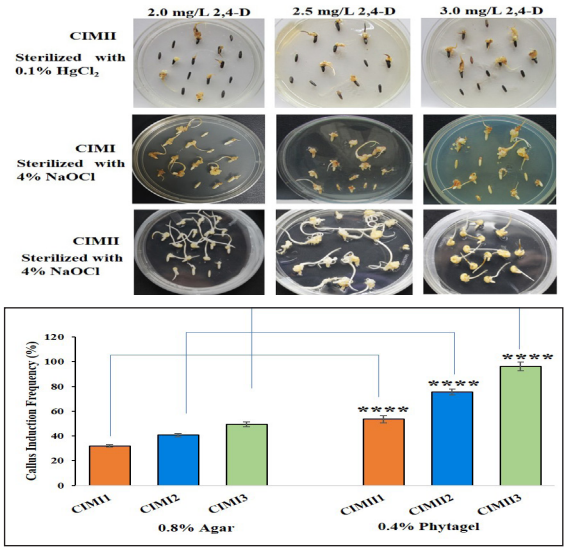
Rooting and hardening

Healthy multiple shoots obtained were transferred to rooting medium (Table1) for induction and proliferation of roots for 15-20 days. The *in vitro* regenerated plants were gently rinsed with the autoclaved distilled water to remove the excess of agar to avoid any fungal contamination. Then the plants were transferred to pots containing soil and vermiculite in the ratio of 1:1 and covered with poly bags to maintain humidity and grown them in the tissue culture room with 26±1˚C and under 16/8 photoperiod with 40 µE mol $m^{-2}s^{-1}$ light intensity. After few days, polybags were removed upon the emergence of new leaves and then transferred to the field for further growth and development

Results and Discussion

Effects of 2,4 D and gelling agent on callus induction

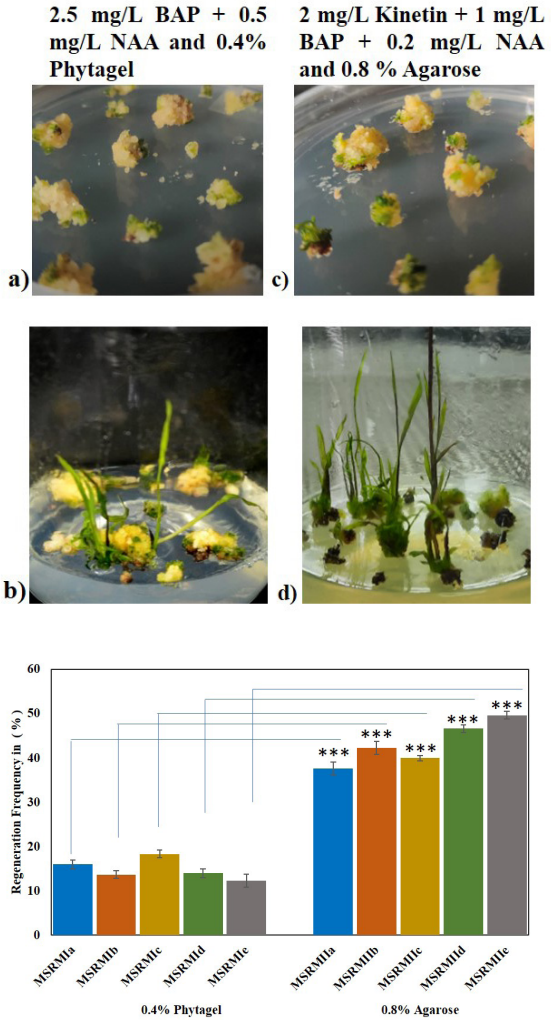
We selected an important Black rice cultivar, i.e., Chakhao amubi for standardization of plant regeneration. Formation of embryogenic calli from seeds is the first step of any rice plant tissue culture system for subsequent regeneration of shoots and roots. We compared the callus induction frequency using the different sterilizing agents, different concentration of 2,4 -D and gelling agents. Initially, when the seeds were sterilized with 0.1% mercuric chloride and inoculated on callus induction media (CIMII1-3) with three different concentrations of 2,4-D no callus induction was observed in any medium (Fig. 1). But when we changed it to sodium hypochlorite and inoculated on CIMI1-3 and CIMII1-3 media, callus induction was initiated after 3 days from scutellar region of the embryo in all the combinations. After 5 days, vigorous callus proliferation was observed and healthy calli were obtained after 15 days of culture. In CIMI, gelling agent used was 0.8 % agar, CIMII medium gelled by 0.4% phytagel and 1,2 and 3 represent with different concentration of 2,4-D, *viz*., 2.0, 2.5 and 3.0 mg/L respectively. The callus induction frequency obtained was 31% in CIMI1, 40% in CIMI2 and 49% (highest) in CIMI3 (Fig. 2). In all the combination of CIMI1-3, cream, friable and unhealthy calli were developed when 0.8% agar gelling agent was used in all the concentration of 2,4-D (Fig 1). In case of CIMII, callus obtained were white compact, globular and healthy in all the combination of 2,4-D. Callus induction frequency obtained was 53% in CIMII1, 75% in CIMII2, and 96% in CIMII3. In all the combinations of CIMI and CIMII, highest callus induction frequency was obtained with CIMII3 when medium was supplemented with 3 mg/L 2,4-D and gelled with 0.4% phytagel (Fig. 1 and 2)). When the concentration of 2,4-D was increased to 3.5 and 4 mg/L, callus induction frequency was decreased to 60%. 2,4-D at 3 mg/L in combination with 0.4% phytagel gelling agent was found to be optimum for callus induction with high frequency. Hence, this concentration of 2,4-D and 0.4% phytagel gelling agent were selected for the subsequent experiments.



Figs. 1 and 2. Standardization of conditions for embryogenic callus induction in Black rice, cv. Chakhao amubi. Effect of 2,4-D, and sterilizing agent on embryogenic callus induction (Fig. 1) and gelling agent on callus frequency (Fig. 2) with their respective media CIMI (1-3) and CIMII (1-3) after 2 weeks of incubation. Data analysed by two-way analysis of variance (ANOVA) was statistically significant at $P < 0.05$.

Effects of phytohormones and gelling agent on regeneration frequency

To obtain high regeneration frequency, MS medium supplemented with different combinations of BAP, Kn and NAA along with two different gelling agents, phytagel and agarose were used (Table1). Two types of regeneration media, MSRMI with 0.4% phytagel and MSRMII with 0.8% agarose were used. Both these media were fortified with different concentrations of BAP, NAA, and Kn. Calli showed maximum regeneration frequency in all the combinations of MSRMII. The regeneration frequency in MSRMI was in the range of 12 to 18%. In case of MSRMI, slightly higher shoot regeneration frequency, 15% and 18% were obtained in MSRMIa and MSRMIc respectively (Fig. 3 and 4).). While in case of MSRMII, high regeneration frequency, 35% to 47% was recorded and out of which maximum regeneration frequency was obtained in MSRMIId with the combination of 2.5 mg/L BAP and 0.5 mg/L NAA and MSRMIIe supplemented with 2 mg/L Kn, 1 mg/L BAP and 0.2 mg/L NAA. It was noticed that the increased concentration of Kn resulted in decrease in shoot regeneration frequency, and medium without BAP led to poor regeneration frequency. In all the combinations, maximum regeneration frequency was obtained in MSRMIId and MSRMIIe, and these concentrations were selected for the further experiments (Fig. 3 and 4).). Initially, green shoot buds appeared after 4-5 days embryogenic callus culture, then these shoot buds were transferred to jam bottles containing the respective medium for their development into shoots. The healthy multiple shoots obtained were transferred to rooting medium (Table1) for induction and proliferation of roots for 15-20 days (Fig. 5a). The regenerated plants were gently rinsed with the sterile distilled water to remove the traces of agar, and then the plants were transferred to pots (Fig. 5b), which were covered with poly bags to maintain humidity and following tissue hardening, they were transferred to the field for further growth and development.



Figs. 3 and 4. Standardization of conditions for plant regeneration in Black rice, cv. Chakhao amubi. Effects of different phytohormones and gelling agent for regeneration in Oryza sativa cv. Chakhao amubi; Embryogenic sub-cultured callus on regeneration media MSRMI and MSRMII, and initiation of shoot buds (Fig. 3a and c) and elongated shoots (Fig. 3b and d); Effects of gelling agents and phytohormones on regeneration frequency (Fig. 4). Data analysed by two-way analysis of variance (ANOVA) was statistically significant at $P < 0.05$.

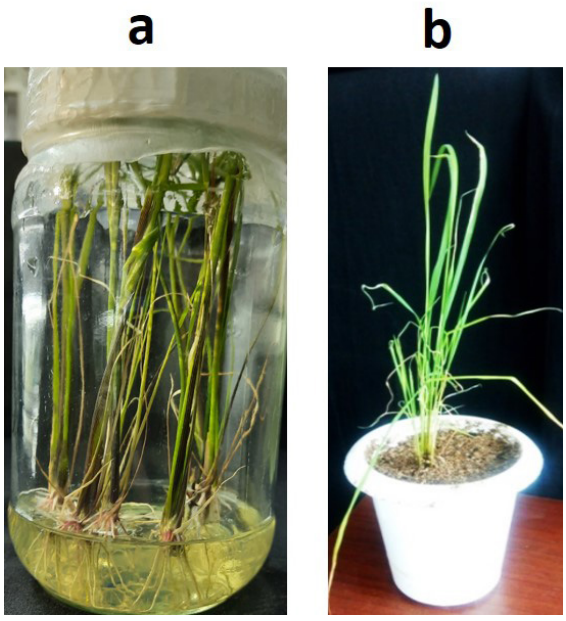


Fig. 5. Rooting of in vitro shoots, and hardening and acclimatation of regenerated plants. Healthy multiple shoots transferred to the rooting medium in the jam bottle containing only half-MS without phytohormones (Fig. 5a) Representative picture of fully regenerated chakhao amubi plant in the pot after 7 days of hardening (Fig. 5b).

Discussion

The most crucial phase in rice tissue culture has long been regarded as embryogenic callus induction. The capacity for callus induction and the frequency of cell regeneration depends on the culture parameters such as the medium composition, genotype, and explant source. Even though there are numerous *in vitro* culture procedures for white rice available, the efforts to develop an efficient tissue culture regeneration protocol for Black rice with high regeneration frequency is absolutely required as the reported frequencies are very low. In this study, Chakhao amubi, an elite, popular and widely cultivated variety of Black rice was considered. Embryogenic callus induction was observed on both CIMI and CIMII. We observed that calli obtained in CIMI were yellowish, friable and unhealthy. On the other hand, calli obtained in CIMII were white, globular, compact and healthy after 15 days of inoculation. Callus induction frequency also varied in all three combinations of CIMI and CIMII of 1,2 and 3. Significant increase in callus induction frequency achieved with using 3 mg/L of 2,4-D in both the case of CIMI and CIMII. 2,4-D is an excellent source of auxin for induction of embryogenic callus, and this is supported by several previous studies (12,15-19). Seed sterilizing agent plays an important role in tissue culture protocol, it not only used for seed surface sterilization but also effects the callus induction and their frequency. Our results did not show any callus induction when the seeds were sterilized with 0.1% mercuric chloride and inoculated on CIM, On the contrary, seeds sterilized with 4% NaOCl showed very efficient callus induction in dark at 28˚C in MS fortified with 3% Maltose, 2.8 g/L proline, 600 mg/L CEH and 3 mg/L 2,4-D. Callus induction frequency also depends on gelling agent (12,20). We observed that when the seeds were inoculated on MS medium gelled with agar, callus obtained were friable, yellow and unhealthy. On the other hand, callus obtained using phytagel containing medium were healthy, compact, white and globular. As per the earlier reports, callus induction frequency also depends on the age of the explants and genotype and different basal medium composition (21-27). Further, we did not observe callus induction with seeds sterilized with mercuric chloride in our initial experiments, but callus induction was observed in CIMI and CIMII when seeds were sterilized with 4% sodium hypochlorite. Therefore, we continued our further standardization with sodium hypochlorite. The maximum callus induction frequency obtained with 3 mg/L 2,4-D as compared to 2.5 and 2 mg/L. According to (28), the optimal dose of 2,4-D for inducing embryogenic callus in indica rice is 0.5 mg/L. Optimal callus formation has also been reported by (29,30), at 2 mg/L of 2,4-D. It was also shown that the concentration of 2,4-D above 2 mg/L also causes reduction in callus induction frequency in Swarna cultivars

(20). Verma et al. (31) and Pawar et al. (32) also noted a decrease in callus induction frequency and callus bulk with an increase in 2,4-D concentration above 2 mg/L. It is evident, and also based on previous studies that the different concentrations of 2,4-D have an impact for callus induction in different rice cultivars. This variance in optimal 2,4-D concentrations for callus induction in rice cultivars imply genotype-dependent responses of the explants to the concentration of 2,4-D in the culture media.

While MS medium supplemented with MS salts, 3 % Maltose, 0.6 g/L CEH, 2.8 g/L proline, 2.5 mg/L BAP and 0.5 mg/L NAA, and gelled by 0.8 % agarose and other combination of 2 mg/L Kn, 1 mg/L BAP and 0.2 mg/L NAA and 0.8 % agarose (pH 5.8) resulted in significant higher regeneration frequency compared with other combinations mentioned in Table 1. There are many previous reports, which suggest that the combination of BAP, Kn and NAA leads to higher regeneration frequency. However, Mostafiz et al. (33) reported that while a combination of MS salts + 2 mg/L BAP + 2 mg/L Kn + 0.5 mg/L NAA resulted in 82 % regeneration in the Malaysian wetland variety MR220- CL2 and 68 % regeneration in the Malaysian wetland varieties MR220 and MR232, it only induced 40 % regeneration in the variety Bario. They attribute the differential reaction to the explant's genotype. Juturu et al. (20) reported that the range of 2-3 mg/L 2,4-D along with 0.5-1.0 mg/L NAA resulted in optimum regeneration frequency. As mentioned above that the gelling agents effect the callus induction frequency, but they also affect the regeneration frequency and similar results were also obtained by Sahoo et al. (12). The use of agarose as a gelling agent in shoot regeneration medium resulted in significant higher regeneration frequency as compared to phytagel and agar gelling agent. Several studies have been published on the effects of gelling agents on shoot regeneration in various rice types (12, 34-37) found no statistically significant differences in the frequency of regeneration of certain Indica rice types when several gelling agents were used in regeneration. Studies have been reported that there is significantly higher value in regeneration frequency in four rice cultivars (IR64, CSR10, Pusa Basmati 1 and Swarna) when 1% agarose used as a gelling agent (12). Mohamed et al. (37) also reported that gelling agents effect regeneration frequency. They found that using gelrite or phytagel as gelling agents increased the frequency of regeneration in the Egyptian rice cultivars Sakha104 and Giza178 as opposed to bacteriological agar. Consequently, just like with plant growth regulators, genotype determines how tissues react to various gelling agents.

Conclusion

We optimised a protocol of plant regeneration of aromatic black rice Chakhao amubi with more than 40% regeneration efficiency. The developed protocol can be used for black rice transformation for its improvement by expressing novel genes that confer useful traits.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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