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Rapid callogenesis and plant regeneration of fine and coarse varieties of rice

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Abstract

In this study, we aimed to develop an effective and genotype-dependent protocol for *in vitro* callogenesis and plant regeneration of two fine rice varieties (Super Basmati and Basmati-515) and one coarse variety (KS-282). According to the results, maximum callus induction (52% of the explants) was observed in case of KS-282 after 11 days of incubation with Murashige and Skoog basal medium (MS, with 30 g/l sucrose and 1.76 g/l Gellan gum), which was supplemented with 3 mg/l of 2,4-dichlorophenoxy acetic acid (2,4-D). In contrast, 41% of the Super Basmati explants produced callus within 17 days, followed by 32% of the Basmati-515 explants (within 11 days), in the aforementioned medium but which is supplemented with 4 mg/l 2,4-D. Indirect shoot formation from callus was found to be the highest in case of KS-282 (24% of callus explants) and Basmati-515 (18% of callus explants) after 21 days of incubation. Next, we tested MS medium without 2,4-D but which was supplemented with 3 mg/l kinetin (Kin), 1 mg/l 6-benzyladenine (BA), and 0.5 mg/l indole-3-acetic acid (IAA). According to the results, 22% of the explants of Super Basmati formed shoots after 19 days on MS medium supplemented with BA instead of 2,4-D at a concentration of 1 mg/l. Roots were formed from shoots on a half-strength MS medium without plant growth regulators after 15 days in KS-282 (93% roots formed), after 19 days in Super Basmati (90% roots formed), and after 23 days in Basmati-515 (86% roots formed). Shoots were effectively multiplied in KS-282, followed by Super Basmati, and Basmati-515 varieties (37, 29, and 23 shoots/shoot, respectively) on a basal MS medium supplemented with 4 mg/l Kin. Rooting was induced within 47, 55, and 57 days for KS-282, Super Basmati, and Basmati-515, respectively. Following acclimatization, the percentage of survival was found to be 71%, 70%, and 65%, which was found to be at-par for all three tested rice varieties.

Key words: 6-benzyladenine, acclimatization, callus, kinetin, *Oryza sativa* L.

Introduction

Currently, the worldwide production of rice is 738.2 million tons per year, covering an area of 160.6 million ha, with Asia being the largest producer (675 million tons in 2016; FAO, 2016). Eight Asian countries, namely, China, India, Indonesia, Bangladesh, Thailand, Vietnam, Burma, and the Philippines are the world's greatest rice producers and Pakistan ranks 10th (FAO, 2016). In Pakistan, rice is the second leading crop after wheat and its cropland covers an area of 2 748 000 ha with an average yield of 2 479 kg/ha and production of 6 811 000 tons in 2016 (Economic Survey of Pakistan, 2015-2016). It also played a vital role in the national economy during 2015-2016, in which rice was accounted for 3.1% of the value added by agriculture and 0.6% of GDP. About 4,262,216 metric tons of rice was exported, and Pakistan earned USD 1.86 billionin export revenue (Economic Survey of Pakistan, 2015-2016). Pakistan exports rice chiefly to United Arab Emirates, Iran, Saudi Arabia, Kenya, and Afghanistan.

Due to the aroma of Basmati rice, which has increased the demand of export of Pakistani rice, new varieties of rice with higher yield will likely be introduced into Pakistan. Super Basmati and Basmati-515 are popular rice varieties and are staple foods in Pakistan. Super Basmati and Basmati-515 were developed in the year

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Table 1. Unique agronomic characteristics of 3 tested rice varieties (Super Basmati, Basmati-515 and KS-282) (Information from Rice Research Institute, Kala Shah Kaku, Lahore, Pakistan)

Characters	Super Basmati	Basmati 515	KS282
Parentage	Basmati 320 × 10486	Super Basmati × Basmati 2000	Basmati 370 × IR 95
Year of release	1996	2011	1982
Plant height [cm]	115	129	106
Maturity days	122	123	101
Stem stiffness	stiff	stiff	semi-stiff
No. of productive tillers	15	14	18
No. of grains per panicle	100	115	122
1000-grain weight [g]	22.85	21.79	26.4
Paddy length [mm]	11.14	11.45	9.82
Head rice recovery [%]	50.5	53	44.89
Paddy yield [t/ha]	2.5-3.0	3.8-4.2	4.62

1996 and 2011, respectively, after crossing two existing highly aromatic Basmati varieties (Table 1). Super Basmati showed a yield of 3.7 t/ha during 1995-96 and 3.4 t/ha in the year 2016, whereas Basmati-515 showed a yield of 3.6 t/ha in the year 2016. Similarly, KS-282, which was developed in the year 1982, showed a yield of 5.3 t/ha, whereas in the year 2016, it reached to 3.3 t/ha. It is noteworthy that these varieties have lost their yield potential over time. Therefore, in this study, our primary objective was to find a way to correct the economic traits such as yield and disease resistance of the three selected cultivars, namely, Super Basmati, Basmati-515, and KS-282 using callus-based somaclonal variation, without changing the parentage and quality traits.

Crop improvement through tissue culture is a relatively easy and well-established technique that plays an important role in agricultural production. It also allows for plants to be manipulated in sterile conditions to improve their agronomic traits, often in a more effective way than the conventional plant breeding techniques (Zalc et al., 2004; Wani et al., 2011). Callus can be induced from different plant parts on a nutrient medium containing specific plant growth regulators (PGRs). Callus has been successfully induced from rice seeds (Rashid et al., 2001, 2003; Islam et al., 2004; Niroula et al., 2005; Hussain et al., 2010). It has been shown that rice seeds have a greater potential for callogenesis than nodes or shoot tips of the rice plants (Rashid et al., 2003). Although callogenesis is a successfully utilized tissue culture technique, most protocols developed to date are cultivar-dependent (Ilahi et al., 2005).

Therefore, in this study, we aimed to identify suitable media for rapid callus induction and regeneration of three rice varieties, namely, Super Basmati, Basmati-515, and KS-282. Table 1 lists the primary characteristics of these genotypes.

Material and methods

Plant material

Three rice varieties (KS-282, Super Basmati, and Basmati-515; Table 1) were obtained from the Rice Research Institute, Kala Shah Kaku, Pakistan. Seeds were dehusked manually, washed with distilled water to remove dust and other particles, and surface sterilized with 35% sodium hypochlorite (NaClO) for 10 min on a shaker (model 3020, GFL, Burgwedel, Germany) at 54 rpm. The seeds were then washed in 70% alcohol for 5 min and rinsed thrice with autoclaved distilled water for 5 min each. Sterilization and culture was performed in a laminar air flow cabinet in a plant tissue culture room.

Culture conditions

All culture media (1 l) were solidified with 1.76 g/l Gellan gum (ID: G434) and the pH of the media was adjusted to 5.7 by adding 1 N HCl or 1 N NaOH. All reagents (product code IDs indicated in parentheses) were

Table 2. Treatments and their respective media compositions

Treatment	PGR concentrations in media
Callus induction medium (CIM)	
C1	2 mg/l 2,4-D
C2	3 mg/l 2,4-D
C3	4 mg/l 2,4-D
C4	5 mg/l 2,4-D
Shoot induction medium (SIM)	
SI1	Kin (3 mg/l) + BA (2 mg/l) + IAA (0.5 mg/l)
SI2	Kin (3 mg/l) + BA (1 mg/l) + IAA (0.5 mg/l)
SI3	Kin (2 mg/l) + BA (2 mg/l) + IAA (0.5 mg/l)
SI4	Kin (2 mg/l) + BA (1 mg/l) + IAA (0.5 mg/l)
Shoot multiplication medium (SMM)	
SM1	Kin (1 mg/l)
SM2	Kin (2 mg/l)
SM3	Kin (3 mg/l)
SM4	Kin (4 mg/l)
Root induction medium (RIM)	
RI1	MS/2
RI2	NAA (2 mg/l)
RI3	NAA (1 mg/l)
RI4	IAA (2 mg/l)

2,4-D – 2,4-dichlorophenoxy acetic acid; BA – 6-benzyladenine; IAA – indole-3-acetic acid; Kin – kinetin; NAA – α-naphthaleneacetic acid; in all media – the basal medium was full-strength MS (except for RI1, which was half-strength MS); to all media – agar was added at 1.76 g/l and sucrose was added at 30 g/l

purchased from Phyto Technology Laboratories (Shawnee Mission, KS, USA). The media were then autoclaved at 15 psi for 20 min at 120°C. The seeds (one seed/test tube) were incubated for 15 days in the dark at 25°C, as suggested by Noor and coworkers (2005). Thereafter, test tubes were transferred to light (16 h photoperiod with a photosynthetic photon flux density (PPFD) of 83.6 $\mu molm^{-2}s^{-1}$ provided by white fluorescent tubes) and incubated at 25 ± 2°C (Hussain et al., 2010).

Callus induction

Explants (surface sterilized dehusked seeds) were cultured on callus induction media (CIM), which consisted of Murashige and Skoog (MS; Murashige and Skoog, 1962; ID: M519); 30 g/l sucrose; and 2, 3, 4,or 5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D, ID: D299).

Shoot and root induction

After 2 weeks of incubation, calli were replated on shoot induction media (SIM), which is MS basal media gelled with 1.76 g/l Gellan gum and supplemented with four different concentrations of PGRs (Naqvi et al., 2006; Table 2). After the shoots reached approximately 2-3 cm long, they were transferred on to root induction media (RIM) (Khatun et al., 2003; Table 2). The experiments were performed in a complete randomized design (3 replicates; 50 samples/replication) (CIM, SIM, SM and RIM). All treatment numbers are described in Table 2.

Acclimatization, hardening, and field establishment of in vitro derived plantlets

The hardening and field establishment of regenerated rice plants was based on a protocol published by

Table 3. Analysis of variance for three rice genotypes for several organogenic responses

Source of variation	DF	Mean square						
		Callus induction [%]	Days to callus induction	Shoot induction [%]	Days to shoot formation	Shoot multiplication [shoots/shoot]	Rooting [%]	Days to rooting
Treatments (A)	3	740.917**	251.333**	237.667**	365.583**	658.917**	731.583**	272.917**
Genotypes (B)	2	364.000**	187.750**	129.000**	219.000**	428.250**	230.250**	230.250**
A × B	6	290.667**	53.083**	21.667**	0.333N.S	13.917**	2.583NS	1.917NS
Error	24	3.833	1.833	1.500	1.917	1.833	2.333	2.167
CV		7.23%	5.72%	8.45%	5.18%	7.63%	1.94%	5.95%

CV – coefficient of variation; DF – degrees of freedom; ** – highly significant; NS – non-significant

Puhan and Siddiq (2013). Plantlets with well-developed roots were washed under running tap water and then dipped for a few seconds in an antifungal solution (0.1% (w/v) "Benlate," Du Pont) as fungal endophytes can cause biochemical changes in rice plantlets (Hao et al., 2010). The plantlets were then transplanted into 7-cm diameter pots containing sand and soil (1 : 1 v/v). Then, they were watered with distilled water (250 ml/pot/plant) to which 1.107 g/l of MS medium was added (Akram and Aftab, 2012) and covered with a lightly perforated polythene bag for 2 days to maintain humidity in the pot and to prevent stress due to dehydration (Lavanya et al., 2009). MS-based autoclaved water was used every 7 days to water the plants. The pots were placed in a culture room maintained at 22 ± 3 °C in the light for the next 15 days in the same conditions as for *in vitro* culture. After 15 days, the micropropagated plants were hardened by transferring them to a shade-house which gets diffused natural light and with ambient conditions for up to 6 days (Chandra et al., 2010). After one month, hardened plants were transferred into natural light conditions and then were transferred to larger clay pots (25 cm in diameter) containing sterile soil and were grown until the plants matured.

Statistical analysis

The experiments were laid out as a two-factor completely randomized design (CRD), replicated thrice with 50 samples per replication. Data were analyzed with MSTAT-C software (Russel Freed, Michigan State University, USA) and treatment means were calculated by the least significant difference (LSD) at $\alpha = 0.05$. Table 3 presents the results of analysis of variance for all three rice genotypes for several organogenic responses.

Results

Callus induction

In case of KS-282 explants, 4 mg/l 2,4-D demonstrated induction of callus at a rate of 52% after 11 days of incubation, whereas 5 mg/l 2,4-D poorly induced the formation of callus in KS-282 explants (Table 4). After 17 days of incubation in MS medium supplemented with 5 mg/l 2,4-D, callus was induced at a rate of 41% in case of Super Basmati explants and at a rate of 32% in case of Basmati-515explants (but only after 11 days) (Table 4). In treatment-1 (C1), Super Basmati and Basmati-515 varieties formed the smallest amounts of calli (14% and 12% within 30 and 35 days, respectively; Table 4). Furthermore, as the concentration of 2,4-D was increased, the percentage of callus formation in Super Basmati and Basmati-515 varieties gradually increased, and the number of days to callus formation decreased but an opposite trend was observed for KS-282. The mean of all four treatments of 2,4 D (Table 2) on three rice genotypes (Table 7) showed that KS-282 produced the largest number of calli (32.75%) in 20 days, followed by Super Basmati (26.75%; Fig. 1A) in 23 days, and then followed by Basmati-515 (21.75%) in 28 days. These results show that KS-282 responded well to callus formation relative to the fine rice genotypes. Table 8 shows the mean values for all four treatments in terms of callus induction. Treatment-3 (C3) was found to be the best in terms of callus induction (which produced 35.33% calluses within 18 days) followed by treatment-4 (C4) (which produced 32% calluses within 21 days), followed by treatment-2 (C2) (which produced 26.33% within 25 days), whereas treatment-1 (C1) produced only 14.67% calluses within 30 days of incubation.

Table 4. Callus-related response of three rice genotypes

	Callus induction [%]				Days to callus induction			
Genotypes	C1	C2	C3	C4	C1	C2	C3	C4
KS-282	18 f	38 b	52 a	23 d	26 c	19 e	11 f	25 c
Basmati Super	14 g	22 de	30 c	41 b	30 b	25 c	19 e	17 e
Basmati-515	12 g	19 ef	24 d	32 c	35 a	31 b	24 cd	11 f
LSD ($\alpha = 0.05$)	3.299				2.282			

LSD – least significant difference; treatments C1-C4: see detailed explanation in Table 2

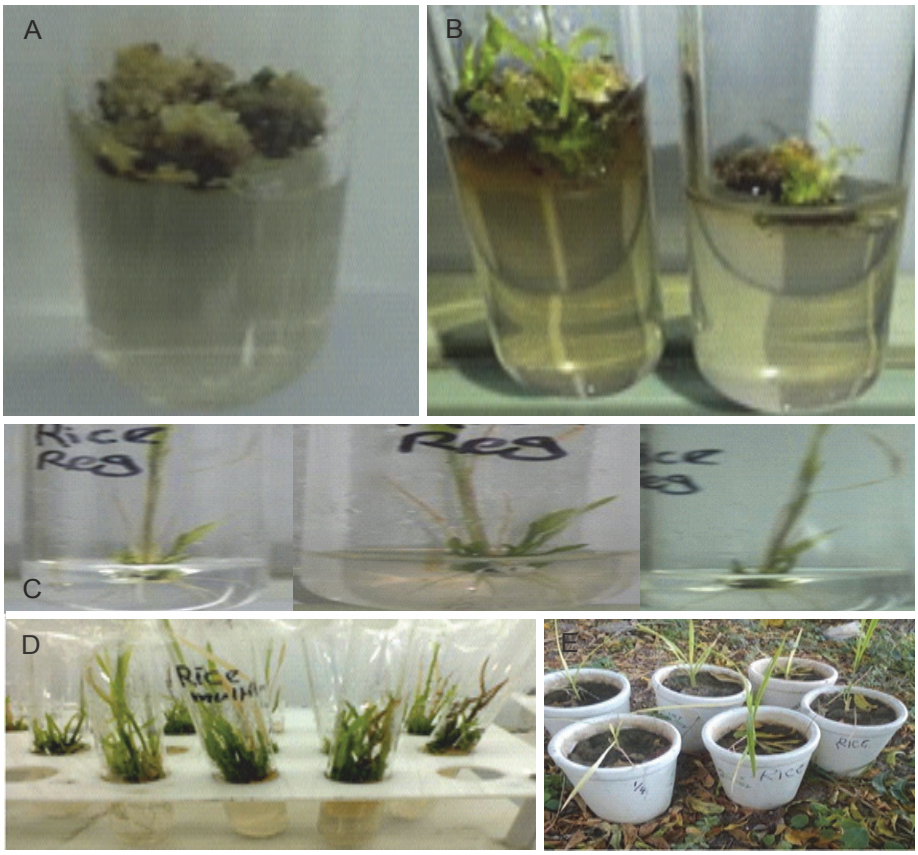

Fig. 1. A) Callus induction on callus induction medium in Super Basmati; B) Shoot induction in shoot induction medium from callus in Basmati-515; C) Individual shoots, separated from callus, rooting on root induction medium: 1 – in Super Basmati, 2 – Basmati-515, and 3 – KS-282; D) Shoot multiplicationof cultivar KS-282 on SM4; E) Acclimatized plants growing in pots

Shoot induction and formation

KS-282 formed the largest number of shoots (24%, Table 5) after 21 days of incubation; through an intermediate callus phase in second treatment of shoot induction (SI2), whereas Super Basmati and Basmati-515 formed the largest number of shoots (22% in 19 days and 18% in 23 days, respectively) from callus in the first treatment of shoot induction (SI1). Table 5 shows different responses to shoot induction observed under different treatments and concentrations for all the tested rice varieties. Table 7 shows mean shoot induction percentages and days to shoot induction for all the tested rice varieties. According to our results, KS-282 showed the highest shoot induction value (17.50%) in 22 days, followed by Super Basmati (15% in 27days) and Basmati-515 (11% in 31 days; Fig. 1B). Treatment-1 (SI1) was

Table 5. Shoot-related response of three rice genotypes

Genotypes	Shoot induction [%]				Days to shoot induction				Shoot multiplication [shoots/shoot]			
	SI1	SI2	SI3	SI4	SI1	SI2	SI3	SI4	SM1	SM2	SM3	SM4
KS-282	19 b	24 a	17 bc	10 ef	14 h	21 fg	25 de	29 c	14 e	19 d	27 b	37 a
Basmati Super	22 a	16 c	13 d	9 f	19 g	26 d	30 c	34 b	10 f	11 f	16 e	29 b
Basmati-515	18 bc	12 de	9 f	5 g	23 ef	29 c	33 b	38 a	7 g	9 fg	11 f	23 c
LSD (α = 0.05)	2.064				2.333				2.282			

LSD – least significant difference; treatments S1-S4: see detailed explanation in Table 2

Table 6. Root-related response of three rice genotypes

Genotypes	Rooting [%]				Days to rooting			
	RI1	RI2	RI3	RI4	RI1	RI2	RI3	RI4
KS-282	93 a	85 c	80 d	74 f	15 g	18 f	21 de	28 c
Basmati Super	90 b	82 d	76 ef	68 h	19 ef	22 d	26 c	31 b
Basmati-515	86 c	77 e	71 g	63 i	23 d	26 c	32 b	36 a
LSD (α = 0.05)	2.574				2.481			

LSD – least significant difference; treatments R1-R4: see detailed explanation in Table 2

Table 7. Organogenic response of three rice genotypes (pooled over all treatment data in Tables 4-6)

Genotypes	Callus induction [%]	Days to callus induction	Shoot formation [%]	Days to shoot formation	Shooting multiplication [shoots/shot]	Rooting [%]	Days to rooting
KS-282	32.75 a	20 c	17.50 a	22 c	24.25 a	83.00 a	21 c
Super Basmati	26.75 b	23 b	15.00 b	27 c	16.50 b	79.00 b	25 b
Basmati-515	21.75 c	28 a	11.00 c	31 a	12.50 c	74.25 c	29 a
LSD (α = 0.05)	3.299	2.282	2.064	2.333	2.282	2.574	2.481

LSD – least significant difference

Table 8. Impact of different treatments on organogenic response of three rice genotypes

Treatments	Callus induction [%]	Days to callus induction	Shooting induction [%]	Days to shoot induction	Shooting multiplication [shoots/shoot]	Rooting [%]	Days to rooting
T_1 = C1, SI1, SM1, and RI1	14.67 d	30 a	20 a	19 d	10.33 d	89.67 a	19 d
T_2 = C2, SI2, SM2, and RI2	26.33 c	25 b	17 b	25 c	13.00 c	81.33 b	22 c
T_3 = C3, SI3, SM3, and RI3	35.33 a	18 d	13 c	29 b	18.00 b	75.67 c	26 b
T_4 = C4, SI4, SM4, and RI4	32.00 b	21 c	8 d	34 a	29.6 a	68.33 d	32 a
LSD (α = 0.05)	3.299	2.282	2.064	2.333	2.282	2.574	2.481

LSD – least significant difference; codes for C, SI, SM and RI media explained in Table 2

found to be the best for shoot induction (19.67%) in 19 days followed by treatment-2 (SI2) (17.33%) in 25 days, treatment-3 (SI3) (13% in 29 days), and treatment-4 (SI4) (only 8% in 34 days) (Table 8).

Shoot multiplication

Shoot multiplication was also studied by culturing one shoot in four different treatment conditions as indicated in Table 2. Shoots were effectively multiplied in KS-282 explants (Fig. 1D) followed by Super Basmati and Basmati-515 varieties (37, 29, and 23 shoots/shoot, respectively) in the fourth treatment conditions of shoot multiplication (SM4) (see Table 5). Shoot multiplication increased when the concentration of kinetin (Kin) was increased. All tested rice genotypes responded well to 4 mg/l of Kin aiding the chance of successful shoot multiplication. KS-282 had the highest shoot multiplication ratio (24.25 shoots/shoot) followed by Super Basmati (16.5 shoots/shoot) and then Basmati-515 (12.5 shoots/shoot) (Table 7). Treatment-4 (SM4) was found to be the best in terms of shoot multiplication (29.67 shoots/shoot) followed bytreatment-3 (SM3) (18 shoots/shoot), treatment-2 (SM2) (13 shoots/shoot), and treatment-1 (SM1) (10.33 shoots/shoot) (Table 8).

Root formation

After the shoots were formed, all four treatment conditions (Table 2) were tested for the root formation efficiency in three tested rice cultivars (Fig. 1C). Only one shoot was placed in one test tube. KS-282 responded best with 93% of the shoots forming roots after 15 days of root induction (RI1) in the first treatment which was also the most suitable for Super Basmati and Basmati-515. In addition, 90% of the Super Basmati shoots formed roots after 19 days in RI1, followed by 82% after 22 days on root induction (RI2) in case of treatment-2 and 76% after 26 days in case of root induction (RI3) treatment-3. For Basmati-515, best rooting (86%) was observed after 23 days of incubation in RI1, followed by 26 days (77%, RI2), and 32 days (71%, RI3) (Table 6). All three genotypes responded poorly to RI4 at treatment-4 (Table 6).

This study indicates that complete rice plantlets (including rooting) can develop within 47, 55, and 57 days as indicated for KS-282, Super Basmati, and Basmati-515, respectively. Table 7 shows the mean rooting percentage and the number of days to rooting for KS-282, Super Basmati, and Basmati-515. KS-282 showed the best rooting (83%) after 21 days of incubation followed by Super Basmati (79%) at 25 days, and Basmati-515 (74.25%) at 29 days. Table 8 shows the mean rooting percentage and days to rooting for four treatments: T1 was found to be the best for rooting (89.67%, 19 days) followed by T2 (81.33%, 22 days), T3 (75.67%, 26 days), and then T4 (68.33%, 32 days).

Discussion

Callus induction

We followed the suggestions made by Khatun and coworkers (2003), Ge and coworkers (2006), and Khaleda and Al-Forkan (2006) with respect to the culture media, PGRs, and other *in vitro* growth conditions. Briefly, dehusked seeds of three rice varieties (KS-282, Super Basmati, and Basmati-515) were cultured on MS medium with various concentrations of 2,4-D (2, 3, 4, and 5 mg/l). Hussain and coworkers (2010) used mature dehusked rice seeds as explants in MS medium and Naqvi and coworkers (2006) used N6 medium, the former being generally superior in terms of callus induction ability across three tested rice varieties (GNY-53, JP-5, and Basmati-370). In this study, our results showed a high frequency of callus induction in MS medium supplemented with different concentrations of 2,4-D, and the callus induction frequency varied for the three studied rice genotypes. Muhammad and coworkers (2014) used rice seeds as explants and studied three coarse rice genotypes (IRRI-6, IRRI-9, and KS-282). According to their results, 93.33% callus initiation in IRRI-6 occurred after 3 days when 2 mg/l 2,4-D was added to N6 medium, followed by 92.22% within 2.33 days in KS-282 at 4 mg/l 2,4-D. IRRI-9 explants formed 91.11% callus in response to 3 mg/l 2,4-D after 2 days. Choice of the culture medium directly impacts the ability of callus induction in rice (Lee et al., 2002), which was confirmed by our results and those of Muhammad and coworkers (2014).

Karthikeyan and coworkers (2009) reported that 2,4-D can induce callus from rice seeds, but the cultivar used was not specified. In our study, we observed that 2,4-D added to MS medium was the key initiator of callus induction; however, the calli responded differently at various levels of 2,4-D in the three tested genotypes (Table 4). Khatun and coworkers (2003) observed callogenesis from mature seeds within 7-11 days in Lx297

(82.5%), IR64-1-1-4 (18.77%), and V19 (6.4%) rice genotypes when 2 mg/l 2,4-D was added to MS medium. In this study, KS-282, Super Basmati, and Basmati-515 required 11-26 (18-52%), 17-30 (14-41%), and 11-35 days (12-32%) to induce callus formation, respectively (Table 4). From the mature seeds of "PAU 201"rice variety, the highest (44.4%) callus induction was achieved on MS medium supplemented with 2.5 mg/l 2,4-D, 0.5 mg/l Kin, 560 mg/l proline, and 30 g/l sucrose, relative to a medium with only 2,4-D (Wani et al., 2011). Rashid and coworkers (2003), using rice seeds as explants, noted that the supplementation of MS medium with 2,4-D at a concentration of 2.0 mg/l induced callus efficiently in KS-282 (31.3%), followed by Basmati-385 (17.6%), and Basmati-370 (6.5%). However, the response of explants to different concentrations of 2, 4-D during the induction of callus formation was genotype-dependent, which was also observed in our study. KS-282 formed the highest amounts of callus (52%) when 4 mg/l 2,4-D was used in MS medium, whereas 2.0 mg/l 2,4-D induced 31.3% of callus formation in the same medium and genotype (Rashid et al., 2003; Table 4). Islam and coworkers (2004) studied the response of three rice genotypes (Pajam, Lucky, and Kalizira) using rice seeds as explants on MS culture medium containing 1 mg/l 2,4-D in the presence of calcium silicate and in various 2,4-D concentrations (1.5, 2, or 2.5 mg/l) in the absence of calcium silicate. It was observed that variety Pajam produced 100% callus, which was significantly higher than the other two tested varieties. Moreover, 1 mg/l 2,4-D in combination with 60 mg/l calcium silicate or 2.5 mg/l 2,4-D alone were found to be the best for callus induction (100%). Bhuiyan and coworkers (2014) found that 3.0 mg/l 2,4-D induced the formation of the largest amount of callus (45.5 and 90%) in the two submergence-tolerant rice varieties, BRRI dhan 52 and FR13A, respectively. Our study revealed that the percentage of callus and the days to callus induction are dependent on a genotype in fine rice cultivars. The coarse rice genotypes formed more callus in fewer days at a low level of 2,4-D as compared to fine rice varieties.

Shoot induction and formation

Plant regeneration ability depends on the genotype and the choice of the shoot induction medium (Hoque et al., 2004). Shoot induction through an intermediate callus phase is a method most commonly used in rice tissue culture studies. Agarwal and coworkers (2006), Rachmawati and Anzai (2006), and Hussain and coworkers (2010) have tested the shoot induction media prepared based on the use of different ratios of BA and NAA in MS medium, whereas Wani and coworkers (2011) used different levels of BA, Kin, and NAA. BA has been effective for the regeneration of shoots in rice (Xue and Earle, 1995; Lee et al., 2002). Hussain and coworkers (2010) observed best shoot formation efficiency (37.45% of explants) through an intermediate callus phase in Basmati-370 when 1 mg/l NAA and 4 mg/l BA were added to MS medium. They concluded that Kin, BA, and IAA are effective in inducing shoots in MS medium similar to the aforementioned studies.

In this study, we further observed that shoots were induced in the coarse rice callus (KS 282) when a medium was supplemented with 3 mg/l BA, whereas fine rice genotypes (Super Basmati and Basmati 515) formed shoots in response to 2 mg/l BA (Table 5). Pons and coworkers (2000) reported that across three rice varieties tested (Senia, Tebre, and Bahia), more shoots were produced from the callus on a medium supplemented with 2 or 3 mg/l BA than when it was supplemented with Kin. Interestingly, when 0.5 g/l of NAA or IAA were added to the media, the response was genotype-dependent. Furthermore, 0.5 mg/l of IAA in combination with other PGRs (BA and Kin) was effective in inducing shoots in both coarse and fine rice genotypes (Table 5). Medhabati and coworkers (2014) observed the maximum shoot regeneration (70%) from callus when 2 mg/l BA and 1 mg/l NAA were added to N6 medium but regeneration decreased when the concentration of BA was increased.

In an attempt to induce shoots, Ilahiand coworkers (2005) transferred the callus of a local rice variety (cv. "Swat-II") onto four different regeneration media (1 – MS + 0.5 mg/l Kin + 0.2 mg/l NAA; 2 – MS + 1.5 mg/l Kin + 0.5 mg/l NAA; 3 – MS + 2.0 mg/l Kin + 0.5 mg/l NAA; 4 – MS + 1.5 mg/l BA + 0.5 mg/l IAA). They observed that when the callus of "Swat-II" was placed on medium 1, moderate regeneration was observed; embryoids formed on medium 2; regeneration was excellent on medium 3; and abnormal small green projections formed from callus on medium 4. In all four media tested, plantlets formed within 4 weeks, but the growth responses were not quantified. Khatun and coworkers (2003) studied shoot formation in four rice genotypes (Lx297, IR64, V19, and IR64-1-1-4). Lx297 was the most

responsive; it formed shoots (13.89%) from callus within 2-3 weeks on SIM medium (MS, 1.0 mg/l MS vitamins, 10.0 mg/l Fe-EDTA, 0.1 g/l *myo*-inositol, 30 g/l sucrose, 0.5 mg/l NAA, 2.0 mg/l Kin). Wani and coworkers (2011) observed maximum (42.5%) direct plantlet formation from callus of a commercial rice variety PAU 201 on MS medium with 2 mg/l BA, 0.5 mg/l Kin, and 0.5 mg/l NAA. The highest percentage of shoot formation (83.4%) in FR13A and (35%) in BRRI dhan 52 rice varieties was observed after 3.5 weeks of culture on MS medium supplemented with 2 mg/l Kin, 2 mg/l BA, and 1 mg/l NAA (Bhuiyan et al., 2014).

In this study, we observed that when MS medium was supplemented with a mixture of Kin, BA, and IAA (Table 1 and Table 5), shoot formation was better in all three tested rice varieties. Shoot induction percentage in coarse rice genotypes was as high as in fine rice varieties.

Shoot multiplication

Our study revealed that Kin played a major role in shoot multiplication in case of both fine and coarse rice varieties (Table 5). Mahajan and coworkers (2013) observed the formation of maximum number of shoots in Ranbir Basmati (20) and in Basmati 370 (18) on MS media supplemented with 0.5 mg/l BA and 0.5 mg/l Kin. Genotype-independent shoot multiplication responses of six rice genotypes (Igra-409, Itape, Fortuna, Taim, Ct6919, and Mocoi) was observed on MS medium supplemented with 5 mg/l BA, ranging from 8-20 shoots/shoot (Medina et al., 2003). In this study, we observed the maximum shoot multiplication when 4 mg/l Kin was used for both fine and coarse varieties of rice (Table 5). Interestingly, shoot multiplication in coarse rice was better than in fine rice, but this feature was genotype-dependent.

Root formation

Our study showed that root formation in all tested conditions was similar for both coarse and fine rice varieties, unlike callus induction and shoot formation. The results of this study also revealed that all three rice varieties responded best to a half-strength MS medium in the early stage of rooting and root formation as well. Bano and coworkers (2005) reported that 10 weeks were required to form rice rooted plantlets in MS medium supplemented with 0.5 mg/l BA and 0.2 mg/l IAA. An effective rooting of Ranbir Basmati and Basmati 370 rice plantlets on MS medium supplemented with 0.2 mg/l NAA (93.48% and 90.21%, respectively) was reported by Mahajan and coworkers (2013). When Medina and coworkers (2003) cultured the shoots of six rice genotypes (Igra-409, Itapé, Fortuna, Taim, Ct6919, and Mocoi) on MS medium lacking PGRs but with 8% (w/v) sucrose, roots developed within a week and a good root system was formed within 3 weeks. The highest rooting efficiency (percentage of shoots with roots) was observed in Mocoi and Itapé varieties, ranging from 65 to 100%. Afrasiab and Rabia (2011) studied two rice genotypes (Super Basmati and IRRI-6) and found that the genotype and medium composition and their interaction affected plant regeneration. In case of Super Basmati, at different concentrations of NAA and BA, 80% of the explants formed shoots and 100% formed roots formed directly from the embryo-derived callus in MS medium supplemented with 1.0 mg/l NAA and 3.0 mg/l BA, whereas in case of IRRI-6, 100% of the explants formed shoots and 80% formed roots directly from callus in the presence of 1.0 mg/l NAA and 5.0 mg/l BA. Noor and coworkers (2005) reported that in case of Super Basmati, 1.0 mg/l NAA and 2.5 mg/l BA resulted in 90% plantlet formation (shoots + roots), whereas in case of Basmati-385, 1.0 mg/l NAA and 5.0 mg/l BA resulted in 83% regeneration on MS medium. As reported by Sikder and coworkers (2006), the highest plantlet regeneration directly from callus was observed on MS medium supplemented with 0.05 mg/l NAA and 5.0 mg/l BA. On PGR-free MS medium, the percentage of shoots forming roots after 5 days of growth in case of FR13A and BRRI dhan 52 rice varieties was found to be 94% and 87%, respectively (Bhuiyan et al., 2014). Sah and coworkers (2014) used PGR-free half-strength MS medium supplemented with 20 g/l sucrose to form successful roots in japonica rice cv. "Kitaake". In this study, a half-strength MS medium and MS with auxins (1 or 2 mg/l NAA, or 2 mg/l IAA) were effective in inducing root formation in both coarse and fine rice genotypes. Similar findings have been reported by Sah and coworkers (2014), Afrasiab and Rabia (2011), and Bano and coworkers (2005). A half-strength MS medium without PGRs is more effective than when combined with NAA, whereas the application of IAA results in earlier root formation for both coarse and fine rice genotypes.

Acclimatization

Acclimatization defines the success of a tissue culture protocol (Deb and Imchen, 2010). In this study,

a total of 256, 146, and 86 callus-derived KS-282, Super Basmati, and Basmati-515 plants were hardened, respectively (Fig. 1E). From them, 182, 102, and 56 plants survived (71%, 70%, and 65%, respectively).

It was observed that the percentage of survival was almost at-par in all three rice genotypes.

Conclusions

An effective, genotype-dependent and rapid protocol allowing a stepwise induction of shoots from callus, root induction, and subsequent acclimatization was achieved for two fine varieties of rice (Super Basmati and Basmati-515) and one coarse variety (KS-282). These varieties are popular in Pakistan, and thus, this protocol represents an effective means allowing a mass production of rice plants in the Asian subcontinent. Thus, based on our results, we conclude that KS-282formed more callus at low 2,4-D concentration (3 mg/l) compared to 4 mg/l 2,4-D for Super Basmati and Basmati-515. A combination of PGRs (Kin, BA, and IAA) in MS medium was found to be effective in shoot formation, supplementation with Kin at 4 mg/l for shoot multiplication, and half-strength MS medium proved to be the best for rooting in case of all three tested rice varieties.

Conflicts of interest and author contributions

The authors declare no conflicts of interest. MIK and MZI planned the experiments. MIK conducted the experiments. MIK and JATdS examined the data, wrote, and edited the manuscript.

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