International Journal of Agriculture System
Vol. 10 Issue 1, June 2022
Nationally Accredited Journal Decree No. 48a/E/KPT/2017
P-ISSN: 2337-9782, E-ISSN: 2580-6815. **DOI: 10.20956/ijas.v10i1.3230**

In Vitro Doubled Haploid Production of Bacterial Blight Resistant Plants from BC_2F_1 Plants (Ranbir Basmati X Pau148) Through Anther Culture

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ABSTRACT

Doubled haploid plants are very important for the development of complete homozygous plants from heterozygous parents in one generation as they possess duplicate copy of haploid chromosome. Haploid production is easily obtained from in vitro anther culture. The present study was undertaken with the objective to develop doubled haploids using anthers for in vitro induction of callus on N_6 medium supplemented with various combinations and concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D) (0.5-2.5 mg L-1), Kinetin (0.5-1.0 mg L-1) and Naphthalene acetic acid (NAA) (2.0 mg L-1) as callus induction medium (CIM). The highest callus induction frequency was obtained when N_6 medium fortified with 2,4-D (2.5 mg L-1), Kinetin (0.5 mg L-1) and NAA (2 mg L-1) of 10.07 per cent. The induced callus was sub cultured for shoot regeneration on Murashige and Skoog medium (MS) supplemented with growth regulators: Kinetin and NAA (0.5 mg L-1 each) in combination with BAP (0.0 - 2.5 mg L-1). MS medium supplemented with NAA (0.5 mg L-1), Kinetin (0.5 mg L-1) and BAP (1.5 mg L-1) was most responsive exhibiting regeneration frequency of 28.1 per cent which resulted in maximum regeneration of green plantlets and only 5.21 per cent of albinos. Individual plantlets were separated and immersed in liquid MS medium augmented with NAA (0.5-1.0 mg L-1) and BAP (0.5-1.0 mg L-1). Maximum rooting was observed in MS medium with NAA (0.5 mg L-1) and BAP (1.0 mg L-1). The survival rate of in-vitro raised plants was 51.51 per cent. Of these surviving plants, 21 plants were observed to have the sterility percentage above 50 percent and hence can be considered as the doubled haploid plants. Plant DH8 is susceptible and DH20 is heterozygous for gene Xa21. Two plants are susceptible for gene xa13.

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Keywords:
Doubled haploids; callus induction; shoot regeneration; anther culture and BB resistant.

1. Introduction

Haploids are plants which possess a single set of gametophytic chromosome number while doubled haploids are those haploids that have undergone duplication of chromosome. Haploid cells or tissues can be used to develop doubled haploids by androgenesis or gynogenesis. Homozygous plants that are produced by conventional breeding method take a little longer than homozygous plant production by *in vitro*

methods. Androgenesis is an *in vitro* technique which can be utilized for the production of complete homozygous lines by haploid and doubled haploids (DHs) in single step. DH techniques have been successfully established in economically important crop species, including major cereals like rice, wheat etc. and cabbages (Wedzony et al., 2009; Germana, 2011) especially in rice to meet the requirements of people, as it is the staple food in South-east Asian countries.

The major advancement in the recent years in the field of rice breeding is the development of anther culture techniques for production of rice. The anther culture response is genotype specific (Jacquard et al., 2006; Nurhasanah et al., 2015) and also depends on the pretreatment (Trejo-Tapia et al., 2002), media composition (Cha-Um et al., 2009) and culture condition (Datta et al., 2005; Wang et al., 2000). The varietal development depends on the adequate numbers of DH plants produced efficiently for field evaluation and selection (Chu et al., 2001; Jiang et al., 2000). Anther culture can be used for development, selection and genetic improvisation of characters for superior performance of homozygous doubled haploid (DH) rice (Mandal et al., 2000). DH lines produced from microspore culture and/or anther culture possess a high potential for genetic improvement as it broadens the genetic diversity resulting in production of homozygous lines within a short period in comparison to numerous cycles of inbreeding or back crossing involved in plant breeding (Morrison and Evans, 1988; Foroughi-wehr and Wenzel, 1989). Anther culture has been extensively used in rice as it speeds the breeding process for production of plants with special agronomic characters which include development of earliness, increased grain weight, superior grain quality, disease resistance (Zhan et al., 1984; Zhang, 1989), dwarf plant type and abiotic stress tolerances (Ruwani et al., 2018).

The composition of nutrient medium provides nutrition to the microspores and also directs the pathways of development initiated by carbon source, macronutrient and micronutrients. Other than nutritional effect carbohydrate source is essential for osmotic effect in anther culture (Bishnoi et al., 2000). Among maltose and sucrose, maltose has been identified as an efficient carbohydrate source for significantly improving androgenesis efficiency and green plant formation in rice (Bojhwani et al., 2001). The ratio of inorganic nitrogen in media (nitrate as potassium nitrate (KNO_3) and ammonium ions as ammonium sulphate [(NH_4) SO_4]) has been observed to be an important determinant for success of anther culture in indica rice (Grimes and Hodges, 1990) and N_6 medium is characterized by having high KNO_3 and (NH_4) ratio. The present study was undertaken keeping in view the essential media requirements for anther culture for the production of doubled haploids.

2. Materials and Methods

Source of explants

The elite plants (BC_2F_1) developed by crossing Ranbir Basmati and PAU148 were sown at School of Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Chatha, Jammu during the year 2017-2018 as shown in Figure 1a. The boots were harvested during morning hours at an early flowering stage with panicles still enclosed within the leaf sheath as shown in Figure 1b. Selection of the spikelet was done on basis of cytological observations or position of the anthers. The spikelets were selected from three parts (top, middle and basal) of each panicle. The

anthers were stained with acetocarmine and observed under a light microscope (100x) in order to check development stage of pollen to be used as explants.

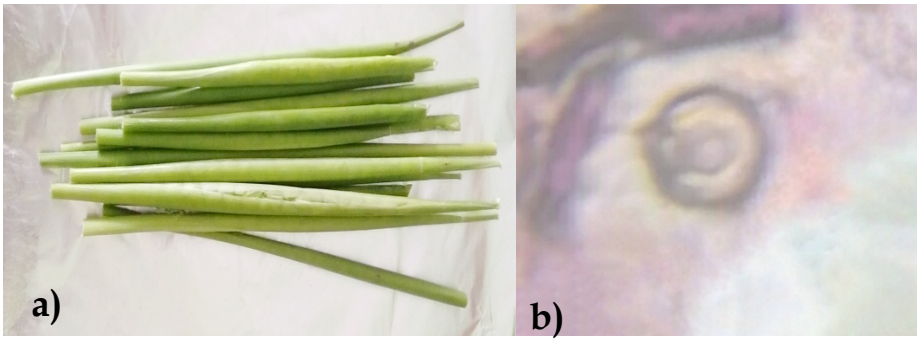


Figure 1. Collection of explant: a) Boots collected with panicles still enclosed, b) Identification of early stages of pollen at 100x.

Surface sterilization of explants

The selected boots were wiped with 70 % (v/v) ethanol (Hi-Media, Mumbai) and kept at 4˚C for 8 days after wrapping in aluminum foil. These panicles were separated from the boots and treated with few drops of detergent (Tween 20) along with Bavistin (0.2%) for 1hr with intermittent shaking. The panicles were then washed under running tap water for 20 minutes in order to remove the traces of detergent. The final surface sterilization of these treated panicles was performed under laminar air flow chamber (Thermo Electron Corporation, Germany). The panicles were first treated with 70% ethanol for few seconds followed by sterilization with 0.1% mercuric chloride ($HgCl_2$) for 10 minutes. These sterilized explants were then finally washed three times with autoclaved double distilled water. Following surface sterilization each spikelet was cut at the basal end holding from the tip to separate the anther lobes from the filaments.

Callus induction and proliferation of cultures

N_6 medium (Chu, 1978) containing maltose (3%) and phytohormones with varying concentrations of 2,4-D, Kinetin and NAA was used for callus induction. The isolated anthers were dusted on N_6 medium supplemented with maltose (3%w/v) as carbon source along with different concentrations and combinations of phytohormones like 2,4-D (0.5 - 2.5 mg L-1); Kinetin (0.5-1.0 mg L-1) and NAA 2.0 mg L-1 (Table 1) and incubated in dark. The observations on frequency of callus induction were recorded after 7-8 weeks of inoculation. In order to obtain sufficient amount of callus mass the anthers showing callus induction were sub-cultured on optimized N_6 callus induction media (CIM).

Regeneration

The shoot regeneration and root induction was performed on Murashige and Skoog medium (1962) fortified with sucrose (3%) and different combinations of growth regulators (Kinetin, NAA and BAP) for shoot and root induction. The anther derived calli were transferred to solidified regeneration Murashige and Skoog medium consisting of 100 mg L^{-1} Myo-inositol, 3% sucrose and 0.8% agar. The growth regulators Kinetin and NAA, each at 0.5 mg L^{-1} in combination with BAP at 0.0 - 2.5 mg L^{-1} was added to the media (Table 2). The observations pertaining to response of calli

were recorded and callus regeneration frequency was calculated. For rooting, the individual plantlets were separated and placed in half MS medium supplemented with growth regulators NAA (0.0 to 1.0 mg L^{-1}) and BAP (0.0 to 1.0 mg L^{-1}) as shown in Table 3.

Media Preparation and Culture Conditions

The pH of media was adjusted to 5.8 with 1 N HCl or 1 N NaOH before adding agar (0.8% w/v). The medium was dispensed in culture vessels and autoclaved at 121°C and 1.05 kg cm^2 for 15 minutes. For callus induction the cultures were kept in dark. The incubation of callus for regeneration was done in culture room 25±2°C with relative humidity of 70% at 16-h of photoperiod provided by cool, white, and fluorescent lamps (1.5 kilolux light intensity).

Acclimatization and Hardening

The rooted plantlets were removed from the test tubes and were thoroughly washed under water to remove traces of media and dipped in Bavistin solution (0.2%) for half an hour. These plantlets were then transferred in pots containing sterilized potting mixture (1:1 ratio of sand and soil) and irrigated with autoclaved ½ MS medium without growth regulators. Hardening was initially done in the laboratory conditions at 24±2˚C, 16 h light and 85-90% RH for transplanting *in vitro* regenerated plants as shown in Figure 2. Then these were transferred to polyhouse under temperature between 26-29˚C with 70-80% RH and then gradually to temperature of 35-37˚C (Figure 2). Plants that survived after hardening were counted for each genotype and survival percentage was calculated. The ploidy level of the plants was judged on the basis of vigor of the plants and sterility/fertility status of the panicles. Those plants were considered haploids in which there was no seed set and general vigor of the plants was weak, while as those with partial to complete seed set and normal vigor were considered doubled haploids.

Figure 2. Surviving doubled haploid plants after hardening.

Data Analysis

All trials of the experiments were repeated thrice. The completely randomized design (Gomez & Gomez, 1984) was used to analyze the data recorded for different parameters. The statistical analysis based on mean values per treatment was made using analysis of variance (ANOVA) technique of CRD and was worked out using OPSTAT software.

Disease scoring

Scoring the disease by measuring the lesion leaf area in per cent at two weeks after inoculation was done by clipping method (Kauffman et. al, 1973). Disease severity was rated as per the scoring system for evaluation of BB resistance as shown in Figure 3. The lesion length (cm) developed below the point of inoculation and was measured with a scale on the 21st day of inoculation. The development of necrotic lesions progressing up to a maximum of 1-5% was considered as resistant (R) reaction. The plants with lesion length more than 26% were considered as susceptible (S) while those with intermediate scores were marked as moderately resistant (6-12%) and moderately susceptible (13-25%).

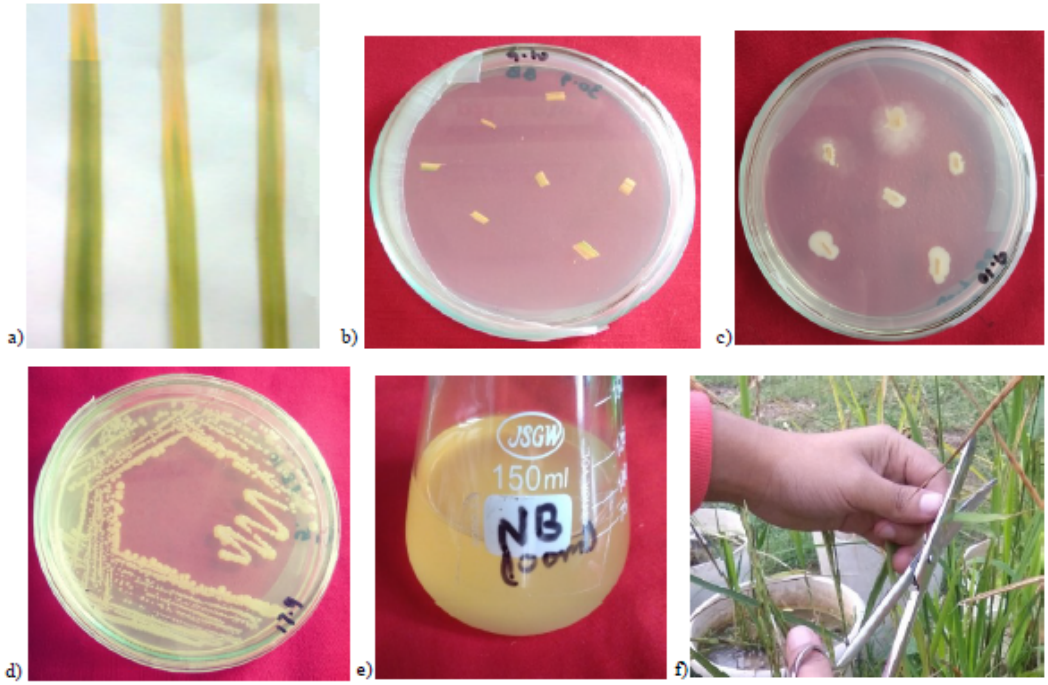


Figure 3. Isolation and infection of DH with Xanthomonas oryzae isolate. (a) Diseased plants taken for isolation of Xoo, (b) Cut sections of leaf for isolation of Xoo, (c) Isolated Xoo further sub cultured, (d) Inoculation of Xoo in NB (Nutrient broth), (e) Infection given to the plants using cut leaf clipping method.

Background selection of DH rice using SSR markers

Molecular markers can be used to identify haploid / double haploid plants at the early stage. All the plants can be differentiated using 6 polymorphic SSR markers RM6318 (199bp), RM224 (157bp), RM104 (222bp), RM1367 (159bp) and RM1135 (148bp).

For the PCR reaction PCR Buffer (3.00 µl), MgCl2 (1.20 µl), dNTPs (0.30 µl), primer (F) and (R) (1.00 µl each), Taq polymerase (0.15 µl) and template DNA 2.00 µl are suspended in sterile water to make the final volume of the reaction to 15 µl. The reaction cycle consists of initial denaturation at 94˚C for 4 min, denaturation of 30-35 cycles at 94˚C for 30 sec (for *Xa21* gene and SSR markers) and 1 sec (for *xa13* gene), annealing at 55˚C for 30 sec (for *Xa21* gene and SSR markers) and 1 sec (for *xa13* gene), extension at 72˚C for 30 sec and final Extension at 72˚C for 7 min (Sharma et al., 2020).

3. Results and Discussion

3.1. Results

3.1.1. Callus induction

First indication of callusing was observed within a few weeks of culturing when anthers turned black or brown from yellow and started swelling. Callus formation started from the cut ends and continued from 4-8 weeks. Embryogenic callus was compact, globular and pale yellow in color as shown in Figure 6a. Media composition especially with regard to combination and concentrations of the growth regulators had a great influence of callus induction frequency. The data represented in Table 1 indicates that the N_6 medium augmented with 2,4-D (2.5 mg L^{-1}), NAA (2.0 mg L^{-1}) and Kinetin (0.5 mg L^{-1}) exhibited the maximum callus induction frequency of 10.07 ± 0.087 percent. Callus induction in N_6 medium with 2,4-D (2.0 mg L^{-1}), NAA (0.1 mg L^{-1}) and kinetin (0.5 mg L^{-1}) was at par with N_6 medium supplemented with 2,4-D (2.5 mg L^{-1}), NAA (1.0 mg L^{-1}) and kinetin (1.0 mg L^{-1}) with callus induction frequency of 8.10 ± 0.176 and 8.26 ± 0.11 percent respectively. However, no callusing was observed in N_6 basal medium supplemented only with 2,4-D (0.5 mg L^{-1}) (Table 1, Figure 4.).

Table 1. Media compositions with varying Growth Regulators

Media	2,4-D (mg L^{-1})	NAA (mg L^{-1})	KN (mg L^{-1})	No. of anthers inoculated (a)	No. of anther producing callus (b)	CIF (b/a x 100)
T_o	0	0	0	450	0	0
T_1	0.5	0	0	468	0	0
T_2	0.5	1.0	0.5	462	7	1.5
T_3	0.5	1.0	1.0	498	10	2.0
T_4	1.0	1.0	0.5	444	10	2.25
T_5	1.0	1.0	1.0	208	6	2.88
T_6	1.5	1.0	0.5	480	24	5.0
T_7	1.5	1.0	1.0	380	5	1.31 ±
T_8	2.0	1.0	0.5	494	40	8.10 ± 0.176
T_9	2.0	1.0	1.0	488	32	6.55 ± 0.148
T_{10}	2.5	1.0	0.5	536	54	10.07 ± 0.087
T_{11}	2.5	1.0	1.0	484	40	8.26 ± 0.11
				5392	**233**	**4.32 ± 0.12**
			C.D. (P = 0.40)	**C.V. = 6.477**		
			SE(m) = 0.136	**SE(d) = 0.192**		

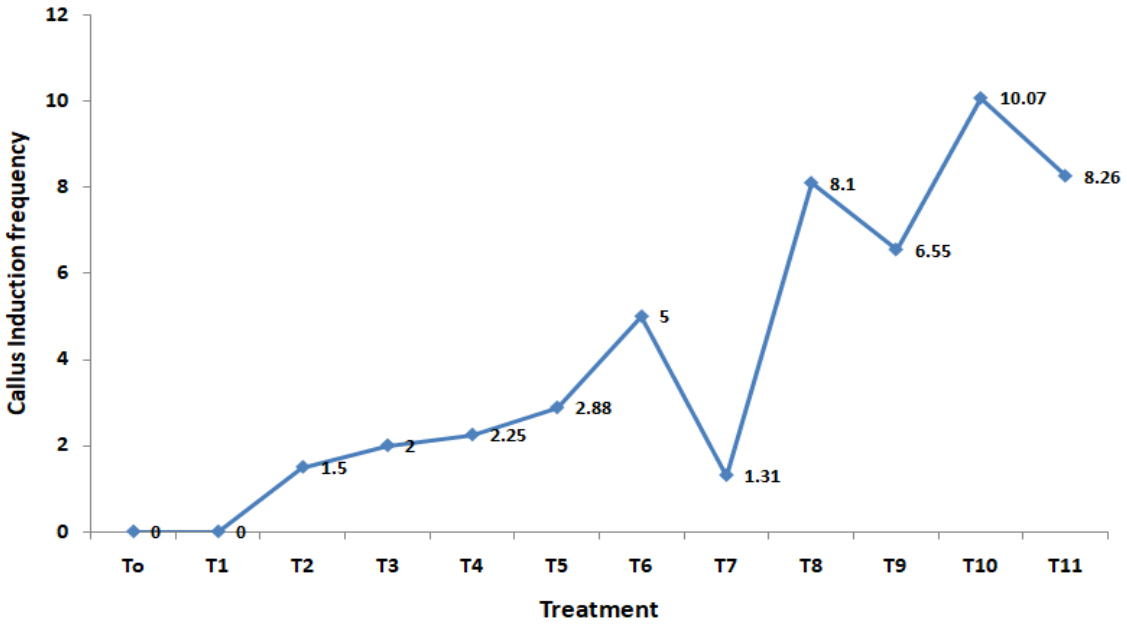


Figure 4. Callus Induction Frequency (CIF) of anther at different concentrations of growth regulators given in Table 1 [2,4-D (0.5 mg L^{-1} to 2.5 mg L^{-1}); Kinetin (0.5 mg L^{-1} and 1.0 mg L^{-1}) and NAA 2.0 mg L^{-1}]

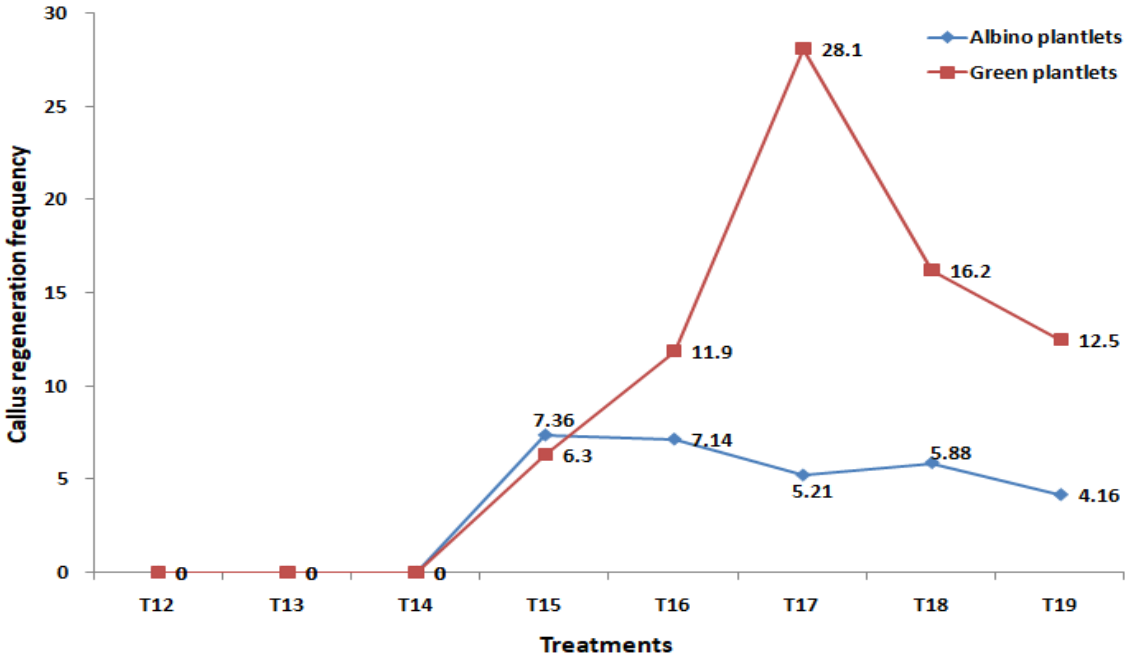


Figure 5. Callus Regeneration Frequency (CRF) of albinos and green plants at different concentrations of growth regulators given in Table 2 [Kinetin 0.5 mg L^{-1}; NAA 0.5 mg L^{-1} and BAP (0.0 mg L^{-1} to 2.5 mg L^{-1})].

3.1.2. Regeneration of plantlets from induced callus

Media composition and growth regulator concentration in culture medium plays an important role in the growth and morphogenesis. Haploid plants were obtained when the induced callus was transferred on MS medium augmented with different growth

regulators for regeneration (Figure 6b). Maximum regeneration frequency of green shoots (28.1 ± 0.18%) was observed after 3-4 weeks on MS medium containing BAP (2.5 mg L^{-1}), Kinetin (0.5 mg L^{-1}) and NAA (0.5 mg L^{-1}) followed by MS medium fortified with BAP (1.5 mg L^{-1}), Kinetin (0.5 mg L^{-1}) and NAA (0.5 mg L^{-1}). Basal medium or absence of cytokinins in MS medium failed to develop any shoot as shown in Table 2. Maximum of seven albinos were obtained in MS medium supplemented with BAP (0.5 mg L^{-1}), Kinetin (0.5 mg L^{-1}) and NAA (0.5 mg L^{-1}) with albino regeneration frequency of 7.36 ± 0.032% (Table 2, Figure 5).

Table 2. Media compositions used for regeneration

Media	BAP (mg L^{-1})	KN (mg L^{-1})	NAA (mg L^{-1})	No. of plants regenerated (b)	No. of albino produced (c)	ARF (c/a x100)	CRF (b/a x 100)
T_{12}	0	0	0	0	0	0	0
T_{13}	0	0	0.5	0	0	0	0
T_{14}	0	0.5	0.5	0	0	0	0
T_{15}	0.5	0.5	0.5	6	7	7.36	6.3
T_{16}	1.0	0.5	0.5	10	6	7.14	11.9
T_{17}	1.5	0.5	0.5	27	5	5.21	28.1
T_{18}	2.0	0.5	0.5	11	4	5.88	16.2
T_{19}	2.5	0.5	0.5	12	4	4.16	12.5
				66	**26**	**4.31± 0.04**	**10.9± 0.10**
For Albinos					**For green plants**		
C.D. (P = 0.173) C.V. = 2.684					**C.D. (P = 0.367) C.V. = 2.250**		
SE(m) = 0.057 SE(d) = 0.081					**SE(m) = 0.121 SE(d) = 0.172**		

Table 3. Rooting efficiency in different plant growth regulators concentrations

Media	NAA	BAP	Rooting efficiency
R1	0.0 mg L^{-1}	0.0 mg L^{-1}	+
R2	0.0 mg L^{-1}	0.5 mg L^{-1}	++
R3	0.5 mg L^{-1}	0.0 mg L^{-1}	+
R4	0.5 mg L^{-1}	0.5 mg L^{-1}	+
R5	0.5 mg L^{-1}	1.0 mg L^{-1}	+++
R6	1.0 mg L^{-1}	1.0 mg L^{-1}	+

The complete regenerated plantlets of optimal length were transferred to the MS media augmented with NAA and BAP for root formation (Figure 6c). Rooting was observed in all the concentrations but the healthy and dense rooting was seen in media concentration with NAA (0.5 mg L^{-1}) and BAP (1.0 mg L^{-1}) (Figure 6c). Out of sixty-six plantlets, thirty-four plants survived during the hardening process with survival rate of 51.51 percent. Amongst the surviving plants, twenty-one plants showed the sterility percentage above 50 percent and hence were considered as the doubled haploids (Table 4, Figure 7).

Table 4. Fertility percent showing the vigor of the plant

Plant no.	Unfilled grains	Filled grains	Total no. of grains	Fertility (%)	Double Haploid (DH) or Haploid (H)
1	75	115	190	60.53	DH
2	94	168	262	64.12	DH
3	61	154	215	71.63	DH
4	35	52	87	59.77	DH
5	162	29	191	15.18	H
6	21	71	92	77.17	DH
7	36	97	133	72.93	DH
8	24	25	49	51.02	DH
9	79	81	160	50.63	DH
10	32	43	75	57.33	DH
11	85	50	135	37.04	H
12	133	136	269	50.56	DH
13	26	32	58	55.17	DH
14	16	114	130	87.69	DH
15	54	153	207	73.91	DH
16	117	40	157	25.48	H
17	65	0	65	0	H
18	64	0	64	0	H
19	170	0	170	0	H
20	39	85	124	68.55	DH
21	35	37	72	51.39	DH
22	210	20	230	8.69	H
23	70	72	142	50.70	DH
24	4	56	60	93.33	DH
25	55	76	131	58.01	DH
26	50	65	115	56.52	DH
27	245	26	271	9.59	H
28	143	25	168	14.88	H
29	197	93	383	24.28	H
30	178	180	358	50.28	DH
31	265	0	265	0	H
32	73	12	85	14.12	H
33	105	36	141	25.53	H
34	20	80	100	80	DH

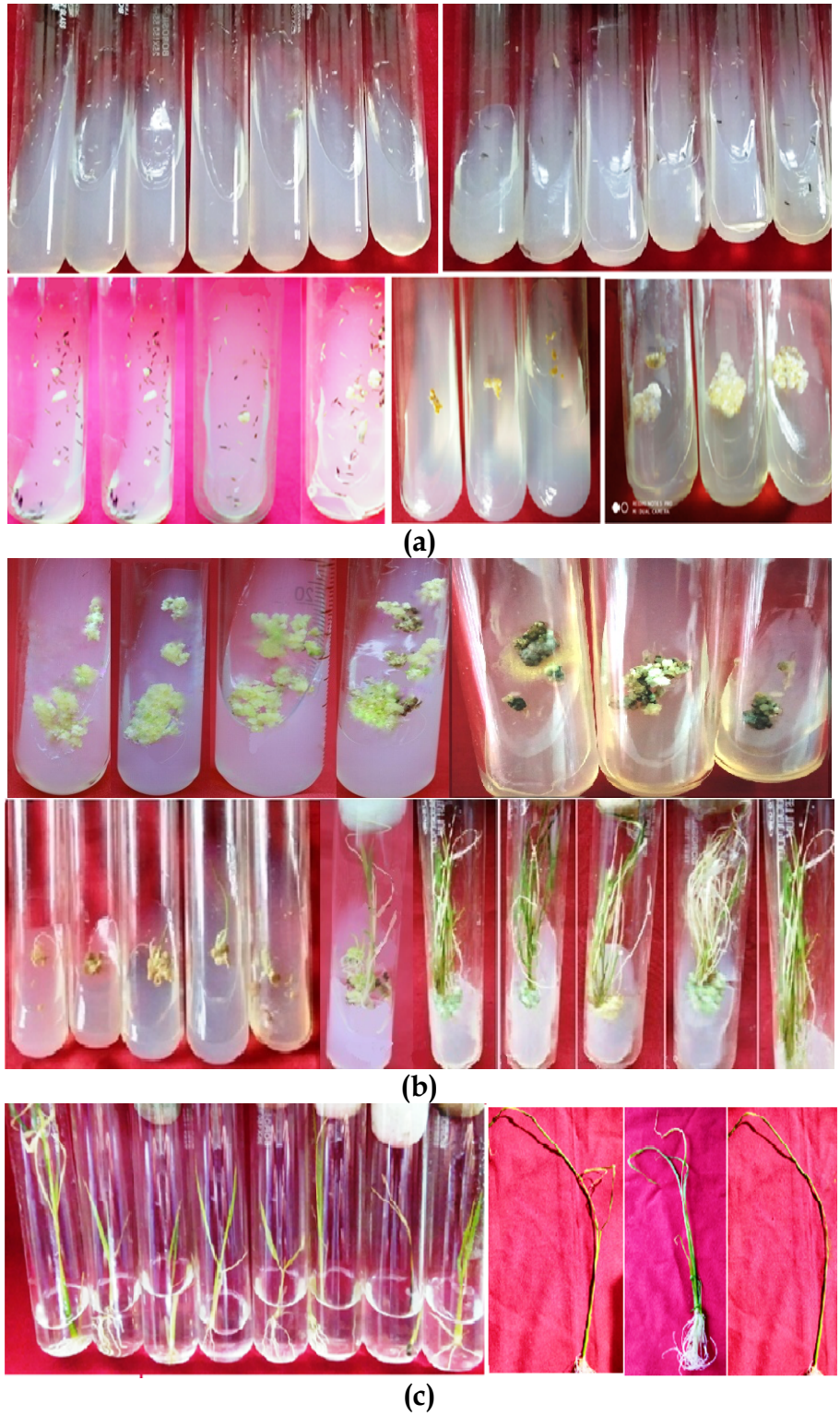


Figure 6. *In-vitro* procedure: **a)** callus induction and callus multiplication, **b)** shoot induction in regeneration media with different concentrations of growth regulators, **c)** root induction in rooting media with different concentrations of NAA and BAP.

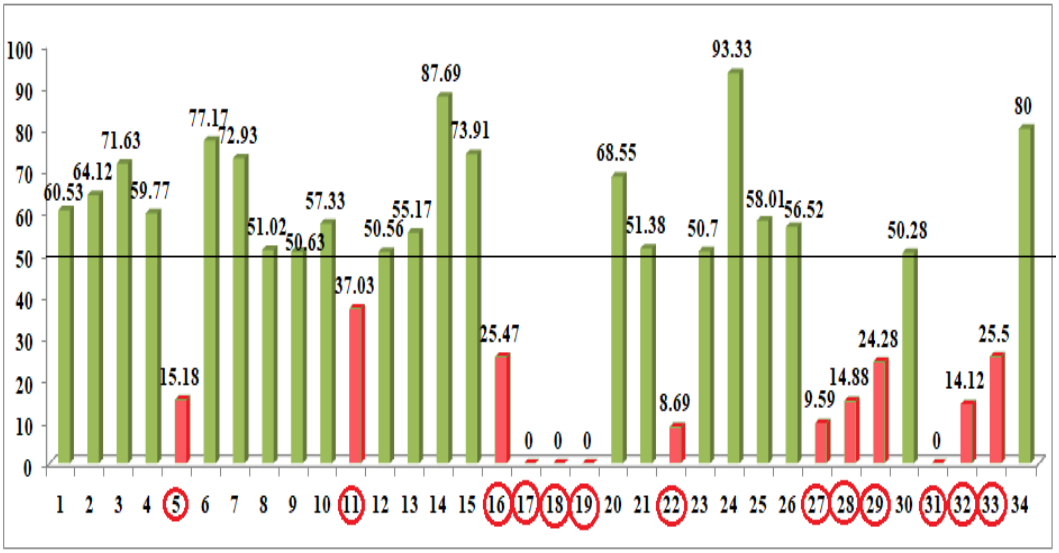


Figure 7. The fertility percent showing the plants that are DH. The bars in red show fertility below 50% showing that they are haploids and that in green are above 50 percent showing they are doubled haploids.

3.1.3. Identification of disease resistance in Double Haploids (DH)

Bacterial infection initiates after 5-6 days of inoculation with suspension culture and after 21 days of inoculation, yellowish-grey colored lesions of bacterial blight infection were seen spreading from point of inoculation towards the base of leaf.

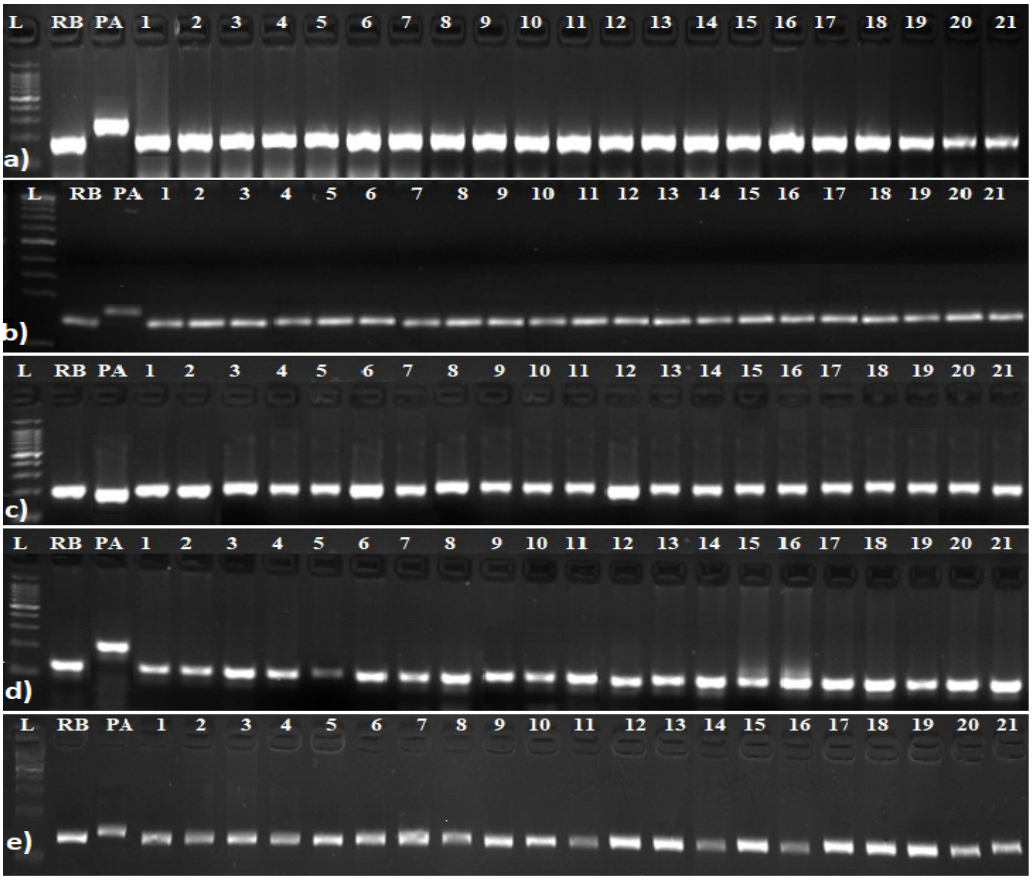
Table 5. Percent disease index (%) of DH plants

S no.	PDI (%)	Reaction
DH 1	4.17	R
DH 2	5.33	R
DH 3	5.00	R
DH 4	3.33	R
DH 5	4.33	R
DH 6	5.00	R
DH 7	1.33	R
DH 8	18.33	MS
DH 9	5.00	R
DH 10	3.90	R
DH 11	2.50	R
DH 12	1.90	R
DH 13	5.49	R
DH 14	4.91	R
DH 15	5.20	R
DH 16	3.82	R
DH 17	1.91	R
DH 18	2.22	R
DH 19	1.63	R
DH 20	6.81	MR
DH 21	3.22	R

Table 5 show that minimum percent disease index of 1.63 percent was recorded in DH19 and the reaction was considered resistant (R) while the maximum percent disease index was observed of 18.33 percent and it was considered as moderately susceptible (MS), followed by PDI of 6.81 percent considered as moderately resistant (MR). Majority of the genotypes expressed resistant response (MR) with PDI of 1 to 5.5%.

The primer pair, *Xa21* pTA248 showed amplicon size of 1000bp and 650bp amplicon in the resistant and susceptible genotypes and the primer pair, xa13-prom show amplicon size of 500bp and 250bp amplicon in the resistant and susceptible genotypes. Plant DH8 is susceptible and DH20 is heterozygous for gene *Xa21*. Plants DH8 and DH20 are susceptible for gene *xa13*.

The androgenic frequency was observed to be 1.41% and haploid regeneration frequency was observed to be 27.30% with the survival rate of 51.51% and DH induction frequency of 61.76% in spontaneously induced DHs. The fertility percentage was observed i.e. above 50% were considered as the doubled haploid plants. These were then confirmed with the markers for BB resistance as well as homozygosity (Figure 8).



(II)

Figure 8. SSR markers used to check polymorphism are: (I.a.) amplification *Xa21* gene and (I.b) amplification of *xa13* gene. (II.a)RM6318 (199bp), (II.b) RM224 (157bp), (II.c) RM104 (222bp), (II.d) RM1367 (159bp) and (II.e) RM1135 (148bp).

3.2. Discussion

From our study we observed highest callus induction frequency of 10.07% when the concentration of 2,4-D was 2.5 mg L^{-1}, that of Kinetin was 0.5 mg L^{-1} and NAA was 2 mg L^{-1}. Similar results were observed by various researchers. Highest callus induction in Azucena rice was obtained on N_6 medium supplemented with 2 mg L^{-1} 2,4-D and 1 mg L^{-1} Kinetin; 1 mg L^{-1} 2,4-D and 2 mg L^{-1} NAA and 0.5 mg L^{-1} Kinetin and 2 mg L^{-1} 2,4-D and 2 mg L^{-1} NAA and 1 mg L^{-1} Kinetin (Sharma et al., 2017). Highest callus induction of callus induction frequency of 6.66% highest in case of Azucena rice variety on N_6 medium supplemented with 1 mg L^{-1} 2,4-D and 2 mg L^{-1} NAA and 0.5 mg L^{-1} Kinetin was observed by Lal et al. (2014). Similarly, the highest callus induction frequency of 4.24% was observed in IKP (*Japonica*) variety on N_6 medium supplemented with 1 mg L^{-1} 2,4-D and 2 mg L^{-1} NAA and 0.5 mg L^{-1} Kinetin by Gueye and Ndoye (2010).

In our study the callus were most responsive in producing green plantlets in the media containing growth regulators in the concentrations of 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} Kinetin + 1.5 mg L^{-1} BAP with regeneration frequency of 28.1%. Highest callus regeneration frequency of 0.33% was observed by Lal et al. (2014) in Azucena rice variety on MS medium supplemented with 80mg Adenine sulphate and phytohormones in the concentrations of 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} Kinetin. Similarly, high regeneration frequency of 5.75% was observed in IKP (*Japonica*) variety by Gueye and Ndoye (2010) on MS medium supplemented with 1 mg L^{-1} BAP and 0.5 mg L^{-1} NAA.

We observed that the rooting was highest in MS media supplemented with 3% Sucrose and, 0.5 mg L^{-1} NAA and 1 mg L^{-1} BAP as growth regulators. Similarly, the highest rooting induction was seen in MS (liquid) media supplemented with 3% sucrose and phyto-hormones 0.5 mg L^{-1} NAA and 1 mg L^{-1} BAP for rooting by Gueye and Ndoye, (2010). The use of IBA (0.5 mg L^{-1}) as phyto-hormone in MS (liquid) media supplemented with 3% sucrose was used for rooting initiation by Ambarwati et al. (2009). The phyto-hormone IAA/IBA (1 mg L^{-1}) in MS (liquid) media supplemented with 3% sucrose was used for rooting purpose by Roy and Mandal (2004). Kaushal et al. (2014) used 1/2 MS without hormones for rooting medium from transferred regenerated green shoots and incubation at 25 ± 1° C, 16/8 hours of light and dark period and relative humidity of 65% (RH).

The introgression has been done before as when multiple genes for bacterial blight resistance *xa*5, *Xa*21 and *xa*33 from RG-9 were introduced into a high yielding and fragrant Manawthukha rice line (MK-75) using marker-assisted backcrossing (MAB) method by Win et al. (2013). Introgression of bacterial blight resistance (*Xa*21 and *xa*13) genes and aroma genes (*fgr*) gene was done into rice (*Oryza sativa* L.) by Salgotra et al., (2012) by a combination of phenotypic selection and MAS led to 20 genotypes out of 29 recombinants. Similarly, improvement of traditional basmati rice varieties (Taraori Basmati and Basmati 386) for bacterial blight resistance at BC_1F_5 possessing a single resistance gene (i.e. either *Xa*21 or *xa*13) from the donor Samba Mahsuri (high-yielding, fine-grain, BB resistant rice) variety was done by Pandey et al. *(*2012).

4. Conclusion

Best response of anther to callus was seen in media composition with N_6 with 4% maltose, 0.8 % Agar, 2.5 mg L^{-1} 2,4-D, 0.5 mg L^{-1} kinetin and 2 mg L^{-1} NAA, with high callus induction frequency of 10.07%. Least response of anther can be seen in media with N_6 with 4% maltose, 0.8 % Agar, 1.5 mg L^{-1} 2,4-D, 1 mg L^{-1} kinetin and 2 mg L^{-1} NAA, with low callus induction frequency of 1.31%. No anther response was seen in media with N_6 with 4% maltose, 0.8 % Agar and 0.5 mg L^{-1} 2,4-D.

Callus were most responsive in producing green plantlets in the media composition containing MS media with 3% Sucrose, 0.8% Agar, 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} kinetin and 1.5 mg L^{-1} BAP. Highest callus regeneration frequency, 28.1% was observed in case of media composition of MS media supplemented with 3% Sucrose, 0.8% Agar, 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} kinetin and 1.5 mg L^{-1}. Of 96 plants induced in MS media supplemented with 3% sucrose, 0.8% Agar, 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} Kinetin and 1.5 mg L-1 BAP is the best media composition with callus regeneration that gave albino plants (5.21%) and green plants (28.1 per cent). No callus responded when media concentration in MS media was 3% sucrose, 0.8% Agar, 0.5 mg L^{-1} NAA and MS media with 3% Sucrose, 0.8% Agar, 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} Kinetin.

The plants obtained have properties of Basmati (the aroma) and also are resistant to the Bacterial blight disease. These are homozygous and hence can be used as pure line parents for the new studies.

Acknowledgements and financial support

Authors would like to thank Dr. R.K. Salgotra for providing seed material for the experiment. This experiment was financially supported by the school of biotechnology, Sher-e-Kashmir University of Agricultural Science and Technology of Jammu.

Conflict of interest

Authors have no conflict of interest.

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