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Development of improved doubled-haploids through anther culture of indica rice (*Oryza sativa* L.)

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ABSTRACT

An intensive study was carried out to achieve green plant regeneration as well as to develop doubled-haploid lines (DHLs) from anthers of advanced breeding line, BR802-78-2-1-1. Response of cultured anthers to callus induction found in KA (no salt) by 2.9% and in KC (5.0 g/l NaCl) by 8.7%. Percent plant regeneration as well as production of green plants increased under salt stress. Data based on seed morphological characteristics of well developed DHLs were subjected to cluster analysis for grouping into types; designated as AC (anther culture) and numbered in ascending order of transferring to earthen pots. At least eight types of variants with a wide range of differences were conceivable including a type similar to the parent, BR802-78-2-1-1. Comparative analysis showed that all the variants had plant height lower than the parent. In respect of effective tillers, an important indicator of grain yield, AC156 and AC192, produced 100% effective tillers and only 4 and 3% grain sterility, respectively. The spikelet number per panicle was 306 and 294 with long-bold and long-slender grain in AC156 and AC192, respectively. The physical characteristics and performance of the AC lines revealed the potentiality of the doubled-haploids in fixing segregating populations. The H1 types will be grown for confirmation of homozygosity in the transplanted aman and boro seasons and further yield trials leading to variety development.

Keywords: Doubled-haploids, anther culture, green plant regeneration, physical properties, ancillary characters, variety development

Abbreviation: BAP: 6-benzylaminopurine, 2,4-D: 2,4-dichlorophenoxyacetic acid, IAA: Indole acetic acid, NaCl: Sodium chloride

INTRODUCTION

Rice (*Oryza Sativa* L.) is the most important food crop of Southeast Asia where almost half the world's population lives. Although rice production has increased almost threefold over the past three decades [1], continued increase in yield productivity and number of varieties is necessary to meet the rising demand of the global population [2]. Development of high yielding rice varieties through tissue culture viz. anther culture accelerates the breeding cycle by reducing the generation needed to fix a population (above F7-F9 generations) in a shorter period of time (F1 or advanced breeding lines) [3]. Anther culture, an unconventional approach, could be a complementary technique along with conventional breeding for rice improvement [2].

In vitro anther culture provides a rapid method for inducing homozygosity [4] and developing true breeding lines in the immediate generation from any segregating population [5], thereby contributes in shortening the breeding cycle of new varieties [6]. This technique also contributes to the development of doubled-haploid or dihaploid lines [7], which are useful for developing mapping populations for molecular analysis [3]. Several superior doubled-haploids have been selected for salinity tolerance [8, 9] and also are now being used as parents in breeding programme [9]. Anther culture technique also offers great opportunities for improving grain quality of rice [10] and production of

female plant in papaya [11]. Albino plant regeneration from anther culture is a crucial problem that interferes in generating a large number of doubled haploids [12, 13 and 14]. Therefore, high rate of green plant regeneration along with better agronomic performance is a prerequisite in anther culture for variety development.

Development of rice varieties using anther culture techniques has been reported in several countries [9, 15 and 10]. Most of the anther culture derived varieties are of the *Japonica* type. The *Indica* type rice is generally recalcitrant to culturability compare to japonica [16] and needs improvement through basic research of culturability. Therefore, overcoming recalcitrant in culturability of Indica rice preferred by South East Asian countries is essential towards variety development.

This study was carried out to enhance culturability, attain higher rate of green plant production and fix advanced breeding lines into homozygous population with the desired characteristics.

MATERIALS AND METHODS

Plant materials:

The advanced breeding line of indica rice, BR802-78-2-1-1 was used in the present study. Plants were grown in the net house under the optimum fertility and management during the month of May to August. Boots with appropriate stage (uninucleate/mid uninucleate at reduction division stages) of anthers in spikelets were collected at booting stage according to the methods of Karim and Zapata, 1994 [17], Shahjahan *et al.*, 1992 [18] and Afza *et al.*, 2000 [19]. The panicles were opened from boots and cut into small pieces. They were surface sterilized by emerging in 70% denatured alcohol (Keru and Co., Bangladesh) for 2-3 minutes and placed onto sterile filter paper in Petri dishes to absorb access alcohol. The spikelets were cut horizontally for getting anthers.

Anther culture and incubation:

The anthers were plated onto callus induction medium (CIM) consisting of modified B5 medium [20] containing 1.0 mg 2,4-D/liter, 0.5 mg IAA/liter and 0.5 mg BAP/liter. The medium was solidified by 8.0 g/liter agar. This medium is designated as KA. A treatment, containing sodium chloride (NaCl) at 5.0 g/l was added to the CIM (KA) prior to anther culture and designated as KC [17]. The CIM was adjusted to pH 5.7 with 1N/0.1N NaOH before autoclaving at 121°C for 15 minutes at 1.05 kg/cm² pressure (15-20 psi). Fifteen ml of autoclaved media (both KA and KC) was dispensed into each sterile glass Petri dishes and cultured anthers on it after cool and solid. The Petri dishes with anthers were sealed by semi-transparent and moisture resistant Parafilm. The cultures were repeated six times and incubated in a dark chamber (incubator) and maintained at temperature of 25°C until callus initiation and subsequent growth of up to appropriate size (2.0mm).

Shoot regeneration and acclimatization

The induced calli with appropriate size (approximately 4-5 fold larger than anther size) were transferred to regeneration medium (RM) consisting of MS medium [21] containing 1.0 mg/l of each Kinetin (K) and Naphtheleneacetic Acid (NAA). The cultures were incubated in a culture room under cool white fluorescent and incandescent lamps (approximately 1000 lux, measured by a lux meter) at 25°C for regeneration. The regenerated plantlets were transferred to MSO medium, without hormone, and incubated at the same culture room for further elongation and rooting. The healthy, green, well-elongated and well-rooted plants were acclimatized to ambient humidity level and transferred to fertile soil in pots. On establishment of the plants for about 2-3 weeks, they are again transferred to big earthen pots in net house and allowed to grow to maturity.

Ancillary characters:

At harvest, data on the various yield-contributing parameters of the anther-derived variants (designated as AC) were collected from one hundred fifteen doubled haploids and 162 haploid lines. The yield contributing parameters are plant height, tiller number, effective tiller percentage, panicle length, spikelet number per panicle, % sterility and 1000-grain weight. Physical properties of grain were also collected to show their size and shape. Percentage mean data on callus induction and both green and albino shoot regeneration were carried out. Almost 100 well-survived and yield contributing AC lines were used for cluster analysis on ancillary characters for grouping into distinct types to show the differences of DHLs.

Statistical Analysis:

A completely randomized design (CRD) was used for all experiments. Descriptive statistics such as mean and standard error were used for percentage of callus induction, shoot regeneration as well as green and albino plant production. Similar analysis was carried out for yield contributing five ancillary characters (e.g. plant height, % effective tiller, number spikelet/panicle, % sterility and 1000grain weight) and physical properties of rice grain

(grain length and breadth) of anther derived DHLs. Cluster analysis was also done to show the grouping of promising lines.

RESULTS

Callus Induction:

Anthers of BR802-78-2-1-1 cultured in KA and KC media (Fig. 1a) started responding by callus inducing (induction of undifferentiated mass of tissue) after two weeks of culture. Initially anthers divided into the middle furrow and calli initiated from inside. Two different types of calli initiated from anthers: (i) compact and whitish (ii) loose and light yellowish. Callus induction from anthers varied in KA and KC media. Higher percentage of callus (8.7%) was initiated from KC medium where 5.0 g/l NaCl was added, whereas, fewer calluses (2.9%) was found to initiate in KA (without addition of salt) in BR 802-78-2-1-1. The effect of salt on callus induction is shown in Fig. 2.

Shoot regeneration:

Shoot primordia were initiated from compact and whitish callus of anthers in shoot regeneration medium. Loose-whitish or loose-light yellowish calli did not response to shoot primordia initiation. Shoot primordia initiated from BR802-78-2-1-1 after 5-7 days of transfer in shoot regeneration medium. Calli with size of 2.0 mm or over initiated shoot primordia. No shoot primordium was seen to initiate from callus smaller than 2.0 mm. Shoot regeneration varied in both KA and KC media. Calli, induced from KC medium, showed higher regeneration (55%) compared to KA (45%). The effect of media on the shoot regeneration is shown in Fig. 3. The regenerated shoots elongated well on MSO medium (without hormone) as well as vigorous rooting was found in regenerated shoots when transferred to MSO medium.

Green and albino plant regeneration:

Calli, induced from KA and KC medium, responded differently into green and albino plant production. Better performance in green plant regeneration was found in calli induced from KC medium (Fig. 1b) in comparison to KA. However, albino plant regeneration was higher from calli induced in KA medium (Fig. 1c) rather than KC. Ninety six percent (96%) green plant was regenerated in BR 802-78-2-1-1 from KC medium whereas, only 63% was regenerated from KA (Fig. 3). On the other hand, 37% albino plant was regenerated from KA media, whereas, 4% was regenerated from KC in the same line (Fig. 3). The combined effect of KA and KC media also showed higher regeneration of green plants (80%) compared to albino plant regeneration, 20% (Fig. 3).

Vigorous shooting in number was found when they were albinos. Extensive rooting also initiated from albino plants. Similar rooting was also seen in green plants (Fig. 1d). Most of the green and albino plants were healthy and well elongated. Less number of poor healthy plants was found in BR 802-78-2-1-1. The poor healthy anther derived lines initiated roots with lower numbers.

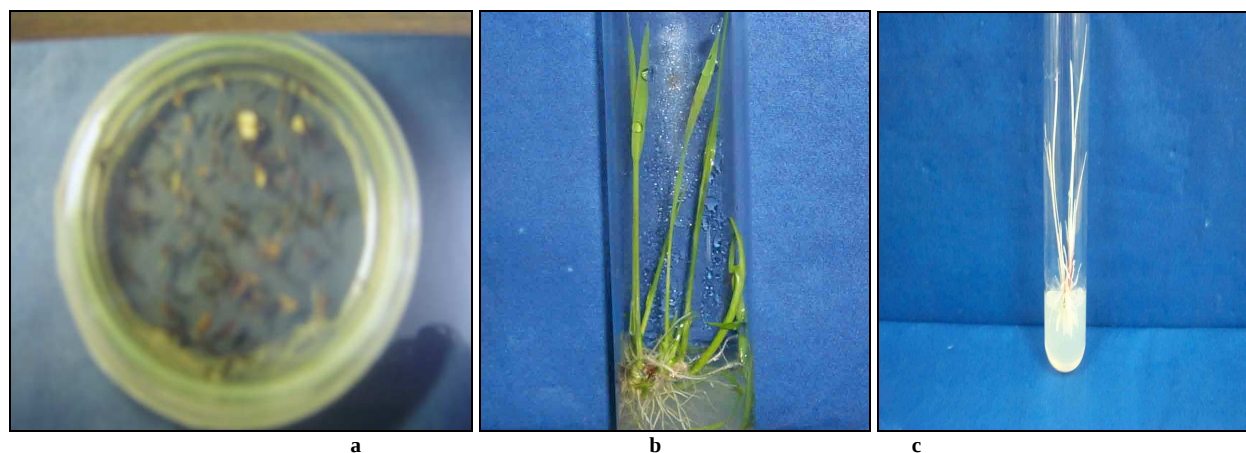




Fig. 1: Anther culture and regeneration. (a) Anthers plated on medium with salt and initiated callus (b) Green plants regenerated under salt stress (c) Albino plants regenerated from KA medium (d) Vigorous rooting observed in anther derived plants (e) Variants with plant height and (f) Variation in length of panicles

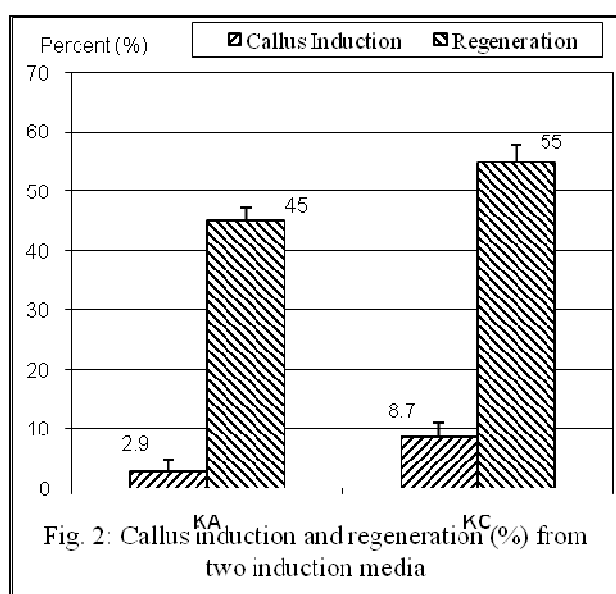


Fig. 2: Callus induction and regeneration (%) from two induction media

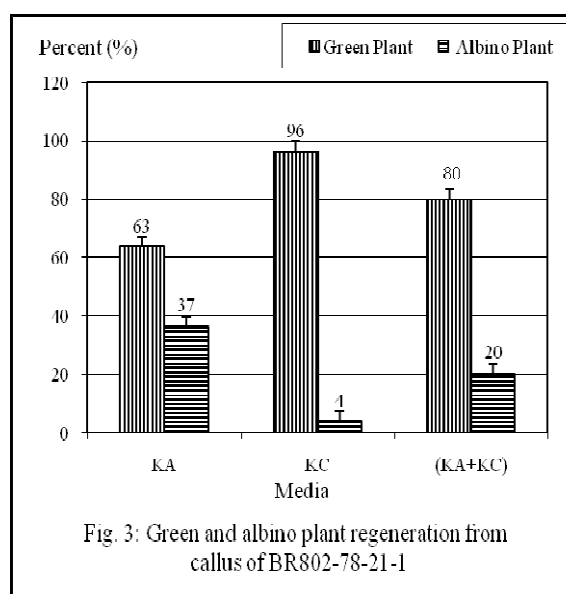


Fig. 3: Green and albino plant regeneration from callus of BR802-78-21-1

Acclimatization:

All the healthy, green, well-elongated and well-rooted plants were acclimatized well to ambient humidity level. Over 79%-acclimatized plants survived well after transfer to soil in pots. No albino plants survived in soil as well. The poor healthy plants died off during acclimatization process.

Table 1: Ancillary characters of 7 variants (H0) of BR 802-78-2-1-1 including parent

Sl. Nos.	Type Name	Plant Height (cm)	% Effective Tiller	No spikelet/ panicle	% Sterility	1000 grain Weight (gm)
1	Parent	119±4.3	81±1.4	227±2.1	17±2.2	27.63±2.3
2	AC135 (dihaploid)	98±3.1	91±0.7	138±2.5	65±2.4	39.08±1.3
3	AC156 (dihaploid)	98±2.4	100±0.0	306±3.1	4±1.1	23.97±1.1
4	AC192 (dihaploid)	94±3.7	100±0.0	294±2.1	3±1.4	19.15±1.3
5	AC230 (dihaploid)	98±2.4	86±0.8	69±2.3	12±2.1	29.4±1.2
6	AC10 (dihaploid)	74±3.9	84±0.6	140±2.5	14±1.6	26.42±2.5
7	AC114 (dihaploid)	81±2.1	95±1.2	153±4.3	31±1.8	17.70±1.2
8	AC167 (dihaploid)	100±2.9	83±0.9	294±5.2	4±0.8	27.01±2.2
9	AC71 (haploid)	63±2.5	0.0	372±3.3	100±0.0	0.0

Ancillary characters are average mean of 9-14 tillers/hill.

Ancillary characters of AC derived plants:

Data on the various yield-contributing ancillary characters were collected on all the anther-derived plants, designated as AC of BR802-78-2-1-1. One hundred fifteen (115) doubled-haploids and 162 haploids were grown to maturity in pot under net house condition. The doubled-haploids were seen to fall into seven (7) noticeable types depending primarily on the height of plant (Fig. 1e), length of panicle (Fig. 1f), grain type and spikelet number per

panicle (Table 1). These noticeable types are AC10, AC114, AC135, AC156, AC230, AC167 and AC192. Among these noticeable types, the effective tiller percentage was 100% in AC156 and AC192, a higher figure than the parent (81%) (Table 1). Two of these types such as AC156 and AC192 have spikelet number of 306 and 294, respectively. These lines also have grain sterility of around 3 to 4%. On the other hand, the parent type showed 227 spikelets per panicle with 17% grain sterility (Table 1).

All the AC types including haploids were shorter in size than the parent (Table 1). Parent size was 119 cm high; however, most of the types ranged from 93 to 98 cm. Lowest height was found in AC10 (74.0 cm). All the haploid plants were 60-65 cm in height. Percentage effective tiller was also found higher in AC135 and AC 114 (>90%) (Table 1).

The thousand-grain weight of these seven (7) selected lines ranged from about 17.70 to 39.08 grams. Among these AC lines, though AC156 and AC192 showed better ancillary characters, especially in % effective tiller, number spikelet/panicle and % sterility compared to the parent, their 1000-grain weight was less than the parent. On the other hand, AC135 and AC230 found better in 1000-grain weight e.g. 39.08 and 29.40 gram, respectively compared to parent (Table 1).

Physical properties of AC lines:

Of the seven types, the grain size, shape and type were observed to be long bold to long slender and medium bold (Table 2). Among the different grain sizes, AC230, AC135, AC167 and AC156 showed higher grain length compared to the parent; on the other hand, AC192, AC10, and AC114 showed grain length lower than the parent. Based on grain size we found long size of grains in AC135, AC156, AC192, AC230 and AC167 including parent. However, medium size was found in AC10 and AC114. Similarly, based on length/breadth ration AC135, AC156, AC10, AC114 and AC167 including parent type grains were bold shaped while AC192 and AC230 showed slender shape.

Table 2: Physical Properties of anther derived variants of BR 802-78-2-1-1

Sl. Nos.	Plant Type	Grain Length (mm)	Grain size	Grain Breadth (mm)	Length/Breadth (L/B) ratio	Size and shape
1	Parent	6.63±0.3	Long	2.53±0.2	2.62	Bold
2	AC135	7.76±0.2	Long	2.83±0.1	2.74	Bold
3	AC156	6.80±0.3	Long	2.57±0.1	2.65	Bold
4	AC192	6.37±0.2	Long	1.93±0.2	3.30	Slender
5	AC230	8.10±0.1	Long	2.10±0.3	3.86	Slender
6	AC10	5.83±0.2	Medium	2.03±0.1	2.87	Bold
7	AC114	5.30±0.1	Medium	2.00±0.2	2.65	Bold
8	AC167	7.65±0.3	Long	2.59±0.1	2.95	Bold
9	AC71	Haploid	-	-	-	-

Grain length and breadth are average mean of 20 grains. Long = >6.0 mm, Medium = 5.0-5.9 mm, Short = <5.0 mm; Slender=>3.0 L/B, Bold= 2.0-2.9 L/B and Round=<2.0 L/B

The above anther-derived lines, especially AC156 and AC192 appear very promising. The ancillary characters revealed the potentiality of these selected doubled haploids in attaining the desired yield for them to be recommended as varieties (Figs. 4 and 5). These lines will be tested in the T. Aman and Boro seasons of Bangladesh to check their performance and adaptability.

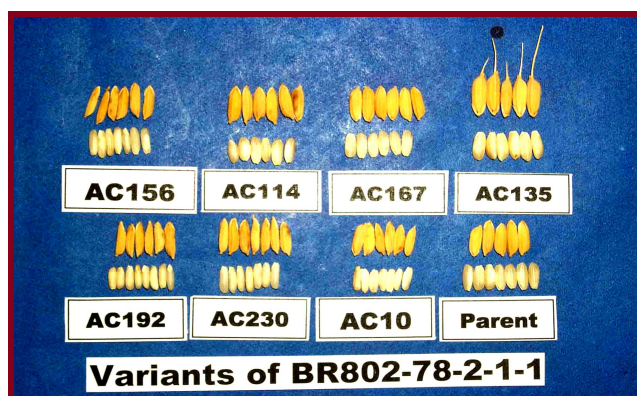




Fig.4. Anther derived variants observed in rice spikelet. Fig.5. Physical properties of Anther derived grains

Cluster Analysis:

Cluster analysis of 100 H0 lines showed remarkable variation among the doubled haploids derived from BR802-78-2-1-1. The characters analyzed were panicle length, spikelets per panicle, percent sterility, length and breadth of grain. The derivatives of BR802-78-2-1-1 (H0 population) formed 10 clusters. The highest number of derivatives was in Cluster 3, followed by Cluster 1. Only 2 derivatives (AC260 and AC90) were found in Cluster 8 and 9, respectively. Our finding shows that in each cluster the characteristics were comparable and the promising derivatives fell into different cluster groups. The characteristics of each group were different thereby allowing selection of the desired anther derived lines belonging to each cluster will be considered in variety development (Table 3).

DISCUSSION

Initiation of callus with totipotent traits is prerequisite to regenerate plants with high rates. Medium is an important and essential factor towards achieving this goal. Anthers of advanced indica lines, BR802-78-2-1-1 initiated callus better rate in KC medium supplemented with NaCl 5.0 mg/l instead of KA (without NaCl). It suggests that anthers could show better performance in embryogenic callus induction under salt stress. Wang *et al.*, 2011 [15] suggests that media composition play a vital role in embryogenic callus initiation in rice. Similar result was found in barley (*Hordeum vulgare* L.) anther culture on embryo yield and green plant production [13], in rice [14, 15] and in wheat genotypes [22]. Opposite response was found in seed culture of japonica varieties Taipae 309 with salt stress [17], probably due to different explant or varieties used.

Table 3: Cluster analysis of anther derived plants and their grouping

Sl. Nos.	Cluster Groups	Anther derived regenerated plants belong to each cluster
1	I	AC34, <u>AC10</u> , AC18, AC27, AC66, AC72, AC86, AC22, AC117, AC26, AC3, AC11, AC50, AC79, AC171, AC185, AC69, AC28, AC40, AC24, AC165, AC37, AC91, AC5
2	II	AC77, AC184, AC70, AC78, AC38, AC52, AC57, AC73, AC59, AC82, AC136, AC245, AC75, AC116, AC239, AC41, <u>AC167</u>
3	III	AC74, AC183, AC64, AC248, AC63, AC76, AC55, AC20, AC43, AC219, AC247, AC123, AC44, AC25, AC8, AC21, AC12, AC13, AC37, AC29, AC33, AC1, AC6, <u>AC135</u> , AC34
4	IV	AC42, AC187, AC100, AC49, AC51, AC53, AC97, AC14, <u>AC156</u>
5	V	AC68, AC81, AC161, AC9, AC54, AC80, <u>AC114</u> , AC58
6	VI	AC94, AC195, AC96
7	VII	AC93, AC89, AC88, AC98, <u>AC230</u> , AC32, AC62
8	VIII	AC260
9	IX	AC90
10	X	AC2, AC31, <u>AC192</u> , AC99, AC146

Callus with minimum size 2.0 mm was able to initiate shoot primordial. This achievement further confirms the report published by Afza *et al.*, 2000 [19]. Callus size less than 2.0mm not initiating shoot primordia suggests unsuitable size for regeneration. Loose whitish or loose and light yellowish calli were unresponsive to shoot primordia initiation indicating such type of calli is non-embryogenic.

Albino plant regeneration is a common phenomenon of anther culture in rice [23]. Similar result was observed in rice [7, 14] and maize [24]. It may be due to the partial development of plastid in cell or its genome or changes in DNA of plastids in anther derived plants [23]. Sun *et al.*, 1979 [25] 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. It also identified the absence of ribosomes in ill-developed plastids as another cause of albinism in rice [25].

Enhancement of green plant regeneration is a major achievement of this study, on the other hand, higher rate of albino plant production from anther is a crucial problem [23]. In this experiment we triumphed over this crucial problem by regeneration of high rates of green plant in KC medium with salt stress. Control medium (KA), on the other hand, produced almost nine times more albino plants in BR802-78-2-1-1. This suggests that salt stress has a key role in enhancement of green plant regeneration as well as decrease albino plant regeneration.

Plants along with remarkable ancillary and better physical characters of grain are prerequisite towards rice variety development [8, 10]. We found plants with seven different types of remarkable variants compared to the parent, BR 802-78-2-1-1 indicating that anther culture could be an alternative for variety development. Senadhira *et al.*, 2002 [9] developed first salt tolerant rice cultivar through anther culture. Production of slender rice (high grain quality) has commercial acceptance over the world like Basmati. Two types of DHLs, AC230 and AC192 found from this result are of slender/fine grain quality. Although these are not as slender as Basmati, conventional breeding between DHLs (AC230 and AC192) and Basmati could produce better slender type of indica rice varieties which will bring better future in rice development.

CONCLUSION

The above results showed cultural conditions molds the performance of a variety in media; in this case media composition as reflected by different callus induction and regeneration. It is therefore essential to test the right kind and amount of nutrients, vitamins, and hormones to be used for *in vitro* rice culture in order to get the best response leading to higher rates of plant production through anther culture. Stress of salt (NaCl) showed a high impact in initiation of higher amount of embryogenic callus and regeneration of green plants. The regeneration of agronomically promising lines from BR802-78-2-1-1 suggests the potentiality of anther culture leading to variety development.

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