ORIGINAL PAPER

Comparative study of tissue culture response of some selected basmati rice cultivars of Pakistan

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Key Message Four basmati rice cultivars of Pakistan were evaluated by their tissue culture responses. Basmati 370 and Super basmati were found to be efficient for callus formation and *in vitro* regeneration, respectively.

ABSTRACT This study was conducted to select the best tissue culture responsive basmati rice cultivar among Basmati 370, Basmati 385, Super basmati and Shaheen basmati. N6 and MS media having four levels of 2, 4-D (1.5, 2.0, 2.5 and 3.0 mg/l) and three levels of agar (4.0, 5.0 and 6.0 g/l) were used in order to evaluate the most appropriate level required for calli formation. Basmati 370 showed the best response as compared to other cultivars on both MS and N6 media. The most cultivars behaved well for callus induction at N6 media, 5.0 g/l agar and 2.0-2.5 mg/l 2, 4-D. Basmati 370 showed callus formation; 74.0-85.07%, Super basmati showed 65.5-75.17%, Basmati 380 showed 72-79% and Shaheen basmati showed 53.0-67.87% callus formation. For regeneration, various treatments (0.5/2.0, 1.0/4.0, 1.5/5.0 mg/l) of NAA/BAP were used. Super basmati exhibited the best response for regeneration (58.33%) at 1.0/5.0 NAA/BAP. Based on our findings, Super basmati was found to be the best tissue culture responsive cultivar. These results will be helpful in unveiling many aspects of callus induction and regeneration and have potential use in genetic improvement of rice by employing various techniques of genetic transformation including *Agrobacterium*-mediated transformation and gene gun method.

Keywords: Basmati 370, Basmati 385, Callus, Regeneration, Super basmati, Shaheen basmati

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INTRODUCTION

Rice scientifically known as *Oryza sativa* (L.) is considered as a staple food crop for more than 50% population of the world, thus, making it a second most important cereal grain after wheat (Long-ping et al., 2014; Zahra et al., 2015; Akhter et al., 2015). It belongs to family Poaceae and has three major subspecies i.e. *japonica*, *indica* and *javanica*. The most cultivated subspecies in the world is *indica* that added 90% of total rice production of the world (Sharan et al., 2004). The three types of rice i.e. basmati, coarse, and short grain cold tolerant rice are being cultivated in Pakistan. After wheat, rice is 2nd staple food for the people of Pakistan that occupies approximately 2.58 million hectares with 5.54 million tons paddy production (Shakoor et al., 2015; Ahmed et al., 2015; Mahmood et al., 2016). Basmati rice has the best quality and aromatic which is exported to other countries (Rabbani et al., 2010; Abbasi et al., 2015).

The production of rice is declining due to urbanization, environmental as well as biotic factors. It has been estimated that the population of the world will be increased up to 9 billion in 2050 (Kajala et al., 2011). These

circumstances warn us to improve the yield of rice through the latest biotechnological approaches in addition to conventional approaches so that the requirements will be fulfilled (Kajala et al., 2011).

Genetic transformation is one the techniques of biotechnology which is being used by the researchers to improve the crop yield. This technique is dependent on tissue culture that is based on different factors such as source and type of explants, types of media, growth regulators, gelling agent and genotype (Joyia & Khan 2013, Mehmood et al., 2013; Shah et al., 2013; 2014a, b; 2015a).The tissue culture of rice is genotype dependent.

Therefore, the current research study was planned to optimize the most suitable level of phytohormones and agar for calli induction and *in vitro* shoot regeneration and to select the most tissue culture responsive cultivar comparing four basmati cultivars (Basmati 370, Basmati 385, Super basmati and Shaheen basmati). Our findings will be useful for tissue culture of different other rice cultivars. The best tissue culture responsive cultivar can be used in genetic transformation experiments for the improvement of rice yield.

MATERIALS AND METHODS

Media preparation

MS basal media (Murashige & Skoog, 1962) and N6 media (Chu, 1978) enriched with four treatments of 2, 4-D (1.5, 2.0, 2.5 and 3.0 mg/l), and agar (4, 5 and 6 g/l) were prepared separately and used for callus induction. MS basal media was supplemented with different combinations of BAP/NAA (4.0/0.5, 5.0/1.0 and 6.0/1.5 mg/l) were used as regeneration media. pH 5.75-5.8 was maintained for each media. Subsequently, the media was sterilized by autoclaving at 121˚C temperature for 20 min.

Sterilization of seeds

Mature seeds of four basmati cultivars; Basmati 370, Basmati 385, Super basmati and Shaheen basmati were de-husked, rinsed with autoclaved distilled water for dust removal. Ethanol (70%) was used to sterilize the seeds for 1 min time duration, and 50% Clorox (Sodium hypochlorite) was also used for fifteen minutes. Subsequently, the seeds were washed away 3-4 times with autoclaved water to eliminate the Clorox. At the end, the sterilized seeds were placed on tissue paper to suck the extra water.

Callus induction

The sterilized seeds were inoculated on callus induction media under controlled conditions for a period of two weeks. The propagated embryogenic calli were placed on maintenance media (callus induction media) for 3-4 days for further propagation.

Regeneration and acclimatization

After three weeks, the embryogenic calli were shifted on regeneration media for shoot formation. After shoot growth, plantlets were transferred to rooting media (MS media fortified with 0. 5 mg/l NAA) for two weeks. The plantlets bearing roots transferred to tubes containing tap water for acclimatization for one week before shifting of plantlets to the soil. At the end, healthy plantlets were transferred to green house.

Statistical analysis

The study design was Completely Randomized Design (CRD). Data was recorded in percentage for each treatment and then ANOVA was employed. Means having significant differences were compared at $P<0.05$ with Duncan's Multiple Range Test (DMRT) through M-STAT-C software.

RESULTS

Response of cultivars on callus induction media

The effect of cultivars on callus induction media has been shown in table 1. All the cultivars exhibited different responses showing significant differences with respect to callus induction frequency. Basmati 370 gave the best callus induction frequency on both MS and N6 media. Super basmati was significantly better than Shaheen basmati on both types of media. These results indicated that each cultivar had different capacity for callus induction; it may be due to difference in genetic makeup among different cultivars. In the present study, response of 2, 4-D for callus induction was checked in four cultivars of rice. The results revealed that 2.5 mg/l and 2.0 mg/l 2, 4-D were significantly better ($P<0.05$) for callus induction of rice cultivars than that of 1.5 mg/l and 3.0 mg/l 2, 4-D on MS media, while 2.5 mg/l 2, 4-D was found to be the best on N6 media (Table 2). It is clear from these results that the most basmati cultivars showed excellent response for callus formation on callus induction media supplemented with 2.0-2.5 mg/l 2, 4-D.

Response of cultivars on MS media fortified with different levels of agar

The table 3 indicated that Basmati 385 showed the best performance for callus formation (76.28%) at 5 g/l agar. Basmati 370 and Super basmati gave the maximum callus induction frequency (76.62 and 68.23%), respectively) at 6 g/l agar. Shaheen basmati revealed the best response of callus induction frequency (62.15%) at 4 g/l agar. However statistically there was no significant ($P>0.05$) effect among different levels of agar on callus induction in basmati cultivars (Table 3).

Response of cultivars on N6 media fortified with different levels of agar

The effect of agar on callus induction of basmati rice cultivars on N6 media has been shown in table 4. The results revealed that the highest callus induction frequency (85.07%) was obtained by Basmati 370 followed by Basmati 385 at 5 g/l agar, while Super basmati produced 75.17% callus induction frequency on N6 media fortified with 6 g/l agar. Hence, Basmati 370 was proved to be best cultivar for callus formation at 5 g/l agar. The results also indicated that less amount of agar (4 g/l) was not suitable for callus induction as compared to 5 and 6 g/l agar (Table 4).

Interaction of cultivars and MS-supplemented 2, 4-D media for calli formation

It was observed that there were no significant consequences for interaction of 2, 4-D and cultivars on callus formation on MS media because probability value was greater than that of 0.05 alpha level. Table 5 showed that Basmati 370 produced 82.99% callus induction at 2.0 mg/l 2, 4-D followed by Basmati 385 that yielded 76.97% callus induction at 2.5 mg/l 2, 4-D.

Interaction of cultivars and N6-supplemented 2, 4-D media for calli formation

Table 6 indicated that 68.74% calli was produced by Shaheen basmati, 82.4% by Basmati 385, 75.92% by Super basmati and 83.17% by Basmati 370 at 2.5 mg/l 2, 4-D. But statistically, interaction of 2, 4-D with cultivars had no significant ($P>0.05$) difference for callus induction on N6 media.

Regeneration of plantlets

Regeneration of all the cultivars under investigation was optimized on MS media having various combinations of NAA and BAP. The best regeneration response was achieved on MS media supplemented with 1.0 mg/l NAA and 5.0 mg/l BAP. The highest regeneration frequency (58.33%) was recorded in Super basmati followed by Shaheen basmati that gave 35% regeneration frequency on MS basal media having 1.0 mg/l NAA and 5.0 mg/l BAP. Green spots appeared on some calli and then shoots were regenerated from these green spots (Fig. 1). The results revealed that MS media supplemented with 1.0 mg/l NAA and 5.0 mg/l BAP was found to be optimum for successful regeneration and Super basmati showed the best regeneration response (Table 7). These plantlets were shifted on rooting media for roots formation.

Table 1 Response of basmati rice cultivars for callus induction (%) on MS and N6 basal media

Cultivars	Callus induction frequency (%)	
	N6 basal media	MS basal media
Basmati 370	77.99[a]	75.17[a]
Basmati 385	75.03[a]	72.58[a]
Super basmati	70.89[b]	66.26[b]
Shaheen basmati	61.74[c]	60.01[c]

Similar superscript letters represent no significant differences, while diverse letters are exhibiting significant differences. DMRT was employed at 0.05 probability level. LSD value for MS media is 3.65, while that of N6 media is 3.21.

Table 2 Callus induction response (%) of basmati rice cultivars at different levels of 2, 4-D

2, 4-D (mg/l)	Callus formation (%)	
	MS basal media	N6 basal media
1.5	63.74[b]	64.60[d]
2.0	72.32[a]	73.80[b]
2.5	72.33[a]	77.57[a]
3.0	65.63[b]	69.67[c]

Similar superscript letters represent no significant differences. DMRT was employed at 0.05 probability level. LSD value for MS media is 3.654, while that of N6 media is 3.219.

Table 3 Callus induction response (%) of basmati rice cultivars on MS media enriched with different levels of agar

Cultivars	Mean callus induction frequency (%)		
	4 g/l agar	5 g/l agar	6 g/l agar
Basmati 370	72.76[a]	76.12[a]	76.62[a]
Basmati 385	68.80[a]	76.28[a]	72.66[a]
Super basmati	63.02[a]	67.53[a]	68.23[a]
Shaheen basmati	62.15[a]	60.59[a]	57.29[a]

Similar superscript letters represent no significant differences. LSD value is 21.92 at 0.05 probability level

Table 4 Callus induction response (%) of basmati rice cultivars on N6 media supplemented with different levels of agar

Cultivars	Mean callus induction frequency (%)		
	4 g/l agar	5 g/l agar	6 g/l agar
Basmati 370	74.56[bc]	85.07[a]	74.33[bc]
Basmati 385	73.29[bcd]	79.05[b]	72.74[bcd]
Super basmati	65.10[e]	72.39[cd]	75.17[bc]
Shaheen basmati	53.12[f]	67.87[de]	64.24[e]

Similar superscript letters represent no significant differences. LSD value is 5.57 at 0.05probability level

Table 5 Interaction of 2, 4-D and basmati rice cultivars for callus induction (%) on MS media

Cultivars	2, 4-D			
	1.5 mg/l	2.0 mg/l	2.5 mg/l	3.0 mg/l
Basmati 370	68.82[ab]	82.99[a]	76.70[ab]	72.16[ab]
Basmati 385	69.48[ab]	74.80[ab]	76.97[ab]	69.07[ab]
Super basmati	62.03[ab]	68.75[ab]	71.06[ab]	63.19[ab]
Shaheen basmati	54.63[b]	62.73[ab]	64.58[ab]	58.10[ab]

Similar superscript letters represent no significant differences. LSD value is 21.92 at 0.05 probability level

Table 6 Interaction of 2, 4-D and basmati rice cultivars for callus induction (%) on N6 media

Cultivars	2, 4-D			
	1.5 mg/l	2.0 mg/l	2.5 mg/l	3.0 mg/l
Basmati 370	69.25^{ab}	81.28^{a}	83.17^{a}	78.24^{a}
Basmati 385	71.11^{ab}	73.41^{ab}	82.45^{a}	73.14^{ab}
Super basmati	67.59^{ab}	72.45^{ab}	75.92^{a}	67.59^{ab}
Shaheen basmati	50.45^{b}	68.06^{ab}	68.74^{ab}	59.72^{ab}

LSD value is 19.31 at 0.05 probability level. Similar superscript letters represent no significant differences

Table 7 Regeneration (%) on MS media enriched with NAA and BAP

Varieties	NAA/BAP		
	0.5/4.0 mg/l	1.0/5.0 mg/l	1.5/6.0 mg/l
Basmati 370	6.66^{g}	21.66^{cd}	8.33^{fg}
Basmati 385	6.66^{g}	31.66b^{c}	10.00^{efg}
Super basmati	18.33^{def}	58.33^{a}	21.66^{cd}
Shaheen basmati	16.66^{defg}	35.00^{b}	20.00^{de}

Similar superscript letters represent no significant differences

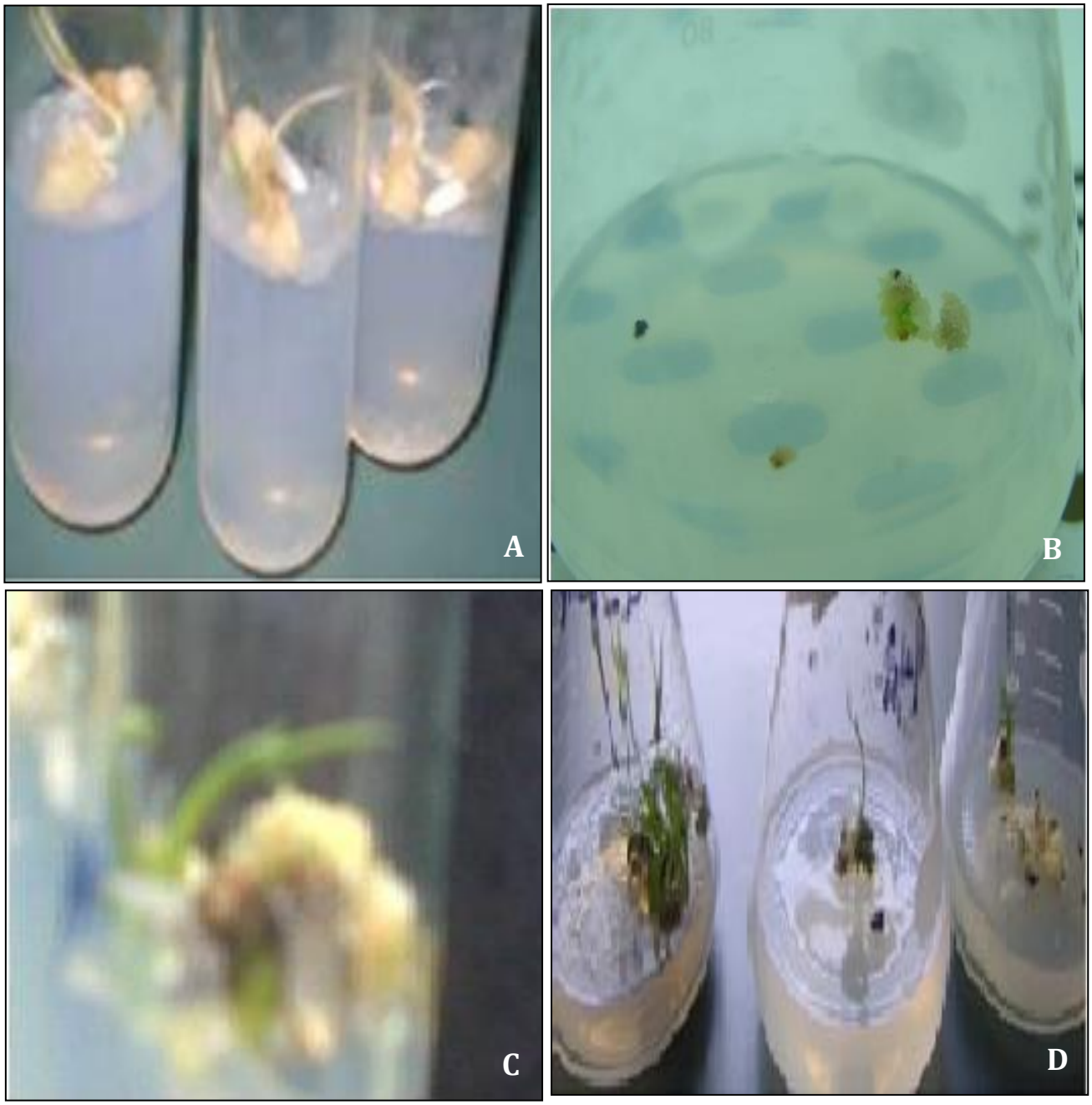

Fig. 1 (A) Callus induction (B) Calli showing green spots (C) Calli showing regeneration (D) Regeneration of plantlets

DISCUSSION

In present era of biotechnology, various techniques of genetic engineering are in practice throughout the world to improve genetic bases of cereals including rice. All these techniques primarily rely on successful regeneration of a healthy plant via tissue culture technique after transformation of an explant/callus phase (Bano et al., 2005; Shah et al., 2015b; 2016).

During this study, we found that all the cultivars under investigation showed higher percentage of callus induction on N6 medium as compared to MS media. Maximum percentage of calli were obtained at 2.0 mg/l and 2.5 mg/l 2, 4-D. Our findings were also confirmed by the previous research studies by Rashid et al. (1996); Rashid et al. (2001); Rashid et al. (2003); Sharan et al. (2004). Our findings are contradictory to Noor et al. (2005); Zaidi et al. (2006). These differences may be due to the use of different media, cultivars and their compositions. All the cultivars formed good quality calli at 5 g/l agar except Super basmati that gave good response at 6 g/l agar. Difference in callus induction frequency at the same concentration of 2, 4-D under similar conditions showed that callogenesis was affected by cultivar (Ali et al., 2004). Different research group investigated various factors affecting rice tissue culture including gelling effect such as agarose, phytagel and agar. In this research study, it was found that optimum levels of agar were suitable for embryogenic callus induction and proliferation, but its high concentrations favored the regeneration.

In regeneration experiment, regeneration media having different treatments of BAP/NAA were used. In this experiment, 1.0 mg/l NAA and 5.0 mg/l BAP was confirmed to be optimum for regeneration of calli. The most calli showed a positive response for shoot formation on regeneration media containing NAA and BAP. The role of auxins and cytokinins has been appreciated in plant tissue culture by Woodward et al. (2005). It was remarkable to note that the effect of cytokinins in tissue culture has been stressed more as compared to auxins. In previous studies, it has been reported that presence of any cytokinin like BAP or kinetin was inevitable for the regeneration medium (Kalaiarasi et al., 2014), while Pons et al. (2000) reported that in case of auxins like NAA and IAA, genotype played a dominant role for regeneration. In the present study, Super basmati showed the best response for shoot formation (58.33%) as compared to other cultivars. These results are supported by Rashid et al. (2000; 2001); Noor et al. (2005).

CONCLUSION

From this study, it is concluded that each cultivar has different response for callus induction and regeneration thereby each cultivar showed its own optimum level of 2, 4-D for the best callus induction. The level of 2, 4-D (2-2.5mg/l) was found to be the optimum level for the highest callus formation. This study also indicated that the very low level of 2, 4-D (1.5 mg/l) was not suitable for callus formation. Only optimum concentration favored the callus induction. Super basmati showed the best response for regeneration among all other cultivars. It would be considered as a model cultivar for tissue culture as well as transformation of genes. The finding of this study would be helpful for tissue culture of rice and its genetic improvement applying different transformation techniques. It is also suggested that Super basmati would be considered as a model cultivar for development of stress tolerance in rice through the latest biotechnology approach.

Author Contribution Statement Muhammad Arshad and Ghulam Muhammad Ali conceived and designed the research project. Khalid Mehmood conducted experiments and wrote the manuscript. Shaukat Ali contributed in optimizing various parameters for the evaluation of the best basmati rice cultivar i.e. technical assistance during the operation of experiments. Mazher Qayyum edited the article.

Conflict of Interest The authors declare that they have no conflict of interest.

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