Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; SP5: 65-69

(Special Issue- 5)
Advances in Agriculture and Natural Sciences for Sustainable Agriculture
(October 12 &13, 2018)

In Vitro callus induction and plant regeneration in basmati rice (*Oryza Sativa* L.) varieties

Shilpy Singh, Arun Kumar, Vivek Rana, Ashwini Kumar and V Rakesh Sharma

Shilpy Singh
Department of Agricultural Biotechnology, S.V.P University of Agri. and Tech, Meerut, Uttar Pradesh, India

Arun Kumar
Department of Agricultural Biotechnology, S.V.P University of Agri. and Tech, Meerut, Uttar Pradesh, India

Vivek Rana
Department of Agricultural Biotechnology, S.V.P University of Agri. and Tech, Meerut, Uttar Pradesh, India

Ashwini Kumar
Department of Agricultural Biotechnology, S.V.P University of Agri. and Tech, Meerut, Uttar Pradesh, India

V Rakesh Sharma
Department of Horticulture, S.V.P University of Agri. and Tech, Meerut, Uttar Pradesh, India

Correspondence
Arun Kumar
Department of Agricultural Biotechnology, S.V.P University of Agri. and Tech, Meerut, Uttar Pradesh, India

Abstract
Rice is the most important staple food for a large part of the world's population. Its production needs increase to meet the predicted demands of increasing population. Biotechnology provides a novel approach for rice improvement through transformation technique. Studies related to the *In-vitro* culture of plant require a basic protocol for callus induction and subsequent plant regeneration. Efficient callus induction and plant regeneration are pre-requisite for transformation. In the present study, an efficient callus induction and regeneration system was established for basmati rice varieties viz. Basmati-370, Pusa Sugandha-4, Vallabh Basmati-22 and Taroari. A number of factors such as media composition, different concentrations and combinations of hormones were studied. MS media supplemented with 2.0 mg/l of 2, 4-D produced the most desired calli. The callus induction frequency was found to be highest for Pusa Sugandha-4 while Basmati-370 showed the highest regeneration frequency on MS medium supplemented with 2.0 mg/l BAP and 1mg/l of NAA.

Keywords: Rice, Oryza sativa, In vitro culture, Callus induction, Regeneration

Introduction
Rice (*Oryza sativa* L. 2n=24) is the second most cultivated cereal crop belongs to family Poaceae, which is predominant staple food in at least 33 developing countries.Domesticated rice comprises two species of food crops i.e. *Oryza sativa* and *Oryzaglaberrima* (Ray, 1985) [22]. These plants are native to tropical and subtropical southern Asia and south eastern Africa. The sub species of *Oryza sativa* i.e. *Indica*, *Japonica* and *Javanica*are most commonly used for cultivation. *Indica* is irrigated rice of warm tropical zones with long, thin and flat grai ns. *Japonica* is an irrigated rice of temperate zone, with medium or short grains, also called round grains, and is a rainfed lowland rice of warm tropical zones. Among them the *indica* type rice accounts for 80% of the rice cultivated area in the world (Hiei *et al*., 2015) [10].
Rice is providing 27% of energy supply, 20% of protein and 3% of fat and also accounts for more than 65% of caloric intake providing 23% of global human per capita energy (Khush, 1996) [13]. India ranks second in rice production across the world and produced over 109.7 million tonnes during the crop year 2016-17 and occupies 39 million hectare area of harvesting (www.agricoop.nic.in; Final Estimate, 2016-17). To full fill the global food demands, the production of grain needs to increase up to 50% more because it has been predicted that the world population will exceed 8 billion people by 2025 (Khush, 2005) [12]. Due to its economic importance as a staple food for 2.7 billion people, rice has been a more attractive target for developing transgenic rice (Cho *et al.*, 2004) [6].
The production of rice is constrained by various biotic and abiotic stresses. Biotic stresses include diseases and pathogens. Abiotic stresses which adversely affect the rice crop and cause extensive losses to the yield of rice are unfavorable soil, temperature, salinity, drought, acidity, cold, iron toxicity and submergence under water conditions (Onaga and Wydra, 2016) [20].
Basmati rice varieties are economically important due to high quality, long grain and aroma. Current Basmati varieties possess excellent cooking and eating qualities (Awan *et al.*, 1998) [3], however, the grain yield of these varieties is low. Basmati rice varieties are generally poor combiners. It is very difficult to combine characteristics of basmati rice varieties in the genetic

background of other varieties through conventional breeding techniques. Availability of diversified germplasm is the basic requirement for success of any breeding program. Rice improvement by conventional approaches is limited by natural incompatibilities and by the time- scale of delivery of exploited germplasms from most breeding programmes. Thus there is a need to use *in-vitro* approaches for crop improvement. Recent development in the area of crop biotechnology have provided a large number of tools and techniques which are more efficient in generating genetic variability and making selection procedures more precise and reproducible. To increase productivity by advances in biotechnology is most viable option, which provides a novel and powerful way to minimize the loss of productivity through transformation technique rather than traditional plant breeding methods.

The suitable plant regeneration methods are requires for the successful application of plant tissue culture techniques for crop improvement. Bajaj, (1991) [4] reported that callus cultures could be raised easily from any part of the rice plant and induced to regenerate complete plants. Studies related to the *In-vitro* culture of plant require a basic protocol for callus induction and subsequent plant regeneration. The ability of plant regeneration from embryogenic callus of rice is also influenced by the genotype (Davoyan, 1987) [7]. The present study was to develop an effective protocol for optimum callus induction and complete plant regeneration, Optimization of *In-vitro* conditions using mature seeds as explant for improvement in callus induction and plant regeneration and Comparative studies on callus induction and regeneration responses for different basmati rice varieties.

Materials and Methods

Preparation and Sterilization of Tissue Culture Media

Tissue culture media consist of various inorganic and organic nutrients. Inorganic nutrients include minor elements (Zn, B, Mn etc.,) and major elements (Na, K, P, Ca, Mg, S etc.). Sucrose is added asorganic nutrients for supplying carbohydrate source in media..MS media supplemented with different concentration of growth hormones was used for callus induction and plant regeneration. After adding the all macro and micro-nutrients, vitamins and carbohydrate sources in double distilled water, the pH was adjusted to 5.8with 1N HCL or 1N NaOH prior to sterilization. After this, 8gm/l agar was added for solidifying the media. Media was poured in conical flask and covered with aluminum foil and then sterilized at 15 lbs psi for twenty minutes at 121°C temperature in autoclave. Sterilization of thermolabile components such as phytohormones and vitamins, stocks were prepared in autoclaved water and filter sterilized through Millipore membrane (0.22-0.45mm pore size) and then added into autoclaved media before pouring it into the petriplates or culture tube.

In vitro tissue culture of rice and Sterilization of Explant

The seed was selected as the most preferable explant for callus induction and regeneration in the present study, the healthy seeds of four Basmati rice varieties i.e., Basmati-370 (B-370), PusaSugandha-4 (PS-4), Vallabh Basmati-22 (VB-22) and Taroari were dehusked and soaked in distill water for 15-30 minutes. After this, seeds were treated with 70% ethanol for 2 minuteand2% sodium hypochlorite for 20 minutes followed by4-5 rinsed with sterile distilled water under laminar air flow. Seeds were dried using filter papers before inoculation.

Callus Induction

The seeds of all the four varieties were aseptically inoculated on MS (Murashige and Skoog, 1962) [17] basal media supplemented with 30 g/l sucrose along with different concentrations of 2, 4-D (1.5, 2.0 & 2.5 mg/l) and 50mg/l tryptophan, 300 mg/l casein hydrolysate, 150mg/l asparagines, 100 mg/l myo-inositol and 8gm/l agar and incubated at 25°C ± 2 under dark conditions for callus induction. After three weeks, the percentage frequency of explants producing embryogenic callus was determined. Only proliferated embryogenic calli (compact, fragile, nodular & pale yellow) derived from the scutellum were separated with sterile forceps and scalpel and non-embryogenic part were discarded. Calli were sub-cultured on the maintenance media after every fifteen days. The frequencies of callus induction were determined as follows:-

$$\textbf{CIF (\%)} = \frac{\text{No. of explants producing callus}}{\text{No. of explants plated}} \times 100$$

Where: CIF= Callus Induction Frequency

Plant Regeneration

Embyogenic calli were inoculated for plant regeneration on regeneration media i.e. MS salts and vitamins supplemented with different consuntrations of BAP (1.5, 2.0, 2,5) and NAA (0.5, 1.0, 1.5) along with30gm/l sucrose, 300 mg/l casein hydrolysates, 50 mg/l tryptophan, 150 mg/l asparagine, 100 mg/l myo-inositol and 8 gm/l agar. Cultures were placed on 25°C ± 2°Cand 10/14 hr light/dark regime under fluorescent light in growth room for 2-3 weeks. After shoot regeneration, plantlets were transferred to MS media without growth hormones for root initiation. Regeneration frequency of each variety was determined after eight week time period as follows:-

$$\textbf{PRF (\%)} = \frac{\text{No. of calli regenerated plantlets}}{\text{No. of calli plated for regeneration}} \times 100$$

Where PRF = Plant Regeneration Frequency

Results and Discussion

Experiments for callus induction, regeneration and transformation in rice were conducted in four Basmati rice varieties i.e. Basmati-370, Pusa Sugandha-4, Vallabh Basmati-22 and Taroari. Callus production and its subsequent regeneration is a prerequisite for transformation technique (Bhaskaran and Smith, 1988) [5]. Callus induction frequency, regeneration and transformation frequency are affected by a number of factors such as explant, media composition, different concentrations and combinations of hormones, age of callus etc.

Callus Induction

Choice of explant, utilization of basal media, growth regulators, source of carbon and amino acids are important considerations for improving callus induction efficiencies.

Factors Affecting Callus Induction

Choice of explant

The choice of suitable explant is an important factor for transgenic plant production. Some researchers used immature embryo derived calli (Hiei and Komari, 2008) [10] but other found that calli initiated from mature seed scutellum was excellent starting material for transformation of rice by

Agrobacterium due to their compactness (Cho *et al.*, 2004; Lin and Zhang, 2005) [6, 15]. In the present study, calli derived from mature seeds of all rice varieties were found to have embryogenic potential as reported by Lin and Zhang, (2005) [15].

MS media was used for callus induction from mature seeds. Callus formation varied among the rice varieties tested. The callus formation was observed at about fifteen days after culture. The callus was initiated from the scutellum region of the seeds. The primary callus was later proliferated into yellowish to white callus after 2-3 weeks (Fig.1). The callus induction frequency was found to be 88.08 % for PS-4, 84.34% for B-370, 79.99% for VB-22 and 74.55% for Taroari (Tab.1& Graph 1).

Callus formation in the present study was found to be genotype dependent. The significant differences between four varieties for callus induction under the same nutritional conditions indicate that callus induction efficiency is genotype specific. These results are in accordance with Shanthi *et al.*, (2010) [25] who reported that rice varieties differed in the degree of callusing. Similar observations were reported by Nguyen, (1984) [18], Khanna and Raina, (1998) [11] and Abassi *et al.* (2000) [1]. It signifies that tissue culture conditions need to be studied for individual genotypes.

Effect of different concentrations of 2, 4-D on callus induction

2, 4-dichlorophenoxyacetic acid (2, 4-D) has been used as the only growth regulator in callus induction media (Upadhyaya *et al.*, 2015) [27]. Mostafiz and Wagiran, (2018) [16] also reported that 2,4-D is indispensable to callus induction. To determine the optimum concentration of plant growth regulators, 2, 4-D were used with different concentrations (1.5 mg/l, 2.0 mg/l and 2.5 mg/l). The best response of callus induction and proliferation for all the four basmati rice varieties was observed when 2 mg/l of 2, 4-D was used in the medium (Table 1 & Graph 1). These results was in confirmation with Revathi and Pillai, (2011) [23] who cultured the rice explants on MS medium supplemented with five different concentrations of 2,4-D and found that MS media supplemented with 2.0 mg/l of 2,4-D produced the most desired calli. Sikdder *et al.*, (2006) [26] also found that MS medium supplemented with 2.0 mg/l of 2, 4-D is suitable for callus formation. Ge *et al*., (2006) [8] also reported 84.16% callus induction frequency at 2 mg/l concentration of 2,4-D.

Table 1: Callus induction frequency of basmati rice varieties on different concentrations of 2,4-D.

Variety		B-370		PS-4		VB-22		Taroari	
Treatments (2,4-D in mg/l)	Inoculated explants	% Contamination	% Callus induction frequency	% Contamination	% Callus induction frequency	% Contamination	% Callus induction frequency	% Contamination	% Callus induction frequency
T1 (1.5 mg/l)	175	32.53	65.77	26.77	70.77	25.66	68.09	32.77	65.77
T2 (2.0mg/l)	175	28.38	84.34	30.55	88.08	30.15	79.99	27.33	74.55
T3 (2.5 mg/l)	175	35.88	68.06	28.08	72.06	26.55	72.65	35.43	68.06

Callus Morphology

Embryogenic calli were selected on the basis of their physical properties as described by Cho *et al.* (2004) [6]. During callus induction experiments, two types of calli were distinguished as embryogenic and non-embryogenic callus. Non-embryogenic calli were devoid of embryoid like structures and were unable to develop into plantlets when maintained on the same MS media. B-370 and PS-4 showed more potential for callogenesis (Table 3). The callus of B-370 and VB-22 was found more embryogenic than that of PS-4 and Taroari. After three weeks, it was found that calli of all varieties showed growth and increase in size.

Table 3: Genotypic responses of four Basmati rice varieties to callus induction

Varieties	Callus growth	Morphology of Callus
B-370	+++	Whitish pale, Compact, Embryogenic
PS-4	+++	Yellowish white, Friable, Somewhat compact. Embryogenic
VB-22	++	White, Granular, Compact, Embryogenic
TAROARI	+	Whitish pale, Embryogenic with some Non-embryogenic part

Callus Size

+ - Small Size, ++ - Medium Size, +++ - Large Size

Regeneration

Several reports showed that plant regeneration frequency in rice is affected by many factors such as genotype, development stage of callus in the explant and hormonal composition of the medium (Niroula *et al*., 2005) [19]. The types and concentrations of cytokinins combined with NAA have been considered to be important factors on shoot regeneration of both *indica* and *Japonica* rice (Lee *et al.*, 2002 [14] and Saha *et al*., 2017 [24]). Supplementation of 2 mg/l of BAP has found to be more effective on regeneration of rice (Mostafiz and Wagiran, 2018) [16]. Several reports also showed that the presence of cytokinin is essential in promoting plant regeneration from cultured cells.

Comparison of regeneration efficiencies of four rice varieties

In the present study, maintained calli were transferred to regeneration media containing basic MS salts and vitamins with different concentrations of BAP (1.5mg/l, 2.0mg/l, 2.5mg/l) and NAA (0.5mg/l,1.0mg/l,1.5mg/l) along with 30 gm/l sucrose, 300 mg/l casein hydrolysates and 8 gm/l agar. On regeneration medium, the embryogenic calli tended to show green spotting and shoot regeneration with less browning (Cho *et al.*, 2004) [6]. The calli having high regeneration potential formed green tissue or green spots on the callus surface with fast growth for 23-26 days of cultivation (Fig.3) and consequently shoot emerged at 30-35 days (Fig.2). The calli of low regeneration varieties showed callus browning with relatively slow growth and ultimately died. Regeneration efficiencies of the four Basmati varieties were found to be 60% for B-370, 56% for PS-4, 48% for VB-22 and 52% for Taroari (Table 2).

Table 2: Plant regeneration frequency of basmati rice varieties on different concentrations of BAP and NAA

Variety		B-370		PS-4		VB-22		Taroari	
Treatments (BAP + NAA in mg/l)	Inoculated callus	Regenerated Plantlets	% Regeneration frequency	Regenerated Plantlets	% Regeneration frequency	Regenerated Plantlets	% Regeneration frequency	Regenerated Plantlets	% Regeneration frequency
T1 (1.5+0.5)	50	22	44.00	21	42.00	20	40.00	22	44.00
T2 (2+1)	50	30	60.00	28	56.00	24	48.00	26	52.00
T3 (2.5+1.5)	50	18	36.00	20	40.00	18	36.00	17	34.00

Regeneration frequency of B-370 was found to be highest among the four Basmati rice varieties (Graph 2). Rashid *et al.* (2003) reported 57.14% regeneration in Basmati-370. On the other hand, VB-22 and Taroari showed low *in-vitro* regeneration responses as compared to B-370 and PS-4 with low rates of green spots and shoot regeneration along with browning of callus. Callus browning, green spotting and plant formation varied depending on the rice variety which indicated that *in-vitro* differentiation of these varieties is under genetic control.

Conclusion

The best response of callus induction and proliferation for all the four basmati rice varieties was observed when 2 mg/l of 2, 4-D was used in the medium. Callus formation in the present study was found to be genotype dependent. The highest regeneration frequency was observed on MS medium supplemented with 2mg/l BAP and 1mg/NAA. The regeneration frequency found to be 60% for B-370, 56% for PS-4, 48% for VB-22 and 52% for Taroari.

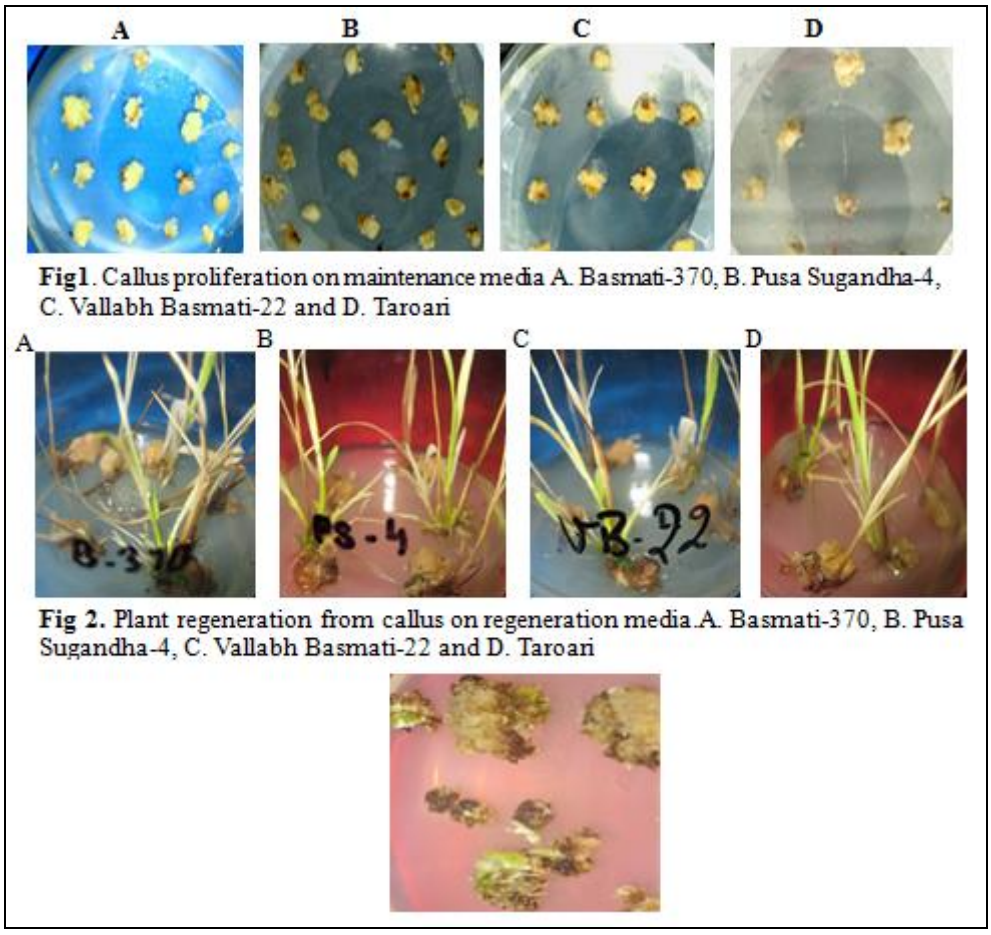
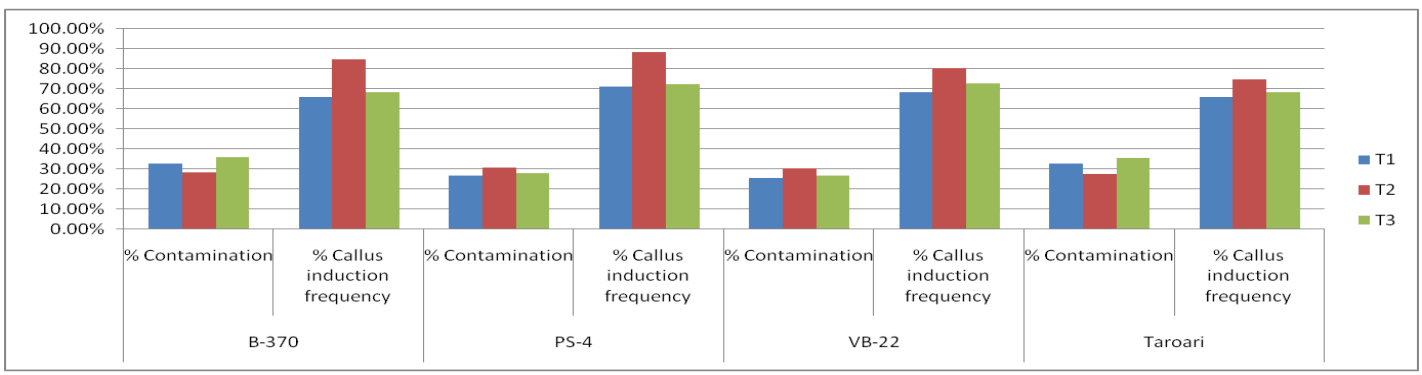


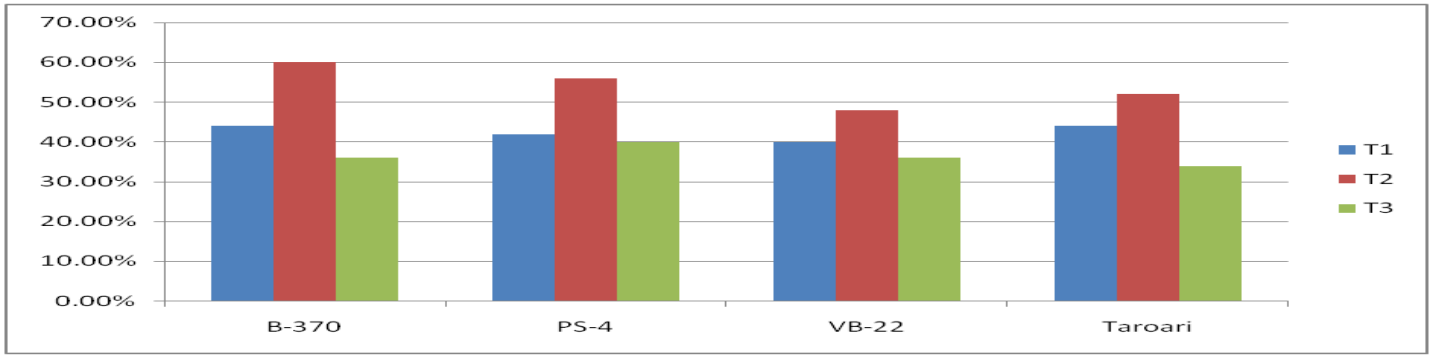
Fig1. Callus proliferation on maintenance media A. Basmati-370, B. Pusa Sugandha-4, C. Vallabh Basmati-22 and D. Taroari

Fig 2. Plant regeneration from callus on regeneration media.A. Basmati-370, B. Pusa Sugandha-4, C. Vallabh Basmati-22 and D. Taroari

Fig 3: Appearance of green spots on callus on regeneration media



Graph 1: Callus induction frequency of basmati rice varieties on MS media



Graph 2: Plant regeneration frequency (%) of basmati rice varieties

References

1. Abbasi FM, Rashid H, Qurashi A. Regeneration efficiency and embryogenic callus production of three cultivars of rice. Pak. J Agric. Res. 2000; 16:97-99.
2. Agricoop (2016-2017). http://agricoop.nic.in/sites/default/files/Annual_rpt_2016-17_E.pdf
3. Awan IU, Sulemani MJ, Ahmad K, Baloch MS. Physio-chemical properties, adoption and productivity of some fine (Basmati) rice varieties in Dera Ismail Khan. Pak. J Bio. Sci. 1998; 1:264-266.
4. Bajaj YPS. Biotechnology in rice improvement. In Biotechnology in Agriculture and forestry. Y.P.S Bajaj. *Rice sprin.-Ver*. 1991; 14:1-18.
5. Bhaskaran S, Smith RH. Enhanced somatic embryogenesis in *sorghum bicolor* L. from shoot tip culture. In-vitro Cell Dev, Biol. 1988; 24:65-70.
6. Cho JH, Lee JY, Kim YW. Improvement of shoot regeneration from scutellum -derived callus in rice. *Korean* J Crop. Sci. 2004; 49:52-60.
7. Davoyan EL. Genetic determination of the process of callus formation and induction of regenerates in the tissue culture of rice. *Genetika*, USSR. 1987; 23:303-310.
8. Ge XJ, Chu JH, Lin YJ, Wang SP. A tissue culture system for different germplasms of *indica* rice. Plant Cell Rep. 2006; 25:392-402.
9. Hiei Y, Komari T. Agrobacterium-mediated transfor mation of rice using immature embryos or calli induced from mature seed. *Nat. Protoc.* 2008; 3(5):824-34.
10. Hiei Y, Ishida Y, Komari T. Rice, indica (*Oryza sativa* L.). Methods Mol. Biol. 2015; 1223:155-67
11. Khanna HK, Raina SK. Genotype X culture media interaction effects on regeneration response of three indica rice cultivars. *Plant Cell Tiss. Org. Cult.* 1998; 52:145-153.
12. Khush G. What it will take to Feed 5.0 Billion Rice Consumers in 2030, *Plant Mol. Bio.* 2005; 59:1-6.
13. Khush GS. Prospects and Approaches to Increasing the Genetic Yield Potential of Rice. *Rice Research in Asia: Prog. and Prior.* CABI, U.K. and IRRI, Manila, 1996.
14. Lee K, Jeon H, Kim M. Optimization of a mature embryo based *in-vitro* culture system for high frequency somatic embryogenesis callus induction and plant regeneration from *japonica* rice cultivars. *Plant Cell Tiss. Org. Cult.* 2002; 71:237-244.
15. Lin YJ, Jhang Q. Optimising tissue culture conditions for high efficiency transformation of *indica* rice. *Plant Cell Rep.* 2005; 23:540-547.
16. Mostafiz SB, Wagiran A. Efficient Callus Induction and Regeneration in Selected Indica Rice. *Agro.* 2018; 8:77.
17. Murashige T, Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant.* 1962; 15: 473-497.
18. Nguyen VU. The use of tissue culture in plant breeding in Vietnam. *Biot. in Inter. Agri. Res.* IRRI, Los Banos, Philippines. 1984; 45-48.
19. Niroula RK, Sah BP, Bimb HP, Nayak S. Effect of genotype and culture media on callus induction and plant regeneration from matured rice grain culture. J Inst. Agric. Anim. Sci. 2005; 26:21-26.
20. Onaga G, Wydra K. Advances in Plant Tolerance to Abiotic Stresses. In book: *Plant Gen.* 2016; 1: 9.
21. Rashid H, Abbasi FM, Quraishi A. Plant regeneration from seed derived callus of three varieties of basmati rice. *Plant Tissue Cult.* 2003; 13(1):75-79.
22. Ray JK. Rice Research Institute in India. Indian Council of Agricultural Research, New Delhi, India. *Intro. to Bot. of the Rice Plant.* 1985; 2:5.
23. Revathi S, Pillai MA. *In vitro* callus induction in rice (*Oryza sativa* L.). *Res. in Plant Bio.* 2011; 1:13-15.
24. Saha D, Bhavya C, Ashok TH. Effect of genotypes and different concentration of growth regulator on callus induction and plant regeneration through anther culture of rice. J of Pharm. and Phytoc. 2017; 6:1354-1358.
25. Shanthi P, Jebaraj S, Geetha S, Aananthi N. Effect of growth regulators on callus induction in Rice embryo culture. Elect. J of Plant Breed. 2010; 1(4):1200-1207.
26. Sikder MBH, Sen PK, Mamun MA, Ali MR, Rahman SM. *In Vitro* Regeneration of Aromatic Rice *(Oryza sativa* L.). Int. J of Agri. and Bio. 2006; 6:759–762.
27. Upadhyaya G, Sen M, Roy A. In vitro callus induction and plant regeneration of rice (*Oryza sativa* L.) var. 'Sita', 'Rupali' and 'Swarna Masuri. Asian J of Plant Sci. and Res. 2015; 5:24-27.