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Research Article

Optimization of tissue culture protocol for rapid regeneration of traditional therapeutic rice genotype 'Kavuni'

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Abstract
Kavuni, one of the traditional rice genotypes possessing blackish purple grain is known for its nutritional and therapeutic properties. Genetic improvement using biotechnological tools and generating variability through genome editing necessitates development of a rapid, reproducible and efficient regeneration protocol. Callus induction from immature embryo explants of Kavuni was found at its maximum in NB media containing 2 mg l^{-1} 2,4-D**,** 1 mg l^{-1} NAA, 1 mg l^{-1} 6-BA, 2% sucrose, 1% glucose. After 3 weeks, embryogenic calli were transferred to pre-regeneration media (NB-PR) containing 2 mg l^{-1} 2,4-D**,** 1 mg l^{-1} NAA, 1 mg l^{-1} 6-BA, 3% maltose and 0.3 g l^{-1} glutamine which increased its regeneration frequency. Embryogenic calli failed to regenerate in a commonly used regeneration media (RNM) containing1 mg l^{-1} NAA, 3 mg l^{-1} 6-BA, 3% maltose and 0.3 g l^{-1} glutamine. Out of several combinations of regeneration media tried, regeneration was found to be maximum (80%) in the media combination # IV containing 3 mg l^{-1}6-BA, 0.5 mg l^{-1} kinetin, 3% maltose and 0.3 g l^{-1} glutamine which produced an average of 12 shoots per calli. Regenerated shoots were transferred to half MS basal media for rooting and finally hardened under greenhouse conditions.

Key words
Therapeutic rice, Kavuni, Regeneration, Protocol

Introduction

Rice is the predominant staple food for two-third of world's population and it plays a vital role in food security across the globe. Asia accounts for 90% of total production and consumption of rice in the world. Rice production must reach 800 million tons by 2030 to meet the demands of global population (Hossain and Narciso, 2004). This necessitates development of high performance rice varieties with enhanced tolerance against major biotic/abiotic stresses. Most of the high yielding rice varieties popularly grown and consumed do not possess high nutritional and therapeutic phytochemicals, which are abundant in traditional rice varieties/landraces. Traditional landraces also called as 'folk varieties' are considered a warehouse of novel genes controlling stress tolerance, valuable nutritive and therapeutic traits. Traditional rice genotypes *viz*., Njavara from Kerala (Mohanlal *et al.*, 2011; Varghese *et al.*, 2014) and Kavuni(Valarmathi *et al.*, 2014) from Tamil Nadu were reported to possess anti-diabetic, anti-cancerous and other medicinal/therapeutic properties. However, these traditional rice are poor in agronomic attributes *viz*., photosensitivity (one season/crop per year), low yield (1 – 1.5 t/ha), red pericarp, bold grain, poor cooking quality *etc*., After the 'First Green Revolution', with the introduction of modern high-yielding hybrid varieties in agriculture, traditional varieties are being abandoned and this threatens future rice production (Rekha *et al.*,2011). Hence traditional rice is gaining momentum among consumers because of the extensive health benefits they provide, thus rice is being regarded as 'functional food' and 'nutraceutical food' in recent times (Bhat and Riar, 2015).

'Kavuni', a blackish purple traditional rice genotype of Tamil Nadu, is popular among farmers for its unknown medicinal properties. A recent study on the nutritional profile of Kavuni revealed that its grains are rich in iron, zinc, calcium, copper, sodium, potassium and magnesium (Valarmathi *et al.*, 2014). It is also found to possess several health advantages like low levels of total soluble sugars, fat and high levels of dietary fiber, protein content, β-carotene, lutein, polyphenols, high anti-oxidant activity and inhibitory activity against enzymes *viz.*α-amylase and α-glucosidase, which will reduce diabetic complications. Inspite of the above advantages, Kavuni is not widely cultivated because of its undesirable phenological properties such as photosensitivity, long duration, poor tillering and low yield than the present elite rice cultivars. In any crop improvement programme, for application of any biotechnological techniques like recombinant DNA technology, genetic engineering and genome editing, presence of an efficient and rapid tissue culture protocol is a pre-requisite (Gosal and Kang, 2012; Sikdar *et al.*,

2015). So far, no reports are available on the successful tissue culture and regeneration protocol in Kavuni. Hence the present study was undertaken to develop an efficient *in-vitro* regeneration protocol in Kavuni which will pave way for unraveling molecular complexity behind key therapeutic properties through genetic engineering.

Materials and Methods

Maturing panicles of the therapeutic rice, Kavuni were collected 12-14 d after pollination from Paddy Breeding Station, TNAU, Coimbatore. Immature embryos were manually de-husked using forceps without causing any damage to the embryo(Fig. 1A, 1B) and surface sterilized with 70 % ethanol (v/v) for 60-90 seconds, followed by 3.5 % sodium hypochlorite plus a drop of tween-20 for 5-10 minutes. Finally it was rinsed five times using sterile distilled water.

Following the sterilization procedure, immature seeds were placed in a sterile 90-mm petriplate and immature embryos were isolated aseptically under a stereo-zoom microscope using sterile forceps. The excised embryos were placed on 0.8% agar plates to prevent desiccation. Then the immature embryos were cultured for callus induction on NB media (Hiei and Komari, 2008) containing N6 major salts, B5 minor salts, B5 vitamins, Fe-EDTA, amino acids – proline (0.5 g l^{-1}), casein hydrolysate (0.5 g l^{-1}), hormones - 2,4-D (2 mg l^{-1}), NAA (1 mg l^{-1}), 6-BA (1 mg l^{-1}), sugars – sucrose (20 g l^{-1}), glucose (10 g l^{-1}) and 0.8 % (w/v) agarose (Fig. 1C). The embryos were incubated at 25°C in dark. After seven days, the elongated shoots were removed and embryos were transferred to same fresh media and incubated under continuous illumination (5000 lux) for 14 days at 32°C for callus induction(Fig. 1D).

The creamish - white, friable embryogenic calli were transferred to pre-regeneration media NB-PR (Hiei and Komari, 2008) containing N6 major salts, Fe-EDTA, B5 minor salts, B5 vitamins, amino acids - proline (0.5 g l^{-1}), casein hydrolysate (0.5 g l^{-1}), glutamine (0.3 g l^{-1}), maltose (30 g l^{-1}), hormones - 2,4-D (2 mg l^{-1}), 6-BA (1 mg l^{-1}), NAA (1 mg l^{-1}) and 0.8% (w/v) agarose and incubated under continuous illumination (5000 lux) at 32°C for 7 days.

Embryogenic calli which turned into golden yellow in pre-regeneration media were selected and placed on to regeneration media RNM (Hiei and Komari, 2008) containing N6 major salts, Fe-EDTA, B5 minor salts, B5 vitamins, amino acids -proline (0.5 g l^{-1}), casein hydrolysate (0.5 g l^{-1}), glutamine (0.3 g l^{-1}), maltose (30 g l^{-1}) and hormones 6-BA (3 mg l^{-1}), NAA(1 mg l^{-1}), 0.8% (w/v) agarose and incubated under continuous illumination (5000 lux) at 32°C. Within 8-10 days, green spots appeared on calli but failed to produce shoots even after prolonged incubation and upon repeated sub culturing on the same media.

To achieve successful regeneration, five different combinations of regeneration media differing in their hormonal composition, amino acid, sugars and gelling agent were tried. The non-regenerating green calli were sub-cultured on to various regeneration media combinations I-V (Table 1) and incubated under continuous illumination (5000 lux) at 32°C. The calli were monitored continuously for any signs of regeneration. Number of shoots formed per callus was noted for each of the combinations and regeneration frequency (%)was calculated using the formula (Zaidi *et al.*, 2006)

$$\text{Regeneration frequency (\%)} = \frac{\text{Number of microcalli regenerating shoots}}{\text{Number of microcalli incubated}} \times 100$$

Individual shoots were excised carefully from the regenerating shoot clusters and transferred into rooting media containing half MS (Murashige and Skoog, 1962) supplemented with sucrose, inositol, proline, casein hydrolysate and gelling agents 0.4% agar (w/v) and 0.2 % (w/v) phytagel and incubated under continuous illumination (5000 lux) at 32°C. After root initiation, the plantlets were transferred from the petriplates to test tubes containing same rooting media and incubated under same conditions for development of complete root system. Plants with well-developed roots and shoots were hardened in clay soil (initially in protrays and later transplanted to plastic pots).

Results and Discussion

Regeneration is affected by a number of intrinsic factors such as genotype, explant, physiological status of the explants and extrinsic factors like hormones, culture media composition and culture conditions (Rueb *et al.*, 1994; Ge *et al.*, 2006). Among all the factors, genotype, nutrient media and their interaction are found to have profound effects on regeneration of rice cultures (Khanna and Raina, 1998). Many protocols have been established to efficiently regenerate rice plants through indirect regeneration from a variety of explants like mature seeds (Abe and Futsuhara, 1986; Sah and Kaur, 2014; Mohd Din *et al.*, 2016; Wani *et al.*, 2011), mature embryos (Azria and Bhalla, 2000; Seraj *et al.*, 1997), immature embryos (Koetje *et al.*, 1989; Seraj *et al.*, 1997), leaf base (Ramesh *et al.*, 2009), coleoptile (Oinam and Kothari, 1995; Sahrawat and Chand, 2001) and root segments (Abe and Futsufara, 1984; Mandal*et al.*, 2003). Taking into consideration the recalcitrant nature of traditional *indica* rice varieties to tissue culture and transformation

protocols and suitability of explant for rapid, large scale and high frequency of transformation, immature embryos were used as explant for callus induction (Aldemita and Hodges, 1996; Hiei *et al.*, 1997; Hiei and Komari, 2006).

Fresh immature embryos were collected from maturing Kavuni plants (12-15 days after flowering) and used for callus induction. Media supplemented with synthetic auxin 2,4-D is inevitable for callus induction in rice cultures (Joyia and Khan, 2013; Karthikeyan *et al.*, 2009; Lin and Zhang, 2005). It was also reported that when 2,4-D was combined with 6- BA and NAA, callus induction was better in certain *indica* rice varieties (Ali *et al.*, 2004). Immature embryos of Kavuni formed good, embryogenic calli on callus induction media supplemented with 2.0 mg l^{-1} 2,4-D, 1.0 mg l^{-1} NAA and 1.0 mg l^{-1} 6-BA(Fig. 1E). The calli were transferred to pre-regeneration media prior to regeneration media since it is reported to enhance the regeneration frequency of immature embryo derived calli(Hiei and Komari, 2006). In this study, embryogenic calli when transferred to pre-regeneration media, it proliferated into large golden yellow calli and it was then transferred to regeneration media for shoot formation. It was observed that green spots started appearing on the calli but it did not show any signs of shoot initiation even after repeated sub-culturing on same media. This clearly indicated the need for optimizing regeneration media for rapid and efficient regeneration of Kavuni calli. The non-regenerating green calli were sub cultured into five different combinations of regeneration media (I – V) to identify the optimal media for shoot induction and elongation.

Among different combinations used, it was observed that combinations IV and V produced more number of green buds immediately within a week after transfer and micro-shoots started emerging from these green buds(Fig. 1F). Media combination I produced more green buds but failed to produce green shoots and all calli dried gradually. Media combination IV showed maximum regeneration frequency of 80 % followed by combination V which showed 33.33 % regeneration, while in combinations I, II and III no regeneration were recorded. In combination IV, micro-shoots elongated to form healthy, robust shoots within 2-3 weeks and also produced a large number of secondary shoots with an average of 12 shoots per callus.

The amino acid proline plays a major role in initiation and maintenance of embryogenic calli in rice callus cultures (Toki *et al.*, 2006). An effective organic supplement, casein hydrolysate enhances the formation of embryogenic calli and helps in regeneration of shoots (Hiei *et al.*, 1994; Toki, 1997). A balance between auxin and cytokinin is the key for initiation of regeneration in calli(Lee and Huang, 2014). It was reported that the frequency of shoot regeneration was enhanced upon addition of cytokinin (6-BA) and auxin (NAA) in rice callus cultures (Sah *et al.*, 2014; Wani *et al.*, 2011). But in this study, increased shoot induction and elongation was observed (media combination IV) in the presence of cytokinins, kinetin and 6-BA which did not contain any auxin. The cytokinins, kinetin and 6-BA causes rapid cell division and plays a major role in development of shoot buds and shoots in rice callus (Gosal and Kang, 2012; Sikdar *et al.*, 2015). The combined effect of all the supplements led to higher regeneration frequency of Kavuni calli in combination IV.

All the regenerated shoots produced proliferous adventitious roots in the rooting medium and *in-vitro* regenerated Kavuni plants were hardened in the greenhouse (Fig. 1G, 1H, 1I). It is proposed that the best regeneration media among all tested combinations is combination IV containing N6 major salts, Fe-EDTA, B5 minor salts, B5 vitamins, proline (0.5 g l^{-1}), casein hydrolysate (0.5 g l^{-1}), glutamine (0.3g l^{-1}), maltose(30 g l^{-1}) and hormones 6-BA(3 mg l^{-1}), kinetin (0.5 mg l^{-1}) and 0.8% (w/v) agarose. In conclusion, a highly efficient regeneration media was optimized for rapid regeneration of immature embryo derived calli of kavuni. This is the first report on *in-vitro* regeneration of traditional therapeutic rice genotype Kavuni. This method will serve as a basis for developing genetically modified Kavuni rice either through genetic engineering or genome editing.

References

Abe, T. and Futsufara, Y. 1984. Varietal difference of plant regeneration from root callus tissues in rice. *Japanese Journal of Breeding*,**34**(2): 147-155.

Abe, T. and Futsuhara, Y. 1986. Genotypic variability for callus formation and plant regeneration in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics*,**72**(1): 3-10.

Aldemita, R. R. and Hodges, T. K. 1996. Agrobacterium tumefaciens-mediated transformation of japonica and indica rice varieties. *Plant*,**199**(4): 612-617.

Ali, S. Xue, Q. Z.and Zhang, X. Y. 2004. Assessment of various factors involved in the tissue culture system of rice.*Rice science*, **11**(5-6): 345-349.

Azria, D. and Bhalla, P. L. 2000. Plant regeneration from mature embryo-derived callus of Australian rice (*Oryza sativa* L.) varieties. *Australian journal of agricultural research*,**51**(2): 305-312.

Bhat, F. M. and Riar, C. S. 2015. Health Benefits of Traditional Rice Varieties of Temperate Regions. *Medicinal & Aromatic Plants*,**04**(03).

Ge, X. Chu, Z. Lin, Y. and Wang, S. 2006. A tissue culture system for different germplasms of indica rice. *Plant Cell Reports*, **25**(5): 392-402.

Gosal, S. S. and Kang, M. S. 2012. Plant tissue culture and genetic transformation for crop improvement. In: Improving crop resistance to abiotic stress, T. Narendra, S. G. Sarvajeet, A. F. Tiburcio, T. Renu (Eds.), Wiley-VCH, Weinheim,p. 357–387.

Hiei, Y. and Komari, T. 2006. Improved protocols for transformation of indica rice mediated by Agrobacterium tumefaciens. *Plant Cell, Tissue and Organ Culture*,**85**(3): 271-283.

Hiei, Y. and Komari, T. 2008. Agrobacterium-mediated transformation of rice using immature embryos or calli induced from mature seed. *Nature Protocols*, **3**(5): 824-834.

Hiei, Y. Komari, T. and Kubo, T. 1997. Transformation of rice mediated by Agrobacterium tumefaciens. *Plant molecular biology*,**35**(1-2): 205-218.

Hiei, Y. Ohta, S. Komari, T. and Kumashiro, T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. *The Plant Journal*,**6**(2): 271-282.

Hossain, M. and Narciso, J. 2004. Global rice economy, long term perspectives. Paper presented in the FAO Conference,"Rice in Global Markets and Sustainable Production Systems" held in Rome on 12-13 February, 2004 to celebrate the International Year of Rice 2004.

Joyia, F. A. and Khan, M. S. 2013. Scutellum-derived callus-based efficient and reproducible regeneration system for elite varieties of indica rice in Pakistan. *International Journal of Agriculture and Biology*,**15**(1): 27-33.

Karthikeyan, A. Pandian, S. T. and Ramesh, M. 2009. High frequency plant regeneration from embryogenic callus of a popular indica rice (*Oryza sativa* L.). *Physiology and Molecular Biology of Plants*,**15**(4): 371-375.

Khanna, H. K. and Raina, S. K. 1998. Genotype x culture media interaction effects on regeneration response of three indica rice cultivars. *Plant Cell, Tissue and Organ Culture*,**52**(3): 145-153.

Koetje, D. S. Grimes, H. D. Wang, Y. C. and Hodges, T. K. 1989. Regeneration of Indica Rice (*Oryza sativa* L.) from Primary Callus Derived from Immature Embryos. *Journal of Plant Physiology*,**135**(2): 184-190.

Sah, S. K. Kaur, A. and Sandhu, J. S. 2014. High frequency embryogenic callus induction and whole plant regeneration in japonica rice cv. Kitaake. *Journal of Rice Research*, **2**(2): 1-5.

Lee, S.T. and Huang, W.L. 2014. Osmotic stress stimulates shoot organogenesis in callus of rice (*Oryza sativa* L.) via auxin signaling and carbohydrate metabolism regulation. *Plant Growth Regulation*,**73**(2): 193-204.

Lin, Y. J. and Zhang, Q. 2005. Optimising the tissue culture conditions for high efficiency transformation of indica rice. *Plant Cell Reports*,**23**(8): 540-547.

Mandal, A. B. Maiti, A. and Biswas, A. 2003. Somatic embryogenesis in root derived callus of indica rice. *Plant Tissue Culture*,**13**(2): 125-133.

Mohanlal, S. Parvathy, R. Shalini, V. Helen, A. and Jayalekshmy, A. 2011. Isolation, characterization and quantification of tricin and flavonolignans in the medicinal rice Njavara (*Oryza sativa* L.), as compared to staple varieties. *Plant foods for human nutrition*, **66**(1): 91-96.

Mohd Din, A. R. Iliyas Ahmad, F. Wagiran, A. Abd Samad, A. Rahmat, Z. and Sarmidi, M. R. 2016. Improvement of efficient in vitro regeneration potential of mature callus induced from Malaysian upland rice seed (*Oryza sativa* cv. Panderas). *Saudi Journal of Biological Sciences*, **23**(1): S69-S77.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum*, **15**(3): 473-497.

Oinam, G. S. and Kothari, S. L. 1995. Totipotency of coleoptile tissue in indica rice (*Oryza sativa* L. cv. CH 1039). *Plant Cell Reports*, **14**(4): 245-248.

Ramesh, M. Murugiah, V. and Gupta, A. K. 2009. Efficient in vitro plant regeneration via leaf base segments of indica rice (*Oryza sativa* L.). *Indian Journal of Experimental biology*, **47**: 68-74.

Rekha, T. Martin, K. P. Sreekumar, V.B.and Madassery, J. 2011. Genetic diversity assessment of rarely cultivated traditional indica rice (*Oryza sativa* L.) varieties. *Biotechnology Research International*.

Rueb, S. Leneman, M. Schilperoort, R. A. and Hensgens, L. A. M. 1994. Efficient plant regeneration through somatic embryogenesis from callus

induced on mature rice embryos (*Oryza sativa* L.). *Plant Cell, Tissue and Organ Culture,* **36**(2): 259-264.

Sahrawat, A. K. and Chand, S. 2001. High-frequency plant regeneration from coleoptile tissue of indica rice (*Oryza sativa* L.). *In Vitro Cellular and Developmental Biology-Plant,* **37**(1): 55-61.

Seraj, Z. I. Islam, Z. Faruque, M. O. Devi, T. and Ahmed, S. 1997. Identification of the regeneration potential of embryo derived calluses from various indica rice varieties. *Plant Cell, Tissue and Organ Culture,* **48**: 9-13.

Sikdar, S. U. Zobayer, N. Nasrin, S. and Prodhan, S. H. 2015. Agrobacterium-mediated PsCBL and PsCIPK gene transformation to enhance salt tolerance in indica rice (*Oryza sativa*). *In Vitro Cellular & Developmental Biology - Plant,* **51**(2): 143-151.

Toki, S. 1997. Rapid and efficient Agrobacterium-mediated transformation in rice. *Plant Molecular Biology Reporter,* **15**(1): 16-21.

Toki, S. Hara, N. Ono, K. Onodera, H., Tagiri, A. Oka, S. and Tanaka, H. 2006. Early infection of scutellum tissue with Agrobacterium allows high-speed transformation of rice. *The Plant Journal,***47**(6): 969-976.

Valarmathi, R. Raveendran, M. Robin, S. and Senthil, N. 2014. Unraveling the nutritional and therapeutic properties of 'Kavuni' a traditional rice variety of Tamil Nadu. *Journal of Plant Biochemistry and Biotechnology,* **24**(3): 305-315.

Varghese, G. Jose, M. Dinesh Raj, R. Bocianowski, J. Thomas, G. and Omanakumari, N. 2014. Quantitative and molecular analyses reveal a deep genetic divergence between the ancient medicinal rice (*Oryza sativa*) Njavara and syntopic traditional cultivars. *Annals of Applied Biology,* **164**(1): 95-106.

Wani, S. H. Sanghera, G. S. and Gosal, S. S. 2011. An efficient and reproducible method for regeneration of whole plants from mature seeds of a high yielding Indica rice (*Oryza sativa* L.) variety PAU 201. *New biotechnology,* **28**(4): 418-422.

Zaidi, M. A. Narayan, M. Sardana, R. Taga, I. Postel, S. Johns, R. MacNulty, M. Mottiar, Y. Mao, J. loit, E. and Altosaar, I. 2006. Optimizing tissue culture media for efficient transformation of different indica genotypes. *Agronomy Research,* **4**(2): 563-575.

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Table 1.Different combination of modified regeneration media used in the study

Combination I		**Combination II**		**Combination III**		**Combination IV**		**Combination V**	
10x N6 major salts	100ml	10x N6 major salts	50ml	10x N6 major salts	50ml	10x N6 major salts	100ml	10x N6 major salts	100ml
100x Fe-EDTA	10ml	100x Fe-EDTA	10ml	100x Fe-EDTA	10ml	100x Fe-EDTA	10ml	100x Fe-EDTA	10ml
100x B5 minor salts	10ml	100x N6 minor salts	10ml	100x N6 minor salts	10ml	100x B5 minor salts	10ml	100x B5 minor salts	10ml
100x B5 vitamins	10ml	100x N6 vitamins	10ml	100x N6 vitamins	10ml	100x B5 vitamins	10ml	100x B5 vitamins	10ml
100mg/l NAA	20ml	100mg/l Kinetin	10ml	100mg/l Kinetin	5ml	100mg/l Kinetin	5ml	100mg/l NAA	20ml
100mg/l 6BA	60ml	Sucrose	20g	Maltose	20g	100mg/l 6BA	30ml	100mg/l 6BA	60ml
Maltose	30g	Sorbitol	30g	Sorbitol	30g	Maltose	30g	Maltose	30g
Proline	0.5g	Casein hydrolysate	1.0g	Casein hydrolysate	1.0g	Proline	0.5g	Proline	1.0g
Casein hydrolysate	0.5g					Casein hydrolysate	0.5g	Casein hydrolysate	0.5g
Make up the volume to 990ml. Adjust pH to 5.8		Make up the volume to 900ml. Adjust pH to 5.8		Make up the volume to 900ml. Adjust pH to 5.8		Make up the volume to 990ml. Adjust pH to 5.8		Make up the volume to 990ml. Adjust pH to 5.8	
Agarose type I	7g	Gelrite	4g	Gelrite	4g	Agarose type I	7g	Agarose type I	7g
Autoclave @ 121°C for 15 mins		Autoclave @ 121°C for 15 mins		Autoclave @ 121°C for 15 mins		Autoclave @ 121°C for 15 mins		Autoclave @ 121°C for 15 mins	
30g/l Glutamine	10ml	10x AA aminoacid (pH 5.8)	100ml	10x AA aminoacid (pH 5.8)	100ml	30g/l Glutamine	10ml	30g/l Glutamine	10ml
		Filter sterilized		Filter sterilized					

*N6 Major salts, N6 minor salts, N6 vitamins, B5 minor salts, B5 vitamins, AA aminoacid(Hiei and Komari, 2008).

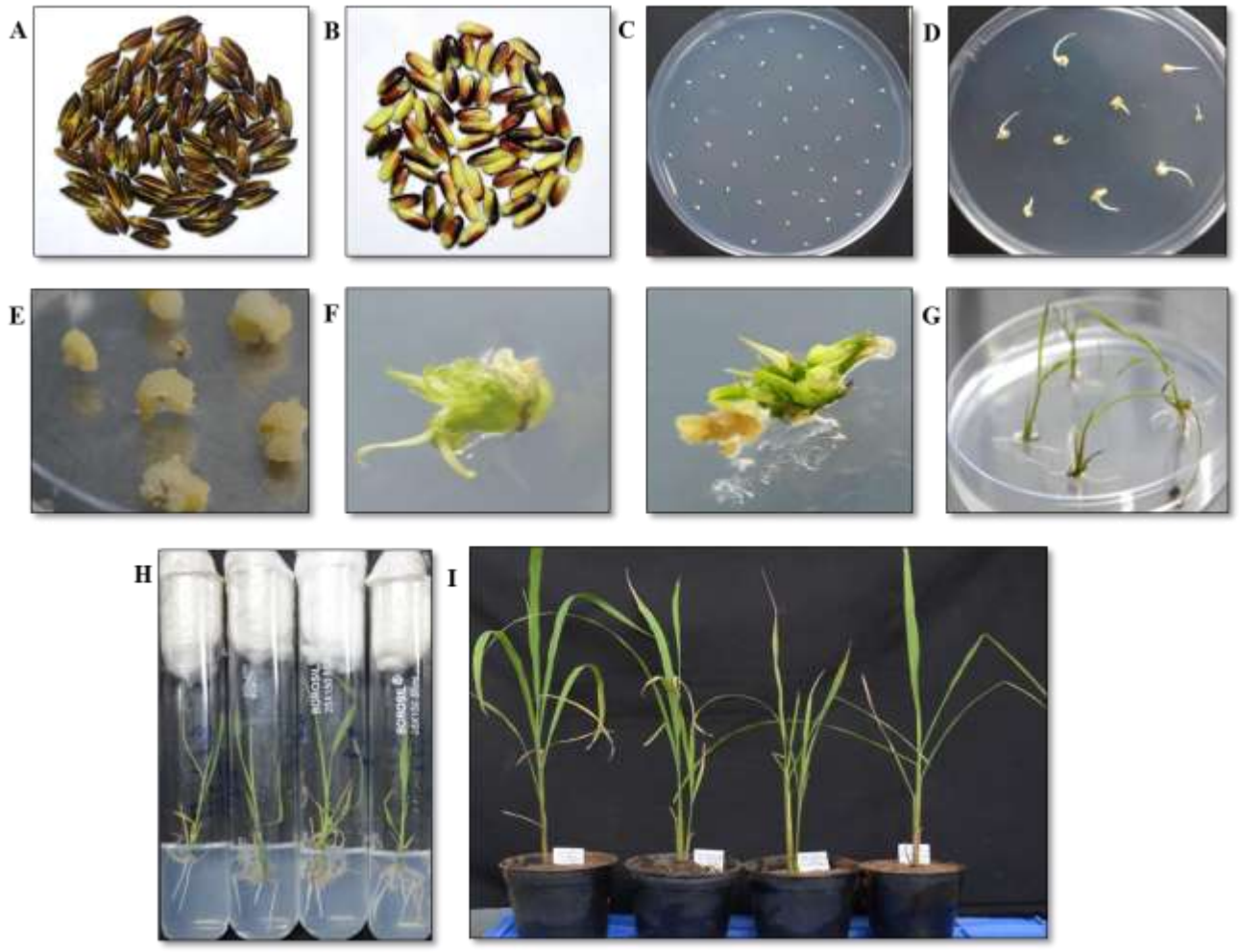

Fig.1. Regeneration of traditional therapeutic rice genotype 'Kavuni'

(A) Immature seeds of kavuni**(B)** Dehusked immature seeds **(C)** Immature embryos of kavuni **(D)** Shoots elongated from immature embryos after 7 days **(E)** 3 week-old embryogenic calli **(F)** Callus regeneration – micro-shoots emerged from calli **(G)** Rooting in petriplate**(H)** *In-vitro* regenerated rice plantlets in test tubes **(I)**Tissue culture regenerated kavuni plants in the green hou


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