Biocatalysis and Agricultural Biotechnology 18 (2019) 100999

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Micropropagation of traditional deep water rice (*Oryza sativa* L.) cv. TNR1 for viable seed production and germplasm conservation

Ramakrishnan Rameshkumar[a], Alagarsamy Karthikeyan[b], Periysamy Rathinapriya[a], Manikandan Ramesh[a,*]

[a] Department of Biotechnology, Science Campus, Alagappa University, Karaikudi, Tamil Nadu, 630 003, India
[b] State Key Laboratory of Tea Plant Biology & Utilization, Anhui Agricultural University, Hefei, Anhui Province, 230036, China

ARTICLE INFO

Keywords:
Traditional crop cultivars
Germplasm conservation
Seed recalcitrance
Deep water rice
Micropropagation
Genetic resources

ABSTRACT

Extinction of traditional crop varieties is becoming a common scenario in the world due to adoption of hybrid/improved varieties. Conservationists are very busy with protection of those verities for novel genes to develop agriculture through molecular breeding. However conservation feasibility is always questionable for traditional seeds because of recalcitrance and low viability in nature. This is the first report on the protection of traditional deep water rice (*Oryza sativa* L.) cultivar, TNR1 from possible extinction using callus induction and regeneration system. TNR1 is known for its traditional (antidiabetic) and genetic (salinity and submergence resistance genes) importance. The matured embryos were inoculated on LS medium prepared with 2.5 mg L^{-1} 2, 4-D and 0.1 mg L^{-1} KN showed 74.5% of embryogenic callus induction. The calli was regenerated on MS medium fortified with 1.5 mg L^{-1} NAA and 3.0 mg L^{-1} BAP and obtained 83.4% of regeneration. Regenerated plants were successfully acclimatized in the field with 100% survival. The germination percentage of tissue culture derived seeds was found to be high (84.2%) compared to traditional seeds (36.4%). The outcome of this study revealed that, tissue culture enhanced the viability of recalcitrant seeds. In future, this protocol will be an invaluable tool for both transgenic plant production or conservation of threatened traditional rice germplasms.

1. Introduction

In ancient times, thousands of rice cultivars were cultivated in the Asian continent because of its ability to grow in a wide range of lands which are susceptible to various biotic and abiotic stresses (Jackson, 1995; Siwakoti and Tiwari, 2007; Al-Atawneh et al., 2008). Rice (*Oryza sativa* L.) is the staple food for more than 3 billion people of the earth and over 90% of rice is manufactured from Asia and still required for ever rising population (Hossain and Narciso, 2004; Khush, 2005). According to FAO statistics, rice production has to be increased 50% by 2025 due to annually increasing 1.8% of new rice consumers (Khush and Virk, 2000). To achieve this, an efficient rice production system is required with least available resources such as land and water (Karthikeyan et al., 2009). Rice grown in the deepwater environment distinguishes itself from recent rice varieties by its capability to survive upto one month in water depths of over 50 cm (http://www.plantphysiol.org/content/118/4/1105 Catling, 1992). China and India are the top two countries in rice production and formerly they are having local collections of 30,000 rice cultivars (Breiholz et al., 2011). These traditional crops have evolved to survive in various environmental anxieties by genetic variation and its seeds naturally, has good grain quality, needs less cost for farming and resistance to multiple stresses (Oka, 1988; Puckridge et al., 2000; Sommut et al., 2004; Song et al., 2005). Wild and traditional crops are considered to be storehouse of novel genes which are having yield promoting potential and multiple stress resistance (Khush et al., 1990; Jena and Khush, 1990; Brar et al., 1996; Siwakoti and Tiwari, 2007; Al-Atawneh et al., 2008). Plant breeders have explored these genes from traditional genetic resources for developing the new crop verities. For instance, *SUB1* and *Saltol* genes were notified from the traditional Indian rice cultivar for its superiority in submergence and salinity respectively. Then, the anti-flood rice was developed from *SUB1* gene through breeding and it was successfully introduced in inundate susceptible lands of India (Swarna Sub1), Bangladesh (Samba Mahsuri) and Philippines (IR64-Sub1)

Abbreviations: cv, Cultivar; CWR, Crop Wild Relative; 2,4-D, 2,4-Dichlorophenoxyacetic acid; BAP, 6-benzylamino purine; DWR, Deep water rice; FW, Fresh weight; GP, Germination percentage; KN, Kinetin; LS, Linsmaier and Skoog medium; MS, Murashige and Skoog medium; NAA, α-napthaleneacetic acid; RH, Relative humidity; TNR1, Thalainayar 1

* Corresponding author.
E-mail address: mrbiotech.alu@gmail.com (M. Ramesh).

https://doi.org/10.1016/j.bcab.2019.01.037
Received 30 October 2018; Received in revised form 7 December 2018; Accepted 22 January 2019
Available online 01 February 2019

(Septiningsih et al., 2009; IRRI, 2013). Another case, the grassy stunt virus-resistant rice variety was produced from crop wild relative (CWR), *Oryza nivara* (Khush, 1977). These two examples proved that, the conventional cultivars having the genetic basis for rice agronomic characters improvement through breeding or transgenic technology. However, such important crops are under vulnerable condition and unfortunately countless traditional cultivars are already extinct from the nature (IRRI Better varieties, 2013). The UN was highly concerned on ever decreasing global biodiversity therefore, it is noticed that the year 2011–2020 as decade of biodiversity. As a result, the conservationists are working hard to conserve gradually vanishing biodiversity of many crop varieties (Brown, 1997; Anupam, 2012).

In our research findings, the Cauvery delta region originated traditional deep water *indica* rice cv. TNR1 (*Oryza sativa* L.) was replaced by modern cultivars. Deepwater rice cv. TNR1 (Thalainayar 1) is an ancient popular local cultivar of Tamil Nadu Cauvery delta region (India). TNR1 cv. is moderate yielding, submergence tolerance, salinity resistant and irrigated lowland rice evolved by pure line parentage (RKMP, 2011). In India, about 16.1 million ha of rice lands are purely submerged by flooding and according to World Bank Report (2008), about 19–40 million ha of geographic area marked as flood susceptible (Radhakrishnan, 1983; World Bank, 2008). Especially, Tamil Nadu (India) covers 43% of rice lands are under flood prone region (Thiyagarajan and Kalaiyarasi, 2010). During monsoon season, 12000 ha of agriculture lands were submerged. At the time of submergence, pure line parentage, deepwater *indica* rice cv. TNR1 (long, bold red rice and yield's 0.8–1.0 tons ha^{-1}), alone survived and feeds the farmers (Radhakrishnan, 1983). The brown rice has unique taste, texture and nutrition than white rice. Normally, the presence and absence of bran layer decides the colour and nutritional content of rice (IRRI Rice facts, 2013). Cooked TNR1 meal is red in colour and it contains less sugar compare to other rice cultivars which are traditionally used as a food for diabetic patients (Farmers opinion, unpublished data). Nowaday's, cultivation of this rice cv. was stopped due to low yielding and lack of new technologies for improving its productivity. But low yielding ancestor cultivars are having great numbers of stress resistant genes (Ali et al., 2006). This is one of the direct evidence in departure of traditional rice farming from agriculture and it is common observable fact in various countries also. For instance, in Thailand, deep water rice cultivation was up to 88% in 1993 but in 2001 it was reduced to 52% similarly in Nepal Seti river valley of kaski district previously used to cultivate above 75 traditional rice cultivars but at present, only 11 traditional cultivars are on hand to cultivate (Puckridge et al., 2000; Sommut et al., 2004). Balanced varieties were restored with modern cultivars. Farmers frequently used new cultivars instead of traditional cultivars as it leads to rapid depletion of ancestral genetic resources from the nature. Since, the modern cultivars provide higher yield through the support of two-fold fertilizers, pesticides, herbicides and irrigation (Puckridge et al., 2000; Sommut et al., 2004). Therefore, there is an urgent need to recognize and conserve vulnerable crop cultivars for future food demand.

The aspiration of the current study was to develop efficient callus mediated regeneration system and evaluation of seed viability between *in vitro* derived seeds and natural seeds of TNR1. Through this protocol with some modification we can regenerate other endangered or recalcitrant traditional rice cv. for crop improvement or conservation approaches. Many wild cultivar are recalcitrant in nature and it is a common feature of many riparian and tropical species (Hong et al., 1998). Those seed materials are unsuitable for germplasm conservation, so micropropagation is versatile tool to establish the recalcitrant explants into viable state. However, optimization of *in vitro* regeneration protocol is a challenging task which is often limited by seed recalcitrant, explant's genotype and nutrient media composition (Mandal and Gupta, 1995; Khanna and Raina, 1998; Ramesh et al., 2009). There are several reports evidencing that rice can be regenerated from various parts such as leaf bases, root segments, immature embryos and mature embryos (Li et al., 1993; Azria and Bhalla, 2000; Mandal et al., 2003; Ramesh and Gupta, 2006; Ramesh et al., 2009). While plenty of literatures are available for rice tissue culture but *indica* rice culture is always problematic due to its recalcitrant nature (Ramesh and Gupta, 2006; Karthikeyan et al., 2009; Zuraida et al., 2011). However, the recalcitrance surmount through auxin 2,4-D treatment, it leads to embryogenic callus formation and successive regeneration (Morita et al., 1999; Lee et al., 2002; Ramesh and Gupta, 2006; Karthikeyan et al., 2009). Seed germination analysis was further demonstrated that seeds from *in vitro* grown plants having more than two-fold of viability than natural seeds. The present study reveals that, recalcitrant TNR1 seeds are the sufficient materials for germplasm conservation which can be rejuvenated through micropropagation. From this protocol, we can obtain healthy plantlets through callus induction and regeneration system which gives viable seeds for farming or preserving ever declining genetic resources through germplasm conservation.

2. Materials and methods

2.1. Plant material

Deep water *indica* rice cv. TNR1 (Thalainayar 1) seeds were obtained from Agriculture Extension Centre, Thalainayar village, Nagapattinam, India. After removing the seed coat, healthy, intact red kernelled grains were used as starting material for culture initiation. In order to reduce surface rigidity of the seeds, it was soaked in sterile distilled water for overnight at 4 °C. Vernalized seeds were disinfected with 70% (*v/v*) ethanol for 1 min and 0.1% (*w/v*) mercuric chloride for 4 min. Subsequently, seeds were rinsed thoroughly in sterile water four to five times to remove chemical traces and blot dried using Whatman No.1 filter paper.

2.2. Embryogenic callus induction

The surface sterilized seeds were transferred to Linsmaier and Skoog (LS; Linsmaier and Skoog, 1965) medium which comprises of 1.0 $mg\,L^{-1}$ thiamine-HCL, 30 $g\,L^{-1}$ sucrose, 4.5 $g\,L^{-1}$ ClariGel™ with various combinations of 2,4-dichlorophenoxyacetic acid (2,4-D; 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 or 5.0 $mg\,L^{-1}$) and kinetin (KN; 0.1, 0.2, 0.3, 0.4 or 0.5 $mg\,L^{-1}$) for embryogenic callus induction. Medium pH was adjusted to 5.8 with the help of 0.1 N NaOH and autoclaved at 121 °C for 15 min. Initiated cultures were maintained in complete darkness at 26 ± 2 °C for 21 days. After three weeks of incubation, callus was subcultured on the for raising the callus mass and viability. The callus induction efficacy was assessed based on the fresh weight (FW) determination. Callus cultures were periodically subcultured on the same medium for two weeks to maintain the proliferation rate of the callus and monitored for every 3 days to observe any morphological changes.

2.3. Plantlet regeneration

The seed derived embryogenic callus was laid on Murashige and Skoog (MS; Murashige and Skoog, 1962) regeneration medium provided with different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 or 5.0 $mg\,L^{-1}$) of 6-Benzylaminopurine (BAP) or incombination with 1-Naphthaleneacetic acid (NAA; 0.5, 1.0, 1.5, 2.0, 2.5 or 3 $mg\,L^{-1}$), for shoot differentiation. Also, MS medium prepared with 30 $g\,L^{-1}$ sucrose and 4.5 $g\,L^{-1}$ ClariGel™ as a sugar and solidifying agent respectively. Cultures were maintained in 16 h light and 8 h dark phase at 26 ± 2 °C for 42 days. Coconut water (2, 4, 6, 8 or 10%, *v/v*) was used as a natural additive in both callus induction and regeneration phases to promote growth and freshness of cultures. Cultures were periodically subculured on the same medium at once in two weeks for multiple shoot induction with maturation. Further, calli derived shoot cultures were maintained in same medium for rooting. After complete rooting, it was transfer for acclimatization.

2.4. Hardening

In vitro cultured plantlets with well developed roots were taken out carefully from the container and washed with sterile distilled water to remove traces of media on the root surfaces. The young plantlets were transferred to plastic pots packed with autoclaved mixture of sand, vermicompost and horticulture soil in 1:1:1 (*w/w/w*) ratio. These plantlets were initially maintained in the plant growth chamber (Sanyo versatile environmental test chamber, MLR 351H, Japan) with 16/8 h (light/dark) at 25 ± 1 °C for 2 weeks under cool white fluorescent light lamp with 150 μmol photons m^{-2} s^{-1} and 60% relative humidity (RH). Then, gradually plants were transferred to the shade house and maintained for 2 weeks with natural conditions. The well-adapted plants were shifted to field conditions for establishment, maturation and viable seed setting.

2.5. Collection of in vitro derived seeds and natural seeds

In order to assess the seed viability, field established plants were noticed for seed set production. The healthy, viable seeds were harvested from randomly selected five micropropagated and traditional crops of TNR1. For seed viability, Hay et al. (2013) protocol was followed with slight modifications. Initially, 30 seeds from each micropropagated and traditional seeds were maintained in hot air oven for 7 days at 50 °C for ripening also to remove the moisture. Then these seeds were kept in air tight container for further experiments.

2.6. Seed viability assay

In order to break the seed dormancy, both (micropropagated and traditional) seeds were soaked in sterile distilled water for 16 h at 4 °C. Then, seeds were spread over the two layers of Whatman No.1 filter paper and placed in Petri plates. The plates were filled with 10 ml of sterile water and maintained in a growth chamber (SANYO, Versatile Environmental Test Chamber, Japan, Model No. MLR 351 H) with the environmental parameters of 65% RH, 12 h light/day and 30 °C. Germination started on next day onwards and further continued up to 15 days. Plants with normal shoots with roots were scored as germinated. After complete regeneration, germination percentage (GP) was calculated by following formula:

GP = (number of seeds germinated) / (total number of seeds inoculated) × 100

2.7. Statistical analysis

All the experiments were carefully designed and performed in triplicate manner with 150 explants per trial. The different growth variables including percentage of callus induction, callus fresh weight, regeneration was evaluated after the second subculture. Further, seed viability was tested after 15^{th} day of seed germination. Finally, the data were analysed using one-way analysis of variance (ANOVA), the significance of variations within means was carried out at $P \leq 0.05$ using Duncan's multiple range test with Statistical Package for Social Science, version 17.0 for Windows, SPSS Inc. (SPSS Inc. 2003; IBM).

3. Results

3.1. Effects of 2,4-D and KN on embryogenic callus induction

Seeds were inoculated on LS medium augmented with different concentrations of 2,4-D (0.5–5.0 mg L^{-1}) and KN (0.1–0.5 mg L^{-1}) independently or in combination for embryogenic callus induction (Fig. 1 and Table 1). Friable, white to yellow embryogenic callus started to produce from seeds after 14 days of dark incubation at 26 ± 2 °C. Among the different concentrations of 2,4-D and KN tested for embryogenic callus induction, LS medium supplemented with 2.5 mg L^{-1} 2,4-D and 0.1 mg L^{-1} KN showed maximum percentage (74.4%) of callus induction whereas 2.5 mg L^{-1} 2,4-D alone showed 63.2% of callus induction after 28 days (Fig. 1 and Table 1). The callus induction was observed after 14 days of dark incubation from the explants were cultured on LS medium (Fig. 2a and b). Friable, white to yellow embryogenic callus was produced after 28 days of dark incubation (Fig. 2c and d). Addition of 2,4-D upto 2.5 mg L^{-1} showed increased callus induction frequency however, above the optimum concentration of 2,4-D (2.5 mg L^{-1}) reduced the callus induction percentage and became necrosis (Fig. 1).

The effect of KN on callus induction was shown in Table 1. In contrast to 2,4-D, increased concentration of KN showed poor callus induction frequency. These experiments proposed that, LS medium supplemented with 2.5 mg L^{-1} 2,4-D and 0.1 mg L^{-1} KN was the best for callus induction from mature seeds of TNR1 (Table 1). Additionally, these results demonstrated that 0.5–2.5 mg L^{-1} 2,4-D was the favourable concentration for inducing the regenerable, friable, white to yellow embryogenic callus from the mature zygotic embryos of TNR1 (Table 1). Similarly, low concentration of KN also promotes the callus induction frequency compared to 2,4-D alone (Table 1).

3.2. Effects of BAP and NAA on in vitro regeneration

The seed derived embryogenic callus was cultured on MS medium supplemented with different concentrations BAP (0.5–5.0 mg L^{-1}) and NAA (0.5–3.0 mg L^{-1}) individually or in combination for shoot differentiation. Among the various concentrations of BAP tested, MS medium containing with 3.0 mg L^{-1} BAP produced 66.2% of regeneration after 41 days of light incubation (Table 2). Compare to BAP alone, the combination of NAA showed highest percentage of regeneration up to 83.4% (Table 2). Among the combinations of BAP and NAA, 3 mg L^{-1} BAP and 1.5 mg L^{-1} NAA showed high frequency regeneration through direct organogenesis (Table 2). Above the optimum concentrations, BAP (3.0 mg L^{-1}) and NAA (1.5 mg L^{-1}) showed decreased percentage of regeneration (Table 2). In the regeneration medium, greenish was observed on 9^{th} day of light incubation (Fig. 2e) and white to yellow embryogenic calluses were gradually developed into bright green shoots (Fig. 2f). *In vitro* produced shoots were cultured in the same regeneration media for six weeks then subcultured for another two weeks for development of good rooting system (Fig. 2g). The well rooted plants were initially hardened into pots (Fig. 2h) and maintained in the shade house then transferred to the field. The combination of 3 mg L^{-1} BAP and 1.5 mg L^{-1} NAA was showing the most effective regeneration, resulting in 83.4% of the tested calli developing into shoots and roots (Table 2). The brief data point out that, mixture of NAA and BAP and their concentrations and appropriate proportion was important factor for shoot regeneration from embryogenic callus of TNR1.

3.3. Effects of coconut water on embryogenic callus induction and regeneration

In order to increase the viability of the explants, coconut water was examined in callus induction and regeneration medium. It decreases the rate of browning and necrosis in cultured plant. Various concentrations of coconut water (2, 4, 6, 8 and 10%, *v/v*) were supplemented in this test. Among them, 10% (*v/v*) of coconut water increased the callus mass and freshness than other concentrations. The addition of coconut water to the callus induction medium promoted a quick response to micropropagation with increased activeness and survivability (Fig. 2d). Above 10% (*v/v*) of coconut water, no significance was observed in callus induction and regeneration phase (Data not shown).

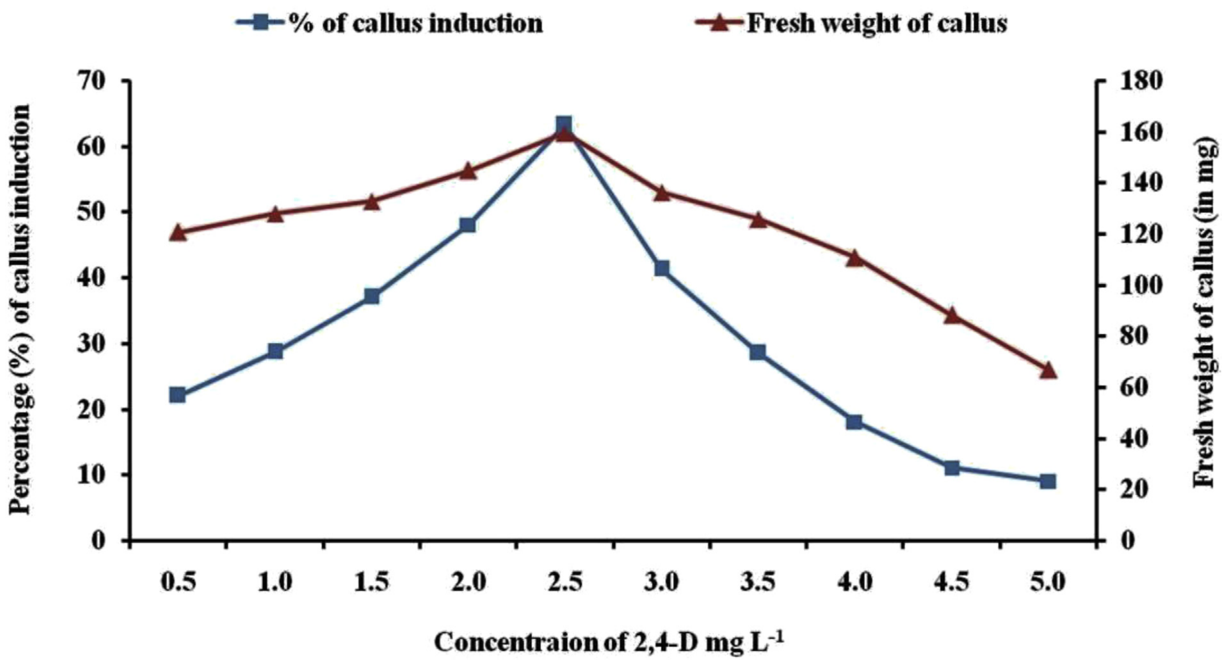

Fig. 1. Effect of 2,4-D on embyogenic callus induction in deep water rice cv. TNR1 on LS medium.

Table 1
Effect of 2,4-D and KN on embryogenic callus induction of Deep water rice cv. TNR 1 on LS medium.

Plant growth regulators (mg L^{-1})		Percentage of embryogenic callus induction (Mean ± SD)	Fresh weight of callus (mg) Mean ± SD
KN	2, 4-D		
0.1	2.5	74.47 ± 0.42^{e}	173.07 ± 0.42^{e}
0.2	2.5	67.23 ± 0.55^{d}	162.63 ± 0.21^{d}
0.3	2.5	52.07 ± 0.25^{c}	152.37 ± 0.31^{c}
0.4	2.5	43.63 ± 0.40^{b}	140.80 ± 0.50^{b}
0.5	2.5	28.30 ± 0.26^{a}	128.10 ± 0.36^{a}

The data are presented as means (± SD) of three independent experiments. The 150 explants per experiment were tried for optimum callus induction. Mean values in each column followed by the same letters are not significantly different by Duncan's multiple range test at $P \leq 0.05$.

3.4. In vitro rooting

In vitro regenerated plantlets with 5–10 cm long roots were hardened to plastic pots filled with an autoclaved mixture of sand, vermicompost and horticulture soil in 1:1:1 (*w/w/w*) ratio (Fig. 2). The regenerated young plantlets were maintained in the shade house with 100% of survival and they were allowed for seed setting. The field acclimatized plants were looking healthy and produced panicles with seeds.

3.5. Effects of germination in micropropagated and traditional seeds

In vitro plant derived seeds were harvested and used for germination study. This experiment illustrated that, tissue culture seeds has more than twofold of germination (84.2%) capacity than stored indigenous seeds (36.4%) (Fig. 3). These viable seeds can be stored in germplasm conservation bank or they can be used for farming.

4. Discussion

Numerous indigenous crops are in the threatened condition due to the various natural and anthropogenic activities, the rice variety TNR1 is an one among them. In this study, the seeds have been used as a explant to achieve a successful embryogenic callus induction and plant regeneration in recalcitrant deep water *indica* rice cv. TNR1. The effective and rapid regeneration protocol reported here has the best regeneration capability over the previously published reports of rice via organogenesis. In earlier studies, rice regeneration have been achieved by using various explants such as stem node, leaf base, anther and mature seed (Furuhashi and Yatazuva, 1964; Guha-Mukerjee, 1973; Genovesi and Magil, 1982; Karthikeyan et al., 2009, 2011; Priya et al., 2011a,b; Krishnan et al., 2013). However, mature zygotic embryos has also been efficiently used as an explant source for callus regeneration in rice (Rueb et al., 1994; Lee et al., 2002; Karthikeyan et al., 2009; Saika and Toki, 2010), sorghum (Rao et al., 1995), lawngrass (Asano et al., 1996), maize (Huang and Wei, 2004), barley (Manoharan and Dahleen, 2002), oats (Choi et al., 2000) white pine (Tang and Newton, 2005), wheat (Mendoza and Kaeppler, 2002; Moghaieb et al., 2010), finger millet (Satish et al., 2016a) and foxtail millet (Satish et al., 2016b). Embryogenesis and plant regeneration systems developed from mature zygotic embryos of recalcitrant seeds of deep water *indica* rice cv. TNR1 through this study may beneficial for *in vitro* and transformation studies.

In vitro regeneration protocol was limited by several factors like genotype, the source of the explant, nutrients, hormone composition and type of explants (Ramesh and Gupta, 2006; Karthikeyan et al., 2009). Most of the *indica* rice cultivars were less responded to tissue culture conditions compared to *japonica* cultivars which are conquered with our protocol (Karthikeyan et al., 2009). Thus, an attempt was taken to rejuvenate the deep water *indica* rice cv. TNR1 through callus mediated regeneration system. Auxin has been considered as an efficient plant growth regulator affecting cell division, differentiation and morphology in the induction of somatic embryogenesis (Pescador et al., 2012). The exogenous supplementation of optimum levels of 2,4-D into the medium resulted in prolific callus formation in various rice explants (Morita et al., 1999; Lee et al., 2002). Generally, pale yellow embryogenic callus was initiated from a scutellar region of the zygotic embryo of TNR1 (Wani et al., 2011). In our study, dry, compact and nodular structured embryogenic callus was obtained in the LS media supplemented with 2,4-D. Similar results have been reported in rice (Karthikeyan et al., 2011). In order to induce the efficient embryogenic

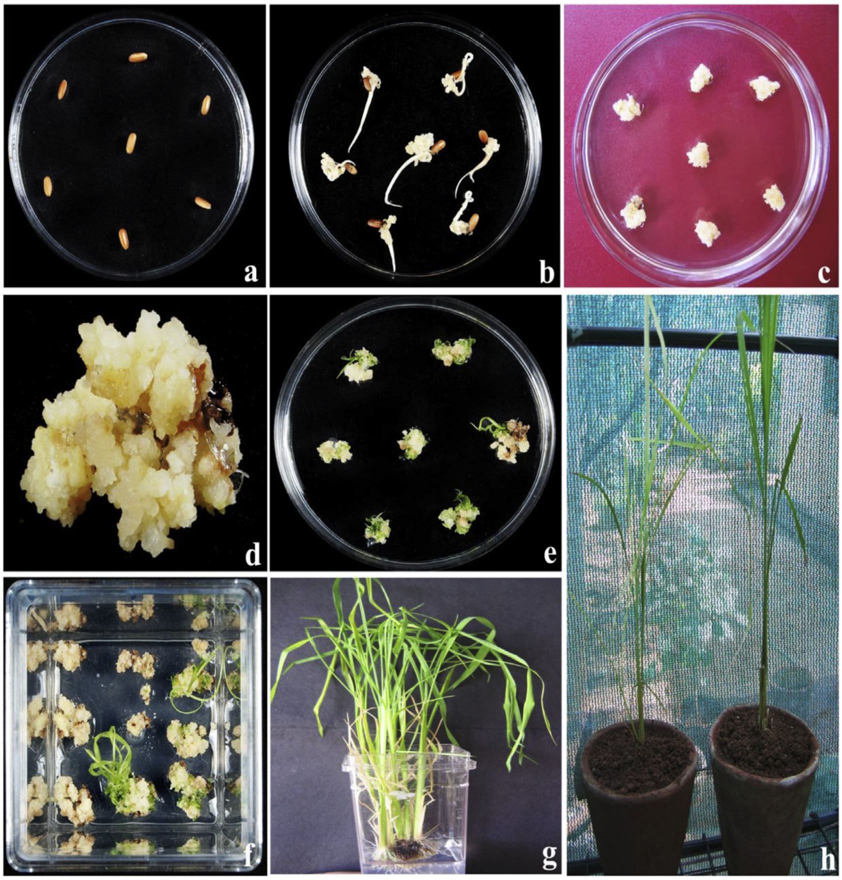

Fig. 2. *In vitro* developmental stages of Deep water rice cv. TNR1 (a) Intact red mature embryos of TNR1 on LS media (b) 2nd week of embryogenic callus initiation (c) Subcultured embryogenic callus (d) Enlarged view of friable pale to yellow embryogenic callus (e & f) Emergence of green shoot sprouts in embryogenic callus on MS media (g) Complete plantlet regeneration after 41 days (h) Gardened TNR1 plants in the shade house. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Effect of BAP and NAA on regeneration of embryogenic callus of Deep water rice cv. TNR1 on MS medium.

Plant growth regulators (mg L^{-1})		Percentage of regeneration (Mean ± SD)	Average shoot length (cm) Mean ± SD
BAP	NAA		
0.5	–	21.57 ± 0.51^{b}	7.20 ± 0.40^{a}
1	–	27.97 ± 0.35^{d}	11.17 ± 0.32^{d}
1.5	–	37.93 ± 0.31^{e}	14.80 ± 0.20^{e}
2	–	46.73 ± 0.40^{g}	17.90 ± 0.36^{f}
2.5	–	56.30 ± 0.36^{i}	21.73 ± 0.31^{h}
3	–	66.27 ± 0.25^{j}	24.13 ± 0.21^{i}
3.5	–	52.17 ± 0.32^{h}	18.93 ± 0.25^{g}
4	–	44.23 ± 0.45^{f}	14.43 ± 0.31^{e}
4.5	–	26.73 ± 0.45^{c}	10.47 ± 0.31^{c}
5	–	16.00 ± 0.30^{a}	8.50 ± 0.26^{b}
3	0.5	71.77 ± 0.21^{m}	28.60 ± 0.46^{l}
3	1	77.27 ± 0.31^{n}	34.83 ± 0.40^{n}
3	1.5	83.47 ± 0.35^{o}	42.37 ± 0.45^{o}
3	2	71.50 ± 0.30^{m}	31.27 ± 0.47^{m}
3	2.5	64.83 ± 0.55^{l}	25.87 ± 0.40^{k}
3	3	52.23 ± 0.40^{k}	18.73 ± 0.25^{j}

The data are presented as means (± SD) of three independent experiments. The 150 explants per experiment were tried for optimum callus induction. Mean values in each column followed by the same letters are not significantly different by Duncan's multiple range test at $P \leq 0.05$.

callus, different concentrations of 2,4-D were studied individually also in combination with KN. The embryogenic callus induction percentage was higher in the LS medium augmented with 2,4-D and Kn. The addition of KN in combination with 2,4-D has been reported to improve the embryogenic callus proliferation by influencing the cell division and it increase cytoplasmic contents in some of the species (Bhaskaran and Smith, 1990; Rao et al., 1988; Ceasar and Ignacimuthu, 2010; Verma et al., 2011; Wani et al., 2011; Satish et al., 2016b). In accordance with the previous reports, the current study showed a significant enhancement on embryogenesis in recalcitrance seeds of deep water *indica* rice cv. TNR1 on LS medium supplemented with 2.5 mg L^{-1} 2,4-D and 1 mg L^{-1} KN.

The higher level 2,4-D supplementation in the culture medium leads to necrosis and the browing of callus. The addition of coconut water in the medium helps to overcome the necrosis and callus browing during the embrogenesis (McCann et al., 1988; Karthikeyan et al., 2011). However, in the present study the improved embryogenic callus induction and plant regeneration of recalcitrant *indica* rice cv. TNR1 was stimulated by the inclusion of coconut water in the culture medium. Similar results have been reported for enriched embryogenesis by the addition of coconut water in plant species such as sugarcane (Ho and Vasil, 1983; Desai et al., 2004), cacao (Pence et al., 1980), mango (Litz et al., 1982), date palm (Al-Khayri, 2010).

The embryogenic callus was regenerated using MS medium supplemented with various concentrations and combinations of auxin and cytokinin. The combination of BAP and NAA showed maximum regeneration frequency from seed derived callus as similar to previous reports (Khush, 2005; Ramesh and Gupta, 2006). In our case, we got high frequency regeneration at 3.0 mg L^{-1} BAP and 1.5 mg L^{-1} NAA in MS media. BAP is the most effective cytokinin used for shoot regeneration of monocot plants (Ramakrishnan et al., 2013). BAP along with lower concentrations of NAA (1.5 mg L^{-1}) showed the increased regeneration frequency. In this current study, combination of BAP with NAA increased the shoot induction, prolification and plant regeneration. Similarly, BAP along with lesser concentrations of auxin promoted a mass of green multiple shoots in finger millet (Satish et al., 2016a) and foxtail millet (Satish et al., 2016b). In previous studies, a combination of BAP and NAA has been successfully used for efficient shoot

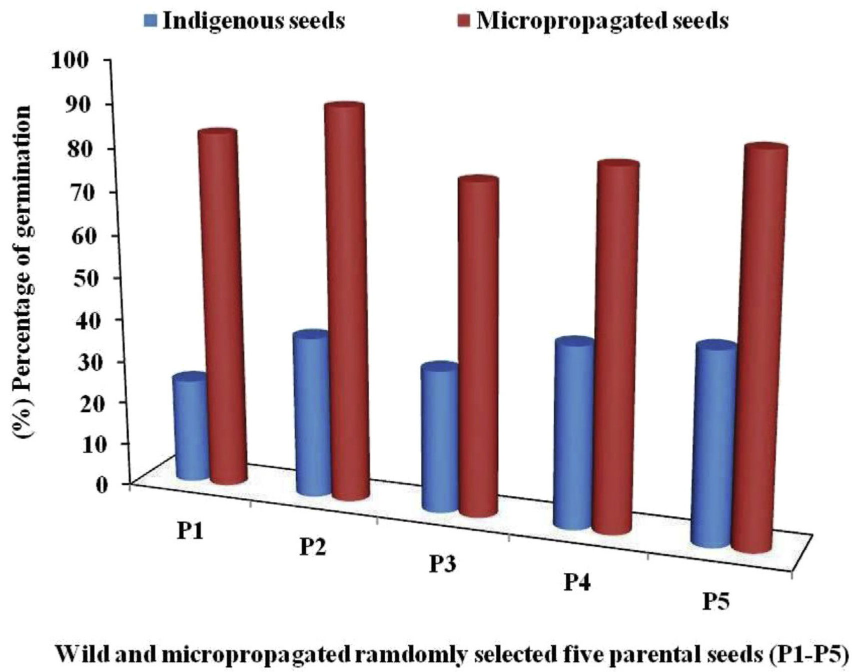

Fig. 3. Comparison of germination percentage between indigenous and micropropagated seeds of deep water rice cv. TNR1.

induction and plant regeneration in various crops (Xu et al., 1984; Kaur and Kothari, 2004; Khush, 2005; Ramesh and Gupta, 2006; Karthikeyan et al., 2009; Wang et al., 2011).

5. Conclusion

In summary, we have developed a rapid and efficient regeneration protocol for producing viable seeds in an indigenous Cauvery Delta originated DWR rice TNR1. This study could be a important biotechnological approach in conservation of endangered rice cultivars. The presence of various stress resistance genes in this traditional crop, will assist the developing technologies to produce new cultivars with high yielding and multi-stress resistance.

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgements

The authors, Dr. M. Ramesh and Mr. R. Rameshkumar sincerely acknowledge the computational and bioinformatics facility provided by the Alagappa University Bioinformatics Infrastructure Facility (funded by DBT, GOI; File No. BT/BI/25/012/2012,BIF). The authors also thankfully acknowledge DST-FIST (Grant No. SR/FST/LSI-639/2015(C)), UGC-SAP (Grant No. F.5-1/2018/DRS-II(SAP-II)) and DST-PURSE (Grant No. SR/PURSE Phase 2/38 (G)) for providing instrumentation facilities.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bcab.2019.01.037.

References

Al-Atawneh, N., Amri, A., Assi, R., Maxted, N., 2008. Management plans for promoting *in situ* conservation of local agrobiodiversity in the West Asia centre of plant diversity. In: Maxted, N., Ford-Lloyd, B.V., Kell, S.P., Iriondo, J., Dulloo, E., Turok, J. (Eds.), Crop Wild Relative Conservation and Use. CABI Publishing, Wallingford, UK, pp. 338–361.

Ali, A.J., Xu, J.L., Ismail, A.M., Fu, B.Y., Vijaykumar, C.H.M., Gao, Y.M., Domingo, J., Maghirang, R., Yu, S.B., Gregorio, G., Yanaghihara, S., Cohen, M., Carmen, B., Mackill, D., Li, Z.K., 2006. Hidden diversity for abiotic and biotic stress tolerances in the primary gene pool of rice revealed by a large backcross breeding program. Field Crop. Res. 97, 66–76.

Al-Khayri, J.M., 2010. Somatic embryogenesis of date palm (*Phoenix dactylifera* L.) improved by coconut water. Biotechnology 9, 477–484.

Anupam, P., 2012. Indigenous Rice: Ignored in Conventional Agriculture Crop Culture: Biotechnology Biodiversity, Centre for Advanced Research and Education (CARE). Saha Institute of Nuclear Physics (SINP), Kolkata, India, pp. 29–38.

Asano, Y., Katsumoto, H., Inokuma, C., Kaneko, S., Ito, Y., Fujiie, A., 1996. Cytokinin and thiamine requirements and stimulative effects of riboflavin and α-ketoglutaric acid on embryogenic callus induction from the seeds of *Zoysia japonica* Steud. J. Plant Physiol. 149, 413–417.

Azria, D., Bhalla, P.L., 2000. Plant regeneration from mature embryo-derived callus of Australian rice (*Oryza sativa* L.) varieties. Aus. J. Agric. Res. 51, 305–312.

Bhaskaran, S., Smith, R.H., 1990. Regeneration in cereal tissue culture: a review. Crop Sci. 30, 1328–1337.

Brar, D.S., Dalmacio, R., Eloloran, R., Aggarwal, R., Angels, R., Khush, G.S., 1996. Gene Transfer and Molecular Characterization of Introgression from Wild *Oryza* Species into Rice. Rice Genetics III. pp. 477–486 (In 2 parts).

Breiholz, J., Plotz, T., Amend, T., 2011. Mountain Gods and Wild Rice. Agrobiodiversity as a Basis for Human Existence. Contributions from China. GIZ, Beijing Available: http://www.conservation-development.net/index.php?L=2&ds=365, Accessed date: 5 March 2013.

Brown, L.R., 1997. The Agricultural Link: How Environmental Deterioration Could Disrupt Economic Progress. Worldwatch Institute, Washington, DC Worldwatch Paper number 136.

Catling, D., 1992. Rice in Deep Water. MacMillan Press, London.

Ceasar, S.A., Ignacimuthu, S., 2010. Effects of cytokinins, carbohydrates and amino acids on induction and maturation of somatic embryos in kodo millet (*Paspalum scorbiculatum* Linn.). Plant Cell Tissue Organ Cult. 102, 153–162.

Choi, H.W., Lemaux, P.G., Cho, M., 2000. High frequency of cytogenetic aberration in transgenic oat (*Avena sativa* L.) plants. Plant Sci. 156, 85–94.

Desai, N.S., Suprasanna, P., Bapat, V.A., 2004. Simple and reproducible protocol for direct somatic embryogenesis from cultured immature inflorescence segments of sugarcane (*Saccharum spp*.). Curr. Sci. 25, 764–768.

Furuhashi, K., Yatazuva, M., 1964. Indefinitely growing rice callus in tissue culture. Kagaku 34, 623.

Genovesi, A.D., Magil, C.W., 1982. Embryogenesis in callus derived from rice microspores. Plant Cell Rep. 1, 257–260.

Guha-Mukerjee, S., 1973. Genotypic differences in the *in vitro* formation of embryoids from rice pollen. J. Exp. Bot. 24, 139–142.

Hay, F.R., de Guzman, F., Ellis, D., Makahiya, H., Borromeo, T., Sackville, H.N.R., 2013. Viability of *Oryza sativa* (L.) seeds stored under genebank conditions for up to 30 years. Genet. Resour. Crop Evol. 60, 275–296.

Ho, W.J., Vasil, I.K., 1983. Somatic embryogenesis in sugarcane (*Saccharum officinarum* L.): growth and plant regeneration from embryogenic cell suspension cultures. Ann. Bot. 51, 719–726.

Hong, T.D., Linington, S.H., Ellis, R.H., 1998. Compendium of Information on Seed

Storage Behaviour. Royal Botanic Gardens, Kew, UK.

Hossain, M., Narciso, J., 2004. Global rice economy: long-term perspectives, paper presented to the FAO rice conference on rice in global markets and sustainable production systems. Rome, Italy. pp. 12–13 February, 2004.

Huang, X.Q., Wei, Z.M., 2004. High-frequency plant regeneration through callus initiation from mature embryos of maize (*Zea mays* L.). Plant Cell Rep. 22, 793–800.

IRRI - International Rice Research Institute (2013) Available at: http://irri.org/resources/publications/annual-reports/annual-report-2013.

Jackson, M.T., 1995. Protecting the heritage of rice biodiversity. Geojournal 35, 267–274.

Jena, K.K., Khush, G.S., 1990. Introgression of genes from *O. officinalis* to cultivated rice, *O.sativa*. Theor. Appl. Genet. 80, 737–745.

Karthikeyan, A., Pandian, S.K., Ramesh, M., 2011. Transgenic indica rice cv. ADT 43 expressing a Δ1-pyrroline-5-carboxylate synthetase (P5CS) gene from Vigna aconitifolia demonstrates salt tolerance. Plant Cell Tissue Organ Cult. 107, 383–395.

Karthikeyan, A., Pandian, S.T.K., Ramesh, M., 2009. High frequency plant regeneration from embryogenic callus of a popular indica rice (*Oryza sativa* L.). Physiol. Mol. Biol. Plants 15, 371–375.

Kaur, P., Kothari, S.L., 2004. In vitro culture of kodo millet: influence of 2,4-D and picloram in combination with kinetin on callus initiation and regeneration. Plant Cell Tissue Organ Cult. 77, 73–79.

Khanna, H.K., Raina, S.K., 1998. Genotype and culture media interaction effects on regeneration response of three *indica* rice cultivars. Plant Cell Tissue Organ Cult. 52, 145–153.

Khush, G.S., Bacalangco, E., Ogawa, T., 1990. A new gene for resistance to bacterial blight from *O. longistaminata*. Rice Genet. News Lett. 7, 121–122.

Khush, G.S., 1977. Disease and insect resistance in rice. Adv. Agron. 29, 265–341.

Khush, G.S., 2005. What it will take to feed 5.0 billion rice consumers in 2030? Plant Mol. Biol. 59, 1–6.

Khush, G.S., Virk, P.S., 2000. Rice breeding: achievement and future strategies. J. Crop Improv. 27, 115–144.

Krishnan, S.R., Priya, A.M., Ramesh, M., 2013. Rapid regeneration and ploidy stability of 'cv IR36' *indica* rice (*Oryza sativa*. L) confers efficient protocol for *in vitro* callus organogenesis and *Agrobacterium tumefaciens* mediated transformation. Botanical Studies 54, 47.

Lee, K., Jeon, H., Kim, M., 2002. Optimization of a mature embryo-based in vitro culture system for high-frequency somatic embryogenic callus induction and plant regeneration from *japonica* rice cultivars. Plant Cell Tissue Organ Cult. 71, 237–244.

Li, L., Qu, R., de Kochko, A., Fauquet, C., Beachy, R.N., 1993. An improved rice transformation system using the biolistic method. Plant Cell Rep. 12, 250–255.

Linsmaier, E.M., Skoog, F., 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plantarum 18, 100–127.

Litz, R.E., Knight, R.L., Gazit, S., 1982. Somatic embryos from culture ovules of polyembryonic *Mangifera indica* L. Plant Cell Rep. 1, 264–266.

Mandal, A.B., Maiti, A., Biswas, A., 2003. Somatic embryogenesis in root derived callus of *indica* rice. Plant Tissue Cult. 13, 125–133.

Mandal, N., Gupta, S., 1995. Effect of genotype and culture medium on androgenic callus formation and green plant regeneration in *indica* rice. Indian J. Exp. Biol. 33, 761–765.

Manoharan, M., Dahleen, L., 2002. Genetic transformation of the commercial barley (*Hordeumvulgare* L.) cultivar Conlon by particle bombardment of callus. Plant Cell Rep. 21, 76–80.

McCann, A.W., Cooley, G., Van Dreser, J., 1988. A system for routine plantlet regeneration of sunflower (*Helianthus annuus* L.) from immature embryo-derived callus. Plant Cell Tissue Organ Cult. 14, 103–110.

Mendoza, M.G., Kaeppler, H.F., 2002. Auxin and sugar effects on callus induction and plant regeneration frequencies from mature embryos of wheat (*Triticumaestivum* L.). In Vitro Cell. Dev. Biol. Plant, vol. 38, 39–45.

Moghaieb, R.E.A., El-Arabi, N.I., Momtaz, O.A., Youssef, S.S., Soliman, M.H., 2010. Genetic transformation of mature embryos of bread (*T. aestivum*) and pasta (*T. durum*) wheat genotypes. GM Crops 1, 87–93.

Morita, S., Kaminaka, H., Masumura, T., Tanaka, K., 1999. Induction of rice cytosolic ascorbate peroxidase mRNA by oxidative stress; the involvement of hydrogen peroxide in oxidative stress signalling. Plant Cell Physiol. 40, 417–422.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plantarum 15, 473–497.

Oka, H.I., 1988. Origin of Cultivated Rice. Japan scientific soc. Japan press.

Pence, V.C., Hasegawa, P.M., Janick, J., 1980. Initiation and development of asexual embryos of *Theobroma cacao* L. in vitro. Z. Pflanzenphysiol. 98, 1–4.

Pescador, R., Kerbauy, G.B., Ferreira, D.M., Purgatto, E., Suzuki, R.M., Guerra, M.P., 2012. A hormonal misunderstanding in *Accasellowiana* embryogenesis: levels of zygotic embryogenesis do not match those of somatic embryogenesis. Plant Growth Regul. 68, 67–76.

Priya, A.M., Pandian, S.K., Ramesh, M., 2011a. Effect of NaCl on in vitro plant regeneration from embryogenic callus cultures of'cv IR 64'indica rice (Oryza sativa L.). Afr. J. Biotechnol. 10, 6947–6953.

Priya, A.M., Pandian, S.K., Ramesh, M., 2011b. Efficient *in vitro* plant regeneration through leaf base derived callus cultures of abiotic stress sensitive popular Asian *indica* rice cultivar IR 64 (*Oryza sativa* L.). Acta Biol. Hung. 62, 441–452.

Puckridge, D.W., Kupkanchanakul, T., Palaklang, W., Kupkanchanakul, K., 2000. Production of rice and associated crops in deeply flooded areas of Chao Phraya delta. In: Proceedings of the 2000 International Conference: the Chao Phraya Delta: Historical Development, Dynamics and Challenges of Thailand's Rice Bowl. 12th–15th December 2001. Kasetsart University, Bangkok, Thailand, pp. 51–85.

Radhakrishnan, R., 1983. Genetic evaluation and utilization deep water. Screening rice verities for deep water tolerance. International Rice Research News Lett 8, 5.

Ramakrishnan, M., Ceasar, S.A., Duraipandiyan, V., Daniel, M.A., Ignacimuthu, S., 2013. Efficacious somatic embryogenesis and fertile plant recovery from shoot apex explants of onion (*Allium cepa*. L.). In Vitro Cell. Dev. Biol. Plant, vol. 49, 285–293.

Ramesh, M., Gupta, A., 2006. Genetic transformation of embryogenic callus cultures of *indica* rice using particle bombardment and selection of stably transformed hygromycin resistant plants. Asian J. Microbiol. Biotechnol. Environ. Sci. 8, 617–622.

Ramesh, M., Murugiah, V., Gupta, A.K., 2009. Efficient *in vitro* plant regeneration via leaf base segments of *indica* rice (*Oryza sativa* L.). Indian J. Exp. Biol. 47, 68–74.

Rao, A.M., Kishor, P.B., Reddy, L.A., Vaidyanath, K., 1988. Callus induction and high frequency plant regeneration in Italian millet (*Setariaitalica*). Plant Cell Rep. 7, 557–559.

Rao, A.M., Sree, K.P., Kishor, P.B.K., 1995. Enhanced plant regeneration in grain and sweet sorghum by asparagine, proline and cefotaxime. Plant Cell Rep. 15, 72–75.

RKMP (2011) http://www.rkmp.co.in/content/tnr-1.Contributed.by.rkmp.tn on 19 July 2011.

Rueb, S., Leneman, M., Schilperoort, R.A., Hensgens, L.A., 1994. Efficient plant regeneration through somatic embryogenesis from callus induced on mature rice embryos (*Oryza sativa* L.). Plant Cell Tissue Organ Cult. 36, 259–264.

Saika, H., Toki, S., 2010. Mature seed-derived callus of the model *indica* rice variety Kasalath is highly competent in *Agrobacterium*-mediated transformation. Plant Cell Rep. 29, 1351–1364.

Satish, L., Rathinapriya, P., Ceasar, S.A., Rency, A.S., Pandian, S., Rameshkumar, R., Subramanian, A., Ramesh, M., 2016b. Effects of cefotaxime, amino acids and carbon source on somatic embryogenesis and plant regeneration in four Indian genotypes of foxtail millet (Setaria italica L.). Vitro Cell. Dev. Biol. Plant., vol. 52, 140–153.

Satish, L., Rathinapriya, P., Rency, A.S., Ceasar, S.A., Pandian, S., Rameshkumar, R., Ramesh, M., 2016a. Somatic embryogenesis and regeneration using Gracilaria edulis and Padina boergesenii seaweed liquid extracts and genetic fidelity in finger millet (Eleusine coracana). J. Appl. Phycol. 28, 2083–2098.

Septiningsih, E.M., Pamplona, A.M., Sanchez, D.L., Neeraja, C.N., Vergara, G.V., Heuer, S., Ismail, A.M., Mackill, D.J., 2009. Development of submergence tolerant rice cultivars: the Sub1 locus and beyond. Ann. Bot. 103, 151–160.

Siwakoti, M., Tiwari, S., 2007. Emerging needs of wetlands protection for the conservation of wild rice biodiversity in Nepal: a case study from Lumbini area. Sci. World 5, 95–99.

Sommut, W., Bose, M.L., Singh, V.P., Hossain, M., 2004. Agriculture and livelihoods in the flood-prone ecosystem in Thailand. Kasetsart J./Nat. Sci. 25, 69–89.

Song, Z., Li, B.O., Chen, J., LU, B.R., 2005. Genetic diversity and conservation of common wild rice (*Oryza rufipogon*) in China. Plant Species Biol. 20, 83–92.

Tang, W., Newton, R.J., 2005. Plant regeneration from callus cultures derived from mature zygotic embryos in white pine (*Pinus strobus* L.). Plant Cell Rep. 24, 1–9.

Thiyagarajan, K., Kalaiyarasi, R., 2010. Status Paper on Rice in Tamil Nadu. Tamil Nadu Agricultural University, Coimbatore. http://www.rkmp.co.in/sites/default/files/ris/rice-state-wise/Status%20Paper%20on%20Rice%20in%20Tamilnadu.pdf.

Verma, D., Joshi, R., Shukla, A., Kumar, P., 2011. Protocol for *in vitro* somatic embryogenesis and regeneration of rice (*Oryza sativa* L.). Indian J. Exp. Biol. 49, 958–963.

Wang, M.Z., Pan, Y.L., Li, C., Liu, C., Zhao, Q., Ao, G.M., Yu, J.J., 2011. Culturing of immature inflorescences and *Agrobacterium*-mediated transformation of foxtail millet (*Setaria italica*). Afr. J. Biotechnol. 10, 16466–16479.

Wani, S.H., Sanghera, G.S., 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, 418–422.

World Bank, 2008. Climate Change Impacts in Drought and Flood Affected Areas: Case Studies in India. Report No. 43946-IN. World Bank Group, Washington, DC, pp. 1–162.

Xu, Z.H., Wang, D.Y., Yang, L.J., Wei, Z.M., 1984. Somatic embryogenesis and plant regeneration in callus cultured immature inflorescence of *Setaria italica*. Plant Cell Rep. 3, 149–150.

Zuraida, A.R., Naziah, B., Zamri, Z., Sreeramanan, S., 2011. Efficient plant regeneration of Malaysian *indica* rice MR 219 and 232 via somatic embryogenesis system. Acta Physiol. Plant. 33, 1913–1921.