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Research Article

Optimisation of plant tissue culture conditions in a popular semi-dwarf *indica* rice cultivar ADT 39 for effective *Agrobacterium*-mediated transformation

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Abstract
The *in vitro* culture conditions plays a critical role in promoting callus and shoot induction in rice during *Agrobacterium*-mediated transformation. In this research, an attempt was made to optimize the key factors affecting the efficiency of *Agrobacterium*-mediated transformation in ADT 39 rice cultivar. Among the different combinations of the NB-Asmedia tried with varying concentrations of casein hydrolysate from 0.25 g l^{-1} to 1.25 g l^{-1}, the media containing 0.5 g l^{-1} casein hydrolysate supplemented with 2 mg l^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D), 1 mg l^{-1}6-Benzylaminopurie (6-BAP) and 1mg l^{-1} Naphthalene Acetic Acid (NAA) was found to be the best with callus induction frequency of 94.44 per cent. Incubation in NB-As media after infection for 20 min. considerably reduced the *Agrobacterium* overgrowth after co-cultivation. For shoot induction, regeneration media RNMH30 containing varied concentrations of 6-BAP from 2 mg l^{-1} to 4 mg l^{-1} was tried, the media containing 3.0 mg l^{-1} 6-BAP added with 1 mg l^{-1} NAA recorded the highest shoot induction frequency of 71.11 per cent. The overall transformation efficiency in ADT 39 with the optimized culture conditions was found to be 7.96 per cent.

Key words: ADT 39, *Agrobacterium*-mediated transformation, Callus induction frequency, Shoot induction frequency, Transformation efficiency.

INTRODUCTION

Rice being the second most leading staple food crop of the world helps to meet the daily nutritional requirements of nearly half of the world's population (Bouis *et al*., 2017). Direct and indirect gene transfer methods help to develop transgenic rice lines which play a crucial role in crop improvement (Tyagi *et al.,* 1999, Visarada and Sarma 2004). *Agrobacterium*-mediated transformation is more preferred over other genetic transformation methods in rice because of its high efficiency, large size and low copy number of T-DNA inserts that are being transferred into the host genome. It involves co-cultivation of the rapidly dividing competent embryonic cells from immature embryos with *Agrobacterium* in the media containing Acetosyringone (Hiei *et al.,* 1997). ADT 39 is a popular semi dwarf *indica* rice cultivar with white medium slender grains which is most widely grown in Southern India, especially Tamil Nadu and Pondicherry (DRDPAT, 2002). ADT 39 is a cross between IR 8 and IR 20 and was released as a variety in the year of 1988. With an average yield of 5.8 t/ha, this variety is well suited for growing in late thaladi or *Kharif* season and is resistant to blast, bacterial leaf blight and brown spot diseases.

The amenability of plants to tissue culture protocols is crucial to carry out an effective and successful *Agrobacterium*-mediated transformation. The culture media composition and other factors affecting T-DNA delivery should be optimized for each and every individual rice variety. This optimization needs to be carried out in a genotype dependent manner in order to facilitate an effective *Agrobacterium*-mediated genetic transformation (Hiei and Komari 2008, Sahoo *et al.,* 2011, Shweta *et al.,* 2020).

The *japonica* rice cultivars are more amenable to *Agrobacterium*-mediated transformation as compared to the *indica* rice cultivars which are recalcitrant to plant tissue culture protocols (Lin and Zhang, 2005). In spite of the limited response of the *indica* rice cultivars, *Agrobacterium*-mediated transformation using immature rice embryos was found to be successful in developing transgenic rice plants with minimum transformation efficiency as compared to its *japonica* counterpart (Aldemita and Hodges, 1996; Hiei and Komari, 2006).

Up to date, there have been no reports on the optimization of *Agrobacterium*-mediated transformation in ADT 39 rice cultivar using immature embryos. Therefore, the current study was focused on optimizing the media composition and duration of incubation as the key factors affecting T-DNA delivery(Wu *et al.,* 2003) for improving the transformation efficiency.

MATERIALS AND METHODS

ADT 39 panicles were collected 15 days after flowering from Paddy Breeding Station, Coimbatore. The immature seeds free of pests and diseases were de-husked by using forceps and transferred to petri plates under sterile conditions inside a laminar air flow chamber (**Fig. 1a)**. After being rinsed with 70% ethanol for 1 min and 1.5% Sodium hypochlorite for 3 min, the de-husked seeds were washed three times with sterile distilled water for 5 min to eliminate excess sterilizing agents which may hinder regeneration of the embryos. The embryos were isolated from the sterilized immature seeds with the help of stereo zoom microscope by using flame sterilized forceps under aseptic conditions. It was then placed on a 0.8% agar plate which prevents them from drying out **(Fig. 1b)** and were transferred to a 1.5 ml sterile eppendorf tube with 1 ml of sterile water. These embryos were then subjected to heat treatment at 42°C for 30 min and kept on ice for 1 min before being centrifuged at 1100rpm for 10 min. All the immature embryos were transferred to NB-As media after removal of excess water under aseptic conditions inside the laminar air flow chamber.

*Agrobacterium*culture harbouring the pRGEB32 vector construct carrying the *hpt* gene internally (Xie *et al.,* 2015, Andrew-Peter-Leon *et al.,* 2020) was streaked on Luria Bertani plates supplemented with antibiotics rifampicilin, tetracyclin and kanamycin, and the plates were incubated at 25°C for 3 days. A loopful of culture was taken from the 3 days old *Agrobacterium* culture and suspended in 1 ml of AA infection media (Hiei and Komari, 2008) in a 1.5 ml eppendorf tube under aseptic conditions to obtain an OD_{600} of 1. Five µl of the prepared *Agrobacterium* infection media was used to infect each of the immature embryos placed on the NB-As (Hiei and Komari, 2008) medium. The plates were then covered with brown paper and incubated at 28°C in the dark for 10, 20, and 30 min. The bacterial suspension should be completely dried in order to prevent bacterial overgrowth after infection (Hiei and Komari, 2008). The infected embryos were then transferred to fresh NB-As media containing N6 major salts, Fe-EDTA, B5 minor salts, proline, plant growth regulators such as 2,4-D (2 mg l^{-1}), NAA (1mg l^{-1}) and BAP (1mg l^{-1}), sugars which includes glucose and proline, and varying concentrations of casein hydrolysate. The plates were then incubated at 25°C for 7 days.

After infection with *Agrobacterium*, the embryos were transferred to five different combinations of NB-As media containing 0.25 g l^{-1}, 0.50 g l^{-1}, 0.75 g l^{-1}, 1.00 g l^{-1} and 1.25 g $l^{-1}$of caseinhydrolysate respectively with three replications for each **(Fig. 1c)**. The plates were then incubated at 25°C and the callus induction frequency was calculated (Zaidi *et al.,* 2006) after 7 days of incubation.

The plumule was removed from the callus obtained after co-cultivation **(Fig. 1d)** with the help of flame sterilized forceps and the healthy callus was transferred to CCMC media (Hiei and Komari, 2008) in the resting phase under aseptic conditions inside a laminar airflow chamber **(Fig. 1e)**. The plates were then incubated at 32°C under continuous illumination with a light intensity of 5000 luxfor 7 days. Afterwards, the well grown embryos were cut into small pieces and moved on to the second phase of resting which lasts for 10 days. The well proliferated callus from the resting stage was transferred to CCMCH50 (Hiei and Komari, 2008) selection media containing the selectable marker hygromycin (50 mg/l) and incubated at 32°C for 10 days **(Fig. 1f)**. The surviving embryos were transferred to the second phase of selection in the fresh selection media and incubated for 7 days at 32°C. The yellowish friable calli obtained after the selection was transferred to NBPRCH40 media (Hiei and Komari, 2008) and incubated for 7 days at 32°C under continuous illumination **(Fig. 1g)**.

The healthy compact calli from pre-regeneration media were transferred to five different combinations of RNMH30 medium (Hiei and Komari, 2008) containing various concentrations of 6- BAP(2 mg l^{-1}, 2.5 mg l^{-1}, 3mg l^{-1}, 3.5 mg l^{-1} and 4 mg l^{-1}), N6 major salts, Fe-EDTA, B5 minor salts, 0.5 g l^{-1} casein hydrolysate, 0.5 g l^{-1} proline, 30 g l^{-1} maltose and 1 mg l^{-1} NAA **(Fig. 1h)**. The plates were then incubated for 14 days at 32°C at 5,000 lux in order to induce shoots. After 14 days, the number of shoots produced from individual callus **(Fig. 1i)** and the

shoot induction frequency was calculated (Zaidi *et al.,* 2006).

The regenerated shoots from RNMH30 media were transferred to MSIH30 (Hiei and Komari, 2008) half MS rooting media and incubated at 32°C for 14 days **(Fig. 1j)**. The plants were then transferred to pro-trays in the greenhouse for primary hardening **(Fig. 1 k & l)**. After isolating the DNA from the regenerated plants by Cetyltrimethyl Ammonium Bromide(CTAB) method (Sambrook and Russell, 2006), the putative transgenic plants were confirmed by carrying out PCR analysis using

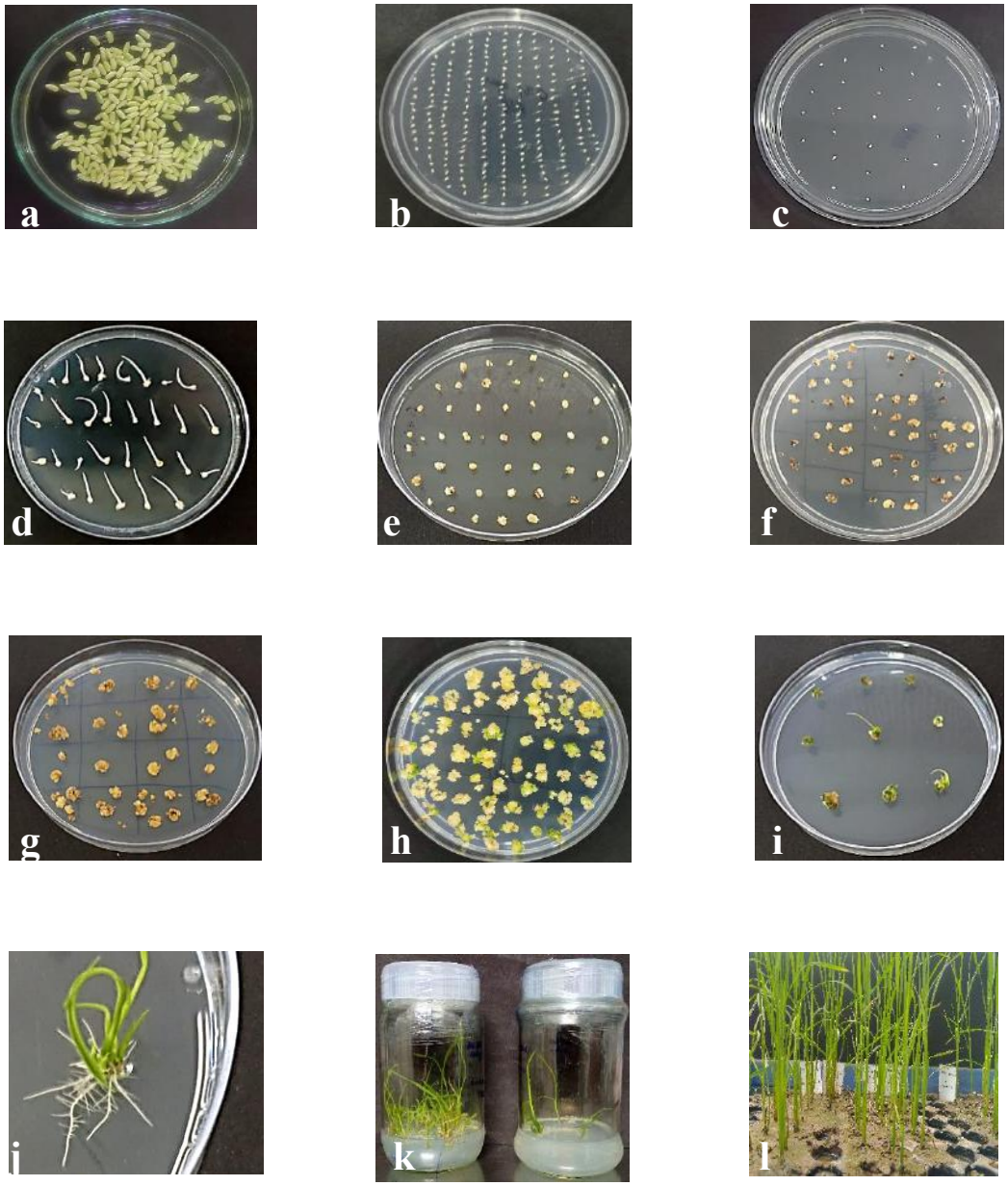

Fig. 1. The different stages involved in Agrobacterium-mediated transformation in ADT 39.
a) De-husked rice seeds in petriplate containing water. b) Isolated embryos placed in 0.8% agar. c) Embryos placed on NB-As media after infection. d) Callus with plumule after seven days of infection. e) Callus placed on resting media. f) Embryos after two rounds of selection. g) Callus placed on pre-regeneration media after selection. h) Greening of embryos in regeneration media. i) Induction of shoots from callus in regeneration media. j) Roots emerging from callus in rooting media on petriplate. k) Rooting of shoots in jam bottles. l)Transgenic ADT 39 plants transplanted in the greenhouse.

hpt gene specific primers designed using Primer3web (https://primer3.ut.ee/) online primer designing tool (Nagaraj *et al.,* 2019). The 686 bp *hpt* gene was amplified by setting up a 20 µl PCR reaction mixture containing 1 µl of plant genomic DNA, 1 µl of each of forward and reverse primers, 10 µl of smart Prime master mix and 7 µl of sterile water, and the PCR amplification was carried out in Eppendorf Mastercycler Pro 384 thermal cycler **(Table 1)**. The number of callus regenerated into transgenic plants and the total number of embryos co-cultivated with *Agrobacterium* was used to calculate the transformation efficiency in the ADT 39 rice cultivar (Kumar *et al.*, 2005)**.**

The data obtained from this experiment were analysed by using OPSTAT statistical analysis package. The means were determined and the Duncan's Multiple Range Test was performed at P≤0.05 to understand the significant difference between the treatments.

RESULTS AND DISCUSSION

The efficiency of *Agrobacterium*-mediated transformation in rice depends on many factors like the media composition, culture density and duration of infection with *Agrobacterium* suspension (Leon *et al.,* 2020). As the efficiency of genetic transformation is dependent on genotype, there is a need to optimize all these factors. In this study, ADT 39 immature embryos were infected with *Agrobacterium* suspension (OD_{600}=1) and incubated at different durations of 10, 20 and 30 min in dark at 25°C. The minimal bacterial overgrowth was observed at 20 min of incubation with an efficiency of 51.10 per cent **(Table 2)**.

Casein hydrolysate is a major source of amino acids, calcium, vitamins, and many micronutrients. It also serves as a plant growth promoter and thereby helps in callus induction (Khaleda and Al-Forkan, 2006). It was evident from the present study that NB-As media containing 0.50 g l^{-1} of casein hydrolysate was optimum to induce callus from the immature embryos of ADT 39 during *Agrobacterium*-mediated transformation **(Table 3)**. Out of five different treatments, the treatment T2 was found to be the best one with 94.44 per cent callus induction frequency and resulted in the formation of healthy whitish creamy textured calli but there was no significant difference between treatments T2 and T3.T3 containing 0.75 g/l of caseinhydrolysate had a callus induction frequency of 91.11 per cent. It was also observed that either decreasing or increasing the optimum concentration of casein hydrolysate (0.5 g l^{-1}) has decreased the callus induction frequency in ADT 39 **(Table3)**.

Table 1. Details of the primers used for amplification of *hpt* gene in the transgenic plants

Gene	Primer	Sequence of the primer (5'- 3')	PCR Profile	Amplicon length
hpt	Forward	GCTGTTATGCGGCCATTGGTC	94°C for 5 min: 1 cycle	
	Reverse	GCCTCCAGAAGAAGATGTTG	94°C for 45 sec 57.8°C for 45 sec } 30 cycles 72°C for 45 sec	686 bp
			72°C for 10 min: 1 cycle	

Table 2. Effect of different durations of incubation time after infection during co-cultivation in ADT 39

Treatment	Duration of incubation	Number of embryos inoculated per replication	Embryos without *Agrobacterium* overgrowth (%)
T1	10 minutes	30	27.78[a]
T2	20 minutes	30	51.10[a]
T3	30 minutes	30	13.33[a]

Numbers in the columns followed by the same letters are not significantly different at P≤0.05.

Table 3. Effect of various concentrations of casein hydrolysate on callus induction in ADT 39

Treatment	Casein hydrolysate (g l^{-1})	Number of embryos inoculated per replication	Callus induction frequency (%)
T1	0.25	30	72.22[b]
T2	0.50	30	94.44[a]
T3	0.75	30	91.11[ab]
T4	1.0	30	82.217[ab]
T5	1.25	30	74.44[b]

Numbers in the columns followed by the same letters are not significantly different at P≤0.05.

A widely used cytokinin, 6-Benzylamino Purine (6-BAP), is known to be important in stimulating callus differentiation into shoots and therefore aiding in the regeneration of the calli (Rueb *et al.,* 1994). This is the first report on the genetic transformation of ADT 39. With a view to find the optimized condition for regeneration, different concentrations of 6-BAP ranging from 2.0 mg l^{-1} to 4 mg $l^{-1}$was tested and found that RNMH30 media containing 2.5 mg/l and 3 mg/l of 6-BAP showed the highest regeneration efficiency of 67.77 and 71.11 per cent, respectively **(Table 4)**. All the regenerated plants obtained from the callus was found to be positive for *hpt* gene **(Fig.2)** which confirms the presence of the transgene. The mean transformation efficiency in ADT 39 by *Agrobacterium*-mediated transformation after optimizing the culture media conditions and the duration of incubation after infection was found to be 7.96 per cent **(Table 5)**. Using different binary vectors, Hiei and Komari (2008) reported a transformation frequency of 3-13 independent transgenic plants for Type 4 and Type 5 (Non-Kasalath variety) and 10-18 independent transgenic plants for Type 6 (Kasalath variety) *indica* rice cultivars.

In this study, the transformation frequency of ADT 39 using pRGEB32 as a binary vector was found to be 9-13 individual transgenic plants. Thus, from this research, an efficient protocol to carry out *Agrobacterium*-mediated transformation in a popular semi-dwarf *indica* rice cultivar ADT 39 was devised and interestingly it was found to be in compliance with the protocol suggested by Hiei and Komari (2008).

Table 4. Effect of different concentrations of 6-BAP on shoot induction in ADT 39

Treatment	Concentration of 6-BA (mg l^{-1})	Number of calli inoculated per replication	No. of shoots per callus	Shoot induction frequency (%)
T1	2.00	30	9^{b}	54.44^{ab}
T2	2.50	30	10^{ab}	67.77^{a}
T3	3.00	30	13^{a}	71.11^{a}
T4	3.50	30	12^{ab}	62.21^{ab}
T5	4.00	30	10^{ab}	42.21^{b}

Numbers in the columns followed by the same letters are not significantly different at P≤0.05.

Table 5. Transformation efficiency in ADT 39

S.No.	Number of embryos co-cultivated	Number of embryos survived after 2 rounds of selection	Number of calli regenerated	Transformation efficiency (%)	Mean transformation efficiency (%)
1	95	12	8	8.42	7.96
2	70	8	5	7.14	
3	36	5	3	8.33	

Numbers in the columns followed by the same letters are not significantly different at P≤0.05.

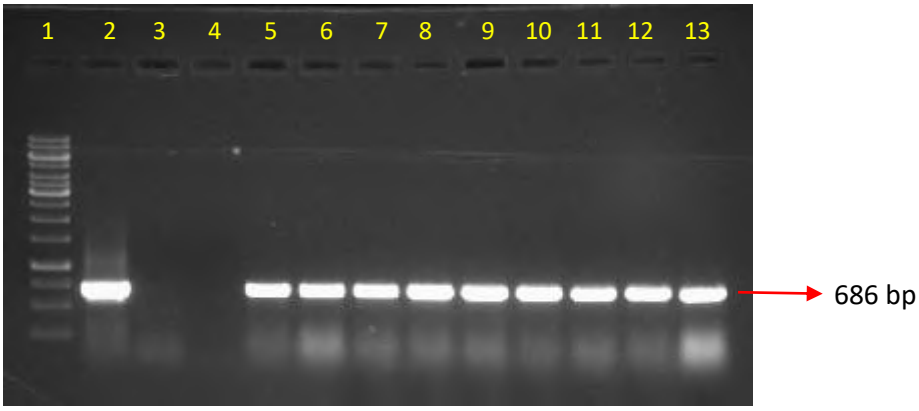

Fig. 2. PCR amplification using *hpt* gene specific primers
Lane 1- 1kb DNA ladder; Lane 2- Positive control (pRGEB32); Lane 3- Negative control (Sterile water); Lane 4- Negative control (Non transgenic ADT 39); Lane 5 to 13- Putative transgenic ADT 39 lines.

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