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Research Article

Protocol optimization for rapid and efficient callus induction and *in-vitro* regeneration in rice (*Oryza sativa* L.) *cv*. CO 51

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Abstract
CO 51 is a recently developed semi dwarf, short duration variety of rice popularly grown in Tamil Nadu. Optimization of tissue culture protocol for the variety was one of the most important steps required; in order to make the variety suitable for genetic transformation and gene editing techniques. Immature embryos of CO 51 have been used as explant for callus induction. The immature embryos were cultured on callus induction media (NB media) containing Naphthalene Acetic Acid (NAA) 1 mg l^{-1}, 6-Benzylaminopurine (6-BA) 1 mg l^{-1} and various concentrations of 2,4 Dichlorophenoxy acetic acid (2,4-D). NB media supplemented with 2.5 mg $l^{-1}$of 2,4-D have been found to show the highest callus induction frequency that is 94.4%. The calli showing desired features were transferred from NB media to pre-regeneration media (NB-PR) having 2,4-D (2 mg l^{-1}), 6-BA (1 mg l^{-1}), NAA (1 mg l^{-1}) where the calli tuned golden-yellow. These calli were then transferred to regeneration media (RNM media) supplemented with 6-BA (3 mg l^{-1}) along with various concentrations of NAA. RNM media with1 mg l^{-1} NAA showed the highest frequency of shoot induction that is 72.2% with an average of 12 shoots per callus.

Key words
CO 51, callus induction, *in-vitro* regeneration, 2,4-D, NAA, 6BA

INTRODUCTION

CO 51 is one of the newer rice variety of Tamil Nadu. It was developed from rice varieties ADT 43 / RR 272 – 1745 at Tamil Nadu Agricultural University and is proposed to be suitable for cultivation in nearly all the districts of Tamil Nadu. It is a short duration (105 -110 days), semi dwarf variety with moderately resistant to disease like blast and also to insects like Brown plant hopper and Green leaf hopper which are severe problems in several districts of the state. The rice grains are medium slender and white in colour. The high milling percentage (68.5%), head rice recovery (63.2%) and yield makes it desirable from commercial point of view. Apart from the above mentioned traits it has intermediate amylose content which is responsible for giving rice its soft and slightly sticky texture preferred by the inhabitants of Tamil Nadu (Robin *et al*., 2019). Being short in duration, the variety can be utilized extensively in crop improvement programmes which involve recombinant DNA or gene editing technologies. However, before taking into account recombinant DNA or gene editing techniques for the crop improvement it becomes important to develop a rapid tissue culture protocol for an efficient regeneration of the concerned variety (Gosal and Kang, 2012; Sikdar *et al.,* 2015).

In plant tissue culture system, callus induction and regeneration are two fundamental aspects that needed to be optimized for successful genetic transformation

of the plant (Li *et a*l., 2007; Seraj *et al*., 1997). Several factors including genotype of the plant, explant type, source of carbohydrate, basal salts present in medium and plant growth regulators have been found to play the major role in callus induction and regeneration of a plant (Rueb *et al*., 1994). Out of all the naturally occurring plant hormones, auxin, cytokinin and the interaction between them are considered to be the most important ones as they are involved in growth regulation and also organized development in plant tissue culture (Evans *et al*., 1981; Vasil and Thorpe 1994).

Till date many reports have been published on optimizing the tissue culture protocol of various rice cultivars using growth regulators however, no report has been published regarding the successful regeneration of CO 51 rice variety *in-vitro.* Therefore, the present study was conducted to develop a rapid and an efficient *in-vitro* regeneration protocol for CO 51 so that it can be used for genetic transformation, as well as molecular biology studies.

MATERIALS AND METHODS

Panicles of rice variety CO 51 approaching hard dough stage that is 14-17 days post-heading was collected from paddy field located at Paddy Breeding Station, TNAU, Coimbatore. The green immature seeds free from any infestation were selected and were de-husked manually using a pair of forceps.

The seeds were then transferred to a sterile petri-plate of size 90 mm under aseptic condition and were surface sterilized for 60 seconds using 70% ethanol (v/v) followed by sterilization with 1.5% (v/v) Sodium Hypochlorite added with 2 drops of tween-20 for 3 minutes. The seeds were finally rinsed 3-4 times with sterile distilled water to remove the remnants of surface sterilizing agents.

The surface sterilized seeds were placed under a stereo-zoom microscope. The immature embryos were carefully isolated using a pair of sterile forceps and placed over 0.8% agar to avoid it's desiccation during the process of isolation. The embryos were then transferred to 1.5 ml micro-centrifuge tube and rinsed with sterile distilled water. After rinsing the embryos for 2-3 times 1 ml of sterile distilled water was added to it and the embryos were placed at 42°C in a water bath for 30 minutes. This was followed by placing the embryos in ice for a minute and then centrifuging them at 1100 rpm for 10 minutes. To determine the optimum concentration of auxin (2,4-D) required for callus induction the pre-treated embryos were cultured on callus induction NB media (Hiei and Komari, 2008) containing N6 major salts, B5 minor salts, Fe EDTA, B5 vitamins, casein hydrolysate (0.5 g l^{-1}), proline (0.5 g l^{-1}), glucose (10 g l^{-1}), sucrose (20 g l^{-1}), hormones - NAA (1 mg l^{-1}), 6-BA (1 mg l^{-1}), along with 2,4-D at 5 different concentrations that is 1.0, 1.5, 2.0, 2.5 and 3.0 mg l^{-1} and 0.8 % (w/v) agarose (**Fig. 1 a**). The embryos were incubated in dark for 7 days at 25° C. Thereafter, the embryos were sub-cultured over the same media after removing the plumule (**Fig. 1 b**) and were incubated at 32° C for 14 days under continuous illumination for callus induction (**Fig. 1 c**). Callus induction frequency was calculated using the following formula (Zaidi *et al.,* 2006)
Callus induction frequency (%) =

$$\frac{\text{Number of explants producing callus}}{\text{Total number of explants cultured}} \text{ X } 100$$

The concentration of 2,4-D showing the best growth of calli among all 5 concentrations was selected based on callus induction frequency and creamish- white, friable calli were further transferred to pre-regeneration NB-PR media (Hiei and Komari, 2008) containing N6 major salts, B5 minor salts, Fe EDTA, B5 vitamins, casein hydrolysate (0.5 g l^{-1}), proline (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 0.5% (w/v) gelrite and was incubated for 7 days at 32°C under continuous illumination (5000 lux).

Golden-yellow friable calli were selected from pre-regeneration media (**Fig. 1 d**) and were transferred to RNM media (Hiei and Komari, 2008) comprising of N6 major salts, Fe-EDTA, B5 minor salts, B5 vitamins, proline (0.5 g l^{-1}), casein hydrolysate (0.5 g l^{-1}), glutamine (0.3 g l^{-1}), maltose (30 g l^{-1}) hormones 6-BA (3 mg l^{-1}) along with NAA at 5 different concentrations that is 0.5, 1.0, 1.5, 2.0, 2.5 mg l^{-1} and agarose. The calli were incubated under continuous illumination (5000 lux) at 31° C for 14 days and were continuously observed for emergence of shoot (**Fig. 1 e and Fig. 1 f**) The concentration of NAA best suited for regeneration was selected based upon the number of shoots per calli and the regeneration frequency. The regeneration frequency was calculated by the formula (Zaidi *et al.,* 2006)

Regeneration Frequency (%) =

$$\frac{\text{Number of calli producing shoots}}{\text{Total number of calli incubated}} \text{ X } 100$$

From the regenerated calli, individual shoots are carefully taken out under stereo zoom microscope and were placed gently over half MS (Murashige and Skoog, 1962) supplemented with sucrose, casein hydrolysate myo-inositol, proline, and gelling agents 0.4% agar (w/v) and 0.2 % (w/v) phytagel and incubated under continuous illumination (5000 lux) at 32°C (Hiei and Komari, 2008). Once the roots start emerging, the plants were transferred from plates (**Fig. 1 g**) to bottles having rooting media of same composition and were incubated under the same condition (**Fig. 1 h**). The plants with well-developed root system were transferred in clay soil under green housing condition for hardening (**Fig. 1 i**).

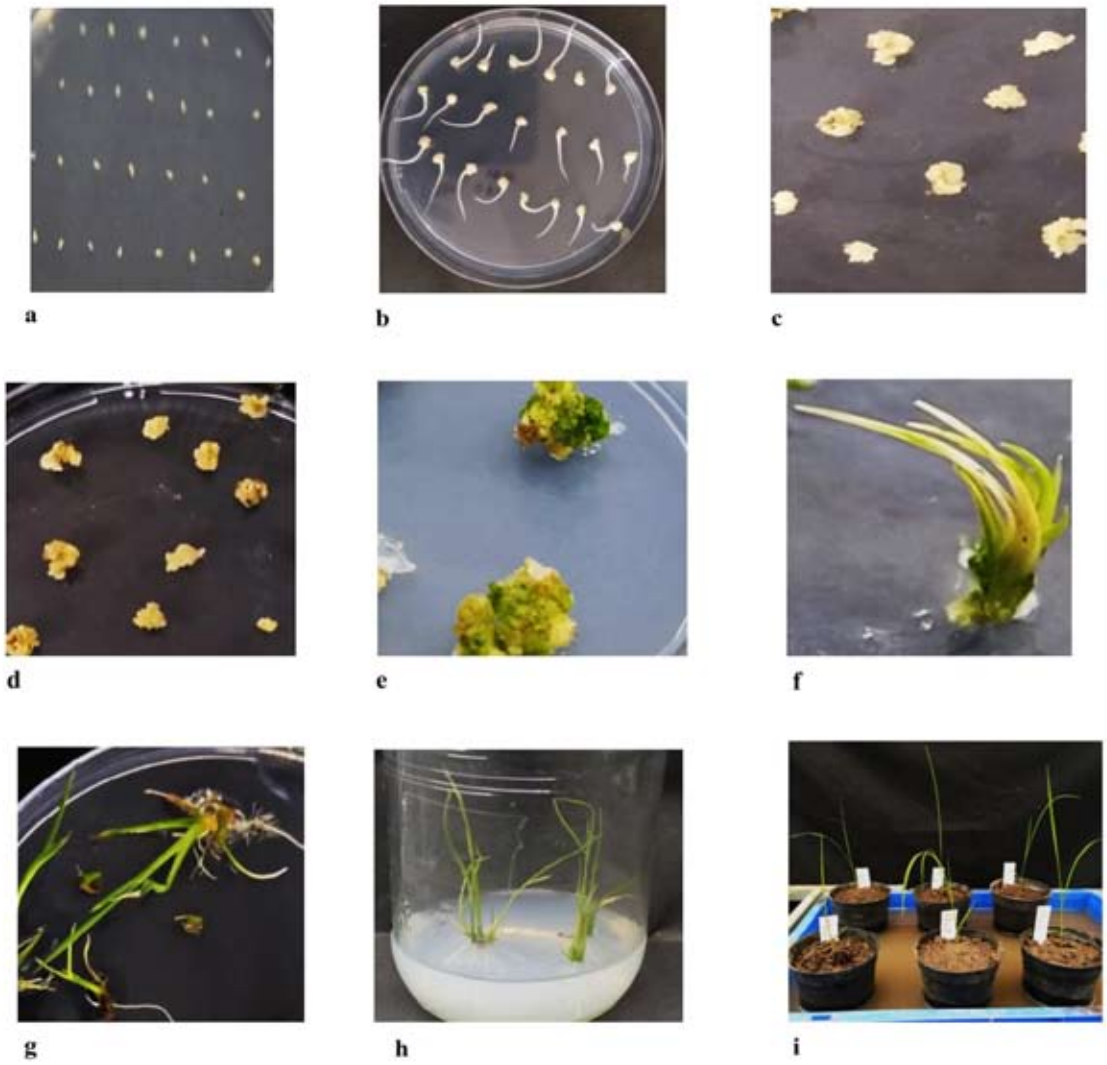

Fig.1. Callus induction and in-vitro regeneration of CO 51 rice variety

(a) Immature embryos of CO 51 (b) Plumule emergence from immature embryos after 7 days (c) 3 week-old embryogenic calli (d) Golden yellow callus as developed on pre-regeneration media (e) Greening of callus on regeneration media (f) Shoot induction (g) Rooting in petriplate (h) Rooting in jam bottles (i) Tissue culture regenerated CO 51 plants in the green house.

RESULTS AND DISCUSSION

It has been evidently reported in various studies that out of all the auxins 2,4-D is the most suitable one for callus induction in rice (Chen *et al*, 1974., Bajaj, 1991). In the present study we have analysed different concentrations of 2,4-D for inducing callus from immature embryos (**Table 1**). As per the results obtained, the callus induction frequency ranged from 81.1% to 94.4% across the various concentrations of 2,4-D. T_5 having the concentration 3.0 mg l^{-1} of 2,4-D showed the lowest percentage of callus induction whereas T_4 (NB supplemented with 2.5 mg l^{-1} of 2,4-D) showed the highest value in terms of percentage of callus induction that is 94.4% however, it did not showed any significant difference when compared to callus induction frequency obtained from T_3 (NB supplemented with 2.0 mg l^{-1} of 2,4-D). This is found to be in agreement with Hiei and Komari, 2006 where 2.0 mg l^{-1} of 2,4-D was considered suitable for callus induction. This indicates that 2.0 mg l^{-1} of 2,4-D can also be considered as optimum for callus induction in CO 51 rice varieties. It was also observed that the frequency of callus induction first increased with increase in concentration of 2,4-D, reached the maximum and then started decreasing. NB- medium supplemented with 1.5 mg l^{-1} to 2.5mg l^{-1} of 2,4-D showed the best results in terms of morphological features. Calli generated on these were creamy white in appearance and were bigger and healthier when compared to the rest of the treatments.

Table 1. Effect of different concentrations of 2,4-D on callus induction of CO 51

Treatments	Concentration of 2,4-D (mg l^{-1})	No. of immature embryos inoculated per replication	Callus induction Frequency (%)
T1	1.0	30	84.4^{bc}
T2	1.5	30	87.8^{b}
T3	2.0	30	92.2^{a}
T4	2.5	30	94.4^{a}
T5	3.0	30	81.1^{c}

Means inside the columns with the same superscript do not vary significantly at $P \leq 0.05$

As per Hiei and Komari, 2006 regeneration frequency of the calli derived from immature embryo can be enhanced by transferring the proliferated calli to pre-regeneration medium prior to regeneration medium. Based upon which the calli were transferred to pre regeneration medium supplemented with 2,4-D (2 mg l^{-1}), 6-BA (1 mg l^{-1}) and NAA (1 mg l^{-1}).The calli that turned golden yellow in colour and appeared friable was transferred further to regeneration media for shoot induction.

RNM media supplemented with1.0 mg l^{-1} of NAA was found to show the best result in terms of percentage of shoot induction that is 72.2% and the number of shoots per calli that is 12 (**Table 2**) which was in agreement with that mentioned by Hiei and Komari, 2006. Thus, we consider 1.0 mg l^{-1} of NAA to be ideal for regeneration in CO 51 rice variety. Apart from this the result also revealed that with the increase in concentration of NAA the shoot induction frequency decreased this could possibly suggest that higher concentration of NAA inhibits the accumulation of cytokinin and thus morphogenesis is also affected (Gasper *et al*, 1996).

Table 2. Effect of different concentrations of NAA on shoot initiation of CO 51

Treatments	Concentration of NAA (mg l^{-1})	Shoot induction Frequency (%)	No. of shoots per callus
T1	0.5	55.6^{c}	12^{a}
T2	1.0	72.2^{a}	12^{a}
T3	1.5	64.4^{b}	7^{b}
T4	2.0	35.6^{d}	5^{b}
T5	2.5	34.4^{d}	5^{b}

Means inside the columns with the same superscript do not vary significantly at $P \leq 0.05$

The results of the present study indicates that NB medium supplemented with 2.5 mg l^{-1} of 2,4-D showed the highest callus induction frequency from immature embryo of CO 51 rice but it did not showed any significant variation from the concentraion (2.0 mg l^{-1} 2,4-D) published by Hiei and Komari 2006 therefore, NB medium supplemented with 2.0 mg l^{-1} of 2,4-D can be considered optimum for callus induction in CO 51. RNM medium having 1 mg l^{-1} NAA was found to be most effective for regeneration of proliferated calli. The results of the above experiment thus helped us in identifying the optimum concentration of 2,4-D and NAA for callus induction and regeneration respectively and also helped us identify that the protocol published by Hiei and Komari 2008 can be successfully used to conduct *Agrobacterium* mediated transformation in immature embryos of CO 51 without having to make any specific changes in it.

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