

Optimizing the protocol for *in vitro* regeneration through callus in rice varieties, viz , Ratnagiri-8, Karjat Shatabdi and Karjat-3

Abstract

The investigation entitled “*In vitro* regeneration studies in rice (*Oryza sativa* L.)” was carried out in tissue culture laboratory at Plant Biotechnology Centre, College of Agriculture, Dapoli-Ratnagiri (M.H.) in Completely Randomized Design with 3 replications. The aim of the research was to set up the *in vitro* regeneration protocol in the three rice varieties, viz, Ratnagiri-8, Karjat Shatabdi, Karjat-3 with an objective to investigate suitable callus induction medium and develop an efficient regeneration technique for genetic transformation. Mature seed embryo was used as explant for callus initiation. The callusing ability of the varieties was tested on 12 medium combinations with different concentrations of 2,4-D and NAA, their combinations and one control treatment. Medium combination T₂: MS + 2,4-D (2.0 mg/l) + NAA (0.5 mg/l) gave the highest callus induction in Ratnagiri-8 and Karjat Shatabdi with a callus induction frequency of 66.67 % and 53.33 % respectively and callus weight of 0.367 g and 0.290 g respectively. Karjat-3 showed highest callus induction for medium combination T₃: MS + 2,4-D (2.0 mg/l) + NAA (1.0 mg/l) with a callus induction frequency of 68.33 % and callus weight of 0.392 g. Embryogenic, soft, friable callus with granular texture and yellowish white colour was obtained from all media combinations in all three varieties. The regeneration ability of the varieties was tested on 15 medium combinations with different concentrations of BAP and Kinetin, their combinations and one control treatment. The shoot and root induction was obtained on same medium. The highest regeneration was observed for T₅ : MS + BAP (2.5 mg/l) in Ratnagiri-8 and Karjat Shatabdi with a shoot induction frequency of 83.33% and 73.67% respectively and root induction frequency of 76.33% and 64.67% respectively. In Karjat-3, highest regeneration was observed for T₆: MS + BAP (3.0 mg/l) with a shoot and root induction frequency of 78.33% and 69.00% respectively.

Key words: Callus induction, regeneration, shoot induction, root induction.

1. Introduction

Roughly half of the world population, including virtually all of East and Southeast Asia, is wholly dependent upon rice as a staple food; 95 percent of the world's rice crop is eaten by humans (Anonymous, 2024). There is an everyday increasing demand of rice production for increasing population in the developing countries. The option for increasing the cultivated area seems to be of less value as agricultural lands are being converted to residential areas. Being a staple food for most of the developing worlds, nutritional improvement of rice can also help in decreasing the evil of malnutrition in the developing worlds. The most viable option, therefore, is to increase the productivity by utilizing the novel biotechnological tools. These days, traditional plant breeding methods aim to improve things by leveraging different aspects of biotechnology, such as introducing new genes through genetic transformation, producing male sterile lines through protoplast fusion, achieving rapid homozygosity through haploid generation, and increasing trait variability through somaclonal variation (Hoque *et al.*, 2007).

Genetic engineering is strongly dependent on genotype and availability of an efficient *in vitro* plant regeneration method. Although rice tissue culture has been the subject of numerous publications, regeneration in *indica* rice remains a challenging endeavor due to significant genotype dependence (Wani *et al.*, 2011). In general, because of poor regeneration abilities, *indica* cultivars are recalcitrant to various biotechnological advances (Kumar *et al.*, 2008). Therefore, identification and screening of useful cultivars for embryogenic callus formation and subsequent *in vitro* plant regeneration are key steps in rice genetic improvement programme through application of biotechnology (Hoque *et al.*, 2004). *In vitro* plant regeneration in rice has been obtained from almost all types of explants. However, there was significant diversity in embryogenic callus formation, somatic embryogenesis, and subsequent plant regeneration across various origins. Compared to other explants, using mature seed embryos as starting material offers a clear benefit for *in vitro* regeneration in rice. The use of mature seed embryos as starting material has distinct advantage for *in vitro* regeneration over other explants in rice (Wani *et al.*, 2011). Dehusked rice seed culture is a valuable technique to exploit somaclonal variation. However, factors such as plant genotype, the culture methods, selection of explant, the media and the culture conditions influence culture efficiency thereby limiting its application. Moreover, embryogenic calli obtained from mature seed embryos are efficient in *indica* rice transformation. Consequently, the primary stages in crop

plants that can be altered by biotechnological methods and to take advantage of somaclonal diversity are callus formation and regeneration.

Genotype and nutrient media are the most important factors which affect callus induction and subsequent plant regeneration.

Keeping in view, the above important aspects, the present investigation was carried out with the objectives to investigate suitable callus induction medium in rice and development of *invitro* regeneration techniques in rice which can be utilized for future genetic transformation technology.

2. Materials and Methods

The experiment was conducted in tissue culture laboratory at Plant Biotechnology Centre, College of Agriculture, Dapoli-Ratnagiri in Completely Randomized Design with three replications.

2.1 Plant material and explant

Mature seed embryo was used as explant for the three varieties, *viz*, Ratnagiri-8, Karjat Shatabdi and Karjat-3 developed by DBSKKV, Dapoli-Ratnagiri, Maharashtra, India.

2.2 Media preparation and sterilization

The basal medium developed by Murashige and Skoog (1962) was used with certain additions of various concentrations and combinations of PGR. After addition of various kinds of adjuvants (after bringing stock solutions to room temperature) to MS basal medium as per requirement, the pH of medium was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl.

The final volume was adjusted as required and then media was dispensed in suitable container and heated and then 2.6 g/l agar and 1 g/l gelrite was added to the medium and heated until boiled.

The medium was poured in sterilized glass test tubes and sealed with non absorbent cotton plug. The culture tubes were then sterilized by autoclaving the tubes using horizontal steam sterilizer at 121°C and 15 lbs/in² pressure for 20 min. After sterilization the medium was

allowed to solidify and culture tubes were stored in undisturbed place for at least 2 days before use to check for any contamination.

2.3 Sterilization of seeds

Mature embryos were used as initial explants. Explants were brought to laboratory and husk from the seed was removed and the seeds were taken in 1 sterilized glass jar. The seeds were then washed with distilled water to remove the dirt present on the seeds. The seeds were dipped in polysolvent Tween 20 (1%) for 20 min. The solution was discarded and seeds were washed once with distilled water. The seeds were then treated with Bavistin (0.1%) and streptomycin (0.05%) solution for 30 min. The solution was discarded and explants were again washed with distilled sterilized water for 2 times. Next steps were performed in Laminar Air Flow cabinet

The culture tubes of media combinations, glass jars with distilled water and solutions required for sterilization were placed in Laminar Air Flow bench and exposed to UV rays for 15 min. for sterilization. Explant containing glass jar was brought to Laminar air flow bench and all explants were transferred in pre sterilized empty glass jar. These explants were again treated with Bavistin (0.1%) and streptomycin (0.05%) solution for 30 min. The solution was discarded and explants were washed with DSW for 2 times. Then explants were treated with 70% ethyl alcohol for 45 seconds. The solution was discarded and explants were washed with DSW for 2 times. The explants were then treated with 0.1 % of Mercuric Chloride (HgCl_2) solution for 4 min. The solution was discarded and explants were finally washed with DSW for 6 times. Finally the explants were inoculated on medium for callus induction.

2.4 Inoculation of seeds and incubation

The treated explants were inoculated on callus induction media (20 seeds/ treatment) in culture tubes containing MS basal medium with different concentrations and combinations of PGR using aseptic culture technique. The culture tubes were then incubated in culture room in dark conditions and observed for callus establishment.

Following medium combinations were used for callus establishment through Embryo culture:

List 1 : List of treatment details used for the study

Sr. No.	Treatments (mg/l)
T ₀	Control
T ₁	MS + 2,4-D (2.0)
T ₂	MS + 2,4-D (2.0) + NAA (0.5)
T ₃	MS + 2,4-D (2.0) + NAA (1.0)
T ₄	MS + 2,4-D (2.0) + NAA (1.5)
T ₅	MS + 2,4-D (2.0) + NAA (2.0)
T ₆	MS + 2,4-D (2.0) + NAA (2.5)
T ₇	MS + 2,4-D (2.5)
T ₈	MS + 2,4-D (2.5) + NAA (0.5)
T ₉	MS + 2,4-D (2.5) + NAA (1.0)
T ₁₀	MS + 2,4-D (2.5) + NAA (1.5)
T ₁₁	MS + 2,4-D (2.5) + NAA (2.0)
T ₁₂	MS + 2,4-D (2.5) + NAA (2.5)

The per cent callus induction was calculated as follows:

$$\text{Callus induction frequency (\%)} = \frac{\text{No. of seeds with calli}}{\text{No. of seeds inoculated}} \times 100$$

2.5 Sub culturing of callus for proliferation :-

The inoculated explants were observed for callus induction and calli were sub cultured on media showing highest callus induction frequency for proliferation.

2.6 Inoculation of callus for regeneration:-

The callus proliferated on nutrient medium was cut into pieces of 100 mg and inoculated on regeneration media (20 callus/treatment) and observed for shoot initiation and multiplication and root induction.

The following medium combinations were used:

List 2 : Medium combinations used for the study

Sr. No.	Treatments (mg/l)
T ₀	Control (only MS basal medium)
T ₁	MS + BAP (0.5)
T ₂	MS + BAP (1.0)
T ₃	MS + BAP (1.5)
T ₄	MS + BAP (2.0)
T ₅	MS + BAP (2.5)
T ₆	MS + BAP (3.0)
T ₇	MS + Kin (0.5)

T ₈	MS + Kin (1.0)
T ₉	MS + Kin (1.5)
T ₁₀	MS + Kin (2.0)
T ₁₁	MS + Kin (2.5)
T ₁₂	MS + BAP (2.5) + Kin (1.0)
T ₁₃	MS + BAP (2.5) + Kin (1.5)
T ₁₄	MS + BAP (3.0) + Kin (1.0)
T ₁₅	MS + BAP (3.0) + Kin (1.5)

The results regarding *in vitro* plantlet regeneration in 15 medium combinations for the three varieties were determined with respect to shoot induction frequency and root induction. The regeneration frequency was calculated as follows:

$$\text{Regeneration frequency (\%)} = \frac{\text{No. of calli inoculated with shoot/root}}{\text{No. of calli inoculated}} \times 100$$

2.7 Statistical analysis :-

The study was conducted under well defined controlled laboratory conditions. Hence, Completely Randomized Design (CRD) was applied for the experiment and data was analysed by following the standard methods (Panse and Sukhatme, 1967).

3. Results and Discussion

3.1 Callus induction:

3.1.1 Medium combination showing highest callus induction and Callusing ability (Callus induction frequency) of varieties on different media combinations:

No callus induction was observed for control treatment T₀. The callus induction frequency decreased with increasing concentrations of 2, 4-D above 2.0 mg/l and NAA @ 0.5-1.0 mg/l. Treatment T₂ and T₃ performed better than others. T₂ was significantly superior over all other treatments in Ratnagiri-8 and Karjat Shatabdi with a callus induction frequency of 66.67% and 53.33% respectively (Table 1) and callus weight of 0.367g and 0.290g (Fig 1), respectively and it was followed by T₃ with a callus induction frequency of 61.67% and 43.67% (Table 1) and callus weight of 0.339g and 0.263g, respectively (Fig1). However, in Karjat-3 the highest callus induction and weight was obtained in T₃ with a callus induction frequency of 68.33% and weight 0.392g followed by T₂ with a frequency of 62.67% and weight 0.364g (Table 1 and Fig 1). The lowest callus induction and weight was recorded for T₁₂ in all the three varieties with a frequency

of 14.67%, 11.67% and 21.67% respectively and weight of 0.234 g, 0.198 g and 0.242 g respectively (Table 1 and Fig 1).

Irrespective of media combinations and with respect to varieties the highest callus induction and weight was observed in Karjat- 3 followed by Ratnagiri-8 and Karjat Shatabdi.

The results proved that callus induction depends on plant growth regulators. 2,4-D is the most preferred auxin for callus establishment. Khan *et al.* (2019) reported optimum concentrations of 2,4-D for callus induction @2.0 mg/l. Here, better callus induction was observed for 2,4-D concentration @ 2.0 mg/l. The callusing frequency decreased after increasing the concentration of 2, 4-D above 2.0 mg/l and this results were in accordance with Kartikeyan *et al.* (2009), Libin *et al.* (2012), and Rashid *et al.* (2021).

2,4-D in combination with other PGR enhances callus induction. It was discovered that combination of 2, 4-D at 2.0 mg/l with NAA @ 0.5 mg/l and 1.0 mg/l induces better callusing. Similar trend was observed for concentrations of NAA as observed in 2, 4-D. The callus induction frequency decreased with increasing concentrations of NAA after 1.0 mg/l. This results also were in accordance with Islam *et al.* (2014), Roly *et al.* (2014) and Din *etal.* (2015).

Also, it was observed that the weight of callus was directly proportional to callus induction frequency and the callus weight decreased with increasing concentrations of 2,4-D and NAA above 2.0 and 0.5-1.0 mg/l. These results were in accordance with Thadavong *et al.* (2002), Summart *et al.* (2008), Kartikeyan *et al.* (2013) and Poeaim *et al.* (2016).

Observations	Callus induction frequency (%)			Days required for callus induction			
	Treatments	Ratnagiri-8	Karjat Shatabdi	Karjat-3	Ratnagiri-8	Karjat Shatabdi	Karjat-3
T ₀		0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a

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Table 1: Observations recorded for callusing ability

T ₁	48.67 ^g	34.00 ^{ef}	52.33 ^{gh}	33.67 ^{de}	40.33 ^{de}	37.00 ^{de}
T ₂	66.67 ^k	53.33 ^j	62.67 ^j	28.33 ^b	33.33 ^b	33.33 ^{bc}
T ₃	61.67 ^j	43.67 ⁱ	68.33 ^k	30.00 ^{bc}	36.33 ^{bc}	31.67 ^b
T ₄	56.67 ⁱ	39.67 ^h	58.33 ⁱ	31.67 ^{cd}	38.33 ^{cd}	35.00 ^{cd}
T ₅	52.33 ^h	37.33 ^{gh}	55.67 ^{hi}	32.33 ^{cd}	39.00 ^{cd}	35.67 ^{cde}
T ₆	50.00 ^{gh}	35.00 ^{fg}	53.67 ^{gh}	33.33 ^{de}	40.00 ^{de}	36.67 ^{de}
T ₇	47.33 ^{fg}	32.67 ^{ef}	50.67 ^{fg}	34.00 ^{de}	41.00 ^{de}	37.67 ^{def}
T ₈	45.00 ^{ef}	31.33 ^{de}	48.33 ^{ef}	34.67 ^{def}	41.33 ^{de}	38.33 ^{ef}
T ₉	41.67 ^e	28.33 ^d	43.67 ^e	36.00 ^{efg}	42.67 ^{ef}	40.33 ^{fg}
T ₁₀	37.33 ^d	22.67 ^c	39.33 ^d	37.33 ^{fg}	44.67 ^f	41.67 ^{gh}
T ₁₁	33.33 ^c	19.67 ^c	34.00 ^c	39.00 ^g	45.33 ^{fg}	44.00 ^h
T ₁₂	14.67 ^b	11.67 ^b	21.67 ^b	44.33 ^h	48.33 ^g	47.00 ⁱ
CV	3.52	4.66	3.66	4.14	3.60	3.58
SE(m)	0.87	0.81	0.96	0.76	0.78	0.73
CD at 1%	3.41	3.17	3.76	3.00	3.08	2.86
F test	SIG	SIG	SIG	SIG	SIG	SIG

(Values are the means of three replicates. The data was analysed by one way ANOVA and is significant at $p \leq 0.05$)

Means within columns with the same superscript are not significantly different at $P \leq 0.05$ by using DMRT.)

3.1.2 Days required for callus induction

The days required for callus establishment were inversely proportional to callus induction frequency. The minimum number of days for callus induction was observed for treatment T₂ in Ratnagiri-8 (28.33 days) and Karjat Shatabdi (33.33 days) and for T₃ (31.67 days) in Karjat-3. In Ratnagiri-8 and Karjat Shatabdi, T₂ was at par with T₃ for early callusing for 30.00 and 36.33 days respectively while in Karjat-3 T₃ was at par to T₂ (33.33 days) for early callus induction. In all the three varieties maximum number of days for callus induction was recorded for T₁₂ requiring 44.33, 48.33 and 47.00 days respectively (Table 1).

Considering the varieties, minimum number of days for callus induction was observed in Ratnagiri-8 (28.33 days) followed by Karjat-3 (31.67 days) and Karjat Shatabdi (33.33 days).

Hence, an average of 30 days were required for callus establishment. Similar findings were reported by Thadavong *et al.* (2002), Carsono *et al.* (2006), Kartikeyan *et al.* (2009), Tiwari *et al.* (2012), Poaimet *et al.* (2016) and *et al.* (2018).

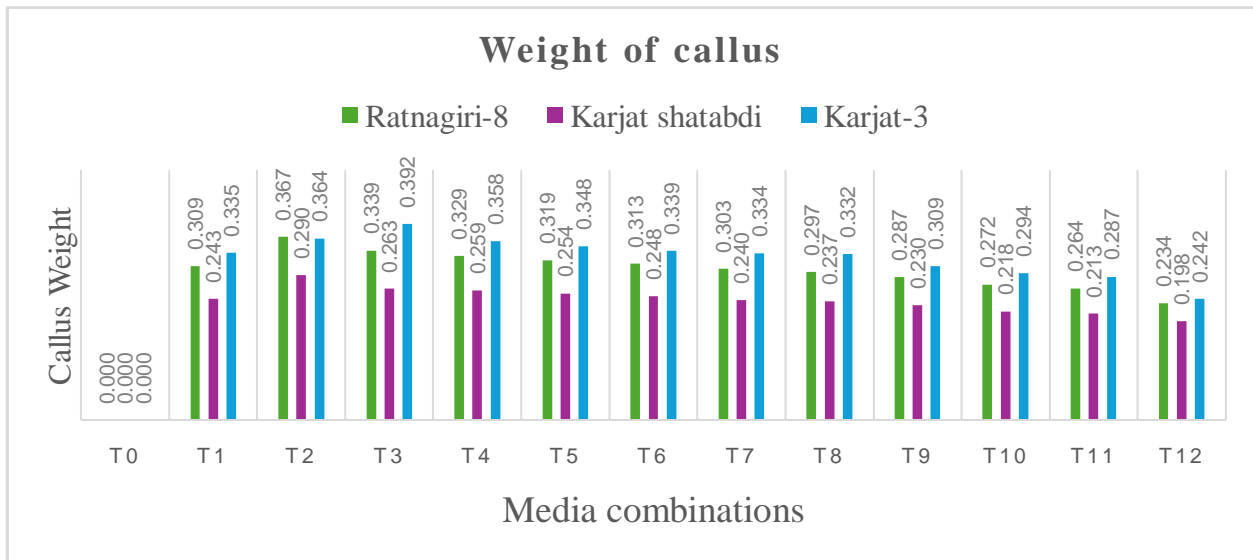


Fig1: Weight of callus on different media combinations

3.1.3 Nature of callus

No callusing was observed in control treatment T₀. In all the three varieties embryogenic soft and friable callus which was granular in texture with a yellowish white colour was obtained from all the media combinations.

However, callus produced at the concentration of 2, 4-D @ 2.0 mg/l exhibited bigger size and higher weight. The results were similar to the findings previously reported by Summart *et al.* (2008), Ho *et al.* (2018), Libin *et al.* (2012) and Rashid *et al.* (2021).

3.2 *In vitro* Plantlet Regeneration

3.2.1 Plantlet Regeneration (Regeneration frequency) of varieties in different media combinations

Shoot induction and root induction was observed on same medium combination. No regeneration was observed in control treatment T₀. The highest regeneration was observed in Ratnagiri-8 followed by Karjat-3 and Karjat Shatabdi.

Treatment T₅ and T₆ performed better. T₅ was significantly superior over all other treatments in Ratnagiri-8 and Karjat Shatabdi with a shoot induction frequency of 83.33% and 73.67%, respectively and root induction frequency of 76.33% and 64.67%, respectively and it was followed by T₆ with a shoot induction frequency of 79.67% and 70.33% respectively and root induction frequency of 72.67% and 60.33% respectively. While Karjat-3 had highest shoot and root induction for T₆ with a frequency of 78.33% and 69.00%, respectively followed by T₅ (74.33% and 65.33%) (Table 2 and 3).

The minimum shoot induction was observed for T₁ in Ratnagiri-8 (62.00%) and Karjat Shatabdi (54.33%) and for T₇ in Karjat-3 (58.67%). The minimum root induction was observed for T₁ in Ratnagiri-8 (55.00%) and Karjat-3 (50.33%) and for T₇ in Karjat Shatabdi (43.67%) (Table 2 and 3).

Irrespective of media combinations the highest shoot induction was recorded in Ratnagiri-8 (83.33%) followed by Karjat-3 (78.33%) and Karjat Shatabdi (73.67%). Irrespective of media combinations the highest root induction was recorded in Ratnagiri-8 (76.33%) followed by Karjat-3 (69.00%) and Karjat Shatabdi (64.67%).

Tariq *et al.* (2008) reported shoot and root induction on N₆ medium supplemented with 2.5 mg/l BAP and 1.0 mg/l NAA. Poaimet *et al.* (2016) also reported that both shoot and root was induced on same MS Basal medium in combination with 2 mg/l BAP and 5.2 g/l phytigel. Similarly, in the present study it was revealed that the shoot and root induction was obtained from same medium (MS Basal medium supplemented with 2.5-3.0 mg/l BAP). The results are similar to the findings previously reported by Illahiet *et al.* (2005), Islam *et al.* (2005), Biswas *et al.* (2007), Kartikeyan *et al.* (2009), Libinet *et al.* (2012), Suraiya *et al.* (2018) and Trunjaruen *et al.* (2020).

Thadavong *et al.*(2002) reported that shoot regeneration is achieved in the medium supplemented with low ratio of auxin to cytokinin while high auxin to cytokinin ratio induces root formation. However, in the present case increase in the endogenous level of auxins in the callus during proliferation on MS medium supplemented with 2,4-D 2.0 mg/l in combination with 0.5-1.0 mg/l NAA resulted in root induction along with shoot initiation. Notably, root induction occurred alongside shoot initiation despite the absence of auxins in the regeneration medium. Therefore, it can be implicated that the proliferation of callus on half strength of medium used for callus induction before transferring to regeneration medium can result in better and early regeneration.

However, the results are in little contrast to the findings of Biswas *et al.* (2007), Suraiya *et al.* (2018) and rueb *et al.* (2020) which indicated that higher shoot induction was obtained from combination of BAP and Kinetin @ 2.0-2.5 mg/l and 1.0-2.0 mg/l, respectively.

Table 2: Observations for shoot induction:

Observations Treatments	Shoot induction frequency (%)			Days required for shoot induction		
	Ratnagiri-8	Karjat Shatabdi	Karjat-3	Ratnagiri-8	Karjat Shatabdi	Karjat-3
T ₀	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a
T ₁	62.00 ^b	54.33 ^b	61.33 ^{bc}	22.67 ^g	25.67 ^{fg}	24.00 ^{gh}
T ₂	64.67 ^{bc}	57.00 ^{bc}	64.00 ^{cd}	22.00 ^{fg}	24.67 ^{ef}	23.33 ^{fg}
T ₃	72.67 ^{ef}	61.67 ^{def}	67.67 ^{ef}	20.33 ^{def}	23.67 ^{c-f}	22.00 ^{ef}
T ₄	75.00 ^{fg}	64.67 ^{fg}	70.67 ^{fgh}	19.33 ^{cd}	23.00 ^{cde}	20.67 ^{cde}
T ₅	83.33 ^j	73.67 ^j	74.33 ⁱ	16.67 ^b	20.33 ^b	19.33 ^{bc}
T ₆	79.67 ⁱ	70.33 ⁱ	78.33 ^j	18.00 ^{bc}	21.67 ^{bc}	18.00 ^b
T ₇	64.33 ^{bc}	54.67 ^b	58.67 ^b	21.67 ^{efg}	27.00 ^g	25.33 ^h
T ₈	74.67 ^{fg}	63.00 ^{ef}	66.67 ^{de}	18.33 ^c	23.33 ^{cde}	22.00 ^{ef}
T ₉	72.00 ^{ef}	61.33 ^{de}	68.33 ^{ef}	19.00 ^{cd}	24.00 ^{def}	21.33 ^{de}
T ₁₀	69.67 ^{de}	58.67 ^{cd}	63.00 ^c	20.00 ^{de}	25.00 ^{efg}	23.33 ^{fg}
T ₁₁	67.33 ^{cd}	56.00 ^{bc}	61.00 ^{bc}	21.67 ^{efg}	25.67 ^{fg}	24.00 ^{gh}
T ₁₂	78.67 ^{hi}	69.00 ^{hi}	69.67 ^{efg}	18.00 ^{bc}	22.00 ^{bcd}	21.67 ^{ef}
T ₁₃	76.00 ^{gh}	66.33 ^{gh}	72.33 ^{ghi}	19.33 ^{cd}	23.00 ^{cde}	20.33 ^{cde}
T ₁₄	74.33 ^{fg}	62.67 ^{ef}	73.00 ^{hi}	20.67 ^{def}	24.33 ^{ef}	19.67 ^{bcd}
T ₁₅	71.67 ^{ef}	59.00 ^{cd}	70.67 ^{fgh}	21.33 ^{efg}	25.00 ^{efg}	21.00 ^{cde}
CV	2.01	2.28	2.14	3.62	3.81	3.68
SE(m)	0.79	0.77	0.79	0.39	0.49	0.43
CD at 1%	3.04	2.98	3.04	1.51	1.91	1.68
F test	SIG	SIG	SIG	SIG	SIG	SIG

(Values are the means of three replicates. The data was analysed by one way ANOVA and is significant at $p \leq 0.05$

Means within columns with the same superscript are not significantly different at $P \leq 0.05$ by using DMRT.)

3.2.2 Number of days required for shoot and root induction

In variety Ratnagiri-8 and Karjat Shatabdi, the minimum number of days for shoot and root induction was observed in T_5 requiring 16.67 and 20.33 days, respectively for shoot induction and 10.33 and 14.00 days, respectively for root induction (Table 2 and 3). The minimum number of days required for shoot and root induction was observed for T_6 in Karjat-3 (18.00 days and 12.67 days). The maximum number of days for shoot induction was recorded for T_1 in Ratnagiri-8 (22.67 days) and for T_7 in Karjat Shatabdi (27.00 days) and Karjat-3 (25.33 days). The maximum number of days for root induction was recorded for T_1 in all three varieties requiring 17.00, 20.33 and 19.00 days respectively. (Table 2 and 3).

Considering the varieties, early shoot and root induction was observed in Ratnagiri-8 followed by Karjat-3 and Karjat Shatabdi.

These results were similar to the findings of Rueb *et al.* (1994), Saharan *et al.* (2004), Kartikeyan *et al.* (2009), Alam *et al.* (2012) and Silva *et al.* (2015).

However, these are in contrast with the findings reported by Tariq *et al.* (2008), Islam *et al.* (2014) and Din *et al.* (2015) which shows that it takes a minimum of 3-4 weeks for plant regeneration.

Table 3: Observations for root induction:

Observations Treatments	Root induction frequency (%)			Days required for root induction		
	Ratnagiri-8	Karjat Shatabdi	Karjat-3	Ratnagiri-8	Karjat Shatabdi	Karjat-3
T_0	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a
T_1	55.00 ^b	45.00 ^{bc}	50.33 ^b	17.00 ^j	20.33 ⁱ	19.00 ^h

T ₂	57.67 ^b	47.67 ^{cd}	53.33 ^{bc}	15.67 ⁱ	19.33 ^{ghi}	18.00 ^{gh}
T ₃	64.00 ^{cde}	52.33 ^{fg}	57.00 ^{de}	14.33 ^{fgh}	17.00 ^e	16.67 ^{efg}
T ₄	68.33 ^{fgh}	56.00 ^{hi}	61.67 ^{fgh}	13.00 ^{de}	15.67 ^{cd}	15.33 ^{cde}
T ₅	76.33 ^j	64.67 ^l	65.33 ⁱ	10.33 ^b	14.00 ^b	14.00 ^{bc}
T ₆	72.67 ⁱ	60.33 ^k	69.00 ^j	11.67 ^c	15.33 ^c	12.67 ^b
T ₇	57.33 ^b	43.67 ^b	56.33 ^{cd}	16.67 ^j	19.67 ^{hi}	18.00 ^{gh}
T ₈	67.00 ^{efg}	54.33 ^{ghi}	61.67 ^{fgh}	13.33 ^{ef}	16.67 ^{de}	16.00 ^{def}
T ₉	65.67 ^{def}	51.67 ^{fg}	63.33 ^{ghi}	14.00 ^{efg}	17.33 ^{ef}	15.00 ^{cd}
T ₁₀	63.33 ^{cd}	48.33 ^{de}	60.00 ^{efg}	14.67 ^{ghi}	18.67 ^{gh}	16.67 ^{efg}
T ₁₁	61.67 ^c	45.00 ^{bc}	58.33 ^{def}	15.33 ^{hi}	19.00 ^{gh}	17.33 ^{fg}
T ₁₂	71.33 ^{hi}	59.67 ^{jk}	59.67 ^{ef}	12.00 ^{cd}	15.67 ^{cd}	16.67 ^{efg}
T ₁₃	69.00 ^{gh}	57.00 ^{ij}	61.33 ^{fgh}	13.00 ^{de}	17.00 ^e	16.00 ^{def}
T ₁₄	66.67 ^{efg}	53.33 ^{fgh}	63.67 ^{hi}	14.00 ^{efg}	18.33 ^{fg}	15.33 ^{cde}
T ₁₅	63.33 ^{cd}	51.00 ^{ef}	61.33 ^{fgh}	15.33 ^{hi}	19.00 ^{gh}	16.33 ^{def}
CV	2.15	2.64	2.43	3.29	2.78	4.25
SE(m)	0.76	0.75	0.79	0.25	0.26	0.37
CD at 1%	2.94	2.92	3.06	0.97	1.02	1.44
F test	SIG	SIG	SIG	SIG	SIG	SIG

(Values are the means of three replicates. The data was analysed by one way ANOVA and is significant at $p \leq 0.05$

Means within columns with the same superscript are not significantly different at $P \leq 0.05$ by using DMRT.)

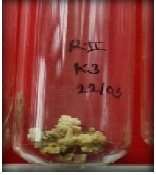
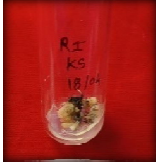
Plate 1 : Regeneration protocol in three varieties

Ratnagiri-8

Karjat Shatabadi

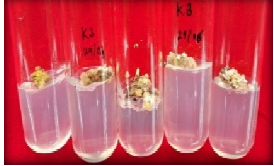
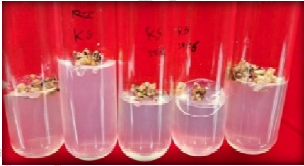
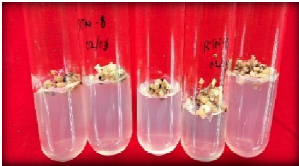
Karjat-3

Callus induction on medium



T₂ : MS + 2,4-D (2.0 mg/l) + NAA (0.5 mg/l)

Callus proliferation on medium



T₂ : MS + 2,4-D (2.0 mg/l) + NAA (0.5 mg/l)

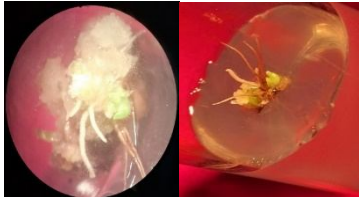
T₂ : MS + 2,4-D (2.0 mg/l) + NAA (0.5 mg/l)

T₃ : MS + 2,4-D (2.0 mg/l) + NAA (1.0 mg/l)

Transfer of proliferated callus on regeneration medium



Shoot and root induction on regeneration medium



T₅ : MS + B₆

T₆ : MS + BAP (3.0 mg/l)

Plantlet regeneration



Conclusion

From the experiment, it is concluded that *in vitro* regeneration in rice depends on optimum concentrations of PGR and genotype. In the varieties, highest callus induction was observed in Karjat-3 followed by Ratnagiri-8 followed by Karjat Shatabdi. However, early callusing was observed in Ratnagiri-8 followed by Karjat-3 followed by Karjat Shatabdi. Callusing ability of the varieties determined the media combination which can be used to obtain highest callus induction. Embryogenic soft and friable callus with yellowish white colour was obtained from all the media combination in all three varieties. Earlier studies reported that callus induction depends on concentration of 2,4-D. In the present study it was revealed that maximum callus establishment was obtained at concentration of 2,4-D @ 2.0 mg/l in combination with low concentrations of NAA @ 0.5 mg/l and 1.0 mg/l. Best plantlet regeneration was observed at 2.5-3.0 mg/l concentration of BAP supplemented in M.S. basal medium. Ratnagiri-8 showed highest regeneration followed by Karjat-3 and then Karjat Shatabdi. It can be implicated that the proliferation of callus on half strength of medium used for callus induction before transferring to regeneration medium can result in better and early regeneration. This study had set the protocol for *invitro* regeneration in given rice varieties which can be further utilized for genetic transformation studies.

Disclaimer (Artificial intelligence)

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- 2.
- 3.

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