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

Abstract

The investigation entitled "*Invitro* 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 varietieswas tested on 12 medium combinations with different concentrations of 2,4-D and NAA,their combinations and one control treatment. Medium combination T_2: 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 and0.290 g respectively. Karjat-3 showed highest callus induction for medium combination T_3: 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 media 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 T_5 : 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 T_6: 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.

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1. Introduction

Roughly one-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. The conventional plant breeding processes are directed today towards the improvement by utilizing various features of biotechnology which includes introduction of novel genes by genetic transformation, protoplast fusion to produce male sterile lines, haploid generation for attaining rapid homozygosity and somaclonal variation for introducing increasing trait variability (Hoque *et al*., 2007).

Genetic engineering is strongly dependent on genotype and availability of an efficient *in vitro* plant regeneration method.Numerous reports been published regarding the rice tissue culture, but there is a lot of genotype dependence and regeneration in *indica* rice is still a difficult task (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 *invitro* plant regeneration are key steps in rice genetic improvement programme through application of biotechnology (Hoque *etal*., 2004). *In vitro* plant regeneration in rice has been obtained from almost all types of explants. However, significant variation was observed in embryogenic callus production, somatic embryogenesis and subsequent plant regeneration from different origins. The use of mature seed embryos as starting material has distinct advantage for *invitro* 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 it's application. Moreover, embryogenic calli obtained from mature seed embryos are efficient in *indica* rice transformation. Therefore, production of callus and its subsequent regeneration are the prime steps in crop plant to be manipulated by biotechnological means and to exploit somaclonal variation.

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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 riceanddevelopment of *invitro*regeneration techniques in rice which can be utilizedfor futuregenetic 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.

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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.

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The medium was poured in sterilized glass test tubes and sealed with non absorbent cotton plug.The culture tubes were then sterilization by autoclaving the tubes using horizontal steam sterilizer at 121°C and 15 lbs/in^2 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

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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

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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 andall 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 andexplants 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. Finallythe explants were inoculated on medium for callus induction.

2.4 Inoculation of seeds and incubation

The treated explants were inoculated on callus induction media 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)

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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:

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 prolifered on nutrient medium was cut into pieces of 100 mg and inoculated on regeneration media 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)

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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:

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

2.7Statistical 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:

3.1.1Medium 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 T_0. 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 T_2 and T_3 performed better than others. $T_2$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 wasfollowed by T_3 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 T_3 with a callus induction frequency of 68.33% and weight 0.392g followed by T_2 with a frequency of 62.67 % and weight 0.364g (Table 1 and Fig 1). The lowest callus induction and weight was recorded for T_{12} in all the three varieties with a frequency of 14.67%, 11.67% and 21.67% respectively and weight of0.234 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.

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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 withincreasing 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).

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Observations	Callus induction frequency (%)	Days required for callus induction

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Table 1: Observations recorded for callusing ability

Treatments	Ratnagiri-8	Karjat Shatabdi	Karjat-3	Ratnagiri-8	Karjat Shatabdi	Karjat-3
T_0	0.00	0.00	0.00	0.00	0.00	0.00
T_1	48.67 (44.24)	34.00 (35.67)	52.33 (46.34)	33.67	40.33	37.00
T_2	66.67 (54.74)	53.33 (46.91)	62.67 (52.34)	28.33	33.33	33.33
T_3	61.67 (51.75)	43.67 (41.36)	68.33 (55.76)	30.00	36.33	31.67
T_4	56.67 (48.83)	39.67 (39.04)	58.33 (49.80)	31.67	38.33	35.00
T_5	52.33 (46.34)	37.33 (37.66)	55.67 (48.25)	32.33	39.00	35.67
T_6	50.00 (45.00)	35.00 (36.27)	53.67 (47.10)	33.33	40.00	36.67
T_7	47.33 (43.47)	32.67 (34.86)	50.67 (45.38)	34.00	41.00	37.67
T_8	45.00 (42.13)	31.33 (34.04)	48.33 (44.04)	34.67	41.33	38.33
T_9	41.67 (40.20)	28.33 (32.16)	43.67 (41.36)	36.00	42.67	40.33
T_{10}	37.33 (37.66)	22.67 (28.43)	39.33 (38.84)	37.33	44.67	41.67
T_{11}	33.33 (35.26)	19.67 (26.33)	34.00 (35.67)	39.00	45.33	44.00
T_{12}	14.67 (22.52)	11.67 (19.97)	21.67 (27.74)	44.33	48.33	47.00
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**

(Figures in parenthesis are arcsine transformed values)

3.1.2 Days required for callus induction

The minimum number of days for callus induction was observed for treatment T_2 in Ratnagiri-8 (28.33 days) and Karjat Shatabdi (33.33 days) and for T_3 (31.67 days) in Karjat-3. In Ratnagiri-8 and Karjat Shatabdi, T_2 was at par with T_3 for early callusing for 30.00 and 36.33 days respectively while in Karjat-3 T_3 was at par to T_2 (33.33 days) for early callus induction. In all the three varieties maximum number of days for callus induction was recorded for T_{12} requiring 44.33, 48.33 and 47.00 days respectively (Table 1).

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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 *etal.*(2012), Poeaim*et al.*(2016) and *et al.* (2018).

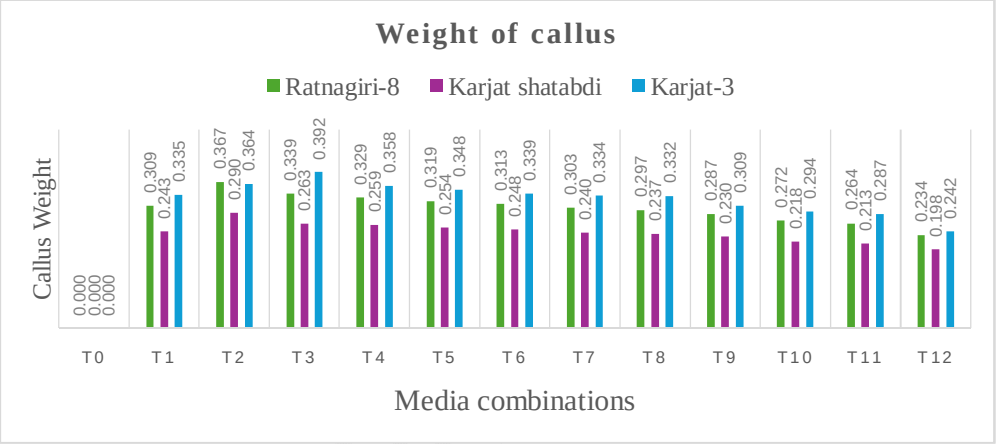

Fig1: Weight of callus on different media combinations

3.1.3 Nature of callus

No callusing was observed in control treatment T_0. 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 T_0.The highest regeneration was observed in Ratnagiri-8 followed by Karjat-3 and Karjat Shatabdi.

Treatment $T_5$and T_6 performed better. T_5 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 %, respectivelyand it was followed by T_6 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 $T_6$with a frequency of78.33% and 69.00%, respectively followed by T_5 (74.33% and 65.33%) (Table 2and 3).

The minimum shoot induction was observed for T_1 in Ratnagiri-8 (62.00%) and Karjat Shatabdi (54.33%) and for T_7 in Karjat-3 (58.67%). The minimum root induction was observed for T_1 in Ratnagiri-8 (55.00%) and Karjat-3 (50.33%) and for T_7 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%).

Similar findings were reported by Tariq *et al*. (2008) and Trunjaruen*et al.* (2020). However they were in 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.

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Table 2: Observations for shoot induction:

Observations	Shoot induction frequency (%)			Days required for shoot induction		
Treatments	Ratnagiri-8	Karjat Shatabdi	Karjat-3	Ratnagiri-8	Karjat Shatabdi	Karjat-3
T_0	0.00	0.00	0.00	0.00	0.00	0.00
T_1	62.00 (51.94)	54.33 (47.49)	61.33 (51.55)	22.67	25.67	24.00
T_2	64.67 (53.53)	57.00 (49.02)	64.00 (53.13)	22.00	24.67	23.33
T_3	72.67 (58.48)	61.67 (51.75)	67.67 (55.35)	20.33	23.67	22.00
T_4	75.00 (60.00)	64.67 (53.53)	70.67 (57.21)	19.33	23.00	20.67
T_5	83.33 (65.91)	73.67 (59.13)	74.33 (59.56)	16.67	20.33	19.33
T_6	79.67 (63.20)	70.33 (57.00)	78.33 (62.26)	18.00	21.67	18.00
T_7	64.33 (53.33)	54.67 (47.68)	58.67 (49.99)	21.67	27.00	25.33
T_8	74.67 (59.78)	63.00 (52.54)	66.67 (54.74)	18.33	23.33	22.00
T_9	72.00 (58.05)	61.33 (51.55)	68.33 (55.76)	19.00	24.00	21.33
T_{10}	69.67 (56.58)	58.67 (49.99)	63.00 (52.54)	20.00	25.00	23.33
T_{11}	67.33 (55.14)	56.00 (48.45)	61.00 (51.35)	21.67	25.67	24.00
T_{12}	78.67 (62.49)	69.00 (56.17)	69.67 (56.58)	18.00	22.00	21.67
T_{13}	76.00 (60.67)	66.33 (54.53)	72.33 (58.27)	19.33	23.00	20.33
T_{14}	74.33 (59.56)	62.67 (52.34)	73.00 (58.69)	20.67	24.33	19.67
T_{15}	71.67 (57.84)	59.00 (50.18)	70.67 (57.21)	21.33	25.00	21.00
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**

(Figures in parenthesis are arcsine transformed values)

3.2.2Number of days required for shoot and root induction

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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 2and 3).The minimum number of days required for shoot and root induction was observed for T_6in 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 2and 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	**Root induction frequency (%)**			**Days required for root induction**		
Treatments	Ratnagiri-8	Karjat Shatabdi	Karjat-3	Ratnagiri-8	Karjat Shatabdi	Karjat-3
T_0	0.00	0.00	0.00	0.00	0.00	0.00
T_1	55.00 (47.87)	45.00 (42.13)	50.33 (45.19)	17.00	20.33	19.00
T_2	57.67 (49.41)	47.67 (43.66)	53.33 (46.91)	15.67	19.33	18.00
T_3	64.00 (53.13)	52.33 (46.34)	57.00 (49.02)	14.33	17.00	16.67
T_4	68.33 (55.76)	56.00 (48.45)	61.67 (51.75)	13.00	15.67	15.33
T_5	76.33 (60.89)	64.67 (53.53)	65.33 (53.93)	10.33	14.00	14.00
T_6	72.67 (58.48)	60.33 (50.96)	69.00 (56.17)	11.67	15.33	12.67
T_7	57.33 (49.22)	43.67 (41.36)	56.33 (48.64)	16.67	19.67	18.00

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T_8	67.00 (54.94)	54.33 (47.49)	61.67 (51.75)	13.33	16.67	16.00
T_9	65.67 (54.13)	51.67 (45.96)	63.33 (52.73)	14.00	17.33	15.00
T_{10}	63.33 (52.73)	48.33 (44.04)	60.00 (50.77)	14.67	18.67	16.67
T_{11}	61.67 (51.75)	45.00 (42.13)	58.33 (49.80)	15.33	19.00	17.33
T_{12}	71.33 (57.63)	59.67 (50.57)	59.67 (50.57)	12.00	15.67	16.67
T_{13}	69.00 (56.17)	57.00 (49.02)	61.33 (51.55)	13.00	17.00	16.00
T_{14}	66.67 (54.74)	53.33 (46.91)	63.67 (52.93)	14.00	18.33	15.33
T_{15}	63.33 (52.73)	51.00 (45.57)	61.33 (51.55)	15.33	19.00	16.33
CV	2.15	2.64	2.43	3.47	2.78	4.25
SE(m)	0.76	0.75	0.79	0.26	0.26	0.37
CD at 1%	2.94	2.92	3.06	1.02	1.02	1.44
F test	**SIG**	**SIG**	**SIG**	**SIG**	**SIG**	**SIG**

(Figures in parenthesis are arcsine transformed values)

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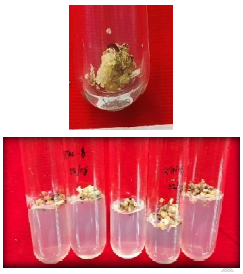
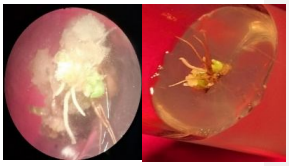
Plate 1 : Regeneration protocol in three varieties

	Ratnagiri-8	Karjat Shatabadi	Karjat-3
Callus induction on medium			
	T_2 : MS + 2,4-D (2.0 mg/l) + NAA (0.5 mg/l)		
Callus proliferation on medium			
	T_2: MS + 2,4-D (2.0 mg/l) + NAA (0.5 mg/l)	T_2: MS + 2,4-D (2.0 mg/l) + NAA (0.5 mg/l)	T_3 : MS + 2,4-D (2.0 mg/l) + NAA (1.0 mg/l)
Transfer of prolifered callus on regeneration medium			
Shoot and root induction on regeneration medium			
	T_5 : MS + B/	T_6 : MS + BAP (3.0 mg/l)	
Plantlet regeneration			

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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. The callus was prolifered on the respective medium for each varieties.The days required for callus establishment were inversely proportional to callus induction frequency. The callus weight was directly proportional to callus induction frequency.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.This study had set the protocol for *invitro*regeneration in given rice varieties which can be further utilized for genetic transformation studies.

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