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Optimizing the Protocol for *In vitro* Regeneration through Callus in Rice Varieties, *viz;* Ratnagiri-8, Karjat Shatabdi and Karjat-3

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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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 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 and 0.290 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 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 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.

Keywords: 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 (Aananthi & Anandakumar, 2020). 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 & Mansfield, 2004). *In vitro* plant regeneration in rice has been obtained from almost all types of explants (Hoque et al., 2013). 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 it's 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 *in vitro* 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 & 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 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 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 prolifered 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 & 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 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 was followed 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 (Fig. 1). 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 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.

Table 1. Observations recorded for callusing ability

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}
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.)

The results proved that callus induction depends on plant growth regulators. 2,4-D is the most preferred auxin for callus establishment. Khan (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 Karthikeyan 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 et al. (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), Karthikeyan et al. (2009) and Poeaim et al. (2016).

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 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).

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 & Yoshida (2006), Karthikeyan et al. (2009), Tiwari et al. (2012), Poeaim et al. (2016) and Suraiya et al. (2018).

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 %, respectively and 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 of 78.33% and 69.00%, respectively followed by T_5 (74.33% and 65.33%) (Tables 2 and 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%) (Tables 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. Poeaim 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 phytagel. 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 Ilahi et al. (2005), Islam et al. (2005), Biswas & Mandal (2007), Karthikeyan et al. (2009), Libin et al. (2012), Suraiya & Wagiran (2018) and Trunjaruen et al. (2020).

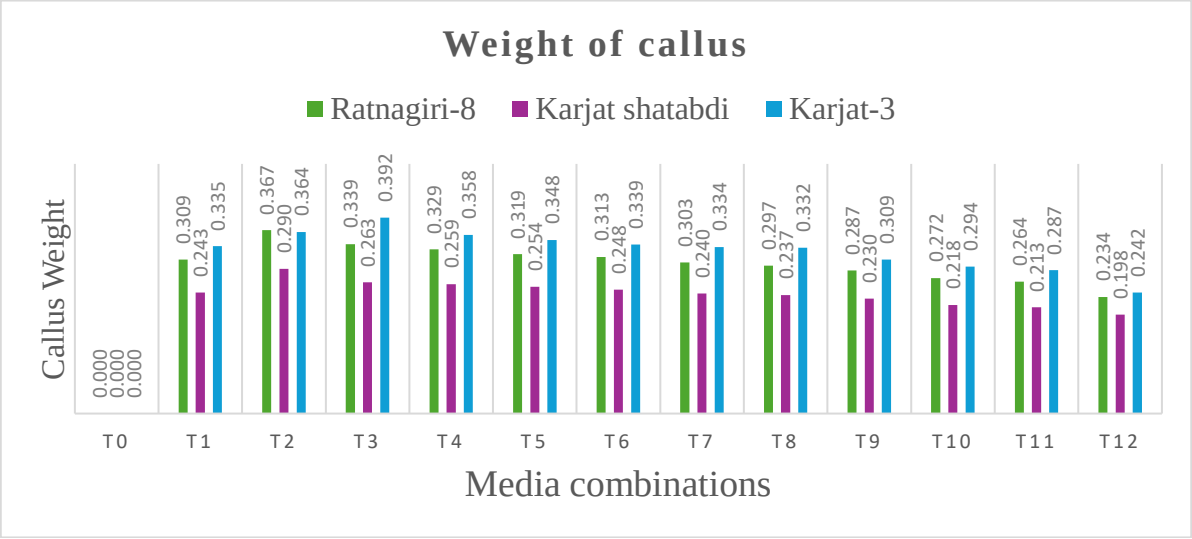

Fig. 1. Weight of callus on different media combinations

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^{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 ≤ 0.05 Means within columns with the same superscript are not significantly different at P ≤ 0.05 by using DMRT.)

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^{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 ≤ 0.05 Means within columns with the same superscript are not significantly different at P ≤ 0.05 by using DMRT.)

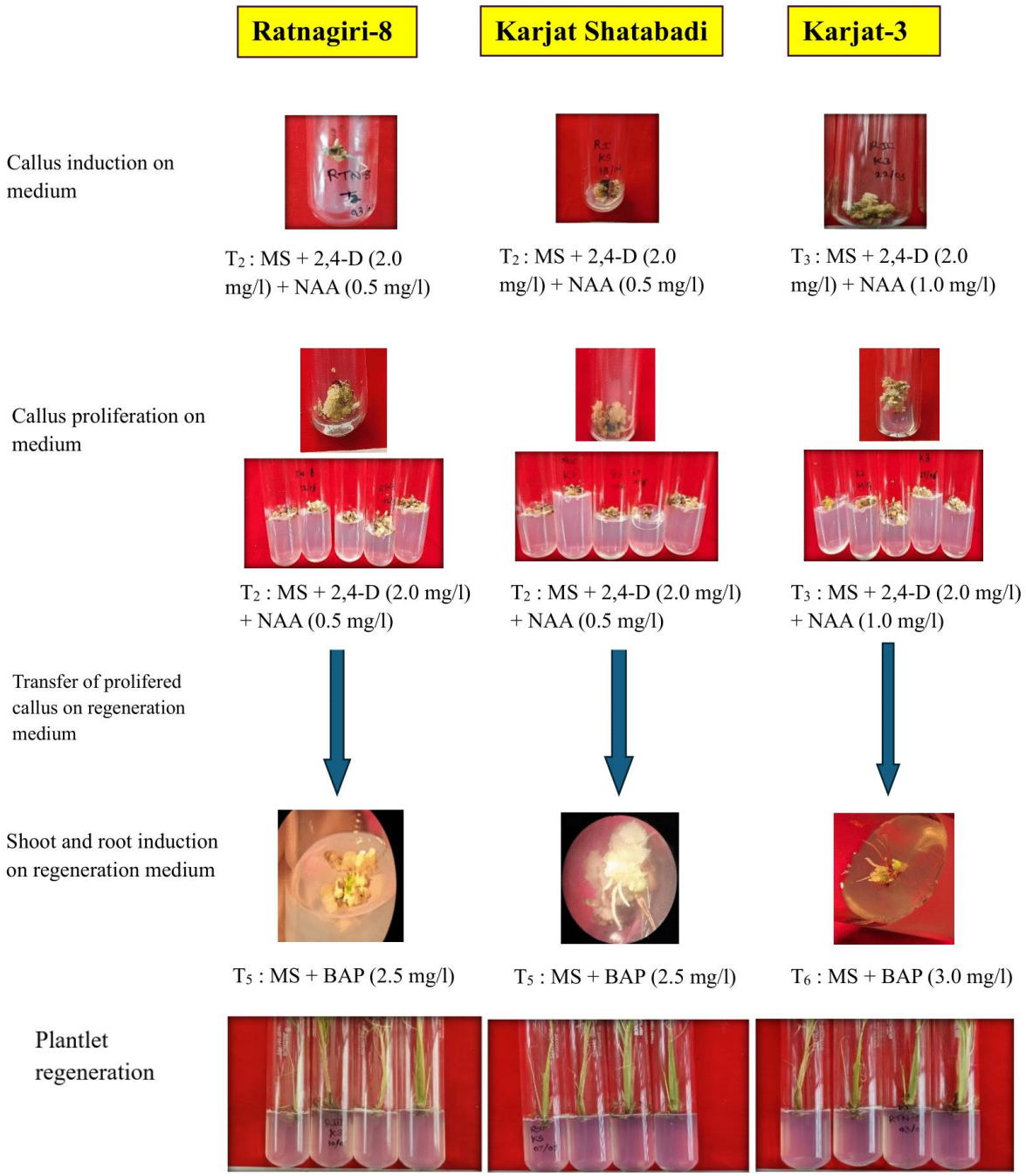

Plate 1. Regeneration protocol in three varieties

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 before transferring to regeneration medium on half strength medium used for callus induction can result in better and early regeneration.

However, the results are in little contrast to the findings of Biswas & Mandal (2007), Suraiya &

Wagiran (2018) and Rueb et al. (1994) 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.

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 (Tables 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 (Tables 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), Karthikeyan 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.

4. 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 before transferring to regeneration medium on half strength medium used for callus induction can result in better and early regeneration. This study had set the protocol for *in vitro* regeneration in given rice varieties which can be further utilized for genetic transformation studies.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declares that, NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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