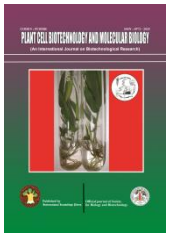

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Investigation of Callus Induction Medium and Callusing Ability in Three Rice Varieties, *viz*, Ratnagiri-8, Karjat Shatabdi and Karjat-3

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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 present investigation 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 with the aim to set up the regeneration protocol in the three rice varieties, *viz*, Ratnagiri-8, Karjat Shatabdi, Karjat-3 with an objective to investigate suitable callus induction medium which can be used to develop an efficient *in vitro* regeneration technique through callus for the selected varieties. Mature seed embryo was used as explant for callus initiation.

The callusing ability of the varieties was tested on 12 media combinations with different concentrations of 2,4-D and NAA, their combinations and one control treatment. The highest callus induction was recorded for medium combination T_2: MS + 2,4-D (2.0 mg/l) + NAA (0.5 mg/l) in Ratnagiri-8 and Karjat Shatabdi with a frequency of 66.67 % and 53.33 % 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 frequency of 68.33 %. Similarly, the highest callus weight was recorded for T_2 in Ratnagiri-8 (0.367 g) and Karjat Shatabdi (0.290 g) for T_3 in Karjat-3 (0.392 g). Embryogenic, soft, friable callus with granular texture and yellowish white colour was obtained from all media combinations in all three varieties.

Keywords: Callus induction; regeneration; medium; rice.

ABBREVIATIONS

DSW : Distilled Sterilized Water
PGR : Plant Growth Regulators
2,4-D : 2,4 dichlorophenoxyacetic Acid
NAA : Naphthalene Acetic Acid
MS : Murashige and Skoog

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 [1].

Genetic engineering is strongly dependent on genotype and availability of an efficient *in vitro* plant regeneration method. Suitable plant regeneration methods are required for successful application of plant tissue culture techniques for crop improvement. The ability of plant regeneration from seed-derived callus of rice is influenced not only by physiological factors but also by genotypes. Among these factors, the genotype of plants is a strong determinant of the regeneration ability from seed callus and this character is under genetic control [2].

Several genetic studies have been performed to improve the regeneration ability from seed derived calli in rice. However, the use of tissue culture in rice improvement is limited as the regeneration can be obtained only in limited number of genotypes [3]. Therefore, identification and screening of useful cultivars for embryogenic callus formation and subsequent *in vitro* plant regeneration key steps in rice genetic improvement programme through application of biotechnology [4]. Callus cultures are extremely important in plant biotechnology. Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of shoots, roots or somatic embryos from which whole plants can subsequently be produced. Callus cultures can also be used to initiate cell suspensions, which are used in a variety of ways in plant transformation studies. Moreover, embryogenic calli obtained from mature seed embryos are efficient in *indica* rice transformation [5].

Factors such as plant genotype, the culture methods, selection of explant, the media and the culture conditions influence culture efficiency of which genotype and nutrient media are two of the

most important factors which affect callus induction and subsequent plant regeneration.

Keeping in view, the above important aspects, the investigation was carried out with the objectives to investigate the callusing ability and suitable callus induction medium in selected rice varieties so as to develop *in vitro* regeneration techniques through callus.

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 [6] 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 (20 seeds/treatment) 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.

List 1. Following medium combinations were used for callus establishment through Embryo culture

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:

Callus induction frequency (%) =

$$\frac{No\ of\ seeds\ with\ callus}{No\ of\ seeds\ inoculated} \times 100$$

2.5 Subculturing of Callus for Proliferation

The inoculated explants (20 seeds/treatment) were observed for callus induction and calli were subcultured on media showing highest callus induction frequency for proliferation.

2.6 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 [7].

3. RESULTS AND DISCUSSION

3.1 Medium Combination Showing Highest Callus Induction and Callusing Ability (Callus Induction Frequency) of Varieties on Different Media Combinations

The results of 12 medium combinations with different concentrations of 2,4-D and NAA and their combinations and one control treatment are presented in Table 1, with respect to callus induction frequency and number of days required for callus induction and weight of callus. 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, followed by T_3 with a callus induction frequency of 61.67% and 43.67% respectively. However, in Karjat-3 the highest callus induction was obtained in T_3 with a callus induction frequency of 68.33% followed by T_2 with a frequency of 62.67 %. The lowest callus induction was recorded for T_{12} in all the three varieties with a frequency of 14.67%, 11.67% and 21.67% respectively (Table 1 and Fig. 1).

Irrespective of media combinations and with respect to varieties the highest callus induction was observed in Karjat- 3 (68.33 %) followed by Ratnagiri-8 (66.67 %) and Karjat Shatabdi (53.33%).

However, for early callusing T_2 was at par with T_3 in Ratnagiri-8 and Karjat Shatabdi while in Karjat-3, T_3 was at par with T_2 for minimum days for callus induction.

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. [8] reported optimum concentrations of 2,4-D for callus induction. Here, better callus induction was observed for 2,4-D concentrations @ 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. [9], Libin et al. [10] and Rashid et al. [11].

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 above 1.0 mg/l. This results also were in accordance with Islam et al. [12] and Roly et al. [13], Din et al. [14].

3.2 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 requiring 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 and Fig. 2).

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. [15], Carsono et al. [16], Kartikeyan et al. [9], Tiwari et al. [17], Poeaim et al. [18] and Ho et al. [19].

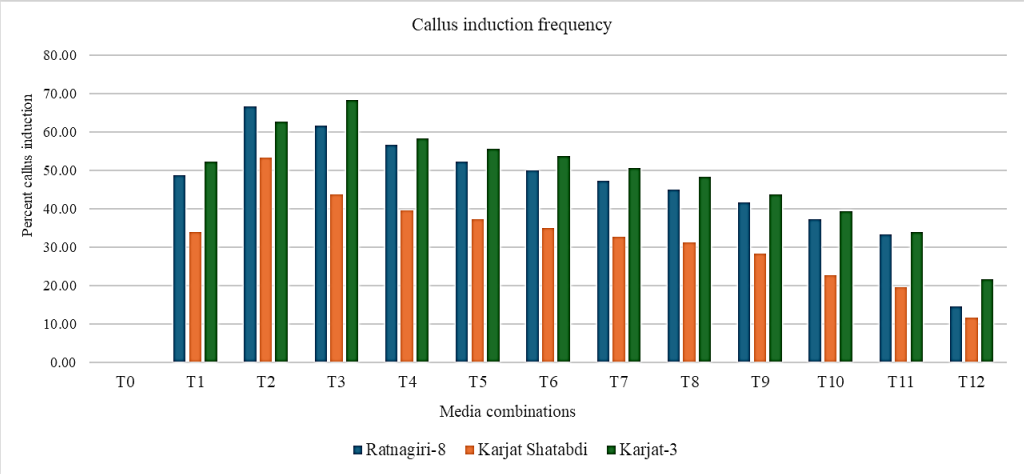

Fig. 1. Callus induction frequency of varieties on different media combination

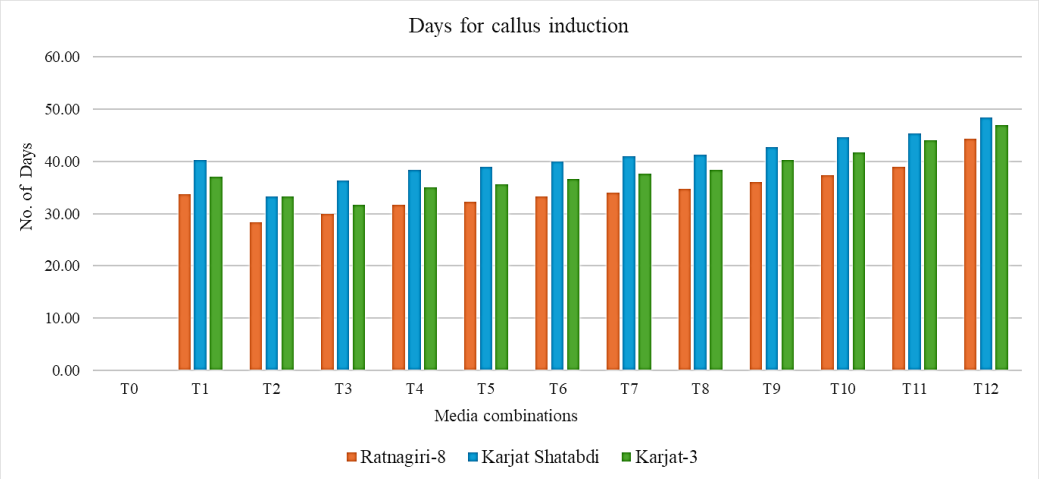

Fig. 2. Days required for callus induction on different media combinations

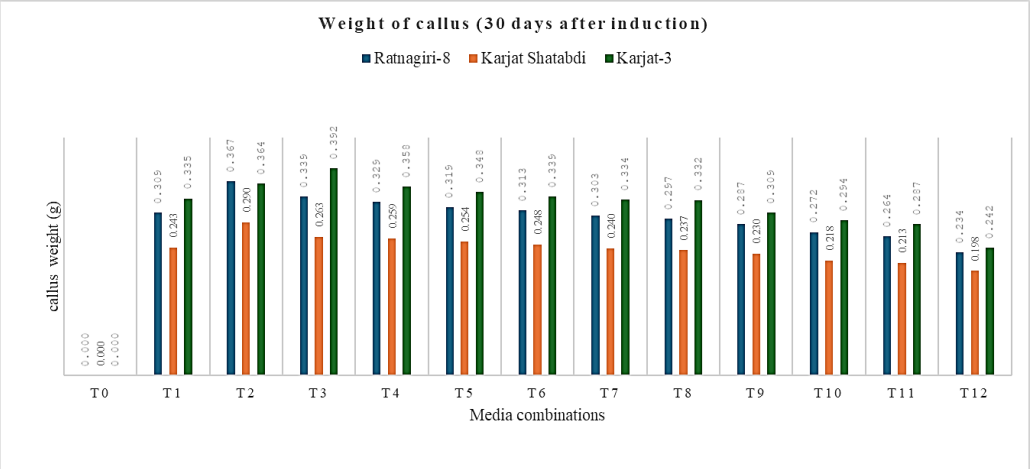

Fig. 3. Weight of callus on different media combinations

Table 1. Comparison of callus induction between rice varieties

Observations	Callus induction frequency (%)			Days required for callus induction			Weight of callus (g)(30 days after induction)		
Treatments	**Ratnagiri-8**	**Karjat Shatabdi**	**Karjat-3**	**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}	0.000^{a}	0.000^{a}	0.000^{a}
T_1	48.67^{g}	34.00^{ef}	52.33^{gh}	33.67^{de}	40.33^{de}	37.00^{de}	0.309^{efg}	0.243^{efg}	0.335^{de}
	(44.24)	(35.67)	(46.34)						
T_2	66.67^{k}	53.33^{j}	62.67^{j}	28.33^{b}	33.33^{b}	33.33^{bc}	0.367^{i}	0.290^{h}	0.364^{e}
	(54.74)	(46.91)	(52.34)						
T_3	61.67^{j}	43.67^{i}	68.33^{k}	30.00^{bc}	36.33^{bc}	31.67^{b}	0.339^{h}	0.263^{g}	0.392^{f}
	(51.75)	(41.36)	(55.76)						
T_4	56.67^{i}	39.67^{h}	58.33^{i}	31.67^{cd}	38.33^{cd}	35.00^{cd}	0.329^{gh}	0.259^{fg}	0.358^{e}
	(48.83)	(39.04)	(49.80)						
T_5	52.33^{h}	37.33^{gh}	55.67^{hi}	32.33^{cd}	39.00^{cd}	35.67^{cde}	0.319^{fgh}	0.254^{efg}	0.348^{e}
	(46.34)	(37.66)	(48.25)						
T_6	50.00^{gh}	35.00^{fg}	53.67^{gh}	33.33^{de}	40.00^{de}	36.67^{de}	0.313^{efgh}	0.248^{efg}	0.339^{de}
	(45.00)	(36.27)	(47.10)						
T_7	47.33^{fg}	32.67^{ef}	50.67^{fg}	34.00^{de}	41.00^{de}	37.67^{def}	0.303^{efg}	0.240^{def}	0.334^{de}
	(43.47)	(34.86)	(45.38)						
T_8	45.00^{ef}	31.33^{de}	48.33^{ef}	34.67^{def}	41.33^{de}	38.33^{ef}	0.297^{def}	0.237^{cde}	0.332^{de}
	(42.13)	(34.04)	(44.04)						
T_9	41.67^{e}	28.33^{d}	43.67^{e}	36.00^{efg}	42.67^{ef}	40.33^{fg}	0.287^{cde}	0.230^{cde}	0.309^{cd}
	(40.20)	(32.16)	(41.36)						
T_{10}	37.33^{d}	22.67^{c}	39.33^{d}	37.33^{fg}	44.67^{f}	41.67^{gh}	0.272^{cd}	0.218^{bcd}	0.294^{c}
	(37.66)	(28.43)	(38.84)						
T_{11}	33.33^{c}	19.67^{c}	34.00^{c}	39.00^{g}	45.33^{fg}	44.00^{h}	0.264^{c}	0.213^{bc}	0.287^{c}
	(35.26)	(26.33)	(35.67)						
T_{12}	14.67^{b}	11.67^{b}	21.67^{b}	44.33^{h}	48.33^{g}	47.00^{i}	0.234^{b}	0.198^{b}	0.242^{b}
	(22.52)	(19.97)	(27.74)						
CV	3.52	4.66	3.66	4.14	3.60	3.58	4.193	4.525	4.099
SE(m)±	0.87	0.81	0.96	0.76	0.78	0.73	0.007	0.006	0.007
CD at 1%	3.41	3.17	3.76	3.00	3.08	2.86	0.027	0.023	0.028
F test	**SIG**	**SIG**	**SIG**	**SIG**	**SIG**	**SIG**	**SIG**	**SIG**	**SIG**

** CV – Co-efficient of variation *SE± - Standard Error * CD – Critical Difference*

(Figures in parenthesis are arcsine transformed values)

(Values are the mean of three replicates. The data was analyzed by one way ANOVA and is Significant at $p \leq 0.05$

Means within the coloum followed by different superscripts are significantly different according to ANOVA and Duncan's Multiple Range Test ($P < 0.05$))

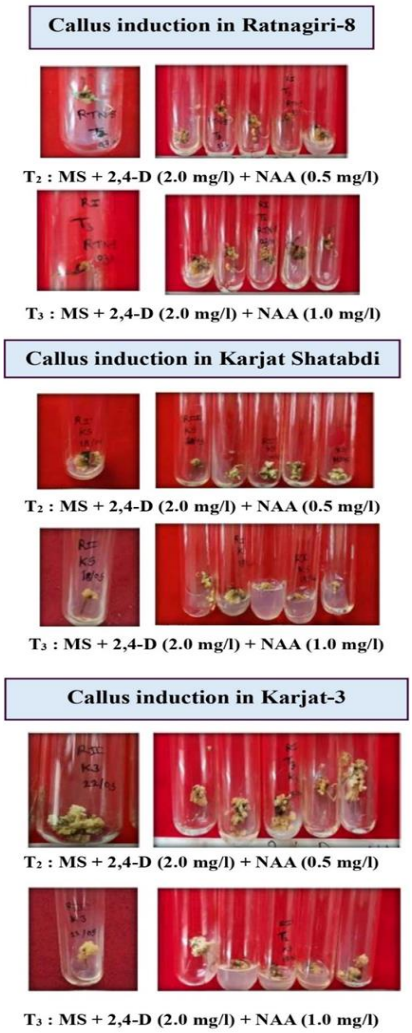

Plate 1. Callus induction

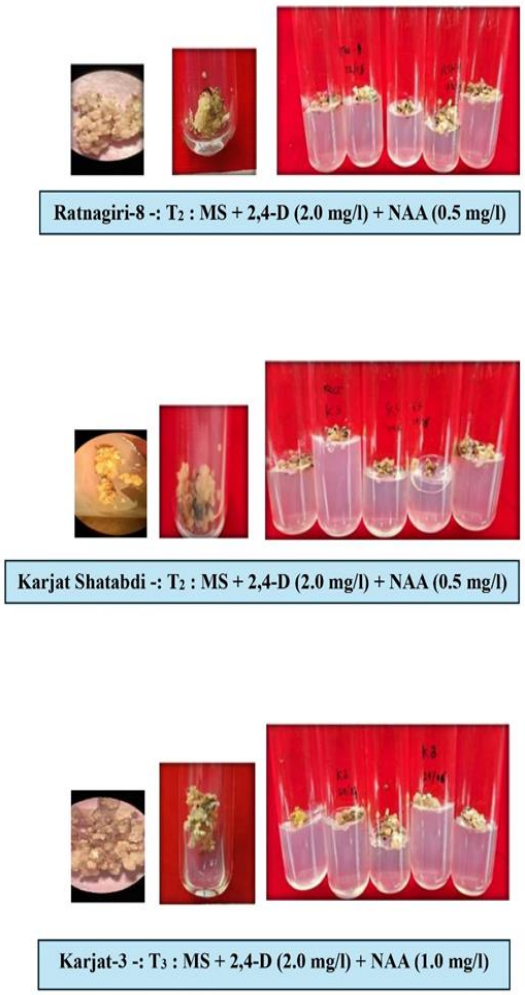

Plate 2. Callus proliferation on medium in three rice varieties

3.3 Weight of Callus (g) (30 Days After Induction)

It was observed that the weight of callus was directly proportional to callus induction frequency. Callus weight decreased for MS medium supplemented with 2,4-D above the concentration of 2.0 mg/l in combination with NAA above the concentration of 0.5- 1.0 mg/l.The highest callus weight in Ratnagiri-8 and Karjat Shatabdi was observed in T_2 of 0.367 g and 0.290 g respectively followed by T_3 with a callus weight of 0.339 g and 0.263 g respectively. In Karjat-3, the highest callus weight was recorded in T_3 (0.392 g) followed by T_2 (0.364 g). The minimum callus weight was recorded in T_{12} for all three varieties with a callus weight of 0.234 g, 0.198 g and 0.242 g respectively. (Table 1 and Fig. 3).

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

It was observed that the callus weight gradually decreased after increasing the concentration of 2,4-D above 2.0 mg/l and at higher NAA concentrations. These results were in accordance with Thadavong et al. [15], Summart et al. [20], Kartikeyan et al. [9], Hoque et al. [21] and Poeaim et al. [18].

3.4 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 to 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. [20], Ho et al. [19], Libin et al. [10], Rashid et al. [11], Wani et al. [22].

4. CONCLUSION

From the experiment, it is concluded that callus induction in rice depends on optimum concentrations of PGR and genotype. In the varieties, Karjat-3 expressed highest callus induction followed by Ratnagiri-8 and then Karjat Shatabdi. However, early callusing was observed in Ratnagiri-8 followed by Karjat-3 and Karjat Shatabdi. Callusing ability of the varieties determined the medium combination which can be used to obtain high 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. This study had set the protocol for callus establishment in selected rice varieties which can be further utilized for *in vitro* regeneration and genetic transformation studies.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare 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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