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STUDY ON CALLOGENESIS AND ORGANOGENESIS IN LOCAL CULTIVARS OF RICE (*ORYZA SATIVA* L.)

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Abstract

Callogenesis and organogenesis studies were carried out for three important cultivars of rice i.e., Basmati-370, DR-82 and IR-6. 2,4-D @ 2.0 mg l^{-1} on MS medium proved to be the best for callus formation in all cultivars. Maximum callus was produced by DR-82 followed by Basmati-370 and IRRI-6, respectively. In order to get maximum regeneration frequency, different factors (age of calli and addition of GA3 in regeneration medium) were optimized. Different concentrations and combinations of hormones i.e., NAA 1.0 mg l^{-1}, BAP 2.0-5.0 mg l^{-1} and GA3 0.5 mg l^{-1} or kinetin 0.5-2.0 mg l^{-1} were used on MS medium. Calli were shifted to different regeneration media to evaluate the plant regeneration frequency in tested rice cultivars as an interaction with all the cultivars. Basmati-370 showed regeneration efficiency (40.0%) on RM3 (NAA 1.0 mg l^{-1} + BAP 5.0 mg l^{-1} + GA3 0.5 mg l^{-1}) while 80.0% and 65.0% was observed on the same medium in DR-82 and IRRI-6, respectively. Maximum plant regeneration as a whole on different regeneration media was noted in IRRI-6, followed by DR-82 and Basmati-370, respectively. It was also observed that 25 days old calli had more plantlet formation as compared to 17, 21 and 29 days old calli. Among the rice cultivars response of different calli ages on regeneration efficiency was of the order of DR-82>IRRI-6>Basmati-370. Maximum regeneration frequency and multiple shoot formation were observed on medium containing GA3 in addition to BAP and NAA from 25 days old calli.

Introduction

In Pakistan three types of rice is cultivated i.e. Basmati type (aromatic), medium long grain rice (IRRI type) and cold tolerant which are bold and short grained. Cultivation of these cultivars is area specific, depending upon the environmental conditions of the area (Salim *et al*., 2003). Abundant supplies from the year, 2006 excellent crop should enable Pakistan to maintain exports at 3.5 million tones. In the last recent years, the focus has shifted to use rice as a model monocotyledon system because of its economic and nutritional importance around the world (Khush, 1997), similar to the use of *Arabidopsis* as a model for dicotyledons.

The potential for callus induction and regeneration in rice tissue culture depends on the type and physiological status of the explants, the composition and concentration of the basal salt, and the organic components and plant growth regulators in the culture medium. Genotype is also an important factor in addition to plant growth regulators (Abe & Futsuhara, 1986). Asian rice group *japonica* was more responsive to callus induction and regeneration as compared to the *indica* group of rice (Abe & Fursuhara, 1984, 1986; Reddy *et al*., 1985; Kavi Kishor & Reddy, 1986; Mikami & Kinoshita, 1988).

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The influence of nutrient composition and plant growth regulators of the culture media are also very important factors for rice tissue culture. A number of media formulations have been established and published. A widely used basal medium for rice somatic culture is MS (Murashige & Skoog, 1962).

Plant growth hormones play a key role in rice tissue culture, in which a high auxin or cytokinin ratio usually is used for initiation of the embryogenic callus, while a low ratio is used for the regeneration of plantlets. It is suggested that plant growth hormones function by mediating the signal transduction cascade that leads to reprogramming of the expression of embryogenic genes (Dudits *et al*., 1995). Auxins, especially 2,4-D, are essential for induction and proliferation of the callus, but they also prevent slow regeneration, which could lead to the loss of embryogenic competence. Cytokinin may also increase the growth rate of pre-embryogenic masses (Kommamine *et al*., 1992).

Synthetic auxin 2,4-D is important to initiate and sustain embryogenic callus growth in rice and has been used as the only growth regulator in callus induction medium (Seraj *et al*., 1997; Khanna *et al*., 1998; Lee *et al*., 2002; Ozawa *et al*., 2003; Lin & Zhang 2005). It is also reported that combination of 2,4-D with kinetin was more effective in producing an embryogenic callus while 2,4-D alone only produced a non-embryogenic calli (Wu *et al*., 2002; Fan *et al*., 2002; Wang *et al*., 2004). There are also few reports which suggested that the addition of NAA and/or IAA could enhance the quality of the initiated callus (Reiffers & Freire, 1990; Tian, 1994; Trejo- Tapia *et al*., 2002).

It is generally held that genotype remains the major limiting factor restricting callus induction and regeneration. A number of genotype dependent tissue culture media for rice have been optimized and published in literature (Lin & Zhang, 2005).

The objective of the present study was to observe the effect of GA3 on callogenesis and organogenesis in three commercial grown rice cultivars of Pakistan. It was also aimed for establishing the most appropriate protocol for inducing embryogenic calli and regenerating them that would be durable and further applicable for creating resistance to biotic and abiotic diseases *via* genetic engineering.

Materials and Methods

Plant material and sterilization: Three rice (*Oryza sativa* L.) cultivars among which one Basmati rice i.e. Basmati-370 and two coarse cultivars i.e. DR-82 and IRRI-6 were used in the present study. Mature seeds after dehusking were washed by sterile distilled water. Surface sterilization was done with 70% v/v ethanol for 30 seconds then rinsed with sterile distilled water and sterilized with 50-70% v/v Clorox™ with constant stirring for 20 minutes. Explants were washed three times with autoclaved distilled water at a regular interval of 5 minutes after surface sterilization.

Inoculation and culture conditions: Laminar airflow cabinet was used for inoculation of explants on media. Flame sterilization of forceps was done during the work by dipping in 70% rectified spirit. Every experiment was repeated three times. Incubation of cultures was done in culture room at 25 ± 2˚C, 16-hr photoperiod at 48 μmol m^{-2} s^{-1}.

Plant tissue culture medium: MS medium (Murashige & Skoog, 1962) with 1.5, 2.0 and 3.0 mg l^{-1} 2,4-D, 3% sugar and 6 g l^{-1} agar was used for embryogenic callus induction. MS medium with different concentrations and combinations of naphthalene acetic acid (NAA), benzyl amino purine (BAP), Kinetin (Kin) and gibberlic acid (GA3), 3% sucrose, 3% sorbitol, 2 g l^{-1} casine hydrolysate and 4 g l^{-1} gelrite (solidifying agent) was used for plant regeneration from calli.

Embryogenic callus production: Seeds were incubated for three weeks on callus inducing media and then the proliferated calli derived from the scutellum were separated and shifted on MS media supplemented with 1.5, 2.0 and 3.0 mg l^{-1} 2,4-D for maintenance and embryogenic calli production. List and composition of media used for callus induction is given in Table 1.

The frequency of callus induction was calculated by the formula:

$$\text{Callus induction frequency (\%)} = \frac{\text{Number of seeds producing callus}}{\text{Number of seeds inoculated}} \times 100$$

Regeneration: Embryogenic calli were transferred on regeneration media with different combinations and concentrations of hormones (Table 2). These cultures were placed in growth room and observed during 8-10 weeks. Calli of different ages were used for evaluation of the best calli age for organogenesis studies.

The regeneration frequency was observed for callus browning, green tissue formation and shoot/plantlet production. Different media used for evaluation of regeneration efficiency are listed in Table 2.

The frequency of plant regeneration was calculated by the formula:

$$\text{Plant regeneration frequency (\%)} = \frac{\text{Number of calli regenerated plantlets}}{\text{Number of calli plated for regeneration}} \times 100$$

Statistical analysis: Callus induction, callus browning, production of green tissue or spots and plantlet formation among the three rice cultivars were statistically analyzed using Analysis of variance technique with a computer program MSTAT-C for all the treatments along with three replicates. Treatment means were compared using Factor CRD (b) and Anova-1.

Results and Discussion

Plant tissue culture techniques have become a powerful tool for studying and solving basic and applied problems in plant biotechnology. Highly efficient tissue culture system is a prerequisite for a elevated efficiency of transformation. For this purpose a number of factors i.e., callus induction, regeneration and combination of hormones are considered critical. Establishment of a suitable system for plant regeneration of rice calli derived from mature seed is an essential requirement for further genetic studies using callus as the target tissue for genetic transformation studies. Therefore, in order to enhance an efficient regeneration frequency in local rice cultivars (Basmati-370, DR-82 and IRRI-6), age of callus and combinations of hormones with GA3 were studied which was not previously reported.

Factors affecting tissue culture studies

Hormones inducing callus formation: Genotype response was observed on callus induction and callus proliferation among all the three rice cultivars. Seeds were shifted on callus induction media supplemented with 1.5, 2.0 and 3.0 mg l^{-1} 2,4-D. Most compact

calli were induced on MS media supplemented with 2.0 mg l^{-1} (Table 4). The significant differences among the three cultivars for callogenesis under the same nutritional conditions indicated that callus induction frequency is genotype specific (Table 3). Pictorial description for callus induction on 0.0 and 2.0 mg l^{-1} 2,4-D is given in Figure 5. Our findings are in agreement with Hoque *et al*., (2007) and Agrawal *et al*., (2006). They reported that effect of rice genotype on callus induction is significant in different rice cultivars.

Table 1. List and composition of media used for callus induction.

Media	Composition
CI-1	MS salts and vitamins + 3% sugar + 4 g l^{-1} gelrite or 6 g l^{-1} agar + 1.5 mg l^{-1} 2,4-D, pH 5.78-5.80.
CI-2	MS salts and vitamins + 3% sugar + 4 g l^{-1} gelrite or 6 g l^{-1} agar + 2.0 mg l^{-1} 2,4-D, pH 5.78-5.80.
CI-3	MS salts and vitamins + 3% sugar + 4 g l^{-1} gelrite or 6 g l^{-1} agar + 3.0 mg l^{-1} 2,4-D, pH 5.78-5.80.

Table 2. List and composition of media used for regeneration.

Media	Composition
RM1	MS salts and vitamins + 3% sugar + 3% sorbitol + 2 g l^{-1} casine hydrolysate (RM0) + NAA 1.0 mg l^{-1} + BAP 1.0 mg l^{-1} + 4 g l^{-1} gelrite, pH 5.78-5.80
RM2	RM0 + NAA 1.0 mg l^{-1} + BAP 2.5 mg l^{-1}
RM3	RM0 + NAA 1.0 mg l^{-1} + BAP 5.0 mg l^{-1}+ GA3 0.5 mg l^{-1}
RM4	RM0 + NAA 1.0 mg l^{-1} + Kin 0.5 mg l^{-1} + BAP 2.0 mg l^{-1}
RM5	RM0 + NAA 1.0 mg l^{-1} + Kin 2.0 mg l^{-1}

Table 3. Effect of rice cultivars and different 2,4-D concentrations on callus induction.

Cultivars	Treatments (2,4-D conc. mg l^{-1})			Means
	1.5	2.0	3.0	
Basmati-370	75.8 c*	87.5 b	70.8 d	74.0 B
DR-82	83.9 b	92.1 a	78.7 c	84.8 A
IRRI-6	62.5 e	75.0 c	68.3 d	72.6 B
Means	78.0 B	84.9 A	68.6 C	

*Any two means not sharing a letter common in a row or column differ significantly at 5% probability level i.e. $\alpha = 0.05$

Table 4. Effect of rice cultivars and different solidifying agents on callus induction.

Cultivars	Treatments (2,4-D conc. mg l^{-1})			Means
	Basmati-370	DR-82	IRRI-6	
Agar	67.7^{NS}	77.1	57.9	67.6 C
Gelrite	82.3	93.7	78.4	84.8 A
Means	75.0 B*	85.4 A	68.2 C	

NS = Non Significant

*Any two means not sharing a letter common in a row or column differ significantly at 5% probability level i.e. $\alpha = 0.05$

Effect of solidifying agents: Use of gelrite and agar were compared for obtaining better callus induction. Results showed that gelrite proved better than agar to be used in rice callogenesis as it provides more water stress. High frequency of callus induction i.e. 84.8% was obtained at 4.0 g l^{-1} of gelrite and 67.6% at 6.0 g l^{-1} of agar (Table 4). Our findings are in confirmatory with Lee *et al*., (1999) as they reported that water stress treatment improved the frequency of somatic embryogenesis and callogenesis in rice. The comparative description of callus induction on agar and gelrite is given in Figure 5.

Combination of hormones for organogenesis: Organogenesis is the development of plants through different tissue and organs, which are used as explant. Selecting the most appropriate medium and assessing the genotype performance for *in vitro* responses are essential requirements for this purpose (Carsono & Yoshida, 2006). In order to get maximum regeneration frequency different factors which affect regeneration were optimized. Different concentrations and combinations of hormones i.e., NAA 1.0 mg l^{-1}, BAP 2.0-5.0 mg l^{-1} and GA3 0.5 mg l^{-1} or kinetin 0.5-2.0 mg l^{-1} were used in MS media with basic salts and vitamins, supplemented with 3% sucrose, 3% sorbitol, and 2.0 g l^{-1} casine hydrolysate (Table 2).

Callus browning was observed on different regeneration media (Table 2). As a whole maximum browning was noted in Basmati-370 (43.0%) followed by IRRI-6 (30.0%) and DR-82 (29.0%), respectively (Table 5). Among all the media tested, RM-3 (NAA 1.0 mg l^{-1} + BAP 5.0 mg l^{-1} + GA3 0.5 mg l^{-1}) showed the best results for plant regeneration from scutellum derived calli of all rice cultivars followed by RM-2 (NAA 1.0 mg l^{-1} + BAP 2.5 mg l^{-1} + Kin 2.5 mg l^{-1}). Agrawal *et al*., (2006) also described regeneration from calli at these hormones but at different concentrations in MS medium. Addition of BAP in regeneration medium positively effects on regeneration frequency and plant production; also confirmed by Lee *et al*., (1989). It was observed that RM-3 showed maximum plantlet formation (61.6%), while RM-5, RM-4, RM-1 and RM-2 showed 8.3%, 20.0%, 33.3% and 41.6% callus regeneration efficiency respectively (Fig. 2). Thadavong *et al*., (2002) also stated that efficient plant regeneration is influenced by hormonal composition of media. Present study results are totally in contrast with Agrawal *et al*., (2006) who reported that the effects of media on regeneration were non-significant. The reason may be the different composition of culture media and genotypes as well. To evaluate the plant regeneration frequency in rice plant genotype as an interaction with all the cultivars, calli were shifted to different regeneration media. Like the calli greening on different regeneration media (Table 6), Basmati-370 also showed better regeneration efficiency (40.0%) as plantlet formation on RM3 (NAA 1.0 mg l^{-1} + BAP 5.0 mg l^{-1} + GA3 0.5 mg l^{-1}) while 80.0% and 65.0% was observed on the same medium in DR-82 and IRRI-6, respectively (Table 7). Maximum plant regeneration as a whole on different regeneration media was noted in IRRI-6 (40.0%), followed by DR-82 and Basmati-370, respectively (Fig. 1). Our results are unique in the sense that regeneration from rice was reported with different combinations of NAA, BAP and Kinetin whereas; in our study GA3 has significantly enhanced plant regeneration in combination with BAP and NAA.

It was concluded from the study that all the cultivars showed different regeneration potentials on the same medium. Comparative description for calli greening, calli regeneration and plantlet formation from different rice cultivars on different regeneration media is given in Fig. 6.

Table 5. Effect of different rice cultivars and regeneration media on callus browning.

Cultivars	Different Regeneration Media					Means
	RM-1	RM-2	RM-3	RM-4	RM-5	
Basmati-370	35.0 ef*	30.0 f	10.0 h	55.0 c	85.0 a	43.0 A
DR-82	10.0 h	15.0 gh	20.0 g	45.0 d	55.0 c	29.0 B
IRRI-6	20.0 g	10.0 h	10.0 h	40.0 d	70.0 b	30.0 B
Means	21.6 C	18.3 C	13.3 C	46.6 B	30.0 A	

*Any two means not sharing a letter common in a row or column differ significantly at 5% probability level i.e. $\alpha = 0.05$

Table 6. Effect of rice cultivars and different regeneration media on callus greening.

Cultivars	Different Regeneration Media					Means
	RM-1	RM-2	RM-3	RM-4	RM-5	
Basmati-370	20.0 f*	25.0 ef	40.0 c	20.0 f	10.0 g	23.0 B
DR-82	35.0 cd	40.0 c	83.0 a	30.0 de	20.0 f	41.6 A
IRRI-6	60.0 b	65.0 b	65.0 b	25.0 ef	30.0 de	49.0 A
Means	38.3 B	43.3 B	62.7 A	25.0 C	20.0 C	

*Any two means not sharing a letter common in a row or column differ significantly at 5% probability level i.e. $\alpha = 0.05$

Table 7. Interaction of rice cultivars and different regeneration media on plantlet formation.

Cultivars	Different regeneration media				
	RM-1	RM-2	RM-3	RM-4	RM-5
Basmati-370	20.0 fg*	25.0 f	40.0 de	15.0 gh	0.0 i
DR-82	35.0 e	45.0 d	80.0 a	25.0 f	10.0 h
IRRI-6	45.0 d	55.0 c	65.0 b	20.0 fg	15.0 gh

*Any two means not sharing a letter common in a row or column differ significantly at 5% probability level i.e. $\alpha = 0.05$

As a whole cultivar and treatment means are given in figure 1&2.

Table 8. Effect of rice cultivars and different ages of calli on calli greening.

Cultivars	Different ages of calli (days)				Means
	17	21	25	29	
Basmati370	25.0 d*	45.0 b	35.0 c	15.0 e	30.0 B
DR-82	10.0 e	55.0 a	45.0 b	35.0 c	36.2 AB
IRRI-6	10.0 e	35.0 c	60.0 a	45.0 b	37.5 A
Means	15.0 C	45.0 A	46.6 A	31.6 B	---

*Any two means not sharing a letter common in a row or column differ significantly at 5% probability level i.e. $\alpha = 0.05$

Table 9. Effect of rice cultivars and different ages of calli on calli browning.

Cultivars	Different ages of calli (days)				Means
	17	21	25	29	
Basmati-370	30.0 bc*	15.0 ef	20.0 de	45.0 a	27.5 A
DR-82	20.0 de	15.0 ef	10.0 f	35.0 b	20.0 B
IRRI-6	45.0 a	25.0 cd	15.0 ef	20.0 de	26.2 AB
Means	31.6 A	18.3 B	15.0 B	33.3 A	

*Any two means not sharing a letter common in a row or column differ significantly at 5% probability level i.e. $\alpha = 0.05$

Table 10. Interaction of rice cultivars and different ages of calli on plantlet formation.

Cultivars	Different ages of calli (days)			
	17	21	25	29
Basmati-370	15.0 d*	40.0 b	25.0 c	10.0 d
DR-82	15.0 d	40.0 b	55.0 a	25.0 c
IRRI-6	10.0 d	25.0 c	45.0 b	25.0 c

*Any two means not sharing a letter common in a row or column differ significantly at 5% probability level i.e. $\alpha = 0.05$

As a whole cultivar and treatment means are given in figure 3&4.

Suitable calli age for regeneration: Moreover, different ages of calli were used during study for getting high regeneration potential. Scutellum derived calli of different ages i.e., 17, 21, 25 and 29 days were placed on RM3 (NAA = 1.0 mg l^{-1} + BAP = 5.0 mg l^{-1} + GA3 = 0.5 mg l^{-1}), which already tested and proved good for regeneration purpose (Fig. 2). Highest plantlet formation i.e., 55.0% was observed in DR-82 from 25 days old calli followed by IRRI-6 (Table 10). The study resulted that three weeks old calli of rice were most suitable for regeneration. These results are in confirmatory to the results of Rashid *et al*., (1996). Before the germination started, callus greening as well as callus browning was also noted from different calli ages (Tables 8 & 9). As a whole, it was observed that 3 weeks (21-25 days) old calli showed more plantlet formation as compared to 17 and 29 days old calli (Fig. 4), but Agrawal *et al*., (2006) described that the effect of development stage of rice callus was non-significant on regeneration potential. Among the rice cultivars response of different calli ages on regeneration efficiency was of the order of DR-82>IRRI-6>Basmati-370 (Fig. 3). Other scientists Agrawal *et al*., (2006) and Valdez *et al*., (1996) also reported that large variabilities in callus growth and plant regeneration potential were revealed among the rice cultivars.

Conclusion

It is concluded from the research carried out that appropriate tissue culture studies are required to transform recalcitrant rice cultivars for various agronomic traits, which are required for their improvement. There are number of factors that affect the callogenesis and organogenesis of rice. It was observed that GA3 in addition to BAP and NAA has significantly enhanced the regeneration in local rice cultivars. The parameters studied in the present work are also helpful for developing a reproducible genetic transformation system in rice. Further more, it can be concluded that tissue culture techniques/protocols developed in the present study will remain a method of choice to improve the production and to develop disease resistant cultivars of rice and other cereal crops in Pakistan.

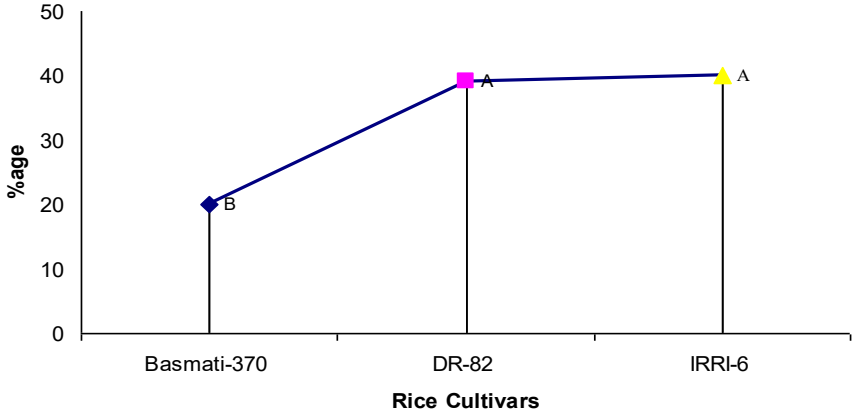


Fig. 1. Effect of different rice cultivars on plantlet regeneration from calli.

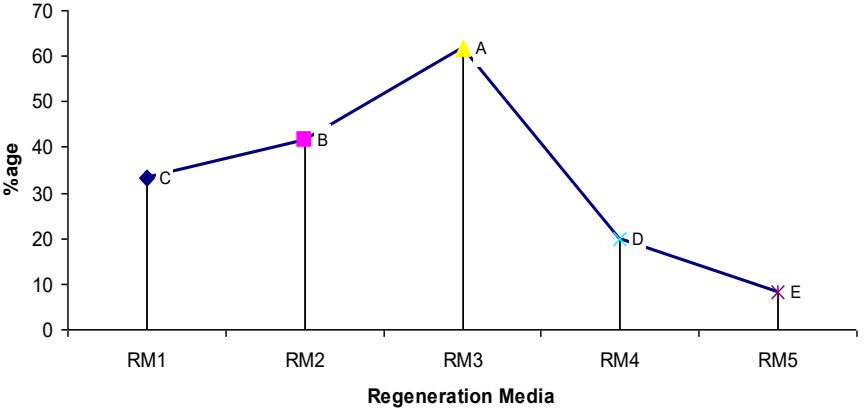


Fig. 2. Effect of different regeneration media on plantlet regeneration from calli.

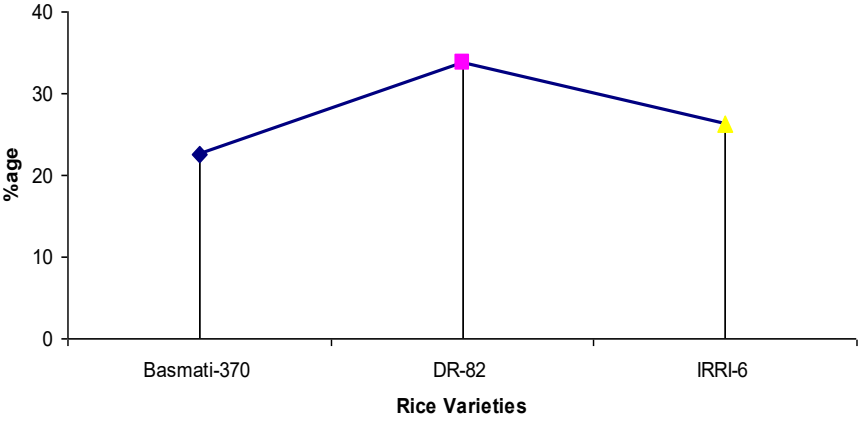


Fig. 3. Response of rice cultivars of calli of different ages on calli regeneration.

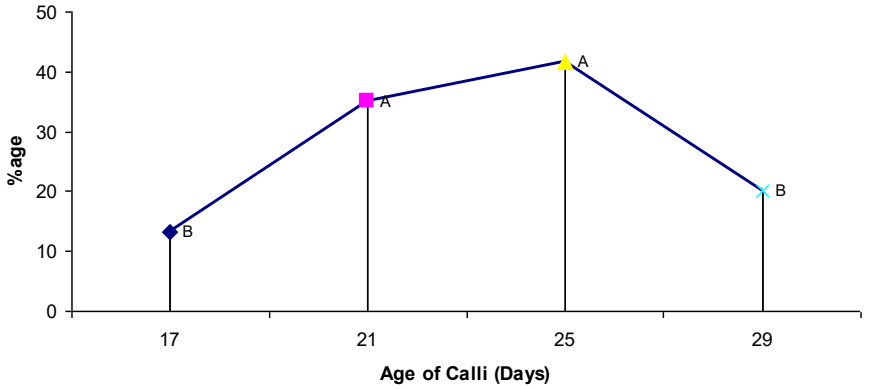


Fig. 4. Effect of different ages of rice calli on plantlet formation at regeneration media.

Comparative description of rice callogenesis and organogenesis

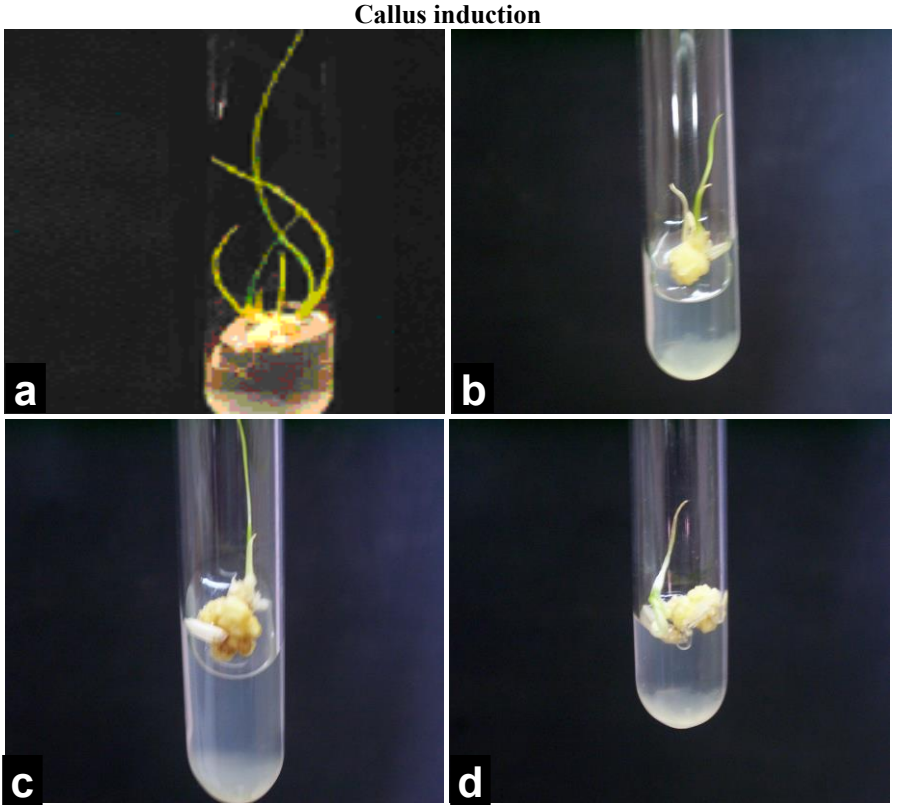
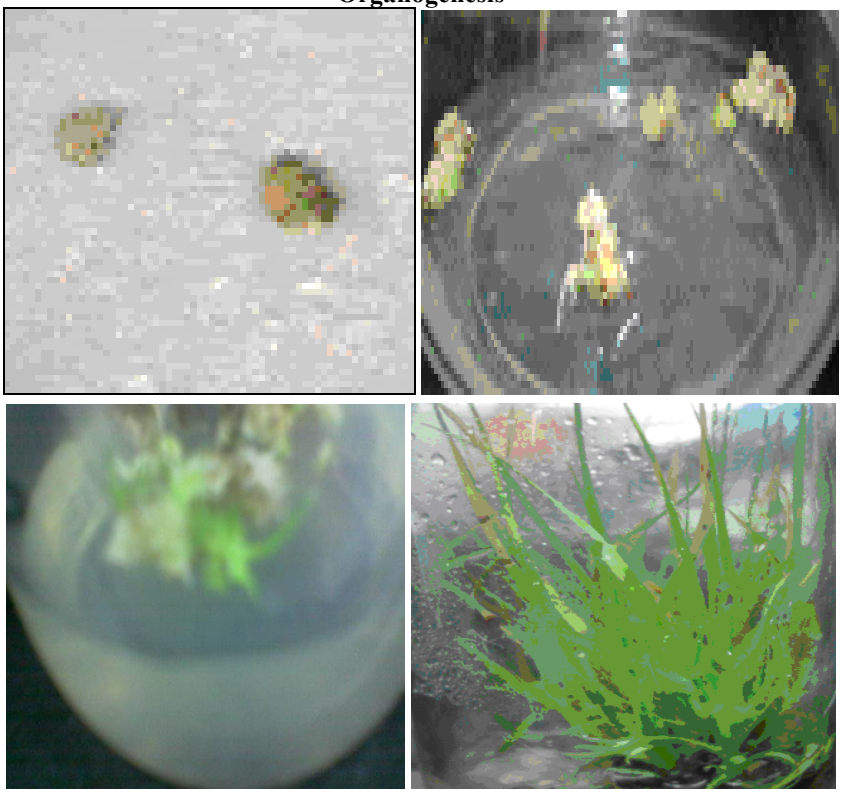
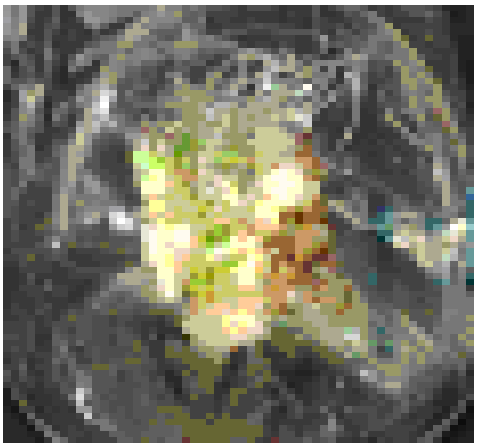


Fig. 5. Effect of 2,4-D, agar and gelrite on callus induction in rice.
(a) Plant formation on MS media containing 2,4-D at 0.0 mg l^{-1} in Basmati-370
(b) Callus induced on MS media containing 2,4-D at 2.0 mg l^{-1} in Basmati-370
(c) Callus induced on MS media containing gelrite 4 g l^{-1} in DR-82
(d) Callus induced on MS media containing agar 6 g l^{-1} in DR-82

Organogenesis



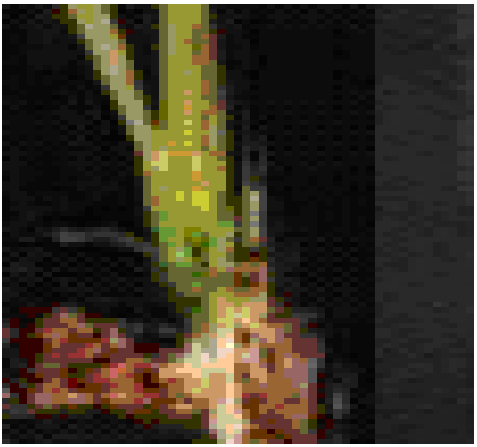
(a) Three weeks old calli showing greening on regeneration media
(b) Increase in size and emergence of root and shoot after 10-12 days
(c) Plantlet development from calli
(d) A number of plans developed from single callus



Cultivar: IRRI-6
Nutritional Status: RM-3
(NAA 1.0 mg l^{-1} + BAP 5.0 mg l^{-1} + GA3 0.5 mg l^{-1})

Cultivar: IRRI-6
Nutritional Status: RM-2
(NAA 1.0 mg l^{-1} and BAP 2.5 mg l^{-1} + Kin 2.5 mg l^{-1})

Cultivar: DR-82
Nutritional Status: RM-1
(NAA 1.0 mg l^{-1} and BAP 1.0 mg l^{-1})



Cultivar: Basmati-370
Nutritional Status: RM-2
(NAA 1.0 mg l^{-1} and BAP 2.5 mg l^{-1} + Kin 2.5 mg l^{-1})

Cultivar: IRRI-6
Nutritional Status: RM-4
(NAA 1.0 mg l^{-1} +Kin 0.5 mg l^{-1} + BAP 2.0 mg l^{-1})

Cultivar: DR-82
Nutritional Status: RM-2
(NAA 1.0 mg l^{-1} and BAP 2.5 mg l^{-1} + Kin 2.5 mg l^{-1})

Fig. 6 .Regeneration comparisons of the rice cultivars on MS basal regeneration media with given hormonal concentrations.

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