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Influence of Additional Nutrients and Gelling Agents on in Vitro Response of Selected *Indica* Rice Varieties

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Abstract

Indica rice varieties are recalcitrant to culture and hence the culture media should be supplemented with additional nutrients to provide energy and osmotic potential for best in vitro response. Combinations of plant growth regulators have profound influence on callus induction and regeneration potential of the selected genotypes. In addition, concentration and choice of gelling agents also have their effect on regeneration of *indica* rice varieties. Impact of L-Proline, and Casein Hydrolysate on tissue culture response of selected *indica* rice varieties is discussed and the best choice of gelling agent and their in vitro response is elucidated.

Keywords: Casein Hydrolysate, Gelling Agents, *Indica* Rice, L-Proline, Plant Growth Regulators, *in Vitro* Response

1. Introduction

Despite of the recalcitrant nature of the rice genotypes (Thokozani et al., 2013), efforts are being made by various research groups to improve the rice tissue culture protocols (Aldemita & Hodges, 1996; Cho et al., 2004; Afolabi et al., 2008). Variations in genotypic potential and differences in growth pattern of various explants are the main reasons for the continued efforts of the rice researchers (Saharan et al., 2004; Islam et al., 2005; Khaleda & Al-Forkan, 2006; Zuraida et al., 2010; Islam et al., 2014). Considering the fact that genotypic differences do exist in *indica* rice varieties (Yinxia & Te-chato, 2012), other endogenous factors (Haque et al., 2003) like improving the media strength by utilization of additional nutrients (Din et al., 2016), combination of various Plant Growth Regulators (PGRs) and utilization of proper gelling agents to support the medium are given key importance in this investigation to study *in vitro* response of selected *indica* rice varieties (Pawar et al., 2015).

Callus which leads to organic regeneration and differentiation are called as embryogenic callus (Ikeuchi et al., 2013; Benlioglu et al., 2015). Moreover, management of pre and post transformants is quite essential to achieve successful events in transgenic experiments (Visarada & Sarma, 2002; Abiri et al., 2015). Artificial culture medium is not autotrophic hence energy, osmotic potential and carbohydrate source should be supplied to the medium (Yaseen et al., 2013). Growth, development and morphogenesis of cells and tissues *in vitro* is maintained by proper composition of mineral nutrients (Sivanesan & Park, 2014).

Additional nutrient supplements like L-Proline and Casein Hydrolysate (CH) have reported to increase callus induction (Lin & Zhang, 2005). Proline is an α-amino acid that is essential for embryogenic callus formation, growth, and primary metabolism (Szabados & Savoure, 2010; Che Radziah et al., 2012; Pawar et al., 2015). In the present investigation attempts were made to enrich the conventional MS media with additional nutrients like L-Proline and CH and the *in vitro* response of selected *indica* rice varieties was studied.

Combination of PGRs plays a pivotal role in cell growth, division and differentiation to form shoots and roots during regeneration stage (Dahot, 2007). Cytokinins decrease the apical dominance and increase adventitious shoot formation (Ngomuo & Ndakidemi, 2013). Auxin to cytokinin ratio will help in somatic embryo development, root and shoot initiation (Azizi et al., 2015). PGRs have profound influence on somatic embryo formation. *In vitro* response of selected *indica* rice varieties under the influence of additional hormones in contrast with hormonal free medium is studied.

Gelling agents provide support for the medium and maintaining proper concentration of gelling agent is critical in supporting the growth of explants during callus induction and regeneration stages. Selected rice genotypes used in

this study showed varied tissue culture response with respect to modern gelling agents like gelrite, clarigel and phytagel compared to conventional agar. Variations in their *in vitro* response were recorded and the consequences were discussed.

Tissue culture response in rice is variety specific (Khanna & Raina, 2002; Zaidi et al., 2006; Chaitanya et al., 2013; Sai Krishna et al., 2018). The outcome of this study would help in formulating amenable media which could support the tissue culture response of most popular rice varieties, and would further help in regeneration of transformants into potential green shoots in transformation experiments.

2. Materials and Methods

2.1 Genotypes

The rice cultivars selected for the study are elite *indica* rice varieties Swarna, Gayatri, Samba Mahsuri, Pooja, Tapaswini, Sahabhagidhan, IR-64, Pusa Basmati1 and Basmati 370. Swarna, a widely grown variety in eleven states of India, is highly popular with a yield potential of 8.0 t/ha (Rao et al., 1983). It is also being widely grown in Bangladesh and Myanmar suggesting its wide adaptability (Baisakh et al., 2001). Gayatri, a high yielding cultivar released from NRRI, is widely grown in shallow and medium low land ecology in Eastern India (Das, 2012). Samba Mahsuri (BPT, 5204) is one of the India's most popular and highly prized rice varieties because of its high yield 4.5 to 5.0 t/ha and excellent cooking quality (Reddi et al., 1979). Pooja, a high yielding cultivar released from National Rice Research Institute (NRRI), is widely grown in shallow and medium low land ecology in Eastern India (Das, 2012). Shabhagidhan is a popular variety suitable for upland, rainfed direct seeded as well as transplanted conditions. It is released for cultivation in state of Jharkhand and Odisha. It bears golden husked long bold grains and has an average productivity of 3.8-4.5 t/ha (Ravindra Babu et al., 2016). Tapaswini is an elite *indica* rice variety with a yield potential of 5.0 t/ha (Panda, 2000; Dokku et al., 2013). IR-64 is one of the mega rice varieties released by International Rice Research Institute (IRRI) in 1985 (Mackill & Khush, 2018) and it is a prominent High Yielding Variety during the green revolution with a yield potential of 8 t/ha (Peng et al., 2000). Pusa Basmati1 is highly popular because of its long slender grains and pleasant aroma. Cooked rice of Pusa Basmati 1 is endowed with desirable traits like soft texture and tenderness. It has a yield potential of 4.5 t/ ha (Siddiq, 1990). It is extensively grown in the Basmati region of India. Basmati 370 is an aromatic variety and showed good tissue culture response (Raina et al., 1987).

Mature dehusked grains of the selected rice genotypes were washed with sterile distilled water and were surface sterilized successively with, 70% ethanol for two min, sodium hypochlorite (contains 4% (v/v) active chlorine) for 15 min and with 0.1% (w/v) aqueous mercuric chloride solution for 5min with intermittent repeated washings with sterile distilled water (Vijayachandra et al., 1995).The kernels were inoculated in culture tubes containing semisolid callus induction (CI) medium MS (Murashige & Skoog, 1962) supplemented with 2, 4-D (2.0 mg l^{-1}) and Kn (0.5 mg l^{-1}) and were evaluated for their potential to support callus induction and subsequent green plant regeneration. Calli developed on these media were transferred onto MS regeneration medium supplemented with phytohormones [NAA (0.5 mg l^{-1}) + Kn (0.5 mg l^{-1}) + BAP (1.5 mg l^{-1})]. Callus induction (CI) and regeneration frequencies (RF) under the influence of additional nutrients and PGRs were recorded and statistical analyses were performed using SAS software (Chaitanya et al., 2013) in separate experiments.

3. Results & Discussion

3.1 Somatic Embryogenesis and Influence of Additional Nutrients

The feasibility of induction of somatic embryogenesis in rice cultures was evaluated in selected rice varieties *viz*., Gayatri, Swarna, Samba Mahsuri, Pooja, Tapaswini and Sahabhagidhan. In Tapaswini, Swarna, Samba Mahsuri, Pooja and Gayatri, pre globular- pro embryos (PGPEs) were found in the embryogenic portion of the primary callus induced after 7-10 days on MS medium supplemented with 2, 4-D (2.0 mg l^{-1}) and Kn (0.5 mg l^{-1}). These structures could undergo subsequent developmental stages like complete globular, heart and torpedo shapes in subcultures. Moreover, somatic embryoids (SEs) were observed to be originating from the peripheral as well as deep-seated portions of the calli, but independent and free from the surrounding callus tissues.

However, when the embryogenic calli with these structures were sub cultured on fresh medium with (2.0 mg l^{-1}) 2, 4-D, a major portion of the embryoids got dissipated to form callus. But, addition of L-Proline (500 mg l^{-1}) in the medium was observed to be better for long term maintenance of embryoids in culture. In Gayatri and Swarna, viable embryoids were observed even in 6-7 month old cultures (after 5-6 passages). Among the genotypes, highest frequency of somatic embryoids (both as % calli with embryoids and number of SEs per unit callus ~5mm dia) was observed in Tapaswini closely followed by Swarna, Sambamahsuri, Pooja, Gayatri and Sahabhagidhan in that order (Table 1). Addition of L-Proline had positive and significant influence on somatic embryogenesis in all the genotypes. It is interesting to note

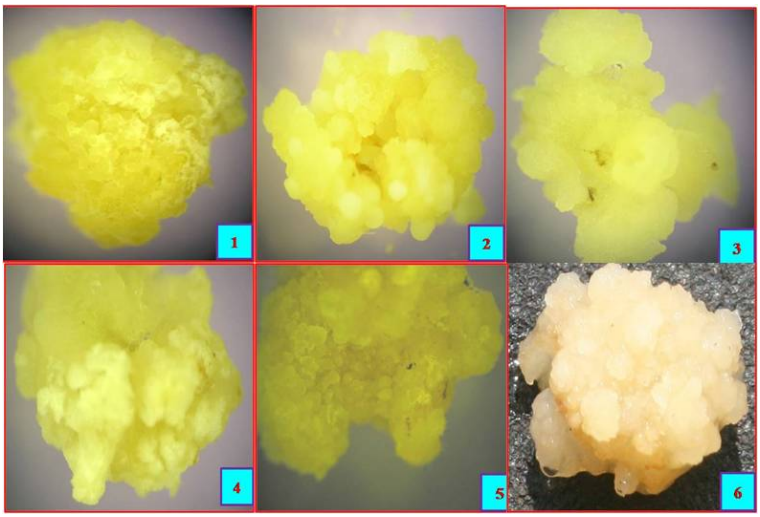
that even though callus induction capabilities of Gayatri and Sahabhagidhan are low, they have good regeneration capabilities.

Table 1. Influence of additional nutrients on callus induction and regeneration of various cultivars

S.No	Genotypes		Frequency of calli with somatic embryoids (%)				
			MS_K	MS_C	MS_P	MS_0	MSR
		SEF	48.34	58.7	53.9	16.8	62.93
1	Gayatri	SD	2.114	1.11	1.35	2.35	2.31
		SE	1.21	0.64	0.78	1.36	1.33
		SEF	77.7	78.4	77.46	26.1	78.0
2	Swarna	SD	2.2	0.91	1.69	2.36	2.60
		SE	1.27	0.52	0.97	1.36	1.50
		SEF	76.3	79.6	77.96	26.9	76.53
3	Samba Mahsuri	SD	1.8	1.05	2.51	2.48	1.42
		SE	1.03	0.60	1.44	1.43	0.82
		SEF	72.23	75.7	75.76	22.43	72.63
4	Pooja	SD	2.159	1.05	1.81	2.07	2.47
		SE	1.24	0.60	1.04	1.20	1.43
		SEF	97.4	98.34	96.8	31.46	82.33
5	Tapaswini	SD	1.90	0.94	1.38	0.66	1.457
		SE	1.10	0.54	0.80	0.38	0.84
		SEF	25.5	36.56	34.03	9.46	33.3
6	Sahabhagidhan	SD	2.2	1.06	1.60	0.94	2.65
		SE	1.27	0.61	0.92	0.54	1.53

SEF: Somatic embriod frequency; SD: Standard Deviation; SE: Standard Error. MS_K = MS + 2, 4-D (2.0 mg l^{-1}) + Kn (0.5 mg l^{-1}); MS_P = MS_K + L-Proline (500mg l^{-1}); MS_C = MS_K + Casein hydrolysate (300mg l^{-1}); MS_O = Hormone free media: MSR= MS basal salts + NAA (0.5 mg l^{-1}) +kn (0.5 mg l^{-1}) + BAP (1.5 mg l^{-1}).

Addition of casein hydrolysate (300mgl^{-1}) also had profound influence on the frequency of the calli with somatic embryoids and thereby average number of somatic embryoids per callus also increased. These somatic embryoids were observed in microscope and they varied in their morphology from genotype to genotype (Figure 1). Influence of casein hydrolysate on somatic embryogenesis is par with that of the L-Proline, but in case of Gayatri and Sahabhagidhan its influence was greater than that of L-Proline (Figure 2).



1) Pooja; 2) Gayatri; 3) Swarna; 4) IR-64; 5) Sahabhagidhan; 6) Tapaswini

Figure 1. Morphological shapes of callus of different genotypes

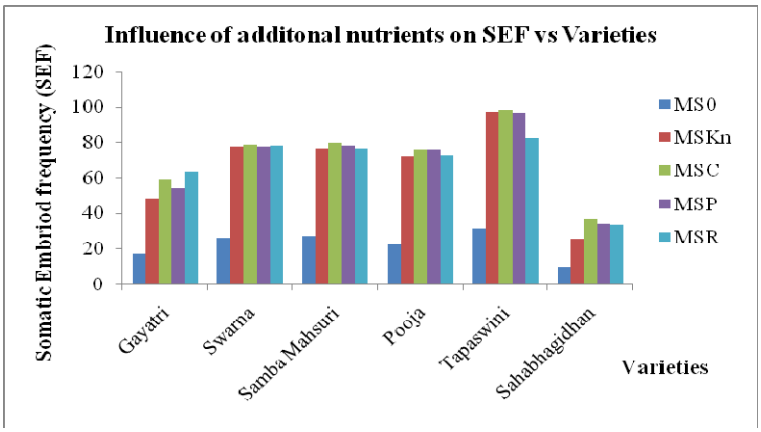


Figure 2. Influence of additional nutrients on somatic embriod frequency (SEF) during callus induction and regeneration in presence and absence of hormones in different cultivars

MS_O = Hormone free media; MS_{Kn} = MS + 2, 4-D (2.0 mg l^{-1}) + Kn (0.5 mg l^{-1}); MS_C = MS_K + Casein hydrolysate (300mg l^{-1}); MS_P = MS_K + L-Proline (500mg l^{-1}); MSR= MS basal salts + NAA (0.5 mg l^{-1}) +kn (0.5 mg l^{-1}) + BAP (1.5 mg l^{-1});

The analysis of variance of the data suggests that significant differences exist between treatments, genotypes and also interaction between genotypes and the treatments (Table 2 (a, b)).

Table 2. Anova on formation of somatic embryoids in different genotypes with the influence of additional nutrients on:

a. Somatic embryoid formation

Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	0.7612	< 0.0001	****	Yes	
Media	0.9464	< 0.0001	****	Yes	
Varieties	97.87	< 0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	182.3	10	18.23	F (10, 36) = 6.459	P < 0.0001
Media	226.7	2	113.4	F (2, 36) = 40.15	P < 0.0001
Varieties	23443	5	4689	F (5, 36) = 1661	P < 0.0001
Residual	101.6	36	2.823		

MS: Mean square; SS: Sum-of-squares; df: degrees of freedom

b. Regeneration

Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	3.546	< 0.0001	****	Yes	
Media	75.78	< 0.0001	****	Yes	
Varieties	20.24	< 0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	869.4	5	173.9	F (5, 24) = 39.76	P < 0.0001
Media	18582	1	18582	F (1, 24) = 4248	P < 0.0001
Varieties	4964	5	992.8	F (5, 24) = 227.0	P < 0.0001
Residual	105.0	24	4.374		

3.2 Influence of Additional Nutrients on Callus Induction Rate

Attempts were made to study the influence of additional supplements on callus induction frequencies, in addition to the constituents of the MS medium. Use of casein hydrolysate was found to be beneficial for generation of embryogenic calli in *japonica* (Hiei et al., 1994; Toki, 1997) as well as in *indica* rice varieties (Zhang et al., 1996).

Combination of eighteen amino acids present in casein hydrolysate was able to substitute nitrogen source for the growth of embryos (Verbruggen & Hermans, 2008). CH is also a rich source of vitamins, calcium, phosphate, and several microelements and helps in maintaining the cell for longer period (Siripornadulsil et al., 2002). A specimen analysis of the casein hydrolysate published by the manufacturers indicates that the quantity of nitrogen supplied by 400mgl^{-1} of casein hydrolysate was equivalent to that supplied by 2.0mM sodium nitrate. Gorham (1950) showed that casein hydrolysate was effective as a nitrogen source for the growth of *Lemna minor* in dark. In the present study, casein hydrolysate (300mgl^{-1}) was tested at callus induction level and the results suggest a positive role of casein hydrolysate on enhancement of callus growth both qualitatively and quantitatively. The results recommend the usage of casein hydrolysate essentially for callus induction as it plays a pivotal role in providing additional nitrogen source to the calli and helps in growth of cells.

L-Proline is an important amino acid used for enhancement of somatic embryogenesis and callus growth in rice. Proline induced stimulation of somatic embryogenesis has been well documented in plant systems like rice, maize, cat grass and other crop plants (Shetty & McKersie, 1993; Afsharsterle et al., 1996; Suprasanna et al., 1997; Murch et al., 1999; Siripornadulsil et al., 2002). The use of proline in the medium has been reported to be effective for the initiation and maintenance of embryogenic calli (Datta et al., 1992; Kishor et al., 1999). Keeping in view of the suggested role of proline and its analogs in stimulating auxin induced embryogenesis; in the present study, L-Proline (1gl^{-1}) was supplemented to the media used for callus induction and the results suggest the induction of good quality callus (Table 1). Shetty & McKersie (1993) found that proline and thioproline significantly contributed to enhanced embryogenic response (embryogenic callus development and somatic embryos). It is opined that alterations in proline metabolism and its potential regulation of associated purine metabolism are important in directing cell differentiation i.e., auxin-induced somatic embryogenesis. Amino acids play a crucial role as organic sources of nitrogen during invitro growth and development of plant cells. They are readily available and assimilated at a faster rate than the inorganic sources of nitrogen. L-Proline acts as organic source of nitrogen. The observations of the study recommend the use of proline in the media as an essential requirement to provide nitrogen source as well as to maintain osmotic balance in the cells.

3.3 Influence of Gelling Agents on Callus Induction and Regeneration

An experiment was conducted with the objective of enhancing the rates of regeneration in *indica* rice varieties. Here, four different gelling agents *viz.,* agar, gelrite, clarigel and phytagel were evaluated in both callus induction and regeneration steps using the following rice genotypes *viz.,* Swarna, Gayatri, Sambamahsuri, IR-64, Pusa Basmati 1 and Basmati 370. All the four gelling agents support callus induction but the frequency is high with gelrite closely followed by clarigel, agar and phytagel, in that order, (Table 3) irrespective of the cultivar (Figure 3).

The analysis of variance of the data suggest that significant differences exist between treatments and varieties with no interaction between genotypes and treatments for callus induction with various gelling agents (Table 4).

Of the four gelling agents evaluated for their influence on regeneration, the frequency is high on agar followed by gelrite, clarigel and phytagel in that order (Table 5) (Figure 4). The analysis of variance data suggests that differences in regeneration rates are significant between the treatments (different gelling agents) while the differences were not significant between different genotypes evaluated (Table 6).

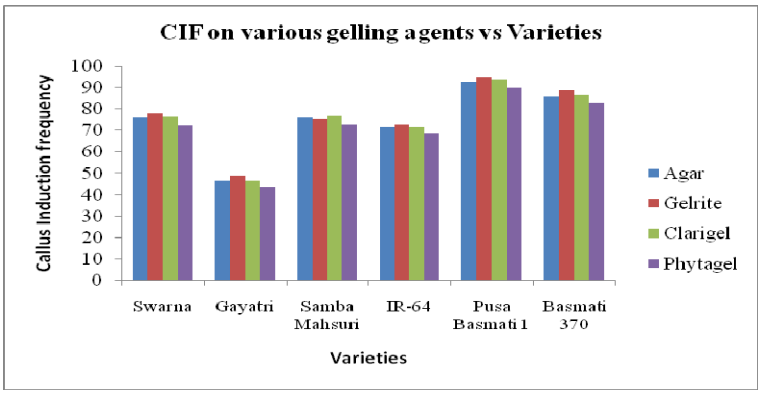


Figure 3. Influence of various gelling agents on Callus Induction frequency (CIF)

Table 3. Callus induction frequencies on various gelling agents

S.N	Genotype		Callus induction frequency (CIF)			
			Agar	Gelrite	Clarigel	Phytagel
		CIF	75.6	77.6	76.37	72.37
1	Swarna	SD	2.10	1.05	2.80	1.96
		SE	1.21	0.60	1.61	1.131
		CIF	46.36	48.64	46.5	43.74
2	Gayatri	SD	2.20	0.75	3.3	1.95
		SE	1.27	0.43	1.90	1.12
		CIF	75.64	75.37	76.4	72.5
3	Samba Mahsuri	SD	2.11	0.85	2.90	1.75
		SE	1.22	0.49	1.67	1.01
		CIF	71.56	72.53	71.47	68.6
4	IR-64	SD	2.05	0.94	2.70	2.2
		SE	1.18	0.54	1.56	1.27
		CIF	92.54	94.53	93.5	89.66
5	Pusa Basmati 1	SD	1.70	1.30	3.16	2.25
		SE	0.98	0.75	1.82	1.30
		CIF	85.67	88.36	86.47	82.4
6	Basmati 370	SD	2.11	0.80	3.30	1.67
		SE	1.21	0.46	1.90	0.96

CIF: Callus induction frequency; SD: Standard Deviation; SE: Standard Error

Table 4. Anova of callus induction data with different gelling agents

Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	0.1010	0.9984	ns	No	
Solidifiers	1.367	< 0.0001	****	Yes	
Varieties	97.12	< 0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	15.73	15	1.048	F (15, 48) = 0.2291	P = 0.9984
Solidifiers	212.8	3	70.94	F (3, 48) = 15.50	P < 0.0001
Varieties	15120	5	3024	F (5, 48) = 660.8	P < 0.0001
Residual	219.7	48	4.576		

MS: Mean square; SS: Sum-of-squares; df: degrees of freedom

Table 5. Regeneration frequencies of cultivars on various gelling agents

S.No	Genotype		Regeneration frequency (REF)			
			Agar	Gelrite	Clarigel	Phytagel
		REF	64.57	63.87	62.5	62.4
1	Swarna	SD	6.30	4.43	4.25	2.76
		SE	3.63	2.56	2.45	1.59
		REF	79.44	76.57	75.54	75.4
2	Gayatri	SD	4.70	5.30	3.70	3.10
		SE	2.71	3.06	2.14	1.79
		REF	46.47	45.73	45.34	45.4
3	Samba Mahsuri	SD	4.16	4.90	4.15	3.40
		SE	2.40	2.82	2.39	1.96
		REF	42.0	41.47	41.67	48
4	IR-64	SD	3	4.90	4.152	8.92
		SE	1.73	2.83	2.39	5.15
		REF	84.5	82.37	81.4	81.57
5	Pusa Basmati 1	SD	2.30	5.15	3.91	3.25
		SE	1.33	2.97	2.26	1.87
		REF	75	74.5	71.53	74.53
6	Basmati 370	SD	1	5.3	2.42	2.81
		SE	0.57	3.06	1.39	1.62

REF: Regeneration frequency; SD: Standard Deviation; SE: Standard Error

Table 6. Anova of regeneration data with different gelling agents

Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	0.7055	0.9623	ns	No	
Solidifiers	0.2944	0.4507	ns	No	
Varieties	93.74	< 0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	123.2	15	8.211	F (15, 48) = 0.4289	P = 0.9623
Solidifiers	51.40	3	17.13	F (3, 48) = 0.8948	P = 0.4507
Varieties	16364	5	3273	F (5, 48) = 170.9	P < 0.0001
Residual	919.0	48	19.15		

MS: Mean square; SS: Sum-of-squares; df: degrees of freedom

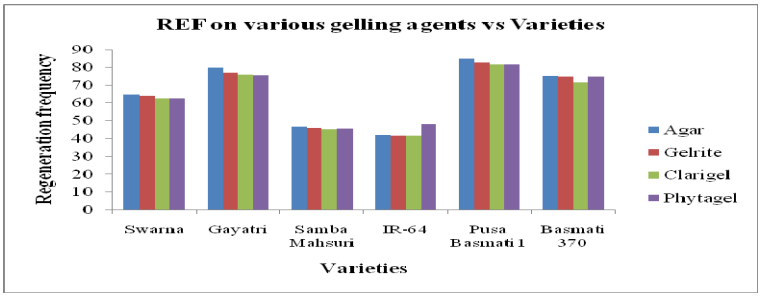


Figure 4. Influence of various gelling agents on Regeneration frequency (REF)

4. Influence of Gelling Agents

Agar is extracted from the seaweed *Gelidium* and it forms a good support for media (Armisen & Gaiatas, 2009). Commercially available gelling materials like clarigel (Hi-media) and gelrite (Sigma-Aldrich) are also good for callus induction and especially gelrite forms a clear and transparent media which is helpful in clearly identifying the newly emerging callus. Importance of media being transparent is more conspicuous while working with very small explants like immature embryos, anthers and embryogenic calli especially during transformation experiments using gene gun etc.

Gellan gum is a widely used gelling agent in plant tissue culture (Puchooa et al., 1999) that is marketed under various trade names including Gelrite, Phytagel and Kelcogel. It is an exo polysaccharide that encapsulates cells of the bacterium *Sphingomonas paucimobilis* and *Pseudomonas elodea*, from which it is obtained by industrial fermentation. The structure, physico-chemical properties and the rheology of solutions of gellan gum and related polysaccharides has been reviewed by Banik et al. (2000). Gellan gum consists of a linear repeating tetra saccharide of D-glucose, D-glucuronic acid, D-glucose and L-rhamnose.

An important aspect here to note is that plants regenerate well and produce intense roots only when the gelling agent is added in proper concentrations and it is found in the study (Table 5) that plants regenerate well when traditional agar is used rather than the commercial gelling agents in contrast to callus induction. In our experiments, a concentration of $10gl^{-1}$ of agar for callus induction and $9gl^{-1}$ for regeneration and $8gl^{-1}$ for rooting media, were used so as to create a porous environment for different stages but in comparison, only $2.6gl^{-1}$ of gelrite would be sufficient for callus induction and regeneration. High concentration of gelling agent was found to improve plantlet regeneration in this study, which corroborates the findings of earlier reports in rice (Jain et al., 1996; Khaleda & Al-Forkan, 2006). Recently plant derived phytagels are also available as gelling agents. Earlier it was reported (Suprasanna et al., 2000) that the same gelling agent at various concentrations have profound influence in retention of water and regulation of moisture regime of the medium and for this reason, addition of agar in correct proportions is needed to meet the requirements at different stages of culture i.e. callus induction, regeneration and rooting of plantlets generated in tissue culture.

Transformation experiments require the addition of antibiotics to the media for selection and here it is found that some commercial gelling agents do not support the dissolution of some antibiotics, in such case use of traditional agar would be ideal choice as a gelling agent.

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Conflict of interests

The authors declare that there is no conflict of interests regarding publication of this paper.

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