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Effect of acetosyringone and age of callus on *Agrobacterium*- mediated transformation of rice (*Oryza sativa* L.) calli

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Abstract
The present investigation was carried out to standardize the age of callus (days), acetosyringone concentration (µM) and acetosyringone incubation period (min) prior to colonization for achieving highest transformation frequency (%). Scutellum-derived calli of an indica rice variety Ratnagiri-711 that was found highly regenerable in previous studies were used as explants. The disarmed hyper virulent *Agrobacterium tumefaciens* strain EHA 105 harboring pBinBt3 was used as vector system. Plasmid pBinBt3 contains the *cry2Aa* gene linked to the cauliflower mosaic virus (CaMV) 35S promoter and neomycin phosphotransferase (nptII) gene under the control of nopaline synthase (nos) promoter and terminator. Treated calli were analyzed through PCR for confirming the presence of transgene and transformation frequencies were calculated. All the three factors *viz.* age of calli, acetosyringone incubation period along with different concentrations were found with significant effect on transformation frequency. 35 days old calli combined with acetosyringone incubation period of 45 min prior to colonization with a concentration of 350 µM were recorded the highest transformation frequency. Acetosyringone was found as an essential key factor in achieving the rice transformation.

Keywords: Acetosyringone, callus age, transformation frequency

Introduction
Rice (*Oryza sativa* L.) is one of the most important food crops and is the staple food for more than half of the global population [1]. Rice productivity is greatly affected by both biotic and abiotic stresses. Insect pest attacks and damage is considered as one of the major causes of rice yield losses throughout the rice producing countries of the world [2]. One way to increase the quantity and quality of food is to reduce damages caused by insects, diseases and weeds to crops. Besides the conventional methods, scientists have deployed several strategies using the transgenic approach to confer insect resistance in crop plants. One of the most successful strategies that is deployed is the introduction of *Bt* δ-endotoxin crystal insecticidal protein genes (*cry* genes). Rice is the first cereal crop species for which an efficient transformation protocol mediated by *A. tumefaciens* was developed [3]. The development of methods for the genetic transformation of cereals was delayed for some time as compared to the initial success in dicotyledonous species. The major cause of the delay was the fact that transformation mediated by the soil bacterium *Agrobacterium tumefaciens* was not readily applicable to cereal plants. Acetosyringone that is secreted at wounded site of dicots is known for enhancing the *Agrobacterium*-mediated gene transformation which lacks in case of monocots as wound response and need to be added externally while carrying out infection of explants. The concentration (µM) as well as incubation period (min) of acetosyringone plays an important role in the transformation. The age of a callus, a dedifferentiated phase more prone to get genetically manipulated, play important role to obtain the high transformation frequency. Every age of callus cannot withstand infection as well as cannot prone to get transformed. Hence, the experiments using a *cry* gene were conducted to optimize the age of callus and acetosyringone concentration and incubation period for getting high transformation frequency.
To date, all the successful reports on *Agrobacterium*-mediated transformation of rice have been based on *Agrobacterium* preinduction and/or cocultivation in the presence of AS [3-8] or based on co-cultivation in presence of suspension culture of potato cells, a rich source of phenolic compounds [9]. No transient expression of the GUS gene was observed in the absence of AS even when using a super virulent *Agrobacterium* strain, and 100µM of AS was reported

to be optimum for transient expression in rice [10]. Several other reports are also duly discussed thereafter supporting the findings in present research work.

Material and Methods

Transformation studies were carried out on an indica rice variety Ratnagiri- 711 which was found highly regenerable in previous experiments. Prior to producing transgenic rice plants, preliminary experiments determining the efficiency of calli transformation were conducted. The disarmed hyper virulent *Agrobacterium tumefaciens* strain EHA 105 harboring pBinBt3 was used as vector system. Plasmid pBinBt3 contains the *cry2Aa* gene linked to the cauliflower mosaic virus (CaMV) 35S promoter and neomycin phosphotransferase (nptII) gene under the control of nopaline synthase (nos) promoter and terminator (Fig. 1). Three factors *viz*. age of callus, acetosyringone concentration and acetosyringone incubation period prior to colonization affecting the transformation were optimized. Scutellum-derived calli were used as explants. The various levels of acetosyringone concentrations used in *Agrobacterium* suspension culture for different incubation period prior to colonization are given in Table 1. Also, the calli with different ages (Table 1) were tested for obtaining highest transformation frequency. Calli treated with acetosyringone were co-cultivated for 4 days on co-cultivation medium MS+ 1.5 mg/L 2,4-D+ 0.5 mg/L BAP. PCR analysis of calli after 4 days of co-cultivation was carried out to confirm the presence of transgene using specific primers for *cry2Aa* gene. Observations were recorded on number of calli transformed out of total calli colonized and transformation frequencies were calculated. The whole experiment was analyzed statistically in 3 factorial-CRD estimating the effects of above 3 factors individually as well as in their interactions.

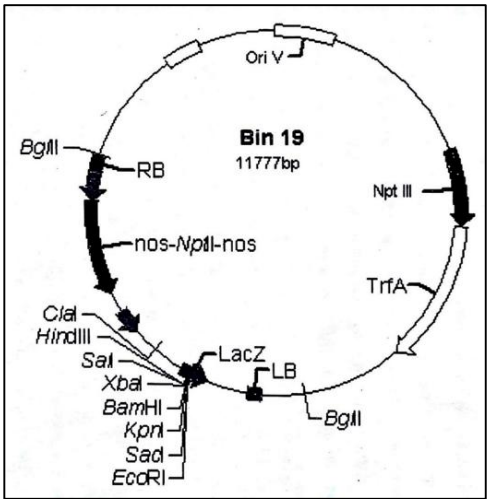


Fig 1: Map of the *Cry2Aa* gene construct in Ti plasmid

Table 1: Different levels of age of callus, acetosyringone concentration and incubation period used in experiment

Level No.	Age of callus (Days)	Acetosyringone incubation period (min)	Acetosyringone conc^{n} (µM)
1	25	15	0
2	30	30	50
3	35	45	100
4	45	60	150
5			200
6			250
7			300
8			350
9			400
10			450
11			500

Results

The results obtained from the experiment determining the effect of acetosyringone concentration (µM)**,** acetosyringone incubation period (min) and age of callus (days) on transformation frequency of rice scutellum-derived calli are presented in Table 2 to 6. Transformation frequencies were estimated as number of transformed calli out of total calli colonized. All the three factors along with their interactions showed significant effect on the transformation frequency (Table 2).

Table 2(a): Anova for effects of acetosyringone and callus age

Source of variation	Degrees of freedom	Mean sum of squares	F value calculated
Age of callus (Days)	3	12,286.55	2,337.77*
Acetosyringone incubation period (min)	3	16,131.25	3,069.30*
Acetosyringone conc^{n} (µM)	9	1,105.68	210.38*
Age of callus × Acetosyringone incubation period	10	6,356.26	1,209.41*
Age of callus × Acetosyringone conc^{n}	30	146.40	27.86*
Acetosyringone incubation period × Conc^{n}	30	382.76	72.83*
Age of callus × Acetosyringone incubation period × Acetosyringone conc^{n}	90	18.65	3.55*
Error	176	5.26	

* significant at 1 %

Table 2(b)

Factors	SE(m)	CD (at 1%)	Significance (at 1 %)
Age of callus (Days)	0.24	0.90	Sig
Acetosyringone incubation period (min)	0.24	0.90	Sig
Acetosyringone conc^{n} (µM)	0.41	1.49	Sig
Age of callus × Acetosyringone incubation period	0.49	1.80	Sig
Age of callus × Acetosyringone conc^{n}	0.81	2.99	Sig
Acetosyringone incubation period × Conc^{n}	0.81	2.99	Sig
Age of callus × Acetosyringone incubation period × Acetosyringone conc^{n}	1.62	5.97	Sig

Effect of age of callus (Days) on transformation frequency

A significantly higher average transformation frequency (34.03 %) was observed when calli of 35 days were used as target tissue (Table 3). It was followed by the calli of 45 days age and recorded the average transformation frequency of 27.96 %. The lowest average transformation frequency (8.86 %) was recorded by 25 days old calli.

Effect of acetosyringone incubation period (min) on transformation frequency

The average transformation frequency was found significantly higher (33.30 %) with the acetosyringone incubation period of 45 min which was followed by acetosyringone incubation period of 60 min (29.03 %) irrespective of its concentration (Table 3). Whereas the acetosyringone incubation period of only 15 min recorded the lowest transformation frequency (2.78 %).

Effect of acetosyringone concentration (µM) on transformation frequency

Acetosyringone concentration showed the significant effect on transformation frequency (Table 3). A significantly higher average transformation frequency (47.50 %) was obtained at acetosyringone concentration of 350 µM proving it to be the ideal concentration for use in rice transformation. It was followed by concentration of 300 µM (40.00 %). It could also be seen from Table 3 that as acetosyringone concentration increases, transformation frequency also increases till reaching to its highest peak at 350 µM and then decreases on further increase in concentration. The lowest average transformation frequency (8.75 %) was found with acetosyringone concentration of 50 µM while no transformation was occurred without use of acetosyringone (0 µM).

Table 3: Interaction effect of age of callus (Days) and acetosyringone incubation period (min) on transformation frequency

Age × IP	Acetosyringone incubation period (min)	I_1	I_2	I_3	I_4	Mean
Callus age (Days)		15	30	45	60	
A_1	25	1.14 (2.26)	6.82 (9.47)	15.46 (18.63)	12.05 (15.17)	8.86 (11.38)
A_2	30	2.05 (3.5)	9.09 (11.71)	24.09 (27.15)	19.55 (22.93)	13.69 (16.32)
A_3	35	4.77 (6.98)	33.18 (33.4)	51.36 (44.57)	46.82 (41.79)	34.03 (31.69)
A_4	45	3.18 (4.87)	28.64 (30.4)	42.27 (39.03)	37.73 (36.25)	27.96 (27.64)
Range	Min	1.14 (2.26)	6.82 (9.47)	15.46 (18.63)	12.05 (15.17)	8.86 (11.38)
	Max	4.77 (6.98)	33.18 (33.4)	51.36 (44.57)	46.82 (41.79)	34.03 (31.69)
Mean		2.78 (4.4)	19.43 (21.25)	33.3 (32.35)	29.03 (29.03)	21.14 (21.76)

Figures in parentheses indicate arcsine transformed value

Interaction effect of age of callus (Days) and acetosyringone concentration (µM) on transformation frequency

The interaction having 35 days old calli with acetosyringone concentration of 350 µM recorded the maximum average transformation frequency (63.75 %) which was significantly different from remaining ones. It was then followed by the two interactions viz. 35 days old calli with 300 µM acetosyringone concentration and 45 days old calli with 350 µM acetosyringone concentration revealing the same average transformation frequency of 56.25 % (Table 4). The calli of all tested ages without use of acetosyringone were found with 0.00 % transformation. The 25 days old calli with the lowest (50 µM) as well as the highest (500 µM) acetosyringone concentration were also found with zero per cent transformation efficiency.

Table 4: Interaction effect of age of callus (Days) and acetosyringone concentration (µM) on transformation frequency

Age × Conc^{n}	Callus age (Days)	A_1	A_2	A_3	A_4	Mean
Acetosyringone Concentration (µM)		25	30	35	45	
Conc^{n}-1	0	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)
Conc^{n}-2	50	00 (0.00)	1.25 (3.23)	20 (23.06)	13.75 (18.74)	8.75 (11.26)
Conc^{n}-3	100	0.63 (1.61)	5 (9.07)	25.63 (26.67)	19.38 (22.75)	12.66 (15.02)
Conc^{n}-4	150	2.5 (5.53)	7.5 (11.32)	29.38 (28.95)	23.13 (25.18)	15.63 (17.75)
Conc^{n}-5	200	8.13 (12.93)	14.38 (18.88)	36.88 (33.36)	30.63 (29.74)	22.5 (23.73)
Conc^{n}-6	250	11.88 (17.15)	18.13 (21.73)	41.25 (37.16)	34.38 (31.93)	26.41 (26.99)
Conc^{n}-7	300	23.75 (26.8)	31.25 (32.87)	56.25 (48.51)	48.75 (43.62)	40 (37.95)
Conc^{n}-8	350	31.25 (33.24)	38.75 (37.97)	63.75 (53.57)	56.25 (48.51)	47.5 (43.32)
Conc^{n}-9	400	15.63 (20.12)	21.88 (24.28)	46.25 (41.67)	38.75 (35.71)	30.63 (30.44)
Conc^{n}-10	450	3.75 (7.84)	8.75 (12.33)	31.25 (30.07)	25.00 (26.37)	17.19 (19.15)
Conc^{n}-11	500	00 (0.00)	3.75 (7.84)	23.75 (25.51)	17.5 (21.49)	11.25 (13.71)
Range	Min	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)
	Max	31.25 (33.24)	38.75 (37.97)	63.75 (53.57)	56.25 (48.51)	47.5 (43.32)
Mean		8.86 (11.38)	13.69 (16.32)	34.03 (31.68)	27.95 (27.64)	21.14 (21.76)

Figures in parentheses indicate arcsine transformed value

Interaction effect of acetosyringone incubation period (min) and acetosyringone concentration (µM) on transformation frequency

The acetosyringone incubation of 45 min with 350 µM concentration was found as the best interaction transforming the 65.00 % calli followed by acetosyringone incubation of 60 min with the same concentration of 350 µM (60.00 %) (Table 5). The 15 min acetosyringone incubation period with concentrations of 50, 100, 150, 200, 450 and 500 have not shown any transformation.

Table 5: Interaction effect of acetosyringone incubation period (min) and acetosyringone concentration (µM) on transformation frequency

Acetosyringone	incubation period (min)	I_1	I_2	I_3	I_4	Mean
Concentration (µM)		15	30	45	60	
Conc^n-1	0	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)
Conc^n-2	50	00 (0.00)	6.25 (10.3)	16.25 (19.79)	12.5 (14.94)	8.75 (11.26)
Conc^n-3	100	00 (0.00)	10 (13.23)	22.5 (25.61)	18.13 (21.25)	12.66 (15.02)
Conc^n-4	150	00 (0.00)	12.5 (14.97)	27.5 (30.41)	22.5 (25.61)	15.63 (17.75)
Conc^n-5	200	00 (0.00)	20 (23.65)	37.5 (37.28)	32.5 (33.99)	22.5 (23.73)
Conc^n-6	250	0.63 (1.61)	25 (28.67)	42.5 (40.41)	37.5 (37.28)	26.41 (26.99)
Conc^n-7	300	10 (16.85)	40 (38.9)	57.5 (49.55)	52.5 (46.5)	40 (37.95)
Conc^n-8	350	17.5 (24.44)	47.5 (43.48)	65 (54.28)	60 (51.09)	47.5 (43.32)
Conc^n-9	400	2.5 (5.53)	30 (32.37)	47.5 (43.47)	42.5 (40.41)	30.63 (30.44)
Conc^n-10	450	00 (0.00)	13.75 (15.8)	30 (32.3)	25 (28.52)	17.19 (19.15)
Conc^n-11	500	00 (0.00)	8.75 (12.33)	20 (22.71)	16.25 (19.79)	11.25 (13.71)
Range	Min	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)
	Max	17.5 (24.44)	47.5 (43.48)	65 (54.28)	60 (51.09)	47.5 (43.32)
Mean		2.78 (4.4)	19.43 (21.24)	33.3 (32.35)	29.03 (29.03)	21.14 (21.76)

Figures in parentheses indicate arcsine transformed value

Interaction effect of age of callus (Days), acetosyringone incubation period (min) and acetosyringone concentration (µM) on transformation frequency

Among all the combinations tested, the interaction consisting of 35 days old calli with acetosyringone incubation period of 45 min prior to colonization and with a concentration of 350 µM recorded the highest transformation frequency attaining the highest peak of 85.00 % across the trend line (Table 6 and Fig. 2) and proved to be the most effective treatment combination. PCR assay of this superior interaction revealing the highest frequency of transformed calli is shown in Fig. 3. It was followed by interaction having 35 days old callus with acetosyringone incubation period of 60 min at a concentration of 350 µM recording the 80.00 % transformation frequency and found at par with the superior combination.

Table 6: Interaction effect of age of callus (Days), acetosyringone incubation period (min) and acetosyringone concentration (µM) on transformation frequency (%)

Age of callus (Days)	Acetosyringone	Conc^n (µM)	AC_1	AC_2	AC_3	AC_4	AC_5	AC_6	AC_7	AC_8	AC_9	AC_{10}	AC_{11}	Mean
	Incubation period (min)		0	50	100	150	200	250	300	350	400	450	500	
25	I_1	15	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	2.5 (6.46)	10 (18.43)	00 (0.00)	00 (0.00)	00 (0.00)	1.14 (2.26)
	I_2	30	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	2.5 (6.46)	7.5 (15.67)	22.5 (28.27)	30 (33.2)	12.5 (20.6)	00 (0.00)	00 (0.00)	6.82 (9.47)
	I_3	45	00 (0.00)	00 (0.00)	2.5 (6.46)	7.5 (15.67)	17.5 (24.67)	22.5 (28.27)	37.5 (37.74)	45 (42.11)	27.5 (31.59)	10 (18.43)	00 (0.00)	15.45 (18.63)
	I_4	60	00 (0.00)	00 (0.00)	00 (0.00)	2.5 (6.46)	12.5 (20.6)	17.5 (24.67)	32.5 (34.73)	40 (39.22)	22.5 (28.27)	5 (12.92)	00 (0.00)	12.05 (15.17)
30	I_1	15	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	7.5 (15.67)	15 (22.78)	00 (0.00)	00 (0.00)	00 (0.00)	2.05 (3.50)
	I_2	30	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	7.5 (15.67)	12.5 (20.6)	27.5 (31.59)	35 (36.26)	17.5 (24.67)	00 (0.00)	00 (0.00)	9.09 (11.71)
	I_3	45	00 (0.00)	5 (12.92)	12.5 (20.6)	17.5 (24.67)	27.5 (31.59)	32.5 (34.73)	47.5 (43.55)	55 (47.85)	37.5 (37.74)	20 (26.55)	10 (18.43)	24.09 (27.15)
	I_4	60	00 (0.00)	00 (0.00)	7.5 (15.67)	12.5 (20.6)	22.5 (28.27)	27.5 (31.59)	42.5 (40.67)	50 (44.98)	32.5 (34.73)	15 (22.78)	5 (12.92)	19.55 (22.93)
35	I_1	15	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	2.5 (6.46)	17.5 (24.67)	25 (29.99)	7.5 (15.67)	00 (0.00)	00 (0.00)	4.77 (6.98)
	I_2	30	00 (0.00)	15 (22.78)	22.5 (28.27)	27.5 (31.59)	37.5 (37.74)	42.5 (40.67)	57.5 (49.3)	65 (53.71)	47.5 (43.55)	30 (33.2)	20 (26.55)	33.18 (33.4)
	I_3	45	00 (0.00)	35 (36.26)	42.5 (40.67)	47.5 (43.55)	57.5 (49.3)	62.5 (52.23)	77.5 (61.69)	85 (67.19)	67.5 (55.24)	50 (44.98)	40 (39.22)	51.36 (44.57)
	I_4	60	00 (0.00)	30 (33.2)	37.5 (37.74)	42.5 (40.67)	52.5 (46.42)	57.5 (49.3)	72.5 (58.37)	80 (63.41)	62.5 (52.23)	45 (42.11)	35 (36.26)	46.82 (41.79)
45	I_1	15	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	12.5 (20.6)	20 (26.55)	2.5 (6.46)	00 (0.00)	00 (0.00)	3.18 (4.87)
	I_2	30	00	10	17.5	22.5	32.5	37.5	52.5	60	42.5	25	15	28.64

			(0.00)	(18.43)	(24.67)	(28.27)	(34.73)	(37.74)	(46.42)	(50.75)	(40.67)	(29.99)	(22.78)	(30.4)
	I_3	45	00 (0.00)	25 (29.99)	32.5 (34.73)	37.5 (37.74)	47.5 (43.55)	52.5 (46.42)	67.5 (55.24)	75 (59.98)	57.5 (49.3)	40 (39.22)	30 (33.2)	42.27 (39.03)
	I_4	60	00 (0.00)	20 (26.55)	27.5 (31.59)	32.5 (34.73)	42.5 (40.67)	47.5 (43.55)	62.5 (52.23)	70 (56.77)	52.5 (46.42)	35 (36.26)	25 (29.99)	37.73 (36.25)
	Range	Min	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	00 (0.00)	2.5 (6.46)	10 (18.43)	00 (0.00)	00 (0.00)	00 (0.00)	1.14 (2.26)
		Max	00 (0.00)	35 (36.26)	42.5 (40.67)	47.5 (43.55)	57.5 (49.3)	62.5 (52.23)	77.5 (61.69)	85 (67.19)	67.5 (55.24)	50 (44.98)	40 (39.22)	51.36 (44.57)
Mean			00 (0.00)	8.75 (11.26)	12.66 (15.02)	15.63 (17.75)	22.5 (23.73)	26.41 (26.99)	40 (37.95)	47.5 (43.32)	30.63 (30.44)	17.19 (19.15)	11.25 (13.71)	21.14 (21.76)

Figures in parentheses indicate arcsine transformed value

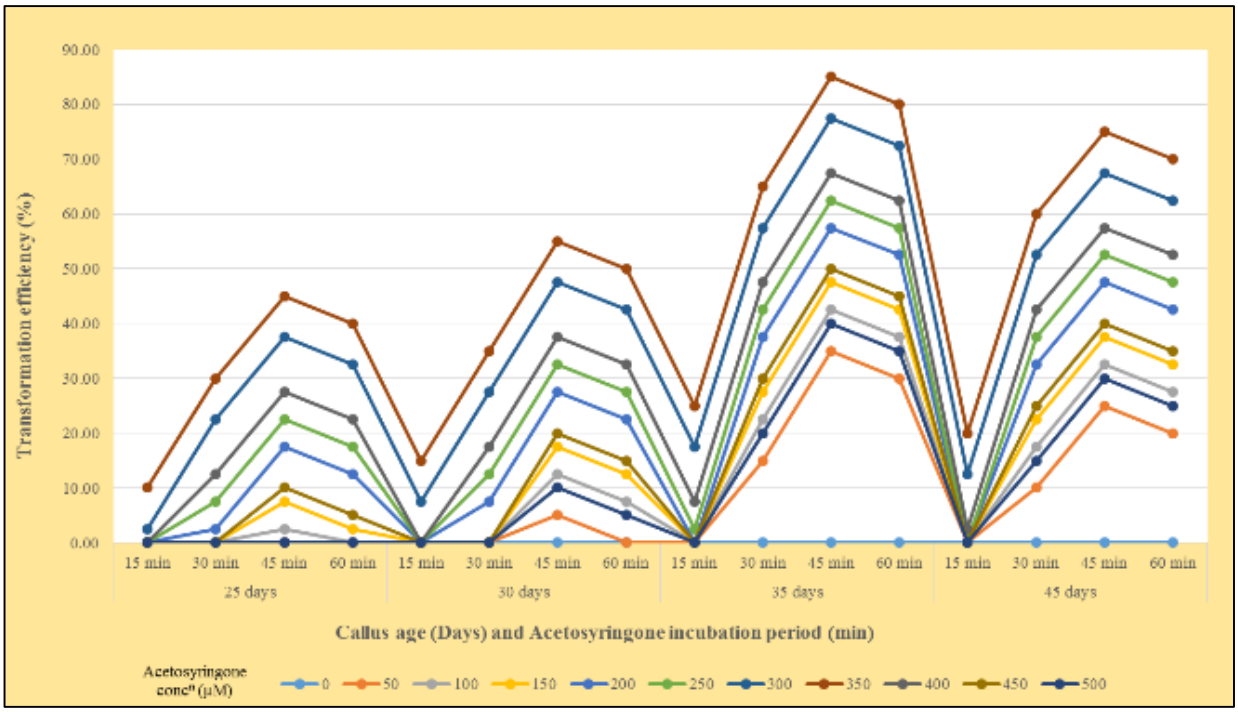
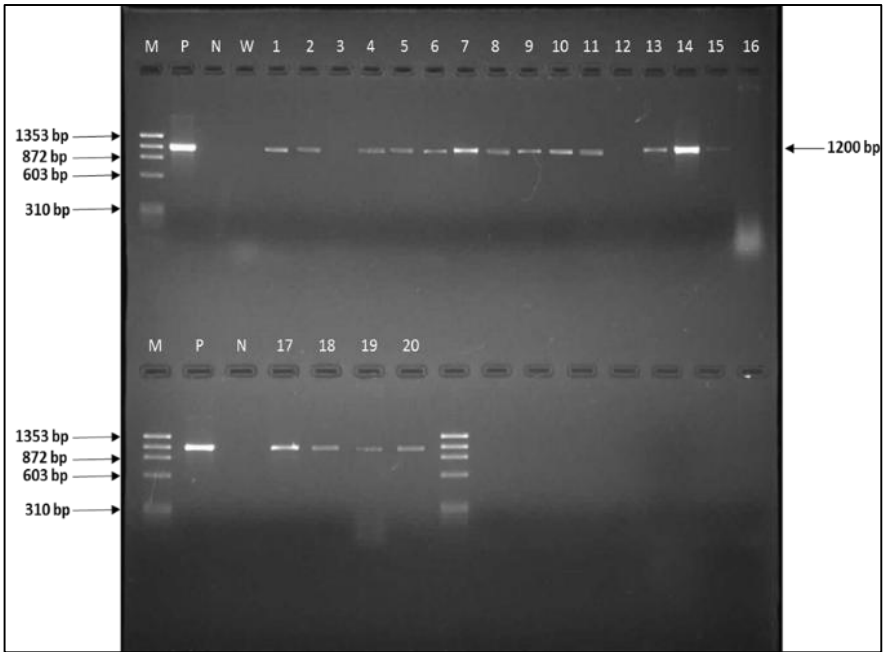


Fig 2: Effect of callus age (Days), acetosyringone concentration (µM) and incubation period (min) on calli transformation frequency (%)



M- Marker (M)
P- Plasmid (Positive control)
N- Normal plant (Negative control)
W- Water (Control)
1, 2, 4-11, 13-15, 17-20 – Transformed calli
3, 12, 16 – Non-transformed calli

Fig 3: PCR assay of rice calli transformed with *cry2Aa* gene and treated with 350 µM Acetosyringone

Discussion

The age of callus (days) and use of acetosyringone (AS) play important role to obtain the high transformation frequency because every age of callus cannot withstand infection as well as cannot prone to get transformed. Further, the Acetosyringone, as a potent *vir* gene inducer is not produced endogenously in monocots as a response to wounding in dicots and need to be supplied exogenously while carrying out infection of explants.

In the present investigation, scutellum derived calli were used for transformation studies. All the 3 factors *viz*. acetosyringone concentration (µM), acetosyringone incubation period (min) and age of callus (days) along with their interactions showed significant effect on the transformation frequency of calli. Among all the combinations tested, 35 days old calli with acetosyringone incubation period of 45 min prior to colonization at a concentration of 350 µM recorded the highest transformation frequency attaining the highest peak of 85.00 % across the trend line and proved to be the most effective treatment combination. No transformation was occurred without use of acetosyringone (0 µM). These findings are in similarity with those of Tripathi *et al*. [11]. Saharan *et al.* [12] also found maximum transformation frequencies in calli of cultivar HKR-126 (44.4%) and HKR- 46 (28.9%) with 3 weeks old calli at an acetosyringone concentration of 400 µM with 30 min incubation prior to colonization and cocultivation for 4-5 days. They also reported that high concentrations of acetosyringone in the *Agrobacterium* culture and co-cultivation medium proved to be indispensable for successful transformation. Manfroi *et al.* [13] also reported the acetosyringone concentration of 400 µM as most favourable for transformation in wheat.

The acetosyringone concentration of 100 µM were reported as optimum by many researchers in callus mediated *Agrobacterium* transformation [14-20]. The same concentration of 100 µM was also reported optimum for attaining *In Planta* transformation in rice [21, 22] as well as in cotton [23, 24].

Several other concentrations of acetosyringone were also reported as effective for achieving high transformation frequency in rice as 50 µM [5, 25, 26]; as 150 µM [27, 28] and as 200 µM [29-33].

However, Puhan *et al.* [34] reported 100% transformation using germinating seed as explant of four genotypes IR64, Jaya, AC41039 and Basmati 370 in absence of acetosyringone in co-cultivation medium further stating that addition of acetosyringone did not show any significant difference in the vector mediated transformation. Its use rather created a problem in controlling the growth of the bacterium difficult to control by cefotaxime. Similar results were also reported by Ananthi *et al.* [26].

The age of the callus is a crucial factor for transformation efficiency. Different reports with different ages stating as optimum were found *viz*. 7-day old [35], 20-21 days old [5, 29]; 4-weeks old [25] and 6-weeks old [26]. Rahman *et al.* [29] reported that young calli of 3 weeks old highly enhanced transformation as compared to older calli of 6 weeks old and above. Callus of more than 6 weeks old are more recalcitrant to *Agrobacterium* infection, due to increased immunity which is acquired by plant as defence to pathogen attack. Callus age less than 3 weeks however, are too friable and fragile to endure bacterial infection. The trend found in present investigation was also in similarity with this but the callus age of 35 days rather than 21 days found with maximum transformation frequency may be due to genotypic differences of varieties. Hiei and Komari [36] reported that fresh and healthy immature embryos ensure successful japonica and indica rice transformation. Young embryogenic callus is also favourable due to its higher regeneration ability as compared to old calli [37].

Conclusion

In present investigation, use of acetosyringone as a *vir* gene inducer was found indispensable in achieving rice transformation. All the three factors viz. age of calli, acetosyringone incubation period along with different concentrations recorded significant effect on transformation frequency. Calli of 35 days age combined with acetosyringone incubation period of 45 min prior to colonization with a concentration of 350 µM were found superior in achieving the highest calli transformation frequency.

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