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Calli mediated regeneration and transformation of Indica rice cultivars, Naveen, IR64 and Swarna

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Abstract
Continuous effort to improve *in vitro* culture and genetic transformation system are needed to increase the rice yield. In this study, three widely cultivated rice varieties (Naveen, IR64 and Swarna) were tested on six different media for callus induction. Similarly, three different regeneration media were examined for their efficiency in shoot induction. The MS medium supplemented with 3mgL^{-1} BAP, 1.0mgL^{-1} kinetin and 0.5 mgL^{-1} NAA showed better regeneration efficiency in IR64 and Swarna. In case of Naveen variety, maximum green shoot regeneration was achieved on MS medium supplemented with 2.0 mgL^{-1} Kinetin and 0.5 mgL^{-1} NAA. All the regenerated plantlets developed good root system in MS medium with 1.0mgL^{-1} NAA. Concentration of acetosyringone (AS) were optimized for Agrobacterium mediated transformation. 100µM AS in co-cultivation media was found to be the optimum concentration for Naveen, whereas 150µM AS was recorded as suitable for IR64 and Swarna. The results show that the modified media significantly improved the callus induction, regeneration and transformation efficiency of all three cultivars tested.

Keywords: Rice, tissue culture, callus induction, MS media, regeneration media, transformation

Introduction
Rice is a major food for half of the world's population, supplying 50-80% of their daily calorie intake (Khush, 2005) [17]. 90% of the rice is grown and consumed in Asia where it provides one-third of total dietary carbohydrate intake. Over the last 40 year the production of rice has kept pace with the increasing population, which is expected to reach around 10 billion by 2050. Thus, there is a need to improve rice productivity for a better life of rice farmers and consumers in less developed countries (Dawe, 2000) [6]. Modern biotechnological tools along with conventional breeding technique will help in the production of more healthier and high yielding plant types, which will help to stand against the present adverse climatic change condition. Development of embryogenic calli and regenerated green plantlet are the major steps for crop plant subjected to genetic modification through biotechnological approach.
It has been reported that Indica varieties are more specific to the tissue culture condition and Agrobacterium-mediated transformation than the Japonica varieties (Ge *et al*., 2006) [8]. Within the rice cultivars, variations have been reported among the genotypes in response to callus formation, plant regeneration and transformation (Seraj *et al*., 1997; Ozawa and Komamine, 1989; Peng and Hodges, 1989) [38, 25, 26]. The frequency of callus formation and plant regeneration is influenced by the interaction of plant genotype and it's *in vitro* culture conditions. The most sensible approach is to improve callus induction frequency and green shoot regeneration by using appropriate media for production of additional number of embryogenic calli and regenerated plants. Hence, the establishment of a robust and reproducible tissue culture system is prerequisite for successful transformation in indica rice.
For this, an efficient tissue culture system needs to be established to achieve transgenic rice. The major objective behind the study was to optimize the tissue culture conditions and to identify suitable reproducible varieties, which were efficient for plant transformation. In this study, emphasis was given on the effect of different nutrition media on callus induction and regeneration of three indica rice cultivars, which were commonly cultivated in Eastern part of India. Elite genotypes could be genetically modified by the transformation of useful genes in a short period of time.

Materials and Methods
Experimental materials
The matured seeds of three rice cultivars (Naveen, IR-64, Swarna) were obtained from gene bank of National Rice Research Institute (NRRI), Odisha, India. The seeds were kept at 37 ºC overnight before starting of the experiment.

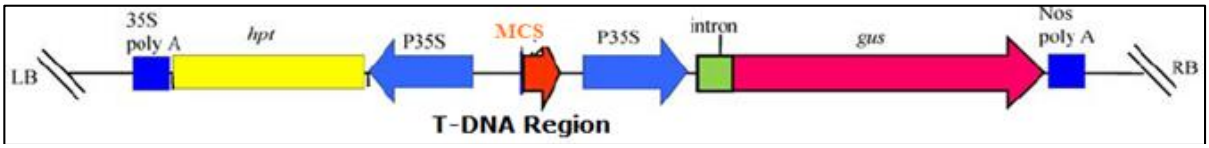

Fig 1: A strain of Agrobacterium tumefaciens, LBA4404 harbouring pCAMBIA 1301contains gene encoding hpt (hygromycin phosphotransferase) and an intron-containing β-glucuronidase (gus-int) in the T-DNA region (Fig.1) conferring resistance to hygromycin.

Callus induction and proliferation

Healthy seeds of three different rice varieties mentioned above were dehusked manually and washed with 4% (v/v) sodium hypochlorite solution containing 2-3 drops of Tween-20 for 20 minutes. The treated seeds were thoroughly rinsed with distilled and then washed thrice with sterilised distilled water, in laminar air flow chamber. The sterilised seeds were transferred to sterile tissue paper to remove moisture. These seeds were then inoculated on sterile MS (Murashige and Skoog, 1962) [24] and N6 (Chu, 1978) [3] medium containing 3.0% maltose and supplemented with, 2.5mg/L 2,4-dichlorophenoxyacetic acid (2, 4-D) and combination of 2,4-D (2.50mg/L) with kinetin (Kin) (0.5mg/L) and 2,4-D (2.5mg/L) with 0.5mg/L kinetin and 0.5mg/L α-naphthalene acetic acid (NAA) separately (Table 1). The pH of the media was adjusted to 5.8 and 8% agar was added before autoclaving it for 15min at 121 ºC. The cultures were incubated at 25±2 ºC in the dark condition. The white, friable embryogenic calli were excised and transferred to the fresh medium containing same concentration of growth regulators for further growth.

Table 1: Different callus induction medium with various concentrations of growth regulators

Media composition	Growth regulators used	Concentration of growth regulators (mg L^{-1})
N1	N6+2,4-D	2.5
N2	N6+2,4-D+KIN	2.5+0.5
N3	N6+2,4-D+KIN+NAA	2.5+0.5+0.5
M1	MS+2,4-D	2.5
M2	MS+2,4-D+KIN	2.5+0.5
M3	MS+2,4-D+KIN+NAA	2.5+0.5+0.5

Regeneration from callus

The subcultured calli were exposed to three regeneration medium with different combination of growth regulators, MS medium with 3mgL^{-1} 6-benzyladeninopurine (BAP) plus 0.5mg L^{-1} NAA, 2mg L^{-1} Kin plus 0.5mg L^{-1} NAA, 3mg L^{-1} BAP plus 1mg L^{-1} Kin plus 0.5mg L^{-1} NAA (Table 2). These inoculated cultures were incubated at 25± 2 ºC under light with a 16/8h day/night photoperiod, with an average irradiance of 50 mmol m^{-2} s^{-1}.

Table 2: Different regeneration medium with various concentrations of growth regulators

Media composition	Growth regulators used	Concentration of growth regulators(mg L^{-1})
RMa	MS+BAP+NAA	3.0+0.5
RMb	MS+KIN+NAA	2.0+0.5
RMc	MS+BAP+KIN+NAA	3.0+1.0+0.5

Rooting and acclimatization

After 21 days of inoculation, the regenerated shoots were excised aseptically and transferred to two different rooting medium, half strength MS medium without growth regulator, half strength MS medium plus 1mg L^{-1} NAA supplemented with 3% sucrose and 0.7% agar. The cultured were kept for a photoperiod for complete initiation of roots. The plants were taken out from the medium and washed under running tap water and then transferred to soil rite for 10 days. Finally the plants were transferred to greenhouse condition.

Transformation

The Overnight culture of LBA4404 harbouring pCAMBIA 1301containing *hpt* and *gus* gene was resuspended in liquid MS medium with an OD<1.0. The subcultured embryogenic calli were co-cultivated in bacterial suspension with different concentration AS (50µM-200 µM) for 20 min. The calli were blot dried on sterile filter paper. These calli were transferred to co-cultivation medium incorporated with various concentrations of AS (50µM-200 µM) with Whatmann #1 filter paper on the surface. The infected calli were incubated at 25± 2 ºC for 72 hours in the dark.

Assay of Putative transformants

After 72 hours of co-cultivation with *Agrobacterium*, the embryogenic calli were subjected to GUS assay following the method of Jefferson (1987) [12]. The samples were incubated in dark at 37 ºC for 24-48 hrs. The transformation frequency was calculated by the ratio between numbers of calli expressing GUS with total number of calli (20 calli per exeperiment, in triplicates) kept for assay.

Statistical analysis

All statistical analysis was performed using Graph Pad Prism 7.04 (USA).

Results

KIN and NAA play variable role on callus induction

For evaluating the effect of growth hormones on callus induction, 2, 4-D was used with MS and N6 basal media. In addition to 2, 4-D, Kinetin and NAA were also used in different combinations. In case of IR64 and Swarna, N1 was noted to be the best among the six different media tested for callus induction. In IR64 variety, N6 medium with only 2,4-D (2.50mgL^{-1}) resulted in 90% callus induction, while MS with 2, 4-D (2.50mgL^{-1}) plus kinetin (0.5mgL^{-1}) showed 80% callus induction efficiency after 10 days of inoculation (Fig. 4b). Comparatively the callus was compact, embryogenic and creamy white in texture when only 2, 4-D was used, but addition of kinetin (0.5mgL^{-1}) and NAA (0.5mgL^{-1}) influenced negatively on induction and compactness of calli. Similarly, in case of Swarna, N6 medium with 2, 4-D (2.50mgL^{-1}) was found to be most suitable (63.33±2.72%) for callusing after 14 days (Fig. 5c). Contrastingly, positive impact of kinetin and NAA addition on induction of calli were observed in Naveen cultivar. N6 medium with 2, 4-D (2.50mgL^{-1}), kinetin (0.5mgL^{-1}) and NAA (0.5mgL^{-1}) produced highest number of calli (70±0.943%) within 14 days of inoculation (Fig. 5a). Moreover, these calli were proliferative and bigger in size (Fig.2c) compared to those induced in other callus induction media.

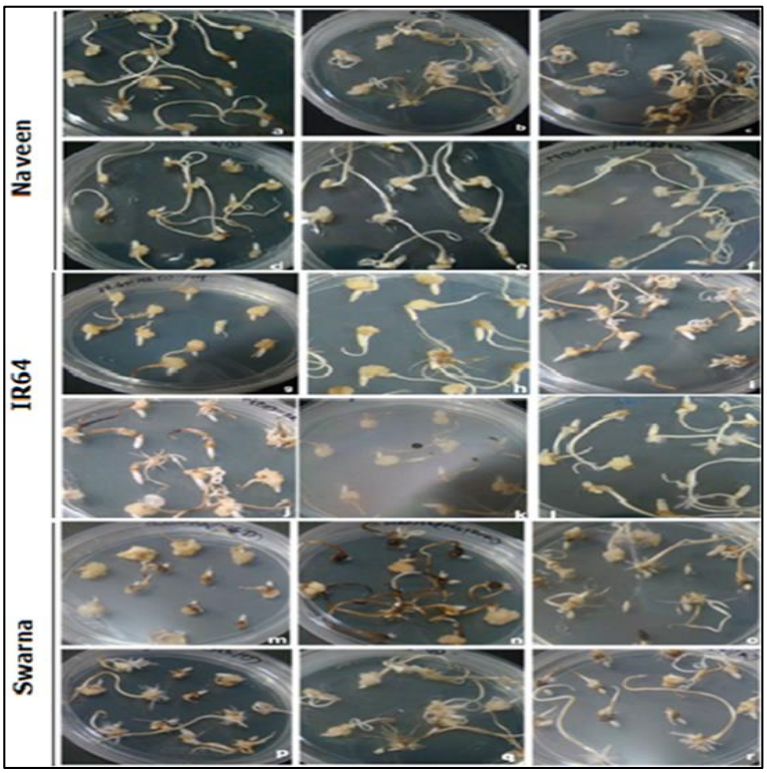

Fig 2: Callus initiation on different induction medium. Three indica rice cultivars i.e. Naveen, IR64 and swarna were used here.(a,g,m) callus induction medium N1(b,h,n) callus induction medium N2 (c,i,o) callus induction medium N3 (d,j,p) callus induction medium M1 (e,k,q) callus induction medium M2 (f,l,r) callus induction medium M3.

Regeneration

Further mature seed derived calli were used for regeneration experiment. Significant differences were noticed between the genotypes on different regeneration media. In case of Naveen variety, maximum green shoot regeneration was achieved on RMb medium (Fig. 5e). RMb media showed 59±0.81% regeneration efficiency within 17-18, whereas only 38±1.63% of calli regenerated on RMc within 20-21 days (Fig. 5e). On the contrary, calli cultured on RMa medium exhibited green spots development and turned green, but no shoots were developed. In case of IR64 and Swarna, highest regeneration frequency was observed on RMc medium (Fig. 3i,o). MS medium supplemented with 3mg L^{-1} BAP plus 1mg L^{-1} Kin and 0.5mg L^{-1} NAA (RMc) regenerated 64.6±1.44% green shoots within 16-18 days (it seems too early) of inoculation of IR64 calli (Fig. 5d), while moderate regeneration (42 ±2.5%) was recorded on RMb within 21 days. Swarna calli showed 35±1.96% regeneration when cultured onto 3mg L^{-1} BAP plus 1mg L^{-1} Kin and 0.5mg L^{-1} NAA (Fig. 5d), but no shoots were found on RMa and RMb medium (Fig. 3m,n). Furthermore, addition of BAP to the medium with kinetin and NAA enhanced multiple shoot regeneration in all three mentioned cultivars, best was found in IR64 cultivar.

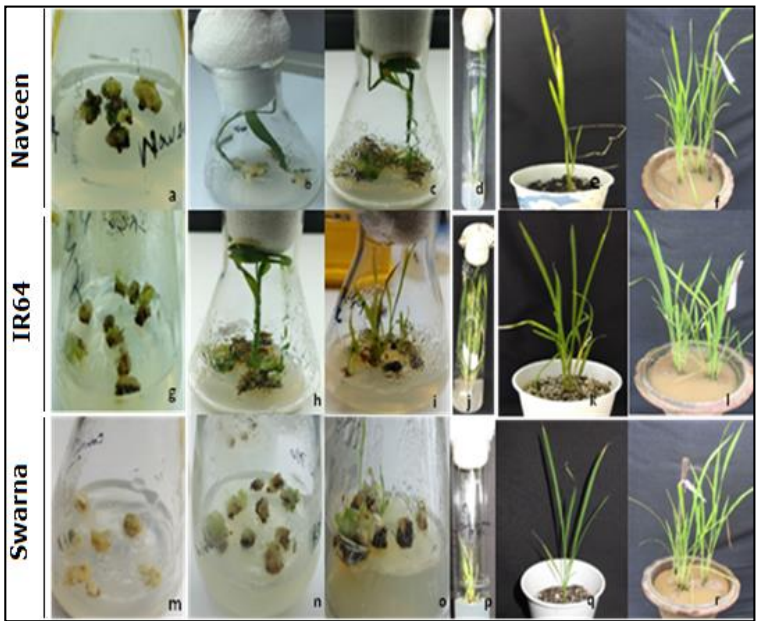

Fig 3: Establishment of plants starting from shoot regeneration to plant acclimatization in green house (a-o). Development of shoots from calli in different regeneration media; RMa (a,g,m), RMb (b,h,n) and RMc (c,i,o). d,j,p - in vitro regenerated shoots in rooting medium. e,k,q-rooted plantlets transferred to soil rite. f,l,r- well rooted plants transferred to pot in green house.

Rooting and acclimatization

Finally elongated shoots were rooted on half-strength MS medium supplemented with and without growth hormone NAA (1.0 mgL^{-1}). When green and healthy regenerated shoots transferred to half-strength MS roots were found to be developed after 2 weeks (Table 3). Profuse rooting was achieved in all three cultivars (IR64, Naveen, Swarna) when cultured onto MS medium supplemented with NAA (1.0 mgL^{-1}) in 14-17 days (Table 3). Maximum rooting (number of roots 24.34±0.720) was achieved in IR64 with supplementation of NAA (1.0 mgL^{-1}) within 14 days (Table 3; Fig. 4) followed by Naveen (number of roots 21.0±0.472) and Swarna (17.70±0.982) cultivars of rice in 15 and 17 days, respectively. The *in vitro* regenerated plantlets after attaining a height of 2–3 inches were then taken out from rooting media and transferred to the natural conditions after acclimatization.

Table 3: Profuse rooting in shoots of Indica rice cultivars cultured on MS culture medium with various concentrations of growth regulators

Name of cultivars	1/2MS+ NAA(1mg/L)		1/2MS	
	Average number of roots	Number of days	Average number of roots	Number of days
Naveen	21.0 ± 0.472	15	9.33±0.72	**17**
IR64	24.34 ± 0.720	14	7.76 ± 0.272	19
Swarna	17.70 ± 0.982	17	6.0 ± 0.471	20

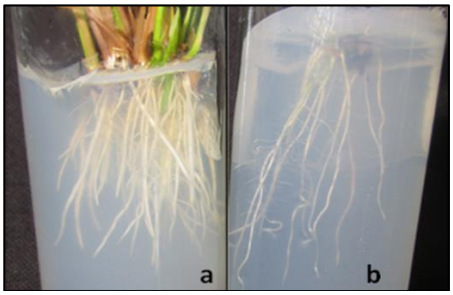

Fig 4: Profuse rooting of IR64 cultured on rooting media (a) MS medium with NAA (1mgL^{-1}) (b) MS medium without growth regulator

Effect of acetosyringone on transformation

Further investigation was carried out to determine the optimum concentration of acetosyringone during co-cultivation, for which various concentrations (50, 100, 150, 200µM) of AS were incorporated separately. Our analysis revealed difference in GUS expression between cultivars in different concentrations of AS. However, significant level of GUS expression was observed at 100 µM AS in case of Naveen and 150 µM in IR64 and Swarna (Fig. 5f) after which GUS expression combined with necrosis was observed.

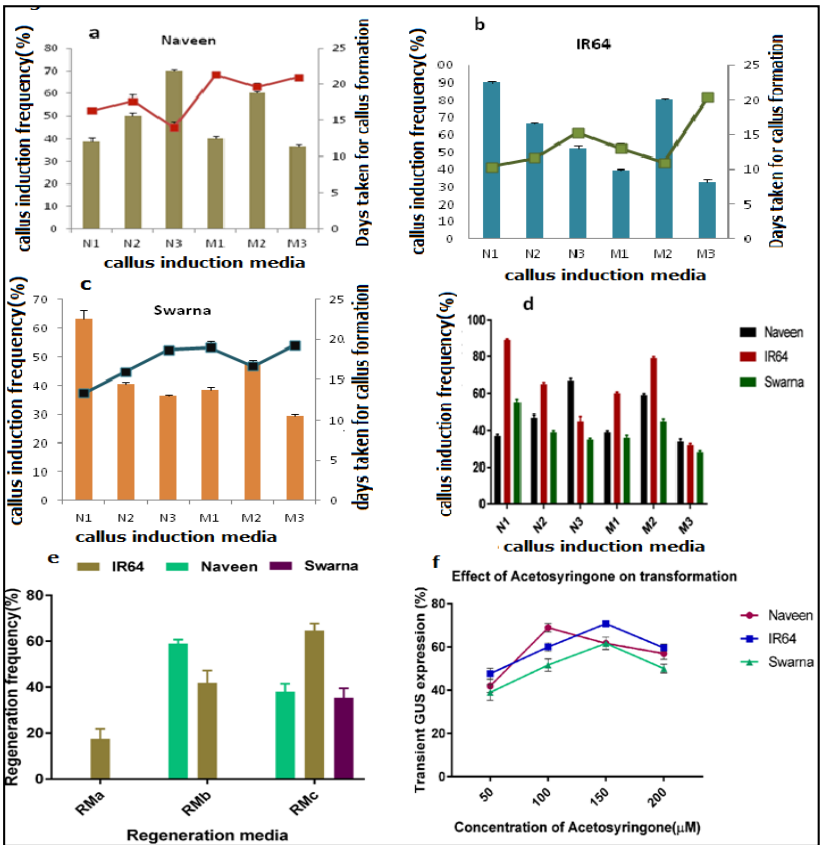

Fig 5: Effect of different growth hormones and acetosyringone in plant tissue culture and transformation. (a,b,c) Effect of different media on callus induction in Naveen, IR64 and (d) Callus induction frequency of three indica rice genotypes (e) Regeneration frequency of calli from each cultivar in three different media with varying hormone concentration (f) Effect of acetosyringone on transformation. Data shown are mean ± standard error of three independent experiments.

Discussion

This research aimed to improve the overall efficiency of callus induction, regeneration and transformation of three different Indica rice cultivars. Each species or genotype lives in an optimal condition that differs from others. So, it is difficult to choose a common media that would be suitable for each plant species and genotype since their nutritional requirement varies. Callus induction as well as regeneration efficacy of explants tissue is influenced not only by type of explants but also by the culture medium with different composition of plant growth hormones (Revathi and Pillai, 2011) [30]. It is likely that one medium cannot provide optimal culturing conditions for different genotypes within the same species. Among all the explants used for embryogenic calli development like leaf base (Ramesh *et al*., 2009) [29], leaf sheath cells (Bhattacharya and Sen, 1980) [2], root (Sticklen, 1991) [37], immature embryo (Datta *et al*., 2000; Datta *et al*., 1999; Koetje *et al*., 1989) [5, 4, 18], and mature seed-derived embryo (Karmakar *et al*., 2016; Molla *et al*., 2013, 2016; Sahoo *et al*., 2011) [13, 23, 22-23, 32], mature seeds derived embryogenic callus culture has been widely used in rice in vitro culture studies because of their easy availability throughout the year. Mature de-husked seeds of three widely cultivated rice genotypes were used as explants to dedifferentiate into calli on six different media composition to study the callus induction efficiency.

Significant differences in callus induction and plant regeneration have been reported among the rice genotypes (Lee *et al*., 1999; Khanna and Raina, 1998; Hartke and Lörz, 1989; Abe and Fustuhara, 1986) [20, 16, 9, 1]. The results of the present investigation also showed that callus induction frequency was genotype dependent and was largely affected by media composition and their interactions. The rate of callus induction frequency in Naveen genotype varied from 36.3% to 70% across the media. Maximum embryogenic calli production (70%) was achieved on N6 media containing 2.5mgL^{-1} 2, 4-D, 0.5mgL^{-1} Kinetin and 0.5mgL^{-1} NAA, which is significantly higher than that achieved with other combinations of MS and N6 media. Here stimulatory effects of kinetin and NAA in combination with 2, 4-D facilitated callus induction within less time period. The callus induction frequency varied from 33% to 90% in the case of IR64 genotype, where as in Swarna, it varied from 29.6% to 63.3%. In both cases, N6 media containing 2.5mgL^{-1} 2, 4-D induced maximum calli. Second highest calli induction efficiency was observed on MS medium supplemented with 2.5 mgL^{-1} 2, 4-D and 0.5mgL^{-1} Kinetin in all three genotypes {i.e. Naveen (60.3%), IR64 (80.3%) and Swarna (48%)}. Most of the earlier studies used MS medium with 2, 4-D as the only growth regulator for the callus induction of Indica rice (Deo *et al*., 2009; Katiyar *et al*., 1999) [7, 15]. Previous works also showed that Indica rice calli were induced from MS media with different concentration of auxin and cytokinin supplement (Sankepally and Singh, 2016; Krishnan *et al*., 2013) [33, 19]. Here, we used N6 along with MS medium with some modification in hormone composition and concentration. From the present study, it was found that N6-based media with different auxin and cytokinin were more productive for calli induction than MS-based media. Significant variation in response to callus induction between three varieties (p<0.001) were observed.

The well-developed embryogenic calli were transferred to regeneration media and incubated in the light. After 7-10 days, green shoot primordia appeared on calli of IR64 and Naveen cultivars on RMc and RMb, respectively. Highest multiple shoots regeneration efficiency (65%) was achieved in IR64 in MS media with BAP (3.0mgL^{-1}), Kinetin (1.0mgL^{-1}), and NAA (0.5mgL^{-1}). The stimulatory effects of BAP in combination with Kinetin and NAA have previously been reported to facilitate regeneration in Indica rice cell cultures (Sahoo *et al*., 2011) [32]. Similarly, MS with Kinetin (2.0mgL^{-1}), and NAA (0.5mgL^{-1}) exhibited greatest percentage of regeneration (64.6%) in Naveen genotype. RMc was found to be suitable for regeneration in Swarna (35%). The observed result revealed that supplementation of BAP was effective for multiple shoot regeneration. Higher concentration of BAP and kinetin may initiate cell division and play a key role in somatic embryogenesis and plant regeneration (Rueb *et al*., 1994) [31]. NAA was used in all three RM because the regenerated plantlets are reported to be weaker without NAA (Rahman *et al*., 2010) [27]. Plant regeneration frequency in different media showed a significant difference (p<0.0001).

The regenerated shoots were transferred to MS basal medium with NAA (1.0mgL^{-1}) and without NAA for root proliferation. Interestingly, the plantlets regenerated from mature embryo-derived calli developed a prolific root system in MS basal medium with NAA (1.0mgL^{-1}) in less time than the media devoid of NAA (Do you have any data showing better root formation in NAA containing media? If so, present the data with a simple table or graph). However, a majority of the earlier studies reported the use of basal MS media without any hormone supplement for root induction (Swain *et al*., 2018; Molla *et al*., 2013, 2016; Sahoo *et al*., 2011) [38, 22, 32]. High auxin concentration leads to the development of a good root system (Sankepally and Singh, 2016) [33]. A significant difference was observed in terms of numbers of roots (p<0.001) in all three cultivars. The number of roots developed on MS basal with NAA was significantly more than that developed on MS basal medium without NAA (p<0.001). However, no significant difference was observed between three cultivars of each treatment (p=0.134). When the in vitro regenerated plantlets of three cultivars transferred to pots, they survived, which implied good adaptability of regenerated plants in ambient environmental conditions.

In monocots, acetosyringone (AS) plays a vital role in transformation (Mohanty *et al*., 1999) [21] that allows Agrobacterium to infect the calli by activating virulence gene expression (Stachel *et al*., 1985) [36]. Hoque *et al*. (2005) [11] reported that the absence of acetosyringone in infiltration media results in no transformation. We observed transient GUS expression in calli in all cultivars but with variation in percent response and staining. Among the various concentrations tested 100µM AS in co-cultivation media showed significant GUS expression in Naveen cultivar compared to higher and lower concentration of acetosyringone (50, 150 and 200µM). In the case of IR64 and Swarna, the highest percentage of transient GUS expression was noticed in 150µM AS on co-cultivation and infiltration media. Significant differences in transient expression were observed among the genotypes in different concentration of AS used (p<0.0001). These data are in consonance with several earlier studies (Karthikeyan *et al*., 2012; Hiei *et al*., 1994) [14, 10]. However, use of 150µM (Sahoo *et al*., 2011) [32] and 200µM (Shri *et al*., 2013; Datta *et al*., 2000) [35, 5] AS has been described in rice transformation. In the present study, in vitro callus induction, regeneration and rooting protocol of three rice cultivars were modified for better performance. Moreover, Agrobacterium-mediated transformation process (co-cultivation) were also standardized that would be helpful in the development of transgenic Indica rice with the

incorporation of important genes for improving rice for different traits.

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