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STUDIES ON THE EFFECT OF GENOTYPE AND EXPLANT TYPE ON CALLOGENESIS AND ORGANOGENESIS IN *INDICA* RICE

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Abstract

The purpose of the research study is to select the best rice explant and variety for *Agrobacterium* mediated genetic transformation studies. *In vitro* callus induction and regeneration frequency of two explants i.e. immature embryo and mature grains of 9 rice varieties (*Oryza sativa* L.*)* was investigated by culturing explants source on N_6 media supplemented with 2, 4-D at 2mg/l for callus induction. Results indicated that mature seed explant produced significantly high number of calli as compared to immature embryo. Two types of calli were distinguished, designated as type-I calli and type-II calli. Regarding the quality of callus, type-I calli (produced from scutellum of mature grains) which were embryogenic produced higher plant regeneration frequency than type-II calli which were non-embryogenic. Mature-seed scutella calli and immature embryo-derived calli (after three weeks of culture) of rice varieties were transferred on regeneration medium i.e., MS salts and vitamins, 3% sucrose, 3% sorbitol, 2g/l casine hydrolysate, NAA 1.0 mg/l, kin 2.5 mg /l and BAP at 0.5mg/l. The highest regeneration capacity was observed in DR-83 from mature seed derived calli followed by Basmati 385. After hardening the plantlets were transferred to soil.

Introduction

Successful genetic transformation in rice is possible when efficient and reproducible plant regeneration protocols are available for the particular cultivar (Rachmawati & Anzai, 2006). In *indica* rice, several applicable tissue culture methods for subculture and regeneration have been reported (Peng & Hodges 1989; Aldemita & Hodges 1996; Rashid *et al*., 1996; Khanna & Raina, 1998). But even though a great deal of progress has been made in *In vitro* studies of rice, the results have been still unsatisfactory in producing transformants as *indica* rice is not only difficult for inducing embryogenic calli but also its calli are easy to get brown (Qian *et al*., 2004). Furthermore *indica* rice is known to have recalcitrant nature of genotypes to regenerate (Aldemita & Hodges, 1996; Rashid *et al*., 1996; Cho *et al*., 2004) and has absence of resistance against diseases of viral, fungal and bacterial origin. The only feasible and economical way for controlling these diseases is to increase the use of resistant varieties. Keeping in view the importance of genetic resistance for disease control, studies have been conducted on screening rice varieties to evaluate their resistance. Considerable efforts are being directed towards the improvement of important agronomic traits of rice through biotechnological techniques (Ge *et al*., 2006). Many reports demonstrated that regeneration from putative transgenic callus were critically lower than expected due to cultivars that showed different regeneration efficiencies depending on the media. The most desirable strategy to improve the regeneration as well as transformation efficiency is the employment of optimized cultural medium capable of 1) producing embryogenic calli showing suitable morphologies, 2) Maintaining and proliferating the calli with viability, 3) Enhancing the frequency of green tissue formation and shoot regeneration with multiple shoots, 4) Reducing callus browning, 5) producing antibiotic selectable marker gene and 6) improving transformation efficiencies in independent events with various rice cultivars *via* optimized regeneration medium for each cultivar that would lead to desirable efficiencies of shoot regeneration to achieve high transformation efficiencies (Cho *et al*., 2004; Wanichananan *et al*., 2010). Production of callus and its subsequent regeneration are the prime steps in crop plant to be manipulated by biotechnological means (Bhaskaran & Smith, 1988) i.e., a prerequisite for a high efficiency transformation technique by Agrobacterium mediated transformation, is a highly efficient and robust tissue culture system. The potential for callus formation and regeneration have been reported to be varietal characteristic and efficient regeneration in *indica* rice still posses a major problem for genetic manipulation through innovative approaches (Toki, 1997). So the present work was carried out to study the effect of cultivar and explant type on *In vitro* regeneration of rice. This study would be helpfull in providing the best variety and explant source of rice for transformation studies.

Materials and Methods

The experimental material consisted of nine rice (*Oryza sativa* L.) varieties including four Basmati rice varieties i.e., Basmati 370, Basmati 385, Basmati 2000 and Super Basmati and five coarse varieties i.e., Pakhal, JP-5, Swat-1, DR-83 and KS-282 supplied from the Rice Breeding Program, NARC, Islamabad. Grains were sterilized by protocol described by Rashid *et al*., (1996). Surface sterilized grains of all varieties were inoculated on solid N_6 (Chu *et al*., 1975) media in a Laminar airflow cabinet. Two grains per test tube were planted. The cultures were incubated in culture room at 20±2°C, 10-h photoperiod at 48-µ mol m^{-2} s^{-1} for three weeks for callus induction.

Immature seeds were harvested from the spikelets and obtained from rice fields of Rice Breeding Program, NARC, Islamabad. They were dehusked, surface sterilized as above. Embryos were then excised and transferred on to the N6 medium with the rounded scuteller side up (scutellar surface side was exposed and the plumule-radical axis was in contact with the medium) or down (the scutellar surface side was in contact with the medium). Callus induction frequency for all varieties was recorded 4-5 days after inoculation. Callus proliferation rate and

callus quality was recorded at 2-3 week after inoculation in all rice varieties for all treatments.

The calli which contained embryogenic potential were designated as Type I (white to light yellow in color, compact and friable) as well as non-embryogenic potential Type II (mucilaginous and smooth). After three weeks the embryogenic part of calli were then inoculated on regeneration media i.e. MS salts and Vitamins (Murashige & Skoog, 1962), 3% sucrose, 3% sorbitol, 2g/l casine hydrolysate, NAA 1.0 mg/l, kin 2.5 mg /l and BAP at 0.5mg/l. These cultures were placed in growth room at 25˚C ± 2˚C with 10-h photoperiod at 48 µ mol m^{-2} s^{-1} for 2-3 weeks. After shoot regeneration, plantlets were transferred to MS media without growth regulators for root initiation. Three observations were recorded for regeneration efficiencies (green spotting, shoot regeneration and callus browning) of each variety on each medium in eight weeks time period. The data of all tissue culture experiments was analyzed in completely randomized design (CRD). Each treatment was replicated thrice and ten test tubes in the case of callus induction and eight flasks in the case of regeneration were used for each replication. The collected data was analyzed by using Minitab 13 statistical software and the means were compared by least significance difference test (LSD) using MSTAT-C at 0.01 level of significance.

Results and Discussion

The choice of a suitable explant source as starting material for infection of *Agrobacterium* is one of the most important factors for generating transgenic plants. Production of embryogenic calli with high regeneration capacity is a prerequisite for highly efficient transformation of rice (Ge *et al*., 2006). Many efforts have been made to identify suitable explants in rice to induce embryogenic calli under appropriate culture condition. A wide range of organs, which include meristematic cells, such as mature seed (Sivamani *et al*., 1996), immature embryo (Lee *et al.*, 2002), have been reported to be good resources for production of embryogenic calli. Although some researches used immature embryo derived calli (Aldemita & Hodges, 1996), but other researchers found that calli initiated from scutellum of mature grains (compared with immature embryo or tissues) was excellent starting material for transformation of rice by *Agrobacterium* (Hiei *et al*., 1994; Rashid *et al*., 1996; Toki, 1997; Cho *et al*., 2004, Ge *et al*., 2006 and Khan *et al*., 2007). In the present study, it was observed that calli initiated from mature grains of all rice varieties had embryogenic potential as reported by Ge *et al*., (2006).

During the study, two different explants, immature embryo and mature seeds were used for callus induction on N_6 (Chu *et al*., 1975) medium containing 2, 4-D at 2mg/l. Average callus induction rate of embryogenic calli of 9 rice varieties was 84.16% from mature grains which showed comparably higher level than that immature embryo i.e., 63.6% (Fig. 1). In general, the proportion of induced calli in each variety from mature seed was higher than that from immature embryo (Table 1). Statistically the effect of variety from both explants was highly significant at 1% level for callus induction ability, whereas effect of explant source and the interaction between variety x explant source was non-significant.

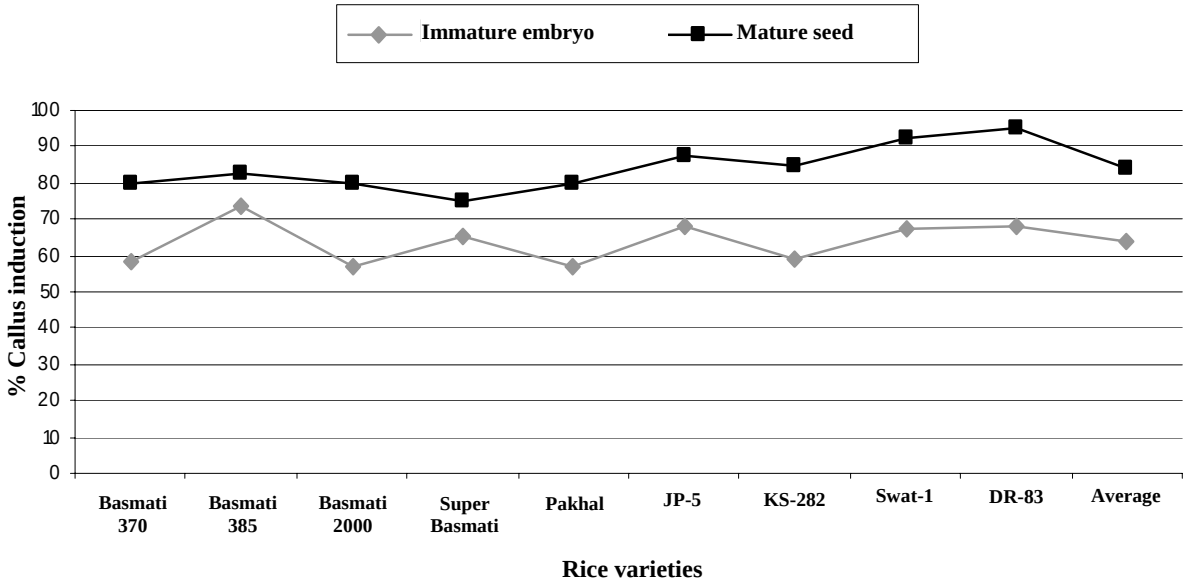

Fig. 1. Average callus induction rate of nine rice varieties from seed and immature embryo and N_6 media.

Two types of calli were distinguished as Embryogenic (Type I) and Non-embryogenic callus (Type II). Immature embryo produced more Type II calli. Calli which were slick and yellowish–compact globular developed from embryo of rice grain and had potential to develop into plantlet when maintained on the same N6 media (Fig. 2a-e). While calli which were comparably whitish, semi-translucent, soft, friable and loose in texture recognized as non-embryogenic (Fig. 2f-l). Non-embryogenic calli were devoid of embryoid-like structures and were unable to develop into plantlets when maintained on the same N6 media. Callus growth was different in almost all the varieties, Basmati 385, Swat-1, DR-83 and JP-5 showed good size calli where as Pakhal Super Basmati and KS-282 showed small size calli (Table 2).

Table 1. Effect of explant source on callus induction among rice varieties.

Rice variety	Average number of callus induction (CI)		Mean of each variety*
	Immature ambryo	Mature seed	
Basmati 370	23.00 ±1.2	32.00 ± 4.2	27.50 cd
Basmati 385	29.00 ± 4.0	33.00 ± 1.5	31.00 abc
Basmati 2000	22.00 ± 2.3	32.00 ± 3.8	27.00 d
Super Basmati	26.00 ± 3.8	30.00 ± 6.1	28 .00 cd
Pakhal	22.00 ± 2.3	32.00 ± 1.0	27.00 d
JP-5	27.00 ± 5.7	33.00 ± 4.6	30.00 abc
KS-282	23.00± 1.2	34.00 ± 7.2	28.50 bcd
Swat-1	27.00 ± 4.2	37.00 ± 3.8	32.00 ab
DR-83	28.00 ± 3.5	38.00m ± 4.6	33.00 a
Mean of each treatment	25.22	33.44	29.33

*LSD 0.01 for varieties = 11.29

The data is average of three replicates ± SE

** Probability was checked by using ANOVA at 0.01 level of significance

Different alphabetic keys notify significant difference

Embryogenic calli were selected on the basis of their physical properties as described by Cho *et al*., (2004). Morphology of embryogenic calli was slick, yellowish-compact type with globular shape, soft and friable. DR-83, Swat-1 and KS-282 showed more potential for callogenesis (Table 1). The significant difference between nine varieties for callogenesis under the same nutritional conditions indicated that callus induction frequency is genotype specific. These findings are in accordance with the reports of several researches (Khanna & Raina 1998; Abassi *et al*., 2000; Noor *et al*., 2005). The callus of Basmati 385, Basmati 2000, Super Basmat, Swat-1 and DR-83 were found more embryogenic than Basmati 370, Pakhal JP-5 and KS-282 (Table 2). The effects of cultivar (1%) and media (1%) were non-significant, whereas cultivar x media interaction had significant effect on callusing. Bano (1997) reported variation in callus proliferation as a result of different combinations and concentrations of growth regulators. To determine the regeneration ability the initiated calli obtained from both explant sources were transferred to a regeneration medium. On regeneration media, Type I calli tended to show regeneration for green spotting and shoot regeneration and less of browning. Generally, the calli having high regeneration potential formed green spotting on the surface with fast growth for 7-10 days of cultivation (Fig. 3a) and consequently, 0.2-1.0 cm size shoots emerged at 14th day (Fig. 3b). The cultures were maintained for one and a half month. However, the calli of type II showed callus browning with relatively slow growth and finally died (Fig. 3c&d). Our results are similar to Rachmawati & Anzai, (2006) who reported that type-I calli produced much higher plant regeneration than type-II calli. In almost all rice varieties, a maximum regeneration was observed from calli derived from mature seed, while calli derived from immature embryo showed poor response. Statistically significant difference in average number of regenerated shoots (Table 3) was observed among varieties and also among explant type. The interaction between variety and explant type was also significant. DR-83 produced highest number of shoot regeneration followed by Basmati 385. The ratio of the three responses (green spotting, plant formation and callus browning) and their changes varied depending on rice variety and kind of medium used. In general, type I calli of rice varieties showed higher rate of shoot regeneration compared to type II calli. Shoot regeneration capacity and plantlet-regeneration capacity were significantly affected by genotype and medium independently as well as by the interaction effect between genotype and medium.

Table 2. Genotypic responses to callus induction.

Rice varieties	Callus growth	Morphology of callus	Effect
Basmati 370	+++	Yellowish-white, soft, friable and somewhat compact	Non-embryogenic (Type II)
Basmati 385	++++	Yellowish-white, compact, relatively dry and nodular appearance callus	Embryogenic (Type I)
Basmati 2000	+++	Copious, compact and embryogenic callus	Embryogenic (Type I)
Super Basmati	++	Soft, friable and somewhat compact	Embryogenic (Type I)
Pakhal	+++	Yellowish, pale, loose texture, watery	Non-embryogenic (Type II)
JP-5	++++	Soft, granular appearance and loose textured callus	Non-embryogenic (Type II)
KS-282	++++	Yellowish, pale, loose texture, watery	Non-embryogenic (Type II)
Swat-1	++++	Whitish green, compact callus	Embryogenic (Type I)
DR-83	++++	Whitish green, compact callus	Embryogenic (Type I)

Sign for callus growth

+ = Small size; ++ = Medium size; +++ = Large size; ++++ = Very large size

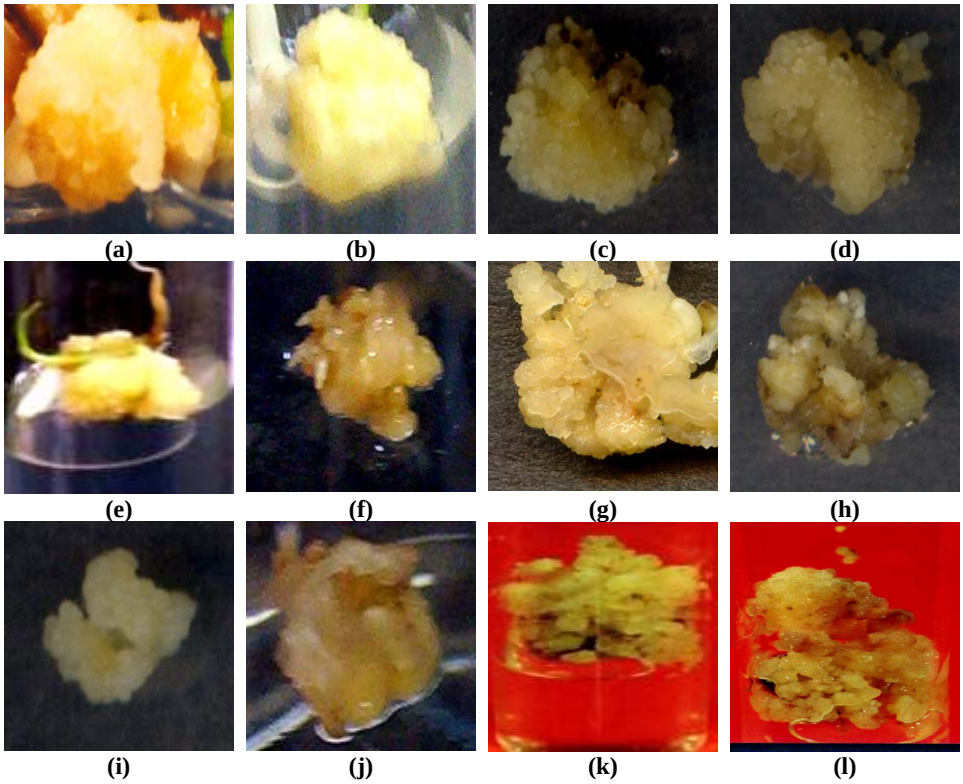

Fig. 2. Embryogenic and non-embryogenic calli
a – e)= Embryogenic calli; f – l)= Non-Embryogenic calli

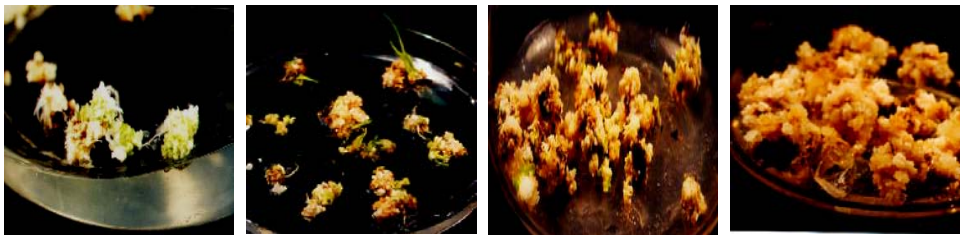

Fig. 3. Regeneration response of the callus
a)= Formation green spotting; b)= 0.2-1.0 cm size shoots emerged; c & d)= The calli of low regeneration varieties showed callus browning with relatively slow growth and finally died

Table 3. Average number of regenerated shoots.

Rice variety	Immature embryo	Mature seed	Average
Basmati 370	2.00 ± 0.6 ^{c}	14.0 ± 2.3 ^{abc}	8.00
Basmati 385	3.00 ± 0.6 ^{bc}	17.0 ± 1.9 ^{a}	10.0
Basmati 2000	3.00± 0.6 ^{bc}	16.0 ± 1.7 ^{ab}	9.50
Super Basmati	2.00 ± 0.6 ^{c}	15.0 ± 0.6 ^{ab}	8.50
Pakhal	4.00± 0.6 ^{bc}	10.0 ± 1.2 ^{bc}	7.00
JP-5	9.00± 0.6 ^{a}	8.00 ± 0.6 ^{c}	8.50
KS-282	2.00± 1.7 ^{c}	14.0 ± 2.9 ^{abc}	8.00
Swat-1	6.00± 0.0 ^{ab}	13.0 ± 0.9 ^{abc}	9.50
DR-83	1.00± 0.6 ^{c}	18.0 ± 1.2 ^{a}	9.50
Mean of each treatment	3.55** ^{b}	14.00** ^{a}	

LSD 0.01 for variety = 9.933
LSD 0.01 for treatment = 6.678
The data is average of three replicates
** Probability was checked by using one way single factor ANOVA at 1% level of significance ± SE
Different alphabetic keys notify significant difference

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