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Effect of Culture Media on Improving Anther Culture Response of Rice (*Oryza sativa* L.)

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***Abstract* – Anther culture has been an effective breeding method to improve desirable traits, since it allows fast achievement of genetically homozygous lines. The present studies were carried out to evaluate the response of rice anthers for high frequency callus induction and green plant regeneration in different culture media and effect of in-culture mannitol treatment in callus induction media. The effect of genotype, media composition and their interaction were significant ($p<0.001$) for both callus induction and green plant regeneration. Among the media, He2 medium produced highest callus induction, green plant regeneration and least albino plant development of 40.64%, 40.93% and 3.72%, respectively. Out of 13 genotypes evaluated, IR58025B with *eui* (25eB) was highly responsive for both callus induction as well as green plant regeneration. Mannitol with a concentration of 100 mg/L enhanced anther response by less than 1.5% compared to untreated anthers. This information can be use in development of improved parental lines through doubled haploidy.**

***Keywords* – Callus Induction, Doubled Haploidy, Medium, Regeneration.**

I. INTRODUCTION

Doubled haploidy through anther culture together with gene stacking for multiple traits is an attractive approach to fix agronomic traits. More than 280 varieties have been produced with the use of doubled haploidy in several crops (http://www.scri.sari.ac.uk/assoc/COST851/COSThome.htm), with majority of the protocols referred to as anther culture [1]. Improvement of an existing parental line by pyramiding or stacking of value- added traits is a time consuming process through conventional breeding. The employment of doubled haploidy and Marker Assisted Selection (MAS) can enhance the efficiency of pyramiding or stacking process. It allows breeders to improve and stabilize the existing parental line with desired traits in a single year, reducing the time required for new variety development by up to five years if through conventional breeding. The breeding efficiency is considerably increased by the use of doubled haploid (DH) population, which express both dominant and recessive genes in their homozygous and heterozygous nature, making selection especially effective for traits controlled by recessive genes and multiple minor effect genes (QTLs).

Rice anther culture response is genotype specific and successful use of anther culture technology in varietal development depends on the efficient production of adequate numbers of DH plants for field evaluation and selection [2 and 3]. The efficiency of this method is influenced by several factors such as genotype of the donor plants [4 and 5], pretreatment [6], media composition [7], and culture condition [8 and 9]. In general, indica cultivars of rice exhibit poorer androgenic response than the japonica cultivars [10]. The nutrient medium not only provides nutrition to the microspores but also directs the pathways of embryo development. The source of carbon, macronutrient (particularly the form in which nitrogen is supplied in the medium), micronutrients and plant growth regulators may determine whether the androgenesis will be initiated or not.

A carbohydrate source is essential in anther culture because of its osmotic and nutritional effects [11]. Maltose has been shown to be superior source of carbohydrate than sucrose for androgenesis in several species, including cereals [12 and 13]. Nitrogen can be supplied to the culture medium in an inorganic or organic form. The inorganic nitrogen is usually introduced in the form of nitrate or ammonium ions while nitrogen in the organic form can be supplied as vitamins and amino acid supplements. The ratio of NO^{3-}:NH_4^+ has been observed to be an important determinant for success of anther culture in indica rice [14]. N6 medium is characterized by having high KNO_3 and $(NH_4)\ SO_4$, has proved to be very efficient for japonica rice anther culture. The indica cultivars require even lower level of $(NH4)^+$ ions. Organic nitrogen supplements such as casein hydrolysate (CH) which is a sources of calcium, several micronutrient, vitamins and amino acids added to the medium have been particularly beneficial for positive anther culture response [15] although a few reports suggest otherwise [16 and 17]. Micronutrients also play an important and sometimes crucial role in normal plant growth and development. Copper and Zinc are two important micronutrients influencing microspore embryogenesis [18]. The effect of plant growth regulators has been widely investigated in anther culture. The type and concentration of growth regulators as well as their interactive presence can be the deciding factors that would influence pollen embryogenesis [19].

Apart from the known inorganic salts and growth substances, various natural extracts such as yeast extract (YE), coconut water (CW) etc., are also claimed to be beneficial for increased callus formation and plant regeneration in anther culture [20 and 21]. However, some studies have shown that high frequency of callus induction and plant regeneration could be obtained on media without any such additives [22 and 23]. Studies showed that ethylene produced by plant cells in closed culture vessels is due to presence of auxin [24], sucrose [25] or calcium [26] in the callus induction medium. Addition of silver nitrate ($AgNO_3$) in the induction medium blocks the inhibitory effect of endogenously produced ethylene from excised anthers [27]. Silva [28] reported that low responsiveness of indica genotype is due to early senescence of cultured anthers and the senescence is likely to be accelerated with *in vitro* generation and

accumulation of the ethylene in sealed culture vessels. Thus the use of $AgNO_3$ as inhibitor of ethylene biosynthesis is likely to be advantageous in rice anther culture. Certain sugar alcohols such as mannitol [29] and sorbitol [30] had beneficial effects on rice anther culture. But the work done on effect of in-culture osmotic treatment on callus induction media is very less. Pande and Bhojwani [31] reported that in-culture mannitol treatment inhibited the androgenesis for most of the cold treated anthers, however promoted androgenesis for un-treated anthers. With increase in the concentration of mannitol, the frequency of number of green calli per 100 responding anther (anther culture efficiency) has decreased. It has been well documented that both attributes callus induction and green plant regeneration are highly influenced by culture components of medium and genetic make up of the genotypes [32, 16 and 17]. Thus, the present study was undertaken with an aim to evaluate the effect of different media and also the impact mannitol concentration on anther culture efficiency.

II. Material and Methods

Plant materials

Seeds of thirteen experimental genotypes (seven parents and six F_1s) were grown at Barwale Foundation farm, following standard agronomic practices (Table 1).

Code	Genotype	Characteristics
PA	Samba Mahsuri Sub-1	Donor for *Sub-1* gene
PB	IR-64 Sub-1	,,
PC	BR-11 Sub-1	,,
PD	TDK-1 Sub-1	,,
PE	Swarna Sub-1	,,
PF	Dular	Donor for *WC* gene
PG	IR58025 with *eui* (25eB)	
SA	25eB x Samba Mashuri Sub-1	F_1 with *eui* x *Sub-1* genes
SB	25eB x IR-64 Sub-1	,,
SC	25eB x BR-11 Sub-1	,,
SD	25eB x TDK-1 Sub-1	,,
SE	25eB x Swarna Sub-1	,,
WF	25eB x Dular	F_1 with *eui* x *WC* genes

Medium

Four basal media viz. N6 [33], B5 [34], He2 [46] and SK1 [16] with certain modifications based on the available literature were used for evaluation of anther culture response. The major modifications made are shown in the Table 2. Each medium supplemented with different concentration of hormones (2, 4-D, NAA, Kinetin), natural additives (YE or CH) and $AgNO_3$ as an ethylene inhibiting agent. MS [36] medium supplemented with 1.5 mg/L BAP, 1mg/L Kinetin and 1mg/L NAA was used for regeneration. To study the impact of mannitol on anther response with N6 medium, different concentrations of mannitol (100 mg/L, 200 mg/L and 300 mg/L) was added on to N6 medium, designated as $N6_{M1}$, $N6_{M2}$ and $N6_{M3}$, respectively.

Table 2: Composition of callus induction media used

Components (mg/L)	N6	B5	He2	SK1
$(NH_4)_2SO_4$	463	134	231	231
KNO_3	3535	3125	3181	3180
KH_2PO_4	400	0	800	540
$NaH2PO_4.H_2O$	0	150	0	0
$MgSO_4.7H_2O$	185	250	3.5	185
$CaCl_2.2H_2O$	166	150	166	440
H_3BO_3	1.6	3	1.6	6.2
$MnSO_4.4H_2O$	22.3	10	22.3	22.3
$ZnSO_4.7H_2O$	8.6	2	1.5	1.5
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.25
KI	0.8	0.8	0.8	0.8
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025	0.025	0.025
$FeSO_4.7H_2O$	27.8	27.8	27.8	27.8
Na_2EDTA	37.5	37.5	37.5	37.5
Thiamine-HCl	2.5	10	10	2.5
Nicotinic acid	2.5	1	0.5	2.5
Pyridoxine-HCl	2.5	1	0.5	2.5
Glycine	2	0	2	2
Inositol	100	100	100	100
2,4-D	1	1	1	1
NAA	1	1	1	1
Kinetin	0.5	0.5	0.5	0.5
Maltose	30	30	30	30
Gelrite	2,700	2,700	2,700	2,700
Yeast Extract	100	0	0	0
Casein Hydrolysate	0	200	200	200
$AgNO_3$	0	8	8	8

Methods

Boots (panicles) from primary tillers of each genotype were sampled at appropriate stage in morning (9 am to 10 am). Usually, the distance between collar of flag leaf and ligule of penultimate leaf of tiller serves as, a reliable guide to anther maturity [11]. Boots were wrapped in muslin cloth, sealed in polythene bags and pretreated at 12° C for 5 days [37]. Cold treated panicles were surface sterilized with 0.1% mercuric chloride for 5 minutes and rinsed several times with sterilized distilled water. Selection of the spikelet was done based on cytological observations or position of the anthers. For rice, the best stage has been described as uni-nucleate to early bi-nucleate stage [38] or, when anthers occupying 1/3 to 1/2 of the spikelet length, is the most suitable stage for anther culture [39]. Following surface sterilization, anthers were dissected out in laminar air flow bench from separated spikelet. Each spikelet was snipped at the base while holding from the tip to detach the anther lobes from the filaments. The released anthers were dropped onto the induction medium contained in petri dishes. Anthers were made to spread evenly by rotating the petri dishes during anther plating. One petri dish constitutes one replication and an average of 4 replicates was cultured for each media. The cultures were incubated at 25 ± 1° C and relative humidity of 65% (RH) in dark for callus induction. The cultured plates were examined periodically

to observe the progress in respect of callus formation. Calli of at least 1-2 mm diameter were transferred to MS regeneration medium and maintained at same temperature, humidity provided with 16/8 hours of light and dark period. The cultures were examined weekly and data on percentage of calli regenerating green and/or albino plants was recorded. Only regenerated green shoots were then transferred to rooting medium (1/2 MS without hormones) under same temperature, humidity and light conditions. Well developed plants with profuse roots were transferred to green house for acclimatization.

Observation on anther response to callus induction was carried out during 60-80 days after anther plating. The frequencies of callus induction and regeneration were estimated as follows: callus induction frequency (CI %) = number of anthers producing calli/number of anthers plated x 100, green (RG) or albino plant (ABN) frequency (%) = number of green or albino plant regenerating calli/ number of calli transferred x 100. Analysis of variance (ANOVA) was conducted using CROPSTAT (version 7. 2. 2007. 3) Computer software for statistical analysis of the data generated during the present study.

III. Results

Approximately 3 weeks after anther plating, some anthers responded by calli emergence from interior of the anthers (*Fig*.1a). Calli produced appeared to be creamy white in colour and compact except for 25eB × Dular (WF) in which friable texture of callus was obtained. Calli of 1-2 mm in size emerging from the cultured anthers were transferred to regeneration medium. Within two to three weeks time, the transferred calli started differentiating into clumps of green spots first (*Fig*.1b) and then into green shoots (*Fig*.1c). Some of the calli, instead of forming green spots exhibited white shoot like structures which subsequently developed albino plants (*Fig*.1d). Only the green plantlets further transferred into rooting media (*Fig*.1e). Well developed plants with profuse roots were acclimatized in green house under controlled conditions (*Fig*.1f).

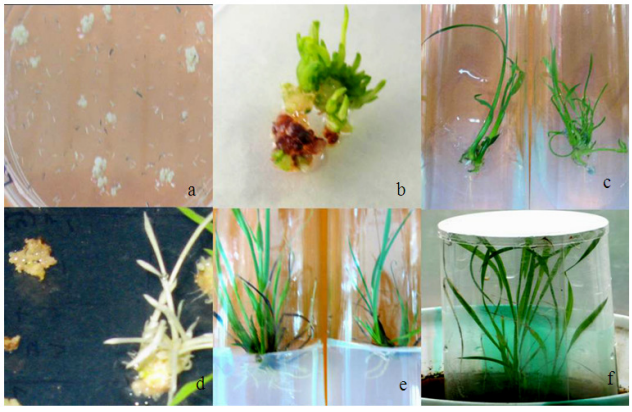

Fig. (1a) Callus induction (1b) Green spots formation (1c) Green shoots regeneration (1d) Albino shoot regeneration (1e) Root development (1f) Acclimatization

The significant effect of genotypes, media and their interaction on callus induction was observed among 13 genotypes by ANOVA ($p<0.001$). The analysis of variance revealed that variations due to genotype and media to be highly significant (*Fig*.2a). Callus was induced in all the genotypes in all media. But the frequency of callus induction was more in F_1 compared to parents except for PG. Depending on the genotype and culture medium, the frequency of callus formation varied from 10.69% for PB in N6 medium to 40.64% for PG in He2 medium. PG was highly responsive to callus induction ranging from 29.59% in N6 medium to 40.64% in He2 medium. The callus induction followed a pattern of development (N6< SK1< B5< He2). Of the four media evaluated, the N6 medium produced less frequency of callus (10.69% to 29.59%) followed by SK1 medium (12.68% to 34.60%) and B5 medium (14.28% to 38.32%). However, He2 medium promoted highest level of callus induction ranging from 17.24% to 40.64%.

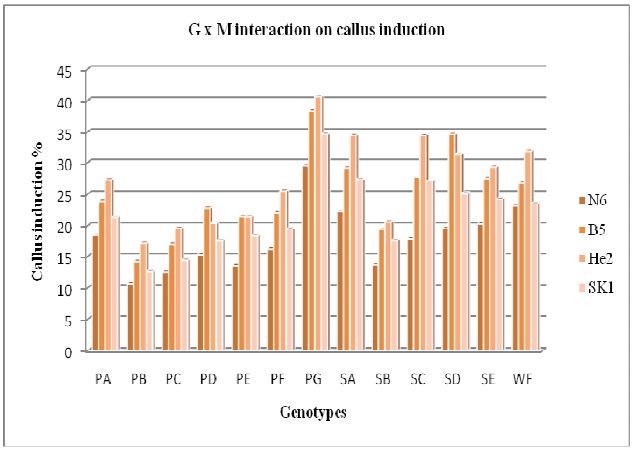

Fig.2a. Response of genotypes to media for callus induction from rice anther

Similarly, significant genotypic difference of green plant regeneration was observed among the genotypes by ANOVA (*Fig*.2b). Similar to callus induction, green spots formation was also higher in F_1 compared to parents except for PG. Highest percentage of green spot formation was observed in PG ranging from 32.86% in N6 medium to 40.93% in He2 medium. N6 medium produced least frequency of green plant regeneration (6.30% to 32.86%), followed by SK1 (8.01% to 35.36%), B5 (9.64% to 36.12%) and He2 (11.43% to 40.93%).

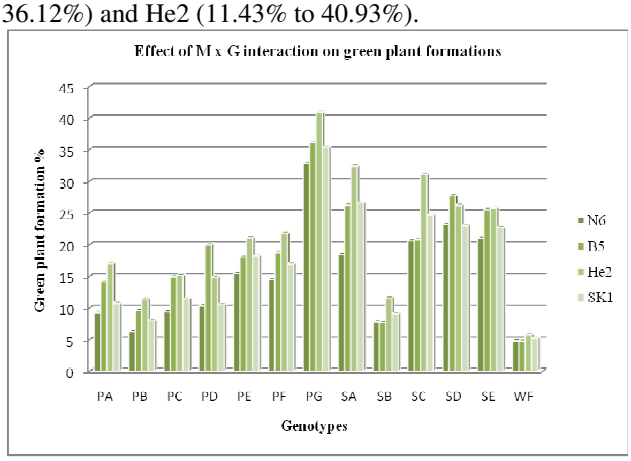

Fig.2b. Response of genotypes to media for green plant regeneration from rice anther

Thus, the green plant regeneration frequency also followed the same pattern as that of callus induction (N6< SK1< B5< He2).

Albinism is a widely stated phenomenon in the rice anther culture of indica, which restricts the use of this breeding technique. The frequency of albino plants developed was lower across the genotypes and media as compared to the frequency of green plants. The albinism followed a trend contrary to callus induction and green spots formation (He2< B5< SK1< N6). Lowest frequency of albino formation was observed in He2 (3.72%), followed by B5 (7.26%), SK1 (7.28%) and 10.65% in N6 medium. The frequency of total regeneration with high green plant and least albino plant recovery was high in He2 medium.

Analysis of variance for the effect of mannitol on anther response showed a significant genotype x mannitol interaction effect. Addition of mannitol into the callus induction media followed a different pattern of anther response for both callus induction and green plant regeneration ($N6_{M3}$ < $N6_{M2}$ < N6 < $N6_{M1}$). Addition of 100mg/L mannitol in to $N6_{M1}$ induced a maximum of 18.60% callus and 16.33% green spots formation, followed by N6 (17.96% and 14.93%), $N6_{M2}$ (13.87% and 11.34%) and least with $N6_{M3}$ (9.14% and 5.41%).

IV. Discussion

Doubled haploidy through anther culture is a time saving and innovative technology that can be utilized for rapid development of improved parental lines. The ability to produce homozygous lines after a single round of recombination saves a lot of time for the plant breeders. Results from the present study revealed that, it is neither medium nor genotype alone but the interaction of genotype and medium that determine the level of anther response. These findings were consistent with the previous reports stating the presence of significant variation in callus induction due to genotype, media composition and their interaction [40 and 41]. The genotype of the pollen plant has greatest influence on the frequency of pollen callus formation [42]. Miah et al. [43] reported that the anther culture response varied from 41% for a japonica cultivar to 0% for an indica cultivar and even among the indica cultivars a considerable variation for pollen callusing and plant regeneration was noted. Similar observation has been noticed in the present study. The different genotypes hold different degrees of potentiality of callus induction in different media (13.72% to 35.79%). We successfully produced calli from all 13 tested genotypes in all four media in contrast to the previous studies. Lentini et al. [17] reported that only one out of 35 indica cultivars exhibited pollen callusing on N6 medium. Guha-Mukerjee [44] reported that only 5 out of 18 indica cultivars showed pollen callusing and callus from only one cultivar differentiated into plants. This slight discrepancy observed in this study, Lentini et al. [17] and Guha-Mukerjee [44] might be due to differences in genetic makeup of the genotypes. In this study, F_1 hybrids showed higher response for anther culture than parents except for PG (recurrent parent), which was the most responsive among the genotypes evaluated. This implies that PG might have an anther culture response enhancing gene/s and the higher response of F_1 was due to transfer of the gene/s from recurrent parent to F_1 hybrid. These findings were consistent with previous reports (Kaushal et al. [37]). Herath and Bandara [35] stating that the difference between indica parents for anther culture response also affected its F_1 hybrid response.

Callus induction and plant regeneration are considered as two distant phases in anther culture process of rice. Both callus induction and green spots formation followed the same pattern of development (N6< SK1< B5< He2). In the present experiment the callus induction was distinctly better in He2 medium except for PD and SD. Overall He2 proved to be superior medium, followed by B5, SK1 showed moderate level, and N6 medium produced lowest frequency of callus induction and green plant regeneration. Although the tested genotypes too perform in N6 medium, but their frequency was less. Chu et al. [45] reported that N6 medium widely used for anther culture was found less suitable for indica rice anther culture. Huang et al. [46] reported that indica genotypes require low $NH4^+$ just half of the concentration required by japonica. The higher concentration of ammonium ions in the N6 medium is less suitable for the tested indica genotypes and hence poor anther response of anthers in N6 medium. The better response of B5 and SK1 seems be to due to lower level $NH4^+$ (1/4 or 1/2 strength compared to N6) in induction media. Whereas, the superior response of anthers in He2 medium might be due to presence of ½ strength of $(NH4)_2SO_4$, $1/50^{th}$ of $MgSO_4$ and 2x of KH_2PO_4 compared to N6 medium. Mandal and Gupta [47] reported that out of 5 different media (N6, modified N6, R3, He2 and He5) higher anther response was obtained from He2 medium. Reddy et al. [48] who studied 8 indica cultivars, found He2 medium to be better than N6 medium. In contrary to this, Rukmini et al. [49] found N6 medium to be superior compared to MO19 and SK1 medium. Silva and Ratnayake [50] reported that callus induction was nearly three times higher on N6 medium than on SK1 medium.

In the perspective of both callus induction and green plant regeneration, the callus generated showed similar level of green plant formation, except for WF. It has been reported that the cultivars that display high callusing ability show the best regeneration frequencies (Javed et al. [51]; Shahnewaz et al. [52]). The results obtained in our studies are consistent with this trend. It was observed that quality of callus play a significant role in plant regeneration. The embryogenic calli which were milky white in colour and compact in texture had excellent regeneration ability. On the contrary, friable calli had poor plant regeneration ability or did not respond at all. These results clearly suggest that the callus induction medium has an influence on the morphogenic competence of the induced callus, determining its regeneration capability. This implies that successes of regeneration dependent on callus formation for all genotypes except for WF. Even though callus induction was high for WF in all four media ranging from 23.14% in N6 to 31.94% in He2 medium, the

regeneration was very low (4.7% in B5 to 5.79% in He2 medium). Talebi et al. [32] and He et al. [53] reported that there are occasions in which genotypes show high callus induction have displayed poor regeneration ability and vice versa. The higher callus induction observed in WF might be due to higher doses of hormone in the induction medium. Application of higher dose of auxin sources can significantly increase the callus induction efficiency, however such calli are embryogenic-less and poor in green plant regeneration. Liang [20] reported that hormone requirement is genotype specific. Therefore, optimum level of auxin in the callus induction media required some degree of compromise between callus induction and regeneration frequency. The poor quality of callus (friable texture) in WF might be one of the reasons behind the poor green plant regeneration. The present results were in agreement with earlier studies stating that the friable callus is considered to be of poor quality with a low potential for regeneration in wheat (Moris and DeMacon [54]). Beside, high callus formation in WF did not result in high levels of plant regeneration efficiency, suggesting that regeneration is independent from callus formation. Results of this study agree with the results obtained by other reporters and support earlier observations that embryoid induction and plant regeneration are independently inherited traits (Deaton et al. [55]; Forough-Wehr et al. [56]). Therefore, media modifications should target the production of embryogenic callus with good regeneration ability rather than simply inducing prolific callusing, from which regeneration would not be possible (Silva [57]).

Addition of $AgNO_3$ as an anti ethylene agent to delay anther senescence had positive impact on anthers response in the present study. Relatively higher frequency of callus induction and regeneration was observed in three media (B5, He2 and SK1) compared to N6 suggested that $AgNO_3$ promotes both callus induction and green plant regeneration. It was speculated that $AgNO_3$ had positive effect on embryogenesis by blocking the inhibitory effect of endogenously produced ethylene in culture vessels. Lentini et al. [17] reported that addition of 10 mg/L of $AgNO_3$, anti ethylene compound to callus induction medium promoted 2 fold increase in pollen callusing frequency (10.1% to 20.06%) and green plant regeneration. Similar positive effect of $AgNO_3$ was reported in anther culture of wheat and brassica (Ghameni et al. [58]; Williams et al. [59]). From the view point of doubled haploid rice breeding, production of green plants with high frequency is a prerequisite. Therefore, priority should be given to the frequency of green plants regeneration rather than high frequency of callus induction.

Albinism is counted as a major problem in anther culture in rice, especially in indica rice (Chen et al. [60]). The frequency of albinos may vary from 5% to 100% (Talebi et al. [32]). In the present study albino development is inversely proportional to green plant regeneration. It followed a trend opposite to callus induction and green spots formation (He2< B5< SK1< N6). Although albino development was observed in all four media, the frequency was about 3fold higher with N6 and 2 fold greater with B5 and SK1 respectively compared to He2 medium (*Fig.*3). The higher frequency of albino formation that was observed in N6 medium might be attributed to genotypes and media composition. The recovery of albino plants from pollen derived calli has been a formidable obstacle to the utilization of rice anther culture for indica rice improvement (Chowdary and Mandal [61]) which might be due to the long culture duration and the genotype. Literature on androgenesis in cereals suggests that albinism could be considerably reduced by shortening the culture period (i.e. frequents subculture). Replenishing the media to avoid depletion of some of the essential micronutrients and balancing the pH often helps in the conditioning of the cultures and their development (Datta [8]). Anther culture efficiency of He2 medium is higher with highest callus induction, subsequent green plant formation and least frequency of albino formation (*Fig.* 3).

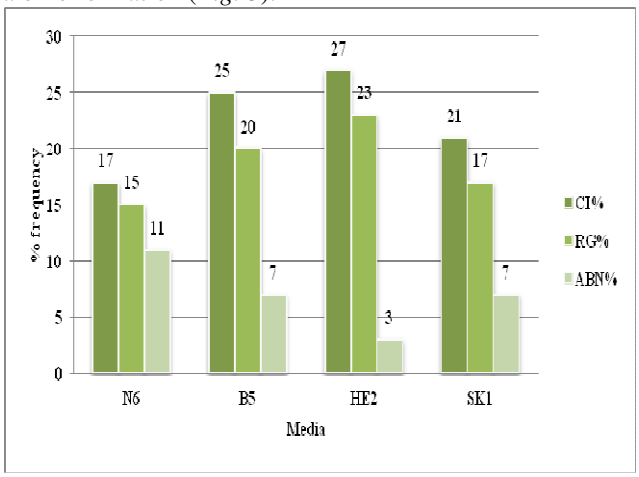

Fig.3. Comparative representation of anther culture efficiency among four media

The present study showed that the anther response was better on $N6_{M1}$ with 100 mg/L mannitol. Compared to control, the response was improved by less than 1.5%. However, mannitol with high concentration in $N6_{M2}$ and $N6_{M3}$ media suppressed the anther response (*Fig.* 4). Mandal and Maiti [62] reported that mannitol as an osmoticum (100 mg/L) induced maximum androgenic calli and regenerants. Similar findings were observed from the present study. Pande [63] reported that in the absence of cold pretreatment, mannitol treatment promoted androgenesis in anther culture of IR43 (3% to 33.4%), however with cold treatment it had no promontory effect. Cold treatment in combination with the osmotic treatment was detrimental. The lesser impact of mannitol in the present study might be due to combined effect of cold and mannitol stress. This implies that the mannitol in this experiment was less effective on genotypes callus induction and green plant regeneration. Genotype being a deciding factor in achieving the success, the components of tissue culture media demonstrated to have crucial role in coaxing an *in vitro* response was evident from the present study.

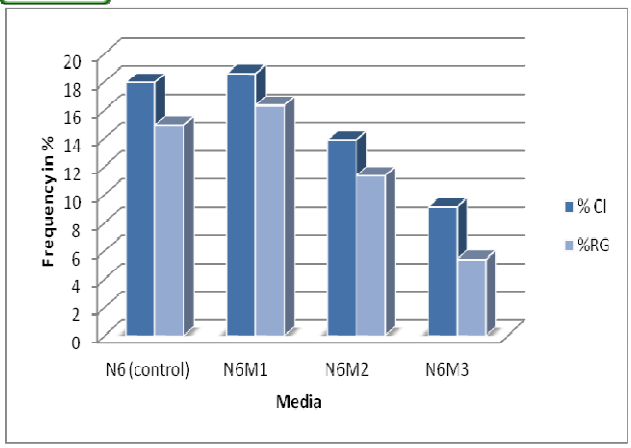

Fig.4. Comparative representation control/ mannitol treatment for improve callus induction and green spots formation

V. Conclusion

To activate the recalcitrance of indica rice for androgenesis, the interaction of genotypes and culture media is important. Among the 4 induction media evaluated, He2 medium produced highest callus induction, green plant regeneration and least of albino formation. Out of 13 genotypes evaluated, the PG exhibited higher frequency of both callus induction and green plant regeneration. Addition of mannitol as in-culture osmoticum in callus induction media also was less effective for improved rice anther culture.

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