RESPONSE OF GENOTYPES TO CULTURE MEDIA FOR CALLUS INDUCTION AND REGENERATION OF PLANTS FROM RICE ANTHERS

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Abstract: *Response of genotypes to culture media for callus induction and subsequent regeneration from rice anthers were investigated at Biotechnology Unit, Khumaltar, Lalitpur, Nepal. Boots of several rice genotypes viz. Bindeshwari, Hardinath-1, Prabhat, Khumal-4, Chhomrong local and Chandnath-3 were cold pretreated at 8±1^{0}C for seven days. Anthers from these boots were aseptically cultured on three different medium designated as Medium **A**: N6 mineral salts and vitamins (2 mg/l each) + myoinositol 100 mg/l + 2,4-D 2.5 mg/l + kinetin 0.5 mg/l + $AgNO_3$ 10 mg/l + maltose 50 gm/l; Medium **B**: N6 mineral salts and MS vitamins + NAA 4 mg/l + Kinetin 2 mg/l + $AgNO_3$ 5 mg/l and sucrose 60 gm/l; and Medium **C**: Medium **B** without $AgNO_3$. Results revealed that the response of genotype to various media compositions were highly significant for response of anthers for callus induction and embryogenic calli formation. The interaction between genotypes and media were also significant. Among media, the frequency of callus induction (calli/anthers) was higher in medium **A** (17.65%) followed by medium **B** (15.74%) and **C** (11.19%). The frequency of albino plants was observed to be higher than the frequency of green plantlets. The frequency of total regeneration and green plants recovery were high in medium **B**. Among genotypes tested over all regeneration was high in rice varieties Chandan nath-3 (14.16%) followed by Chhomrong local (9.89%) and Bindeshwari (8.33%). Significant response of genotypes to media compositions indicated that Bindeshwari (25.0%) followed by Chandan nath-3 (19.33%) can induce higher frequency of plantlets from Embryogenic calli obtained from anthers when cultured in medium **B**. Genotype Prabhat failed to induce any plantlet. This result can be employed for the anther culture work of F_1 hybrids in rice.*

Key words: Embryogenic callus; Silver nitrate ($AgNO_3$); Cold pretreatment; Incubation; Growth environments; Hormones.

Abbreviations: 2,4-D=2,4-dichlorophenoxy acetic acid; NAA=α-naphthalene acetic acid; BAP=6-benzylaminopurine; $AgNO_3$=Silver nitrate

INTRODUCTION

Cereal grains are the staple food for more than half of the world's population, most of them living in Asia and Africa. During the last four decades, increase in cereals' production was made possible by the development of high yielding varieties (HYVs). In one hand, with limited scope for expansion of cereals area, increasing cost of inputs and environmental concerns, the task of further increase in cereals production now is much more complex. In other hand, increasing population need more food grain for their consumptions. To achieve further elevation in cereals' yields, one of the options is to explore the possibility of modifying the present high yielding plant type and the physiological processes.

In this regard, breeding of self-pollinating crops like rice aims to produce pure lines (homozygous for most of the favourable genes) with manifested superior phenotype. Normally, six to nine cycles of selfing are needed to end up homozygous for most of the desirable traits. Thereafter, three to five years are required for field tests before a variety is officially released. That means duration of around 15 years is required for developing a new variety. The breeding objectives are generally not getting fulfilled and efforts become useless if the government's policy and/or consumer and growers demands are modified. Further, the chances of permanent lose of important recessive genes are also very high during the process of handling the segregating populations. Therefore, techniques which help to express recessive alleles and saves from erosion, and generate homozygous lines at the earliest without descending the first hybrid generation for segregation are urgently needed.

In other hands, another approach to raise crop production is through exploitation of hybrid vigour or heterosis. It was only by 1973 that China could successfully exploit hybrid vigour by developing commercial rice hybrids, which yielded 20 per cent higher than semi-dwarf rice varieties (Yuan, 1994). These hybrids could not be adapted in other tropical environments either because of their susceptibility to diseases, pests and their poor grain quality or unavailable freely. This clearly indicates that hybrids should be developed in the background of locally well adapted cultivars. Despite of the yield advantage of hybrids over pure lines, development of rice hybrids in tropics is still limited. Owing to the technical limitations in hybrid seed production and their high cost, poor grain quality, the hybrid rice technology is still facing problems for large-scale adoption by farmers in many countries. Further, the farm-

ers have to buy fresh F_1 hybrid seeds from the company every season which results the costly crop production. Further, the hybrid seeds are also not available in time. Hybrids also gradually replace the local cultivars and genetic resources leading to genetic erosion. Therefore, if the recombinants that perform as good as hybrid can be identified, the problems with hybrid rice technology can be circumvented through biotechnological approach. Biotechnological tools like induction of doubled haploids through anther culture can shorten the required cycles of selfing to bring to homozygous considerably and it is possible to obtain pure lines in less than two generations. Hence several years are exempted required for handling the segregating generations. It also helps to generate somaclonal variants for genetic study and uses in future.

In case of rice, haploids were first produced through anther culture by Niizeki and Oono (1968) and closely followed by Nishi and Mitsuoka (1969) in Japan, and Guha *et al.* (1970) in India. China exploited the technique of anther culture for the first time. Varieties developed through anther culture yielded as high as 10.3 t/ha under moderate fertility. The magnitude of hybrid vigour that could be realized in doubled haploid lines (homozygous) derived through anther culture of hybrid rice can be retained more or less the same level of vigour as the hybrids (Bong and Swaminathan, 1995). Lines derived from pollens of hybrids can perform more ideal than the hybrid itself. In general, in China, yields from some lines were proved to be higher than those of local standard cultivars (Niizeki, 1997). In India, promising lines with earliness and high yield potential were selected from a large number of plants derived from anther culture of several crosses. Parag 401, a semidwarf rice variety developed through anther culture, was released for cultivation on irrigated vertisols of Maharastra State of India (Patil *et al.*,1997). At present improved rice varieties and lines derived from anther culture are widely grown in China, Taiwan, South Korea, Japan, USA, and India and in several other countries (Misso *et al.*, 1991; Zhang, 1992; Mia *et al.*, 1996; Niizeki, 1997; Raina and Zapata, 1997; Gupta, 1999).

The anther culture technique as a tool of Biotechnology is highly useful for plant breeding research work for the development of a novel prominent high yielding variety in the country like Nepal. It is specially very effective for doubled haploid production. Immature anthers containing haploid microspores when cultured at a particular stage of development and under appropriate conditions result in cell division and growth of gametophytic cells leading into plant regeneration. The anther culture system has the unique property of allowing male gametes of a heterozygous parent to develop into haploid or homozygous diploid lines in a single generation as against several generations of selfing. Thus it has become handy to evolve varieties in the shortest period with less effort. However, an effective and promising protocol along with other technical inputs are required for the successful and routinely induction of doubled haploids (Raina, 1989; Misso *et al.*, 1991; Lentini *et al.*, 1995; Zhuo *et al.*, 1996; Laxmi and Reddy, 1996; Niizeki, 1997; Raina and Zapata, 1997; Xie *et al.*, 1997; Chu *et al.*, 1975; Karim and Zapata, 1999). Therefore, this study was conducted with the objectives to standardize the anther culture protocol, identify responsive genotypes for incorporation of responsive gene into promising varieties for future use, stabilize culture and growth room environment and train technical staffs.

MATERIALS AND METHODS

Plant establishment

Thirty day old seedlings of six rice genotypes viz. **Bindeshwari** (released for terai)**, Hardinath-1** (for terai), **Prabhat** (for terai)**, Khumal-4** (for mid hills)**, Chhomrong local** (for medium to high hills)**,** and **Chandan nath-3** (for high hills) were transplanted in the plastic bucket. Each variety was planted with six replications containing single plant/bucket and grown in glasshouse conditions of Biotechnology Unit, Khumaltar during normal rice growing season of 2006. Chemical fertilizers (NPK) at the rate of 60:30:30 kg/ha were applied in each buckets. Various plant protection measures were adopted to maintain the plants as healthy as possible.

Boots collection

Boots from primary and secondary tillers of each variety were sampled at appropriate stage in the morning. The anthers with mid to late uninucleate stages were first determined by cytological test using acetocarmine staining technique. The cytological test result was later traced back with anthers position on spikelet. Most of the boots then harvested when the growth of anthers was reached around one half to one third of spikelet length. Boots were collected in beaker with tap water.

Pretreatment

The material for cold pretreatment was prepared by keeping boots intact with their penultimate leaf sheath and node, and trimming off flag leaf and extra basal nodes. These materials were cleaned and partly sterilized by wiping with tissue paper moistened in 70% ethanol. Bundles of 10-15 boots were then wrapped in plastic wrapper with tissue paper and finally wrapped in aluminum foil and kept at $8\pm2\ ^0C$ for 7 days.

Sterilization

After seven days of pre-treatment, the boots were removed from refrigerator and the only middle portion of spikelets were taken by removing 1/3 lowest and 1/3 upper portion of boot. The remaining panicles were then removed out from boot leaves. A cluster of 3 to 5 spikelets attached to the secondary and or tertiary rachis were cut and collected in a beaker containing 50-100 ml distilled water. The water was drained off and the clump of material was transferred into sterilized beaker under laminar clean air flow cabinet. The final sterilization was performed by immersing in alcohol (70%) for a few seconds and then mercuric chloride solution (0.1%) for 20 minutes. After 20 minutes of immersion with occasional shaking, the mercuric chloride solution was drained off. The whole material was thoroughly washed for at least 3-4 times with sterile double distilled water.

Inoculation of anthers

The excess water after final wash was absorbed using several layer of sterile cheese cloth or filter papers with the help of sterile forceps. Then anthers were isolated by holding spikelets cluster with a sterile forceps on one hand and cut was provided near the basal end of florets with the help of sterile scissors in the other hand. The cut florets were collected in a sterile petri-plate lined with two-three layers of filter paper. Using sterile forceps, anthers of each variety was inoculated uniformly over the surface of the medium by grasping cut floret as open end down and tapping it on petri-dish rim so that all the anthers are dropped on the surface of the medium. Based on the size of the petri-plate and amount of media 80-100 anthers were cultured in each petri-plate. For the study of response of anther to callus induction, the experiment was laid out in completely randomize design with two factors: media as main factor and genotype as subfactor. Anthers cultured in petri-plates were transferred to the dark incubation chamber maintained at 26±1^{0}C with relative humidity maintained above 60 per cent.

Media

Three different media designated as Medium **A**, **B**, and **C** were prepared by supplying the following ingredients: **Medium A**: N6 mineral salts and vitamins (2 mg/l each) + myo-inositol 100 mg/l + 2,4-D 2.5 mg/l + Kinetin 0.5 mg/l + $AgNO_3$ 10 mg/l + maltose 50 gm/l; **Medium B**: N6 mineral salts + MS vitamins + NAA 4 mg/l + Kinetin 2 mg/l + $AgNO_3$ 5 mg/l and sucrose 60 gm/l; and **Medium C**: Medium B without $AgNO_3$. Regeneration was performed in MS medium supplemented with 1 mg/l NAA, 2 mg/l BAP, 0.5 mg/l Kinetin and 20 gm/l sucrose. The pH of each medium was adjusted to 5.8. Media were solidified with agar (0.7 %). All the media were sterilized by autoclaving at 15 psi for 18 minutes. The medium was dispensed at the rate of 15-20 ml per 70 mm petri-plates. The cultured petri-plates were sealed with para-film and incubated under dark in growth room.

Regeneration

Embryogenic calli of 2 to 3 mm in size emerging from the cultured anthers were transferred to regeneration medium in culture tubes (25 x 150 mm). Cultures were maintained in a culture room with temperature at 26±1^{0}C and relative humidity of above 60% under 16/8 hours of light and dark period. Sub-culturing of calli was maintained at 12-15 days intervals in regeneration medium. Well developed plantlets with profuse root were hardened in liquid solution followed by in sterile soil in above mentioned room environments.

Statistical analysis

Observation on response to callus induction was carried out during 30 - 90 days after inoculation considering that each callus piece originated from a single anther. The frequency of callus induction and regeneration was calculated as follows: Callus induction frequency (%) = (number of anthers producing calli/number of anthers cultured) x 100; and regeneration frequency (%) = (number of plants recovered/number of calli cultured) x 100. The frequency data on callus induction were transformed by arcsine "x transformation method for analysis of variance (ANOVA) and interpretation of results. The original frequency data are presented in summary tables and graphs.

RESULTS AND DISCUSSION

Efforts were attempted for improving the efficiency of anther's ability for culture of Nepalese rice germplasm. Response of genotypes to culture media for callus induction and subsequent regeneration from rice anthers were investigated. Boots of six rice genotypes viz. **Bindeshwari, Hardinath-1, Prabhat, Khumal-4, Chhomrong local** and **Chandan nath-3** were cold pretreated at 8±1^{0}C for seven days. Anthers from these boots were aseptically cultured on three different medium designated as Medium **A**, **B** and **C**. Anthers start callusing after four weeks of incubation (**Fig. 1a**). Embryogenic calli of 2 to 3 mm in size emerging from the cultured anthers (**Fig. 1b**) are transferred to regeneration medium. Cultures are maintained in the room temperature of 26±1^{0}C and relative humidity (RH) of above 60% provided with 16 hours of light and 8 hours of dark period a day and night regime. Green (**Fig. 1c**) and albino (**Fig. 1d, e, f**) plantlets are differentiated which are then transferred to rooting media (**Fig. 1g, h**) (½ MS without hormones) under same temperature and light conditions. Well rooted plants are then transferred to liquid nutrient solution and allowed to grow for one week. Later they are shifted to sterile soil in small pots (**Fig. 1i**) under above mentioned growth room environment for hardening and after two weeks they are grown in glasshouse.

Results revealed that the effect of the media compositions on the response of rice genotypes evaluated for callus induction

Table 1: Analysis of variance (ANOVA) of data on frequency of responding anthers to callus induction and embryogenic calli formation

	Source of variance	Degree of freedom	Variance of responding anthers inducing callus	Variance of responding anthers inducing embryogenic callus
1	Treatments	17	800.30**	614.85**
2	Media	2	244.29*	194.81**
3	Genotype	5	2137.74**	1627.51**
4	Media x Genotype	10	242.78**	192.82**
5	Error	32	46.29	33.25
6	Total	49	307.88	235.03

Note: Variance followed by * and ** are significant at 5% and 1% level of probability, respectively.

Table 2: Response of anthers to the frequency of callus induction (%)

Genotype		Media			Mean of Genotype
		A	B	B	
1	Bindeshwari	0.84	0.81	1.16	0.94
2	Hardinath-1	2.54	7.14	0.75	3.47
3	Prabhat	5.74	0.00	0.00	1.91
4	Khumal-4	22.42	3.22	0.34	8.66
5	Chhomrong Local	69.71	52.83	34.23	52.26
6	Chandan nath-3	4.66	30.41	30.64	21.90
	Mean of Media	17.65	15.74	11.19	
	Experimental Mean				14.86
	F test (Treatments)				**
	Media				*
	Genotype				**
	Media x Genotype				**
	CV(%)				39.49
	SEM				13.89

Note: * and ** are indicated for the significance of variance at 5% and 1% level of probability, respectively.

Table 3: Response of anthers to the frequency of embryogenic callus induction (%)

Genotype		Media			Mean of Genotype
		A	B	B	
1	Bindeshwari	0.84	0.81	1.16	0.94
2	Hardinath-1	2.54	7.14	0.37	3.35
3	Prabhat	5.04	0.00	0.00	1.68
4	Khumal-4	19.82	0.69	0.34	6.95
5	Chhomrong Local	55.87	40.57	34.23	43.56
6	Chandan nath-3	4.66	26.05	23.22	17.98
	Mean of Media	14.80	12.54	9.89	
	Experimental Mean				12.41
	F test (Treatments)				**
	Media				**
	Genotype				**
	Media x Genotype				**
	CV(%)				38.29
	SEM				11.77

Note: ** are indicated for the significance of variance at 1% level of probability

were highly significant. Interactions between media and genotypes were significant for callus induction frequency (**Table 1**). The effect of callus induction media tested were similar for induction of embryogenic calli (**Table 1**). All the media across the rice genotypes were able to induce embryogenic calli with a frequency ranging from 0.00 – 69.71 percent. The highest frequency of callus induction was recorded in medium **A** (17.65%) supplemented with 2, 4-D (2.5 mg/l) and $AgNO_3$ (10 mg/l) followed by medium **B** (15.74%) and medium **C** (11.19%) (**Fig. 3**). The significant difference among rice genotypes was observed for callus induction (**Table 1**). Medium **B** was found to be superior for the regeneration of green plants (**Fig. 3**). The frequency of albino plants was observed always high across the genotypes and media as compared to the frequency of green plants. The frequency of total regeneration and green plants recovery were high in medium **B**.

Among rice genotypes, response of anther to callus induction was found superior in rice variety Chhomrong local, released for medium to high hills of Nepal. The frequency of responsive anther in this genotype varied from 34.23 to 69.71% depending upon the media composition (**Table 2**). Chhomrong local also induced higher rate of embryogenic calli (43.56%) ranging from 55.87% in medium **A** followed by medium **B** (40.57%) and **C** (34.23%). Only the calli of two rice varieties namely Chandan nath –3 in medium **B** and **C**, and Khumal----4 in medium **A** were able to regenerate into green plants (**Fig. 3**). The higher rate of regeneration was recorded in genotype Chandan nath-3. The calli induced in medium **B** and **C** were found to be highly responsive. All these regenerated plants were haploid with chromosome constituents of 12. Morphologically these plants were small in size without auricle and ligules, and were completely sterile as compared to their original parent. Only haploid plants with full sterility (**Fig. 1j, k**) were obtained. In general, variety Chandan nath-3 was recorded more responsive to anther culture than the rest of the rice varieties evaluated.

These findings were consistent with the previous reports (Guha-Mukherjee, 1973; Miah *et al*., 1985; Raina, 1989; Quimo and Zapata, 1990; Lentini *et al*., 1995). They reported that the *in vitro* anther culture in rice was significantly affected by rice genotypes and culture medium. Miah *et al.* (1985) reported that anther culture response varied from 41 % for a *japonica* cultivar to 0 % for an *indica* cultivar. As against the finding of Miah *et al.* (1985), even among the *indica* cultivars a considerable variation for pollen callusing and plant regeneration were recorded in this study. Calli from all rice genotypes evaluated in this study were induced using medium **A** in contrast to the report of Lentini *et al*. (1995). They found that only one out of 35 *indica* cultivars exhibited pollen callusing on N6 medium. The rate of callus production in medium **A** varied from 0.84-69.71 percent (**Table 2**). Though medium **A** was found to be very effective for callus induction across the genotypes, the frequency of regeneration was very poor (4.83%) as compared to 20-70% reported by Laxmi and Reddy (1996) using the same medium with 2, 4-D (2 mg/l) (**Fig. 2**).

Successful protocol of anther culture is the prerequisite for induction of green plants with high frequency in the breeding program. Therefore, great attention has to be paid to the frequency of green plants regeneration rather than high rate of callus induction. It is because of genotype and medium with high rate of callus induction may not necessarily yield higher number of green plants as found in present study for genotype Chhomrong local (**Table 2 and Fig. 2**). In this perspective, callus induction medium **B** supplemented with NAA (4 mg/l) + Kinetin (2 mg/l) + $AgNO_3$ (5 mg/l) was found superior. This study also confirmed the results suggested by Chen *et al*. (1991), who reported that callus forming ability from anthers of rice was high in medium supplied with 2, 4-D, but the

Figure 1: Various stages in anther culture.

regeneration ability from these calli was quite low as compared to calli formed on medium supplemented with NAA. However, the above conclusion may not be applicable to all rice cultivars, as genotypic difference in hormone requirement had been reported (Liang, 1978). Thus the higher rate of callus induction and lower rate of regeneration observed in the callus induction medium **A** might be attributed to the use of relatively higher doses of 2, 4-D in this study (**Table 2 and 3, Fig. 3**). This suggested that the exact level of 2, 4-D in the callus induction medium required some degree of compromise between callus induction and regeneration frequency. Relatively higher frequency of callus induction and regeneration from callus induction medium **B** than **C** also suggested that $AgNO_3$ promoted not only callus induction but also help in regeneration of green plants (**Fig. 3**). It was speculated that $AgNO_3$ had positive effect on embryogenesis by blocking the inhibitory effect of endogenously produced ethylene in culture vessels. Lentni *et al*. (1995) and Laxmi and Reddy

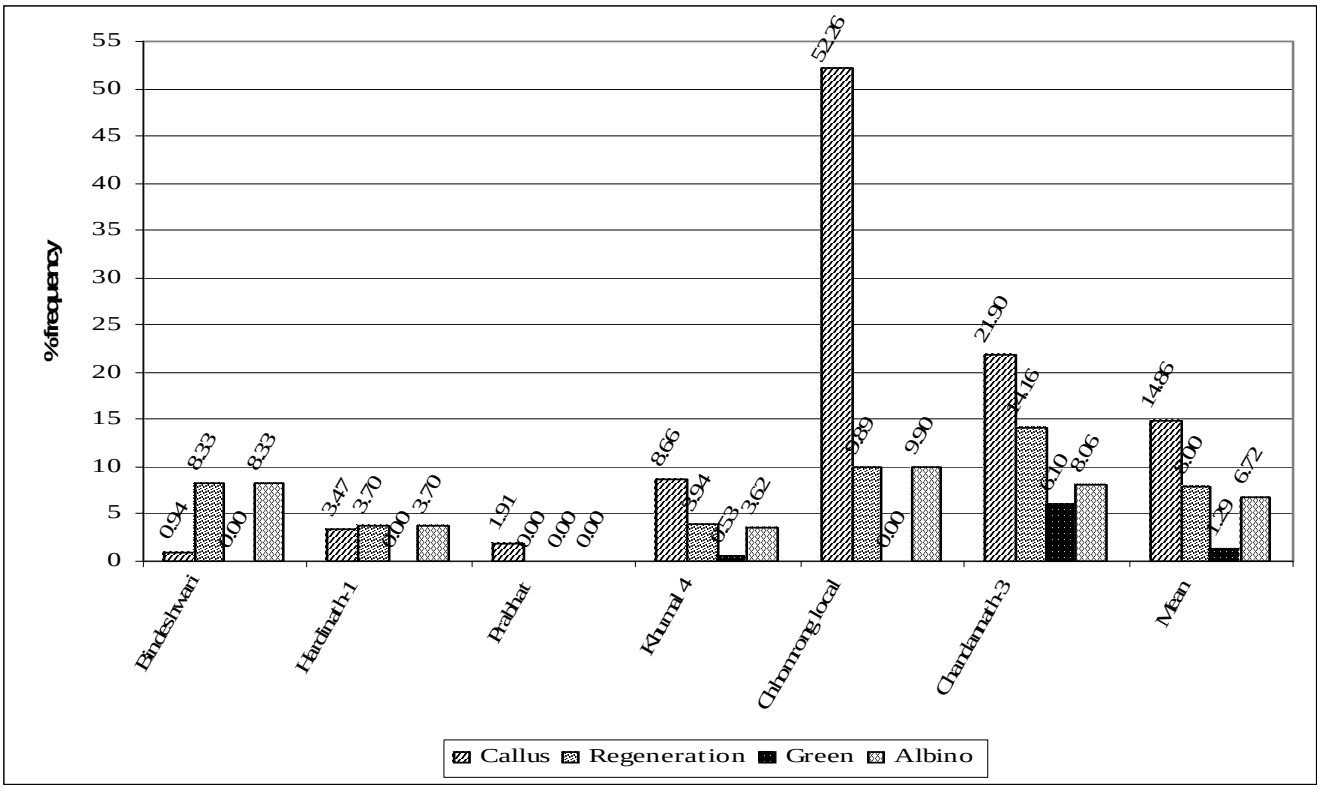

Figure 2: Response of genotypes to media for callus induction and regeneration of plants from rice anthers

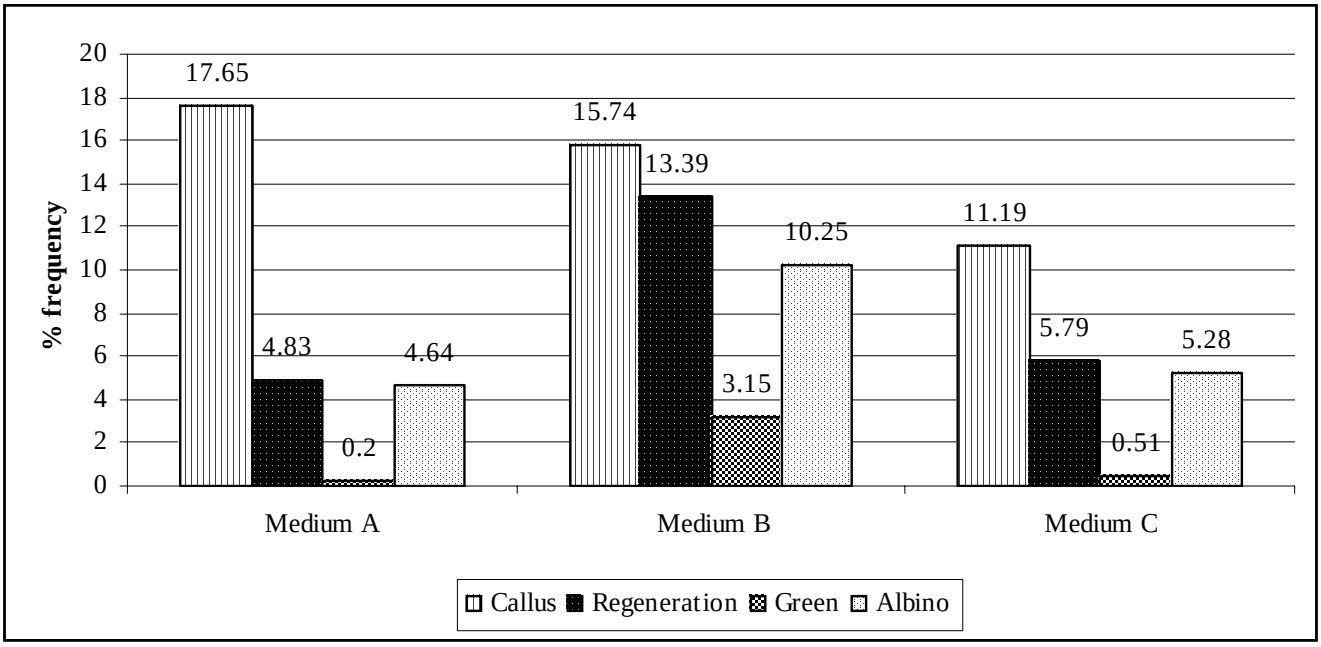

Figure 3: Effect of media on the efficiency of genotypes for callus induction and regeneration of plants from rice anthers

(1996) reported that with the addition of $AgNO_3$ at the rate of 10 mg/l in N6 based medium, the frequency of callus induction and green plant differentiation in *indica* rice was doubled. Similar positive effect of $AgNO_3$ was reported in anther culture of wheat, pearl millet, and *Brassica* (Williams *et al*., 1990; Pius *et al*., 1993; Ghamemi *et al*., 1994).

The higher rate of albino plant production in this study might be attributed to higher rate of 2, 4-D in medium **A** (**Fig. 2, 3 and Table 2, 3**) and long culture duration and genotypes itself. It has been well documented that such factors favors the production of albino plants and sometimes the frequency of albinos production goes up to 100 % (Chen *et al*., 1991). *Indica* rice cultivars are more prone to this problem than *japonica* rice. Several factors, including pre-treatment, culture medium, and the protocol, influence the frequency of albinos. The literature on androgenesis in cereals suggested that albinism could be considerably reduced by shortening the culture period (i.e. frequent subculture for long duration). In this study, varieties released for temperate zone (with cold tolerance gene/s) were found to be more responsive to anther culture than

the varieties released for tropical environments (terai). The scientific reasons of this phenomenon is still not available. However, the possible reasons might be that cold tolerance gene/s either linked with anther culture enhancing gene/s or have favorable effects during cold pretreatment.

CONCLUSION

The result of this study revealed that there are possibilities of enhancing callus induction and subsequent green plant regeneration from rice anthers by manipulating media compositions and identifying superior and responsive genotypes. Rice genotypes showed variable response to media for callus induction and subsequent regeneration from anthers. Therefore, the quality and frequency of callus induction and subsequent plant regeneration could be improved by selecting better responsive rice genotypes like Chandanath-3 and Khumal-4 and medium B. The callus induction medium B would offer great promise for the overall enhancement of ability of anther culture in Nepalese rice. Cold tolerant genotypes can be used in the breeding programme to improve the anther culture ability of rice genotypes for warmer areas.

ACKNOWLEDGEMENT

Nepal Agricultural Research Council (NARC) is highly acknowledged for providing financial support. Mrs Bina Dangol, Technical Staff and Mr. Santosh Nayak, student under internship, are thankful for their untiring technical assistance in completing this study.

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