# Asian Journal of Plant Sciences

ISSN 1682-3974

science alert

ANSInet
an open access publisher
http://ansinet.com

Asian Journal of Plant Sciences 8 (5): 368-374, 2009
ISSN 1682-3974


# Effect of Genotype and Callus Induction Medium on Green Plant Regeneration from Anther of Nepalese Rice Cultivars

[1,2]R.K. Niroula and [1]H.P. Bimb
[1]Biotechnology Unit, Nepal Agricultural Research Council, Khumaltar,
Lalitpur, Nepal, GPO Box 1135, Kathmandu, Nepal
[2]Sant'Anna School of Advance Studies, Martiri della Liberta, 033-56100, Pisa, Italy

**Abstract:** Effects of genotype and medium composition on the frequency of callus induction and green plant regeneration from anther of Nepalese rice were investigated. Cold pretreated anthers from six rice genotypes at 8±2°C for seven days were cultured on three different callus induction media designated as Callus Induction Medium (CIM 1): N6 mineral salts + N6 vitamins (2 mg $L^{-1}$ each) + myoinositol (100 mg $L^{-1}$) + 2,4-D (2.5 mg $L^{-1}$) + KI (0.5 mg $L^{-1}$) + $AgNO_3$ (10 mg $L^{-1}$) + maltose (50 g $L^{-1}$), CIM 2: N6 mineral salts + MS organic salts + NAA (4 mg $L^{-1}$) + Kinetin (KI) (2 mg $L^{-1}$) + Silver nitrate ($AgNO_3$) (5 mg $L^{-1}$) and sucrose 60 (g $L^{-1}$) and CIM 3: CIM 2 without $AgNO_3$. The callus induction frequency was significantly affected by rice genotypes and genotype x medium interactions. The efficiency of callus induction (calli/anther) was higher in CIM 1 (14.1%) followed by CIM 2 (12.54%) and CIM 3 (10.3%). CIM 2 was found to be superior for the recovery of green plants. Among genotypes, only the calli from Chandanath -3 and Khumal-4 were able to differentiate into green plants. In this study the calli induced on medium containing 2, 4-Dichloroacetic acid (2, 4-D) had lower regeneration ability than the medium supplied with α-naptthalene acetic acid (NAA). This study also revealed that the temperate cultivars (hill rice) were more responsive to anther culture than the tropical ones (terai rice).

**Key words:** $AgNO_3$, 2, 4-D, anther culture, albino, calli, *indica* rice

## INTRODUCTION

Anther culture is an important biotechnology technique for immediate fixation of homozygosity and compressing the breeding cycle in rice (*Oryza sativa* L.). Rice cultivars developed using anther culture are said to be possessed traits such as earliness, increased grain yield, resistant to biotic and abiotic stresses and superior quality (Roy and Mandal, 2005). At present improved rice varieties and lines derived from anther culture are widely grown in China, Taiwan, South Korea, Japan, USA, India and in several other countries (Gupta, 1999; Niizeki, 1997). Although, anther culture has been well integrated into rice breeding programs, specially in China, there still remain problems to exploit its full potential. High frequency of callus induction and green plant regeneration is a prerequisite for the successful utilization of *indica* rice anther culture in breeding programs. However, most of the tested *indica* type cultivars produced very high proportion of albino plants, which significantly limits the use of anther culture in rice breeding. In general, *indica* cultivars of rice exhibit poorer androgenic response than the *japonica*, Tongil rice (*japonica*×*indica*) and *javanica* cultivars (Raina, 1997). Beside genotype specificity, *in vitro* anther culture of rice also significantly affected by many factors such as culture medium composition, panicles pretreatment, anther condition, donor plants growing conditions and microspores development stage (Cha-um *et al*., 2009; Datta, 2005; Afza *et al*., 2000).

Since, the last four decades, several efforts have been attempted to improve the overall efficiency of rice anther culture. These include improved method of pretreatment and culture techniques (Trejo-Tapia *et al*., 2002; Niizeki, 1997), formulation and use of different media with varieties of additives (Cha-um *et al*., 2009; Thomas, 2008; Grewal *et al*., 2006; Raina, 1997) and use of clochicine in callus induction medium (Datta, 2005). They reported that their attempts have greatly enhanced the anther culturabiltiy in *indica* rice. Moreover, calcium in the medium is known to stimulate ethylene production in many plant tissues, which on accumulation in culture vessels causes inhibitory effect on callus induction and subsequent regeneration from rice anther culture (Raina, 1997). Some researchers suggested that the pollen

**Corresponding Author:** Raj Kumar Niroula, Crop and Plant Physiology Laboratory, Sant'Anna School of Advance Studies, Martiri della Liberta, 033-56100, Pisa, Italy Tel: +39-329-9298053

callusing could be enhanced with the addition of either calcium ionophore (0.5 μM) or $AgNO_3$ (10 mg $L^{-1}$) in CIM. Lentini *et al.* (1995) reported that with the addition of $AgNO_3$, the frequency of green plant differentiation was also doubled.

Among various factors associated with anther culturability, the most important one is the genotypic difference. Many researchers reported that different rice species, subspecies, or varieties behaved quite differently in response to anther culture. Similarly, culture medium, also strongly influences the anther culture response (Raina and Zapata, 1997). Several problems are still left regarding genotype specificity and media composition in the perspective of rice anther culture. Therefore, this study was undertaken to evaluate the response of Nepalese rice genotypes to anther culture and to select the appropriate medium composition for the enhancement of anther culturability in rice.

## MATERIALS AND METHODS

Six rice cultivars, representing *japonica* (Chandanath-3, and Chomrong Local) and *indica* (Khumal-4, Pravat, Bindeswori, and Hardinath-1) were selected for this study. Among 6 rice genotypes first 3 genotypes belong to hill rice and rest are the tropical rice. Thirty day old seedlings from these varieties were transplanted in the plastic bucket containing well fertilized sterilized soils. Each variety was planted in six buckets containing single plant/bucket. Six staggered plantings at 7 day interval were made to supply the enough amount of anther. Then plants were grown in glasshouse during June - November in 2006 at Nepal Agricultural Research Council (NARC), Lalitpur, Khumaltar, Nepal. Plants were uniformly fertilized with 90-40-30 kg $ha^{-1}$ NPK in the form of urea, triple superphosphate and muriate of potash. As per need various plant protection measures were adopted to maintain the plants as healthy as possible. At booting, boots from primary and secondary tillers containing the anthers with mid to late uninucleate stages (Fig. 3a-c) were harvested based on cytological test and anther position in spikelet. The cytological test was preformed using iron alum haematoxylin staining technique (Chang *et al.*, 1978). Boots were then harvested at 7-8 am when the growth of anthers was reached around one half to one third of spikelet length. Harvested boots were cleaned, partly sterilized in alcohol (70%) and cold pretreated for 7 day at 8±2°C. Pretreated spikelets were harvested only from middle portion of panicle by removing 1/3 of each from lower and upper portion of boot. A cluster of 3-5 spikelets were cut and collected in a beaker for sterilization. Sterilization of spikelet and excision and collection of anther was performed as earlier (Sah and Niroula, 2007).

In each petridish, anthers (90-100) were inoculated uniformly over the surface of the medium. After inoculation, the petridishes were sealed with parafilim and incubated in dark chamber at 25±1°C with relative humidity around 60%. For the study of response of anther to callus induction, the experiment was laid out in Completely Randomize Design (CRD) with unequal replications (Gomez and Gomez, 1984). Three different callus induction media, designated as CIM 1, CIM 2 and CIM 3, were prepared as described earlier (Niroula *et al.*, 2005). CIM 1: N6 mineral salts (Chu, 1978) + N6 vitamins (2 mg $L^{-1}$ each) + myoinositol (100 mg $L^{-1}$) + 2,4-D (2.5 mg $L^{-1}$) + KI (0.5 mg $L^{-1}$) + $AgNO_3$ (10 mg $L^{-1}$) + maltose (50 g $L^{-1}$), CIM 2: N6 mineral salts + Murashige and Skoog (MS) organic salts (Murashige and Skoog, 1962) + NAA (4 mg $L^{-1}$) + KI (2 mg $L^{-1}$) + $AgNO_3$ (5 mg $L^{-1}$) and sucrose (60 g $L^{-1}$) and CIM 3: CIM2 without $AgNO_3$. Two-three subcultures at 12 days interval in CIM followed by regeneration was performed in 1/2 strength MS medium supplemented with sucrose (20 g $L^{-1}$), NAA (1 mg $L^{-1}$), Benzylaminpurinc (BAP) (2 mg $L^{-1}$) and KI (0.5 mg $L^{-1}$). The pH of each medium was adjusted to 5.8 with NaOH or HCl (0.1 M) prior to autoclave and solidified with 0.7% agar.

Embryogenic calli of 2 to 3 mm in size were transferred to culture tube containing regeneration medium. Culture was maintained in incubator at 25±1°C under 16/8 h light/dark regime. Well developed plants with profuse roots were hardened as described by Niroula *et al.* (2005). The ploidy status of regenerated plants was confirmed by counting chromosome number in root tip and in pollen mother cells following usual acetocarmine staining technique (Khan, 1975). Various morphological attributes were also used to verify the ploidy status as suggested by Sah and Niroula (2007). Observation on response to callus induction was carried out at 30-60 days after inoculation considering that each callus piece was originated from a single anther. The frequency of callus induction and regeneration was calculated as follows:

$$\text{Callus induction frequency (\%)} = \frac{\text{No. of anthers producing calli}}{\text{No. of anther plated}} \times 100$$

$$\text{Total regeneration frequency (\%)} = \frac{\text{No. of plants (green + albino) recovered}}{\text{No. of calli cultured}} \times 100$$

$$\text{Green or albino plant frequency (\%)} = \frac{\text{No. of green or albino plants recovered}}{\text{No. of calli cultured}} \times 100$$

$$\text{Anther culturability (\%)} = \frac{\text{No. of green plants recovered}}{\text{No. of anther plated}} \times 100$$

Data on callus induction frequency was transformed by arcsine $\sqrt{x}$ function (Gomez and Gomez, 1984) and analyzed using MINITAB (version 10) and MSTATC statistical packages.

## RESULTS AND DISCUSSION

In order to increase the efficiency of rice anther culture, we tested different varieties of Nepalese rice and three N6 based callus induction media supplemented with two different auxin sources (NAA and 2, 4-D) and silver nitrate. All the media across the rice genotypes were produced culturable calli with a frequency ranged from 10.30-14.14 (Fig. 1, 3d, e). The highest frequency of callus induction was observed in CIM 1 supplemented with 2, 4-D (2.5 mg $L^{-1}$) and $AgNO_3$ (10 mg $L^{-1}$) followed by CIM 2 and CIM 3. The significant effect of rice genotypes and genotype×medium interactions on callus induction was observed (Table 1). Although, the effect of media composition for callus production was found non significant, the effect was apparently seen for green plant regeneration (Fig 1, Table 1, 2). CIM 2 was found to be superior for the regeneration of green plants (Fig. 1). The frequency of albino plants was always high across the genotypes and media as compared to the frequency of green plants (Fig. 1, 2, 4b). Among rice genotypes, better response of anther to callus induction was found in *japonica* variety; Chomrong Local. The percentage of responsive anther in this genotype was varied from 37.7-68.7 depending upon the media composition (Table 2). Only the calli of two rice varieties; Chandanath-3 and Khumal-4 were regenerated into green plants (Fig. 2). The higher rate of regeneration was recorded for *japonica* variety; Chandnath-3 calli induced in CIM 2 and CIM 3 media (Fig. 3f, 4a, b). The over all anther culturability of this genotype was 1.2%. Altogether, we successfully regenerated ten green plants with overall anther culture efficiency across the media and genotypes was 0.22%, by culturing 532 calli derive from 4384 anthers from six rice genotypes (Table 2). No diploid plants with full fertility were recorded. All these regenerated plants were haploid with chromosome constituents 2n = 12. Morphologically these plants were small in size without auricle and ligules and were completely sterile as compared to their original parent (Fig. 4c, d). In general, we found that the *japonica* varieties such as Chandanath-3 was more responsive to anther culture than the rest of the rice varieties.

From the viewpoint of rice breeding through anther culture (doubled haploid), production of green plants with high frequency is a prerequisite. Therefore, the priority should be given to the frequency of green plants regeneration rather than high frequency of callus

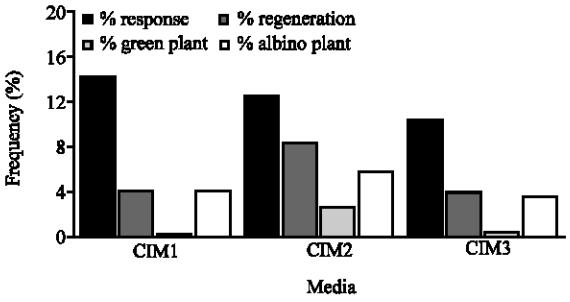


Fig. 1: Overall efficiency of three different media across the rice genotypes used for rice anther culture during experiment

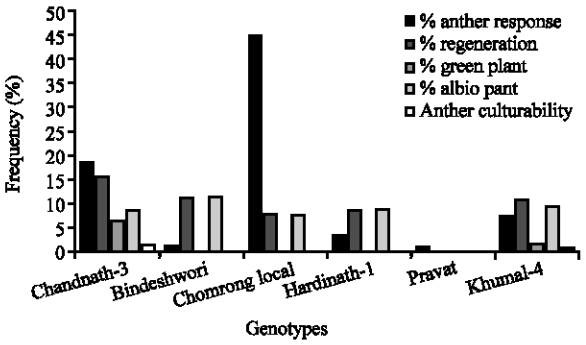


Fig. 2: Response of Nepalese rice genotypes to different parameters of anther culture across the media

Table 1: Influence of genotypes and media on callus induction efficiency of rice anther culture

| SOV | df | Mean square (MS) | LSD (0.5) |
|---|---|---|---|
| Callus induction media | 2 | 2.22 | 4.62 |
| Rice genotypes | 5 | 1819.59** | 6.54 |
| Media×Genotypes | 10 | 243.07** | 10.69 |
| Error | 31 | 41.20 | |

CV = 5.9%. MS followed by ** are significant at 1%

induction. It is because of genotype and medium with high rate of callus induction may not be yielded higher number of green plants as observed in the present study for Chomrong Local (Table 2, Fig. 2). Application of higher dose of auxin source can significantly increase the callus induction efficiency even in recalcitrant rice genotypes, however, such calli are embryogenicless. Hence, higher frequency of calli *per se* is not important in doubled haploid aided rice breeding programme. It has been well documented that both attributes; embryogenic calli induction and subsequent green plant regeneration, are highly influenced by culture components of medium and genetic make up of genotypes (Talebi *et al*., 2007; Raina and Zapata, 1997; Lentini *et al*., 1995).

In the perspective of green plant regeneration, CIM 2 supplemented with NAA (4 mg $L^{-1}$) + KI (2 mg $L^{-1}$)

Table 2: Influence of genotypes and callus induction media on anther culturability of Nepalese rice

| | Genotypes/Media | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Chandanath-3 | | | Bindeshwori | | | Chomrong local | | | |
| Parameters | CIM 1 | CIM 2 | CIM 3 | CIM 1 | CIM 2 | CIM 3 | CIM 1 | CIM 2 | CIM 3 | |
| No. of anther plated | 163.0 | 211.0 | 374.0 | 327.0 | 281.0 | 188.0 | 160.0 | 106.0 | 154.0 | |
| Responsive anther | 8.0 | 57.0 | 113.0 | 3.0 | 4.0 | 2.0 | 110.0 | 56.0 | 58.0 | |
| Response (%) | 4.9 | 27.0 | 30.2 | 0.9 | 1.4 | 1.1 | 68.7 | 52.8 | 37.7 | |
| No. of calli plated | 8.0 | 47.0 | 83.0 | 3.0 | 4.0 | 2.0 | 86.0 | 43.0 | 58.0 | |
| Total regeneration | 1.0 | 9.0 | 11.0 | 0.0 | 1.0 | 0.0 | 5.0 | 3.0 | 6.0 | |
| Regeneration (%) | 12.5 | 19.1 | 13.2 | 0.0 | 25.0 | 0.0 | 5.8 | 7.0 | 10.3 | |
| Green plant produced | 0.0 | 7.0 | 2.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | |
| Green plant (%) | 0.0 | 14.9 | 2.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | |
| Albino plant produced | 1.0 | 2.0 | 9.0 | 0.0 | 1.0 | 0.0 | 5.0 | 3.0 | 6.0 | |
| Albino (%) | 12.5 | 4.3 | 10.8 | 0.0 | 25.0 | 0.0 | 5.8 | 7.0 | 10.3 | |

| | Genotypes/Media | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Hardinath-1 | | | Pravat | | | Khumal-4 | | | |
| Parameters | CIM 1 | CIM 2 | CIM 3 | CIM 1 | CIM 2 | CIM 3 | CIM 1 | CIM 2 | CIM 3 | Total |
| No. of anther plated | 522.0 | 273.0 | 238.0 | 164 | 157 | 163 | 328.0 | 304.0 | 271.0 | 4384.0 |
| Responsive anther | 14.0 | 19.0 | 2.0 | 5 | 0 | 0 | 70.0 | 10.0 | 1.0 | 532.0 |
| Response (%) | 2.7 | 6.9 | 0.8 | 3 | 0 | 0 | 21.3 | 3.3 | 0.4 | 12.1 |
| No. of calli plated | 14.0 | 19.0 | 1.0 | 5 | 0 | 0 | 62.0 | 2.0 | 1.0 | 438.0 |
| Total regeneration | 0.0 | 3.0 | 0.0 | 0 | 0 | 0 | 7.0 | 0.0 | 0.0 | 46.0 |
| Regeneration (%) | 0.0 | 15.8 | 0.0 | 0 | 0 | 0 | 1.3 | 0.0 | 0.0 | 10.5 |
| Green plant produced | 0.0 | 0.0 | 0.0 | 0 | 0 | 0 | 1.0 | 0.0 | 0.0 | 10.0 |
| Green plant (%) | 0.0 | 0.0 | 0.0 | 0 | 0 | 0 | 1.6 | 0.0 | 0.0 | 2.3 |
| Albino plant produced | 0.0 | 3.0 | 0.0 | 0 | 0 | 0 | 6.0 | 0.0 | 0.0 | 36.0 |
| Albino (%) | 0.0 | 15.8 | 0.0 | 0 | 0 | 0 | 9.7 | 0.0 | 0.0 | 8.2 |

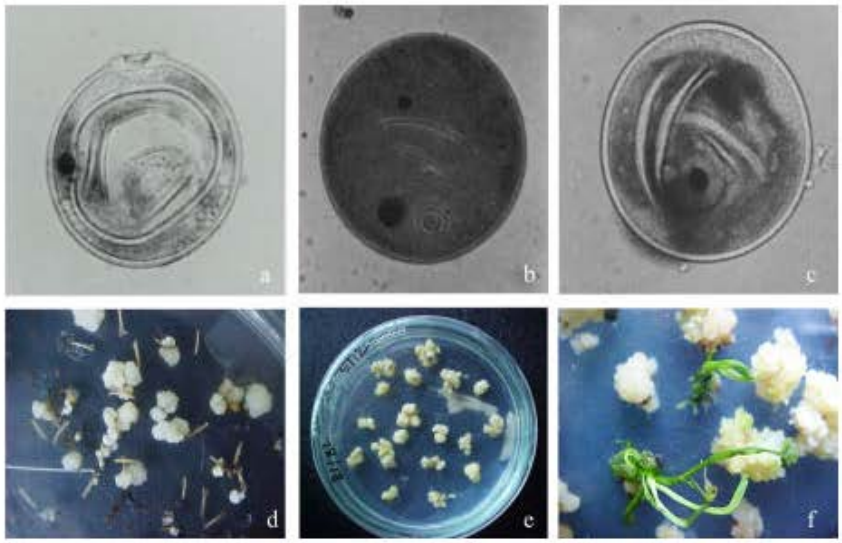


Fig. 3: (a-c) Pollen stages in relation to androgenic response. a: Mid-uninucleate microspore- a large vacuole is formed, nucleus located at one end of the microspore, with small nucleus. b: Bicellular microspore, with a large vegetative and a small generative cell. c: Late uninucleate microspore, vacuole has disappear, nucleus move away from the end of microspore and nucleolus has enlarged. (d-f) Androgenesis in rice anther culture, d: Calli induced from anther culture on CIM 1 from Khumal-4. e: Subculture of induced calli, Chandanath-3. f: Differentiation of green plant from induced calli, Chandanath-3

+ $AgNO_3$ (5 mg $L^{-1}$) was found relatively better. This study also confirmed the results suggested by Chen *et al*. (1991). They reported that callus forming ability from anther of rice was high in 2, 4-D supplied medium, but the regeneration ability from these calli was quite low as compared to calli formed on medium


Fig. 4: Differentiation of green plants from rice anther culture. (a) Generation of rice plant with well developed root and shoot resulted from CIM 2 induced calli in regeneration medium, Chandanath-3. (b) Albino plantlets resulted from CIM 2 induced calli in regeneration medium, Chomrong Local. (c) Fully grown anther culture derived haploid plants of Chandanath-3 and (d) Mother plant, Chandanath-3. Comparatively anther derived plants were small, completely sterile, dark green in color, without auricle and presence of minute ligule than mother plant

supplemented with NAA. However, above conclusion may not be applicable to all rice cultivars, as genotypic difference in hormone requirement have been reported (Liang, 1978). Thus, the higher rate of callus induction and lower rate of regeneration observed in the CIM 1 might be attributed to use of relatively higher doses of 2, 4-D used in this study (Table 2). This suggested that the exact level of 2, 4-D in the CIM required some degree of compromise between callus induction and regeneration frequency.

These findings were consistent with the previous reports (Lentini *et al*., 1995; Raina and Zapata, 1997). 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. Even among the *indica* cultivars a considerable variation for pollen callusing and plant regeneration has been noticed. In this study, we successfully produced calli from all rice genotypes using CIM 1 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. This slight discrepancy observed in this study and of Lentini *et al*. (1995) might be due to differences in genetic make up of genotypes. The rate of callus production in CIM 1 was varied from 0.9-68.7% (Table 2). Though, the CIM 1 was promoted the high frequency of callus induction across the genotypes, the frequency of regeneration was very poor (0.16%) as compared to 20-70% as reported by Laxmi and Reddy (1997) using the medium supplemented with 2, 4-D (2 mg $L^{-1}$) (Table 2, Fig. 1).

Relatively higher frequency of callus induction and regeneration was observed in CIM 2 than CIM 3 also suggested that $AgNO_3$ promotes not only callus induction but also regeneration of green plants (Fig. 1, Table 2). It was speculated that $AgNO_3$ had positive effect on embryogenesis by blocking the inhibitory effect of endogenously produced ethylene in culture vessels. Laxmi and Reddy (1997) and Lentni *et al*. (1995) reported that with the addition of $AgNO_3$ (10 mg $L^{-1}$) 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 and *Brassica* (Ghameni *et al*., 1994; Williams *et al*., 1990). The higher rate of albino plant production in this study might be attributed to higher rate of 2, 4- D in CIM 1 and long culture duration and genotypes themselves. It has been well documented that such factors favors the production of albino plants and sometimes the frequency of albinos production goes upto 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, affected 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). In this study we mostly observed that varieties with cold tolerance gene/s are relatively more responsive to anther culture than the tropical varieties. The absolute cause of this phenomenon is still unknown. However, the possible explanation might be that cold tolerance gene/s either linked with anther culture enhancing gene/s or have favorable effect during cold pretreatment. Thus this study *indica*ted that the possibility of enhancing callus induction and green plant regeneration by exploiting hill varieties of rice and N6 based medium supplemented with $AgNO_3$ (5-10 mg $L^{-1}$).

## CONCLUSION

This study revealed that there are possibilities of enhancing rice anther culturability by manipulating medium compositions and using responsive genotypes. Differential response of rice genotypes to callus induction media was observed in between and within rice subspecies. 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 designated as CIM 2. The Callus Induction Medium (CIM2) would offer great promise for the overall enhancement of ability of anther culture in Nepalese rice. Anther culture responsive cold tolerant genotypes (hill rice) can be used in the breeding programme to improve the anther culture ability of *indica* rice (tropical rice).

## ACKNOWLEDGMENTS

We thank Nepal Agricultural Research Council, Singha Darbar Plaza, Kathmandu, for financial supports and B. Dangol and S. Khanal for their laboratory assistance.

## REFERENCES

Afza, R., M. Shen, F.J. Zapata-Arias, J. Xie and H.K. Fundi *et al*., 2000. Effect of spikelet position on rice anther culture efficiency. Plant Sci., 153: 155-159.

Cha-Um, S., B. Srianan, A. Pichakum and C. Kirdmanee, 2009. An efficient procedure for embryogenic callus induction and double haploid plant regeneration through anther culture of Thai aromatic rice (*Oryza sativa* L. subsp. *indica*). *In vitro* Cell. Dev. Biol. Plant, 45: 171-179.

Chang, H.Y., T.M. Liu and Y.L. Wang, 1978. A preliminary observation on histogenesis and organogenesis of the *in vitro* development from rice microspores into plantlets. Proceedings of the Symposium on Plant Tissue Culture, May 25-30, Peking, China, pp: 126-126.

Chen, C.C., H.S. Tsay and C.R. Huang, 1991. Factors Affecting Androgenesis in Rice (*O. sativa* L.). In: Biotechnology in Agriculture and Forestry Rice, Bajaj, Y.P.S. (Ed.). Vol. 14, Springer Verlag, Berlin, ISBN: 354051810X, pp: 193-215.

Chu, C.C., 1978. The N6 medium and its applications to anther culture of cereal crops. Proceedings of the Symposium on Plant Tissue Culture, May 25-30, Peking, China, pp: 45-50.

Datta, S.K., 2005. *Androgenic haploids*: Factors controlling development and its application in crop improvement. Curr. Sci., 89: 1870-1878.

Ghameni, M., A. Sharrafi and G. Alibert, 1994. The effects of silver nitrate, colchicine, cupric sulfate and genotype on the production of embryoids from anthers of tetraploid wheat (*Triticum turgidum*). Plant Cell Tissue Organ Cult., 36: 355-359.

Gomez, K.A. and A.A. Gomez, 1984. Statistical Procedures for Agricultural Research. 2nd Edn., John Wiley and Sons, New York, ISBN: 978-0-471-87092-0, pp: 197-198.

Grewal, D., R. Gill and S.S. Gosal, 2006. Role of cysteine in enhancing androgenesis and regeneration of *indica* rice (*Oryza sativa* L.). Plant Growth Regul., 49: 43-47.

Gupta, P.K., 1999. Haploidy in Higher Plants: Cytogenetics. 1st Edn., Rastogi Publication, Shivaji Road Meerut, India, ISBN: 81-7133-662-0, pp: 116-119.

Khan, S.H., 1975. A technique for staining rice chromosomes. Cytologia, 40: 595-598.

Laxmi, G.V. and G.M. Reddy, 1997. Anther culture of *indica* rice: Technical improvements in callus induction and green plant regeneration. J. Genet. Breed., 51: 295-302.

Lentini, Z., P. Reyes, C.P. Martinez and W.M. Roz, 1995. Androgenesis in highly recalcitrant rice genotypes with maltose and silver nitrate. Plant Sci., 110: 127-138.

Liang, H.M., 1978. The advance of studies on medium for anther culture of rice in China. Proceedings of the Symposium on Plant Tissue Culture, May 25-30, Peking, China, pp: 57-64.

Miah, M.A.A., E.D. Earle and G.S. Khush, 1985. Inheritance of callus formation ability in anther cultures of rice, *Oryza sativa* L. Theor. Applied Genet., 70: 113-116.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15: 473-497.

Niizeki, H., 1997. Anther (pollen) Culture. In: Science of Rice Plant Genetics, Matsuo T., Y. Futsuhara, F. Kikuchi and H. Yamaguchi (Eds.). Vol. 3, Food and Agriculture Policy Research Center, Tokyo, ISBN: 4-540-96010-5, pp: 691-697.

Niroula, R.K., B.P. Sah, H.P. Bimb and S. Nayak, 2005. Effect of genotype and culture media on callus induction and plant regeneration from matured rice grain culture. J. Inst. Agric. Anim. Sci., 26: 21-26.

Raina, S.K., 1997. Doubled haploid breeding in cereals. Plant Breed. Rev., 15: 141-186.

Raina, S.K. and F.J. Zapata, 1997. Enhanced anther culture efficiency of *indica* rice (*Oryza sativa* L.) through modification of the culture media. Plant Breed., 116: 305-315.

Roy, B. and A.B. Mandal, 2005. Anther culture response in *indica* rice and variations in major agronomic characters among the androclones of a scented cultivars, kernal local. Afr. J. Biotechnol., 4: 235-240.

Sah, B.P. and R.K. Niroula, 2007. Successful regeneration and characterization of anther derived rice hybrid plants from *O. sativa* L. x *O. rufipogon* Griff. Scietnific World, 5: 14-18.

Talebi, R., M.R. Rahemi, H. Arefi, M. Nourozi and N. Bagheri, 2007. *In vitro* plant regeneration through anther cutur of some Iranian local rice (*Oryza sativa* L.) cultivars. Pak. J. Biol. Sci., 10: 2056-2060.

Thomas, T.D., 2008. The role of activated charcoal in plant tissue culture. Biotech. Adv., 26: 618-631.

Trejo-Tapia, G., U.M. Amaya, G.S. Morales, A.D.J. Sanchez and B.M. Bonfil *et al*., 2002. The effects of cold-pretreatment, auxins and carbon source on anther culture of rice. Plant Cell Tissue Organ Cult., 71: 41-46.

Williams, J., D.A.C. Pink and N.L. Biddington, 1990. Effect of silver nitrate on long term culture and regeneration of callus from *Brassica oleracea*. var. *gemmifera*. Plant Cell. Tissue Organ Cult., 21: 61-66.