Biharean Biologist (2009)
P-ISSN: 1843-5637, E-ISSN: 2065-1155

Vol. 3, No.2, Pp.: 119-124
Article No.: 031117

Evaluation of Effective Factors in Anther Culture of Iranian Rice (*Oryza sativa* L.) Cultivars

Nadali BAGHERI, Nadali BABAEIAN-JELODAR and Ali GHANBARI*

Department of Biotechnology and Plant Breeding, College of Agriculture,
University of Mazandaran, Babulsar, Mazandaran, Iran, P.O.Box: 578.
* Corresponding author: A. Ghanbari, Tel: +98-151-3822574, Fax: +98-151-3822577, E-mail: biohort@gmail.com

Abstract. This study was conducted to develop the breeding method of anther culture for pure line induction in Iranian Rice cultivars. Several aspects were studied: the genotype factors, the culture medium, the date of sampling and the kind and content of sugar in culture medium. Results showed that the first date of sampling (middle July) had better callus induction than the second one and has led to increased callus induction of cultivars to 8.1 percent. In the investigation of carbon source, callus induction medium with 4% maltose was significantly better than others of sugar treatments. Also, different treatments of sugar had positive effects on cultivars regeneration, but there were not significant. Results indicated that there were significant differences in callus production among the different cultivars, and the average of callus induction ability ranged from 2.13% to 23.38%. Rashti had the highest callus induction and Amol3 and Amol2 had the lowest callus induction. Reciprocal effects of genotype and medium were significant ($p<0.05$). N_6, Fj_1 and G_1 media had the highest callus induction, respectively. The average of regeneration ranged from 0.0 to 15.2 percent. The maximum plant regeneration percentage was obtained in M_5 regeneration medium.

Key words: Anther culture, Callus, Regeneration, Rice (*Oryza sativa* L.)

Introduction

Haploid production is one of the most important technique in plant breeding and genetic analysis. This is important especially when it would integrate into common breeding methods for desired traits production in plant. One of haploid plant generation method is anther cultures, which today is applied widely in rice breeding. Rice pure line production is performed using anther culture method in a very short time and by confidence of the derived lines genetic purity. Many factors such as genotype, physiological age of donor plant and culture medium are effective in anther culture reaction for androgenic calli and plant regeneration (Mercy 1990). Culture medium and genotype are important factors for callus induction from anther culture.

Nutritional requirements for androgenesis and formed embryos growth are different (Zapata et al. 1990). Since 1983, maltose has been known as a better carbohydrate source in comparison to sucrose for androgenesis in many plant species such as corn (Sun et al. 1993). Studies of barley indicate that in medium, sucrose has analyzed quickly to glucose and maltose as after three weeks from anther culture, that medium was without any sucrose. But maltose analysis had been lesser relative to sucrose in similar time period. Meanwhile sucrose toxicity for androgenesis is due to microspore sensitiveness to fructose no glucose (Last & Brettell 1990). Zapata et al. (1990), perceive that genotype difference is a requirement for stock medium. N_6 medium had the best result in pollen culture of Japonica variety Taipei 309, meanwhile Indica variety IR-43 had the best reaction in E_{10} medium (modified B_5 medium).

Draz et al. (1991a) expressed that callus induction of media have also a role in plant regeneration, each callus medium will have the best reaction with a particular regeneration medium. Kim et al. (1991) reported that the best reaction to anther culture resulted from hybrid of Japonica × Japonica and following it; Indica × Japonica and then Indica × Indica rice cultivars. In a study which was conducted in order to determine callus induction and green regeneration heredity of genotypes derived via diallel analysis of four rice cultivars (two Indica and two Japonica cultivars), it determined that callus induction and green plant regeneration are influenced by genotype and medium × genotype interaction (Quimio & Zapata 1990). Draz et al. (1991b), in order to produce doubled haploid rice lines by Indica with Japonica hybrids anther culture, found out those hybrids which their maternal parent was Japonica cultivar, had more calli than other hybrids. In general, Indica rice and hybrids between Japonica and Indica cultivars had a lower anther culture ability in comparison to Japonica cultivars (Chen et al. 1991).

Culture medium is one of the important factors for callus production from cultured anthers. Nutritional requirements are different for androgenesis and the formed embryos growth (Zapata et al. 1990). Zapata et al. (1990) showed that there are genotypic differences for basic culture medium requirements. N6 culture medium had best results in pollen seed culture of Taipei 309 Japonica cultivar, while IR-43 Indica cultivar had the best reaction in E10 culture medium (changed from of B_5 culture medium). Draz et al. (1991 a) expressed that callus formation culture media had also a role in induction of plant regeneration, each callus culture medium will have the best reaction with a specific regeneration medium. They also showed that induced calli in G_1 culture medium had the best regeneration in M_5, M_3 and M_7 culture media and induced calli in M_7, M_4 and SK_{11} culture media. Also, induced calli in L_8 culture medium had the best regeneration in M_5, M_4, M_3, M_2 and SK_{11} culture media. Concerning the genotype and culture medium effects on callus induction and regeneration of anther culture, Mandel and Gupta (1995) expressed that NAA or 2,4-D by a $2mgL^{-1}$ concentration is effective in callus formation and calli which were induced in culture medium containing NAA, had more green plant regeneration frequency than calli which were induced in culture medium containing 2,4-D. Also, they illustrated that oxin in high concentrations will prevent green plant regeneration.

This study was performed because of rice anther culture importance in agricultural cultivars production and also the role of effective factors on callus induction and regeneration to determine suitable time for sample collection and culture medium for tested genotypes. This study can be a field for biotechnology affairs such as genetic engineering.

Materials and Methods

Plant materials and callus induction

Genotypes used in this study were cultivated in plant per hill and a distance of 25x25 cm, after receiving from Rice Research Institute of Amol (Iran). Anther supplier panicles collection was performed in the early morning and/or in the afternoon and from primary tillers (3-4 original stems in per plant) which flag leaf base distance from the first lower leaf was 5-9 cm (Kasha et al. 1990). Panicles after washing in distilled water were sterilized in 70% percent ethanol. In order to perform the cooling pre-treatment, panicles were kept at 8°C for 8 days (Chaleff & Stolarz 1981, Yamaguchi et al. 1990). For anther culture, after cytology examination, florets that were in an appropriate step of mono/uni-core developments were suitable for culture (Kasha et al. 1990, Armstrong 1995). Florets were sterilized with 1% sodium hypochlorite for 20 min and then were rinsed by sterilized distilled water for three times. Sixty anthers were used in each Petri dish containing 6 ml culture medium. After anthers culture, Petri dishes were sealed by Para-film and were kept in incubator with dark conditions and at 26±1°C (Croughan 1995). Calli were induced after 3-4 weeks. The percentage of callus induction by the induced callus numbers was calculated based on cultured anther numbers (in each Petri dish). After calli reached to a diameter of 2-4 mm, they were transferred to different culture media for re-generation (Yoshida 1995). The percentage of regeneration was calculated by regenerated plant number based on transferred callus to regeneration culture medium. They were kept in light intensity of 3000 Lux for 16 h lightness, 8 h darkness and at 24±1°C (Croughan 1995). After a period of 2-3 weeks, calli started to regenerate.

Date of anther supplier cluster harvest

For performing this study, Iranian Rice cultivars including Rashti, Mohammadi-Chepersar, Ghasroddashti, IT 28 and Amol3 were examined on two dates: middle July and middle August. Also, N6 culture medium with 2 mg/L 2,4-D was used to study the callus induction in these cultivars.

Type and concentration of carbon sources

In this study, Rashti , Mohammadi-Chepersar, Ghasroddashti, IT 28 and Amol3 genotypes were used in N6 Semi-Solid callus induction medium (Chu et al. 1978), along with 2mg/L 2,4-D and by three sugar combinations including 4% maltose, 4% sucrose and 4% maltose+sacrose. For regeneration, MS5 modified culture medium (Murashige & Skoog 1962), was used with 1mg/L Naphthalene Acetic Acid (NAA) and 1mg/L kinetin.

Genotype and culture medium

The used genotypes included Sadri, Rashti, Salari, Chepersar, Ghasroddashti, IR-28 and Amol-3 culture medium and N6 modified culture media (Fj1, Fj4, L8, Fj, and G_1) (Chu et al. 1978). For regeneration of obtained calli, MS modified regeneration culture media (M_7, SK_{11} and M_5) were used which are different in terms of combination and hormone amounts (Murashige & Skoog 1962).

Variance analysis

Since the data was expressed in percentage values, regarding the data percentage ranges, $Arc\sin\sqrt{x}$ angular conversion were used in order to standardize data in data variance analysis. Also, statistical analysis was conducted on data using MSTATC software.

Results

Effect of time of sample collection from farm

The results in Figure 1 show that the studied genotypes that were collected from the farm in the middle of July, had more anther culture reaction than the samples taken in August. Mohammadi-Chepersar and Salari cultivars showed the least difference in callus induction during two stages of cluster or sample collection from the farm. Also, collected samples of anther culture in the first date has caused to increase an average of 8.1 percent callus formation in studied genotypes than on the second one (Fig.1).

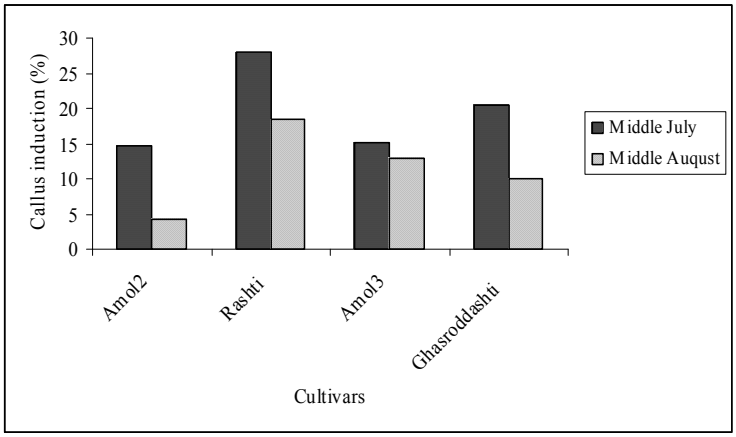

Figure 1. Plant harvest date effect on cultivars callus induction in N6 culture medium.

Effect of type and concentration of sugar

Results shows that callus induction in culture medium containing maltose has been more than other culture media with same concentration of different sugars. So, callus induction percentage of Rashti cultivar has increased, from 21.02% (in culture medium containing 4% sucrose) to 25.3% (in culture medium containing 4% sucrose+maltose) and 29.25% (in culture medium containing 4% maltose). Also, callus induction percentage of Amol-3 cultivar has been increased from 9.78% (in culture medium containing 4% sucrose) to 14.07% sucrose+maltose) and 17.83% (in culture medium containing 4% maltose). Mohammadi-Chepersar, Ghasroddashti and IR-28 cultivars had a better response than other sugar combinations of culture medium containing 4% maltose (Table 1).

Tested cultivars regeneration means were variable in range of 5.0-17.5%. Cultivars regeneration means comparison based on Duncan's multi-ranges test ($P<0.01$) showed that Ghasroddashti cultivar has a significant superiority comparing with other tested cultivars in regeneration culture medium supplemented with 3 sugar types (Table 1). Although, tested cultivars regeneration have increased in retention culture medium containing 4% maltose to regeneration culture medium containing 4% sucrose and 4% maltose+sucrose, sugar effect on cultivars regeneration was not statistically (Fig.2).

Genotypes reaction to callus induction in different culture media

There was a significant difference between genotypes and culture media in terms of callus induction. Rashti, Salari, Mohammadi-Chepersar, Ghasroddashti cultivars (aromatic cultivars) were superior to other culture media in terms of callus induction in N6 culture medium. Among 7 tested genotypes, 5 genotypes (Rashti, Salari, Mohammadi-Chepersar, IR-28, Amol-3 cultivars) were superior to other culture media in Fj_1 culture medium. In terms of callus induction, tested genotypes were superior to other culture media in N_6, Fj_1 and G_1 culture media, respectively. Rashti and Ghasroddashti cultivars showed the most induction percentage and Amol-3 and IR-28 cultivars showed the least induction

Table 1. Effect of sugar kinds and concentrations on callus induction and plant regeneration (In each column, means which have one common letter, based on Duncan's multiple test).

cultivars	Callus induction medium			Regeneration induction medium		
	Sucrose (4%)	Sucrose+Maltose (4%)	Maltose (4%)	Sucrose (4%)	Sucrose+Maltose (4%)	Maltose (4%)
Rashti	21.02 a	25.3 a	29.25 a	10.1 ab	11.8 ab	12.4 ab
Ghasroddashti	17.95 b	19.93 b	23.98 b	15.0 a	16.2 a	17.5 a
Amol2	8.63 c	11.02 d	15.43 c	5.6 b	7.6 b	5.0 b
Amol3	9.78 c	14.07 c	17.83 c	5.02 b	5.0 b	10.0 ab

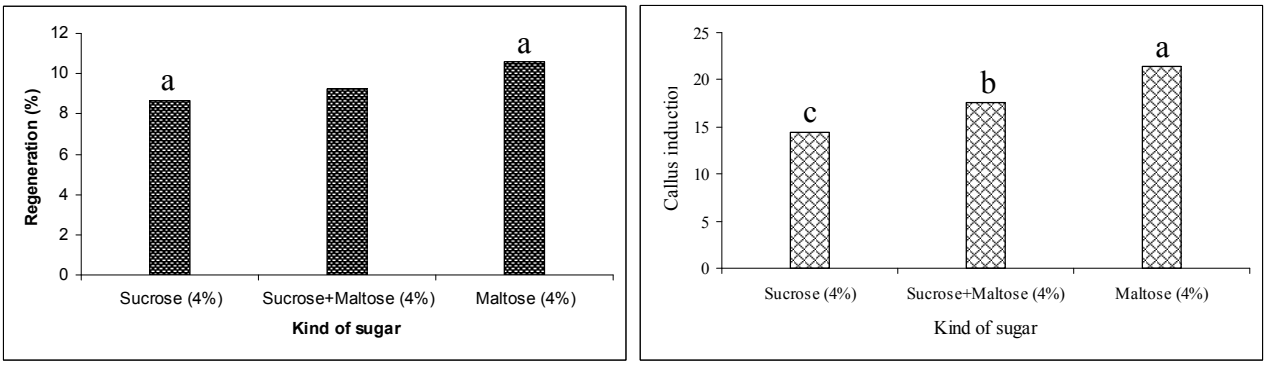

Figure 2. Mean comparison of sugar effect on cultivar callus induction and regeneration cultivars in bases of Duncan's multiple range tests ($p < 0.05$).

Table 2. Mean comparison of tested cultivars callus induction percentage ($p<0.01$).

Culture medium	Cultivars			
N_6	Rashti	Ghasroddashti	Amol3	Amol2
	$\bar{x}$ = 23.4 a	$\bar{x}$ =19.6 b	$\bar{x}$ =6.7 c	$\bar{x}$ = 5.0 c
Fj	Rashti	Ghasroddashti	Amol3	Amol2
	$\bar{x}$ =18.4 a	$\bar{x}$ = 14.6 b	$\bar{x}$ = 7.5 c	$\bar{x}$ = 5.0 d
Fj_1	Rashti	Ghasroddashti	Amol3	Amol2
	$\bar{x}$ = 22.5 a	$\bar{x}$ = 17.1 b	$\bar{x}$ = 9.2 c	$\bar{x}$ = 8.4 c
Fj_4	Rashti	Ghasroddashti	Amol3	Amol2
	$\bar{x}$ = 15.8 a	$\bar{x}$ = 12.5 b	$\bar{x}$ = 3.4 c	$\bar{x}$ = 2.9 c
L_8	Rashti	Ghasroddashti	Amol2	Amol3
	$\bar{x}$ = 18.4 a	$\bar{x}$ = 10.9 b	$\bar{x}$ = 2.9 c	$\bar{x}$ = 2.12 c
G_1	Rashti	Ghasroddashti	Amol2	Amol3
	$\bar{x}$ = 21.3 a	$\bar{x}$ = 17.5 b	$\bar{x}$ = 3.4 c	$\bar{x}$ = 2.9 c

percentage. Mean percent age of callus induction in N_6 and G_1 for studied genotypes are superior in comparison to other media (Table 2).

Regeneration of tested cultivars

In this study, tested cultivars green plant regeneration percentage in different media represents that green plant regeneration range is variable from zero (Sadri & Salari) to 33.4% (Ghasroddashti). Also, regeneration percentage of tested cultivars albino plant in different media shows albino plant regeneration range is variable from 5 (IR-25) to 51.6% (Rashti). Study of green plant regeneration percentage in different media indicates that M_7 (10%), Sk_{11} (21.9%) and M_5 (30.2%) media had maximum green plant regeneration and M_5 (36.9%), M_7 (56.8%) and SK_{11} (63.4%) media had maximum albino plant regeneration percentage, respectively (Table 3).

Regarding these results from the plant regeneration and the culture media regeneration, which are different in terms of components and hormone amounts (M_5 culture medium with 1mg/L Naphthalene Acetic Acid + 4 mg/ L Kinetin, M_7 culture medium with 1mg/L

Naphthalene Acetic Acid + 16mg/L Kinetin and SK_{11} culture medium with 1mg/L Naphthalene Acetic Acid + 1mg/L Kinetin+ 1mg/L Benzyl Amino Purine), it is determined that the more homogenous culture media have more effect in green plant regeneration (Fig. 3).

Discussion

In this study, tested genotypes which sample collection from the farm was in middle July, had more anther culture reaction than in August. Mohammadi-Chepersar and Salari cultivars showed the least difference in callus induction during two stages of cluster or sample collection from the farm. Also, collected samples anther cultures from the first date caused the increase of a mean of 8.1% callus induction in tested genotypes than the second date. These results represent the physiological situation difference of anther supplier varieties and characteristics of rice growth medium temperature in flowering time as an effective factor in anther reaction.

Sugars have two roles in culture medium, both as carbon source and as osmotic pressure regulator, which are both important for callus induction (Bishnoi et al. 2000). Results indicate that sugar type in this experiment had no effect on cultivars regeneration, and at least had no statistically. This point expresses this fact that in anther culture, sugar type in androgenesis is mostly effective on callus induction and had no effect on plant regeneration, which confirms the results of Lentini et al. (1995). As a result, it is suggested that sucrose can be used for rice plant regeneration because of lower price than maltose and in an amount of 3% in culture medium. The above mentioned, stress the importance of carbon source as one of effective factors in rice anthers callus induction and focus on using maltose in an amount of 4% in culture medium. Although if it is inclined to use sucrose in culture medium, regarding the time interval of callus induction in rice androgenesis, which is 3-4 weeks (Yoshida 1995), and in this time interval when sucrose is decomposed in culture medium (Last & Brettell 1990), it is suggested to use more amount of sucrose (60 g/L) for androgenesis or anther culture.

Culture medium and genotype are two important factors for callus induction in rice anther culture. N_6 medium used mainly for the Helijaponica rice (group 6) and for indica rice (group 1) is not suitable (Gosal et al. 1997). The much indica rice is fragrant that belonging

Table 3. Mean comparison of cultivar callus induction frequency for survey genotype× medium interaction effect ($p < 0.05$).

Cultivars	Culture medium					
	N_6	Fj	Fj_1	Fj_4	L_8	G_1
Rashti	23.4 a	18.4 ab	22.5 a	15.8 b	18.4 ab	21.3 a
Ghasroddashti	19.6 ab	14.6 b	17.1 ab	12.6 c	10.8 c	17.5 ab
Amol2	5.1 cd	5.1 cd	8.4 c	2.9 e	2.9 e	3.4 e
Amol3	6.7 cd	7.5 cd	10.1 c	2.91 e	2.1 e	2.95 e

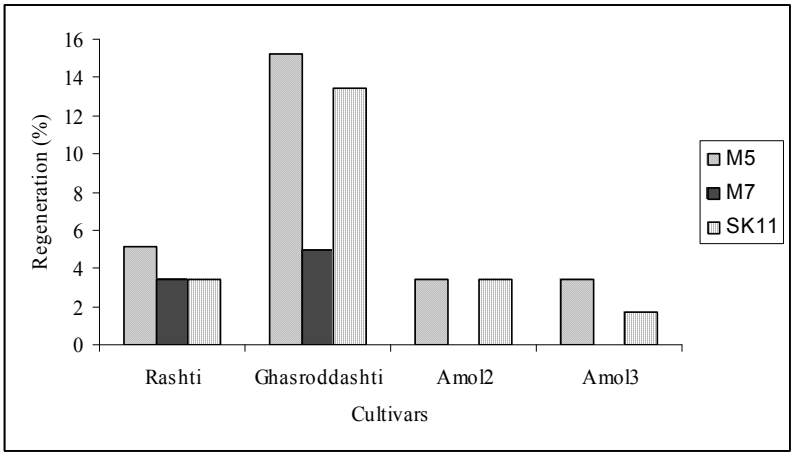

Fig. 3. Green plant regeneration percent in studied cultivars in M_5, M_7 and SK_{11} media.

had to group 5, but according to isosymic studies to Japonica rice is early (Celazman 1987). In these two media (N_6 and G_1) in addition to nitrate and ammonium, there also exist nitrate ions and ammonium that were effective in absorbing growth regulators via cell membrane (Khana & Raina 1998). Also these two media (N_6 and G_1) were in comparison to other media supplemented with 2 mg/L 2-4,D and 2-4,D as an oxin material had basically role in rice anther callus induction on growth regulator.

Albino plant production is one of the main problems in rice anther culture (Bashnoi et al. 2000). We indicated that production of albino plant can decrease by transferring new calli to regeneration medium, in 26°C in incubator and using of more suitable callus induction and regeneration media. Also, in this study, M_5 and SK_{11} culture media which are more balanced than M_7 culture medium in terms of combination and hormone amounts were appropriated reaction.

This study was performed because of rice anther culture importance in agronomical cultivars production. This research can be a preliminary work for biotechnology assays in the case of genetic engineering in rice cultivars.

References

Armstrong, K. C. (1995): Staining procedures for chromosome analysis. Pp. 251-266, In: Gamborg, O. L., Phillips, G. C. (eds.). Plant Cell Tissue and Organ Culture: fundamental methods. Springer-Verleg Berlin Heidelberg, New York.

Bishnoi, V., Jain, R. K., Rohilla, J. S., Chowdhury, V. K., Gupta, K. R., Chodhury, J. B. (2000): Anther culture of recalcitrant Indica × Basmati rice hybrids. Euphytica 114: 93-101.

Chaleff, R. S., Stolarz, A. (1981): Factors influencing the frequency of callus formation among cultured rice (*Oryza sativa* L.) anthers. Plant Physiology 51: 201-206.

Chen, C. C., Tasy, H. S., Huang, C. R. (1991): Factors affecting androgenesis in rice (*Oryza sativa* L.). Pp. 192-215. In: Bajaj, Y. P. S. (ed.). Rice. Biotechnology in Agriculture and Forestry, Vol. 14. Springer, Berlin, Heidelberg, New York.

Chu, C. C., Wang, C. S., Sun, C. S. (1978): The N_6 and its application to anther culture of cereal crops. Pp. 43-50. In: Proceeding of The Symposium On Plant Tissue Culture. Science Press, Peking.

Croughan, T. P. (1995): Anther culture for doubled haploid production. Pp. 143-154. In: Gamborg, O. L., Phillips, G. C. (eds.): Plant Cell Tissue and Organ Culture: fundamental methods. Springer-Verleg Berlin Heidelberg, New York.

Draz, A. E., Zapata, F. J., Khush, G. S. (1991a): Interaction of media for callus induction and for plant regeneration in rice anther culture. International Rice Reserch Newsletter 16: 5, 7-8.

Draz, A. E., Zapata, F. J., Khush, G. S. (1991b): Development of dihaploid rice lines through anther culture (II). International Rice Reserch Newsletter 16: 6-7.

Glaszmann, T. C. (1987): Isozymes and classification of Asian rice varieties. Theoretical and Applied Genetics 74: 21-30.

Gosal, S. S., Sindhu, A. S., Sandhu, J. S., Sandhu-Gill, R., Singh, B., Khehra, G. S., Sidhu, G. S., Dhaliwal, H. S. (1997): Haploidy in rice. Pp. 1-34. In: Mohan Jain, S., Sopory, S. K., Veilleux, R. E. (eds.): Cereals. In vitro Haploid Production in Higher Plant. Vol. 4. Kluwer Academic Publishers, Dordrecht/ Boston/London.

Kasha, K. J., Ziauddin, A., Cho, U. H. (1990): Haploids in cereal improvement anther and microspore culture. Pp. 213-232. In: Gustafson, J. P. (ed): Gene Manipulation Plant Improvement (II). Plenum Press, New York

Khanna, H. K., Raina, S. K. (1998): Genotype culture media interaction effects on regeneration response of three indica rice cultivars. Plant Cell, Tissue and Organ Culture 52: 145-153.

Kim, H. S., Lee, Y. T., Lee, S. Y., Kim, T. S. (1991): Anther culture efficiency in different rice genotypes under different cold pretreatment durations and culture temperatures. Research Reports of the Rural Development Administration, Bio/Technology 33: 5-13.

Last, D. J., Brettell, R. I. S. (1990): Embryo yield in wheat anther culture is influenced by the choice of sugar in the culture medium. Plant Cell Report 9: 14-16.

Lentini, Z., Reyes, P., Martine, C. P., Roca, W. M. (1995): Androgenesis of highly recalcitrant rice genotypes with maltose and silver nitrate. Plant Science Limerick 110: 127-138.

Mercy, S. T. (1990): Basic Studies in Rice Anther Culture 83. Pp. 49-64. Daya Publishing House, New Delhi, India.

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

Quimio, C. A., Zapata, F. J. (1990): Diallel analysis of callus induction and green plant regeneration in rice anther culture. Crop Science 30: 188-192.

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

Rina, S. K. (1997): Doubled haploid breeding in cereals. Plant Breeding Reviews 15: 141-186.

Sandhu, J. S., Gill, M. S., Gosal, S. S. (1993): Callus induction and plant regeneration from cultured anthers of indica rice varieties. Plant Tissue Culture 3: 17-21.

Sun, Z. X., Si, H. M., Cheng, S. H., Zhan, X. Y. (1993): Effect of maltose on efficiency of anther culture of rice. Chinese Journal of Rice Science 7: 227-231.

Yamaguchi, M., Yomoda, A., Hinata K. (1990): Varietals differences in the response to low temperature treatment for callus formation in anther culture of rice. Japan Journal Breeding 40: 193-198.

Yoshida, T. (1995): Relationship between callus size and plant regeneration in rice *(Oryza sativa* L.) anther culture. JARQ, Japan Agricultural Research Quarterly 29: 143-147.

Zapata, F. J., Aldemita, R. R., Ella, E. S., Cho, M. S. (1990): Isolated microspore culture of rice at the international rice research institute. Pp. 311-319. In: Rice Genetics II. Proceedings of the Second International Rice Research Genetics Symposium, 14-18 May 1990, IRRI, Manila.

Submitted: 23 May 2009
/ Accepted: 8 July 2009

Published Online: 10 July 2009