Factors Affecting Androgenesis in Indica Rice

Sant S. Bhojwani, Himani Pande and Anupam Raina

Department of Botany, University of Delhi, Delhi 110007, India
e-mail: ssbhojwani@satyam.net.in

Abstract

For its effective utilization in breeding programmes, the haploid production technique should allow genotype-independent production of large numbers of haploids. Although anther culture has been successfully used to hasten the breeding programmes in several crop species, including rice, there still remain problems to realize its full potential. Unlike the highly responsive model systems, most of the indica cultivars respond rather poorly in anther cultures. There is also concern regarding the gametic spectrum representation by the anther culture-derived doubled haploids. If there is a biased representation of recombinants possessing better culturability, it is important to analyse how seriously it effects the objectives of the breeder.

Whereas in maize androgenesis occurs via embryogenesis, in barley, wheat and rice the microspores divide to form a callus which later differentiate plants. Both the steps require different culture conditions and are affected by the genetic make-up of the plants. Recently, the physiology of the donor plants has been identified as a critical factor in achieving better anther culture efficiency. In this paper the factors affecting androgenesis in rice, particularly indica rice will be discussed.

Genotype

Most of the *in vitro* morphogenic responses are genotype–dependent (Bhojwani and Razdan, 1996). In general, indica cultivars of rice exhibit poorer androgenic response than the japonica cultivars (Hu, 1985, Raina, 1997). 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 observed. Guha-Mukherjee (1973) reported that only 5 out of 18 indica cultivars showed pollen callusing and in only four cases did the calli differentiate plants. Similarly, Lentini *et al*. (1995) reported that only one out of 35 indica cultivars exhibited pollen callusing on N_6 medium. The purple pigmented, coarse-grained indica cv Crossa performed significantly better (40 %) than the fine grained, aromatic cv Basmati-370 (<10 %; Raina, 1989).

Physiology of Donor Plants

The physiological state of the donor plants, which is affected by several factors, has a profound effect on the androgenic response of their microspores. In *Brassica juncea*, a recalcitrant species, we were able to improve the androgenic response from 3 % to 16 % only by late sowing of the donor plants, which probably acted as a stress on the plants (Agarwal and Bhojwani, 1993). Our more recent experience with this oilseed crop is that under controlled growth conditions, in a phytotron or growth chamber, the donor plants can be maintained for a long time without effecting the androgenic potential of their anthers, and the difference between the anthers from main branch and lateral branches of different order is considerably reduced. We do not know of any similar study with rice but there is sufficient evidence to suggest that the growth conditions of the donor plants have significant effect on the yield of androgenic pollen in this crop.

A remarkable effect on the androgenic response of an indica rice cultivar by light and day/night temperature regime was observed by Raina and Zapata (see Raina, 1997). The plants of cv IR43 that reached the panicle emergence stage under long days (>12 h), high solar radiation (>18Mj m^{-2}) and sunshine (>8 h) and day/night temperature (34 ^{o}C/24 ^{o}C) showed highest anther culture response; the response declined with lowering values of these parameters. They also observed that the plants grown in the field were significantly superior in this respect to those grown in the glasshouse or in pots near the field. Superiority of field-grown plants over glasshouse-grown plants has also been reported for other cereals, including maize (Petolino and Thompson, 1987) and wheat (Lu *et al*., 1991).

The physiology of the donor plants can also be altered, and the androgenic response modified, by treating the donor plants with certain chemicals, such as etherel (Zhao et al., 1991, *cited in* Sun, 1999).

Pre-Treatment

Application of a variety of stresses, such as temperature pre-treatment, osmotic shock and sugar starvation, during the labile developmental period of pollen grains is known to be promontory or essential for the induction of androgenesis in several plants, including cereals (Bhojwani and Razdan, 1996; Bhojwani *et al*., 1997). However, the type, duration and the time of application of these pre-treatments may vary with the species or even variety (Datta, 2001).

Low Temperature

The most widely used pre-treatment for androgenesis is the low temperature shock. In most of the published works on androgenesis in rice, panicles were given a cold pre-treatment but the temperature and duration varied. Matsushima *et al.* (1988) had reported that a pre-treatment at 10 ^{o}C for 10-30 days was necessary to induce sporophytic divisions in microspores of the japonica cv Nipponbare. This was subsequently confirmed by several workers for japonica and indica cultivars (Ogawa *et al*., 1992, Datta *et al*., 1990, Raina and Irfan, 1998). Ogawa *et al*. (1995) observed that 28 days of pre-treatment at 10 ^{o}C was optimum for the indica cv IR 24. Gupta and Borthakur (1987) had selected pre-treatment at 10 ^{o}C for 11 days for anther

culture of the indica cultivar khonorullo. Although the frequency of anthers showing pollen callusing after cold-treatment for 25 days was fairly high, most of the plants regenerated from the calli formed after such a long cold pre-treatment were albinos. Similarly, Pande (1997) observed that cold pre-treatment was essential for androgenesis in anther cultures of the indica cv IR43, and 10 ^{o}C for 10 days was most suitable. Pre-treatments longer than 11 days resulted in albino production. Reddy *et al.* (1985) reported that a brief (10 min) exposure to high temperature (35 ^{o}C) before cold-treatment was better for pollen callusing but it adversely affected green plant production.

Osmotic Stress

Another pre-treatment, which can substitute cold treatment for the induction of androgenesis, is osmotic shock. Wei *et al.* (1986), who first time reported *ab initio* microspore culture of barley, followed a protocol involving isolation of microspores in 0.3 M mannitol and treating them in this solution for 3 days. According to them, mannitol pre-treatment for 3 days was essential and for 7 days optimum to induce androgenesis in this system. Roberts-Oehlschlager and Dunwell (1990) had reported that 4 day incubation of barley anthers in a medium containing 3.2 % mannitol raised the pollen callusing response from 23 % to 78 % which was more than cold treatment alone or in combination with mannitol. According to these authors, mannitol stress improves sugar uptake causing build-up of glucose pool in the anther tissue. Similar results were reported by Ziauddin *et al.* (1990). Hoekstra *et al*. (1992) and Cistue *et al.* (1994) confirmed the importance of mannitol pre-treatment. In the latter case, application of 0.7 M mannitol for 3-5 days improved green plant regeneration significantly. Increase in mannitol concentration showed linear increase in the number of dividing microspores and an increase in the proportion of green plants regenerated. The optimum duration of treatment varied with the genotype. Hoekstra *et al*. (1993), who gave mannitol stress (440 mOs kg^{-1}) for 4 days and cultured the microspores on a medium of the osmolarity of 350 mos kg^{-1} at a density of $2x10^{4}$ microspores per ml, obtained 320 embryo-like structures per 1x104 microspores. The yield of green plants increased from 50 % to 97 %.
Recently, Raina and Irfan (1998) have reported that treatment of anthers in 0.4 M mannitol solution was essential to induce androgenesis in microspore cultures of indica and japonica cultivars. For indica cv, pre-treatment at 33 ^{o}C was better than at 25 ^{o}C, with respect to the number of embryo-like structures or calli formation. In the absence of cold treatment, mannitol treatment promoted androgenesis in anther cultures of cv IR43 from 3 % to 33.4 %; with cold treatment it had no promotory effect (Pande, 1997). For wheat microspore culture, Mejza *et al*. (1993) isolated and fractionated microspores in 20 % maltose solution and cultured them on a medium with 9 % maltose. This allowed genotype non-specific regeneration in *ab initio* microspore cultures. Cold treatment in combination with the osmotic treatment was detrimental. Therefore, maltose treatment was given at 25 ^{o}C.

Ogawa *et al*. (1995) reported that sugar starvation of microspores for 3 days could, to some extent, substitute for cold treatment for the induction of androgenesis in microspore cultures of indica rice. Sugar starvation of the anthers of IR43 for 2 days in the beginning of culture caused12-fold increase in the androgenenic response (39 %)of cv IR43 (Figure 1; Pande, 1997). However, cold treatment was superior to sugar starvation. Sugar starvation has been earlier shown to promote androgenesis in tobacco (Aruga *et al*., 1985) and barley (Wei *et al.*, 1986).

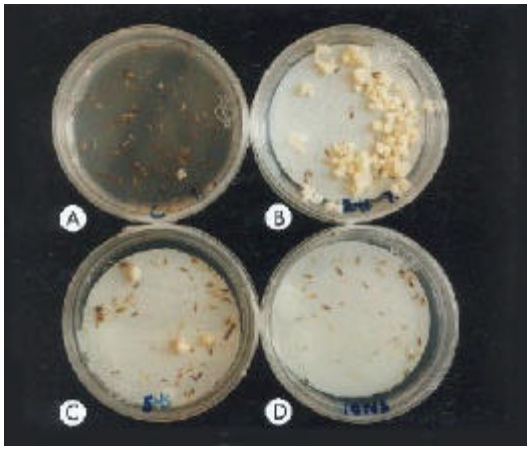

Figure1: **A-D**. Effect of sugar starvation for 0, 2, 5 and 10 days, respectively, on androgenic response in anther cultures of rice cv. IR43. Maximum pollen calli were formed in 2 days-starved cultures.

Culture Medium

Chu (1975) had demonstrated that the level of ammonium nitrogen in the culture medium is critical for androgenesis in rice. On this basis he developed the N_6 medium which has been most widely used for rice anther culture (Raina, 1989, 1997). However, this medium has proved significantly better than most other media for japonica rice. The indica cultivars require even lower NH_4^+ ions. Reddy *et al.* (1985), who studied 8 indica cultivars, found He_2 medium to be better than N_6 medium. He_2 medium is derived from N_6 medium by reducing NH_4^+ to half strength and $MgSO_4$ to $1/50^{th}$ level, and doubling the concentration of KH_2PO_4. In Korea N_6-Y1 medium was recommended for indica-japonica hybrids (Chung, 1987). This medium is essentially the same as N_6 medium except that $(NH_4)_2SO_4$ has been reduced from 3.5 mM to 1.5 mM.

In 1989, Raina reported that a medium with high KNO_3 and NH_4^+ ions completely replaced by 50 mg/l casein hydrolysate (CH) was significantly better than the original MSN medium for indica x indica F_1 hybrids, with regard to the frequency of green plant regeneration. This SK-1 medium was half as effective as N_6 medium so far as the frequency of pollen callusing is concerned but the calli formed on this medium produced twice as many green plants as those on N_6 medium. More recently, Raina and Zapata (1997), based on their detailed study on the medium requirement of the indica cultivar IR43 evolved a new medium called M-019 (*see* Raina and Irfan, 1998). This medium differs from SK-1 medium in substitution of CH by a small amount of $(NH_4)_2SO_4$ and the level of KH_2PO_4 raised from 170 mg/l to 540 mg/l. Ogawa *et al*. (1995) studied the effect of nitrogen source on androgenesis in another indica cultivar IR24, using R-2 medium as the control. R-2 has 40 mM KNO_3 and 2.5 mM $(NH_4)_2SO_4$. When 20 mM KNO_3 was combined with a small amount of $(NH4)_2SO_4$, glutamine or alanine all treatments induced pollen callusing but alanine was the best supplement. It not only induced high frequency androgenesis but also showed maximum regeneration of green plants.

Amino acids have been used as nitrogen source in *in vitro* culture of various tissues, including isolated microspores. Cho and Zapata (1988) reported that proline and glutamine promoted callus formation in microspore cultures of a japonica cultivar. These amino acids also induced a higher degree of plant regeneration and green plant production than the medium containing alanine or no amino acid. Ogawa *et al* (1995) also found glutamine to be

promotory for pollen callusing in microspore cultures of an indica cultivar but alanine was far better than glutamine for plant regeneration and green plant production.

Table 1: Composition of the culture media used for rice anther/microspore culture

Constituents	Media (mg l^{-1})			
	He_2*	MO19**	N_6***	C****
KNO_3	3181.5	3101.0	**2830.0**	3134.0
KH_2PO_4	800.0	540.0	**400.0**	540.0
$(NH4)_2SO_4$	231.0	264.0	**463.0**	231.5
$MgS0_4.7H_2O$	3.5	370.0	185.0	185.0
$CaCl_2.2H_2O$	166.0	**440.0**	166.0	150.0
KI	0.8	0.8	0.8	0.83
H_3BO_3	1.6	6.2	1.6	6.2
$MnSO_4.4H_2O$	4.4	22.3	4.4	22.3
$ZnSO_4.7H_2O$	1.5	8.6	1.5	8.6
$Na_2MoO_4.2H_2O$	-	0.25	-	0.25
$CuSO_4.5H_2O$	-	0.025	-	0.025
$CoCl_2.6H_2O$	-	0.025	-	0.025
$FeSO_4.7H_2O$	167.1	27.8	27.8	27.8
$Na_2EDTA.2H_2O$	223.5	37.3	37.3	37.3
Inositol	-	100.0	-	-
Nicotinic acid	2.5	2.5	0.5	2.5
Pyridoxine HCl	0.5	2.5	0.5	2.5
Thiamine HCl	0.5	1.0	1.0	2.5
Glycine	2.0	2.0	2.0	2.5
$AgNO_3$	-	-	-	10.0
Casein hydrolysate	250.0	-	-	-
Yeast extract	1000.0	-	-	-
Sucrose	30000.0	40000.0	40000.0	-
Maltose	-	-	-	50.0

* Huang et al. (1978)
** Raina & Irfan (1998)
*** Chu et al. (1975)
**** Letini et al. (1995)

Since 1983, maltose has been shown to be a superior source of carbohydrate than sucrose for androgenesis in several species, including cereals (Finnie *et al*. 1989; Last and Brettell, 1990; Pande and Bhojwani 1999). A significant increase in anther culture efficiency and green plant formation in otherwise highly recalcitrant indica rice cultivar occurred when sucrose was replaced by maltose (Raina, 1997).

Lentini *et al*. (1995) reported that on N_6 medium with 146 mM sucrose only one out of 23 indica cultivars exhibited pollen callusing and green plant production. Substitution of sucrose by equimolar amount of maltose enhanced pollen callusing from 6.3 % to 10.1 % and green plant regeneration from 0.6 % to 1 %. Glucose was inhibitory. Earlier, Mejza *et al*. (1993) had observed that isolation and fractionation of microspores in 20 % maltose solution, and their culture on a medium containing 9 % maltose allowed genotype-independent plant regeneration in *ab initio* microspore cultures of wheat. Maltose promoted the direct

embryogenic pathway of plant formation from pollen in wheat (Navarro-Alvarez *et al*., 1994), maize (McDonald, 1992) and barley (Powell *et al*., 1992).

In cultures, sucrose rapidly breaks down to glucose and fructose, so that after 3 weeks of barley anther culture the medium did not contain any sucrose (Last and Brettell, 1990). On the other hand, the hydrolysis of maltose during the same period was below the detectable level. The toxicity of sucrose for androgenesis is due to the sensitivity of microspores to fructose but not to glucose.

Calcium in the medium is known to stimulate ethylene production in many plant tissues. Addition of the calcium ionophore A23187 (0.5 μM) along with $CaCl_2$ (1 mM) enhanced pollen callusing over $CaCl_2$ alone. Addition of 10 mg L^{-1} of $AgNO_3$, an anti-ethylene compound, to the callus induction medium enhanced pollen callusing frequency in indica cultivars from 10.1 % to 20.6 %. With $AgNO_3$ the frequency of green plant differentiation doubled (Lentini *et al*., 1995). It is also known to promote pollen embryo production in anther cultures of Brussels sprouts (Biddington *et al*., 1988).

Microspore Culture

There are many problems associated with raising androgenic haploids by anther culture. The pollen grains within an anther being genetically heterogeneous, the plants arising from an anther would constitute a heterogeneous population. In cereals, where androgenesis occurs *via* callusing, mixing of calli of different pollen origin within an anther lobe enhances the chances of chimeric plant formation. This limitation can be overcome by isolated microspore culture, which offers many other advantages: (a) it provides an efficient system of isolated, haploid, single cells for biochemical and molecular analysis of androgenesis/ embryogenesis, (b) enrichment of androgenic microspore population by gradient centrifugation becomes possible, (c) isolated microspores can be genetically manipulated before culture to obtain solid mutants or transformants, and selection of new genotypes can be made at an early stage. Unlike the earlier belief, isolated microspore culture is less tedious and time consuming than anther culture.

Considering these advantages, during the last decade considerable progress has been made in isolated microspore culture of cereals (Jahne and Lorz 1995, Raina 1997). Plant regeneration from microspore culture has been achieved in barley (Wei *et al*. 1986, Hunter 1987), maize (Coumans *et al.* 1989; Pescitelli *et al*., 1989), wheat (Mejza *et al*. 1993) and rice (Raina and Irfan, 1998).

Most of the work on rice microspore culture involved an anther pre-culture step (Jia *et al*. 1987; Cho and Zapata 1988,1990; Xie *et al*. 1995). Datta *et al.* (1990) following the float culture method to raise microspore cultures of indica rice cultivars (Basmati-370, Chinsurah Boro II, Upland-13, and a local West Bengal cultivar). Anthers from florets exposed to 4 ^{o}C/10 ^{o}C for 10-18 days were cultured in liquid medium. Within 3-4 days the anthers burst and released the microspores in the medium. The released microspores and those within the anthers were collected and plated in fresh liquid medium. A total of 1300 anthers of the indica cultivars yielded 42 green and 13 albino plants. Against this the Japonica cultivars (Nipponbare, Yamabiko, Yamahoushi) yielded 140 green and 63 albino plants from 3380 anthers cultured.

Recently, Raina and Irfan (1998) reported high frequency androgenesis and plant regeneration in *ab initio* microspore cultures of two indica cultivars (IR43, IR54) and one japonica cultivar (T-309). They observed that co-cultivation of microspores with young ovaries of rice (10 ovaries/ml of medium) significantly increased the androgenic response. However, pretreatment of anthers in 0.4 M mannitol solution for 4 days at 25/30 ^{o}C was essential to induce androgenesis. Another critical factor for sporophytic division in *ab initio* cultured microspores was substitution of sucrose by 9 % maltose. The japonica cv T-309 showed up to 70 % division of the microspores. The response of indica cultivars was comparatively poor but some green plants were obtained for all the cultivars (T-309 9 %; IR43 7 % and IR52 2 %). Some of the indica microspore calli turned brown soon after transfer to semi-solid medium for regeneration. Occasionally, browning was also observed in liquid medium. This problem in barley was overcome by adding Ficoll, a high molecular weight polymer, which keeps the anthers and microcalli afloat on the medium.

Ogawa *et al*. (1992,1994, 1995) achieved androgenic plant formation in *ab initio* microspore cultures of the indica cv IR24 without a nurse tissue. The critical factor was cold pre-treatment of the panicles at 10^{o}C for 28 days before isolation of the micropsores (Ogawa *et al*., 1995). In this study the microspores were purified by filtration and enrichment of androgenic grains by using 0/35% Percoll gradient. They obtained 56 colonies per dish containing 5x10^{4} pollen. Only 6 calli exhibited regeneration and 1 out of 37 plants was green.

Albinism

The occurrence of a large proportion of albinos in the pollen plant population is probably the most frustrating feature of androgenesis in its application to rice breeding. The frequency of albinos may vary from 5 % to 100 %. Indica rice cultivars are more prone to this problem than japonica rice.

Several factors, including pre-treatment, culture medium and the protocol, affect the frequency of albinos. The literature on androgenesis in cereals suggests that albinism can be considerably reduced by shortening the culture period. In most of the cereals androgenesis occurs via pollen callusing and the callus needs to be transferred from the induction medium to regeneration medium for obtaining plants. In rice cv IR43, the pollen calli left on the induction medium longer than 30 d after release from the anthers lost their regeneration potential (Pande and Bhojwani, unpublished). Therefore, we routinely transferred the microcalli to regeneration medium 10 days after their release from the anther. A more effective approach to reduce the culture period is to alter the mode of androgenesis from pollen callusing to direct pollen embryogenesis. In barley, by reducing the culture period to 6 weeks by osmotic shock pretreatment and low density microspore plating, Hoekstra *et al*. (1993) could change the proportion of green to albino plants from 1:1 to 34:1.

Generally, liquid medium is used for anther/microspore culture of barley. Consequently, the anthers and large pollen calli released by them sink in the culture medium, and the tissues are exposed to anaerobic conditions. This results in the production of lactate and alcohol dehydrogenase in the tissues which damages the internal structure and DNA of the plastids, leading to albino plant regeneration (Kao *et al*., 1991). Proper aeration of the tissue by addition of Ficoll (40 %) to the induction medium, supplementing the regeneration medium with Ficoll and high sucrose (4.5 %) and gelling the embryo maturation and germination medium with starch and agar, Kao *et al* induced direct embryogenesis in barley, which cut down the period from anther culture to plant regeneration to 6 weeks. It raised the proportion of green plants from 0 % to 50 %. The authors have remarked that the calli should be transferred from induction medium to regeneration medium within 70 days of culture. High Ficoll / high sucrose kept the anthers and calli afloat on the medium and delayed anther dehiscence, maintaining sugar starvation-like condition around the microspores. The plants regenerated through this protocol showed very little or no genetic variation in the field or with 100 RFLP markers.

The stage of pollen at the time of culture may also contribute towards albinism. In rice the cytoplasm of microspores at the tetrad stage is rich in plastids. With further development simplification of the cytoplasm occurs and the granular matrix of the plastids is replaced by starch grains, which has been positively correlated with deletion of pDNA (Kawata *et al*., 1995). Consequently, calli from anthers cultured at the early uninucleate stage of pollen produce only green plants, and the frequency of albinism increased with advancing stage of the pollen (Pandey 1997). Kawata *et al*. (1995) are of the opinion that more than the process of androgenesis, the culture period is responsible for pDNA deletions. The plants regenerated from one-month-old pollen or seed callus did not show pDNA deletion but those from 11-year-old callus showed substantial pDNA deletion. This theory is further supported by the fact that with the passage of time the calli initially producing only green plants may start producing albinos but it never happens the other way round.

Another observation deserving comment in this context is the *in vitro* selection of microspores. The phenomenon of pollen dimorphism has been observed in several species,

including rice. For example, in IR43 15 % of the grains in an anther are large and thin-walled. These grains are normally incapable of forming viable pollen, which may be due to minor genetic abnormalities. However, these so called androgenic grains are most capable of forming pollen plants in cultures. Albinism may be one of the expressions of this genetic deficiency.

Concluding Remarks

Although considerable progress has been made to improve the *in vitro* androgenic response and the feasibility of *ab initio* microspore culture of indica rice cultivars has been demonstrated, the routine application of this technique to rice breeding is still fraught with many problems. Dihaploid breeding has resulted in the production of several improved cultivars and breeding lines of rice but the success is largely restricted to japonica cultivars. Efforts need to be made to reduce the problem of albinism by manipulating the various *in vivo* and *in vitro* factors and achieving direct pollen embryogenesis to reduce the culture period. In many treatments rice pollen produce rounded, smooth and shining embryo-like structures (ELS) which eventually fuse with adjacent ELS' to form macrocalli. It has been possible to change the 'pollen callusing' pathway to a 'pollen embryogenesis' pathway by manipulating the culture conditions (Kao *et al*., 1991). Gynogenesis is an alternative to raise green haploid plants of rice but the number of haploid plants produced by this technique is a limiting factor.

Figure 2: Androgenesis in anther cultures of IR43. The androgenic pollen form shining globular embryo-like structures (**A**), which later fuse to form microcalli (**B**) rather than embryos.

References

Agarwal, P.K. and S.S. Bhojwani (1993): Enhanced microspore embryogenesis and plant regeneration in anther cultures of *Brassica juncea* cv. PR-45. Euphytica 70, 191-196.

Aruga, K., T. Nakajima and K. Yamamoto (1985): Embryogenic induction of pollen grains of *Nicotiana tabacum* L. Japan J. Breed. 35, 50-58.

Bhojwani, S.S., W.Y. Soh and H. Pande (1999): Morphogenesis in haploid cell cultures. In: W.Y. Soh and S.S. Bhojwani (eds.) Morphogenesis in Plant Tissue Cultures. Kluwer, Dordrecht.

Bhojwani, S.S. and M.K. Razdan (1996): Plant Tissue Culture: Theory and Practice, A Revised Edition. Elsevier, Amsterdam.

Biddington, N.L., R.S. Sutherland and H.T. Robinson (1988): Silver nitrate increases embryo production in anther culture of Brussels sprouts. Ann. Bot. 62, 181-185.

Cho, M.S. and F.J. Zapata (1988): Callus formation and plant regeneration in isolated pollen culture of rice (*Oryza sativa* L. cv. Taipei 309). Plant Sci. 58, 239-244.

Chu, C.C., C.C. Wang, C.S. Sun, C. Hsu, K.C. Yin, C.Y. Chu and F.Y. Bi (1975): Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci. Sin. 18, 659-668.

Chu, Q., Z. Zhang and Y. Gao (1985): Cytogenetical analysis on aneuploids obtained from pollenclones of rice (*Oryza sativa* L.). Theor. Appl. Genet. 71, 506-512.

Chung, G.S. (1987): Application of anther culture technique for rice (*Oryza sativa* L.) improvement. Res. Rep. Yeongnam Crop Exp. Sta. (RDA), Milyang. Korea. 36-55.

Coumans, M.P., S. Sohota and E.B. Swanson (1989): Plant development from isolated microspores of *Zea mays* L. Plant Cell Rep. 7, 618-621.

Datta, S.K., K. Datta and I. Potrykus (1990): Embryogenesis and plant regeneration from microspores of both 'indica' and 'japonica' rice (*Oryza sativa*). Plant Sci. 67, 83-88.

Datta, S.K. (2001): Androgenesis in cereals. In: Current Trends in Embryology of Angiosperms (S.S. Bhojwani and W.Y. Soh, eds.). Kluwer Academic Publishers, Dordrecht, pp. 471-488.

Finnie, S.J., B.P. Forster, K.J. Chalmers, A.F. Dyer, R. Wough and W. Powell (1991): Genetic stability of microspore-derived doubled haploids of barley: A cytological, bio-chemical and molecular study. Genome 34, 923-928.

Guha-Mukherjee, S. (1973): Genotypic differences in the *in vitro* formation of embryoids from rice pollen. J. Exp. Bot. 24, 139-144.

Gupta, H.S. and D.N. Borthakur (1987): Improved rate of callus induction from rice anther culture following microscopic staging of microspores in iron alum-haemotoxylin. Theor. Appl. Genet. 74, 95-99.

Hoekstra, S., M.H. van Zijderveld, J.D. Louwerse, F. Heidekamp and F. van der Mark (1992): Anther and microspore culture of *Hordeum vulgare* L. cv. Igri. Plant Sci. 86, 89-96.

Hoekstra, S., M.H. van Zijderveld, F. Heidekamp and F. van der Mark (1993): Microspore culture of *Hordeum vulgare*: L. the influence of density and osmolality. Plant Cell Rep. 12, 661-665.

Hu, H. (1985): Use of haploids for crop improvement in China. Genet. Manipulation Crops Newslett. 1, 11-23.

Hunter, C.P. (1987): Plant generation method. Australian Patent Application No. AU-A-7271. Shell Int. Res.

Jahne, A. & Lorz, H. 1995. Cereal microspore culture. Plant Sci. 109: 1-12.

Kao, K.N., M. Saleem, S. Abrams, M. Pedras, D. Horn and C. Mallard (1991): Culture conditions for induction of green plants from barley microspores by anther culture methods. Plant Cell Rep. 9, 595-601.

Kawata, M., A. Ohmiya, Y. Shimamoto, K. Oono and F. Takaiwa (1995): Structural changes in the plastid DNA of rice (*Oryza sativa* L.) during tissue culture. Theor. Appl. Genet. 90, 364-371.

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

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

Lu, C.S., H.C. Sharma and H.W. Ohm (1991): Wheat anther culture: effect of genotype and environmental conditions. Plant Cell Tiss. Org. Cult. 24, 233-236.

Macdonald, M.V. (1992): Donor plant growth factors affecting anther culture of maize and sweet corn (*Zea mays*). Ann. Bot. 70, 357-363.

Matsushima, T., S. Kikuchi, F. Takaiwa and K. Oono (1988): Regeneration of plants by pollen culture in rice (*Oryza sativa* L.). Plant Tissue Cult. Lett. 5, 78-81.

Mejza, S.J., V. Morgant, D.E. DiBona and J.R. Wong (1993): Plant regeneration from isolated microspores of *Triticum aestivum*. Plant Cell Rep. 12, 149-153.

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. Appl. Genet. 70, 113-116.

Navarro-Alvarez, W., P.S. Baenzigar, K.M. Eskridge, D.R. Shelton, V.D. Gustafson and M. Hugo (1994): Effect of sugars in wheat anther culture media. Plant Breed. 112, 53-62

Ogawa, T., T. Hagio and Y. Ohkawa (1992): Plant regeneration from isolated pollen grains in indica type rice, Japan J. Breed. 42, 675-679.

Ogawa, T., H. Fukuoka and Y. Okhawa (1994): Induction of cell division of isolated pollen grains by sugar starvation in rice. Breed. Sci. 44, 75-77.

Ogawa, T., H. Fukuwa and Y. Ohkawa (1995): Plant regeneration through direct culture of isolated pollen grains in rice. Breed. Sci. 45, 301-307.

Pande, H. (1997): Androgenesis in anther cultures of an indica cultivar of *Oryza sativa* L. Ph.D. Thesis, University of Delhi.

Pande, H. and S.S. Bhojwani (1999): Promotion of androgenesis in rice anther cultures by substitution of sucrose with maltose and mannitol. Biol. Plant. 42, 125-128.

Pescitelli, S.M., J.C. Mitchell, A.M. Jones, D.R. Pareddy and J.F. Petolino (1989): High frequency androgenesis from isolated microspores of maize. Plant Cell Rep. 7, 673-676.

Petolino, J.F. and S.A. Thompson (1987): Genetic analysis of anther culture response in maize. Theor. Appl. Genet. 74, 157-159.

Powell, W.W., T.B. Thomas and D.M. Thompson (1992): The agronomic performance of anther culture derived palnts of barley produced via pollen embryogenesis. Ann. Appl. Biol. 120, 137-150.

Raina, S.K. (1989): Tissue culture in rice improvement: status and potential. Adv. Agron. 42, 339-397.

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.

Raina, S.K. and S.T. Irfan (1998): High-frequency embryogenesis and plantlet regeneration from isolated microspores of indica rice. Plant Cell Rep. 17, 957-962.

Reddy, V.S., S. Leelavathi and S.K. Sen (1985): Influence of genotype and culture medium on microspore callus induction and green plant regeneration in anthers of *Oryza sativa*. Physiol. Plant. 63, 309-314.

Roberts-Oehlschlager, S.L. and J.D. Dunwell, J.D. (1990): Barley anther culture: Pretreatment on mannitol stimulates production of microspore-derived embryos. Plant Cell Tiss. Organ Cult. 20, 235-240.

Sun, Z. (1999): Methods to increasing frequency of rice anther culture, In: Regional (RCA) Training Course on Production and Utilization of Doubled Haploid Lines in Rice, Republic of Korea, 18-30 October, 1999.

Wei, Z.M., M. Kyo and H. Harada (1986): Callus formation and plant regeneration through direct culture of isolated pollen of *Hordeum vulgare* cv. Sabarlis. Theor. Appl. Genet. 72, 252-255.

Ziauddin, A., E. Simion and K.J. Kasha (1990): Improved plant regeneration from shed microspore culture in barley (*Hordeum vulgare* L.) cultivar Igri. Plant Cell Rep. 9, 69-72.