Plant Tissue Cult. **12**(1) : 37-47, 2002 (June)

PTC

Effects of Microspore Stage, Pre- and Post Temperature and Donor's Environment on Maize (*Zea mays* L.) Anther Culture Response

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Key words : Anther culture, Microspore stage, Pre- and post-temperature, Genotypes, TIBA, *Zea mays*

Abstract

An enhanced frequency of embryoids was obtained when anthers of an F_1 (M947 x M948) at mid-uninucleate microspore, were cold shocked at 8 - 10_C for 14 days and heat-preincubated in dark at 27 ± 2_C for 14 days. Effects of genotype, genotype x media and genotype x environment interaction on response was apparent in this study. Embryoids appeared within three - nine weeks of culture on different media. Maximum number of embryoids were obtained when donors were grown in optimum field conditions. The growth of embryoids was very weak and slow when donors were grown in a partially shady garden. There was a small difference in response between 6N1 and YP media and most of the genotypes produced maximum number of embryoids on 0.1 mg/l TIBA supplementation media from both the environments. Maximum plantlet regeneration was obtainted on media supplemented with 1.0 mg/l Kn followed by 0.1 mg/l TIBA. Among 23 genotypes tested, six produced dark green plantlets when donors were grown in the optimum environment whereas two pale green plantlets were produced in the shady environment. Most of the plantlets had narrow and erect leaves.

Introduction

Many factors are responsible for successful anther culture. All investigators, who have reported success in maize anther culture, agreed that the uninucleate microspore is the most responsive stage for culture. However, some have shown a preference for the mid-uninuleate stage, while others preferred the late-uninuleate stage. Temperature shocks are believed to improve androgenesis

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by diverting normal gametophytic development into a sporophytic pathway leading to the formation of haploid embryo like structure (Nitsch et al. 1982). Genovesi (1990) reported highly significant effects of post-treatment with elevated temperature on embryoid formation.

The variation in androgenetic response in anther culture is dependent upon the donor plants' growth environment. This has been reported in a large number of crops, including wheat (Picard and De Buyer 1975), rice (Chaleff and Stolarz 1981) and maize (Genovesi 1990). The donor plants' physiology and vigor were found to profoundly influence anther culture response frequency of maize (Nitsch et al. 1982). Genovesi (1990) stated that anthers of weak donor plants of maize produced only a few embryoids or calli *in vitro*. This is probably indicative of the importance of endogenous factors for anther culture response. He also mentioned the importance of environmental factors, such as the influence of photoperiod, light intensity and quality, temperature and nutrition on the donor plant vigour. Field grown donor plants gave better embryo induction frequencies (Dieu and Beckert 1986). For maize androgenesis, Nitsch et al. (1982) recommended that the source plants be grown under optimal conditions and that pesticide treatment be avoided. Maize anthers also respond differentially to incubation conditions such as temperature and light quality and quantity.

This paper describes the effect of different stages of microspore, pre- and post-temperature treatments and the donor's growing environment on androgenesis via anther culture. The varieties used in the study were different from those reported above. Effects of the genotype and media for embryoid formation and regeneration have also been investigated.

Materials and Methods

Twenty one F_1s developed from inbred lines of International Maize and Wheat Improvement Center (CIMMYT), Mexico and male female crosses of local composite varieties Barnali and Khoibhutta were used.

All F_1s were grown under two environmental conditions at 10 - 14 days intervals avoiding extreme weather. Environment I (Env. I) : the F_1s grown during 1996 - 1997 in the field under optimum condition of Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur. Environment II (Env. II) : the F_1s grown during 1998 - 1999 in the garden of the Department of Botany, University of Dhaka, Dhaka in a partially shady place with only seven hrs direct sunlight. No pesticide or systemic insecticide was used.

Tassels were collected from healthy plants just prior to their emergence from the whorl. The tassels with anthers containing uninucleate microspores were determined by acetocarmine squash and shocked with cold in refrigerator at 8 - 10_C for 14 days unless otherwise stated by sealing them in polyethylene bag.

Tassel with anthers of M947 x M948 having early-, mid- and late-uninucleate or binucleate stages of microspores were selected. The selected tassels were cold-shocked for 0, 7, 14 and 21 days at 8 - 10_C. Anthers were cultured on YP + 0.1 mg/l TIBA and heat-preincubated in the dark at 25 ± 2_C, 27 ± 2_C or 30_C. For each experiment 489 - 495 anthers were used.

The tassels with uninucleate anthers of all the F_1s were surface sterilized with 70% ethyl alcohol for a few seconds followed by 0.1% mercuric chloride for 10 min and then rinsed four - five times with sterile distilled water. Fifty five anthers from main branch were placed on 20- by 100 mm Petri dishes containing 0.7% agar solidified induction media. Total number of anthers used for each genotype and treated were 451 - 509.The media consisted of 6N1 (Genovesi 1990) and YP (Ku et al. 1981) basal salt supplemented with 0.05, 0.1 or 0.15 mg/l 2, 3, 5-triiodobenzoic acid (TIBA). Thiamine-HCl and nicotinic acid at the rate of 1.0 and 0.5 mg/l and sucrose (S), casein hydrolysate (CH), L-proline and activated charcoal (AC) at the rate of 120.0, 0.4, 0.1 and 5.0 g/l, respectively were added to the media. Sealed with parafilm, the anthers were heat pre-incubated for 14 days in the dark at 27 ± 2_C for Env. I and at 25 ± 2_C for Env. II. Then the plates were transferred to cool-white fluorescent light (2000 lux) under a 16 h photoperiod regime at 25 ± 2_C. For microspore stage and pre- and post-temperature treatments, the embryoid induction medium 6N1 with 0.1 mg/l TIBA and others mentioned above was used.

Seven to 12 days embryoids of about 1.5 mm were placed on to five different regeneration media in test tubes viz. 6N1/YP + 0.1 mg/l TIBA + 30 g/l S; 6N1/YP + 1.0 mg/l Kn + 0.005 mg/l abscisic acid (ABA) + 25 mg/l succinic acid + 25 g/l S and MS + 0.5 mg/l 2,4-D + 0.05 mg/l ABA + 20 g/l. Nicotinic acid, CH and L-proline were used at the same rate as in the case of embryoid induction medium except for reduced thiamine HCl (0.5 mg/l). AC was not added to any of the regeneration media. All the culture and regeneration media were adjusted to pH 5.8 before autoclaving.

Results and Discussion

Out of four treatments, tassels cold-shocked at 8 - 10_C for 14 days only produced embryoids or calli. Genovesi and Collins (1982) found that the best

levels of response occur when the genotypes were subjected to cold-shock treatment at 8_C for 14 days. Koinuma et al. (1990) reported that the cold pretreatment of tassels at 8_C for 14 days was more effective than that of seven days. Genovesi (1990) and Choi et al. (1995) have suggested cold-pretreatment of anthers at 10 and 9_C for 14 days, respectively.

Embryoid frequency was the highest (8.54%) when anthers showing mid-uninucleate microspores (Fig. 1a) were cold-shocked at 8 - 10_C for 14 days and heat-preincubated at 27 ± 2_C for 14 days (Fig. 2b). Miao et al. (1981) and Choi et al. (1995) recommended mid-uninucleate of microspore for maize anther culture. Whereas, Ku et al. (1981) and Brettell et al. (1981) have reported best response at vacuolated late-uninucleate microspores. In this study 7.27% response was found at late-uninucleate microspore (Fig. 1b) when the anthers were heat-preincubated at 27 ± 2_C (Fig. 2c). Low frequency of embryoid formation was observed in early uninucleate and binulceate microspores (Figs. 2a, d). Satarova (1994) showed young two-celled pollen grains to be more productive. Dieu and Beckert (1986) obtained best results from tassels pretreated for seven days at 7_C and preincubated for seven days at 14_C. The minimum response was observed at 25 ± 2 and 30_C postincubation in the present study.

After three - nine weeks of culture, embryo-like structures (ELS) were observed on 6N1 and YP supplemented media in both the environments. ELS emerged from the locules rupturing the anther wall (Fig. 1c). The number of embryoids per anther varied from one to four and they grew faster than calli. Loose embryoids were easy to subculture and they differentiated into shoots (Fig. 1d), roots or both. Some ELS proliferated in a bipolar manner in the induction medium (Fig. 1f). Compact ones were not viable when they were subcultured. The majority of the cultured anthers produced embryoids while a few produced non embryogenic calli. Calli became brown and gradually degenerated and died two weeks later.

After two weeks, a green area in the embryoids appeared. New shoots emerged from this green sector. Some embryoids remained undifferentiated and some of the bipolar embryoids showed no further growth. If the main root emerged before shoots, this was always followed by the development of profuse root systems and these embryoids showed incomplete morphogenesis (Fig. 1g). Ting et al. (1981) also reported similar findings. The quality of embryoids should be considered as major factor affecting plantlet regeneration in maize anther culture (Dieu and Beckert 1986).

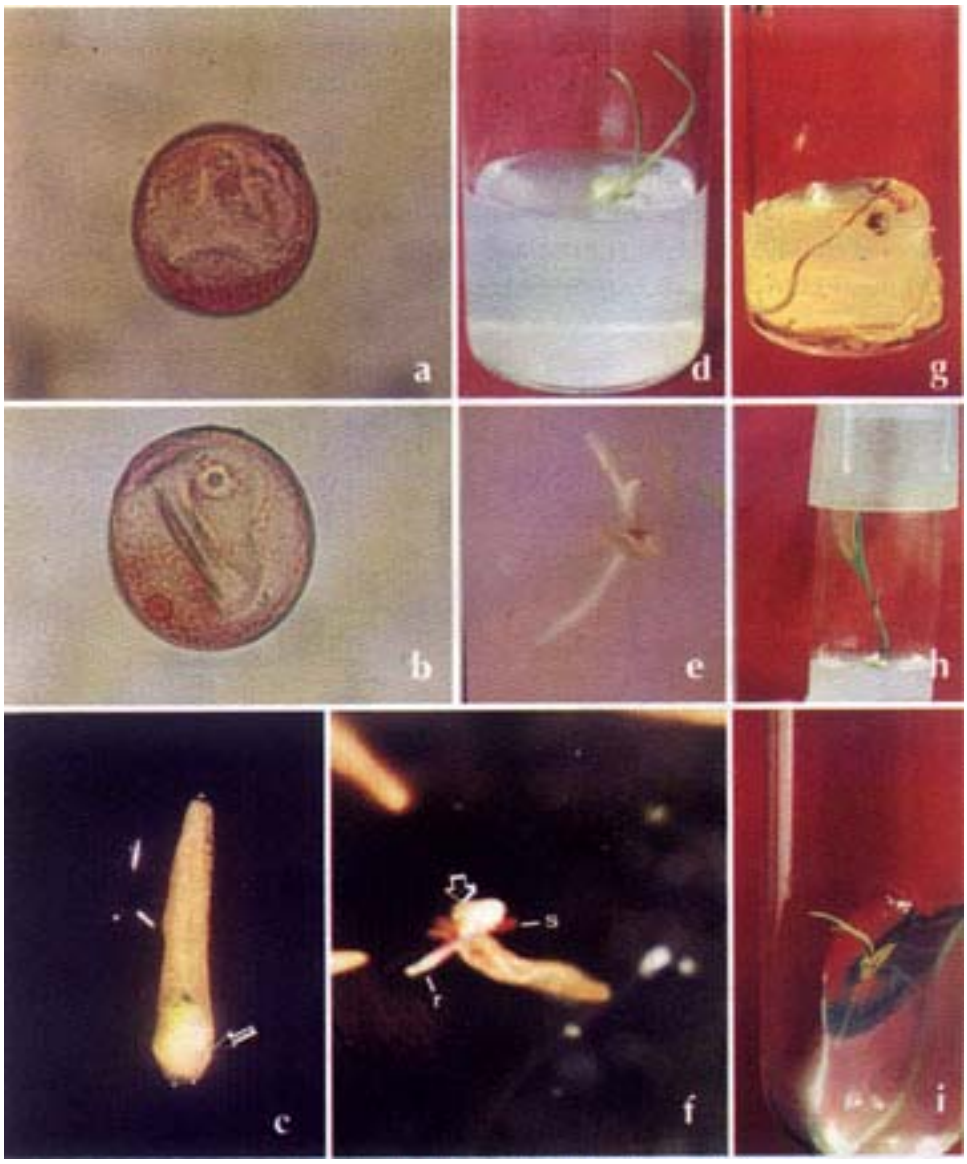

Fig. 1. (a) Mid-uninucleate microspore. (b) Late-uninucleate microspore. (c) Embryoid (arrow) emerging through side-wall of an anther on induction medium. (d) Shoot development without root on regeneration medium. (e) Embryoid with both root and shoot on regeneration medium. (f) Embryoid (arrow) with both root (r) and shoot (s) on induction medium. (g) Development of profuse root systems on regeneration medium. (h) A healthy green plantlet developed on YP + 1.0 mg/l in Env. I. (i) A weak plantlet with pale green leaves developed on 6N1 + 1.0 mg/l Kn in Env. II.

In Env. I, among 23 F_1s, 18 produced at least one ELS. Embryoid regeneration ranged from 0.2 to 5.8% (Table 1). The highest ELS was produced by M947 x M948 (5.8%) on YP + 0.1 mgl TIBA followed by M948 x M955 (3.18%) on YP + 0.1 mg/l TIBA. Ten crosses produced _ 2.0% ELS on 0.1 mg/l TIBA supplemented 6N1 or YP medium.

In Env. II, 14 genotypes produced ELS ranging from 0.2 to 2.0% (Table 1). The highest ELS was produced by Khoibhutta (2.0%) on YP + 0.1 mg/l TIBA followed by M951 ∇ M955 (1.81%) and Khoibhutta (1.8%), respectively on 6N1 + 0.1 mg/l TIBA. Only six crosses could produce _ 1.0% ELS in this environment.

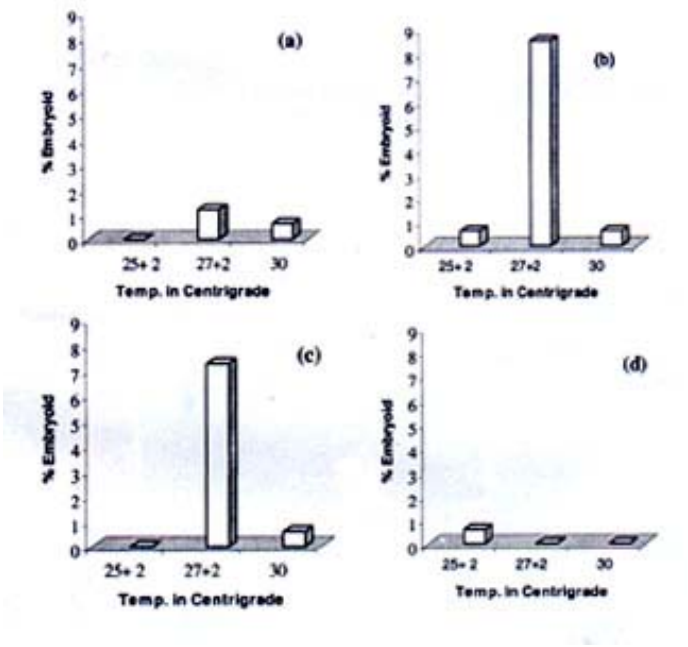

Fig. 2. Effects of stages of microspores and heat-preincubation of plated anthers on embryoid formation frequency. (a) Early-uninucleate micrrospores. (b) Mid-uninucleate microspores. (c) Late-uninucleate microspores. (d) Binucleate microspores.

The results of the study indicated the effect of genotype, genotype ∇ TIBA concentration and genotype ∇ environment interaction on response. Genotype has been considered to be a critical factor in anther culture of maize (Ku et al. 1981, Genovesi and Collins 1982, Dieu and Beckert 1986, Hongchang et al. 1991,

Table 1. Effects of different concentrations of TIBA in 6N1 and YP media on embryoid formation in two environments.

Basal salt	TIBA (mg/l)	Environment I			Environment II			Average % of embryoid in two environments
		No. of anthers/F_1	Embryoid formation		No. of anthers/F_1	Embryoid formation		
			Number	%		Number	%	
6N1	0.05	457 - 509	0 - 8	0 - 1.6	495 - 504	0 - 7	0 - 1.4	0.29
	0.1	466 - 506	0 - 16	0 - 3.2	496 - 504	0 - 9	0 - 1.8	0.82
	0.15	468 - 507	0 - 7	0 - 1.4	497 - 504	0 - 1	0 - 0.2	0.10
YP	0.05	497 - 505	0 - 8	0 - 1.6	495 - 504	0 - 6	0 - 1.2	0.30
	0.1	498 - 506	0 - 29	0 - 5.8	496 - 504	0 - 10	0 - 2.0	0.89
	0.15	496 - 504	0 - 8	0 - 1.6	498 - 504	0 - 2	0 - 0.4	0.12

Petolino and Jones 1986). Kuo et al. (1986) reported that the ability to respond is genotype-dependent. The success of anther culture is dependent upon donor genotype and the genotype ∇ environmental interaction (Choi et al. 1995).

Among the TIBA concentrations studied, 0.1 mg/l TIBA either in 6N1 or YP medium was the best. There was a small difference in response between 6N1 and YP in both the environments. The responding crosses produced maximum embryoids on 0.1 mg/l TIBA supplemented YP (1.33%) or 6N1 (1.11%) media in Env. I (Fig. 3). In Env. II, 0.1 mg/l TIBA supplemented 6N1 medium produced more embryoids as compared to 0.1 mg/l TIBA supplemented YP medium (Fig. 3).

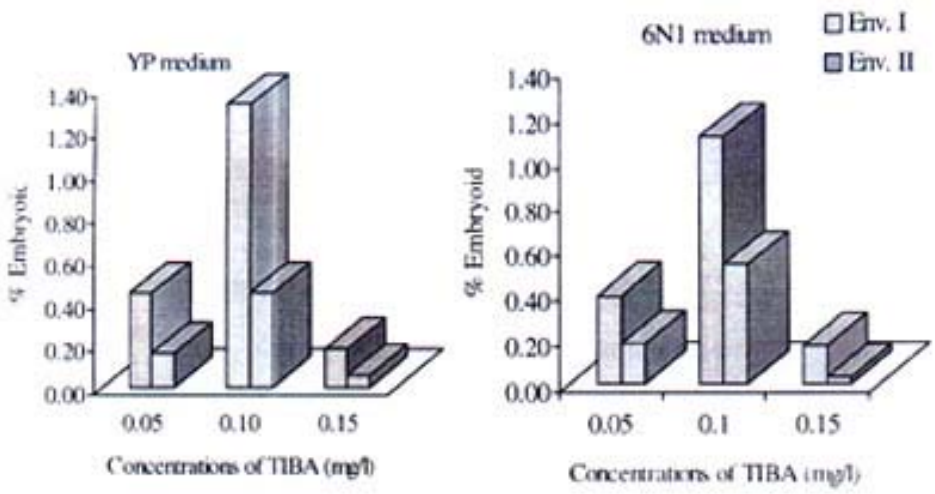

Fig. 3. Embryoid formation frequency at three levels of TIBA supplemented 6N1 and YP media in two environments.

In both the environments, maximum average response was obtained from media containing 0.1 mg/l TIBA (Table 1). Genovesi and Collins (1982) achieved the best response for most of the genotypes on YP + 1.0 mg/l TIBA. Ku et al. (1981) compared anther response on YP basal medium supplemented with 2.0 mg/l 2,4-D, 1.0 mg/l Kn and 2.0 mg/l BAP versus YP supplemented with various levels of TIBA and found the best response (13.1%) on 0.1 mg/l TIBA.

Fig. 3 showed double the embryoid formation in optimum environment as compared with the shady environment. The result confirmed the previous report by Genovesi (1990). The growth of embryoids was also very poor and

slow. The poor performance in response might have been due to the influence of the donor's growing environment. After a series of studies Heberle-Bors (1982 a,b) concluded that growth conditions of donor plants, particularly the light intensity and temperature, affected the frequency of both anomalous pollen *in vivo* and formation pollen embryoids in anther culture, as well as their sexual balance.

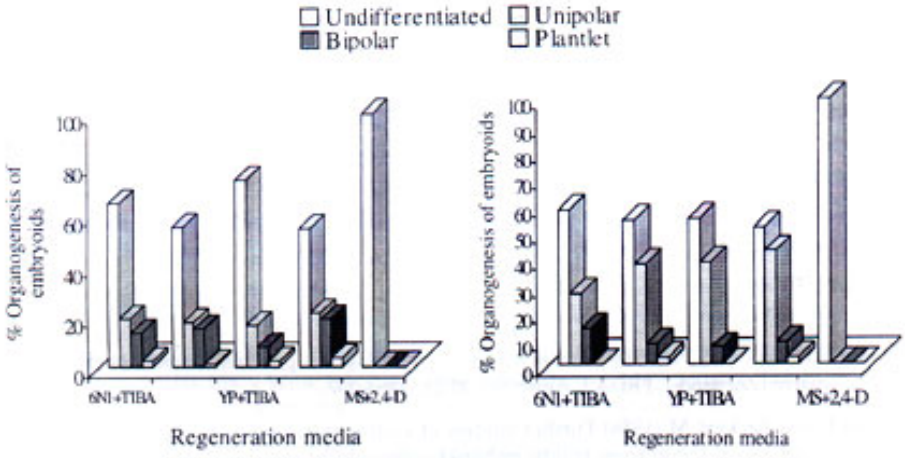

Fig. 4. Organogenic response of embryoids on regeneration media in Env. I (above) and Env. II (below).

Variation in organogenesis was observed on regeneration media. More than 50% embryoids remained undifferentiated in both the environments. In Env. I, plantlets could not be obtained from all bipolar embryoids on regeneration media for undetermined reasons. Maximum plant regeneration was 3.3% which was achieved on YP + 1.0 mg/l Kn followed by 6N1 + 0.1 mg/l TIBA and YP + 0.1 mg/l TIBA (2.2%) and 6N1 + 1.0 mg/l Kn (1.1%) (Fig. 4). Genevosi and Collins (1982) obtained 19.2% plantlets regeneration in Kn supplemented YP medium. Nitsch et al. (1982) reported the highest frequency of plantlets on a 0.1 mg/l TIBA supplemented medium. No differentiation was obtained on MS + 0.5 mg/l 2,4-D. Upon transfer to soil, the anther-derived plants of Env. I became yellow shortly and ultimately died. This is probably due to the poor root system of the regenerants.

In Env. II, plantlet formation occurred only on Kn supplemented 6N1 (2.7%) and YP (2.9%) media (Fig. 4). The frequency of root organogenesis was

high (27.03 - 42.86%) in this environment. The growth of plantlets was very poor and they did not survive in the regeneration medium after one month. All these may be due to the influence of shady environment. Incubation of plated anthers at a lower temperature (25 ± 2_C) also could cause weaker growth of embryoids in Env. II.

Of the 23 F_1s, six were capable of producing plantlets in Env. I as compared to two in Env. II, indicating genotypic and environmental influences on response. However, all plants obtained in Env. I were dark green (Fig. 1h) indicating no chlorophyll deficiency. Brettell et al. (1981) and Nitsch et al. (1982) also obtained green plantlets. In Env. II plantlets were pale green (Fig. 1i). Most of the plantlets had narrow and erect leaves.

References

Brettell RIS, Thomous E and **Wernicke W** (1981) Production of haploid maize plants by anther culture. Maydica **26** : 101 - 111.

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

Choi BH, Park KY, Park RK and **Son YGH** (1995) Haploid production technology in maize (*Zea mays* L.) RDA J. Agric. Sci. **37**(2) : 228 - 237.

Dieu P and **Beckert M** (1986) Further studies of androgenetic embryo production and plant regeneration from *in vitro* cultured anthers in maize (*Zea mays* L.). Maydica **31**: 245-259.

Genovesi AD and **Collins GB** (1982) *In vitro* production of haploid plant of corn via anther culture. Crop Sci. **22** : 1137 - 1144.

Genovesi AD (1990) Maize (*Zea mays* L.): *In vitro* Production of Haploids. *In:* Bajaj YPS (Ed.). Biotechnology in Agriculture and Forestry. Vol. 12. Haploids in Crop Improvement I, Springer-Verlag, Berlin Heidelberg, New York, London, Paris, Tokyo, Hongkong. pp. 176 - 203.

Heberle-Bors E (1982a) *In vitro* pollen embryogenesis in *Nicotiana tabacum* L. and its relation to pollen sterility, sexual balance, and floral induction of the pollen donor plants. Planta **156** : 396 - 401.

Herberle-Bors E (1982b) On the time of embryogenic pollen grain induction during sexual development of *Nicotiana tabacum* L. plants. Planta **156** : 402 - 306.

Koinuma K, Mochizuki N and **Inoue Y** (1990) Embryoid and callus induction and plant regeneration by anther culture of japanese local varieties of maize (*Zea mays* L.) Bullet National Grassland Res. Inst. (Japan). **43**: 13-22.

Hongchang M, Liang GH and **Wassom CE** (1991) Effect of growth regulators and genotypes on callus and embryoid induction from maize anther culture. Plant Breed. **106** : 47 - 52.

Ku MK, Cheng WC, Kuo IC, Kuan YL, An HP and **Hunng CH** (1981) Induction factors and morpho-cytological characteristics of pollen derived plants in maize (*Zea mays* L.). Proc. Symp. Plant Tissue Culture Beijing 1978. Pitman. Boston. pp. 35 - 42.

Kuo CS, Lu WL and **Kui YL** (1986) Corn (*Zea mays* L.) : Production of pure lines through anther culture. *In:* Bajaj YPS (ed.) Biotechnology in Agriculture and Forestry. Vol. **2**. Crops 1, Springer Berlin, Heidelberg, New York, Tokyo, pp. 152 - 164.

Miao SH, Kuo CS, Kwei YL, Sun AT, Ku SY, Lu WL and **Wang YY** (1981) Induction of pollen plants of maize and observations on their progeny. Proc. Symp. Plant Tissue Culture, Beijing 1978, Pitman, Boston. pp. 23 - 33.

Nitsch C, Andersen S, Godrad M, Neutter MG and **Sheridan WF** (1982) Production of haploid plants of *Zea mays* L. and *Pennisetum* through androgenesis. *In:* Earle ED and Demarly Y (eds.) Variability in plant regeneration from tissue culture. Praeger, New York. pp. 69 - 91.

Petolino JF and **Jones AM** (1986) Anther culture of elite genotype of *Zea mays* L. Crop Sci. **26** : 1027 - 1286.

Picard EJ and **De Buyser** (1975) Nouveaux resultants concernant la culture d'antheresin vitro de ble tendure (*Triticum aestivum* L.). Effects d'un choe thermique at de la position de l'anthere dans I'epis. C.R. Acad. Sci. (Paris), **280D** : 127 - 130.

Satarova TN (1994) Specific features of the culture of maize anthers on the example of genotype B14 x Wf 9. Izvestiya - An - Rossijskaya - AN - Seriya - Biologiceskaya (Russian Fed.) **5**: 771-778.

Ting YC, Yu M and **Zheng WZ** (1981) Improved anther culture of maize (*Zea mays* L.). Plant Sci. Lett. **23** : 139 - 145.