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ESTABLISHMENT OF REGENRABLE, EMBRYOGENIC CALLUS CULTURES IN IMPORTANT HETEROTIC HYBRIDS OF MAIZE (*ZEA MAYS* L.)

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ABSTRACT
Efficient protocol for establishment and multiplication of callus cultures, in five heterotic maize hybrids HM-5, HM-8, HM-10, HQPM-1 and HQPM-4 has been developed using anther culture technique. Anthers having uni nucleate pollen grain (stained with 0.1% aceto carmine) were used as explants for anther culture response in all the hybrids. Maximum *in vitro* establishment of explants (90%) in all hybrids was observed by surface sterilization of explants with 0.1% $HgCl_2$ for 10 minutes without giving any pre-treatment. M4 medium having MS basal salts supplemented with 2-4D (0.2 mg/l), Zeatin (1.0 mg/l), $AgNO_3$ (5.0 mg/l) and Sucrose (100 g/l) was found best for callus initiation in all hybrids. Highest percent callus induction response (38.5%) was observed in HM-5 hybrid followed by HQPM-1(32.08%), HM-10(19.8%), HM-8(9.6%) and HQPM-4(4.7%) on M4 medium. Callus multiplication response was observed maximum (17.2%) in HM-5 hybrid followed by HQPM-1(16.8%), HM-10(12.2%), HM-8 (7.1%) and HQPM-4(3.2%) on CM-6 medium having MS basal salts supplemented with Zeatin (5.0 mg/l) alone. MS basal medium was found best for anther culture response, among four basal media tried (MS, N_6, LS, Nitsch). Geno typically hybrid HM-5 was found best for anther culture response followed by hybrids HQPM-1, HM-10, HM-8 and HQPM-4.

INTRODUCTION

Maize (*Zea mays* L., 2n = 20, family *Poaceae*) provides food, feed, fodder and serves as a source of basic raw material for a number of industrial products. Therefore the demand for maize and maize products is increasing across the world and most predominantly in Asia FAO, (1992). Maize is cultivated over 8.17 million hectare with a production of 19.73 million tons in India (Huang *et al.*, 2010**)**. Hybrid lines produced are being used for all the purposes, and these have to be produced every year due to the segregation of genes, taking lot of time, manpower, land usage and a huge amount of money. Farmers have to take/buy seeds of maize hybrids every year repeatedly, because seed obtained from hybrids can not be sown in the next year, because of segregations of genes of F_1 hybrids. All these inputs can be saved by androgenesis of F_1 hybrids, to obtain fixed homozygous doubled haploids of maize lines and is in great demand. Also 4-5 years can be saved by developing doubled haploids and seed can be stored and sown in the next year by the farmers. To obtain doubled haploids in maize lines, establishment of callus cultures of important maize hybrids is first step. Few reports are available for doubled haploid production in maize hybrid lines using anther culture technique. Progress in doubled haploid production using anther culture technique is limited due to strong genotype dependency of tissue culture response (Datta, S.K.,2005). Maize haploid tissue are less sensitive to hormones added in different media combinations as compared to other plant species (Chen *et al.,* 1979), therefore frequency of regeneration of haploid plantlets is very low in maize. Therefore, development of standardized protocol of callus establishment and multiplication in important maize hybrids has been reported. Production of friable embryogenic callus of haploid origin is the most important step in anther culture technique (Spitko *et al.,* 2006; Zhu You-Yin *et al.,* 2008; Sylvester Ebikana Anami *et al.,* 2010). Morphologically different types of primarily induced calli have been observed (Fluminhan A. and De Aguiar-Perecin M. L. R. 1998) by using immature embryo as explants. Barnabas. *et al.,* 2005; Datta S. K., 2005 and Wen-Ping DU *et al.,* 2006 have reported doubled haploid production in maize lines. However, regeneration frequency reported is very low, due to strong genotype dependency of regeneration response in maize. Also, very few reports are available for callus induction studies in heterotic hybrids of maize. To achieve this objective, embryogenic callus initiation and multiplication studies has been conducted in five most important heterotic hybrids (HM-5, HM-8, HM-10, HQPM-1 and HQPM-4), of maize using anther culture technique.

MATERIALS AND METHODS

Seed of five heterotic hybrids (HM-5, HM-8, HM-10, HQPM-1, and HQPM-4) of maize used in the present study was obtained from Regional Research Station Uchani, Karnal and sown in net house of Centre for Plant Biotechnology CCS HAU New Campus Hisar, during end of June, first week of November, and end of February during 2012-2015. Seed of

all hybrids was sown at 5-10 days interval to collect anthers at uninucleate stage regularly Spikelets of all hybrids were collected before 10 a.m. in the morning, and anthers were checked under microscope for uninucleate stage of pollen grains by staining (Clark, G., 1981; Kiernan J. A., 2002) with 0.1% acetocarmine solution. Methodology used for collection of explants, surface sterilization of spikelets having uninucleate anthers, media preparation, inoculation of explants has been followed according to books published by (White, P. R., 1943, Bajaj Y. P. S., 1977 and Singh B. D., 2006). Spikelets bearing anthers at uninucleate stage were used as such and were also given cold pretreatment at 10ºC for 10 -15 days for anther culture response. Both the types of spikelets (as above) were first washed with teepole (5-6 minutes). treated for one minute with 70% alcohol and sterilized using mercuric chloride (0.1%) or sodium hypochloride (0.4%) for different time periods or given pretreatment with bavistin (0.2%) and streptocyclin (0.2%) followed by sterilization with mercuric chloride or sodium hypochloride . The treatments were given for different durations to find out the best combination for surface sterilization of explants (Table 1). The spikelets were finally rinsed with double distilled sterilized water 5-6 times under aseptic conditions. Anthers excised aseptically were inoculated on different callus induction media, having different basal media MS (Murashigae *et al.,* 1962), LS (Linsmaier *et al.,*1965), N6 (Chu *et al.,* 1975) and Nitsch (Nitsch *et al*., 1982) supplemented with different combinations and concentrations of cytokinins, auxins and various growth regulators to study callus induction response. Ninety-seven media combinations were tested for callus induction out of which ten media combinations on which best response was observed are given in Table 2. About 20-25 anthers/ petridish and 5-8 petridish/ hybrid were cultured for callus induction response. The cultures were maintained in dark as well as in light at 25 ± 1ºC for 25- 30 days. Heat treatment (35ºC for 7 days) and cold treatment (10ºC for 10-15 days) was also given to the cultures. After heat and cold treatment cultures were shifted to light conditions (16h/8h) in growth room. Data were recorded for percent callus induction response in each hybrid on each medium after 30 -35 days of culturing. All the cultures having callus induction response were transferred on to different callus multiplication media, having MS basal medium supplemented with different combinations and concentrations of auxins and cytokinins Table-3. The cultures were maintained under 16h photoperiod (16h/8h) at 25 ± 1ºC in culture room. Data were recorded for percent callus multiplication response of each hybrid on each medium after 25-30 days of inoculation.

RESULTS AND DISCUSSION

Results obtained during present study have been explained in accordance with development stage of microspore, i*n vitro* establishment of contamination free cultures and exogenous stimulus inducing androgenic response, because these three physiological factors are important for establishment, multiplication of cultures and achievement of successful androgenic response in maize lines. Genetic factors are important in determining the level of anther culture response (Mary and Mac Donald, 1992 and Datta,S.K., 2005).

For identification of pollen grains at uninucleate stage in

Table 1: Standardization of surface sterilization of anthers in F_1 hybrids of maize.

Sr. No.	Treatment Code	Treatment	Conc. used	Duration (min)	Sterilizing agent (duration)	Survival %
1	T1	Bavistin + Streptocyclin	0.2% + 0.2%	30	$HgCl_2$ (8 min)	17
2	T2	Bavistin + Streptocyclin	0.2% + 0.4%	40	$HgCl_2$ (8 min)	26
3	T3	Bavistin + Streptocyclin	0.4% + 0.4%	30	$HgCl_2$ (10 min)	32
4	T4	Bavistin + Streptocyclin	0.2% + 0.2%	30	4% sodium hypochloride(15 min)	11
5	T5	Bavistin + Streptocyclin	0.4% + 0.4%	40	4% sodium hypochloride (20 min)	13
6	T6	Bavistin + Streptocyclin	0.2% + 0.4%	30	4% sodium hypochloride (30 min)	21
7	T7	-	-	-	0.1% HgC_2 (6 min)	59
8	T8	-	-	-	0.1% HgC_2 (8 min)	81
9	T9	-	-	-	0.1% $HgCl_2$ (10 min)	90
10	T10				0.1% $HgCl_2$ (12 min)	50% cultures turned brown

Table 2 : Composition of callus induction media used and percent callus induction response of anthers in F_1 hybrids of maize.

Sr. No.	Medium Code	Composition of medium Basal medium, Growth hormones/Growth regulators used (mg/L), Carbon Source (g/L)	Callus induction response (%) HM-5	HQPM-1	HM-10	HM-8	HQPM-4
1	M1	MS, 2-4D 1.0, Sucrose 30	17.1	15.3	4.8	10.2	8.7
2	M2	MS, 2-4D 2.0, Sucrose 30 19.2	17.5	13.3	11.7	9.5	
3	M3	MS, 2-4D 0.2 +Zeatin 0.5+$AgNO_3$ 5.0, Sucrose 100	27.3	14.6	6.8	16.2	6.25
4	M4	MS, 2-4D 0.2+Zeatin 1.0+$AgNO_3$ 5.0, Sucrose 100	38.5	32.08	19.8	9.6	4.7
5	M5	MS, 2-4D 0.2 +Zeatin 1.0+$AgNO_3$ 7.5, Sucrose 100	22.5	7.8	4.1	16.3	15.9
6	M6	MS, 2-4D 0.2 +Zeatin 1.5+$AgNO_3$ 7.5, Sucrose 100	31.7	12.9	5	11	6
7	M7	MS, 2-4D 0.2 + Kin 1.0 +$AgNO_3$ 5.0, Sucrose 100	32.7	19.09	19.8	12.7	8.7
8	M8	MS, NAA2.0 +IAA2.0, Maltose130	28.2	0	4.7	13.4	11.8
9	M9	N6, IAA 2.0 +NAA 2.0, Sucrose 30	14.8	10.6	2.8	13.3	13.9
10	M10	Nitcsh, 2-4D 0.2 +Kin 1.0 +$AgNO_3$15.0, Sucrose 100	3.1	0	0	14.6	8.8

Table 3 : Composition of callus multiplication media used and percent callus multiplication response of anthers in F_1 hybrids of maize.

Sr. No.	Medium Code	MS basal medium supplemented with Growth hormones/Growth regulators used (mg/L), Carbon Source, Sucrose(30g/L)	Callus multiplication response (%) HM-5	HQPM-1	HM-10	HM-8	HQPM-4
1	CM1	2-4D 1.0,	9.97	5.2	2.2	6.2	3.9
2	CM2	2-4D2.0	11.9	7.1	5.3	6	-
3	CM3	NAA1.0 +BAP1.0 +Kin.1.0	-	-	-	3.9	-
4	CM4	2-4D2.0 +Proline 2.76 + casein hydrosilate 1.0 g + $AgNO_3$ 0.015	-	-	-	-	-
5	CM5	-	7.1	2.2	-	7.5	-
6	CM6	Zeatin 5.0	17.2	16.8	12.2	7.1	3.2
7	CM7	Kin. 5.0	12.9	2	-	5	-
8	CM8	NAA 0.5 +BAP2.0	-	-	-	-	-
9	CM9	NAA 1.0 +Kin.1.0	5.2	1.1	-	8.9	4.2

anthers, anthers bearing pollen grains from emerging tassels in each hybrid were taken at all stages before 10 a.m. in the morning, till they emerged out completely from the leaf and were checked for uninucleate stage of pollen grains by staining with 0.1% acetocarmine solution under microscope. Maximum percentage (> 70%) of uninucleate stage of pollen grains was found in the anthers bearing pollen grains present on the tassels, which are just emerging from the leaves, in the middle and just below the middle part of the tassel. Similarly, embryogenic response from anthers in maize lines has been obtained (Petolino and Jones, 1989; Obert and Barnabas, 2004; Wen-Ping DU *et al.*, 2006; Mohammadi *et al.,* 2007) using parts of tassels containing mid or late uninucleate microspores.

Surface sterilization of spikelets having anthers at uninucleate stage of pollen grains to obtain *in vitro* contamination free cultures is one of the critical step to initiate callus induction response in maize lines. Results obtained for surface sterilization of spikelets bearing anthers having pollen grains at uninucleate stage have been shown in Table 1. Ten different surface sterilization treatments (Table 1) were tried for surface sterilization of spikelets in all hybrids. Maximum percent (90%) *in vitro* survival of explants, was obtained when spikelets were washed with teepole for five minutes (T9 treatment), and surface sterilized with 0.1% $HgCl_2$ for ten minutes, without giving any pretreatment in all hybrids. Also, 81% (T8 treatment) *in vitro* surviving explants were obtained when spikelets were surface sterilized 0.1% $HgCl_2$ for eight minutes. However, maximum *in vitro* surviving explants were reported by (Jager *et al*., 2010), when tassels were surface sterilized with 0.2% sodium hypochloride for 20 minutes. *In vitro* surviving response obtained was not observed good when spikelets after washing with teepole were given presurface sterilization treatments Table-1 with bavisitin and streptocyclin for different times. As the time for surface sterilization was increased to twelve minutes, explants (50%), Table 1 started turning brown. Mohammadi *et al.,* 2007 reported best surface sterilization response, when parts of tassel containing mid or late uninucleate microspores were surface sterilized with 20% sodium hypochloride for twenty minutes.

Results obtained for callus induction response using anthers having uninucleate pollen grains in the present study in all hybrids have been shown in Table-2**.** Anther culture responsive germplasm is of great interest to commercial maize breeders and has to be identified to get maximum response (Barnabas *et al.*, 2005). Callus induction response was not observed in any maize hybrid when spikelets bearing anthers at uninucleate stage were used giving cold pretreatment at 10º C for 10 -15 days. Heat treatment given to anthers bearing uninucleate pollen grains at 35ºC for seven days after inoculation on different media combinations was found best for callus initiation in all maize hybrids. Obert and Barnabas, (2004) reported best callus induction response using pretreatment of tassels at 7ºC for 7 days. Jumpatong (1996) reported maximum callus induction response by giving heat treatment of tassel at 30ºC for 7-10 days. Pretreatment (7ºC for 10-15 days) to the anthers having uninucleate pollen grains did not give any response for callus induction Calli obtained in all hybrids on various callus induction media were transferred on to nine different callus multiplication media having Ms basal salts supplemented different combinations and concentrations of growth regulators Table 3. Results obtained for callus multiplication response in all maize hybrids have been shown in Table 3. Maximum callus multiplication response (17.2%) was observed on CM-6 medium having MS basal salts supplemented with zeatin alone in HM-5 hybrid, followed by HQPM-1(16.8%), HM-10(12.2%), HQPM-4 (7.1%) and HM-8(3.2%) on the same medium. Somatic embryogenesis and plant regeneration response in tropical maize genotypes have been reported earlier using anther culture technique (Mohammadi *et al.,* 2007 and Sylvester *et al*. 2010). In addition, CM-2 medium having MS basal salts supplemented with 2-4D 2.0mg/l was also found good for callus multiplication response [HM-5(11.9%), HQPM-1(7.1%), HM-10(5.3%),HQPM-4(6.0%),and HM-8(0%)]. Genotypically, hybrid HM-5 was observed to be the best for anther culture response followed by HQPM-1, HM-10, HM-8 and HQPM-4. The effect of donor plant's physiology and vigour on frequency of anther culture response has been explored by Geiger and Gordillo (2009). ZHUYou-yin1 *et al.*(2008) have also observed that percent callus induction response varies with the genotype in maize.

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