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Original Article

Improvement of maize (*Zea mays* L.) anther culture embryogenesis and direct regeneration by differentplant growth regulators and gelling agent

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Abstract

Androgenesis via anther culture or microspore culture is one of the current methods for producing haploid and double haploid plants in maize. To use of this method in maize breeding program it should be able to regenerate enough plantlets. Anthers culture usually is carried out indirectly via callus induction and regeneration on at least two different media. In this study a responsive genotype, ETH-M82, using a new single culture medium was used for embryogenesis and regeneration. We tested different growth regulators (2,4-D; Kinetin; NAA & IAA) in modified YP medium. After6-week, direct regeneration on some treatments was observed. The highest frequency of direct formation of plantlets (in 100 anthers) occurred on medium supplemented with $2mgl^{-1}$ IAA and $2mgl^{-1}$ kinetin (4%).Best results with an average of 3.1plantlets in replication were obtained with the medium solidified with agar, while in difcobactoagar only 1.4 of plantlets in replication was produced. This experiment suggested that agar and plant growth regulators in the medium were beneficial for producing embryo and plantlet from maize anthers.

Keywords: Anther culture,Directregeneration; Plantlet, Zea *mays* L.

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Introduction

Isolated anther culture has become an important tool for the production of varieties produced by doubled haploid procedure in many important crops since the first report describing embryogenesis from the pollen of *Daturainnoxia* [1]. In maize, another culture is an effective technology for the production of DH population [2, 3]. The anther culture response is influenced by the following factors: genotype, environmental factors affecting the physiological stage of anther donor plants, stage of microspore development, and physical and chemical conditions for anther induction and plant regeneration [4].

Maize genotypes differ with respect to their amenability to anther culture [5]. There has been no consensus on a requirement for hormones, the synthetic auxin 2,4-dichlorophenoxy-acetic acid (2,4-D) and the antiauxin 2,3,5-triiodobenzoic acid (2,3,5- TIBA) are both widely used in another culture induction media of maize due to their reliably positive effect on the proliferation of microspores with androgenic fate. The synthetic auxin 2, 4-Dcauses a rapid dose-dependent decrease in the number of dividing cells, cell membrane damage, cytoplasm and nucleoplasm vacuolization, as well as chromatin abnormalities and chromosome aberrations [6-8]. In contrast, it has been hypothesized that endogenous growth regulator levels are sufficient for embryo structure development in maize and exogenous supply is not necessary [9] (Rashid, 1988). The data indicate that thehormone requirement may also be genotype-dependent [10] and the donor plant growth conditions [3, 11] may influence these requirements. Differences in the embryogenic competence of genotypes may be caused by the differential response of the cells to exogenous plant growth regulators, mainly to 2,4-D [12]. One of the major differences between the more and less competent lines in maize is the distribution of IAA within the embryos after culturing them on medium containing 2,4-D [13]. The polar transport of auxin is essential for the establishment of bilateral symmetry during embryogenesis in dicotyledonous and monocotyledonous species [14].

Cultured plant cells show rapid responses even to minor changes in the osmolarity of theirculture media [15] and therefore medium containing mixable osmotic reagents should be provided. A growing plant cell's water uptake is governed by the relative water potential values between the cell and the external medium. The major components of thenutrient medium that influence water availability are the concentrations of the agar, carbon source, and any non-metabolite added as an osmoticum [16].According to Hadeler *et al* [17], the gallant strongly influences cellular differentiation and sensitivity of *in vitro* plant cell cultures to cytokinin. Gelling agents are believed to influence the availability of mineral salts and sometimes may result in

the verification of cultured tissues [18]. Most culture media are solidified with agar, which is obtained from the red alga Rhodophyceae. It is highly stable, clear, non-toxic and resistance to metabolism during culture [19, 20]. Phytagel is an agar substitute produced from a bacterial substrate but results in clumping. Therefore, to prevent clumping, phytagel should be added to rapidly stirring culture medium at room temperature (Sigma Aldrich Catalogue, 2013).Although agar has been for a long time the only gelling agent used to produce solid media, more reproducible results on anther culture have been obtained with Gelrite (gellan gum) and agarose [21]. Phytagel is a gellan gum PS-60 isolated from the bacterium *Pseudomonas elodea*. It is a linear polysaccharide composed of glucuronic acid, glucose, and rhamnose, requiring the presence ofmonovalent and divalent cations for gellation [22]. Since phytagelis purified from a single organism, it is highly purified and does not contain contaminants [23].

The aim of the present study was to examine the potential of the effect of 6 different treatment of plant growth regulator on embryogenesis and direct regeneration and 3 gelling agents; (agaragar, phytagel and difcobactoagar) to the induction media of maize anther cultures to enhance the efficiency of anther response and the frequency of microspore-derived embryo structures.This is the first report that plant growth regulators and gelling agent affects on embryogenesis and plantlet regeneration in the culture of anthers from maize.

Materials and Methods

The responsive maize genotype ETH-M82 (provided kindly by Dr Aulinger, Swiss Federal Institute of Technology, Zurich, Switzerland) was used as anther donor plants. The donor plants were grown in the greenhouse at 25 °C (day) and 15°C (night), a photoperiod of 16 h and a light intensity of 250 $Jmolm^{-2}s^{-1}$.Tassels were collected prior to the emergence of the main leaf blade and checked for microspore development. They were then covered with sterile aluminum foil and kept in the cold and dark (at 7 °C for 10 days) as pretreatment [2].During this period, the microspores in the anthers reached the premitotic developmental stage. Tassel fragments containing anthers in the late-uninuclear microspore development stage (determined by acetocarmine squash) were surface sterilized with 20% sodium hypochlorite for 10 min and then washed three times with sterile distilled water. In first experiment anthers were then dissected under sterile condition and were placed in 55×15 mm plastic petridishes containing 12.5 ml modified YP medium [24] with 60 grl^{-1} sucrose and 6 different concentration of plant growth regulator. The experiment was carried out based on completely randomized design with 5 replications. The units of this study were one Petri dish containing 25 anthers. Analysis of variance was carried out using SPSS statistical software (SPSS, version 10.0). The effect of plant growth regulator was determined by the following 6 treatments:

1- Modified YP without plant growth regulator
2- Modified YP +2 mgl^{-1}kinetin
3- Modified YP +3 mgl^{-1}kinetin
4- Modified YP +2 mgl^{-1}kinetin+2 mgl^{-1} IAA
5- Modified YP +2 mgl^{-1}kinetin+2 mgl^{-1} NAA
6- Modified YP +2 mgl^{-1}kinetin+2 mgl^{-1} 2-4-D

In second experiment, plantlet derived from modified YP medium without plant growth regulator transferred directly to basal MS medium. Each replication with 20 embryo were removed from the 1-monthcultured anthers and transferred directly to the 10 cm plastic petridishes containing 15 ml plant regeneration medium (YPNAS) [24] containing these different gelling agents:

1- 7.5 gl^{-1}agar-agar
2-3 gl^{-1}phytagel
3- 7.5 gl^{-1}difcobactoagar

Plantlet differentiation was carried out under 16-h illumination (50 $mmols^{-1}m^{-2}$ light intensity) at a constant 25 °C. Then, plantlets were placed in 250 ml glass containers, containing 30 ml of hormones-free MS medium for further growth. Well differentiated healthy green plantlets were then transplanted into 10-cm pots containing vermiculite and peat (1:1). Finally, the plants were transferred to soil and grown to maturity in a controlled growth chamber.

Result and Discussion

In this study a responsive genotype, ETH-M82, using a new single culture medium was used for embryogenesis and regeneration. We tested different growth regulators (2.4.D; Kinetin; NAA & IAA) in modified YP medium.

After 6 week direct regeneration on some treatments was observed. The highest frequency of direct formation of plantlets (in 100 anthers) occurred on YP medium supplemented with 2mgl^{-1} IAA and 2mgl^{-1} kinetin (4%).

Androgenesis has been traditionally divided in two main stages, namely induction and regeneration. In the former one, somatic cells acquire embryogenic characteristics by means of a complete reorganization of the cellular state, including physiology, metabolism and gene expression [25]. It is usually after a change in one or more culture conditions [e.g., culture medium, composition of plant growth regulators (PGRs), carbohydrate source, osmoticpotential, etc.] that the induced tissues or cells reach the regeneration stage, in which cells display their embryogenic competence and differentiate into somatic embryos. Level of endogenous hormones is considered to be one of the crucial factors determining embryogenic potential of explants [26, 27].

Table 1. Mean squares (MS) of the ANOVA for embryo induction (T1) and plantlet regeneration (T2) in another culture of *Zea mays*L.(** Significant difference at 1% probability level)

Source of variation (S.O.V)	df	MS for T1	MS for T2
Plant growthregulatore	5	627.08**	16.75**
Error	12	14.92	1.08
Total	17		

Table 2. Mean squares (MS) values for gelling agent effect on plantlet regeneration in the anther culture of *Zea mays* L.

Source of variation (S.O.V)	**df**	**MS**
Gelling agent	2	15.72**
Error	69	1.88
Total	71	

** Significant difference at 1% probability level.

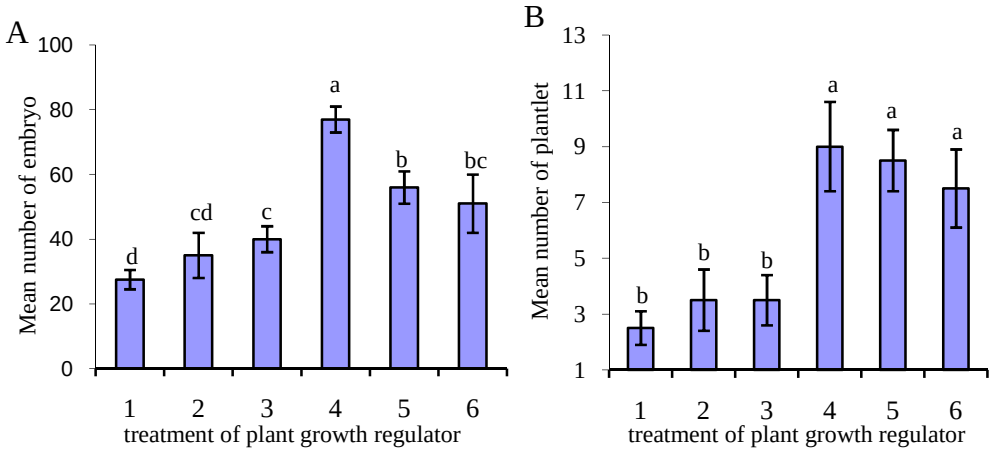

Figure 1. Effect of plant growth regulators onembryo induction (A) and plantlet regeneration (B) in another culture of *Zea mays* cvs. ETH-M82. Means with the same letter are not significantlydifferent at p=0.01.
1- Modified YP without plant growth regulator; 2- Modified YP +2 mgl^{-1} kinetin; 3- Modified YP +3 mgl^{-1} kinetin; 4- Modified YP +2 mgl^{-1} kinetin+2 mgl^{-1} IAA; 5- modified YP +2 mgl^{-1} kinetin+2 mgl^{-1} NAA; 6- Modified YP +2 mgl^{-1} kinetin+2 mgl^{-1} 2-4-D

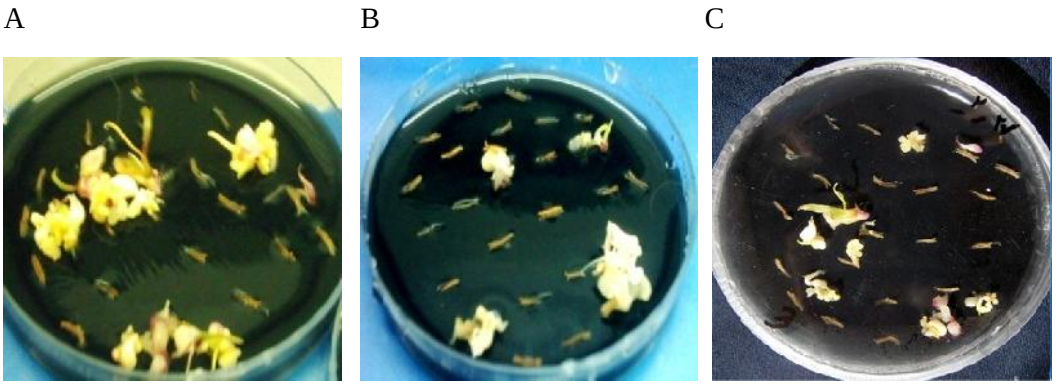

Figure 2. Embryogenesis and plantlet regeneration in maize anther culture. A: Modified YP medium +3 mgl^{-1} kinetin; B: Modified YP medium +2 mgl^{-1} kinetin+2 mgl^{-1} 2-4-D; C: Modified YP medium +2 mgl^{-1} kinetin+2 mgl^{-1} IAA.

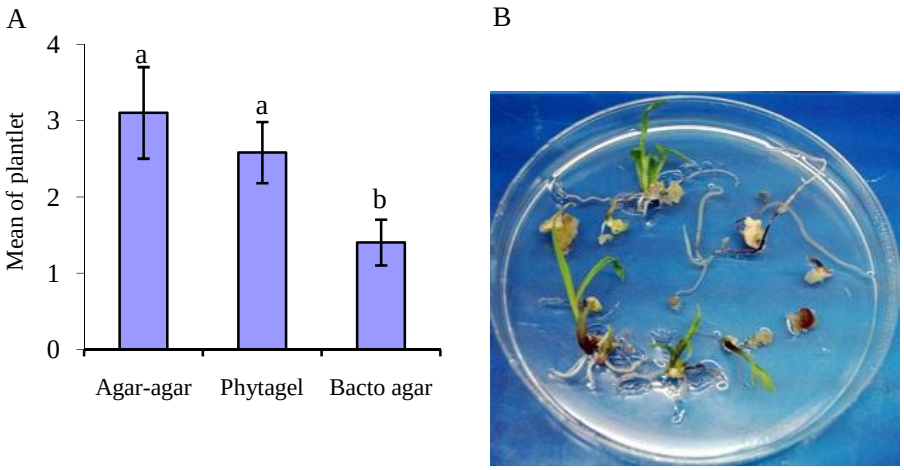

Figure 3. Effect of gelling agent on plantlet regeneration in anther culture of *Zea mays*L.Means with the same letter are not significantly different at p=0.01(A).Plantlet regeneration in maize anther culture in modified YP medium solidified with agar-agar (B).

Various media compositions have been explored to determine the optimum conditions for maize anther culture. High auxin concentrations were reported to increase the anther culture response in maize, and decrease the number of embryo structures [28]. A higher endogenous IAA levelhas been shown to be associated with increased embryogenic response in maize [29]. In this study, a better induction of embryogenesis and direct regeneration of plantlet in maize was obtained by using 2mgl^{-1} kinetin and 2 mgl^{-1} IAA.

In tissue cultures, the gelling agent has generally been considered only a solid support, preventing plant explants

from immersion and suffocation. Moreover, the growth of cultured plant explants may be affected by inhibitive substances released by necrosed plant tissues, and which concentrate near the explants [30]. Those effects depend on the type and concentration of the gelling agent used. Phytagel was reported to provide a better availability of water [31]. Similarly, inhibitive molecules, such as phenols produced by plant explants, are fluently diffused in the medium solidified with phytagel [32]. Moreover, this gelling agent contains a higher level of Ca, K, Mg and Fe ions, compared to the medium solidified with agar, which has three times as many Na ions [33]. In this study, a better induction of regeneration in maize was obtained by using agar as the gelling agent. The influence of agar on several *in vitro* developmental processes has been reported in literature [34, 35]. The results presented here are in agreement with these observations.

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