Effect of induction medium on embryogenesis of maize anther culture and increase of normal haploid plantlet regeneration with wathman filter paper

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Abstract

This study involves *in vitro* androgenesis of *Zea mays* L. via anther culture. In the first experiment, three embryo induction media were tested in ETH-M82 genotype of maize. The results showed that the embryo structures were established 2 to 3 weeks after culturing the anthers. The effect of induction medium was significant for embryo frequencies. The obtained results confirm that the induction medium is one of the major supplements that support the development of anthers finalized either to embryo. IMSS medium with 22.4 embryo like structure (ELs)/replication was better than other medium and N6 medium had low production of embryos. In the second experiment, the analysis of variance showed that the percentage of normal regenerated plants was remarkably influenced by using whatman filter paper on regeneration medium (YPNAS).The use of whatman filter paper on the surface of regeneration medium had substantial increased normal plant production. In fact, the percentage of normal plants was 38% more in the presence of whatman paper on plant regeneration medium. In general, considering the morphology, such *in vitro* normal regenerated plants were very similar to maize seedlings. Such success in producing normal plants in maize anther culture by using whatman paper has not been reported yet.

Keywords: Zea mays; anther culture; embryogenesis; filter paper; regeneration medium

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Introduction

Breeders have long recognized the advantages of doubled haploid (DH) technologies based on the knowledge that several theoretical and practical aspects of plant biology and genetics can take advantage of haploidy technology (Forster and Thomas, 2005). For crop improvement purposes, DH lines are developed mainly to achieve homozygosity in diploid or allopolyploid species, saving several generations in a breeding program and producing new homozygous cultivars or parental lines for F_1 hybrids (Veilleux, 1994). Anther culture

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(androgenesis) defined as a process, which obtain haploid embryo using immature pollens (microspores) in anthers cultivated on nutrition media. This procedure usually needs short time to be conducted (only one generation) and could accelerate the production of new varieties with improved traits (Barakat et al., 2012). Haploid plants are of great importance, which are used in achieving homozygosity in quick way, and facilitating genetic and breeding researches (El-Hennawy et al., 2011).

Therefore, many investigations on the use of doubled haploids through androgenesis, have been carried out (Dieu and Beckert, 1985). Anther culture capacity is characterized by embryo production and plant regeneration. Embryogenesis can be influenced by genetic and environmental factors such as anther pretreatment and embryo induction medium. Androgenic development was first reported in anther culture of *Datura innoxia*, and since that study it has been reported in over 200 species of angiosperms belonging to more than 50 genera and 25 families of dicots and monocots. Numerous factors including genotype of the donor plant, the stage of pollen development, the composition of the nutrient medium, pretreatment of flower buds or inductive treatment of isolated microspores, and the physiological state and conditions of growth of donor plants have been described as affecting factors in induction of androgenesis (Smykal, 2000).

Development of an efficient and cost-effective doubled haploid (DH) production system in maize is the prerequisite for the application of DH technology in a practical breeding program. Anther culture is useful for conventional breeding, mutation breeding, *in vitro* selection and DNA transformation techniques (Karsai and Bedo 1998). In maize, anther culture is an effective technology for the production of DH population (Mohammadi et al., 2007). One of the main approaches have been used to improve the anther-culture-based doubled haploid production system in maize is to identify physiological and environmental conditions that are optimal for anther-culture-based doubled haploid production using the selected genotypes. In other hand, condition of in vitro culture has an important role. Among different condition of in vitro condition, basal medium has very important effect. The most commonly used basal media for anther culture are N6 medium (Chu, 1978), (modified) MS medium (Murashige and Skoog, 1962), and B5 medium (Gamborg et al., 1968), but there are many others. Generally, half-strength MS salt mixtures are suggested for the *Solanaceae*, and N6 medium for the cereals (Chu, 1978).

The majority of somatic embryos (SEs) derived from tissue culture typically fail to mature and/or develop into normal plants. This problem is even more evident in maize, especially haploid maize, that require lengthy culturing times to generate plants, a process that is often conducive to the production of undesirable somaclones. Therefore, increasing the number of SEs that produce plants is an important consideration in developing a tissue culture protocol for plant breeding program. Generally, it is necessary to transfer embryogenic cultures to a medium with reduced hormone concentration and nutrient content, or with no hormone, to initiate and/or mature SEs. Other methods used for these purposes include exertion of stressful conditions, such as heat, cold, and temporary starvation. Subjecting the cultured tissues to periods of stress such as desiccation (Parrott et al., 1988; Timbert et al., 1996; Bomal and Tremblay, 1999) appears to improve the conversion of SEs into mature embryos and subsequently to plants.

There is narrow study about regeneration medium on plantlet regeneration in the culture of anthers from maize. This study was aimed to examine the effect of three embryo induction media (IMSS, YPm and N6) to identify suitable induction medium for maize anther cultures. In addition, this is the first report about using filter paper on regeneration medium on plantlet regeneration in the culture of anthers from maize.

Materials and Methods

First experiment

The ETH-M82 maize genotype (provided by Dr. IE. Aulinger, Swiss Federal Institute of Technology, Zurich, Switzerland) was used as anther donor plants. Seed of this genotype were surfaced sterilized and tested for viability based on ISTA protocols (as described by Eisvand et al., 201;

FarzamiSepehr and Ghorbanli, 2010) and then were grown in a growth room at 25°C (day) and 15°C (night), with a photoperiod of 16 h and a light intensity of 500 $\mu molm^{-2}s^{-1}$. Tassels containing anthers in the late-uninuclear microspore developmental stage (Fig. I) (determined by acetocarmine squash) were collected. As a cold pretreatment, the tassels were covered with an aluminum foil and then kept at 7°C for 10 days (Jumpatong et al., 1996). Tassel fragments were surface sterilized with 2% w/v sodium hypochlorite for 10 min and then washed 3 times with sterile distilled water. The anthers were then dissected under sterile conditions and were placed in 55×15 mm plastic petri-dishes containing 8 ml semi-solid induction media (IMSS); (Saisingtong et al., 1996) and YPm medium (Genovesi and Collins, 1982) and N6 medium (Chu, 1978) and then incubated at 28°C in the dark for one month. The number of responding anthers and the frequency of microspore-derived structures (embryo-like structures; ELSs) were then calculated as a percentage of the cultured anthers.

The experiment was carried out based on a completely randomized design (CRD) with five replications. Each replication consisted of one petri-dish containing 25 anthers. Analysis of variance (ANOVA) was carried out for data with normal distribution using the SPSS statistical software (version 10.0).

Second experiment

The produced ELSs from ETH-M82 genotype were removed from the one-month cultured anthers and transferred directly to the 10 cm plastic petri-dishes containing 15 ml of plant regeneration medium (YPNAS) (Genovesi and Collins, 1982) either with one layer of whatman filter paper on its surface, or without it (control). They were incubated under a 16 h illumination period (50 $\mu mol\ m^{-2}s^{-1}$ light intensity) at 25°C. The regenerated plantlets were then transferred into the 250 ml glass containers containing 30 ml of basal MS medium for further growth. Healthy green plantlets were then transplanted into 10-cm pots containing vermiculite and peat (1:1). Finally, the plantlets were transferred to soil and grown to maturity in a growth chamber. Plants which produced normal male and female inflorescences were self-pollinated and the seed set was recorded. Experiment was considered on the basis of a completely randomized design (CRD) with 36 replications for each treatment. Each replication consisted of one petri-dish containing 20 embryos.

Fig. I. Tassel containing anthers in the late-uninuclear microspore developmental stage (Left), Anthers (Right)

Results

Effect of different media

The results showed that the embryo structures were established 2 to 3 weeks after culturing the anthers (Fig. II). The effect of induction medium was significant for embryo frequencies (Table 1). The results obtained confirm that the induction medium is one of the major supplements that support the development of anthers finalized either to embryo. For this genotype IMSS medium (22.4 ELs/replication) was better than other medium and N6 medium had low production of embryo (Fig. III). The culture medium used for the first successful anther culture of maize was MS (Murashige and Skoog, 1962) basal medium with 12%-24% sucrose, this resulted in a 1% response frequency (i.e., 1 % of the anthers produced at least one embryo).

Fig. II. Embryogenesis in induction medium in maize anther culture.

Effect of filter paper

In the second experiment, the analysis of variance (Table 2) showed that the percentage of normal regenerated plants was remarkably ($P<0.01$) influenced by using whatman filter paper on regeneration medium (Fig. V). In fact, the percentage of normal plants was 38% more in the presence of whatman paper on plant regeneration medium (Fig. IV). Furthermore, it was observed that the whatman paper improved the shoot organogenesis and rhizogenesis, resulted in producing plants having normal shoots and leaves. The regenerants on the medium without whatman paper produced longer and thinner roots, while those on the whatman paper produced the thicker and shorter roots. It seems that the surviving percentage of plants having such latter roots will be increased as they being transferred to *ex vitr*o conditions.

Discussion

In the present study, the type of induction medium showed highly significant effects on the frequency of embryogenic response. In the first experiment, IMSS medium was better than other medium and N6 medium had low production of embryo. Although MS medium was used widely in many plant micropropoagation and callus induction protocols (Kaviani, 2014; Ghasempour et al., 2014), later, the researchers found that N6 medium, originally developed for rice anther culture, was superior to MS medium for maize anther culture (Chu, 1978). Besides MS and N6 basal media, Yu-peror YP (Ku et a1., 1978; Nitsch et a1., 1982; Genovesi and Collins, 1982; Pauk, 1985; Petolino and Jones, 1986; Dieu and Beckert, 1986) and Zheng14 (Ting et al., 1981; Dieu and Beckert, 1986) have also been used. These media differ mostly in the forms of N used in their combination. Maize anther culture media also often contain an organic nitrogen source such as lactalbumin (Ku et a1., 1978), casein hydrolysate (Miao et a1., 1978), or proline (Nitsch et a1., 1982). Anther culture media are generally solidified by adding agar, but the beneficial effect of other solidifying agents, such as starch (potato, wheat, corn or barley starch), gelrite, agarose and ficoll, has been reported. Liquid, semisolid and two-phase systems in which anthers are floated on liquid medium overlying an agar-solidified medium have been tested with different results (Dunwell, 2010).

In the second experiment, the use of whatman filter paper on the surface of regeneration medium had substantial increased normal plant production, shoot organogenesis and rhizogenesis, resulted in producing plants having normal shoots and leaves. Indeed, it was observed that the roots of regenerated plants grown on whatman paper could not penetrate into the medium but just penetrate into the whatman paper. The effects of drying rate and minimum moisture content of somatic embryos

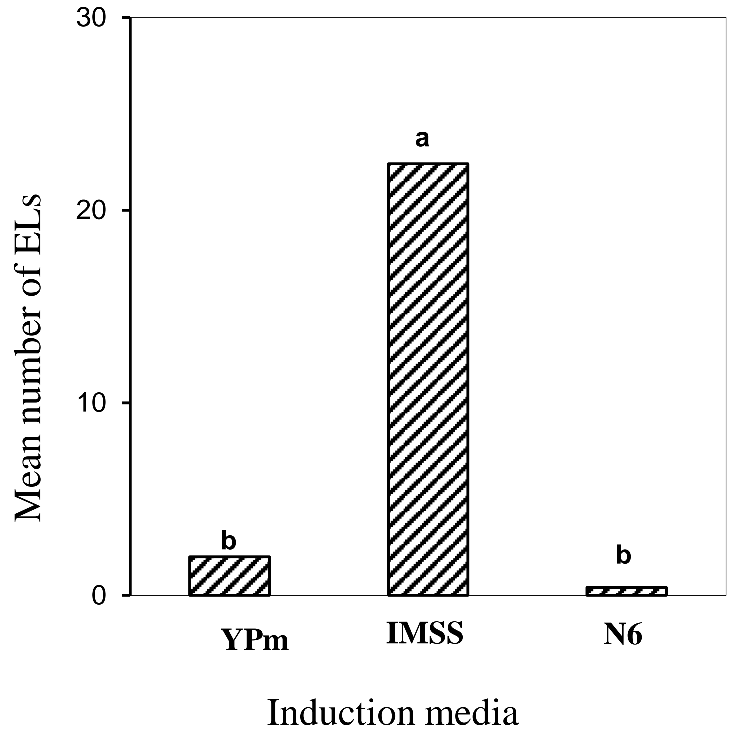

Fig. III. Effect of different induction medium on the ESs production in maize anther culture. Means with the same letter are not significantly different at p=0.01% following Duncan's multiple range test.

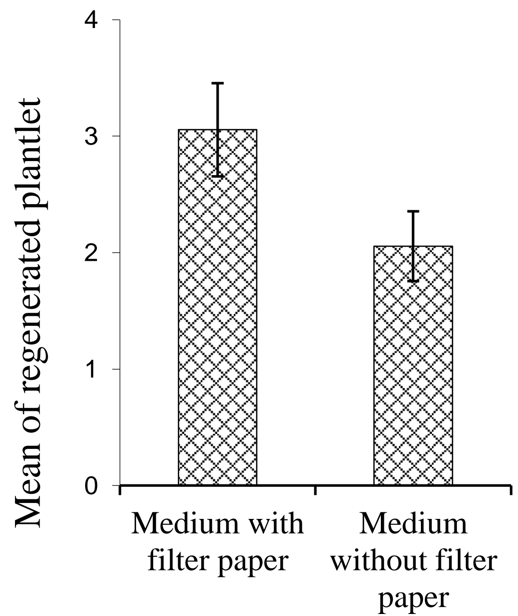

Fig. IV. Differences between regeneration medium with or without wathman filter paper of plantlet regeneration.

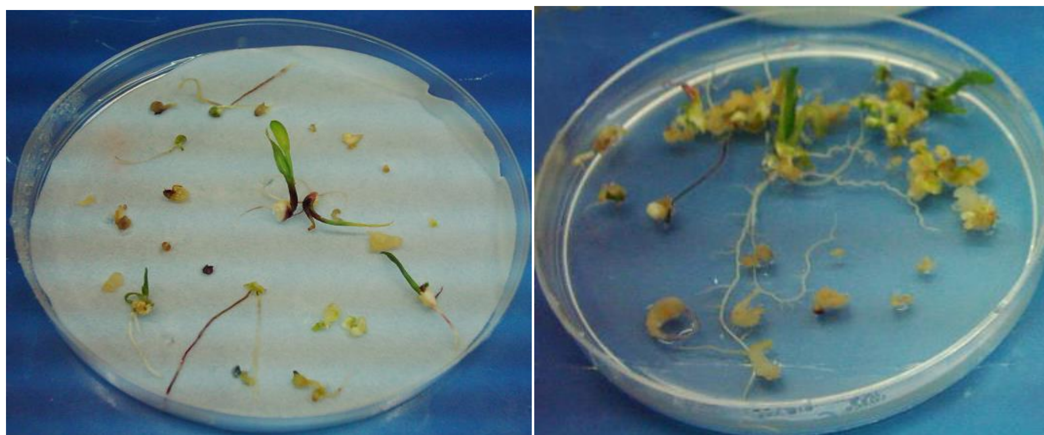

Fig. V. Plantlet regeneration in maize anther culture. Regeneration medium with wathman filter paper (A); Regeneration medium without wathman filter paper (B).

Table 1
Analysis of variance for embryo induction in anther culture of *Zea mays*.

Source of variation (S.O.V)	d.f.	M.S.
Induction medium	2	188.07**
Error	12	22.97

** Significant difference at 1% probability level.

Table 2
Analysis of variance for normal plantlet regeneration in anther culture

Source of variation (S.O.V)	d.f.	M.S.
Wathman paper	1	9.0**
Error	70	1.82

* Significant difference at 5% probability level.

on germination and plantlet development are still not clearly defined. Roberts et al. (1990) showed that somatic embryos of interior spruce, maturated with low osmoticum (3.4% sucrose), reached a moisture content of 69%, and germinated at low frequency (24%). Under their conditions, a partial desiccation leading to 3.3% water loss was necessary to achieve high germination frequency (80%) (Roberts et al., 1990). For hybrid larch, the plantlet regeneration frequency was increased from 42 to 82% after a partial dessiccation reducing the moisture content of the embryos from 83 to 75% (Lelu et al., 1995). Restricting water uptake by using osmotica in the maturation medium could provide a natural drought stress during embryo development (Attree et al., 1995).

Germana (2011) reported that the exploitation of haploid and DHs as a powerful breeding tool requires the availability of reliable tissue culture protocols that can overcome several methodology problems, such as low frequencies of embryo induction, albinism, plant regeneration, plant survival and the genotype-dependent response, in order to improve the regeneration efficiency in a wider range of genotypes. He also reported that there is no single standard condition or protocol for inducing pollen-derived plant formation.

In general, considering the morphology, such in vitro normal regenerated plants were very similar to maize seedlings. Such success in producing normal plants in maize anther culture by using whatman paper has not been reported yet. The results presented here indicate that the best embryo induction can be achieved by use of

IMMS induction medium and use of filter paper on regeneration medium can improve plantlet regeneration in maize anther culture.

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