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REGULAR ARTICLE

SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM IMMATURE EMBRYO INDUCED CALLUS OF MAIZE (*ZEA MAYS* L.)

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ABSTRACT

Somatic embryogenesis is the process by which somatic cells, under induction conditions, generate embryogenic cells, which go through a series of morphological and biochemical changes that result in the formation of a somatic embryo. These characteristics have designated somatic embryogenesis into a model system for the study of morphological, physiological, molecular and biochemical events occurring during the onset and development of embryogenesis in higher plants. In our experiments somatic embryogenesis and plant regeneration was achieved from immature embryos of two maize (*Zea mays* L.) lines A18 and A19. Callus was initiated on N6 medium supplemented with 1 mg.dm^{-3} 2,4-D, N6 salts, 2 % sucrose, 25 mmol.dm^{-3} proline, 100 mg.dm^{-3} casein hydrolysate, N6 vitamins, 10 mg.dm^{-3} silver nitrate, 3g gelrite. Induction of primary callus ranged between 0 and 93%. Generally, three types of callus were formed: embryogenic, non-embryogenic and organogenic callus. Embryogenic callus was formed within two weeks of culture in callus maintenance medium. Induction of embryogenic callus ranged between 0 and 5%. Somatic embryos were matured on N6 medium supplemented with 6% sucrose and 1 mg.dm^{-3} NAA. After transfer of embryogenic calli on regeneration medium containing MS medium supplemented with 2% sucrose, somatic embryos started to form plantlets. Callus initiation and plant regeneration were genotype dependent. Regenerated plants were transferred on the surface of solidified MS medium supplemented with myo-inositol.

Keywords: Somatic embryogenesis, Immature embryos, Callus induction, Maize (Zea Mays L.)

INTRODUCTION

Somatic embryogenesis is developmental process by which somatic cells undergo restructuring to generate embryogenic cells. These cells then go through a series of morphological and biochemical changes that result in the formation of somatic or non-zygotic embryo capable of regenerating plants. Somatic embryogenesis represents a unique developmental pathway that includes a number of characteristic events: dedifferentiation of cells, activation of cell division and reprogramming of their physiology, metabolism and gene expression patterns (**Zimmerman, 1993; Schmidt et al., 1997; Komamine et al., 2005**). Somatic embryogenesis forms the basis of cellular totipotency that is unique to higher plants. Differing from its zygotic counterpart, somatic embryos are easily traceable, culture conditions can by monitored and lack of material is not a limiting factor for experimentation (**Kawara and Komamine, 1995**). Somatic embryogenesis plays an important role in clonal propagation. When integrated with conventional breeding programs and molecular and cell biological techniques, somatic embryogenesis provides a valuable tool to enhance the genetic improvement of commercial crop species (**Stasolla and Yeung, 2003**).

Maize (*Zea Mays* L.) is one of the most important cereal crops and widely cultivated in world today. It is a good source for human food, animal feed and chemical industry. Maize is considered as a major food crop worldwide (**Danforth 2009**). Production of this crop is very important due to increased population, limited land, environmental and biotic stresses. Over the years, conventional breeding has been used as a tool for sustainable production of this crop. Nowadays, biotechnological tools can be helpful to enhance breeding and subsequently maize production.

Plant regeneration through tissue culture of maize was first reported by **Green and Philips (1975)** utilizing immature embryos as the explants. Since the successful plant regeneration has been reported from callus initiating from different tissue sources (**Ting et al., 1981; Rhodes et al., 1986; Conger et al., 1987**). Maize immature embryos were most widely used as initial explant for maize regeneration (**Lu et al., 1982; Lu and Vasil, 1983; Vasil et al., 1984**). However various conditions for somatic embryo induction and regeneration were tested and used, the ability to regenerate embryo derived from callus cultures has been

reported to be dependent on the maize genotype used (**Lee and Phillips, 1987; Obert et al. 2009**).

Hormones are the most likely candidates in the regulation of developmental switches. Auxins and cytokinins are the main growth regulators in plants involved in the regulation of cell division and differentiation. The influences of exogenously applied auxins, preferentially 2,4-dichlorophenoxyacetic acid (2,4-D), on the induction of somatic embryogenesis are well documented (**Dudits et al., 1995**). Auxins promote, mainly in combination with cytokinins, the growth of calli, cell suspensions and organs, and also regulate the morphogenic processes. At the cellular level, auxins control basic processes such as cell division and cell elongation. Since they are capable of initiating cell division they are involved in the formation of somatic embryos.

These characteristics have made somatic embryogenesis a model for the study of morphological, physiological, molecular and biochemical events that occur during the onset and development of embryogenesis in higher plants. It also has potentially rich biotechnological applications such as artificial seed, micropropagation, transgenic plants, etc.

The objective of the present study was to assess callus initiation and plant regenerative response of two inbred lines from immature embryos.

MATERIAL AND METHODS

Plant material

Maize callus induction system was developed using immature embryos. Two maize inbred lines (A18 and A19) were used in our experiments. Plants were grown in experimental field of the Institute of Plant Genetics and Biotechnology in Nitra. Ears were harvested 16 and 20 days after pollination. Size of immature embryos was 1-4 mm. The ears were surface sterilized for 5 min in 70% ethanol and then for 20 min in 40% SAVO. This was followed by three times rinse in sterile distilled water. Immature embryos were aseptically isolated by cutting the tips of the kernels with a scalpel without touching the embryo.

Callus induction and maintenance

The embryos were placed with embryo axis in contact with callus initiation medium N6 (**Chu et al., 1975**). N6 medium supplemented with N6 salts, 2 % sucrose, 25 mmol.dm^{-3}

proline, 1 mg.dm^{-3} 2,4-D, 100 mg.dm^{-3} casein hydrolysate, N6 vitamins, 10 mg.dm^{-3} silver nitrate, 3g gelrite. The media was adjusted to pH 5,8 and autoclaved. Twenty embryos were placed in each Petri dish. Cultures were incubated in the dark at 28 ºC. Percentage of immature embryos forming primary callus was recorded two weeks after culture. The developing callus was sub-cultured after 14 days into the callus maintenance medium as for callus initiation but without silver nitrate.

Type of induced callus (embryogenic, non-embryogenic and organogenic) was determined by examining under the microscope Leica ZOOM 2000 and later on by ability to form appropriate structures.

Embryogenic callus was transferred into embryo maturation medium containing N6 medium supplemented with N6 vitamins, 6% sucrose, 1 mg.dm^{-3} NAA and 3g gelrite for embryo maturation. Cultures were incubated in the dark at temperature of 28 ºC.

Plant regeneration

After two weeks of culture on embryo maturation medium callus with somatic embryos were transferred into regeneration medium containing MS medium (**Murashige and Skoog, 1962**) supplemented with 2% sucrose, 2 g.dm^{-3} myo-inozitol, 3g gelrite for plant regeneration. The cultures were incubated at 28 ºC in the light. Well-rooted plantlets from culture flasks were rinsed with water to remove the media then transferred into pots containing peat moss.

Histological analysis

Embryogenic callus was fixed in Navashins fixative, dehydrated in alcohol alcohol series. After dehydration, the samples were infiltrated with xylene (ethanol : xylene in ratio 3:1, 1:1, 0:1). The samples were filled with paraffin (Paraplast Plus, Sigma) and embedded in paraffin blocks. Cured samples were removed from curing oven, mounted and trimmed for ultramicrotomy CUT 4055 (MicroRec, Walldorf). Semi-thick sections were collected onto glass slides and stained with Safranin – Fast green. Digital images were imprinted with CCD camera SONY DXC- S500 on an Axioplan 2 (Zeiss) microscope.

RESULTS AND DISCUSSION

Indirect somatic embryogenesis

Immature embryos collected from maize ears 16-20 days after pollination were plated on N6 medium. Immature embryos were placed on N6 medium with their embryo axis in contact with medium. The orientation helps to retard the germination of embryos and induce proliferation of scutellar cells (**Green and Phillips, 1975**). Callus initiation from immature embryos was observed after the seventh day of culture on callus initiation medium from the scutellum. This was due to the presence of meristematic cells in the scutellum. **Al-Abed et al. (2006)** reported the presence of the meristematic cells in the scutellum of maize embryos from which callus is induced. The percentage of the primary callus formed to 95% from immature embryos, while embryogenic callus was formed in frequency from 2 to 5%. Embryogenic callus was formed after the first subculture on callus maintenance medium. Somatic embryos were initiated on the surface of the embryogenic callus. Three types of callus were formed: embryogenic, non-embryogenic and organogenic callus. And two types of embryogenic callus were formed, type I and II callus. Type I callus was compact, white in color, while type II was compact and light yellow. The formation of type I and II callus has been reported in maize (**Jiménez and Bangerth, 2001; Tomes and Smith, 1985**). Non-embryogenic callus was also formed. It was soft, watery and yellow in color, which ceased to grow and turned brown in subsequent subculture. The formation of embryogenic and non.embryogenic callus has been reported in other maize genotypes (**Shohael et al., 2003; Jiménez and Bangerth, 2001**) from immature embryos. From organogenic callus mainly roots were formed.

Age and size of the immature embryo was a critical factor in determining the capacity of callus initiation from immature embryo. The percentage of primary and embryogenic callus formed from immature embryos 3-4 mm was lower than that formed from 1-2 mm long immature embryos. This is probably due to reduction in the meristematic activity of the cells with ageing suggesting that the physiological and developmental state of immature embryos is important in determining callus initiation response. According to **Bohorova et al. (1995)**, immature embryos of maize less than 0,5 mm in length did not respond in culture, while **Lu et al. (1983)** reported similar results for embryo less than 1 mm.

Many studies have shown that 2,4-D is an important factor in the initiation and proliferation of primary and embryogenic callus from immature embryos of maize (**Carvalho et al. 1997; Bohorova et al., 1995**). The optimum 2,4-D concentration for the initiation of

embryogenic callus was 1 mg.dm^{-3}. High concentration of 2,4-D applied to induction media reduced the percentage of embryogenic callus formed suggesting that 2,4-D had inhibitory effect at higher concentration.

Somatic embryos were matured when embryogenic callus was transferred to N6 medium with high concentration of sucrose (6%) and 1 mg.dm^{-3} NAA. High concentration of sucrose has been reported to promote the maturation of maize somatic embryos (**Ratif et al. 2006; Ge et al. 2006; El-Itriby et al. 2003; Bronsema et al. 1997**). When embryogenic callus with somatic embryos were transferred into the regeneration medium, they turned green and shoots were observed within two weeks. Some of the somatic embryos did not regenerate into plantlets.

In vitro regenerated plants with well-developed roots were transferred into the pots containing peat moss for hardening.

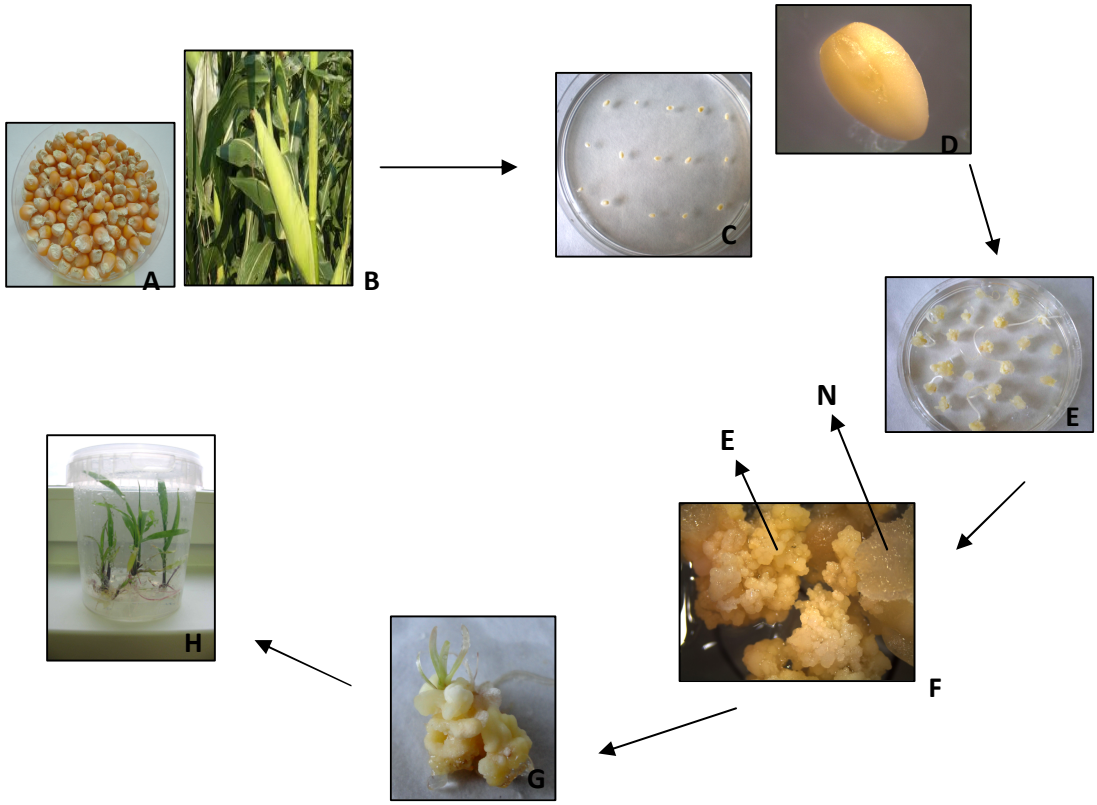

Figure 1 Somatic embryogenesis and plant regeneration from immature embryos of maize. **A:** Seeds of maize **B**: Cob of Zea mays L. 20 days after pollination; **C-D**: Immature embryos on callus initiation medium Nitsch N_6 after first day of culture; **E**: Callus initiation after 14 days culture; **F**: E- Embryogenic callus with somatic embryo at globular stage on the surface and N- non-embryogenic callus; **G**: Embryo maturation in N6 medium supplemented with 6% sucrose and 1 mg.dm^{-3} NAA showing first leaf with coleoptile; **H**: *In vitro* rooting of maize regenerants in $^1/_2$ MS medium supplemented with 0,8 mg.dm^{-3} IBA.

Structural characteristics of cells

Embryogenic cells show characteristics common to meristematic cells – high division rates, a dense cytoplasm with several starch grains, large nuclei and prominent nucleoli, small vacuoles and thick cell walls.

Groups of early pro-embryogenic cells are covered by a network of fibrillar material forming an outer continuous layer. This layer is called the extracelluar matrix surface network (ECMSN) and has been found in many dicotyledonous, monocotyledonous and gymnosperm plant species. An ECMSN was observed preferentially in early embryogenic stages including globular embryos and gradually disappeared when protodermis was formed in torpedo-stage embryos (**Šamaj et al., 2006**).

Histological analysis showed that meristematic cells appeared first on the peripheral sides of the callus, later on formed clusters of meristematic cells. Formation of somatic embryos was accompanied by formation of protodermis, which covered developing structure. Somatic embryos in globular stage were still cowered with protodermis and were located on periphery of the callus.

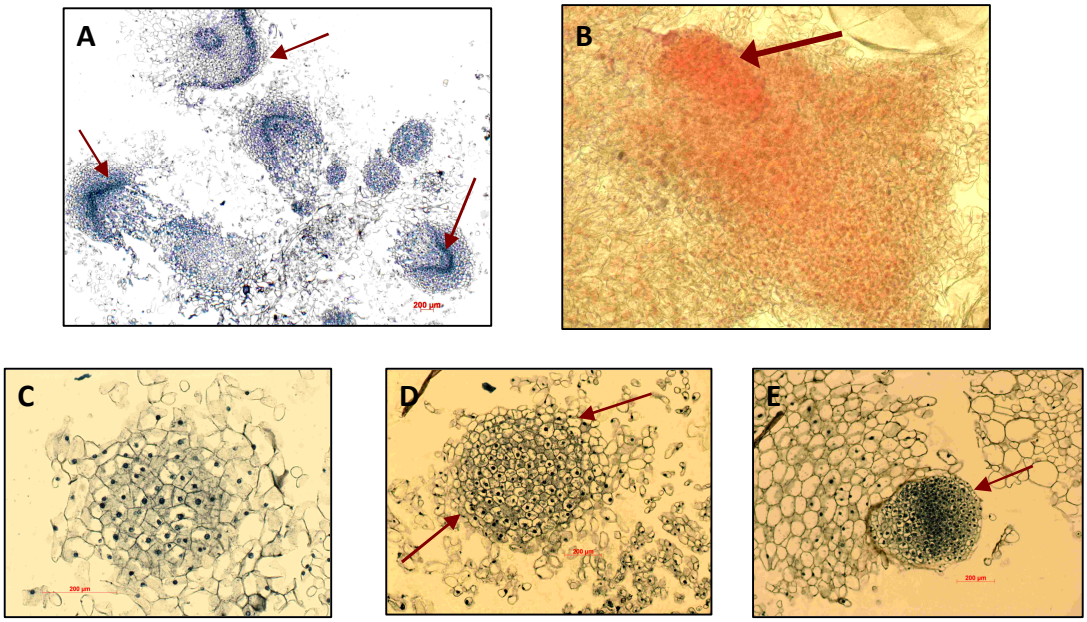

Figure 2 A: Embryogenic callus showing meristematic cells on the peripheral sides; **B:** Cluster of meristematic cells; **C:** Meristematic cells differentiate in callus; **D:** Meristematic cells covered by protodermis; **E:** Early embryogenic stages including globular embryo.

CONCLUSION

In this study we established a reproducible regeneration system of maize genotypes through somatic embryogenesis from immature embryos. Somatic embryogenesis is an important step in any successful plant transformation scheme, because from the callus each transformed cell has the potential to produce a plant. Our results showed, that the genotype, 2,4-D concentration and age of explants had an effect on callus initiation, somatic embryo formation and plant regeneration. Further studies on optimization of plant regeneration from immature embryos of maize are in progress.

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