Maize Journal (April 2021) 10(1): 6-12

RESEARCH REVIEW PAPER

In vitro regeneration in maize (*Zea mays* L.)

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Abstract: Maize is a versatile cereal crop having the highest genetic potential, production, and productivity. In the past few decades, plant tissue culture and transformation approaches have played an important role in maize improvement via introducing beneficial transgene(s) or modulating the expression of the endogenous gene(s), etc. However, the capability of *in vitro* regeneration in maize is highly influenced by genotypes, type of explants, media compositions among others. Some genotypes are more amenable to tissue culture producing embryogenic calli, while others are recalcitrant to tissue culture. Genotypic differences in morphogenesis and organogenesis are generally reported that might be possible due to differences in endogenous hormone levels. The *in vitro* regeneration potential in maize is usually decreased during the channelized path of tissue maturation, therefore embryogenic callus is mostly achieved from immature zygotic embryos. The present article aimed to provide the current state of the art in maize somatic embryogenesis. Further, the article describes the procedure for maize whole plant regeneration from embryogenic callus.

Keywords: Maize · Somatic embryogenesis · Whole plant regeneration

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Received: 21 November 2020/ Accepted: 12 March 2021

Introduction

Conventional plant breeding has contributed exceptionally to crop improvement. Nonetheless, it has a major limitation to introduce desired traits into crop plants through genetic crossing due to requirements of species compatibility and the dearth of germplasm diversity within the species. However, the advent of plant tissue culture and transformation methods have made it possible to insert beneficial foreign gene(s) into crop plants i.e. into sexual incompatibility species. Plant tissue culture techniques are useful to multiply and propagate plants under *in vitro* conditions on a rapid and large scale, irrespective of season, with less space requirement and in a shorter time frame. These techniques have been utilized for micro-propagation, genetic transformation, double haploid line production, storage of plant cells and organs, biosynthesis of secondary metabolites, etc. Thus, plant tissue culture and transformation methods have played a pivotal role in gene-function studies and crop improvement (Agarwal *et al*., 2018; Kumar *et al*., 2018).

Maize (*Zea mays* L.) is the most widely grown cereal crop in the world with wide adaptability under diverse ecologies. It is consumed as feed and food, and also has myriads industrial applications, including the production of bioethanol and starch. It is one of the most extensively studied crops. The successful development of a tissue culture and transformation system for maize helped to develop genetically modified (GM) or transgenic maize having one or more novel traits that may not occur naturally in the species (Kumar *et al*., 2020). To date, nearly 250 transgenic events in maize have been commercialized (ISAAA database 2019; https://www.isaaa.org/), hence tissue culture and transformation played a major role in the genetic improvement of maize in the past two decades. The *in vitro* regeneration in maize is highly

dependent on genotypes and influenced by many factors like media, hormone composition, temperature, and light influx.

In vitro regeneration

The regeneration ability in maize is highly dependent on the genotype as only a few genotypes are amenable for tissue culture and transformation. *In vitro* regeneration in maize was first reported by Green and Phillips (1975) using immature embryos as explants. Generally, regeneration can be achieved directly (without callus formation) or indirectly (with callus formation followed by somatic embryogenesis) (Figure 1). It has been shown that callus induction and *in vitro* regeneration is highly challenging in maize as compared to other cereal crops (Jia *et al*., 2008; Zhao *et al*., 2008; Martinez and Wang, 2009; Anami *et al*., 2010; Rakshit *et al*., 2010; Chu *et al*., 2011; Yadava *et al*., 2017). Many factors influence callus production in maize such as genotype, source and stage of explant, and growth medium components. Several reports are available in temperate, subtropical, and tropical maize germplasm for embryogenesis and indirect organogenesis (Armstrong, 1999; Ahmadabadi *et al*., 2007; Aguado-Santacruz *et al*., 2007; Rakshit *et al*., 2010; Tiwari *et al*., 2015). In comparison to indirect regeneration, relatively lesser research has been carried out on *in vitro* regeneration via the direct organogenesis pathway. Factors affecting whole plant regeneration in maize are described below:

Genotype and explant

Different tissues are used as a source of explants for maize regeneration by different groups globally. The regeneration in temperate, subtropical, and tropical maize germplasm has been achieved using different explants such as immature embryo, nodal culture, leaf, and mature embryo, etc. (Armstrong, 1999; Bohorova *et al*., 1999; Ahmadabadi *et al*., 2007; Aguado-Santacruz *et al*., 2007; Rakshit *et al*., 2010; Malini *et al*., 2015; Tiwari *et al*., 2015). However, immature embryos are the most preferred explants for producing embryogenic callus and hence indirect regeneration in maize (Armstrong, 1999; Aguado-Santacruz *et al*., 2007; Yadava *et al*., 2017; Agarwal *et al*., 2018). This is due to the higher callusing and regeneration efficiency of immature embryos as the regeneration capacity is reduced as tissue mature. However, immature embryos are usually not available throughout the year, and culturing them is also laborious. The age of explants like immature embryos also influences the efficiency of callus induction and hence regeneration capacity (Abhishek *et al*., 2014).

Apart from explants type, the genotype of the explants is also shown to affect the regeneration potentiality in maize. The nuclear genes have been implicated in controlling regeneration capability (Tomes and Smith, 1985; Hodges *et al*., 1986). Further, it has been proposed that at least one gene or a block of genes control the somatic embryogenesis of maize in tissue culture (Willman *et al.*, 1989). Bohorova *et al.* (1995) showed the effect of various maize genotypes on somatic embryogenesis. Abhishek *et al*. (2014) reported the effect of different genotypes on embryogenic type II callus production and whole plant regeneration in tropical maize. Thus, significant differences have been reported in callus induction, embryogenic type II calli production, and regeneration potential between various explants and genotypes in maize.

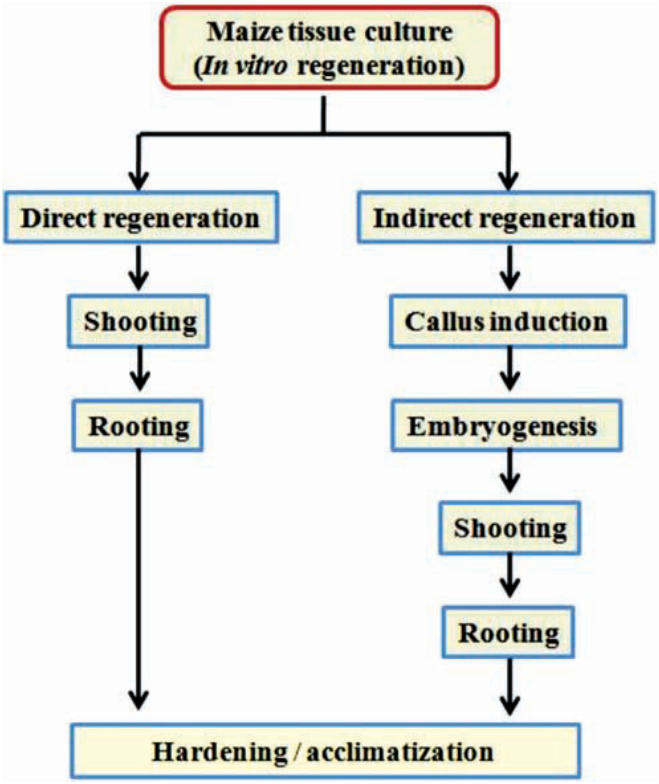

Figure 1. Schematic representation of various routes of maize tissue culture procedure

Media composition and hormone concentration

Optimization of tissue culture medium components is of utmost importance for the establishment of a reproducible *in vitro* regeneration system. To date, a range of basal nutrient media has been utilized for tissue culture in various cereal crops and most of the cases. Murashige and Skoog (MS)-based medium (Murashige and Skoog, 1962) is found superior, as it promotes rapid callus growth, embryogenic callus development, and whole plant regeneration (Hanzel *et al*., 1985; Luhrs and Lorz, 1987; Bregitzer 1992; Rakshit *et al*., 2010). The first somatic embryos in maize were produced in the year 1975 by Green and Phillips from embryo scutellar tissues. In maize mostly, N6 (Chu), MS or Linsmaier and Skoog (LS)-based culture media have been utilized for *in vitro* regeneration and transformation at different stages. Optimization of various media components, *viz*., carbon source, vitamins, amino acids and concentration of plant hormones (growth regulators) in the tissue culture medium is imperative for using these media. Depending upon maize genotypes and explants stage, various reducing and non-reducing carbon sources such as glucose, fructose, galactose, and sucrose have been tested in the tissue culture media. However, sucrose is the most widely used carbon source (Yadava *et al*., 2017). Amino acids such as *L*-asparagine, *L*-glutamine, *L*-arginine, *L*-proline, and *L*-cysteine, etc. being a source of organic nitrogen have been used in tissue cultures to augment somatic embryogenesis and regeneration (Armstrong and Green, 1985; Claparols *et al*., 1993; Kim and Moon, 2007). Various vitamins such as thiamine, ascorbic acid, pyridoxine, riboflavin, myo-inositol, folic acid, niacin, pantothenic acid, biotin, etc. have also been tested in tissue culture. Vitamins, in combination with other media components, have been shown to improve callus growth, somatic growth, embryonic development, and rooting via affecting secondary metabolite production and cell signaling pathway (Abrahamian and Kantharajah, 2011). Plant growth regulators, mainly cytokinins [6-benzyloaminopurine (BAP), kinetin and Zeatinetc. and auxins like indole-3- acetic acid (IAA), indole-3-butricacide (IBA), 2,4-dichlorophenoxy-acetic acid (2,4-D), *Dicamba* (3,6-dichloro-2-methoxybenzoic acid) and naphthalene- acetic acid (NAA)] are used in different combinations and concentrations during maize tissue culture by researchers as they played a very critical role in callus induction, multiplication, shoot development and root development (Bohorova *et al*., 1999; Frame *et al*., 2000; Rakshit *et al*., 2010; Abhishek *et al*., 2014; Tiwari *et al*., 2015; Muppala *et al*., 2020).

Callus induction and embryogenic callus production

In tropical maize factors like genotypes, media, source of auxin and their concentrations significantly affect callus induction frequency (Rakshit *et al*., 2010). Proline (a source of nitrogen supply and osmoprotectant) and casein hydrolysate are considered very essential amino acids for callus formation but their high concentration may adversely affect callus proliferation (Zhao *et al*., 2008; Joshi *et al*., 2010; Wang *et al*., 2012). The callus obtained from immature and mature embryos were supplemented generally with 2,4-D or Dicamba in MS or N6 basal media for the production of regenerable callus (Furini and Jewell, 1994; Rooz 2002; Huang and Wei, 2004; Rakshit *et al*., 2010) (Figure 2). In maize, two types of embryogenic callus i.e. Type I and type II are obtained by *in vitro* culture of different explants. Former is slow-growing, compact/non-friable, harder with characteristic yellow color while later is fast-growing, friable, soft with characteristic white color (Carvalho *et al*., 1997). Type II callus is more regenerable and best for obtaining a large number of calli via sub-culturing due to its friable nature (Omer *et al*., 2008; Manivannan *et al*., 2010).

MS salts have a higher concentration of inorganic nitrogen, but a lower ratio of nitrate to ammonium (NH_4^+) compared to N6 salts (Armstrong *et al*., 1991; Elkomin and Pakhhomova, 2000). It has been shown that the use of MS salts as callus induction media leads to compact Type I callus due to lower nitrate and high NH_4^+ levels, whereas, use of N6 salts lead to friable Type II callus due to high nitrate level and low NH_4^+ level (Elkonin and Pakhomova, 2000; Yadava *et al*., 2017). The callus induction media having 2,4-D, proline, and casein hydrolysate when supplemented with $AgNO_3$ have shown to induction of type II callus from immature embryos (Armstrong *et al*., 1991; Songstad *et al*., 1991 and El-itriby *et al*., 2003). The regeneration ability of the callus is highly genotype-specific and also requires specialized skills to visually select the regenerable portion of the callus. A generalized flow diagram of the indirect embryogenesis pathway of maize tissue culture is given in Figure 2.

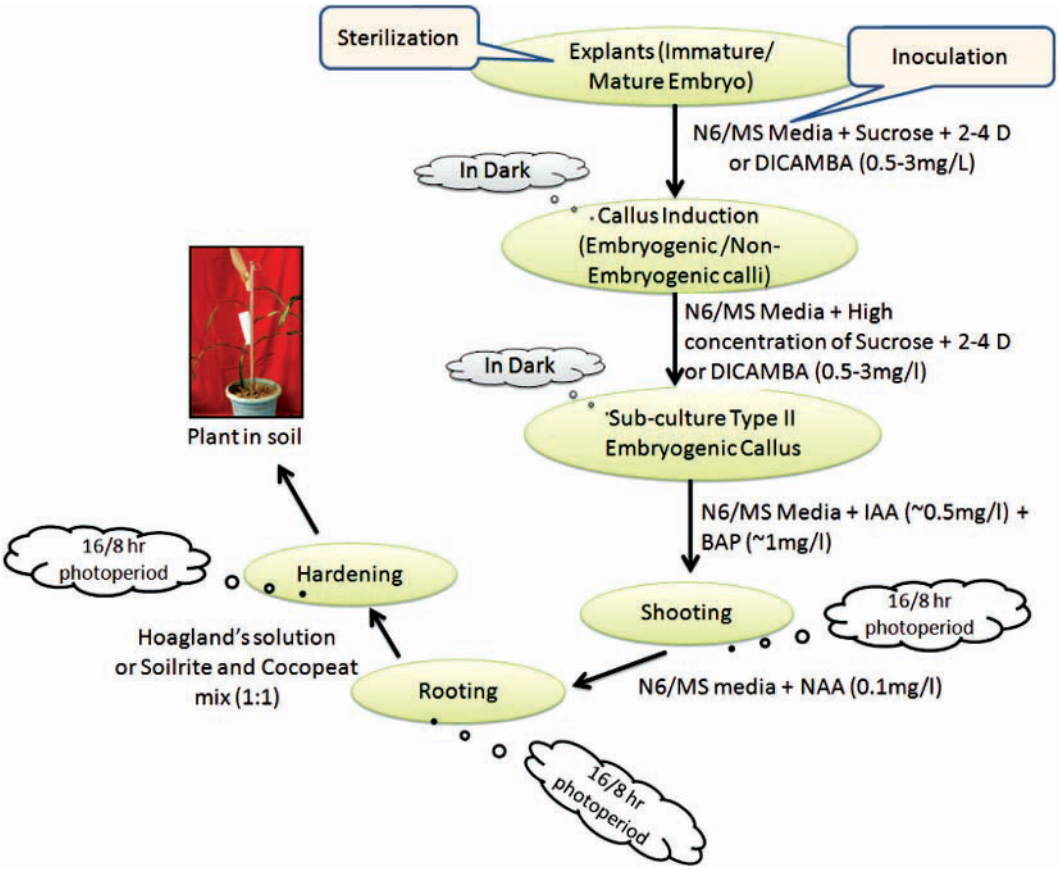

Figure 2. Flow diagram depicting the indirect embryogenesis pathway of maize tissue culture

Organogenesis and regeneration

Phytohormones play a crucial role *in vitro* organogenesis during tissue culture. Organogenesis involves three distinct phases – in the first phase, plant cells are de-differentiated to attain organogenic competence; in the second phase, de-differentiated cells are determined for specific organ formation, and in the third phase organ morphogenesis continue. In cereals, the initiation of somatic embryogenesis has been shown to depend on cytokinins. The development of a white, nodulated embryogenic callus in somatic embryogenesis and the formation of green buds during organogenesis suggests divergent modes of differentiation during morphogenesis.

In maize, usually, the application of auxins and cytokinins are essential for successful shoot regeneration and hence for the successful development of plantlets. It has been shown that the phytohormones such as kinetin and BAP are important along with 2,4-D during *in vitro* regeneration for an efficient shoot regeneration/shooting from immature and mature embryos derived calli (Ozcan, 2002; Wang *et al*., 2006; Jia *et al*., 2008; Zhao *et al*., 2008; Rakshit *et al*., 2010; Wang *et al*., 2012; Tiwari *et al*., 2015; Muppala *et al*., 2020). Depending upon genotype and explants, root regeneration can be done either in a hormone-free medium or by applying auxins (mostly NAA) (Figure 2). Diagrammatic representation of various stages during embryogenic calli formation and *in vitro* regeneration in maize by utilizing immature embryos as explant source is given in Figure 3.

Conclusion

In vitro callusing and regeneration is highly challenging in maize compared to other crops like rice. Among the various explants, the immature embryo is preferred for regeneration in maize due to its better regeneration potential and reproducibility than other explants. However, the availability of immature embryos throughout the year is a major limitation in the rapid development of improved maize cultivars via genetic transformation. Further, *in vitro* regeneration is highly dependent on genotypes and also is influenced by different media components such as phytohormones (mainly auxins and cytokinins), vitamins, amino acids (mainly proline and casein hydrolysate), etc. Despite all these challenges, maize is the prime target crop

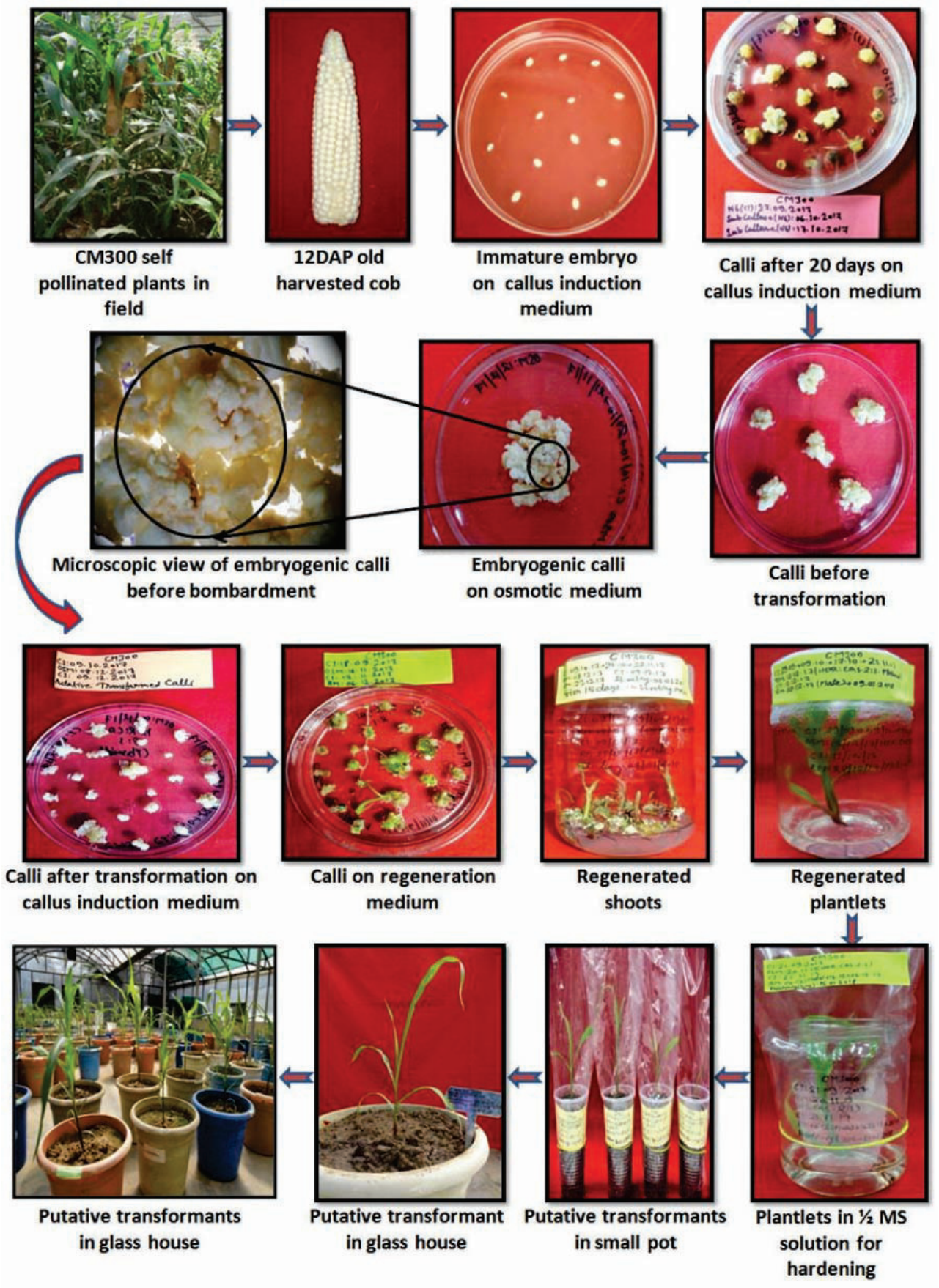

Figure 3. A pictorial representation of embryogenic calli formation and *in vitro* regeneration in CM300 inbred maize line using immature embryos as explant source

for tissue culture and transformation and hence the maximum number of genetically modified (transgenic) events belonging to various traits have been approved and commercialized in maize globally. Therefore, tissue culture to develop transgenic maize having desired useful trait(s) is a forefront technology for maize improvement.

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