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Regeneration and transformation of mature embryos of B73 maize line

BACHELOR THESIS

Author: **David Ušák**

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Prohlašuji, že jsem bakalářskou práci vypracoval samostatně s vyznačením všech použitých pramenů a spoluautorství. Souhlasím se zveřejněním bakalářské práce podle zákona č. 111/1998 Sb., o vysokých školách, ve znění pozdějších předpisů. Byl jsem seznámen s tím, že se na moji práci vztahují práva a povinnosti vyplývající ze zákona č. 121/2000 Sb., autorský zákon, ve znění pozdějších předpisů.

V Olomouci dne ....…

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Abstrakt

Genetická transformace rostlin je klíčová metoda základního i aplikovaného výzkumu rostlin. I když byly v minulosti vyvinuté univerzální, vysoce efektivní transformační systémy pro desítky modelových rostlin, transformace kukuřice jako zástupce jednoděložných rostlin zůstává stále neúčinná. Transformace explantátů odvozených ze zralých semen pomocí *Agrobacterium* již byla popsána dřív, avšak žádný efektivní transformační protokol nebyl dosud publikován.

Cílem této bakalářské práce, bylo ověřit dostupné regenerační systémy pro regeneraci meristematických kultur, odvozených ze zralých embryí zaužívané linie kukuřice seté, B73. Regenerační efektivita po 13 týdnech kultivace *in vitro* a následném přesunu do půdy *ex vitro*, dosáhla 0,9 – 5,9%. Tranzientní a stabilní transformace pomocí *Agrobacterium*, nesoucí vektor pCAMBIA 1303 s genem *GUSA*, byla provedena pro otestování susceptibility odvozených explantátů vůči dostupným transformačním systémům. GUS histochemický test byl vykonán pro zjištění eficience tranzientní a stabilní transformace, která dosáhla 80%, respektive 55,7%. Následné pokusy regenerovat transformované explantáty v dospělé rostliny byly ale neúspěšné.

Klíčová slova	kukuřice, transformace, *in vitro* regenerace, zralá semena, B73, *Agrobacterium tumefaciens*
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Abstract

The genetic transformation of plants is the key method in both basic and applied plant research. Even if there have been universal, high-efficiency transformation systems developed for dozens of model plants, maize, among other monocotyledonous species still remains recalcitrant to most efforts. The use of *Agrobacterium* for the transformation of explants derived from mature seeds, has already been proposed in the past, however, no effective protocol has been established yet.

In this bachelor thesis, the focus was to examine available regeneration systems for the regeneration of mature embryo-derived meristem cultures of a well-established maize line B73. Regeneration rate of 0.9 – 5.9% was reached after up to 13 weeks of *in vitro* cultivation and subsequent soil transfer. Both transient and stable *Agrobacterium*-mediated transformation with pCAMBIA 1303 harboring *GUSA*, was performed to analyze the amenability of the explants to the existing transformation systems. GUS histochemistry assay was used to test both transient and stable transformation rate, which reached 80% and 55.7%, respectively. Subsequent attempts to regenerate mature plants containing the transgene were unsuccessful.

Keywords	maize, transformation, *in vitro* regeneration, mature seeds, B73, *Agrobacterium tumefaciens*
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AIMS OF THE THESIS

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PRACTICAL PART

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2. Verification of the enzymatic pretreatment effect on transformation.
3. Comparison of the transformation efficiency utilizing various *Agrobacterium tumefaciens* strains.

1 INTRODUCTION

With the progress of the global climate change, the fluctuations in plant growth conditions all around the world are to be expected. In the modern biotechnology research, there is a focus on the improvement of agriculturally important crops like maize, to make them able to adapt to such environmental changes. Maize is a cereal crop grown worldwide for the production of food, livestock feed and also biomass for the industrial use. As such, the failure of the attempts to adapt maize to a gradually changing environment could threaten worldwide food security.

Genetic engineering of plants proved itself useful in generating new cultivars with beneficial traits quicker than traditional plant breeding, with a more specific way of genome alterations. Genetic transformation enables researchers and plant breeders to introduce foreign genes into an organism, that give it an advantage over wild-type lines and even elite hybrid lines used nowadays. However, there are still barriers to be overcome in the development of transgenic maize: most notably genotype-dependency of the transformation outcome, regeneration of stable transformants using plant tissue culture and the labor-intensity of protocols used, which prevent the universal adaptation of transgenic maize techniques.

The focus of this thesis is to provide reviewed summary of methods available nowadays for maize *in vitro* regeneration and transformation, along with their application in regeneration and transformation of maize cultivar B73, utilizing mature seeds as the starting material. Additionally, a novel approach in improving regeneration rate, the *BBM/WUS* system, is analyzed.

2 CURRENT STATUS OF THE MAIZE RESEARCH

2.1 Importance of Maize as a culture crop

Maize (*Zea mays*, family *Poaceae*), is one of the most essential plants in the world. It is the second most produced crop (after sugar cane) and the most produced cereal in the world, with over 1 billion metric tonnes (MT) produced in 2016 (FAO, 2018). Geographically, most of the produce origins from Americas and South East Asia, with USA, China and Brazil accountable for over 65% of corn produced worldwide in 2018, and with USA being the main producer and consumer of maize in the world (USDA, 2020). It has remained one of the most important crops for direct human consumption, even more so in African and Central American countries Lesotho, Malawi, Zambia, Zimbabwe, South Africa and Mexico, where daily per capita consumption of maize exceeds 200 g (Ranum *et al.*, 2014). Corn is also used as an essential part of livestock feed as 36% of globally produced corn is utilized as animal feed; in USA the percentage reaches 67 % (Cassidy *et al.*, 2013). Such importance is furthermore supported by corn usage in fuel industry for bioethanol production – 98% of all used plant species share in USA, 70% share in China and Canada (Balat and Balat, 2009) and by cornstarch, corn oil and corn sweetener production – with amount of corn consumption by weight of 6, 1.6 and 21.1 MT respectively – a share of 7.9% of total field corn use in USA (USDA, 2019).

2.2 Maize as a model organism in contemporary research

The origins of maize used as a culturally significant crop date back to roughly 9000 years ago to Central American region, most notably the contemporary Mexico. Even if there are many different theories on the actual genetic predecessor of maize, the most recognized hypothesis postulates that it evolved from teosinte, a collective of wild maize relatives (Mangelsdorf and Reeves, 1938; Wellhausen *et al.*, 1952; Galinat, 1971; Matsuoka *et al.*, 2002; Sánchez González *et al.*, 2018). The breeding effort undergone by locals to domesticate corn resulted in a phenotype modification from a vigorous, tall, multiple-stalk grass into a robust, single-stalk plant with naked grains on a girthy ear aligned in several rows (Wellhausen *et al.*, 1952; Doebley *et al.*, 2006; Hake and Ross-Ibarra, 2015).

The basis of maize research stems from the works of late-19th century researchers de Vries and Correns, who utilized its large size and distinctive phenotype for the study of xenia; a natural imprinting phenomenon occurring in plants, where the filial generation of fruit or seeds (in this case corn kernel) is affected by the pollen of the parental plant (Bulant and Gallais, 1998). Their successors' research – the studies of E.M. East and R.A. Emerson were similarly focused on Mendelian genetics, heritability and plant breeding. The main focus of maize research throughout 20^{th} century was the development of lines with high adaptability to different environments and also large amount of grains which can be further processed without any decrease in the final kernel quality. High adaptability traits are crucial for developing beneficial corn lines. Cultivars that possess those traits can be grown in conditions ranging from dry to humid, from freezing to scorching. Such traits encompass resistance to abiotic stresses like drought, flooding, soil salinity or biotic stresses of fungal, bacterial or even animal parasites.

In plant research nowadays, maize is an important model organism, demonstrated by its various advantageous attributes. Firstly, its inherent genetic diversity and cosmopolitan nature of growing enables the plant to thrive in conditions present at the geographical latitude of 40°S to 58°N, therefore it can be field-grown by researchers all around the world (Troyer, 1996). Its inherent physiological characteristic of being monoecious plant possessing unisexual flowers distincts maize from other cereals with bisexual flowers, which have to undergo tedious emasculation to prevent self-pollination in laboratory conditions. Moreover, maize produces hundreds of seeds upon a single crossing, providing plant breeders with virtually unlimited amount of seeds for further labor (Strable and Scanlon, 2009; Wallace *et al.*, 2014).

The genome of maize contains approximately 32 000 genes dispersed among 2.5 billion base pairs (bp) – a size comparable to human genome with 25 000 genes and 2.9 billion bp. However, in maize there is an incredibly high portion of non-coding sequences (introns), which allow increased level of variation between lines, varying from single nucleotide polymorphisms (SNP) to larger-sized sequences – an advantage for breeding new exploitable lines. The most studied lines of maize are inbreds (lines created by crossing genetically similar organisms) – cultivars Mo17, W22 or B73, which has also been in 2009 the first fully-sequenced maize genotype (Schnable *et al.*, 2009).

2.3 Maize tissue culture

Due to the immense strain on breeding new crop cultivars without using time-consuming traditional methods, there has been a focus on establishing techniques that utilize plant at a cellular or tissue level rather than the plant as a whole. Plant tissue culture, which can be described as *in vitro* culturing of plant cells under strictly-set, reproducible and sterile conditions, has proven imperative in establishing protocols for quick cultivation of mature plants from singular tissues, their multiplication by clone production, flanking the seed-stage of a plant or for the propagation of plants from naturally seldom-germinating seeds (Hussain *et al.*, 2012). Indeed the ability to regenerate various explants like immature embryos (IEs) (embryos obtained from undeveloped corn ears), mature embryos (MEs), leaves, meristematic tissues, anthers or protoplasts remains crucial in providing large amounts of plants to be utilized by numerous biotechnological techniques (Pathi *et al.*, 2013).

The general process of *in vitro* tissue ontogenesis incorporates these steps: I. Isolation of explant tissue from the plant of origin; II. Adaptation of cells to growth medium and propagation into the callus cells (masses of dedifferentiated, totipotent cells whose main feature is that they can be regenerated into a whole plant); III. Differentiation of callus culture into organ primordia; IV. Regeneration of a fertile mature plant. If the cell cultures are subucultured on a regular basis, they may be preserved *in vitro* indefinitely (Efferth, 2019).

2.3.1 Media composition in maize tissue culture

In order to secure vigorous cell division and tissue propagation of explants, one must consider the optimal *in vitro* culturing conditions. Moreover, to reduce the risk of contamination, the plant is grown in a contained environment. Therefore, the exchange of gases in the cultivation vessel is limited, which requires the incorporation of all necessary nutrients directly in the growth media. Most of the growth media used nowadays are based on traditional media developed in the mid-20th century, derived from media used for whole-plant cultivation. Basing their observations on reviewing known growth media used at the time, mostly for the growth of higher plants like Knop‘s solution, White’s medium or Uspenski and Uspenskaia solution, Murashige and Skoog (MS) formulated new defined medium (Murashige and Skoog, 1962), which contained as much as 25-fold increase in some nutrients concentration, especially NH_4NO_3. This helped to steer the focus from prevalently used complex media containing protein hydrolysates and extracts, whose batch-to-batch composition varied. Thus, MS medium allowed new plant tissues to be cultured and remains the most used plant growth medium to this very day (Thorpe, 2007). An alternative to MS medium, N6 salts medium, was formulated by Chu et al., which contains a different NH_4^+ ion source – $(NH_4)_2SO_4$ (Chu *et al.*, 1974). Nevertheless, in a study conducted to compare their effect on embryogenic callus induction frequency and transformation frequency, it was demonstrated that using MS medium achieved superior results (Frame *et al.*, 2006).

The composition of medium used in any protocol is strict and requires the containment of macronutrients, micronutrients, amino acids, carbon sources, vitamins and growth factors altogether. The representative compounds and their respective concentration may differ, however the exclusion of any of the major components usually results in poor growth performance or even plant death. Saad depicts a comprehensive list of the most commonly used medium components in contemporary research, providing guidance on optimal concentration of each compound (Saad and Elshahed, 2012).

2.3.2 Plant growth regulators in maize tissue culture

Because maize micropropagation protocols encompass several steps necessary for the proper development of a mature plant, different growth media, varying in the nutrient composition, and even more so in the additional factors, such as plant hormones, antibiotics or transformation promoters, are used in each step. Pioneer studies by Skoog and Miller (Skoog and Tsui, 1948; Miller *et al.*, 1955) led to the discovery of auxins and cytokinins, respectively, allowing even non-meristematic tissues to be cultured. The addition of these phytochemicals to a growth medium promotes cell expansion (auxins), cell division (cytokinins), cell elongation (gibberelins) or cell growth inhibition (abscisic acid) (Jones *et al.*, 1988). However, it was manifested that the hormones‘ ratio rather than a total concentration affects the micropropagation of the cultured tissues

(Allan, 1981). In maize regeneration studies nowadays, the most used plant regulators are auxins 2,4-Dichlorophenoxyacetic acid (2,4-D), 1-Naphtaleneacetic acid (NAA) and cytokinins 6-Benzylaminopurine (BAP) or kinetin (KIN) (Gould *et al.*, 1991; Ahmadabadi *et al.*, 2007; Abebe *et al.*, 2008; Kotchoni *et al.*, 2012; Pathi *et al.*, 2013).

Since each phytohormone affects different cell processes, their addition to the media is determined by the media purpose. The ratio of auxin concentration to cytokinin concentration is the main determinant of the tissue fate of the cultivated explant. By cultivating the explant on media with high auxin-to-cytokinin ratio, the induction of root growth is promoted. On the other hand, the cultivation on media with high cytokinin-to-auxin ratio results in the shoot formation from the cultivated tissue (Motte *et al.*, 2014). Maintaining a neutral ratio is crucial in callus development *in vitro* (Muoma *et al.*, 2008; Ikeuchi *et al.*, 2013). However, the hormone concentration ratio in media varies by each regeneration protocol, and further composition adjustments might be required when employing different maize cultivars, explants or dissimilar hormones (even though they would belong to the same group of auxins, cytokinins, etc.).

2.3.3 Indirect maize regeneration

Although most of the explants used in maize tissue culture experiments are of pluripotent origin, to generate a mature plant while preventing necrosis, one should preserve the proliferative nature of explant cells during its adaptation to growth on a medium. This is usually done by inducing calluses (unorganized masses of plant cells), that are grown on solidified growth media amended with plant hormones (Green and Phillips, 1975). Maize callus initiation from any explant is based on early protocols utilizing IEs, and the basic course of action follows the induction of primary callus from scutellar region by culturing on growth medium with increased auxin concentrations and subsequent embryogenic callus induction by addition of cytokinins like BAP to subculture medium.

In order to succeed in regenerating a mature plant, the calli must undergo further morphogenetic changes. These can be of two types: organogenesis and embryogenesis (Chieng *et al.*, 2014). Organogenesis encompasses the formation of unipolar organs, such as shoots, roots or leaves, from the callus tissue, while embryogenesis follows the induction of bipolar somatic embryos (Bhatia and Bera, 2015). Based on this, there are two described types of maize calli (Figure 1), classified according to their morphological and physiological differences – type I is an organogenic callus comprised of compact, more differentiated greenish cells, while type II remains embryogenic, with less associated friable cells, its color ranging from white to yellow (Sidorov *et al.*, 2006; Wang *et al.*, 2009).

After obtaining a viable population of calli, the organogenetic pathway seeks to regenerate a mature plant in two separate (and chronological) stages: shoot formation (caulogenesis) and root formation (rhizogenesis). In maize regeneration protocols, shoot formation is the predominant starting phase, developing shoot system that enables the future plantlet to produce its own energy by photosynthesis. For shoot formation, media differing in auxin and cytokinin concentrations are used. Subsequently, root formation by subculturing on rooting media is promoted. To provide enough space for vertical plantlet growth, the rooting media cultivation is usually carried out in Erlenmeyer flasks as opposed to Petri dishes. Plant growth regulators-free medium is then used to develop plantlets that can be regenerated into fully grown plants (Green and Phillips, 1975; Wang, 1987; Zhong *et al.*, 1992; Wei and Huang, 2004; Guruprasad *et al.*, 2016).

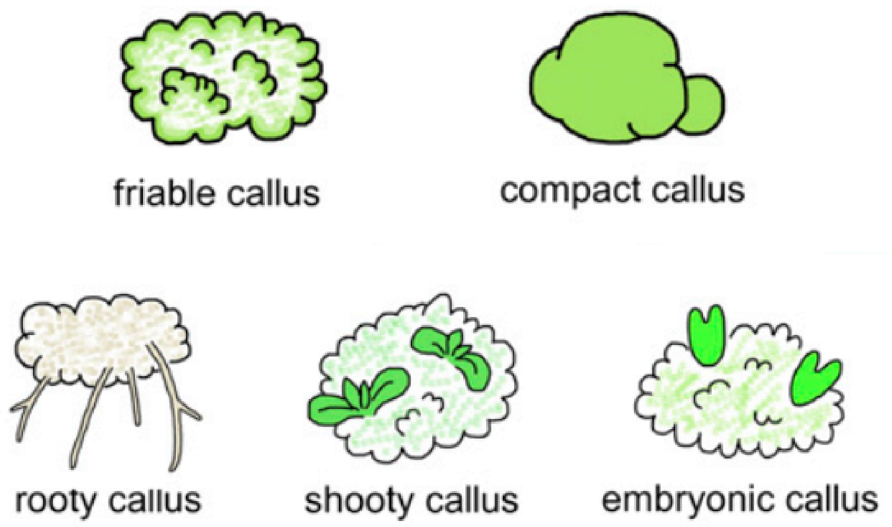

Figure 1: Main types of plant calli, as described by (Ikeuchi *et al.*, 2013). Friable type II calli often possess increased regeneration capability, compared to compact type I calli. After receiving proper stimuli (e.g. by the incorporation of phytohormones in growth media), these can be further reformed into shooty, rooty or embryonic calli.

A process of somatic embryogenesis – embryo development from a single non-zygotic cell, which resembles a zygotic embryo both morphologically and by the patterns of gene expression – can be used for the regeneration of both callus types. Nevertheless, because of its longer-lasting regeneration capability, type II callus is employed more routinely. Somatic embryogenesis enables to regenerate plants with both root and shoot system developing at the same time, effectively shortening the protocol length (Derkach *et al.*, 2017). However, to overcome the possible poor performance (and response to the media) of the developing somatic embryos (SEs) of various maize cultivars used in the research, the development of a proficient maize line to use in *in vitro* experiments was needed. This led to the establishment of a purpose-specific Hi-II hybrid line (descended from inbred lines' A188 x B73 cross), whose *in vitro* embryo regeneration rate may be as high as 68% (Abdel-Rahman and Widholm, 2010). Indeed, several papers describe using of Hi-II line in their callus culture protocols (Frame *et al.*, 2000, 2002, 2011; Zhao *et al.*, 2001; Wang and Frame, 2009; Lee and Zhang, 2014). Despite that, Hi-II line is not very convenient for maize breeding and transformation experiments, as its poor agronomic performance prevents extensive application in practice (Garrocho-Villegas *et al.*, 2012; González *et al.*, 2012; Pathi *et al.*, 2013).

2.3.4 Direct maize regeneration

An alternative maize regeneration system, which flanks the callus stage of indirect regeneration, enables an even more significant decrease in the time needed for mature plant establishment. The starting explants are typically zygotic or meristematic in origin, as the totipotent state of the tissue is necessary to control direct morphogenesis (Elhiti and Stasolla, 2011). Even though, the addition of plant hormones in the growth media is mandatory to maintain the cell fate of cultured tissues. After formation of the organ primordia or somatic embryoids, the regeneration pathway is largely similar to indirect regeneration systems (as described before). To this date, direct regeneration of split nodes (Mushke *et al.*, 2016) and shoot tips (Zhong *et al.*, 1992; Sairam *et al.*, 2003) was described.

2.3.5 *Baby boom*/*Wuschel* system

One of the main challenges to overcome in maize tissue culture, is the overtime loss of totipotency of zygotic or meristematic cells in the developing explants. This is predominant in MEs, who have higher plant-hormone requirements for callus or somatic embryogenesis induction (Elhiti and Stasolla, 2011; Delporte *et al.*, 2014). This has been, of course, resolved by the addition of growth regulators like auxins and cytokinins directly to the growth media, however, it still remains not-100%-effective system. A supplementary method, first proposed in 1997, theorized that the introduction of genes responsible for plant hormone synthesis could not only increase *in vitro* regeneration capabilities, but also improve transformation efficiency (TE) of plants (Ebinuma *et al.*, 1997; Sugita *et al.*, 2000). It was also suggested, that the use of such transformation vectors could bypass the need for a selectable marker gene for the identification and selection of transformants. Such marker genes need to be eliminated after the selection step, while their removal adds to the labor intensity of the protocol (Richael *et al.*, 2008).

In maize, such problems could be alleviated by the use of *Baby Boom* (*BBM*)/*Wuschel* (*WUS*) system. *WUS* is a well-known gene, naturally expressed in the organizing center (OC) in the shoot apical meristem (SAM) of all plants, where it directs the fate of future meristematic cells by maintaining basal stem cell population (Laux *et al.*, 1996; Mayer *et al.*, 1998; Somssich *et al.*, 2016). As such, it was hypothesized, that its overexpression in explants *in vitro* could lead to the cell dedifferentiation of the subcultured tissues and subsequent somatic embryo formation. This was later confirmed in studies by (Zuo *et al.*, 2002; Bouchabké-Coussa *et al.*, 2013). Similarly *BBM* encodes transcription factor *Apetala2 Family/Ethylene Responsive Element Binding Factor* (*AP2/ERF*), which affects processes like cell proliferation and differentiation, embryogenesis and apomixis, along with increasing TE in various plants, both monocots and eudicots (Boutilier *et al.*, 2002; Passarinho *et al.*, 2008; Jha and Kumar, 2018), including maize (Salvo *et al.*, 2014).

Based on these findings there was a study published, which describes the overexpression of *Baby Boom* (*BBM*) and *Wuschel2* (*WUS2*) genes, controlled by maize *Ubiquitin* (*pUBI*) and *Agrobacterium nopaline synthase* (*pNOS*) promoters, positively affecting the TE in maize. The starting explants for *Agrobacterium*-mediated transformation were both IEs and MEs, with successful transformation of some recalcitrant inbred lines such as the Pioneer inbred PHH5G. It was also proven that both morphogenes are needed for the somatic embryogenesis induction. Two years later, the same research group published a follow-up study, transforming 7 maize inbred lines, including the recalcitrant B73, using the same set of genes. Reportedly, *BBM* was under the control of *Zea mays* phospholipid transferase protein promoter and *WUS2* driven by maize auxin-inducible promoter *pAXIG1*. The transformation frequency ranged from 9 to 96%, depending on the maize genotype used (Lowe *et al.*, 2016, 2018). Alternatively, an article specializing on using marker-independent transformation of sorghum and B73 inbred maize using *pUBI::BBM* and *pNOS::WUS2* containing Ti-plasmid was released (Mookkan *et al.*, 2017).

The main idea of these experiments is to use transformation (*Agrobacterium*-mediated or biolistic) to stably introduce morphogenes into the plant genome, while being driven by a specific set of promoters. These promoters need to be inducible either by the addition of a supplementary nutrient in the medium (e.g. plant hormones) or different growth stages during plant ontogenesis. Such activation/deactivation of gene expression allows the controlled induction of organogenesis, somatic embryogenesis or callus formation, depending on the experimental design, surpassing the deviations in explant adaptation to growth media due to genotype differences, ontogenetic stages or simple variation between individual plants (Sultana and Gangopadhyay, 2018).

2.4 Genetic transformation of maize

Genetic transformation is a molecular biology method, during which a target cell is altered by introducing a foreign DNA molecule into the cell itself. To successfully produce transformed cells, the process must undergo two crucial events: 1. endogenous introduction of the foreign DNA and 2. incorporation of the molecule into the genome. These events are called transient and stable transformation, respectively (Altpeter *et al.*, 2016). There have been ongoing studies on transformation since 1920s, starting with Griffith's studies of *Streptococcus pneumoniae* natural transformation capabilities (Griffith, 1928). However successful experiments with artificial plant transformation date back only to 1980s, when the construction of first *Agrobacterium*-mediated transgenic plants was reported (Barton *et al.*, 1983; Herrera-Estrella *et al.*, 1983; Hoekema *et al.*, 1983; Fraley *et al.*, 1983). The main difference between traditional breeding methods and transformation, is that transformation enables precise introduction of gene of interest into a plant, which is modified in a more controllable way. Moreover, a genetic transformation protocol may use the introduction of foreign genes (genes from unrelated species) into the organism, therefore it utilizes larger pool of the available genes.

The attempts to transfer foreign genes into maize were performed as early as in 1960s (Coe and Sarkar, 1966), however the first successfully transformed maize experiment was reported in 1986 (Fromm *et al.*, 1986) and no fertile pollen-producing plant had been recovered until 1990 (Gordon-Kamm *et al.*, 1990). The first transgenic maize plant, launched commercially in 1996 in USA, was Bt Corn – corn cultivar resistant to European Corn Borer parasites, and by 2015, there were 143 approved transgenic maize events, with approximately 30% of the area of all biotechnologically-enhanced crops utilized for genetically-modified maize cultivation (James, 2015; Yadava *et al.*, 2017).

2.4.1 Transformation methods

Because of the striking differences between all the model organism plants, there have been developed several ways of introducing the foreign DNA molecule into the organism. Even though particle bombardment and *Agrobacterium*-mediated transformation protocols have been available since 1970s, there is not a universal, all-encompassing technique available for every plant system yet. Nowadays, there are three main methods of genetic transformation of maize – electroporation, particle bombardment and *A. tumefaciens*-mediated transformation (Bennetzen and Hake, 2009; Rivera et al., 2012; Figure 2).

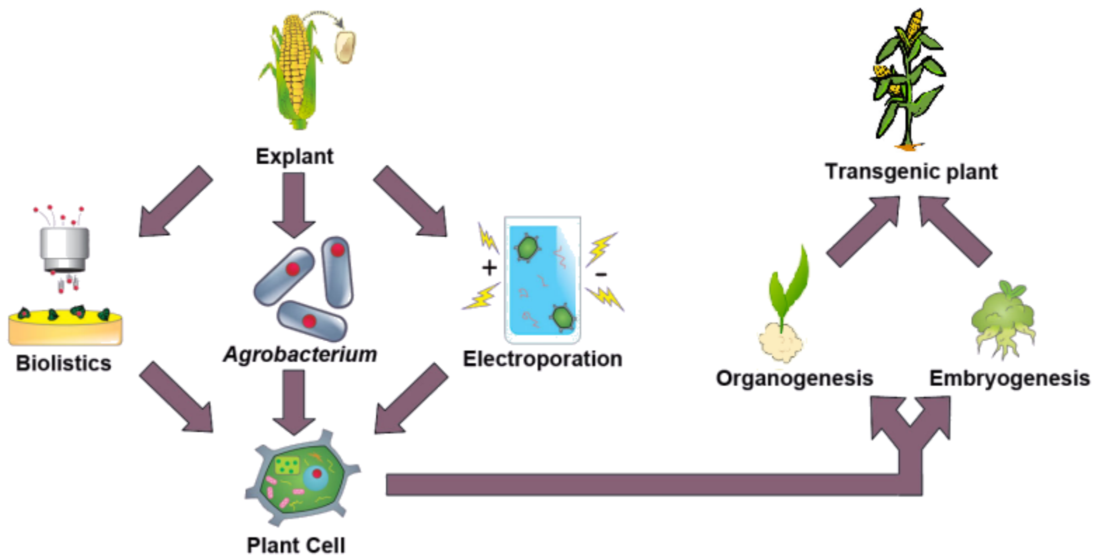

Figure 2: Simplified diagram of basic maize transformation methods employed nowadays. Note that prior to transformation, some protocols require *in vitro* cultivation of explants to obtain plant material amenable for transformation. Adapted from (Castellano-Hernández *et al.*, 2011).

2.4.1.1 Electroporation of protoplasts

The basis of electroporation method lies in the application of the electrical current to a suspension culture of maize protoplasts – cells that lack cell wall – to physically damage the cytoplasmic membrane, which becomes permeable for DNA molecules available in the solution. It belongs among the earliest methods used to transfer foreign genes of interest into maize cells, with Fromm creating the first transformed maize culture (Fromm *et al.*, 1986) and Rhodes regenerating the first maize transgenic plant (Rhodes *et al.*, 1988). The preparation of maize protoplasts is typically done by isolation from a callus culture and subsequent cell wall degradation using cellulase and pectinase enzyme cocktail (Imbrie-Milligan and Hodges, 1986; Planckaert and Walbot, 1989); the calli are usually derived from IEs, grown in an explant culture *in vitro* (Efferth, 2019). An alternative way to disrupting cell membrane using electric current, is to use a chemical polyethyleneglycol (PEG). It is an organic substance, non-toxic to plant cells, which acts in a similar manner of disrupting the plasmalemma (Bates, 1992). In practice, Golovkin employed PEG electroporation to introduce foreign vector harboring methatrexate (MTX) resistance into HE/89 maize hybrid line protoplasts, which were then regenerated into fertile plants on N6 salts-based medium and screened for resistance to MTX (Golovkin *et al.*, 1993). Other papers were also concerned with developing transgenic maize cells, introducing chimeric neomycine resistance gene (neomycine phosphotransferase II; E.C 2.7.1.95) (D'Halluin *et al.*, 1992; Sukhapinda *et al.*, 1993), trehalose-6-phosphate synthase gene (E.C 2.4.1.15) from *Arabidopsis thaliana* (Almeida *et al.*, 2007) or phosphinotricine-N-acetyltransferase (bialaphos herbicide resistance; E.C. 2.3.1.183) (Laursen *et al.*, 1994).

Unfortunately, electroporation still remains quite harsh on the used cells and protocols requiring electricity treatment remain labor-intensive, being even more difficult in monocotyledonous plants, whose somatic cells such as mesofyll cells cannot be used to isolate protoplasts. This forces any researcher to resort to working with cumbersome embryogenic cell cultures. Moreover, culturing protoplasts for a long time usually results in the loss of regeneration capacity which projects the method largely ineffective (Wang *et al.*, 2009; Yadava *et al.*, 2017).

2.4.1.2 Biolistic transformation

A somewhat more effective approach to transfer systems is particle bombardment, also known as a biolistic treatment method. The first gene guns were invented roughly 30 years ago (Sanford *et al.*, 1987), and it took only a decade to establish the first protocols of using microprojectiles harboring nucleic acid molecules in artificial endogenous transport of foreign DNA into maize cell cultures (Fromm *et al.*, 1990). A typical gene gun employs inert metal particles like gold (more uniform in shape and size) or tungsten (less expensive and slightly phytotoxic) coated with DNA (Oneto *et al.*, 2011), which are "fired" into the tissue at high velocity using high-pressure helium as the carrier substance. The mechanism of the bombardment is ensured by a plastic macrocarrier carrying the particles, which is driven by the gas to a stopping screen – the inertial force propels the microparticles towards the sample (Bio-Rad, 2019a). Particles then penetrate the tissue without inflicting fatal damage to the cells themselves (Oneto *et al.*, 2011). The undoubting advantage of this method is its universal nature, making it able to transform virtually any cell type or subcellular compartment of nearly any plant species (Vain *et al.*, 1995). The prime explants being targeted in biolistic transformation protocols in maize are IEs, which can be bombarded directly (Gordon-Kamm *et al.*, 1990; Walters *et al.*, 1992; Hill *et al.*, 1995; Frame *et al.*, 2000) or through suspension culture system initiated from the IE (Gordon-Kamm *et al.*, 1990; Fromm *et al.*, 1990).

Even if the majority of biolistic transformation experiments use high-pressure gene guns (HPGG), such as PDS-1000/He originally trademarked by Bio-Rad (Hercules, CA), that facilitate anorganic microparticles of diameter between 0.6-1.6 µm (Bio-Rad, 2019b), there has been an intriguing research into the use of bacterial cells as the vectors for the bombardment protocol. The bacteria adopted were *Escherichia coli* and *Agrobacterium tumefaciens*, which were fired by Helium-based HPGG to transform tobacco (*Nicotiana tabacum*) cells. Reportedly hundreds of transient transformants were produced (Rasmussen *et al.*, 1994).

Despite the efficiency of microparticle bombardment of maize cells being highly genotype-dependent, affected by the developmental state, projectile accessibility or the ability of the transformed cells to regenerate using tissue culture methods, biolistic transformation is often reported as the major transformation method to be used on maize (Wang *et al.*, 2009), with several studies reporting successfully generated transgenic cell lines using this method (Cao *et al.*, 1990; Hill *et al.*, 1995; Jardinaud *et al.*, 1995; Kausch *et al.*, 1995; Zhong *et al.*, 1996; Brettschneider *et al.*, 1997; Bohorova *et al.*, 1999; Wright *et al.*, 2001; El-Itriby *et al.*, 2003; Wang and Frame, 2009; Du *et al.*, 2014).

2.4.1.3 *Agrobacterium*-mediated transformation

The most widely employed biological method, *Agrobacterium*-mediated transformation is another method of gene transfer applicable in plant biology research. *Agrobacterium tumefaciens*, which is a rod-shaped gramnegative soil bacterium, is used as the vector mediator, inserting the foreign DNA molecule inside the cell. First reported in 1907, Smith acknowledged that a new type of plant tumor – a so-called crown gall tumor – is caused by *A. tumefaciens* (back then called *Bacterium tumefaciens*) (Smith, 1907), however the molecular causative agent behind the uncontrolled proliferation of tumor cells was reported many years later in 1970s (Chilton *et al.*, 1977; Márton *et al.*, 1979).

In order to introduce its plasmid, carrying T-DNA, inside the cell, *Agrobacterium* naturally utilizes open wounds to surpass cell walls. After wounding, the damaged root cells of dicotyledonous plants produce and accumulate phenolic secondary metabolites like acetosyringone or hydroxyacetosyringone that protect the site from nematodes, insects, fungi or bacteria (Lattanzio *et al.*, 2006; Bhattacharya *et al.*, 2010). However, these compounds attract nitrogen-fixing symbiotic bacteria like *Agrobacterium* and *Rhizobium* which are allowed to enter the tissue. This happens by inducing pathogenic *VIR* genes located on the tumor-inducing Ti plasmid along with a T-DNA segment to be integrated into plant cell's own genome (Engström *et al.*, 1987; Bourras *et al.*, 2015), containing genes for the biosynthesis of opines like nopaline or octopine – nutrients crucial for the bacterial survival, whose type determines the *Agrobacterium* strain (Gordon and Christie, 2014).

However, since monocotyledonous species do not have the same wounding response as dicotyledonous species (they do not secrete acetosyringone along with other plant phenolics) (Raja *et al.*, 2010), cereals including maize are especially recalcitrant to *Agrobacterium tumefaciens*. It was demonstrated in the past, that the cereals are insusceptible to *Agrobacterium*, not being its natural host organism (Fromm *et al.*, 1986; Ji *et al.*, 2013; Hiei *et al.*, 2014; Singh and Prasad, 2016; Anand and Jones, 2018). Moreover, differences in monocotyledon cell wall composition, mismatched receptor proteins for *VIR* genes, Ti plasmid selectivity, along with the transgene inactivation by methylation, mutually contribute to the inherent resistance to *Agrobacterium* (Sood *et al.*, 2011).

Nevertheless, the discovery of the inter-species DNA transfer provided a promising system in gene transfer, although some modifications were necessary to achieve its full potential. It has been well-known that *Agrobacterium* pathogenic infection kickstarts uncontrollable cell division, driven by the synthesis of auxins like indole-3-acetic acid (IAA) or cytokinins like trans-zeatin. whose biosynthetic pathway is encoded by the Ti plasmid (Regier and Morris, 1982; Liu *et al.*, 1982; Bevan and Chilton, 1982). Therefore, the resulting root tumor growth would limit the method's application quite extensively. As a result, all oncogenes (genes responsible for the tumor formation) had to be eliminated from the plasmid artificially.

Because the incorporation of T-DNA inside the plant genome is uncommon, scientists had to develop a system which would enable researchers an easy identification of transformed plants over the non-transformed. The most convenient way was to insert a marker gene – a gene that helps to determine whether the foreign DNA was introduced – flanked by a plant regulatory sequence, into the T-DNA region along with the gene of interest. Such marker genes may have a biochemical effect, giving the transformed plants an advantage over the non-transformed (antibiotic or herbicide resistance) or spectroscopic, which makes the transformed plants visually distinct to non-transformed (for example the enzyme β-glucuronidase catabolizing the conversion of a colorless substrate into a colored product) (Yuan, 2012).

In addition to unmodified Ti plasmids containing unwanted genes responsible for phytohormone synthesis, they are also very large in size, ranging from 200 to 800 kbp, therefore there is only a small chance of any plasmid containing unique restriction sites necessary for a quick and simple cloning of genes of interest into the molecule. To overcome these problems, a binary vector system was developed. It utilizes a small binary vector containing a transgene along with a plant selectable reporter gene, flanked together by left and right border sections (conserved 25bp direct repeat sequences), a mutual origin of replication (ori site) for both *Escherichia coli* and *Agrobacterium* and a bacterial selection marker. The transgene introduction takes place in *E. coli* after which the cells are selected by growing on a medium containing antibiotic to which transformed colonies contain resistance. A functioning plasmid is subsequently cloned into *Agrobacterium* cells possessing shortened helper vector accommodating *G* genes necessary for the successful introduction of T-DNA from the binary vector into the plant cell (Frandsen, 2011; Yuan, 2012; Lacroix and Citovsky, 2013). Other techniques and vector systems used in *Agrobacterium*-mediated plant transformation are described in (Gelvin, 2003).

The first maize transformation experiments utilizing *Agrobacterium* date back to 1990s (Gould *et al.*, 1991; Shen *et al.*, 1993; Ritchie *et al.*, 1993), over a decade after the first highly-efficient transformation experiments from the start of 1980s (Márton *et al.*, 1979; An, 1985), with the results being somewhat unremarkable. The first research group establishing high TE of 5-30% (Ishida *et al.*, 1996) listed several factors like explant type, bacterial strain, medium composition or bacterial composition as critical to achieve high TE, which proved the need for the further optimization of this method (Wang *et al.*, 2009). To surpass the challenges presented by the resistance of maize to

Agrobacterium, several research groups attempted to establish efficient transformation systems by studying agroinfiltration and microinjection of bacteria into developing shoot meristems (Escudero *et al.*, 1996), effective preparation of suitable explants (Zhong *et al.*, 2018), co-cultivation time effect on the TE (Didoné *et al.*, 2018) or growth medium concentration and composition (Frame *et al.*, 2006; Souza *et al.*, 2017). Routine transformation protocols, most with considerable transformation frequencies, were established (Frame *et al.*, 2002, 2011; Sidorov *et al.*, 2006; Ishida *et al.*, 2007; Ahmadabadi *et al.*, 2007; Lee and Zhang, 2014; Cho *et al.*, 2014; Anand *et al.*, 2018; Du *et al.*, 2019).

2.4.2 Explant source's role in transformation

Since transformation performance of maize depends on *in vitro* tissue culture methods, there should be consideration put in choosing and culturing the right plant tissue while preparing maize transformation protocols. There is a wide range of explants available from a single plant, however the research is focused on explants with a high capacity to proliferate (stem cells) in simulated conditions *ex vivo*. The main need for the use of stem cells is the capability of quick regeneration of transformed, fully-mature plant from tissues that are limited in size (Singh and Prasad, 2016). Totipotency, the ability of a cell or tissue to form any tissue of an organism by specific differentiation and therefore grow a whole organism (Condic, 2014) is a crucial characteristic of explants like IEs, root and shoot apical meristems or nodal regions. Indeed, they remain the most used source in maize transformation experiments (Wang *et al.*, 2009).

2.4.2.1 Immature vs mature embryos as the starting material

The most developed system of maize transformation, utilizing IEs, has been in use for 30 years since the advent of maize transformation experiments. Immature maize embryo's endosperm has been used as an explant in plant tissue culture systems since 1940s (Ahsan *et al.*, 2000; Thorpe, 2007). IEs are undeveloped maize seed parts, which are harvested from undeveloped corn ears by manual excision 12 to 18 days after pollination (DAP) (Schlappi and Hohn, 1992; Garrocho-Villegas *et al.*, 2012). However, the period-sensitive nature of IE maturation does not enable researchers to store the explants in artificial conditions (refrigerated), which forces study groups to exploit valuable greenhouse space and additional skilled staff for year-round corn plant cultivation to ensure stable supply of quality IEs. Nevertheless, IEs have been crucial in maize transformation research and continue to be used to this very day, with reports of successful biolistic (Songstad *et al.*, 1996; El-Itriby *et al.*, 2003; Xu *et al.*, 2010) and *Agrobacterium* transformation systems (Schlappi and Hohn, 1992; Frame *et al.*, 2011; Didoné *et al.*, 2018).

Realizing the disadvantages posed by IEs as the explants of choice for calli cultures, scientists were eager to establish tissue culture systems from alternative organs prone to transformation and regeneration efforts. MEs have several advantages over IEs: after harvest, they can be easily stored for practically indefinite amount of time; there is no need for their laborious extraction; after maturation, all of the embryos are in the same developmental state unlike IEs where the embryos growing in a mid-part of the ear are more-developed than embryos at both ends, therefore their use provides higher yield per single corn knob (Garrocho-Villegas *et al.*, 2012). Currently, there are two ways how MEs can be efficiently subcultured on media: by producing either scutellum-derived embryogenic calli or meristematic multi-shoot cultures (MSCs) from apical protomeristems of germinating seedling (Torney *et al.*, 2007).

2.4.2.2 Transformation of mature maize seeds as starting materials

Despite the numerous aforementioned advantages of MEs for the effortless use as an explant, there has only been a handful of successful experiments utilizing fully-developed corn seed for transformation. The experiments utilize the germination of seed and the subsequent excision of nodal parts that contain apical and adventitious meristems which are subcultured to induce the growth of embryogenic calli. The calli are then transformed by particle bombardment or by co-cultivation with *A. tumefaciens* (Wang *et al.*, 2003; Sidorov *et al.*, 2006). An alternative protocol, that describes *A. tumefaciens* transformation of non-germinated maize seedlings and their subsequent germination in the soil, was also published (Wang *et al.*, 2007).

2.4.2.3 Shoot apical meristem transformation

The direct targeting of apical meristem as the explant-of-choice for transformation, tries to capitalize on the proliferative nature of germ-cells in plants' apices. Located at the far end of plants' stem, the SAM is comprised of small amount of totipotent cells. These stem cells are further organized in three layers: L1, L2 and L3, the last one not being present in cereals (Figure 3). The main function of SAM is to establish new organs – leaves, flowers and floral organs – during the ontogenesis of a plant (Takacs *et al.*, 2012; Murray *et al.*, 2012). Because of SAM's natural plasticity in *in vitro* cultures, SAM can be easily propagated into a callus, with the potential for an indefinite sustainability of its proliferative character.

There are several methods available for the transformation of shoot apical meristem, however only two methods utilize mature seed as the starting materials. First method follows the excision of SAM from the mature zygotic embryo, which is subsequently transformed; while the second requires germination of the seed and micropropagation of meristematic tissues to establish calli cultures – also called as shoot meristem cultures (SMCs) (Sticklen and Oraby, 2005). SMCs have been used in several experiments targeting a wide variety of maize lines. Among the first studies, the biolistic method was tested on 16 genotypes with the relative efficiency of 0.6 to 6.7% (Zhong *et al.*, 1996). Further research proved that SAM is indeed a potential explant for transformation (Zhang *et al.*, 2002; O'Connor-Sánchez *et al.*, 2002; Sairam *et al.*, 2003).

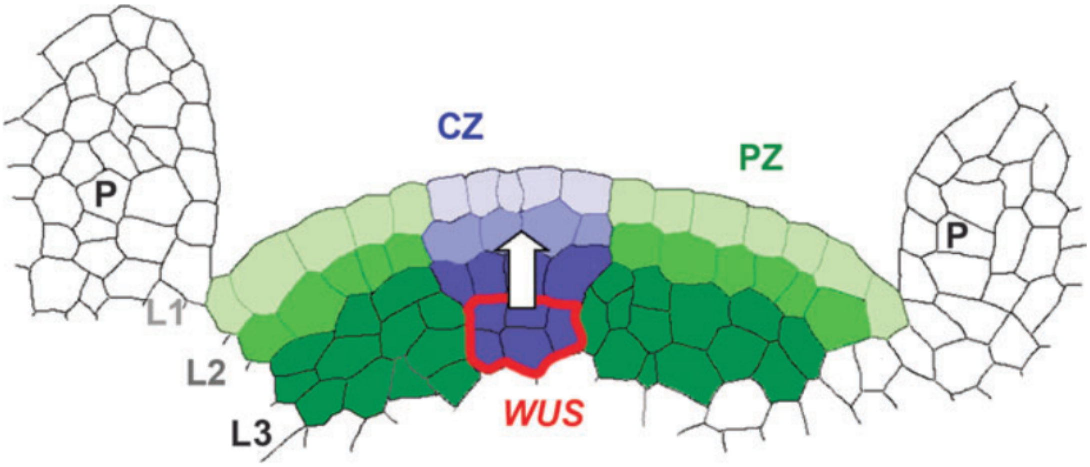

Figure 3: Structure of shoot apical meristem. Cell layers L1, L2 and L3 are indicated by the increase in color saturation. The location of *Wuschel* expression of is marked by a red circle. CZ = central zone; PZ = peripheral zone; P = leaf primordia. Adapted from (Sablowski, 2007).

2.4.3 Transgenic maize applications

The use of genetic engineering methods in maize research undoubtedly provides farmers and breeders with new potential transgenic lines, that usually harbor one or more advantageous genes for the plant. Considering that the ability of a maize cultivar to be transformed is genotype-dependent, and most of the agronomically-interesting lines remain recalcitrant to transformation efforts, the introduction of such advantageous genes into elite germplasm is still carried out by recurrent breeding, with several selection cycles needed to develop a commercially-applicable line.

Since 1996, the release year of the first genetically-modified maize cultivar – the Roundup-Ready corn (Lebrun *et al.*, 1997), which harbors the resistance to glyphosate, there has been an outbreak of newly-developed transgenic maize varieties, and by 2020, 238 maize varieties were released (ISAAA, 2020, as of 22/2/2020). Among the possible advantageous traits commonly inserted into maize and plants in general are herbicide resistance, pathogen resistance to pests like European corn borer (Bt Corn) (Koziel *et al.*, 1993), viral resistance (e.g. to maize dwarf mosaic virus) (Murry *et al.*, 1993) and drought resistance (Wang *et al.*, 2015). Despite the development of genetically modified maize being a time-saving process of procuring advantageous crops, the beneficial genes from wild-growing mutants can also be introduced by traditional breeding methods. Indeed several research groups reported the establishment of maize lines possessing profitable traits, without using genetic engineering methods (Parker *et al.*, 1990; Newhouse *et al.*, 1991; Abel *et al.*, 2001). Some traits like European corn borer resistance, however, are polygenic, which means that to obtain the full level of resistance, the breeder must succeed in transferring all the critical genes during the crossing; the difficulty is increased even more by the fact that naturally-occurring mutations are often recessive by nature, which further prevents a simple selection process. Additionally most of the maize lines growing in nature are of poor agronomic performance and are therefore inadequate for quality germplasm development (Scott and Pollak, 2005).

Nowadays, maize remains the crop with the most approved transgenic events (either singular or two or more events stacked together), the two most used traits introduced into maize being herbicide and insect resistance. It is the second-most planted GM crop (after soybean), growing on 33% of the total GM crops-planted area – 60.6 million hectares accounting for as much as 26% of the total corn-planted area. Out of all cultivated transgenic corn, 12% is herbicide tolerant, 10% insect-resistant and 78% had both traits incorporated (ISAAA, 2016; Pellegrino *et al.*, 2018).

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

Maize immature seeds of elite inbred genotype B73 were used as the main plant material, obtained from Agricultural Research Service of U.S. Department of Agriculture (Accession number PI550473).

3.1.2 Bacterial strains

Agrobacterium tumefaciens strains AGL-1, LBA4404 and EHA 105, kindly provided by Ing. Vojtěch Hudzieczek, Ph.D. from the Institute of Biophysics of the Czech Academy of Sciences, were used for transformation experiments. Each strain contained pCAMBIA 1303 plasmid harboring β-glucuronidase reporter gene (*GUSA*) under the control of *CaMV35S* promoter. Bacterial colonies were stored in glycerol stocks at -80 °C.

3.1.3 Chemicals

- Ampicillin (AMP), Fluka
- Acetosyringone (AS), Sigma-Aldrich
- Beef extract, HiMedia
- 6-Benzylaminopurine (BAP), Sigma-Aldrich
- 5-Bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc), ThermoFisher Scientific
- Casein hydrolysate, Duchefa
- Cellulase, Sigma-Aldrich
- 2,4-Dichlorophenoxyacetic acid (2,4-D), Duchefa
- Disodium phosphate – Na_2HPO_4, Sigma-Aldrich
- Ethanol – CH_3CH_2OH, Lach-ner
- Ethylenediaminetetraacetic acid (EDTA), Sigma-Aldrich
- Gelrite, Duchefa
- Kanamycin (KAN), Fluka
- Kinetin (KIN), Duchefa
- Krystal Sanan bleach, Cormen

- Monosodium phosphate – NaH_2PO_4, Sigma-Aldrich
- 2-(N-morpholino)ethanesulfonic acid (MES), Duchefa
- Murashige and Skoog (MS) basal salts. Duchefa
- 1-Naphthaleneacetic acid (NAA), Sigma-Aldrich
- Pectinase, Sigma-Aldrich
- Peptone, HiMedia
- Phytoagar. Duchefa
- Potassium ferricyanide – $K_3[Fe(CN)_6]$, Sigma-Aldrich
- Potassium ferrocyanide – $K_4[Fe(CN)_6]\cdot 3H_2O$, Sigma-Aldrich
- Rifampicine (RIF), Sigma-Aldrich
- Sodium chloride – NaCl, Cormen
- Spectinomycine (SPE), Sigma-Aldrich
- Sucrose, Lach-ner
- Timentin (TIM), Duchefa
- Triton X-100, Sigma-Aldrich
- Tween-20, Sigma-Aldrich
- Yeast extract, Duchefa

3.1.4 Solutions and culture media

All media dissolved in sterile water.

- CSMD9B:
  MS (4.4 $g.l^{-1}$), sucrose (30 $g.l^{-1}$), casein hydrolysate (500 $mg.l^{-1}$), BAP (2 $mg.l^{-1}$), 2,4-D (0.5 $mg.l^{-1}$) – pH 5.8 (KOH 1 $mol.l^{-1}$), gelrite (2.8 $g.l^{-1}$)
- SMC REG:
  MS (4.4 $g.l^{-1}$), sucrose (30 $g.l^{-1}$) – pH 5.8 (KOH 1 $mol.l^{-1}$), gelrite (2.8 $g.l^{-1}$)
- GERM M:
  MS (4.4 $g.l^{-1}$), sucrose (30 $g.l^{-1}$), BAP (3 $mg.l^{-1}$), 2,4-D (5 $mg.l^{-1}$) – pH 5.8 (KOH 1 $mol.l^{-1}$), gelrite (2.8 $g.l^{-1}$)
- CPM Dark:
  MS (4.4 $g.l^{-1}$), sucrose (30 $g.l^{-1}$), BAP (1 $mg.l^{-1}$), 2,4-D (2 $mg.l^{-1}$) – pH 5.8 (KOH 1 $mol.l^{-1}$), gelrite (2.8 $g.l^{-1}$)

- <u>REGEN M:</u>
  MS (4.4 $g.l^{-1}$), sucrose (30 $g.l^{-1}$), NAA (0.5 $mg.l^{-1}$), BAP (2 $mg.l^{-1}$), KIN (1 $mg.l^{-1}$) – pH 5.8 (KOH 1 $mol.l^{-1}$), gelrite (2.8 $g.l^{-1}$)
- <u>YEB medium:</u>
  Yeast extract (1 $g.l^{-1}$), sucrose (5 $g.l^{-1}$), peptone (5 $g.l^{-1}$), $MgCl_2$ (0.5 $mg.l^{-1}$), beef extract (5 $g.l^{-1}$) – pH 6.8 (KOH 1 $mol.l^{-1}$), bactoagar (15 $g.l^{-1}$) (omit for liquid variant), RIF (0.5 $mg.l^{-1}$), KAN (1 $mg.l^{-1}$)
- <u>Infection medium:</u>
  MS (4.4 $g.l^{-1}$), sucrose (30 $g.l^{-1}$), casein hydrolysate (500 $mg.l^{-1}$), BAP (2 $mg.l^{-1}$), MES (4264 $mg.l^{-1}$), 2,4-D (0.5 $mg.l^{-1}$), AS (100 $\mu mol.l^{-1}$) – pH 5.4 (KOH 1 $mol.l^{-1}$)
- <u>Enzymatic pretreatment solution:</u>
  Pectinase (0.03 $g.ml^{-1}$), cellulase (0.03 $g.ml^{-1}$), dissolve in Infection media
- <u>Co-cultivation medium:</u>
  MS (4.4 $g.l^{-1}$), sucrose (30 $g.l^{-1}$), casein hydrolysate (500 $mg.l^{-1}$), BAP (2 $mg.l^{-1}$), 2,4-D (0.5 $mg.l^{-1}$), L-cysteine (300 $mg.l^{-1}$), AS (100 $\mu mol.l^{-1}$) – pH 5.8 (KOH 1 $mol.l^{-1}$), gelrite (2.8 $g.l^{-1}$)
- <u>Resting medium:</u>
  MS (4.4 $g.l^{-1}$), sucrose (30 $g.l^{-1}$), casein hydrolysate (500 $mg.l^{-1}$), BAP (2 $mg.l^{-1}$), 2,4-D (0.5 $mg.l^{-1}$), AMP (500 $mg.l^{-1}$) – pH 5.8 (KOH 1 $mol.l^{-1}$), gelrite (2.8 $g.l^{-1}$)
- <u>Washing medium:</u>
  MS (4.4 $g.l^{-1}$), sucrose (30 $g.l^{-1}$), casein hydrolysate (500 $mg.l^{-1}$), BAP (2 $mg.l^{-1}$), 2,4-D (0.5 $mg.l^{-1}$), AMP (500 $mg.l^{-1}$), TIM (100 $mg.l^{-1}$) – pH 5.8 (KOH 1 $mol.l^{-1}$)
- <u>GUS staining solution</u>
  Triton X-100 (0.1% v/v), EDTA (10 $mmol.l^{-1}$), potassium ferrocyanide (2 $mmol.l^{-1}$), potassium ferricyanide (2 $mmol.l^{-1}$), Na_2HPO_4 (100 $mmol.l^{-1}$), NaH_2PO_4 (100 $mmol.l^{-1}$), X-Gluc (2 $mmol.l^{-1}$)

3.1.5 Equipment and devices

- air displacement micropipette, Nichipet
- analytical balance, Sartorius spol. s.r.o.

- autoclave, BMT Medical Technology s.r.o.
- benchtop orbital shaker, Thermo Fisher Scientific s.r.o.
- digital camera Nikon D5600, Nikon Corporation
- electronic balance, Cole-Parmer
- laminar flow box, Thermo Fisher Scientific s.r.o.
- microbiological incubator, Laboratorní přístroje Praha
- multifunction centrifuge, Jouan
- pH meter, WTW s.r.o.
- plant LED incubator, Thermo Fisher Scientific s.r.o.
- vacuum controller, KNF Neuberger Inc.

3.2 Methods

All work was carried out under sterile conditions in flow-box, unless stated otherwise.

3.2.1 Seed sterilization

Immature maize seeds were incubated for up to 3 days in sterile water. Water was decanted and the seeds were sterilized in sterile 50 ml centrifuge tube with 70% ethanol for 3 minutes, followed by 50% Krystal Sanan (local brand bleach) for 30 minutes. The bleach was removed and seeds were washed 4 times with sterile water to remove the excess detergent from the tube. Using sterile Petri dish as a cutting board and a scalpel, the seed tip cap was cut off and the embryo was cut out from the seed. Excess endosperm was removed from the embryo. Radicle was cut off and the remaining embryo was put in Petri dish, filled with distilled water to prevent drying out. The dissected embryos were subsequently sterilized in sterile 50 ml centrifuge tube using 15% bleach and washed 4 times by sterilized water. The embryos were placed in the groups of six per one Petri dish to the respective germination medium, with the scutellar side facing upwards, and cultivated at 28 °C, 16 h photoperiod with 140 $\mu mol.m^{-2}.s^{-1}$ light intensity (same conditions were used in all experiments, except where noted).

3.2.2 Martinez protocol

3.2.2.1 Shoot meristem culture preparation

For germination, CSMD9B medium was used. After 2 weeks cultivation, when the seedlings were at least 2-3 cm long, the shoots and scutellum were cut off, with the remaining shoot apical meristem located between the primordial leaf bases being cultivated on fresh CSMD9B medium. The process was repeated twice for 4-5 weeks, until calli of 3 cm diameter were formed. New developing shoots should be observed before continuing further.

3.2.2.2 Shoot meristem culture regeneration

The newly-grown SMCs were transferred to regeneration medium (SMC REG) to induce roots formation. Explants were subcultured every two weeks till a developed root system is visible. Plantlets were then transferred to a pressed, wet soil mixture of 1:1 peat moss and perlite for mature plant regeneration, and covered with plastic foil for 1 week to keep optimal humidity. After a week, to prevent plants drying out, each pot's soil moisture was checked every 1-2 days and watered accordingly. After 3 weeks, developing plants were transferred to larger pots.

3.2.3 Split internode approach

Sterile maize seeds were germinated on GERM M for 2-3 weeks to grow plantlets of significant size. The noticeably yellow bulged internodes were cut out and split in two using scalpel. Afterwards, they were placed on CPM Dark with the cut-side down to help establish better nutrient uptake from the medium, and cultivated at 28 °C in the dark to form an embryogenic callus. After 3 weeks or after the sizable calli were propagated, they were transferred to REGEN M to develop shoot system of 2-3 cm length. Afterwards, the calli were transferred to SMC REG to develop new root. Adaptation of the developing plantlets to soil was carried out as described above.

3.2.4 *Agrobacterium tumefaciens*-mediated transformation

3.2.4.1 Bacteria culture preparation

A. tumefaciens glycerol stock, stored at -80 °C, was removed from the freezer into a rubber test tube container chilled at -20 °C. Using bacterial loop, the slightly thawed cultures were streaked on solid YEB medium and cultivated at 28 °C for up to 24 h, until isolated colonies were visible. The isolated colonies were inoculated using sterile toothpick or cotton bud into liquid YEB medium, submerged under the liquid surface. *Agrobacterium* was grown on liquid medium in a rotary flask shaker at 28 °C, 250 rpm for 24-28 hours, until OD_{600} = 1.2 was reached. The optical density was measured by pipetting 1 ml of bacterial culture into a plastic cuvette in sterile conditions, and using spectrophotometer, the absorbance at 600 nm was measured. Liquid YEB media was used as a blank.

Bacterial cultures were centrifuged at 15 °C, 2268 x*g* for 15 min and resuspended in Infection media, the OD_{600} adjusted to 0.8. Calli, obtained from the plant tissue culture experiments, were cut under sterile conditions to ~3 mm pieces using scalpel, and placed into sterile 50 ml centrifuge tube, which was subsequently filled with 20 ml enzymatic pretreatment solution and incubated for 9 minutes. After the incubation, the digesting solution was removed and explant slices were rinsed thoroughly with Infection media. 20 ml of *Agrobacterium* solution was then added into centrifuge tubes. Alternatively, the enzyme pretreatment step was omitted.

Sliced embryogenic calli incubated with *Agrobacterium* in Infection medium were placed in a desiccator for 30 min in 4900 Pa vacuum to promote the transformation. Afterwards, Infection medium was drawn off and the calli pieces were dried on sterile filter paper to remove excess *Agrobacterium*. Under sterile conditions, pieces were cultivated on Co-cultivation medium for 5 days.

3.2.4.2 GUS histochemistry assay

GUS staining protocol was based on (Pečinka *et al.*, 2009). After 5 days of co-cultivation, the slices were washed using Washing solution and placed in a 5 ml plastic test tube. They were pressed inside using a nylon mesh, to keep them submerged and GUS staining solution was added. In a vacuum of 49 Pa in a desiccator, they were stained for 15 min and then moved to 37 °C incubator for 24 h or overnight, in the dark. GUS staining solution was drained and replaced with 70% ethanol to destain chlorophyll. Ethanol was changed every 2-3 hours. After 24-48 h, the pieces were observed under stereo-microscope and the number of successful transformants was recorded. Transformation frequency was calculated as a number of *GUSA* expressing calli divided by total number of assayed pieces, per each repeat.

3.2.4.3 Transformed callus slices regeneration

Calli slices, cultivated on Co-cultivation medium, were washed with Washing solution and moved to Resting media for one week. Antibiotics were used to inhibit potential development of *Agrobacterium* colonies and prevent bacterial overgrowth. After 7 more days, calli were subcultured on REGEN M (As described in split internode approach) to develop new shoot system. 3 weeks later, the surviving calli slices were transferred to CPM Dark to induce callus formation. The regeneration efficiency of stable transformants was recorded after 6 weeks of cultivation on CPM Dark.

4 RESULTS AND DISCUSSION

4.1 Shoot meristem culture regeneration

4.1.1 Martinez protocol

This protocol was based on work by (Martínez Nicolás, 2008). All of the surviving maize SMCs, cultivated for 11 weeks, were transferred to pots and grown at 28 °C, 16/8 h photoperiod for 12 weeks, being watered accordingly. The regeneration efficiency was determined as the percentage of plantlets surviving the whole protocol. Out of the total number of 222 sown embryos divided into 37 Petri dishes, 5 plantlets with developed root and shoot system from *in vitro* cultivation were moved to the pots, with 2 plants surviving the harsher *ex vivo* conditions. Most of the *in vitro* cultured explants were lost either due to necrosis (as the result of poor adaptation to subculturing on new media), or contamination. Poor adaptation to soil was also the main factor affecting the regeneration efficiency of pot-grown plants, resulting in the death of three regenerants. The regeneration efficiency reached 0.9%.

4.1.2 Split internode approach

All work was performed according to (Pathi *et al.*, 2013). Maize calli, developed by meristem culture from bulged internodes, underwent shoot induction and rooting, induced by the subcultivation on REGEN M and SMC REG, respectively, in the consecutive protocol steps (Figure 4A-E). Newly-formed plantlets (after 10 weeks cultivation), ready for the transfer to the soil, were moved to the pots filled with a mixture of peat moss and perlite. It was managed to develop 12 plantlets successfully responding to caulogenesis and rhizogenesis. A total of 12 pots were sown, each containing a regenerating plantlet (Figure 4F). Out of the 202 cultivated zygotic embryos, this accounted for 5.9% regeneration efficiency.

The prime cause of callus death during the *in vitro* phase was necrosis, induced by subculturing on media, which resulted in the small amount of plantlets available for *ex vitro* transfer. In comparison to the original protocol, which described the total period of 2 months (or 50-56 days) needed to regenerate a viable plantlet from a mature seed, the total *in vitro* cultivation time was longer by approximately 14 more days (split internode protocol) and 21 days (Martinez protocol), respectively. This was due to the repetition of the rooting step by cultivation on SMC REG, as the roots had not been developed yet, in both situations. The reported regeneration rate was 90% for the same REGEN M composition used, nevertheless, in the original study, local Indian maize cultivar HPQM-1 was used in comparison to the line B73, utilized in this thesis (Pathi *et al.*, 2013). Similarly, another Indian cultivar, HQPM-5, was used in mature seeds regeneration study, where it was managed to reach plantlet stage in 9 weeks of *in vitro* cultivation (Mushke *et al.*, 2016). (Abebe *et al.*, 2008) reported a similar regeneration time of 9-12 weeks to obtain viable plantlets (lines KAT and CML 216). However, to this date, B73 maize regeneration utilizing mature seeds as the starting material was reported only twice, firstly by (Wang, 1987) with the regeneration rate reaching 4-5% − similar to results in this study; and also by (Martínez Nicolás, 2008), in a work which was a basis for this thesis. In the latter, no regeneration efficiency data was recorded.

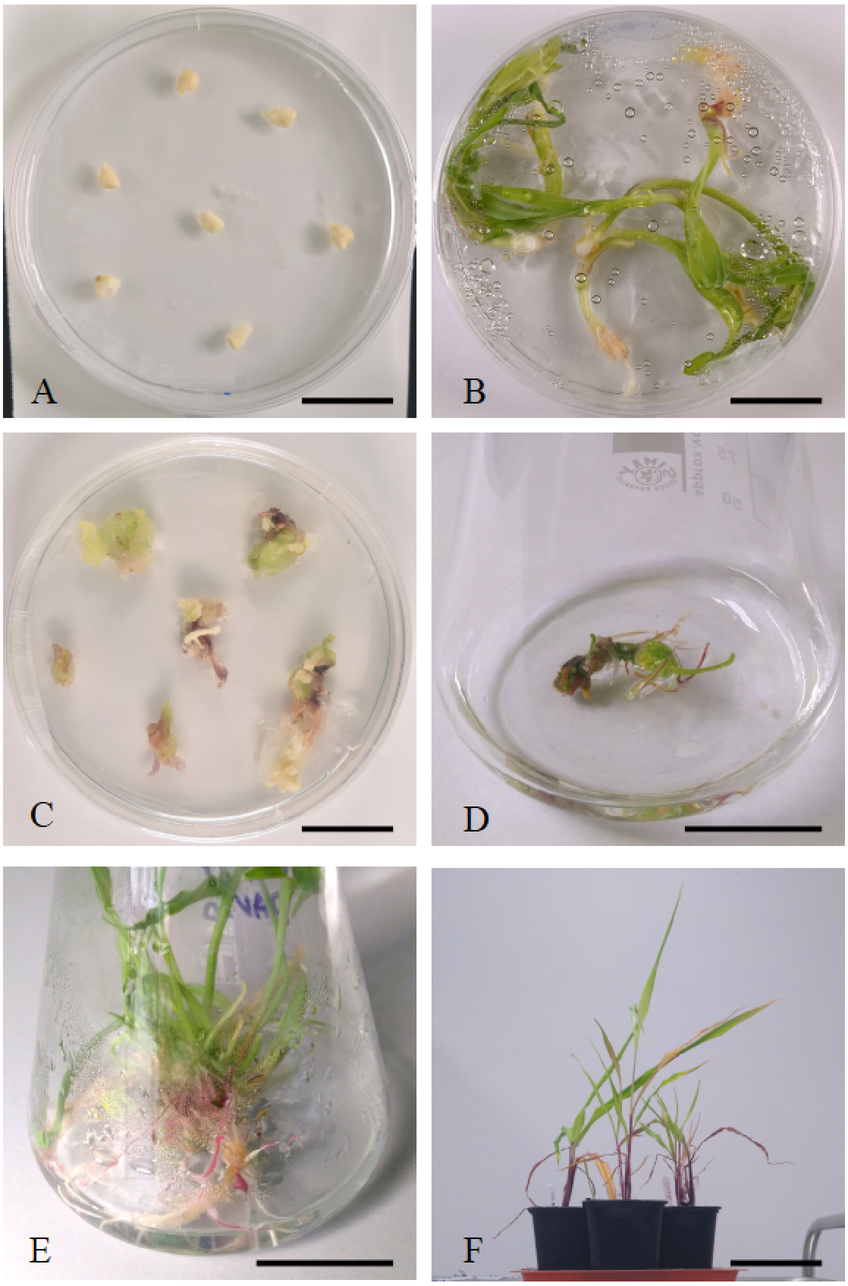

Figure 4: Respective steps of maize mature embryo regeneration **A:** Freshly sterilized, dissected and sown mature embryos, cultivated on GERM M medium; week of cultivation (WOC) = 0. **B:** Development of plantlets from the germinated embryos, grown on GERM M medium; WOC = 2. **C:** Proliferative embryogenic calli, induced by the cultivation on CPM Dark medium in dark. The calli shown are already transferred to REGEN M; WOC = 4. **D:** Plantlets with developing shoot system, induced by REGEN M cultivation, transferred to sterile Erlenmayer flask containing SMC REG; WOC = 6. **E:** Fully-developed plantlet with newly-induced root system, prior to transfer to soil; WOC = 10. **F:** Young maize plantlet after 8 weeks of cultivation in soil. Scale = 25 mm, except F, where scale = 100 mm.

4.2 *Agrobacterium tumefaciens*-mediated calli transformation

4.2.1 Strain effect on transient transformation assay

3 *A. tumefaciens* strains, AGL-1, LBA 4404 and EHA 105 were used for *Agrobacterium*-mediated transformation with *pCAMV35S::GUSA:tNOS* on pCAMBIA 1303 (KAN^R bacteria, HYG^R plants). A total number of 20 calli, each dissected into pieces 3 mm in diameter, were used for the assay of the strain performance on the efficiency of transformation. No enzymatic treatment was used prior to infection. Transient GUS assay was carried out for each strain 5 days after the infection and subsequent co-cultivation on Co-cultivation media. Even though no specific transformation frequency was measured, no histochemical signal was observed on explants transformed by *Agrobacterium* strains LBA 4404 and EHA 105. GUS reporter system expression was considerably high in the explants transformed by AGL-1, with the majority of the calli containing transformed cells. The strain effect was further validated by performing the same transformation protocol on *Arabidopsis thaliana* leaves and friable *Nicotiana tabacum* calli, with the same outcome.

Even though LBA 4404 and EHA 105 strains are one of the major *Agrobacterium* strains used in maize transformation protocols, which report TE of up to 95% (Zhao *et al.*, 2001; Ishida *et al.*, 2007; Zhi *et al.*, 2015; Du *et al.*, 2019), there has not yet been a significant number of studies focused on the transformation of MEs. In the sole study in which B73 was also transformed (IEs), 0% transformation frequency was similarly described (Frame *et al.*, 2006).

When applying the infection media containing AGL-1, a significant increase in transformation frequency was likewise described in a comparative study on different *Agrobacterium* strains effect on TE (Cho *et al.*, 2014). In another article published in 2014, 90% TE (AGL-1) was reported, with LBA 4404 transforming only approximately 11% of the MSCs. Several parameters' effects on maize TE, also assessed in the study, were described, most notably explant age, media composition and *Agrobacterium* strain. It was proposed, that strains like AGL-1 (nopaline strains) might be better options when transforming apical meristem-derived explants, in comparison to octopine strains (like LBA 4404) (Cao *et al.*, 2014). Therefore, AGL-1 could be a prime *Agrobacterium* strain-of-choice in SMC transformation studies. Notwithstanding, EHA 105 is also a nopaline strain and no transformants were generated by it in this thesis' experiment. Thus, the vulnerability of the explant to transformation efforts is indeed dependent on the combination of factors like explant genotype, growth media composition and explant origin, not only *Agrobacterium* strain used (Yadava *et al.*, 2017).

4.2.2 Transient transformation efficiency assay

In further transformation protocols, only *A. tumefaciens* strain AGL-1 was used. The lytic enzyme pretreatment protocol was based on (Du *et al.*, 2019). A total amount of 48 calli were used for the transformation protocol, which were sliced into 210 pieces of ~3 mm diameter. Enzymatic pretreatment effect on the transient TE was assayed, with 106 calli slices being treated with pectinase-cellulase cocktail for 9 min prior to the *Agrobacterium* infection; this step was omitted for the rest 104 pieces. Each group was divided into 4 Petri dishes of around 25 pieces per setting, to represent the respective repeats (Table 1). For the control group, the additional lysis step was exchanged for basal Infection media incubation. GUS histochemical assay was used to assess transient TE.

Unlike the original work, no significant change in transformation frequency was recorded after digesting the explants. However, in the article, different maize cultivar (Hi-II) was used, with IEs as the starting material (Du *et al.*, 2019). They also noted, that prolonged enzymatic lysis (over 9 min) had a negative effect on TE. Furthermore, pretreating the calli with unamended infection media for the same period, resulted in lowered TE. Therefore it can be presumed that the positive effects of cellulase/pectinase treatment on B73 calli were negated by the negative effects of additional transformation protocol step, effectively canceling out each other.

Table 1: Enzymatic pretreatment effect on the transient *Agrobacterium*-mediated transformation efficiency of sliced maize calli. Transient transformation efficiency calculated as mean frequency ± SD.

	+ ET		- ET	
Repeat	# of infected calli slices	# of GUS expressing calli slices	# of infected calli slices	# of GUS expressing calli slices
1	31	25	34	30
2	18	14	22	18
3	25	18	21	18
4	32	28	27	23
Transient transformation efficiency		79.5 ± 5.6%		85.2 ± 2.3%

Afterwards, the transient transformation experiment was performed with the exclusion of enzymatic pretreatment step, as it did not provide an increase in TE in the first transient TE assay. 90 calli, sliced into 198 pieces were transformed using *A. tumefaciens* strain AGL-1 and after 5 days of co-cultivation, GUS expression assay was carried out to determine transient transformation frequency (Figure 5). For the control group, the infection step (30 min incubation with *A. tumefaciens* in the desiccator) was exchanged for 30 min incubation in the basal Infection media, also in the desiccator. A total number of 3 Petri dishes (alongside 2 control groups) were assayed as the respective repeats (Table 2).

Compared to the original thesis (Martínez Nicolás, 2008), on which the transformation protocol in this work was based, it was managed to increase the TE approximately tenfold. An alternative hypervirulent strain (AGL-1) was used in comparison to EHA 101. Furthermore, the OD_{600} for AGL-1 was set to reach 1.2 prior to liquid YEB media incubation, and 0.8 for Infection media resuspension step (as compared to 1.0 and 0.6, respectively). These differences, along with the increased co-cultivation time, might be responsible for the higher number of transformation events in the sliced calli. On the other hand, it was observed that AGL-1 needed more time to grow colonies on solid YEB media, with distinct colony formation developing as late as 36 h after Petri dish inoculation. This could result in unplanned delay of transformation protocols, which usually require *Agrobacterium* colony to establish in 24 h time.

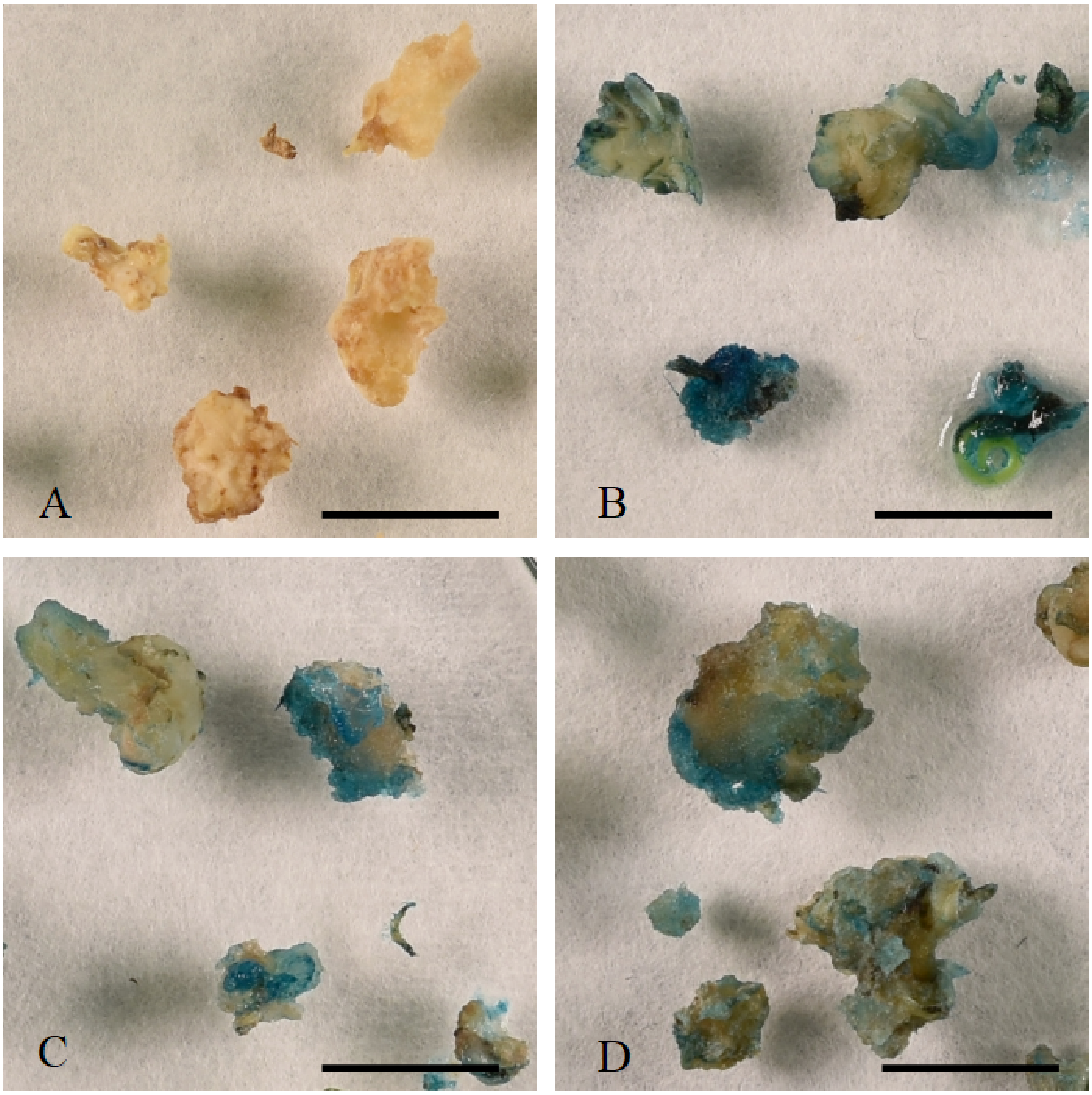

Figure 5: Transient GUS expression in sliced SMCs after 5 days co-cultivation with *Agrobacterium*. **A.** Control. **B.** Repeat group #1. **C.** Repeat group #2. **D.** Repeat group #3. Scale = 10 mm.

Table 2: Transient *Agrobacterium*-mediated transformation efficiency of sliced maize calli. Transient transformation efficiency calculated as mean frequency ± SD.

	Transiently transformed calli slices		Control	
Repeat	# of infected calli slices	# of GUS expressing calli slices	# of infected calli slices	# of GUS expressing calli slices
1	30	25	25	0
2	42	34	35	0
3	66	50	-	-
Transient transformation efficiency		80.0 ± 3.2%		0 ± 0%

4.2.3 Stable transformation efficiency assay

90 calli were sliced into 224 pieces and subsequently infected with *A. tumefaciens* for 30 min. The explants were co-cultivated on Co-cultivation media for 5 days and transferred to Resting media afterwards, to eliminate *Agrobacterium*. After 7 days on the Resting media, the slices were incubated in GUS staining solution and after 48 h, GUS expression assay was performed (Figure 6, Table 3).

GUS expression analysis was used as the means to analyze stable transformation efficiency, as it was not managed to recover any plants from regeneration experiment (see below). The assay was performed 7 days after co-cultivation, with the assumption that most non-integrated T-DNA molecules are degraded or attenuated by that time (Bartlett *et al.*, 2014; Philips *et al.*, 2019).

Table 3: Stable *Agrobacterium*-mediated transformation efficiency of sliced maize calli. Stable transformation efficiency calculated as mean frequency ± SD.

	Stably transformed calli slices		Control	
Repeat	# of infected calli slices	# of GUS expressing calli slices	# of infected calli slices	# of GUS expressing calli slices
1	47	24	49	0
2	54	33	23	0
3	51	28	-	-
Stable transformation efficiency		55.7 ± 4.1%		0 ± 0%

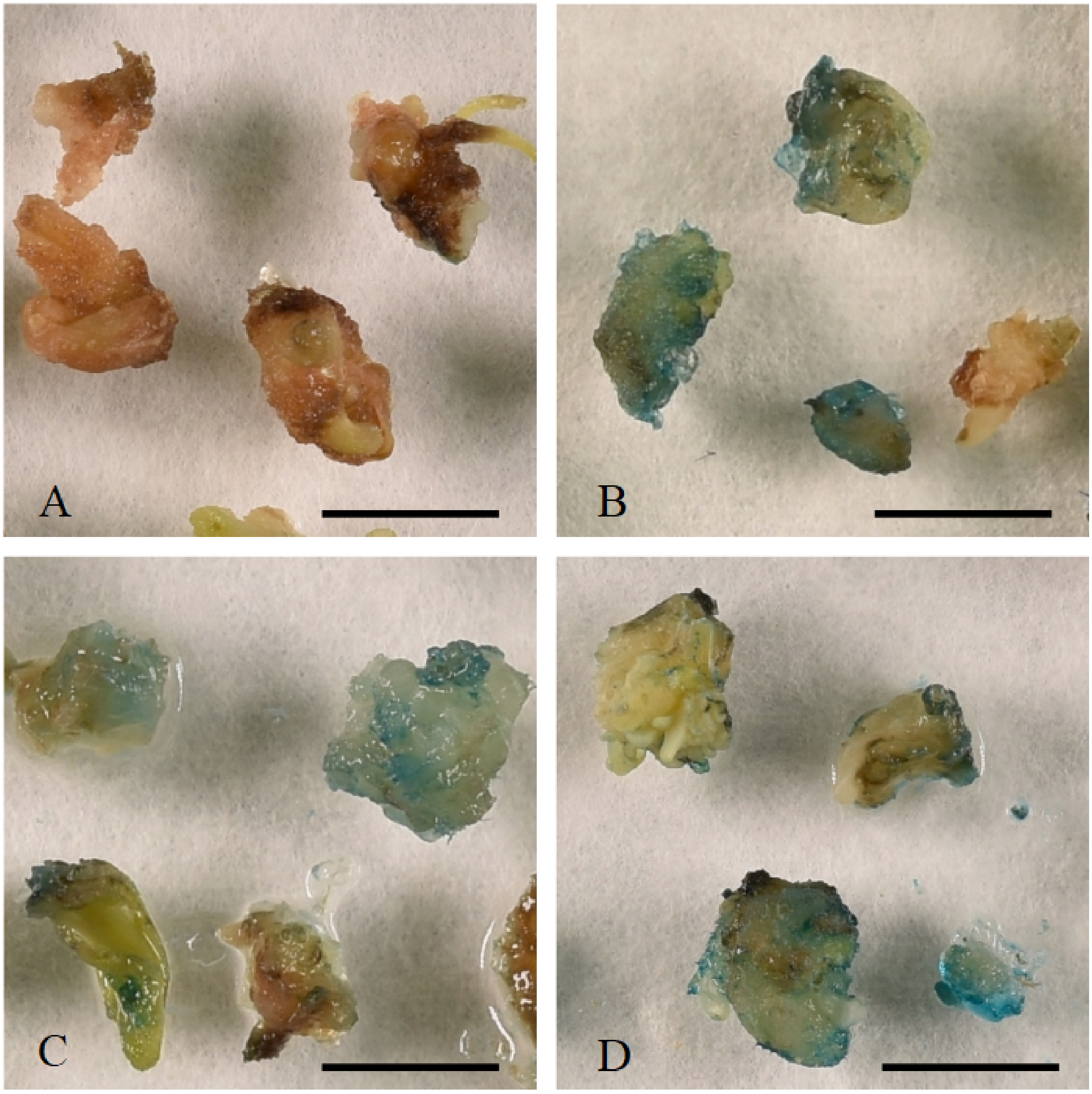

Figure 6: Stable GUS expression in sliced SMCs after 5 days co-cultivation with *Agrobacterium* and 7 days on Resting media afterwards. **A.** Control. **B.** Repeat group #1. **C.** Repeat group #2. **D.** Repeat group #3. Scale = 10 mm.

4.2.4 Transformed callus slice regeneration

A total number of 30 calli slices, transformed by *A. tumefaciens* strain AGL-1, were used for regeneration of mature plantlets. After 7 days of cultivation on Resting media, approximately 40% of slices failed to respond to the growth media and died. The rest of the calli slices were subcultured on REGEN M to try to induce new shoots growth, however no visible shoots were established even after three weeks. Ultimately, the calli slices were subcultured on CPM Dark in order to promote tissue dedifferentiation into a callus. The calli slices' color turned from yellowish white to dark green, which was maintained for two more subcultivation repeats (a total of 10 weeks since the transfer from the Co-cultivation media), however the calli did not manage to develop and all of the explants died as well.

No stable transformant regeneration was similarly described in (Martínez Nicolás, 2008), on which the regeneration protocol was based. Utilizing calli induction media developed by (Pathi *et al.*, 2013) resulted in no difference in the regeneration. Even though no plant was successfully recovered, approximately half of the cultivated sliced calli survived the subcultivation to REGEN M. The ability to regenerate the transformed SMCs should therefore be determined by the explants' proliferative capacity post transformation, rather than ineffective regeneration media used. However, the explants still lacked sufficient phytochemical cues from the media to redirect cell fate and induce caulogenesis. Further modifications and refinement of the media formulation are henceforth needed for the protocol improvement and eventual fertile regenerant production. Alternatively, there were few studies which managed a successful regeneration of transformants (Ishida *et al.*, 2007; Zhang *et al.*, 2007; Vega *et al.*, 2008), however the starting explants were typically IEs of various lines, different from B73 MEs used in this thesis and (Martínez Nicolás, 2008).

5 CONCLUSION

The main focus of this bachelor thesis was the regeneration and transformation of maize as an archetypal monocotyledonous plant and model organism. Due to its irreplaceable position in contemporary maize research, B73 inbred cultivar was used as the sole studied line in this work. The utilization of this line was further cemented by the fact, that no efficient protocol combining regeneration and *Agrobacterium*-mediated transformation, utilizing mature B73 maize seeds, has ever been published. Therefore, it was sought to establish a working system on this line as well.

Two different regeneration protocols were carried out and subsequently compared. Regeneration rate of 0.9 and 5.9% was reached, respectively. Either of the approaches used did not provide a significant improvement in the outcome, with lower efficiency as compared to the original studies. The regeneration of mature seeds-derived B73 explants therefore still remains elusive. Experiments focused on transforming calli explants, utilized different *Agrobacterium* strains and pretreatment options. Transformation of explants was demonstrated only with strain AGL-1, with transient TE reaching 80% and stable TE reaching 55.7%. Experimenting with enzymatic lysis prior to inoculation did not yield compelling results, on the contrary, the TE was recorded lower than compared to the control. The attempted regeneration of transformed explants was not successful.

Nevertheless, even if it was not managed to establish an efficient B73 mature seed regeneration and transformation protocol, the TEs achieved in this thesis were significant. Unlike that, the regeneration efficiency of mature seed-based protocols is generally low, which makes it the limiting factor of such protocols. Therefore, it is crucial to develop techniques enabling quick and efficient *in vitro* regeneration of mature seed-derived explants, providing a simple and economical way of generating transgenic maize plants for both research and commercial application.

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7 LIST OF ABBREVIATIONS

- 2,4-D	2,4-Dichlorophenoxyacetic acid
- AMP	Ampicillin
- AS	Acetosyringone
- *AP2*	*Apetala2* family
- BAP	6-Benzylaminopurine
- bp	Base pairs
- *BBM*	*Baby Boom*
- CPM	Callus proliferation medium
- DAP	Days after pollination
- EDTA	Ethylenediaminetetraacetic acid
- *ERF*	*Ethylene responsive element binding factor*
- GM	Genetically modified
- GUS	β-glucoronidase
- HPGG	High pressure gene gune
- HYG	Hygromycine
- IAA	Indole-3-acetic acid
- IE	Immature embryo
- KAN	Kanamycin
- KIN	Kinetin
- ME	Mature embryo
- MES	2-(N-morpholino)ethanesulfonic acid
- MS	Murashige and Skoog
- MSC	Multi-shoot culture
- MT	Metric tonne
- MTX	Methatrexate
- NAA	1-Naphthaleneacetic acid
- *NOS*	*Agrobacterium nopaline synthase*
- OC	Organizing center
- PEG	Polyethyleneglycol
- RIF	Rifampicine

-	SAM	Shoot apical meristem
-	SE	Somatic embryo
-	SMC	Shoot meristem culture
-	SNP	Single nucleotide polymorphism
-	SPE	Spectinomycine
-	TIM	Timentin
-	TE	Transformation efficiency
-	*UBI*	*Ubiquitin*
-	WOC	Week of cultivation
-	*WUS*	*Wuschel*
-	X-Gluc	5-Bromo-4-chloro-3-indolyl-β-D-glucuronide
-	YEB	Yeast extract beef