

## SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM AN ELITE TROPICAL MAIZE INBRED LINE

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*Revista Brasileira de Milho e Sorgo*, v.15, n.3, p. 366-376, 2016

**ABSTRACT** – The improvement of tropical maize inbred lines by genetic transformation techniques remains a difficult task since not all genotypes are capable of regenerating efficiently *in vitro*. The objective of this study was to evaluate three different callus induction media, based on N6 or MS salts containing either 2,4-D (0, 2.5, 5.0, 10.0, 15.0, 30.0 mg.L<sup>-1</sup>) or Dicamba (0; 0.25; 0.5; 1.0; 2.0; 4.0 mg.L<sup>-1</sup>) in the production of embryogenic callus from immature zygotic embryos of the tropical maize inbred line L3. Callus maturation was tested in MS medium containing 60 g.L<sup>-1</sup> sucrose and supplemented with different combinations of BAP (0; 0.1; 0.5; 1.0 mg.L<sup>-1</sup>), NAA (0; 1.0 mg.L<sup>-1</sup>) and CuSO<sub>4</sub> (0; 1.25 mg.L<sup>-1</sup>). The L3 inbred line presented higher capacity for Type II callus formation on N6 medium content 10 mg.L<sup>-1</sup> 2,4-D. For the maturation of callus, absence of plant growth regulators and addition of CuSO<sub>4</sub> allowed higher percentage of regeneration. The protocol developed presented 85% production of Type II embryogenic callus and 45% plant regeneration.

**Keywords:** 2,4-D, dicamba, embryogenic callus, *Zea mays*.

## EMBRIOGÊNESE SOMÁTICA E REGENERAÇÃO DE PLANTAS A PARTIR DE UMA LINHAGEM DE MILHO TROPICAL ELITE

**RESUMO** – O melhoramento de linhagens de milho tropical através de técnicas de transformação genética continua a ser uma tarefa difícil uma vez que nem todos os genótipos são capazes de regenerar eficientemente *in vitro*. O objetivo deste estudo foi avaliar três meios diferentes para a indução de calos embriogênicos, baseados em N6 ou MS sais contendo 2,4-D (0; 2,5; 5,0; 10,0; 15,0; 30,0 mg.L<sup>-1</sup>) ou Dicamba (0; 0,25 ; 0,5; 1,0; 2,0; 4,0 mg.L<sup>-1</sup>) na produção de calos embriogênicos a partir de embriões zigóticos imaturos da linhagem de milho tropical elite L3. A maturação dos calos foi testada em meio MS com 60 g.L<sup>-1</sup> de sacarose suplementado com diferentes combinações de BAP (0; 0,1; 0,5; 1,0 mg.L<sup>-1</sup>), ANA (0; 1,0 mg.L<sup>-1</sup>) e CuSO<sub>4</sub> (0; 1,25 mg.L<sup>-1</sup>). A linhagem L3 apresentou alta capacidade para produção de calos do Tipo II em meio N6 contendo 10 mg.L<sup>-1</sup> de 2,4-D. Para a maturação dos calos, ausência de reguladores de crescimento vegetal e adição de CuSO<sub>4</sub> possibilitou maior porcentagem de regeneração. O protocolo desenvolvido apresenta produção de 85% de calos embriogênicos do Tipo II e 45% de regeneração de plantas.

**Palavras-chave:** 2,4-D, dicamba, calos embriogênicos, *Zea mays*.

Maize is a crop of great agronomic importance that moves a multibillion-dollar market; also it is extensively used in basic and applied research. Information about the organization and regulation of maize genome are being made often available (Feuillet & Eversole, 2009) and there is a great potential for application of this knowledge in the improvement of this culture through the integration and expression of genes of agronomic interest via genetic transformation.

A primary requirement for genetic transformation of plant cells is the establishment of an efficient *in vitro* plant regeneration system (Frame et al., 2006; Vega et al., 2008; Pitzschke, 2013). Somatic embryogenesis is the most used methodology for the regeneration of transgenic maize cells and it was first described by Green and Philips (1975) using immature maize embryos as explants.

Regeneration is largely influenced by the explant type and the composition of culture medium (Armstrong & Green, 1985; Songstad et al., 1991; Bohorova et al., 1995). Maize explants such as immature inflorescences (Mu et al., 2012), apical meristem (Zhang et al., 2002), mature seeds (Zhao et al., 2008) and immature embryos (Vega et al., 2008) have been used for callus induction and genetic transformation. However, so far the scutellum of immature zygotic embryos is the explant with highest regeneration efficiency, competence to infection by *A. tumefaciens* and generation of transgenic plants (Frame et al., 2006; Vega et al., 2008).

Culture media formulations based on Murashige and Skoog (1962) or Chu et al. (1975) - MS and N6 salts respectively, have been widely used to cultivate maize *in vitro*. Embryogenic callus is normally formed when immature maize zygotic embryos are cultured in these basal media supplemented with auxins, such as 2,4-D or Dicamba

(Green & Phillips, 1975; Armstrong & Green, 1985; Frame et al., 2006; Petrillo et al., 2008).

Maize embryogenic cultures have two predominant forms of callus, Type I and Type II, that differ primarily in their regeneration efficiency during an extended time in culture. Type I callus is compact, yellowish or white in color. Type II is soft, friable and highly embryogenic (Armstrong & Green, 1985). Although both calli are capable of plant regenerating, cultures formed by Type II callus grow faster; can be maintained for a long period of time and form a large number of somatic embryos (Vasil, 2005). These characteristics favor the selection and regeneration of transgenic plants (Ji et al., 2011).

The occurrence of Type II friable embryogenic callus is not as common, only a limited number of maize genotypes are able to express this phenotype in culture medium, especially the hybrid HiII (Armstrong & Green, 1985). Because of this characteristic HiII is one of the most used genotype in genetic transformation protocols. However, Hi-II is not interesting for the evaluation of agronomic characteristics such as improved productivity and tolerance to biotic and abiotic stresses by having a low agronomic performance (González et al., 2012; Que et al., 2014). Backcrosses can be done to transfer the transgene to an elite variety but this process is lengthy and unwanted traits can be transmitted together with the gene of interest.

Advances of *in vitro* culture methodologies and particularly changes in the composition of culture media enabled the regeneration of an increased number of maize genotypes (Frame et al., 2006). Although most of these genotypes are adapted to temperate conditions, tropical genotypes capable of regeneration have also been identified (Petrillo et al., 2008; Anami et al., 2010; González et al., 2012) which

indicates the possibility of directly manipulating tropical elite genotypes via genetic transformation, thus accelerating tropical maize breeding programs.

This study aimed to develop a routine method of induction and regeneration of somatic embryogenic callus for the tropical elite maize inbred line L3 used at Embrapa's Maize and Sorghum breeding program by testing the capacity of immature L3 zygotic embryos to form embryogenic callus on three different callus induction media supplemented with 2,4-D or Dicamba and to regenerate maize plants.

### Materials and Methods

Immature embryos used in these experiments were from the L3 tropical maize inbred line plants grown at Embrapa Maize and Sorghum – MG, Brazil. Ears for embryo extraction were surface-sterilized in half-strength liquid commercial bleach and 0.01% Tween 20, during 40 minutes and then rinsed three times with distilled water. Zygotic immature embryos harvested 10-15 days after pollination, between 1.5 to 2.0 mm long were used as explants. To select a medium able to support the formation of embryogenic callus,

these immature embryos were cultivated with the axis in contact with three different basal media – M1 and M2 media based on N6 salts (Chu et al., 1975), and M3 medium (Carvalho et al., 1997) based on MS salts (Murashige & Skoog, 1962) (Table 1). These media were supplemented with various concentrations of the auxins 2,4-dichlorophenoxyacetic acid (2,4-D) (0; 2.5; 5.0; 10.0; 15.0 and 30.0 mg L<sup>-1</sup>) or 3,6-dichloro-o-anisic acid (Dicamba) (0; 0.25; 0.5; 1.0; 2.0; 4.0 mg L<sup>-1</sup>), totaling 36 treatments. Embryogenic calli developed on these media were transferred every 14 days onto fresh medium and the amount of Type I and Type II calli formed were evaluated after 42 days of cultivation.

This experiment was evaluated using a triple factorial 3x2x6 (Factor 1: Culture media; Factor 2: Type of auxin; Factor 3: Concentration of auxin). The experimental design was completely randomized, with four replicates per treatment. Each repetition consisted of 24 immature embryos, totaling 96 embryos per treatment. The analyzed variable was the percentage of Type I and Type II callus. The software used was the program R (R Development Core Team, 2015).

The most efficient callus inducing medium selected previously was used to generate new calli that

**Table 1.** Composition of the culture media used for induction of somatic embryos in the L3 tropical elite inbred maize line.

Culture Medium	Composition
M 1	N6 salts and vitamins (Chu et al., 1975); 30 g L <sup>-1</sup> sucrose; 100 mg L <sup>-1</sup> hydrolyzed casein; 100 mg L <sup>-1</sup> myo-inositol; 2.9 g L <sup>-1</sup> L-proline; 15 mg L <sup>-1</sup> silver nitrate; 3 g L <sup>-1</sup> Phytigel; pH 5.8.
M 2	N6 salts and vitamins (Chu et al., 1975); 30 g L <sup>-1</sup> sucrose; 100 mg L <sup>-1</sup> de hydrolyzed casein; 2.9 g L <sup>-1</sup> L-proline; 0.01 g L <sup>-1</sup> MES; 5.5 mg L <sup>-1</sup> de glycine; 0.85 mg L <sup>-1</sup> silver nitrate; 3 g L <sup>-1</sup> Phytigel ; pH 5.8.
M 3	MS salts and vitamins (Murashige & Skoog, 1962); 30 g L <sup>-1</sup> sucrose; 0.7 g L <sup>-1</sup> L-proline; 0.5 g L <sup>-1</sup> MES; 0.85 mg L <sup>-1</sup> silver nitrate; 3 g L <sup>-1</sup> Phytigel ; pH 5,8.

were used to select the best media for maturation and regeneration. Sixteen different treatments consisting of maturation medium (MS salts and vitamins, 60 g.L<sup>-1</sup> sucrose, 100 mg.L<sup>-1</sup> myo-inositol, 6 g.L<sup>-1</sup> Phytigel) content 0 or 1.25 mg.L<sup>-1</sup> CuSO<sub>4</sub> (Cho et al., 2014) and combinations of 6-benzylaminopurine (6-BAP) (0; 0.1; 0.5; 1.0 mg.L<sup>-1</sup>) and naphthalene acetic acid (NAA) (0; 1.0 mg.L<sup>-1</sup>) were tested. Calli were kept in these media for 42 days. Callus initiation, maintenance and maturation were carried on in Petri dishes (100x25 mm) at 27±2°C and dark.

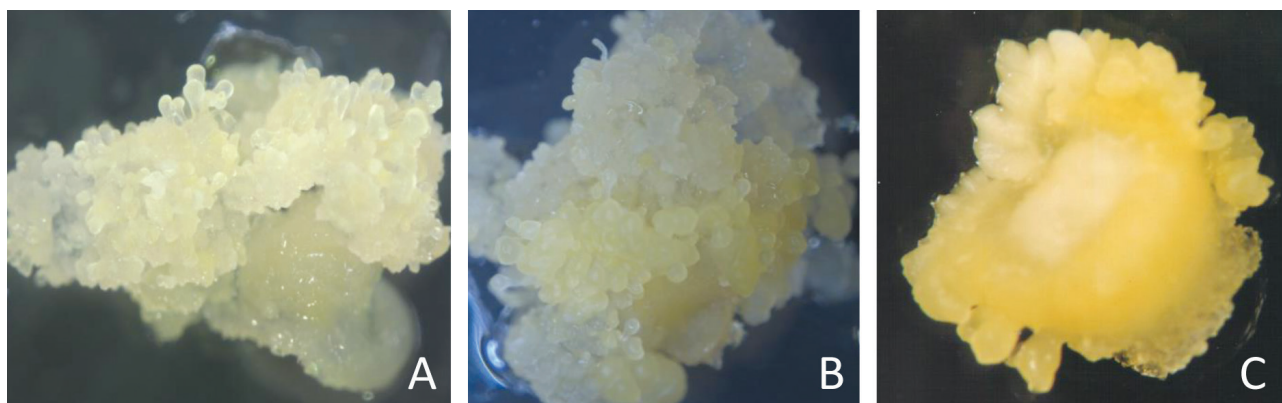
Mature somatic embryos with an opaque and dry appearance were transferred to Magenta jars (Sigma / Brazil) containing MS media without any plant growth regulator and were incubated at 27±2°C, 16 hours photoperiod and light intensity of 36 µmol.m<sup>-2</sup>.s<sup>-1</sup> until germination, approximately two weeks.

To evaluate this experiment it was used a double factorial 8x2 (Factor 1: Plant growth regulator; Factor 2: CuSO<sub>4</sub>) in a completely randomized design, with five replicates per treatment. Each repetition consisted of four somatic embryos, totaling 20 calli per treatment. The variables analyzed were percentage of matured callus and number of germinated plants. The software used was the program R (R Development Core Team, 2015).

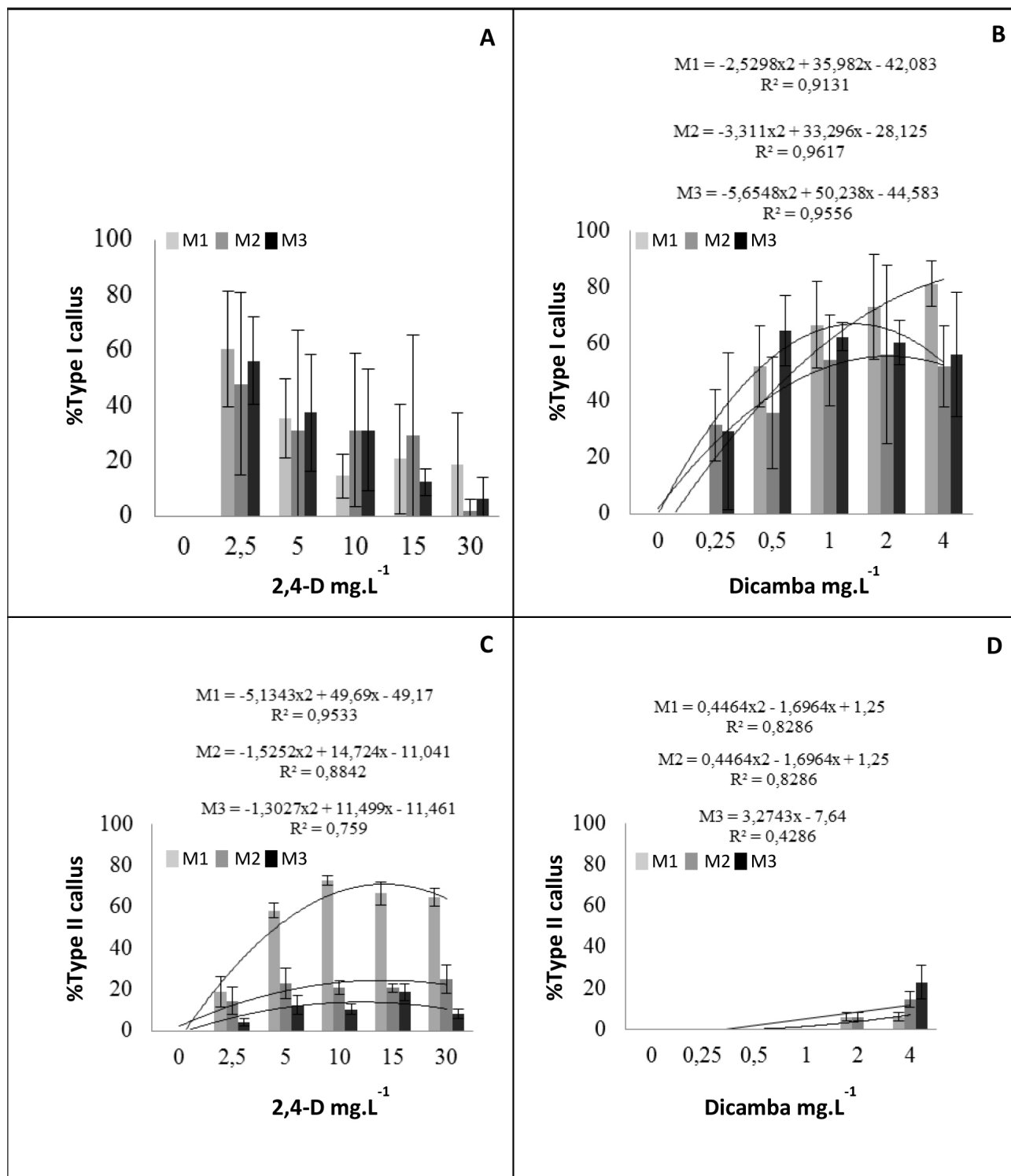
## Results and Discussion

Media composition directly affected the performance of the explant material employed in tissue culture and, according to Fehér (2015) plant embryogenesis can be initiated by a diversity of conditions including several plant hormones and/or stress treatments. Auxins are among the plant growth regulators most frequently used to induce somatic embryogenesis (Fehér, 2015). Therefore, the capacity of the immature zygotic embryos of the tropical inbred line L3 to form embryogenic callus of Type I or II was evaluated in the presence of various concentrations of 2,4-D and Dicamba.

In this work, there was a clear influence of the type and concentration of auxin on callus morphology and somatic embryo production efficiency. Figure 1 exemplifies the morphology of the embryogenic callus found for tropical maize inbred line L3. For the variable Type I callus (Figure 2A and 2B), the statistical analysis pointed out that there was interaction between type and concentration of auxins used in the M1, M2 and M3 basal media. In the 2,4-D treatments the regression test indicated that no curve was adjusted for the results obtained (Figure 2A).



**Figure 1.** Morphology of somatic embryogenic callus. (A) Type II callus from the temperate maize hybrid HiII; (B) Type II callus from the tropical maize inbred line L3; (C) Type I callus from the tropical maize inbred line L3.



**Figure 2.** Percentage of Type I and Type II callus obtained with the media M1, M2 and M3 containing various concentrations of 2,4-D or Dicamba.



Media supplemented with Dicamba caused the highest percentages of Type I callus formation and a quadratic behavior. The formation of Type I callus in M2 and M3 media increased with the concentration of Dicamba up to 1 mg.L<sup>-1</sup>, stabilizing afterwards (Figure 2B). Unlike the behavior observed for M2 and M3, in M1 the elevation in Dicamba concentration was accompanied by an increase in the percentages of Type I callus formation (Figure 2B).

The statistical analysis revealed that there was a triple interaction among the factors – basal media composition, type and concentration of auxins - used to induce Type II callus (Figure 2C and 2D). Higher percentage of fast growing callus, highly friable, presenting numerous embryos in the globular stage - characteristic of Type II callus – were observed in the M1 medium supplemented with 10 to 30 mg.L<sup>-1</sup> 2,4-D (Figure 2C). The greater production of Type II callus occurred at 10 mg.L<sup>-1</sup> 2,4-D. Concentration higher than that did not show improvement of Type II callus production (Figure 2C). For Dicamba (Figure 2D), regardless of the basal media, the minimal amount required for the formation of Type II callus was 2 mg.L<sup>-1</sup>. However, the generation of Type II callus in presence of 10 mg.L<sup>-1</sup> 2,4-D was more expressive than in the presence of 2 mg.L<sup>-1</sup> Dicamba.

Some studies reported that media supplemented with Dicamba have been successfully used in the formation of Type II callus in various maize genotypes and others grasses (Bohorova et al., 1995; Carvalho et al., 1997; Akoyi et al., 2013). Others indicated that 2,4-D is better to induce the formation of Type II callus from maize zygotic embryos (Songstad et al., 1991). In this study, Dicamba was not adequate to achieve high levels of Type II callus formation from immature embryos of the inbred line L3; however, the combination of M1 basal media and 4 mg.L<sup>-1</sup> Dicamba

resulted in more than 80% Type I callus formation for this maize line.

At low concentrations of 2,4-D or in all tested doses of Dicamba there was the predominant formation of Type I callus independent of basal media, whereas at concentrations equal or above 5 mg.L<sup>-1</sup> 2,4-D there was the higher induction of Type II callus when M1 medium was used. Fehér (2015) considers that 2,4-D is one of the most efficient auxin to induce embryogenesis because it is a plant growth regulator and a stress-inducing herbicide at the same time.

The ideal concentration of 2,4-D for the formation of embryogenic calli has fluctuated between 1.5 and 2 mg.L<sup>-1</sup> (Anami et al., 2010; Ombori et al., 2008; Frame et al., 2006). Differences in responses to auxin induction of somatic embryogenesis may occur depending upon the species, genotype or explants used (Fehér, 2015). This fact could be evidenced in this study, where the concentration of 2,4-D used to induce Type II callus in the inbred line L3 was approximately five to seven times higher than the one used by others (Anami et al., 2010; Ombori et al., 2008; Frame et al., 2006) to induce the same kind of callus in different maize genotypes.

Pulianmackal et al. (2014) stated that a complex set of regulatory interactions are involved in somatic embryogenesis. The formation of somatic embryos can be explained by the interaction of the environmental or induction conditions with the endogenous hormone levels that depend on the genotype and developmental phase of the explant (Fehér, 2015).

The interaction between the level of endogenous and exogenous auxins present in the culture media can interfere with ethylene biosynthesis which in turn interferes with the polar transport and asymmetric distribution of endogenous auxin, regulating a series of genes related to embryogenesis such as *wus*,

*agamous like15 (agl15)*, *baby boom (bbm)*, *embryo maker (emk/ail5)*, and *leafy cotyledon (lec)* [reviewed by Pulianmackal et al. (2014)]. Akoyi et al. (2013) mentioned that genotype variations observed among different tropical maize lines with relation to the formation of embryogenic callus could be explained by allelic differences in genes related to the embryogenesis process, such as the ones cited above.

Higher concentrations of L-proline and AgNO<sub>3</sub> present in the M1 medium may have influenced positively the formation of Type II callus. Addition of L-proline to the culture medium had been shown by Armstrong and Green (1985) to allow predictable initiation of Type II callus from immature A188 embryos. Silver ions can prompt somatic embryogenesis in *Zea mays* (Songstad et al., 1991). The exact mechanism by which this happens on plants is unclear (Kumar et al., 2009); one of the hypotheses is by the regulation of ethylene action. As discussed above, ethylene can affect callus growth and somatic embryogenesis *in vitro* (Pulianmackal et al., 2014) and its production is dependent on the level of 2,4-D in the medium (Jiménez, 2005).

Maturation of maize embryos is a process that happens prior to the plantlets germination. During this phase changes in gene expression happens in the cells leading to the accumulation of storage material (Teoh et al., 2013) and acquisition of desiccation tolerance. Maturation of somatic embryos have

been reported in the absence of 2,4-D as well as in the presence of others growth regulators such as cytokines (Ombori et al., 2008; Akoyi et al., 2013), or abscisic acid (ABA). Other components may also be used to promote maturation of embryogenic callus, such as high concentration of sucrose, gelling agents and heavy metals (Fehér, 2015; Cho et al., 2014).

To study the maturation and regeneration of callus from inbred line L3, zygotic immature embryos were cultivated in M1 basal medium supplemented with 10 mg.L<sup>-1</sup> 2,4-D and, approximately 80% of them produced Type II callus. Combinations of BAP, NAA and CuSO<sub>4</sub> added to a higher osmotic media (6% sucrose) were evaluated for the maturation of the Type II callus. The results showed no interaction among these factors. The only factor that impacted the maturation of embryogenic callus was the presence of CuSO<sub>4</sub> in the culture medium (Table 2). These results differ from those found by Ombori et al. (2008) and Akoyi et al. (2013), which reported that BAP and NAA were effective in the maturation of somatic embryos of maize. Over a period of 42 days, there was a higher percentage of maturation in the treatments where CuSO<sub>4</sub> was present (Table 2). Studies have shown that an increased level of the micronutrient copper in the culture medium dramatically improves regeneration from maize callus cultures (Cho et al., 2014). Copper is an essential micronutrient for normal plant development and one of its main functions is as an activator or constituent of enzymes.

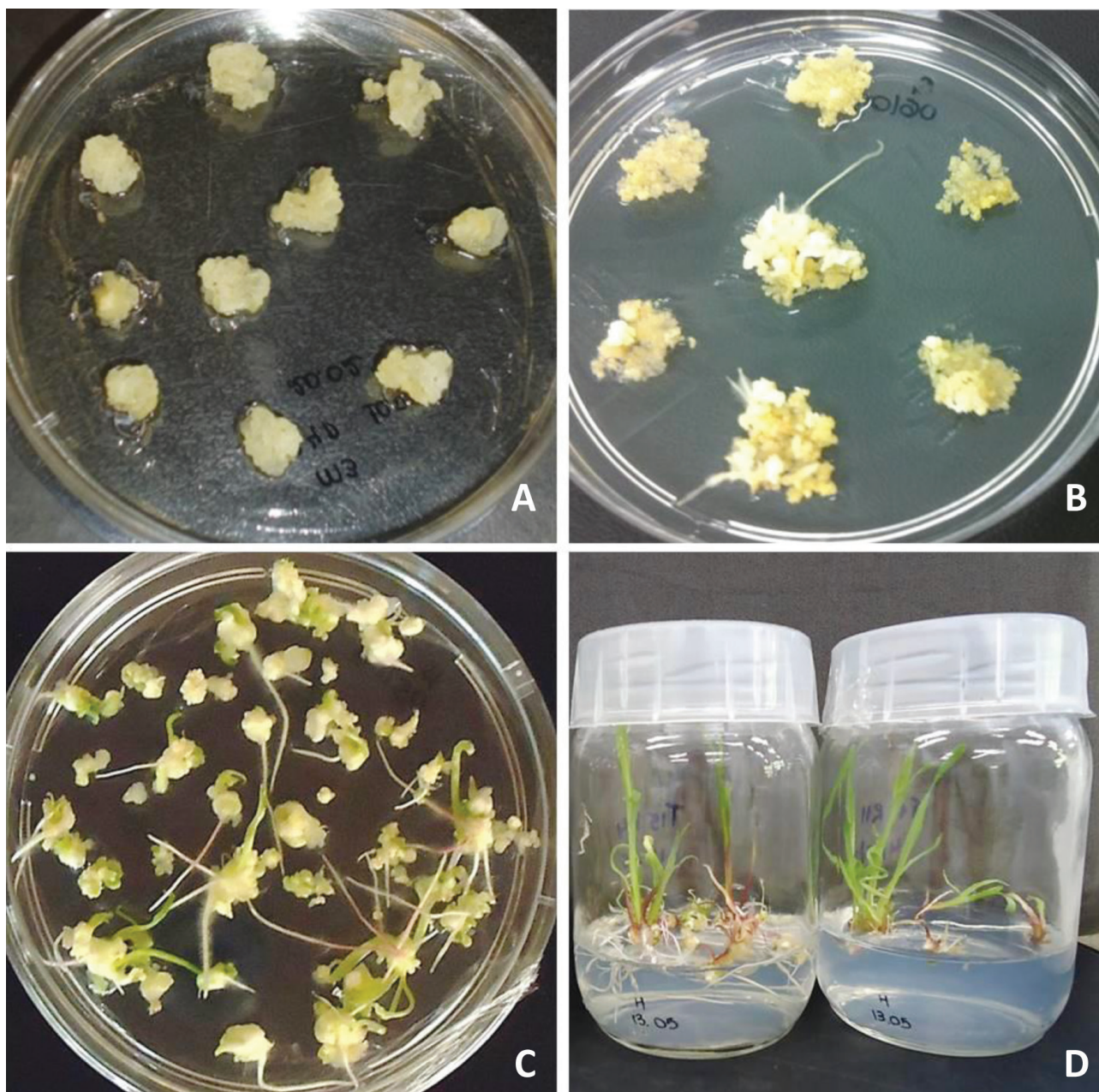
**Table 2.** Average percentage of mature callus and number of maize plants regenerated in the presence or absence of 1.25 mg.L<sup>-1</sup> CuSO<sub>4</sub>.

Treatments	Mature callus (%)	Regenerated Plants (%)
With CuSO <sub>4</sub>	92.5 a	9.0
Without CuSO <sub>4</sub>	76.87 b	4.8
CV(%)	27.44	-

Means followed by the same letter in the column do not differ statistically at 5% by Tukey test.

The L3 inbred line has an efficient level of *in vitro* embryogenic capacity and was able of regenerating into complete and fertile plants (Figure 3). In addition, this tropical line has excellent agronomic fitness.

These characteristics make it promising to be tested on *Agrobacterium tumefaciens* or biolistic transformation protocols, and participate in a commercial transformation pipeline at Embrapa Maize and Sorghum.



**Figure 3.** Callus formation and plant regeneration from L3 tropical elite inbred line. (A) Callus formation after 4 weeks cultivation on N6 medium supplemented with  $10 \text{ mg.L}^{-1}$  2,4-D; (B) Maturation after 3 weeks cultivation on medium content  $\text{CuSO}_4$ ; (C) Callus germination; (D) Regenerated maize plantlet.



### Conclusions

1. M1 medium (N6 basal medium supplemented with proline, silver nitrate, casein hydrolyzate and Dicamba or 2,4-D) is efficient to induce the formation of Type I (4 mg.L<sup>-1</sup> Dicamba) and Type II callus (10 mg.L<sup>-1</sup> 2,4-D) from immature somatic embryos of the tropical maize elite line L3.
2. The optimal 2,4-D concentration to induce Type II embryogenic callus from various maize genotypes is around 2 mg.L<sup>-1</sup>; here it was shown that a much higher concentration is needed to induce Type II callus formation in immature embryos from the tropical line L3 (10 mg.L<sup>-1</sup>). It would be interesting to test whether different concentrations of 2,4-D supplementing the M1 medium would be sufficient for the development of embryogenic calli in others tropical maize genotypes.
3. The presence of CuSO<sub>4</sub> in the culture medium increases the maturation of Type II callus and the regeneration of maize plants.

### Acknowledgements

To Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) for financial support. To Coordenação de Aperfeiçoamento Pessoal de Nível Superior (Capes) for scholarship support of RAVS.

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