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Dicamba Growth Regulator Promotes Genotype Independent Somatic Embryogenesis from Immature Zygotic Embryos of Tropical Maize Inbred Lines

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Abstract: Maize is one of the most important cereal crops in Sub-Saharan Africa and an important source of energy for humans. However, the difference in the dedifferentiation frequency of immature embryos among various genotypes indicates that callus induction and genetic transformation is dependent on the genotype. This phenomenon is an impediment in the fundamental process of improving tropical maize germplasm especially through genetic engineering. Here, five tropical maize (*Zea mays* L.) genotypes, CML 216, CML 144, A 04, E 04 and TL 21, were evaluated for callus induction on MS medium supplemented with the growth regulator dicamba. Embryogenic and non embryogenic callus induction was independent of genotype when young immature embryos, 12 days after pollination (DAP) were used for tissue culture in combination with dicamba. The optimal concentration of dicamba for induction of embryogenic callus in all the genotypes was 3 mg/L, which was also the concentration at which non embryogenic callus formation was lowest. The frequency of embryogenic callus induction ranged from 35% to 79% among the five genotypes and somatic embryos regenerated R_0 shoots that produced normal R_1 progenies. This regeneration method is expected to facilitate the development of a more efficient genotype independent *Agrobacterium*- mediated transformation system for tropical inbred lines.

Key words: Tropical maize, genotype independent, dicamba, somatic embryogenesis.

1. Introduction

Tropical maize is a major commodity in sub-Saharan Africa and Latin America agriculture and a major source of income for the poor resource populations [1]. Though protocols are available for embryogenic calli-mediated tropical maize regeneration, they are mostly variety-dependent [2-5], a phenomenon that has been an impediment to the elemental process of efficient regeneration and breeding of tropical maize lines for agronomic traits through genetic engineering.

During dedifferentiation of the maize immature embryos into callus tissue, the cells acquire high energy charge due to enrichment of pyruvate, glycolysis and gluconeogenesis metabolic pathways [6]. The improvement in embryo cell number and quality as a result of ectopic expression of *Brassica napus* Shoot Meristemless (STM) (BnSTM) was linked to the increased pyrimidine and purine salvage activity

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during the early phases of embryogenesis and the enlargement of the adenylate pool (ATP + ADP) required for the active growth of the embryos. This was as a result of an increase in transcriptional and enzymatic activity of several salvage enzymes, including adenine phosphoribosyltransferase (APRT) and adenosine kinase (ADK) [7]. A number of genes have been found to be expressed during embryogenesis. Genes involved in amino acid and carbohydrate transport and metabolism, cell wall and cell membrane biogenesis and signal transduction mechanism were significantly changed during the dedifferentiation of maize immature embryos [6]. A Somatic Embryogenesis Receptor Kinase (SERK) [8], isoperoxidase, esterase and malate dehydrogenase isoenzymes [9] and higher levels of the primary amine, ethylamine gene [10] have been linked to somatic embryogenesis in many plant species. Allelic variation in some of the above-mentioned genes might explain the difference in dedifferentiation frequency of maize embryos among various genotypes that may cause genetic transformation and callus induction to be genotype dependent [6]. In particular, some tropical maize inbred genotypes fail to induce embryogenic calli and their recalcitrance can only be overcome by a single cross hybrid with better responding genotypes [3].

Induction of somatic embryos is usually promoted by auxins. Exogenously supplied auxins are involved in establishing auxin gradients within plant cells during the induction phase of somatic embryogenesis, essential for initiating dedifferentiation and cell division of already differentiated cells before they can express embryogenic competence [11]. Synthetic auxins normally used are categorized into different classes based on the position of their carboxylic acid moieties on their aromatic rings. The classes include phenoxyalkanoic acids (e.g. 2,4-D), benzoic acids (e.g. dicamba), and the pyridine-carboxylic acids (e.g. picloram) [12]. Among the different auxins, 2,4-D has been most commonly used for somatic embryo induction in tropical maize genotypes [2-4, 13-15], where the response is genotype dependent and often accompanied with negative somaclonal variations. The optimal concentrations for embryogenic callus induction on media containing 2,4-D has been 2 mg/L [3, 5, 16]. Induction of somatic embryos on medium containing dicamba has been reported in well adapted tropical Indian maize inbred lines and South American tropical and subtropical maize genotypes [17]. Media supplemented with dicamba gave a better callusing response in terms of both quality and frequency when compared to 2,4-D in Indian elite tropical maize genotypes [17]. Interestingly, three tropical maize genotypes gave a similar callusing response on MS medium supplemented with dicamba [17], giving an indication of genotype independent response of tropical maize in tissue culture. It is reported that dicamba was superior to 2,4-D in promoting callus induction and production of type II calli, respectively [18]. Indeed, the presence of dicamba in medium reduced the processing of globulin-1 (*Gbl1*)-encoded protein in maize tissue culture [19], pointing to a regulatory role for auxins in the processing of Glb1-encoded polypeptides. In other plant species, thidiazuron (TDZ) was found to have genotype independent effect on callus initiation in sugar beet and rice [20, 21].

Maize inbred line CML 216, CML 144 and TL 21 were chosen because they have previously been used to study somatic embryogenesis in Kenya and hence model for tropical maize regeneration experiments [2, 3, 13]. Genotypes A 04 and E 04 are locally adapted highland maize that grow well in high rain fed environments with superior attributes including good grain quality and varying levels of disease resistance. Here, we report efficient genotype independent callus induction from 12 DAP immature embryos of five maize genotypes using dicamba as a growth regulator. This variety-independent callus induction system may widely be applicable for genetic transformation of tropical maize varieties.

2. Materials and Methods

2.1 Plant Material

Five tropical maize inbred lines, CML 216, CML 144, E 04, A 04 and TL 21 were used in the study. All the genotypes were white seeded. Seeds of the tropical maize (*Zea mays* L.) inbred lines CML 216 and CML 144 were obtained from CIMMYT (Kenya). Local Kenyan tropical inbred lines A 04 and E 04 were supplied by Dr. G.A. Ombakho (Kenya Agricultural Research Institute (KARI), Nairobi, Kenya). TL 21 was supplied by Dr. Jane Ininda, a breeder at KARI. The tropical genotypes were grown under field conditions at Kenyatta University (Nairobi, Kenya) and at KARI in the months of October, November and December 2011. Plants were self pollinated and the whole ears collected 12, 14, 16 to 18 days following pollination as described previously [3].

2.2 Callus Induction and Media Composition

Maize cobs were harvested and washed with 70% (v/v) ethanol for 3 min then rinsed five times with sterilized distilled water. Subsequently, the immature kernels were surface sterilized for 20 min under aseptic conditions using sodium hypochlorite 2.5% (v/v) containing Tween 20 (1 to 2 drops). Immature embryos of 1.0 to 1.5 mm in size were aseptically excised from the surface sterilized kernels under laminar flow and placed with scutellum side up and the embryo axis side down on solid callus induction medium (CIM) (Table 1).

The CIM was based on Murashige Skoog (MS) basal salts and vitamins [22] supplemented with 1 to 5 mg/L dicamba, 0.7 g/L *L*-proline, 30 g/L sucrose, 0.5 g/L MES and 0.85 mg/L silver nitrate ($AgNO_3$) (Table 1). Callus maintenance media included MS salts supplemented with 1 mg/L NAA, 60 mg/L sucrose and 0.7 g/L proline whereas shoot induction medium was based on MS basal salts supplemented with only 30 g/L sucrose. The pH of the media was adjusted to 5.8 prior to autoclaving at 121 °C (108 kPa) for 20 min. Three replicates per treatment were maintained and arranged in a completely randomized design.

Table 1 Media composition for establishing tropical maize tissue culture.

Organic/inorganic supplements	CIM	CMM	SIM
Dicamba (mg/L)	1-5	-	-
NAA (mg/L)	-	1	-
Sucrose (g/L)	30	60	30
L-Proline (g/L)	0.7	0.7	-
$AgNO_3$ (mg/L)	0.85	-	-
pH	5.8	5.8	5.8
Plant agar (g/L)	8	8	8

CIM: callus induction medium; CMM: callus maturation medium; SIM: shoot induction medium.

2.3 Effect of Days after Pollination (DAP)

The effect of DAP on callus induction, precocious germination, necrosis, swelling rhizogenic and dead calli were visually quantified and recorded after immature embryos were incubated in the dark at 28 °C for 2 weeks on CIM. Embryogenic calli were transferred to callus maturation medium (CMM) with the same composition as CIM, but without silver nitrate and an increased sucrose concentration of 60 mg/L to allow for somatic embryo maturation before germination.

2.4 Effect of Genotype on Callus Induction

The effect of genotype on embryogenic and non embryogenic callus induction and multiple shoot induction from immature embryos was studied by culturing immature embryos 12 DAP of the five genotypes on MS medium supplemented with 3 mg/L dicamba. The number of immature embryos forming embryogenic, non embryogenic callus and shoot induction per callus were recorded and compared among all the genotypes.

2.5 Regeneration

Individual clones of matured calli were transferred to hormone free shoot induction medium (Table 1) and cultivated at 28 °C with 16-h light/8-h dark cycle.

The number of shoots formed was recorded after 1 week. Plantlets were transferred to peat moss in 10 cm diameter plastic pots, and covered with a moistened plastic paper, for 3 to 5 days, for gradual acclimatization and hardening in the greenhouse. Hardened plantlets were transferred into 20 L pots containing sterile soil for further development to maturity.

3. Results and Analysis

3.1 Effect of Zygotic Embryo Age on Somatic Embryogenesis

The authors evaluated embryogenic callus induction in five tropical maize genotypes from immature embryos 12 to 18 days after pollination (DAP) ranging from 1 to 1.5 mm in size (Fig. 1A). All cultures were maintained under the same experimental conditions on Murashige and Skoog (MS) medium (Table 1) at 28 °C under white fluorescent light with 16 h photoperiod. Callus initiation from cultured embryos was observed within 1 week following culture with swelling and subsequent formation of mass at the embryo axis side (Fig. 1B). Within two weeks of inoculations, precocious germination was observed on most of the embryos harvested at 14, 16 and 18 DAP and was not observed on embryos harvested 12 DAP at 3 mg/L dicamba (Fig. 2). 12 DAP was determined as the developmental stage for immature zygotic embryos to induce the highest frequency of embryogenic callus at all the growth regulators concentrations (Fig. 2). One way ANOVA revealed that the zygotic embryo age had a significant effect on precocious germination (P = 0.008) and somatic embryogenesis (P = 0.001) but no effect on non-embryogenesis (P = 0.395). In addition to precocious germination, immature embryos collected 14, 16 and 18 DAP displayed other phenotypic characteristics observed in tissue culture including swelling of the immature embryo that becomes unresponsive, pronounced rhizogenecity (Fig. 1C) and in some the swollen mass developed into an irregular callus which turned organogenic (Fig. 1D). The calli generated were maintained on CIM medium for at least for 4 weeks before transferring to callus maturation medium (CMM) (Table 1). During the transfer of calli to SIM, necrosis was observed in some calli, which eventually died. Somatic embryos on SIM (Fig. 1E) markedly prematured by turning green in color and eventually the leaves temporary coiled during shooting (Fig. 1F). Within three week on SIM, clonal plantlets germinated (Fig. 1G) and eventually transferred to the field (Fig. 1H) with no somaclonal variation observed.

3.2 Effect of Genotype on Callus Induction

Immature embryos harvested at 12 DAP induced type II embryogenic callus within 15 days following culture in all the genotypes (Fig. 1B and Fig. 3). A distinction between embryogenic and non-embryogenic callus was performed on the basis of visual observation of calli external characteristics using a light microscope. Embryogenic type II callus were globular (Anami et al., 2010), transparent and hard. These characteristics were used to determine percent somatic embryogenesis. The frequency of callus induction ranged from 35% for TL 21 to 79% for CML 216. Genotypes E 04, CML 144 and A 04 were intermediary (66%, 73% and 74%, respectively) (Table 2). Quantitative analysis of somatic embryogenesis induced from immature embryos harvested 12 DAP from all the genotype and cultured at 3 mg/L dicamba showed that genotype influenced callus induction (P < 0.05, Fig. 3), though in this study, TL 21 genotype is outrightly a recalcitrant KARI line [13] and CML 216 is a good responding line in tissue culture [3]. One way ANOVA using OriginLab data analysis and graphing software showed that callus induction data was significantly drawn from a normally distributed population and that the population mean were significantly different (P < 0.05). However, Tukeys analysis showed that 91% of the callus induction difference of the group means was not significant (P =

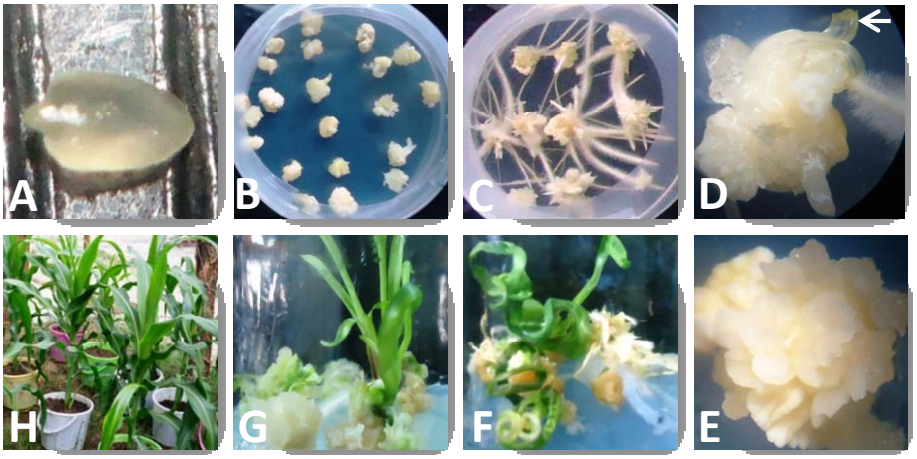


Fig. 1 Somatic embryogenesis from the surface of somatic embryos on MS medium with dicamba and plant regeneration of tropical maize CML 144 genotype. (A) Immature embryo size (1 mm) used in tissue culture (12 DAP). (B) Embryogenic type II calli of CML 144 genotype on CMM. (C) Rhizogenic calli derived from immature embryos. (D) Arrows show organogenic calli derived from immature embryos. (E) Proliferating embryogenic calli from tropical maize immature embryos, 2 weeks after culture on CMM. (F) Regenerating coiled plantlets one week on SIM without dicamba. (G) Plantlets on regenerating medium three weeks after culture. (H) R_1, plants growing in the field.

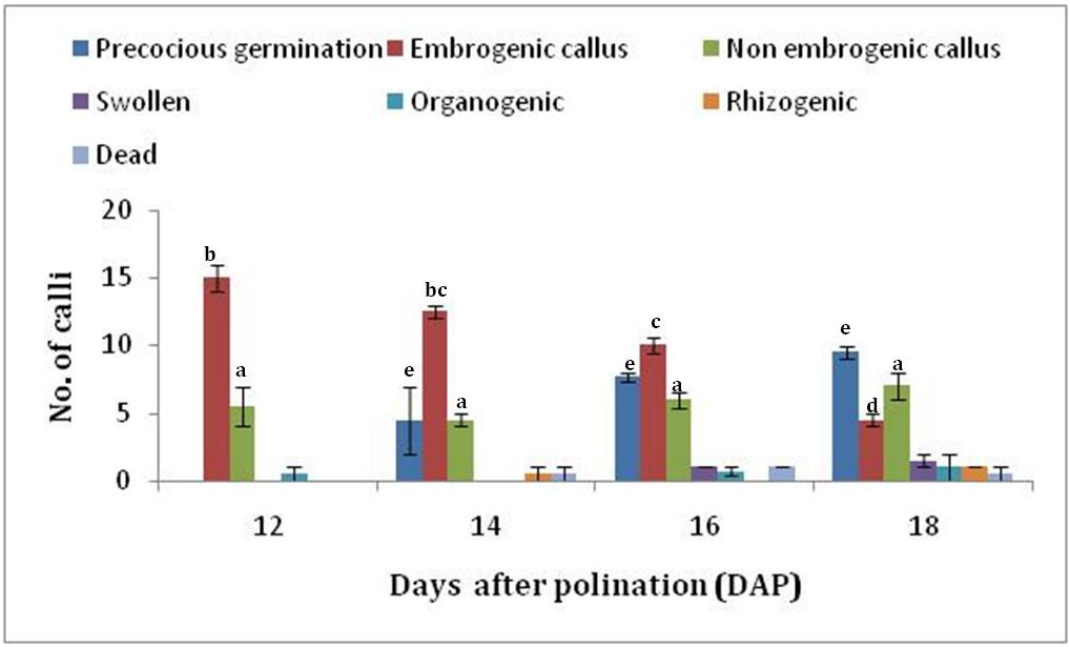


Fig. 2 Effect of DAP on embryogenesis at 3 mg/L dicamba in CML 144. Color interpretation is indicated in the legend at the top. The values are means ± standard error of three replicates per treatment. Values with the same letter are not significantly different by Tukey's pair-wise comparison ($P < 0.05$). Each parameter was statistically analyzed independently from the other.

0) suggesting genotype independent callus induction response. Only 9% of the callus induction difference of the group mean was significantly different at the 0.05 level. Analysis by two-way ANOVA revealed a statistical lack of interaction between genotype and dicamba concentrations for type II embryogenic calli induction ($P = 0.65$) and for non embryogenic calli induction ($P = 0.878$) for immature embryos harvested 12 DAP and cultured at 3 mg/L dicamba concentration, supporting the concept that embryogenic and non embryogenic calli induction on dicamba is independent of genotype.

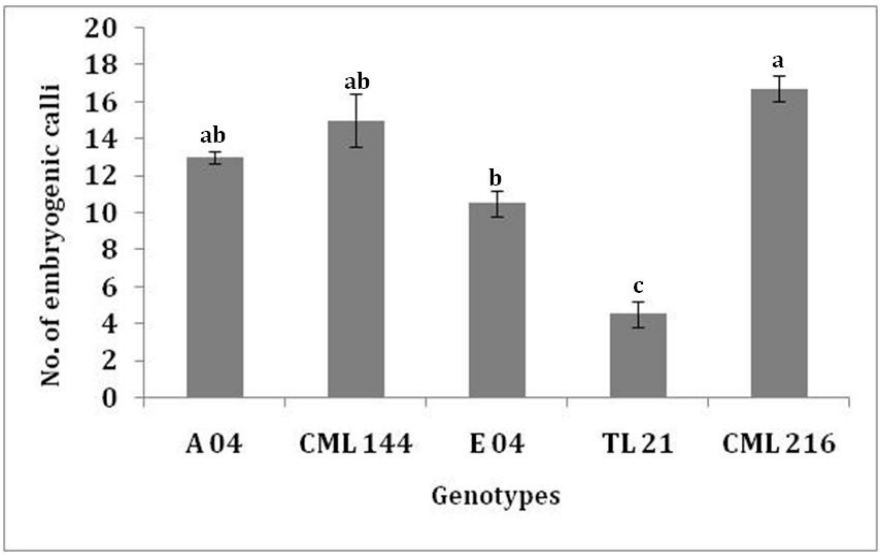
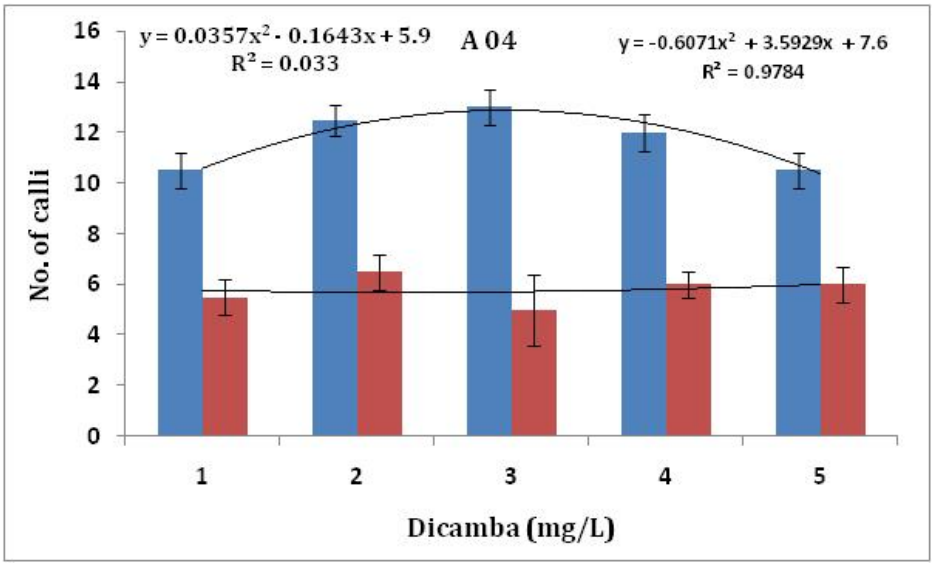


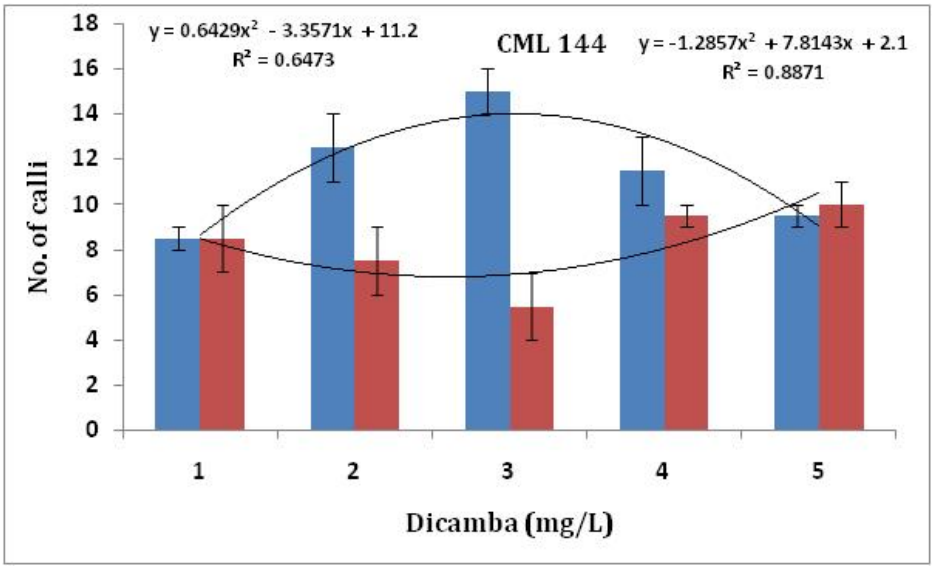
Fig. 3 Average number of type II embryogenic calli from three replicates per treatment induced from immature embryos harvested at 12 DAP in A 04, CML 144, E 04, TL 21 and CML 216 genotypes cultured at 3 mg/L dicamba concentration. Values with the same later are not significantly different by Tukey's pairwise comparison ($P < 0.05$).

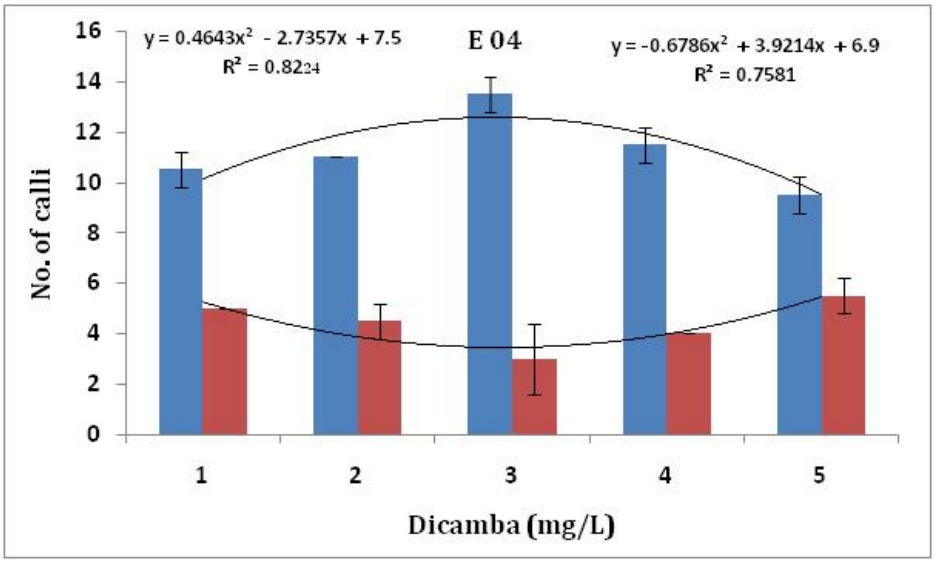
3.3 Effect of Dicamba on Induction of Regenerative Calli

In this study, somatic embryogenesis from immature embryos harvested 12 DAP from all the five genotype seemed independent of genotype and this prompted us to test embryogenesis and non embryogenesis of tropical maize genotypes grown in Kenya on MS medium supplemented with dicamba in order to designate the optimal concentration of the growth regulator for embryogenic callus induction. Callus induction was investigated on callus induction medium (CIM) supplemented with dicamba concentrations ranging from 0, 1, 2, 3, 4 to 5 mg/L. After 5 to 7 days of tissue culture, the scutellum tissue increased in size, 2 weeks later, the coleoptile emerged from the embryo axis site in a number of the immature embryos (precocious germination). Thereafter, the embryo axis site becomes swollen and develops into an irregular callus mass (Fig. 1E) [3]. Finally, embryogenic and non embryogenic calli then begins to form in all the five genotypes. The formation of embryogenic calli increased as the level of dicamba increased in the medium up to 3 mg/L as shown in (Fig. 4). The number of immature embryos forming embryogenic calli was highest at 3 mg/L dicamba. A significant correlation between dicamba concentration and embryogenic calli formation was observed from regression R^2 values of 0.887 for CML 144, 0.8414 for CML 216, 0.9784 for A 04, 0.7453 for TL 21 and 0.7581 for E 04, indicating that the influence of dicamba on somatic embryogenesis process in tropical maize is independent of genotype (Fig. 4 R^2 values on the right).

Interestingly, the number of immature embryos forming non-embryogenic calli was lower than for those forming embryogenic calli at each dicamba concentrations except TL 21 genotype. In this genotype, the number of immature embryos forming non embryogenic calli was high at all dicamba concentrations (Fig. 4 and Table 2). On the other hand, the number of immature embryos forming non-embryogenic calli decreased with the increase in growth regulator concentrations up to 3 mg/L. The number of immature embryos forming non embryogenic calli on medium with dicamba above 3 mg/L increased with the increasing dicamba concentrations (Fig. 4 and Table 2). A correlation between dicamba concentration and non-embryogenic calli formation was observed from regression R^2


y = 0.0357x² - 0.1643x + 5.9
R² = 0.033
A 04
y = -0.6071x² + 3.5929x + 7.6
R² = 0.9784
No. of calli
Dicamba (mg/L)


y = 0.6429x² - 3.3571x + 11.2
R² = 0.6473
CML 144
y = -1.2857x² + 7.8143x + 2.1
R² = 0.8871
No. of calli
Dicamba (mg/L)


y = 0.4643x² - 2.7357x + 7.5
R² = 0.8224
E 04
y = -0.6786x² + 3.9214x + 6.9
R² = 0.7581
No. of calli
Dicamba (mg/L)

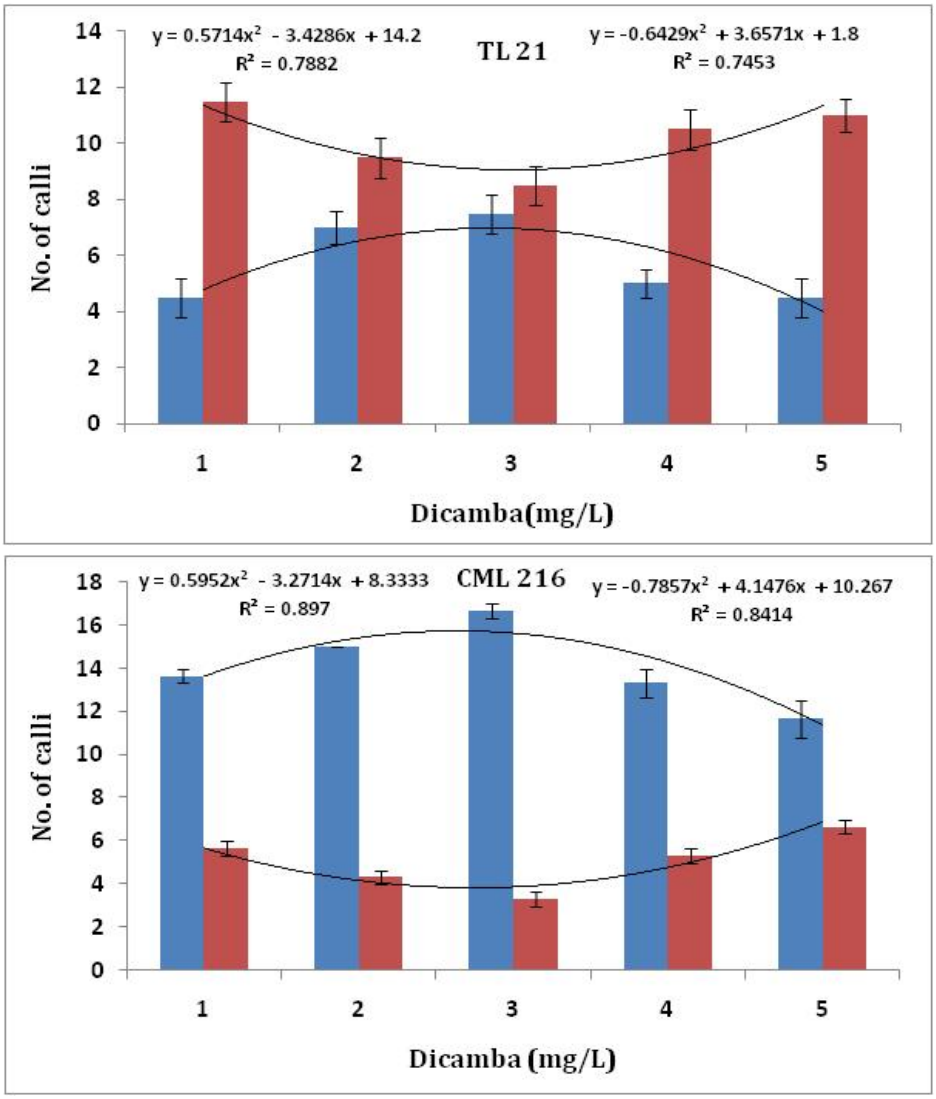


Fig. 4 Effect of dicamba on somatic embryogenesis and non-embryogenesis in five tropical maize genotypes CML 144, CML 216, TL 21, E 04 and A 04. Red bars: Genotype independent hyperbolic functions of the number of immature embryos forming non embryogenic calli; Blue bars: Genotype independent parabolic function of the number of immature embryos forming embryogenic calli. Shown on the left of each graph are the regression values for the non embryogenesis and to the right the regression values of the embryogenic calli formation. The values are means ± standard error.

Table 2 Percentage embryogenic and non embryogenic callus induction in tropical maize genotypes on MS medium supplemented with dicamba (% callus induction ± standard error).

Genotypes	Dicamba, mg/L									
	1		2		3		4		5	
	E	N.E	E	N.E	E	N.E	E	N.E	E	N.E
CML 144	45.94±0.49	45.94±1.49	60.97±1.49	36.58±1.49	**73.17±0.99**	26.82±1.49	54.76±1.49	45.23±0.49	48.71±0.49	51.28±0.99
A 04	63.63±2.4	33.33±0.49	67.56±1.49	35.13±0.49	**74.28±0.0**	28.57±1.99	57.14±0.99	28.57±0.99	52.5±0.49	30±1.99
CML 216	67.21±0.33	27.86±0.33	73.77±0.0	21.31±0.33	**79.36±0.33**	15.87±0.33	65.57±0.66	26.22±0.33	58.33±0.88	33.33±0.33
E 04	52.5±0.49	25±0.0	53.65±0.0	21.95±0.49	**65.85±0.49**	14.63±0.99	53.48±0.49	18.60±0.0	42.22±0.49	24.44±0.49
TL 21	22.5±0.49	57.5±0.49	32.55±0.0	44.18±0.49	**34.88±0.49**	39.53±0.49	23.25±0.0	48.83±0.49	20.45±0.49	50±0.0

E: Embryogenic calli; N.E: Non embryogenic calli; Values in bold show the highest level of somatic embryogenesis at 3 mg/L dicamba.

values of 0.6473 for CML 144, 0.8970 for CML 216, 0.7882 for TL 21 and 0.8824 for E 04, indicating that the influence of dicamba on non embryogenesis in these tropical maize is independent of genotype (Fig. 4, R^2 values on the right) except A 04 which was genotype dependent (Fig. 4, $R^2 = 0.033$).

The number of immature embryos forming embryogenic calli was lower at concentrations of dicamba higher than the 3 mg/L compared to those forming non-embryogenic calli. Those forming non embryogenic calli reduced with increase in the concentrations of dicamba up to 3 mg/L, and then increased with further increase in the concentrations of the growth regulator (Fig. 4 and Table 2). Dicamba at 3 mg/L was therefore considered the optimal concentration for genotype independent embryogenic calli induction (or genotype independent inhibition of non embryogenic calli formation) (Fig. 4 and Table 2).

3.4 Quantitative Analysis of Regeneration

A higher number of shoots per callus were regenerated at the optimal dicamba concentration for all the genotypes except E 04. TL 21 genotype had the highest number of shoots that were produced via organogenesis and were not clonal at the optimal callus induction (Table 3). Regeneration started with the appearance of green coloration on mature embryos within 4-5 days on growth regulator free regeneration medium supplemented with 30% sucrose under continuous illumination. Green shoots formed within 1 week of culture (Fig. 1F). Mature embryos from CMM produced shoots that coiled and eventually developed into normal shoots (Fig. 1F and G). The coiling could have been impacted by the growth regulator dicamba since this phenotype has not been observed in regenerants produced on media supplemented with 2,4-D growth regulator [3]. Indeed analysis of regeneration data by ANOVA showed that genotype significantly influenced regeneration more ($P = 0.003$) than dicamba ($P = 0.012$) though still significantly. The *in vitro* rooted plants were acclimatized by transfer to peat moss-containing pots covered with plastic sheets to maintain high moisture conditions for 3 days. The moisture was gradually reduced over a period of 15 days and plantlets were transferred to large soil-filled 20-liter pots in the open field.

4. Discussion

Green and Phillips [23] were the first to report plant regeneration from tissue culture of maize via somatic embryogenesis which was confirmed microscopically in Refs. [24, 25]. Since then, many published reports are available for tropical maize suggesting successful callus induction and regeneration from immature embryos [3-5, 13, 14, 26] as well as mature embryos [26]. However, callus induction and regeneration either on N6, MS or LS medium supplemented with 2,4-D growth regulator, has strongly been genotype dependent. It is reported that N6 medium supplemented with dicamba gave efficient callus induction and plant regeneration in tropical and

Table 3 Regeneration of tropical maize genotypes calli after culture on MS medium supplemented with dicamba (Mean number of shoots per callus piece (% regeneration efficiency*)).

Genotypes	Dicamba, mg/L				
	1 shoot/calli	2 shoots/calli	3 shoots/calli	4 shoots/calli	5 shoots/calli
CML 144	2/6 (33)	4/7 (60)	5/6 (84)	2/6 (33)	3/6 (50)
A 04	2/6 (33)	3/6 (50)	3/6 (50)	2/5 (40)	2/5 (40)
CML 216	2/6 (33)	5/6 (84)	5/6 (84)	3/6 (50)	3/6 (50)
E 04	2/6 (33)	3/8 (40)	1/8 (18)	1/8 (13)	1/7 (14)
TL 21	7/18 (39)	8/17 (50)	6/10 (60)	5/10 (50)	6/12 (50)

* Regeneration efficiency was computed as total number of shoots produced over total number of calli taken to shoot induction medium × 100).

subtropical maize germplasm [26]. It is also suggested that callus obtained from immature embryos in the presence of dicamba developed into somatic embryos at a higher frequency than the callus obtained with 2,4-D [27]. Furthermore, it is showed that three tropical maize genotypes produced nearly similar callus induction response when cultured on N6 medium with dicamba [17]. MS salts have recently been reported to improve *Agrobacterium* mediated transformation of temperate maize [16]. Therefore, efforts were made to promote callus induction in a genotype independent way in tropical maize genotypes adapted to Kenya using MS media supplemented with different concentrations of the dicamba growth regulator.

Between 8 and 20 days after pollination (DAP), immature embryos provide several discrete developmental stages of meristematic tissue that exhibit varied competence to callus induction and *Agrobacterium* infectivity. Immature embryos at the right developmental stage and size (1 to 1.5 mm) are optimal for successful callus induction and transformation in temperate maize [28]. In tropical maize genotypes, calli have been induced from immature embryos harvested 11 to 21 days after pollination and this has been predominantly genotype dependent [3, 4, 14, 29]. Embryos harvested 14, 16 and 18 DAP germinated at the expense of forming embryogenic calli. This precocious germination is accompanied by reduction in maturation association gene expression during zygotic embryogenesis. The reduction is attributed to lack of endogenous ABA production or sensitivity to external ABA and absence of expression of *Viviparous-1* (*Vp1*) gene [30]. Zygotic embryo maturation is associated with the accumulation of 7S globulin storage protein [31] and a variety of late embryogenesis abundant proteins (LEAs) [32]. Expression of *Vp1* gene is required to prevent maize zygotic embryo germination and in *vp1* mutants, the expression of a 7S globulin gene is blocked [30, 31]. *Vp1* expression declines in cultured embryos [33], which might potentiate precocious germination.

It is also likely that immature embryos collected 14, 16 and 18 DAP had elevated levels of gibberellins [34] and were insensitive to external growth regulator dicamba, which could further contribute to precocious germination. This factor and the likely lack of expression of *Vp1* might have potentiated precocious germination. Precocious germination was not observed on embryos harvested 12 DAP because embryos at this stage have apparently not yet developed the competence to germinate. Immature embryos harvested 12 DAP induced type II embryogenic calli within 15 days following culture in all the genotypes suggesting embryos 12 days after pollination consisted the right developmental stage for embryogenic calli induction that is genotype independent. This data is in line with recent findings that the immature embryo at 12 days after pollination (DAP) showed the highest competence for embryogenesis [35]. Thus, the embryogenic genotype independent responses of immature embryos at 12 DAP may be a pointer to understanding the induction of somatic embryogenesis in tropical maize. Genes associated with the formation of embryonic calli may offer additional insights into the mechanism of somatic embryogenesis. Further research on these genes may determine their role in increasing the rate of induction of embryonic calli.

In tissue culture, the variation in embryogenic calli formation within varieties has been attributed to many factors, of which genotype is the most important [23]. It is for this reason that a protocol for callus induction universally adaptable to a number of tropical maize genotypes is yet to be developed. In this study, genotype independent embryogenic and non embryogenic callus induction in five tropical maize genotypes of sub-Saharan Africa on MS medium has been demonstrated. The results point to the fact that embryogenic and non embryogenic pathways in tropical maize studied are clearly distinct and could be

controlled by different genetic elements or regulators [35]. Tropical maize genotypes have been reported to vary greatly in embryogenic capacity reflecting differences in their ability to activate key elements/genes of the embryogenic pathway. Our data suggest that at 3 mg/L dicamba, key elements of the embryogenic genetic program are activated and may have an inhibitory effect on key elements of the non embryogenic program or their expression is repressed [36] for instance by *ZmSERK* genes [35].

5. Conclusion

In this work, the authors report genotype independent somatic embryos formation on MS medium containing dicamba. In all the genotypes studied herein, dicamba effectively induced somatic embryos from immature zygotic embryos. The optimal concentration for somatic embryogenic callus induction was 3 mg/L in all the genotypes tested. These results indicate a new phenomenon in tropical maize tissue culture where somatic embryogenic, callus induction and non embryogenic callus inhibition seem to be genotype independent on medium containing the auxin dicamba contrary to those induced by 2,4-D. The underlying biochemical/signaling pathways seem to operate distinctly for the two pathways as already reported for 2,4-D [35]. The growth regulator dicamba, however, seems to allow for the rare dissection of the specific cellular events related to the overlapping phases of dedifferentiation, cell cycle reactivation and the acquisition of embryogenic competence in sub-Saharan tropical maize that could not be achieved with 2,4-D [2, 3, 5].

In some Indian elite tropical maize, it is noted a similar independent interaction between auxin treatment and genotypes when using both 2,4-D and dicamba as well, on N6 basal medium [37, 38]. The absolute requirement for exogenous auxin to maintain callus cultures in plant cells is complemented by production of substantial amounts of the native auxin indole acetic acid (IAA) within the cells [36]. Therefore, the application of exogenous auxin and subsequent elevation (fluctuations) of endogenous auxin are both determining factors in the induction of callus tissue and somatic embryogenesis [35, 36]. The implication from this study is that the interaction of the endogenous auxin (IAA) in tropical maize tissues with the exogenous synthetic auxins such as 2,4-D and dicamba may be varying with the type of synthetic auxin, as genotype independent somatic embryogenesis occurred with dicamba yet it has been genotype dependent with 2,4-D.

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