EFFECT OF MEDIA, GENOTYPE AND AGE OF EXPLANT ON CALLUS INDUCTION THROUGH INDIRECT SOMATIC EMBRYOGENESIS IN MAIZE (ZEA MAYS L.)

S.T. Akinyosoye, M.O. Balogun, J.A. Adetumbi, O.D. Amusa, M.O. Olowolafe, & D.J. Ogunniyan

Abstract

Physiological age of explants, basal salt composition and genotype were observed to affect callus formation and plantlet regeneration as means of protocol optimization for indirect somatic embryogenesis. This study examines the efficacy of two basal salt formulations, age of explants and genotype on callus induction and somatic embryogenesis in maize. Excised embryos from surface sterilized kernels harvested at 7, 14, and 21 days after pollination (DAP) were used as explants to initiate callus on varied concentration of Murashige and Skoog basal (MS) and Nitsch (N6) induction media supplemented with 30g/l sucrose, 8 g/l agar, 0.1g/l myo-inositol and 2 mg/l 2,4-D. Results reveals that physiological age of the explant and maize genotypes had significant effect for most of the traits studied. Significant differences were observed among the basal salt media used and their interactions. High degree of callusing was recorded at 14 days after pollination (DAP) while 21 DAP showed high degree of shoot formation, root formation and leaf formation. Both full MS and ½ MS show high degree of callusing and shoot formation while ½ N6 medium favoured callus initiation. Best callus growth was achieved in genotype TZMi-757 at 14 DAP on full MS medium. This protocol for callus induction of these maize genotypes provides a basis for development of genetic transformation to enhance genetic improvement of priority traits.

Keywords: somatic embryogenesis, explants age, basal medium, maize, tissue culture

Introduction

Maize (Zea mays L.) is the third most important cereal crop after wheat and rice in terms of production in the world (IITA, 2009). It is a major cereal crop for livestock feed, human nutrition and important raw material for several agro-based industries in Nigeria (Akande and Lamidi 2006). The production of this crop is on the decrease due to increase population, limited land, environmental and biotic stresses.

Over the years, conventional breeding has been used to develop new varieties with traits that are amenable to these challenges. This has resulted in development of modest increments in yields and improved agronomic characteristics such as disease resistance and drought adaptability in different agroecological zones, as well
as traits such as enhanced levels of macro and micro nutrients (Machuka, 2004). For recalcitrant traits for which improvement through classical breeding holds little promise, molecular breeding methods involving marker assisted selection and genetic transformation via somatic embryogenesis now provide viable alternatives in several crops, including maize (Bruce et al., 2002). However, the pre-requisite for crop genetic transformation is the existence of a reliable plant regeneration system. In cereals such as wheat, barley and maize, immature embryos have been the favourite explant for in vitro culture and plant regeneration (Green and Phillips, 1975; Ray and Ghosh, 1990). It is necessary to further improve regeneration protocols for use in maize improvement programmes to ensure sufficient production (Machuka, 2001). So far almost all maize tissue culture and genetic transformation involves the use of immature zygotic embryos as an explants source for regeneration (Danson et al., 2006; El-Itribi et al., 2003).

Somatic embryogenesis is a developmental process by which somatic cells undergo restructuring to generate embryogenic cells. These cells then go through a series of morphological and biochemical changes that result in the formation of somatic (non-zygotic) embryos capable of regenerating plantlets. Somatic embryogenesis represents a unique developmental pathway that includes a number of characteristic events: dedifferentiation of cells, activation of cell division and reprogramming of their physiology, metabolism and gene expression patterns (Zimmerman, 1993; Komamine et al., 2005). Somatic embryogenesis plays an important role in clonal propagation. When integrated with conventional breeding programs and molecular and cell biological techniques, somatic embryogenesis provides a valuable tool to enhance the genetic improvement of commercial crop species (Stasolla and Yeung, 2003). Among them the genotype and nutrient composition are regarded to be the major sources of variation in in vitro cultures (Khanna and Raina 1998). The combination of minerals required by a particular plant species is usually determined by the empirical manipulation of one or a combination of existing published nutrient medium formulations. Often, only one medium type is used for the duration of culture, though this formulation may not be optimal for the different stages of explants growth and development (Carl and Richard, 2001). However, one of the pre-requisites for genetic improvement of crop plants through genetic transformation is the availability of a reliable protocol for regeneration (Yadav and Padmaja, 2003). Keeping in view the above observations, this study was undertaken to investigate the effect of different basal media, genotypes and age of explants on callus induction in maize with a view to developing protocols for regeneration.

Materials and Method
The experiment was conducted at Biotechnology Laboratory of Institute of Agricultural Research and Training
Five maize genotypes (TZM1-757, TZM1-765, TZM1-759, TZSTR-195 and 450 STR) were obtained from the gene bank of I.A.R.T, Ibadan. Seeds of the five maize genotypes were sown in the field and plants to be used for callus induction were selfed. Seven (7), fourteen (14) and twenty one (21) days after pollination (DAP), cobs were harvested. The seeds were washed with Tween20 under running tap water. They were then disinfected in 70% methylated spirit, 0.1% and 0.2% mercuric chloride respectively and rinsed in three changes of sterile distilled water. Immature embryos were excised from seeds aseptically using forceps in the laminar flow hood and cultured on Murashige and Skoog basal medium (1962) and N6 basal medium (Chu et al. 1975) each modified by adding full and half concentrations of ammonium nitrate and ammonium sulphate respectively. Each was further supplemented with 30g/l sucrose, 2 mg/l 2,4-D and 8 g/l agar. However, MS medium contained 0.1g/l myo-inositol while N6 medium contained 0.05g/l myo-inositol. The size of immature embryos ranged from 0.3mm to 1.8mm.

| Table 1: MS and N6 modified media modified with 2mg/l 2, 4-D and 2 nitrogen regimes |
|-------------------------------|----------------|----------------|----------------|----------------|
| Salts                         | Media          | Full strength | ½ strength     | th             |
| NH₄NO₃                       | MS             | 1650           | 825            |                |
| KNO₃                          | MS             | 1900           | 950            |                |

\[(\text{NH}_4)_2\text{S} \quad \text{N6} \quad 463 \quad 231.5\]
\[\text{O}_4 \quad \text{N6} \quad 2830 \quad 1415\]
\[\text{KNO}_3 \quad \text{N6} \]

Note: Amounts are in mg/l

The cultures were maintained for six weeks during which data were collected on fresh callus weight (mg), days to callus initiation, number of leaves, callus length, callus width; longest distance between one end of the callus and the other in the petri plate was taken as the length, while the shortest distance was taken as the width in millimetre (Akande et al. 2009). The degree of callus, shoot and root formation respectively was recorded as the fraction of the cultured explant on which callus had been induced on a scale of: 0: no callus, 1: lowest (up to 20%), 2: lower (21 to 40%), 3: medium (41 to 60%), 4: high (61 to 80%) and 5: highest (81 to 100%).

**Data Analysis**

Analysis of Variance (ANOVA) was performed on data collected using IBM SPSS version 21.0 software. Difference between treatments was separated by the Duncan Multiple Range test (DMRT) at 5% and 1% levels of significance.

**Results and Discussion**

**Variations in callus induction and plantlet regeneration traits**

Genotype mean squares were significantly different for days to callus initiation, callus formation, shoot formation and root formation, but not significant for callus weight, callus length, callus width, and number of
as traits such as enhanced levels of macro and micro nutrients (Machuka, 2004). For recalcitrant traits for which improvement through classical breeding holds little promise, molecular breeding methods involving marker assisted selection and genetic transformation via somatic embryogenesis now provide viable alternatives in several crops, including maize (Bruce et al., 2002).

However, the pre-requisite for crop genetic transformation is the existence of a reliable plant regeneration system. In cereals such as wheat, barley and maize, immature embryos have been the favourite explant for in vitro culture and plant regeneration (Green and Phillips, 1975; Ray and Ghosh, 1990). It is necessary to further improve regeneration protocols for use in maize improvement programmes to ensure sufficient production (Machuka, 2001). So far almost all maize tissue culture and genetic transformation involves the use of immature zygotic embryos as an explants source for regeneration (Danson et al., 2006; El-ltriby et al., 2003).

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Materials and Method
The experiment was conducted at Biotechnology Laboratory of Institute of Agricultural Research and Training
(L.A.R&T), Obafemi Awolowo University, Moor Plantation, Ibadan, Nigeria. Five maize genotypes (TZMi-757, TZMi-765, TZMi-759, TZSTR-195 and 450 STR) were obtained from the gene bank of L.A.R&T, Ibadan. Seeds of the five maize genotypes were sown in the field and plants to be used for callus induction were selfed. Seven (7), fourteen (14) and twenty one (21) days after pollination (DAP), cobs were harvested. The seeds were washed with Tween20 under running tap water. They were then disinfected in 70% methylated spirit, 0.1% and 0.2% mercuric chloride respectively and rinsed in three changes of sterile distilled water. Immature embryos were excised from seeds aseptically using forceps in the laminar flow hood and cultured on Murashige and Skoog basal medium (1962) and N6 basal medium (Chu et al. 1975) each modified by adding full and half concentrations of ammonium nitrate and ammonium sulphate respectively. Each was further supplemented with 30g/l sucrose, 2 mg/l 2,4-D and 8 g/l agar. However, MS medium contained 0.1g/l myo-inositol while N6 medium contained 0.05g/l myo-inositol. The size of immature embryos ranged from 0.3mm to 1.8mm.

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Results and Discussion
Variations in callus induction and plantlet regeneration traits

Genotype mean squares were significantly different for days to callus initiation, callus formation, shoot formation and root formation, but not significant for callus weight, callus length, callus width, and number of
leaves (Table 2). However, age differences were significant for all traits. Significant differences were observed among the media in callus weight, and root formation, and days to callus initiation.

Genotypes by age interaction were significant for callus width, callus formation, shoot formation, callus weight, callus length, root formation and number of leaves but not significant for other traits (Table 2). Interaction between variety and basal media were significant for callus length, callus width and root formation (Table 2). Also, age by basal media interactions were significant for root formation and callus weight. However, differences among genotype×Age×media were only significant for root formation (Table 2). Results obtained corroborate the findings of Carvalho et al., (1997) who reported that there was a significant interaction between genotype and callus induction medium, showing that different genotypes respond differently to the same type of medium.

**Indirect somatic embryogenesis**
Immature embryos (7-21DAP) were placed on MS and N6 media with their embryo axis in contact with media and embryos initiated callus within 24 hours of inoculation. This was due to the presence of meristematic cells in the scutellum. Undifferentiated mass of cells (callus) were observed on the surface of scutellum. Al-Abed et al., (2006) reports the presence of the meristematic cells in the scutellum of maize embryos from which callus was induced. The results of this study show that the presence 2 mg/l of 2,4-D in culture medium is critical for callus induction and embryogenic callus formation from immature embryos which concurred with the findings of Armstrong and Green, (1985); Bohorova et al., (1995); Binott et al., (2008).

Two types of embryogenic callus were formed; type I and II callus. Type I callus was compact, white in colour (Fig.1b), while type II was compact and light yellow (Fig.1a) and type II callus has high capacity to re-differentiate into plantlets than type I over time. The formation of type I and II callus has been reported in maize (Tomes and Smith, 1985; Jiménez and Bangerth, 2001).
Fig. 1: Somatic embryogenesis from immature embryos of maize
(a) Embryogenic callus with somatic embryo (type 1) (b) Embryogenic callus with somatic embryo (type 1)

Genotype specificity of somatic embryogenesis
Maize lines TZMi-757, TZSTR-195 and 450 STR initiated callus a day earlier than TZMi-756 and TZMi-759 after inoculation. TZMi-757 had highest callus weight (18.76mg) followed by TZSTR-195 (17.05mg) and 450 STR (16.14mg), but lowest callus weight was found in TZMi-759 (14.30mg) and TZMi-765 (13.66mg). TZMi-757 had highest callus width (5.13mm) and lowest was found in TZMi-765 (4.21mm) and TZMi-759 (4.26mm). TZMi-757 showed highest frequency of callus formation with 2.04 (40.80%) and callusing was poorest in TZMi-765 with 1.52 (30.4%). TZMi-757 had highest shoot formation, root formation, and number of leaves with 2.49 (49.80%), 0.47 (9.40%) and 0.66 respectively while TZMi-765 had lowest shoot formation, root formation and number of leaves with 1.43 (28.60%), 0.11 (2.20%) and 0.34 respectively (Table 3). These observations showed that there were interactions among genotypes and media. Hence, genetic factors are considered to be a major contributor to the in vitro response of cultured tissues. This corroborates the studies of Lu et al. (1982); Vasil and Vasil (1984); Jakubeková et al. (2011). In addition, Bohorova et al. (1995) and Jakubeková et al. (2011) reported that factors influencing the expression of totipotency in cell culture are: genotype, composition of plant culture medium, growth regulators, age of embryo and embryo size.

Effect of embryo physiological age on callus induction
Callus was induced from embryos excised from ears at 14 DAP (0.8-1.2mm) and 21 DAP (1.3-1.8mm). Explant at 21 DAP initiated callus earlier than 14 DAP but calli were not initiated in 7 DAP (less than 1mm). Highest callus weight was recorded in 14 DAP (29.99mg) followed by 21 DAP (26.79mg). There was no significant difference found in 14 and 21 DAP for callus length and callus width. Explants aged 14 DAP showed better callus formation with 3.47 (69.40%) than 21 DAP 2.78 (55.60%) while 21 DAP showed better performance for shoot formation with 3.81 (76.20%), root formation of 0.76 (15.20%) and number of leaves (1.22) than explants aged 14 DAP 3.43 (68.60%); 0.24 (4.80%) and 0.60 for the same traits respectively (Table 4).

In this study, it was established that the optimal physiological age for callus induction was 14 DAP. Embryo size contributed very significantly to differences in callus induction among inbred lines (Binott et al., 2008). Result obtained corresponds with the work of Jakubeková et al. (2011) who reports that age and size of the immature embryo is a critical factor in determining the capacity of callus initiation from immature embryo.
According to Bohorova et al., (1995), immature embryos of maize less than 0.5 mm in length does not respond in culture, while Lu et al., (1983) and Jakubeková et al. (2011) report similar results for embryo less than 1 mm. Other researchers had obtained the same results where high rates of embryogenic callus formation were obtained from immature embryos collected 14 days after pollination (Rufuz et al., 1992; Rikiishi et al., 2003; Aguado-Santacruz et al., 2011).

**Effects of different basal media and concentrations on callus induction**

Among the different media; ½ N6 medium favoured callus initiation (1 day) earlier than MS medium (2 days). Full MS had highest callus weight (20.28mg), followed by ½ MS (16.36mg), full N6 (13.74mg) and lowest callus weight was found in ½ N6 (12.63mg). Full MS and ½ MS had highest values for callus length, callus width, callus formation and shoot formation respectively, while ½ N6 had lowest values for callus length, callus width, callus formation, shoot formation and root formation (Table 5). Medium composition is one of the most important factors affecting maize tissue culture (Frame et al., 2006; Binott et al., 2008).

Therefore, choice of medium and genotypes are significantly important in embryogenesis. This was consistent with the studies of Lu et al. (1982); Vasil and Vasil, (1995); Binott et al., (2008). Differences observed in the morphogenic efficiencies between MS and N6 media might be attributed to their respective inorganic nitrogen components and the favourable $\text{NH}_4^+/\text{NO}_3^-$ ratio present in the MS basal formulation. The MS medium contains 60 mM of inorganic nitrogen, compared to 35 mM in N6 (Armstrong and Green, 1985), while the ammonium nitrate ratio is approximately 1:2 in MS compared with 1:4 in N6 (Samoylov et al., 1998). These differences observed in inorganic salts was also reported to be important for achieving efficient somatic embryogenesis and plant regeneration in mexican elite barley cultivars (Aguado-Santacruz et al., 2011). Similarly, this study showed that somatic embryogenesis and regeneration in maize is highly genotype specific, as reported by Hodges et al., (1986); Wenbin et al., (2002); Aguado-Santacruz et al. (2007).

Among them the genotype and nutrient composition are regarded to be the major sources of variation in *in vitro* culture (Khanna and Raina 1998; Rafiq et al., 2005).

**Table 2**: Mean square values of callus weight, callus length, callus width, callus and shoot formation parameters and number of leaves of different maize genotypes, media and their interaction

<table>
<thead>
<tr>
<th>SOV</th>
<th>df</th>
<th>Dcallusinit (days)</th>
<th>Calluswt (mg)</th>
<th>Calluslength (mm)</th>
<th>Calusswidt (mm)</th>
<th>Calform (0-5)</th>
<th>Shootform (0-5)</th>
<th>Rootform (0-5)</th>
<th>Noteaves (number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>4</td>
<td>1.08**</td>
<td>82.07ns</td>
<td>3.57ns</td>
<td>4.79ns</td>
<td>1.42**</td>
<td>5.70**</td>
<td>1.04*</td>
<td>0.39ns</td>
</tr>
</tbody>
</table>

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### Table 3: Mean values of the genotypes for callus weight, callus length, callus width, callus and shoot formation parameters and number of leaves

<table>
<thead>
<tr>
<th>Entry No</th>
<th>Lines</th>
<th>Dcallusinit (days)</th>
<th>Callus wt (mg)</th>
<th>Dcallusinit (mm)</th>
<th>Calenht (mm)</th>
<th>Caluswidth (0-5)</th>
<th>Caliform (0-5)</th>
<th>Shootform (0-5)</th>
<th>Rootform (0-5)</th>
<th>Noleaves</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>TZMi-757</td>
<td>1.47b</td>
<td>18.76a</td>
<td>5.60a</td>
<td>5.13a</td>
<td>2.04a</td>
<td>2.49a</td>
<td>0.47a</td>
<td>0.366a</td>
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</tr>
<tr>
<td>2</td>
<td>TZMi-765</td>
<td>1.71a</td>
<td>13.66b</td>
<td>4.98a</td>
<td>4.21b</td>
<td>1.52b</td>
<td>1.43c</td>
<td>0.11b</td>
<td>0.34b</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>TZMi-759</td>
<td>1.54ab</td>
<td>14.30b</td>
<td>4.96a</td>
<td>4.26b</td>
<td>1.80ab</td>
<td>2.04b</td>
<td>0.28ab</td>
<td>0.46ab</td>
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<tr>
<td>4</td>
<td>TZSTR-195</td>
<td>1.38b</td>
<td>b</td>
<td>5.27a</td>
<td>4.65ab</td>
<td>1.69b</td>
<td>2.21ab</td>
<td>0.23ab</td>
<td>0.52ab</td>
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<tr>
<td>5</td>
<td>450 STR</td>
<td>1.37b</td>
<td>16.14a</td>
<td>5.49a</td>
<td>4.70ab</td>
<td>1.79ab</td>
<td>1.88b</td>
<td>0.23ab</td>
<td>0.49ab</td>
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</tr>
</tbody>
</table>

Means with the same letter(s) in the same column are not significantly different from each other at 5% level of probability. Dcallusinit: days to callus initiation, Calluswt: callus weight (mg), Caluswidth: callus width (mm), Calenht: callus length (mm), Caliform: callus formation, Shootform: shoot formation.
formation, Rootform: root formation
and Noleaves: number of leaves

Table 4: Mean values of age of explants for callus weight, callus length, callus width, callus and shoot formation parameters and number of leaves

<table>
<thead>
<tr>
<th>Age (DA P)</th>
<th>Dcallus (days)</th>
<th>Callus wt (mg)</th>
<th>Caleng th (mm)</th>
<th>Calusw (mm)</th>
<th>Calfor m (0-5)</th>
<th>Shootfo rm (0-5)</th>
<th>Rootfo rm (0-5)</th>
<th>Noleaves</th>
</tr>
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<tbody>
<tr>
<td>7</td>
<td>0.00c</td>
<td>0.00c</td>
<td>0.00c</td>
<td>0.00c</td>
<td>0.00c</td>
<td>0.00c</td>
<td>0.00c</td>
<td>0.00c</td>
</tr>
<tr>
<td>14</td>
<td>3.07a</td>
<td>29.99a</td>
<td>9.61b</td>
<td>8.33b</td>
<td>3.47a</td>
<td>3.43b</td>
<td>0.24b</td>
<td>0.60b</td>
</tr>
<tr>
<td>21</td>
<td>2.16b</td>
<td>26.79b</td>
<td>9.07b</td>
<td>8.00b</td>
<td>2.78b</td>
<td>3.81a</td>
<td>0.76a</td>
<td>1.22a</td>
</tr>
</tbody>
</table>

Means with the same letter(s) in the same column are not significantly different from each other at 5% level of probability.

DAP: Days to pollination,
Dcallini: days to callus initiation,
Calusw: callus weight (mg),
Caluswtd: callus width (mm),
Caleng: callus length (mm),
Calform: callus formation, Shootform: shoot formation, Rootform: root formation

Table 5: Mean values of treatments for callus weight, callus length, callus width, callus and shoot formation parameters and number of leaves

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dcallus ini (days)</th>
<th>Callus wt (mg)</th>
<th>Calen gth (mm)</th>
<th>Calusw (mm)</th>
<th>Calfor m (0-5)</th>
<th>Shootfo rm (0-5)</th>
<th>Rootfo rm (0-5)</th>
<th>Noleaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full MS</td>
<td>1.62a</td>
<td>20.28a</td>
<td>5.76a</td>
<td>5.30a</td>
<td>1.94a</td>
<td>2.27a</td>
<td>0.24ab</td>
<td>0.65a</td>
</tr>
<tr>
<td>1/2 MS</td>
<td>1.50ab</td>
<td>16.36b</td>
<td>5.61a</td>
<td>4.98a</td>
<td>1.94a</td>
<td>2.13a</td>
<td>0.11b</td>
<td>0.40a</td>
</tr>
<tr>
<td></td>
<td>13.74a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Full N6</td>
<td>1.50ab</td>
<td>b</td>
<td>5.06ab</td>
<td>4.19b</td>
<td>1.56b</td>
<td>1.87bc</td>
<td>0.35ab</td>
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<td></td>
<td>1.59a</td>
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<td></td>
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<tr>
<td>1/2 N6</td>
<td>1.31b</td>
<td>12.63c</td>
<td>4.39b</td>
<td>3.63b</td>
<td>b</td>
<td>1.74c</td>
<td>0.41a</td>
<td>0.43a</td>
</tr>
</tbody>
</table>

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Dcallini: days to callus initiation,
Calusw: callus weight (mg),
Caluswtd: callus width (mm),
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Calform: callus formation, Shootform: shoot formation, Rootform: root formation

Conclusion
Genotype, medium, and age of embryo had significant effects on callus, shoot and root formation respectively. The study has shown that 14 DAP favoured high callus formation, 21 DAP favoured shoot formation, root formation and leaves formation while among the different media; full MS and 1/2 MS
favoured callus and shoot formation and ½ N6 medium favoured callus initiation. The best callus growth was achieved in TZMi-757 at 14 DAP on full MS medium. This protocol for callus induction of these maize genotypes provides a basis for development of genetic transformation to enhance genetic improvement of priority traits, so as to ensure sufficient maize production and wider adaptation to biotic and abiotic stresses.

Acknowledgement
This paper is dedicated to the memory of late Prof. (Mrs) S.R. Akande (Head, Biotechnology Unit, Institute of Agricultural Research and Training, Obafemi Awolowo University, Ibadan, Nigeria), who died recently may her soul rest in peace.

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