Callus production and plant regeneration in Egyptian maize genotypes

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ABSTRACT

Five elite Egyptian maize inbred lines (Zea mays L.) were evaluated for their in vitro response and regenerability from immature embryos on four different callus induction media combinations. To study the effect of media composition and genotype on type I or [illegible] II callus production and plant regeneration, MS and N_6 media were used with different con[illegible]tions of silver nitrate, dicamba (10 M) chloramben (10 M) and 2,4-D. Lines Gz643 and Gz639 gave the best response on callus induction medium N_6-Ag and the highest regeneration frequency. Immature embryos from the hybrid Sd7xA188 were used to compare the tissue culture response and regeneration frequency of the hybrid with the commercially important line Sd7. The regeneration frequency was significantly improved in the hybrid (Sd7 x A188).

Key words: *Maize, culture media, immature embryos, embryogenic callus, regeneration.*

INTRODUCTION

In recent years, embryogenic maize tissue culture has been routinely used as targets for producing transgenic plants *via* the biolistic process. The recovery of fertile transgenic plants by this method was first reported by Fromm *et al.* (1990) and Gordon-Kamm *et al.* (1990). Production of genetically transformed plants depends on both the ability to integrate foreign genes into target cells and the efficiency with which plants are regenerated from genetically transformed cells. Numerous maize inbreds and hybrids have been regenerated from embryo derived calli, but efficient plant regeneration has been obtained from relatively a few of them (Bohorova *et al.*, 1995). However, one of the major limitations in the current system is that only tissue from one hybrid germplasm (HiII) and a few inbred lines (such as A188 and H99) can be used for establishment of embryogenic cultures and therefore as a starting material for transformation, (Green and Philips, 1975; Armstrong and Green, 1985; Armstrong *et al.*, 1991). Although these germplasms have good tissue culture responsiveness and transformability, they have poor agronomic characteristics. Therefore, any transgene introduced into these maize lines must be moved into more elite genetic background through conventional backcross breeding. It is therefore highly desirable for researchers and breeders that the genes of interest are introduced into elite lines directly. In addition, many basic biology research requires that transformation is accomplished on inbred lines with specific genetic background.

Maize embryogenic callus can be classified as type I or type II. Type I is a

compact mass of callus that generates somatic embryos showing complex and organized structures which are easily obtained from immature embryos. While type II is friable, embryogenic and maintains the ability to regenerate plants over time. Type II callus tends to be initiated at a lower frequency than type I in embryos and has been obtained from fewer genotypes (Walter *et al.*, 1995 and Carvalho *et al.*, 1997).

Samaj *et al.* (1999) identified the embryogenic units of friable maize callus to be formed as globular or oblong packets of tightly associated meristematic cells. These units are surrounded by conspicuous cell walls visible in light microscopy after staining with basic fuchsin. They used transmission electron microscopy to show that embryogenic cells are rich in endoplasmic reticulum, polysomes and small protein bodies, and that the outermost layer of their cell walls is composed of fibrillar material.

The effect of $AgNO_3$ on the enhancement of type II callus production and the promotion of maize regeneration has been demonstrated by different authors, (Vain *et al.*, 1989a & b; Songstad *et al.*, 1991 and 1992). $AgNO_3$ has been shown to inhibit ethylene action, which was identified as a restrictive factor of type II callus induction, thus promoting an increase in type II callus and plant regeneration. In most experiments, $AgNO_3$ was used with 2,4-D while only Songstad *et al.* (1991) tested the effect of D medium (Duncan *et al.*, 1985), containing dicamba combined with $AgNO_3$ on type II callus induction. Dicamba has been reported to increase somatic embryogenesis in some Gramineae such as maize (Duncan *et al.*, 1985; Bohorova *et al.*, 1995), and wheat (Hunsinger and Schauz, 1987).

Other types of auxin-like plant growth regulators have been shown to be effective in induction of somatic embryogenesis. Close and Ludeman (1987) showed that chloramben induced a higher frequency of somatic embryogenesis than 2,4-D, while chloramben and dicamba induced embryogenesis at relatively high frequencies in all genotypes tested.

This report describes the effects of $AgNO_3$, 2,4-D, dicamba and chloramben as well as their interaction with MS or N_6 medium, on the frequency of somatic embryogenesis, type of callus and plant regeneration from immature embryos of five Egyptian maize inbred lines. This could reflect their usefulness for transformation protocols. The genotype specificity of response under a variety of culture induction conditions is also discussed.

In an attempt to improve the culturability of the commercial inbred line Sd7, crosses were made between plants of Sd7 and the American line A188, previously described as having the best *in vitro* embryogenic response, (Green and Philips, 1975; Armstrong and Green, 1985; Tomes and Smith, 1985; Hodges *et al.*, 1986; Tuberosa and Lucchese, 1989).

MATERIALS AND METHODS

Immature embryos from field-grown plants were used as explant source in all experiments. Seeds from Egyptian maize (*Zea mays* L.) inbred lines Sd62, Sd7, Gz643,Gz613 and Gz639 were kindly provided by Maize Program, Field Crops Research Institute (Table 1). All plants were self-pollinated and when the embryos had attained a length of 1-2 mm (approx. 10-15 days post-pollination), they were aseptically excised and placed in petri dishes with embryo axis in contact with the medium.

To study the effect of Dicamba, $AgNO_3$, 2,4-D and Chloramben on somatic embryogenesis and type of callus, callus

Table (1): Genetic background of the maize genotypes tested.

Code #	Inbred Line	Ancestors
1	Sd 7	(American Early Dent x Composite A4)
2	Gz 613	B73 x Sd7
3	Sd 62	Tepalcinco No.5
4	Gz 639	B73 x Sd62
5	Gz 643 y	B73 x Sd 62

induction was performed using four different media. Two of the media are based on the N6 medium (Chu *et al.*, 1975) while the other two on MS medium (Murashige and Skoog, 1962). N_6-A_g medium is composed of N6 salts and vitamins supplemented with 0.1 g myo-inositol, 25mM L-proline, 1 mg 2,4-D, 10 µM $AgNO_3$, and 2% sucrose (Armstrong *et al.*, 1991). N6-2D medium contains N6 salts and vitamins supplemented with 200 mg casein hydrolysate, 2.302 mg L-proline, 3% sucrose and 2.0 mg dicamba (Bohorova *et al.*, 1999). MS-Ag medium contains MS salts and vitamins supplemented with 100 mg myo-inositol, 150 mg asparagines, 1 mg 2,4-D, 2% sucrose and 10 mg $AgNO_3$ (Vain *et al.*, 1989b). While MS-2C medium is composed of MS salts and vitamins supplemented with 100 mg myo-inositol, 150 mg asparagines, 10 µM chloramben, 2% sucrose and 1 mg $AgNO_3$ (Close and Ludeman, 1987).

Immature embryos from each genotype were cultured on each of the four media in ten replicates with 30 embryos per replicate. Cultures were maintained in dark at 26 transferred to fresh medium every 2 weeks.

Twenty days after plating, the cultures were classified as embryogenic or non-embryogenic and the embryogenic calli were classified as type I or type II. Slow-growing and dark calli were discarded during subculturing. The number of embryogenic calli was scored after two months of culturing. All the calli were used to regenerate plants.

Plant regeneration was started after two months of culturing. Calli were transferred first to medium containing MS inorganic salts and vitamins, 300 mg L-glutamine, 1.0 mg kinetin, 0.5 mg 6-BA and 6% sucrose. After 2 weeks, the calli were transferred to MS medium supplemented with 2% sucrose. Regenerated plantlets were transferred to rooting media composed of half strength MS medium supplemented with 2% sucrose. Plantlets, 5-7 cm in length, were transferred to pots in the greenhouse.

To improve the regeneration frequency of the commercially important lin crosses were made between Sd7 and the American line A188 which has been proven to be the best source for the establishment of embryogenic cultures (Green and Phillips, 1975; Armstrong and Green, 1985; Armstrong *et al.*, 1991 and Rosati *et al.*, 1994). A total of 300 immature embryos were aseptically excised from the hybrid Sd7xA188 and cultured on N_6-Ag medium. Calli were subcultured and regenerated as described with the other lines.

Statistical analysis was performed according to Steel and Torrie (1980), using the SAS computer software (version 5) with associated least significant differences (LSD) function.

RESULTS AND DISCUSSION

Data presented in Table (2) show the effect of media composition on callus induction, embryogenic callus production and regeneration frequency of the lines under investigation.

Table (2): Effect of supplementing the callus induction media with 2,4-D, dicamba, $AgNO_3$, and chloramben on type of callus formed and the regeneration frequency.

Line	Medium	Average no. of Embryogenic Callus	Callus Type	Reg. Freq. (%)
Sd 7	MS-Ag	5.90_g (1.96 %)	I	10.67_{ef}
	MS-2C	15.00_{ef} (5.00 %)	I	9.00_{efg}
	N_6-2D	8.40_g (2.80 %)	N/I	3.33_{fgh}
	N_6-Ag	15.70_{ef} (5.23 %)	I	22.33_d
Gz 613	MS-Ag	5.40_g (1.80 %)	N/I	0.00_h
	MS-2C	12.80_f (4.27 %)	I	12.67_e
	N_6-2D	5.70_g (1.90 %)	I	4.33_{fgh}
	N_6-Ag	1.50_h (0.50 %)	N/I	0.00_h
Sd 62	MS-Ag	19.40_{cd} (6.47 %)	I	23.00_{cd}
	MS-2C	6.70_g (2.23 %)	N/I	2.67_{gh}
	N_6-2D	24.70_{ab} (8.23 %)	I	13.67_e
	N_6-Ag	22.40_{bc} (7.47 %)	I	33.33_b
Gz 639	MS-Ag	17.10_{de} (5.70 %)	I	33.83_b
	MS-2C	6.80_g (2.27 %)	I	$4.00f_{gh}$
	N_6-2D	22.80_{bc} (7.60 %)	I	2.67_{gh}
	N_6-Ag	13.20_f (4.40 %)	I/II	52.66_a
Gz 643	MS-Ag	20.30_{cd} (6.77 %)	I	27.33_{bcd}
	MS-2C	24.00_{ab} (8.00 %)	I	30.67_{bc}
	N_6-2D	17.80_{de} (5.93 %)	I	7.33_{efgh}
	N_6-Ag	27.30_a (9.10 %)	I/II	50.67_a

N: *non-embryogenic calli* I: *Type I calli* II: *Type II calli*
Vertical means with the same letter(s) are not significantly different at Alpha=0.05

Embryogenic callus formation

Excised embryos placed on initiation media displayed visible changes in the scutellum during the first week of *in vitro* culture. The primary growth region was in the scutellum near the coleorhizal end of the embryo, where cells proliferated and grew rapidly. Within two weeks, the scutellar surface became irregular and was transformed into callus tissue. The compact nodular type I callus was formed by all lines tested with varying frequencies.

The inbred line Gz643 revealed the highest response on most of the media used except on N_6-2D. The highest number of embryogenic calli (27.3) was formed by Gz643 on N_6-Ag. While, the number of embryogenic calli formed by Gz639 was significantly higher on medium N_6-2D in comparison with those obtained on the other media. Gz639 and Sd62 were the best lines in forming embryogenic callus on N_6-2D medium (22.80 and 24.70, respectively). However, the number of embryogenic calli formed by Sd62 on medium N_6-Ag was not significantly different from N6-2D. The type of callus produced by these lines (i.e. Gz639, Gz643 and Sd62) was mainly type I as described by Armstrong and Green (1985) and Bohorova *et al.* (1995). Callus type II, which

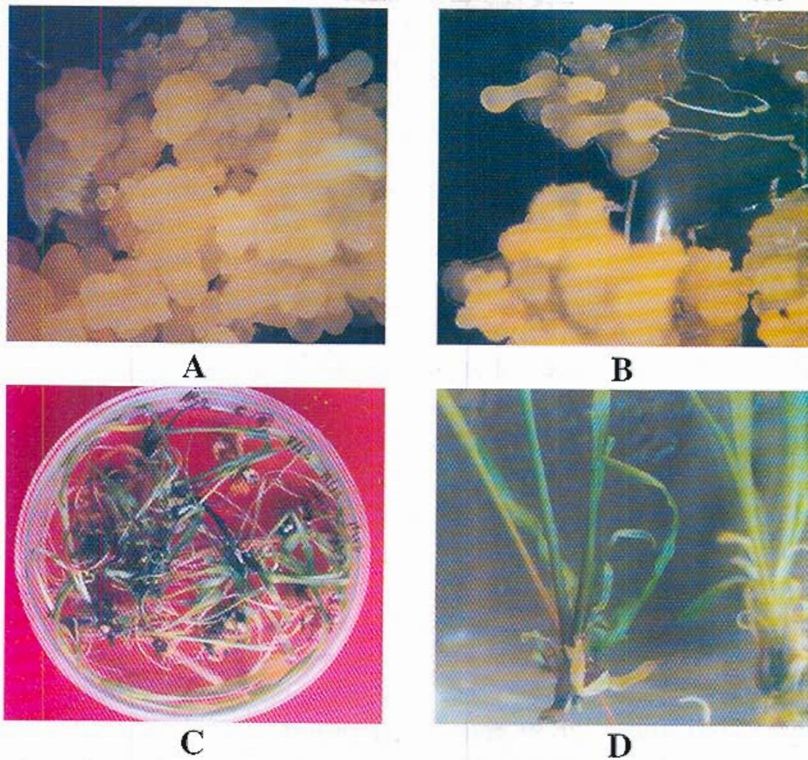

Fig. (1): Somatic embryogenesis and regeneration of plantlets from immature embryos of maize. (A) & (B) embryogenic maize callus on induction media. (C) calli on regeneration media. (D) regenerated maize plantlets.

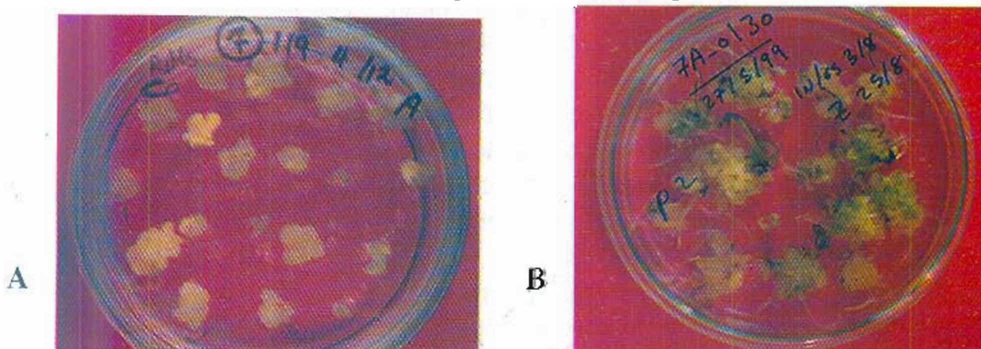

Fig. (2): A) Sd7 on regeneration medium, B) Sd7xA188 on regeneration medium.

is yellowish in colour and contains organized somatic embryos at the coleorhizal end of the scutellum, was formed at a lower frequency by Gz639 and Gz643 (approx. 20-30%). This type of callus was similar to that described by Franz and Schel (1991) for the genotype A188; it was also formed by tropical lines 48, 109 and 705 (Carvalho *et al.*, 1997).

Lines Gz613 and Sd7 gave the lowest number of embryogenic calli on all media. Their response in forming callus was similar on MS-Ag, MS-2C and N_6-2D however; there was a slight improvement of Sd7 on N_6-Ag (15.70). Non-embryogenic soft callus was formed by Gz613 on all media at a high frequency. This type of callus was either non-

regenerable or had a very low regeneration frequency depending on the culture medium used.

From these results it is clear that there was a significant interaction between genotype and callus induction medium, showing that the genotype culturability was dependent on the medium utilized. This is in agreement with the results of Carvalho *et al.* (1997). Moreover, Hodges *et al.* (1986) demonstrated that differences in the effects of media on somatic embryogenesis and plant regeneration indicate the importance of different genes at various stages of regeneration and that there must be genes that are important in controlling regeneration.

Plant Regeneration

The highest significant regeneration frequency was obtained from lines Gz639 and Gz643 when maintained on N_6-Ag medium (52.66% and 50.67%, respectively). Although the number of embryogenic calli formed by Gz639 was significantly higher on N_6-2D, its regeneration frequency was significantly lower when compared with calli initiated on N_6-Ag. This could be attributed to type II callus being formed by Gz639 on N_6-Ag medium, while most of the calli produced by this line on N_6-2D were of the type I. Both Gz639 and Gz643 produced embryogenic type II callus and therefore, showed a high plant regeneration response on N_6-Ag medium. On the same medium, Sd62 gave a relatively high regeneration frequency (33.33%). On the other hand, MS medium supplemented with $AgNO_3$ (MS-Ag) revealed lower regeneration frequency for Gz639, Gz643 and Sd62 (33.83, 27.33 and 23.00%, respectively).

Although the regeneration frequencies of Sd7 and Gz613 were generally very low on the different media compositions, the regeneration frequency of Sd7 was slightly enhanced on N_6-Ag, which indicates that the combination of N6 medium with $AgNO_3$ has an effect on improving the type of callus produced by Sd7. On the other hand, Gz613 was the only line that responded to the addition of chloramben to MS medium (MS-2C) and its regeneration frequency was relatively improved (12.67%) in comparison to its regeneration frequency on the other media.

It is clear that $AgNO_3$ has a remarkable effect on improving the type of callus and the regeneration frequency of maize genotypes, specially when combined with N6 medium supplemented with the auxin 2,4-D (1mg/l).

The present results indicate that the choice of medium is significantly important when dealing with different genotypes. This is consistent with the studies of Lu *et al.* (1982) and Vasil and Vasil (1984). In addition, Bohorova *et al.* (1995) showed that, under appropriate culture conditions, embryogenic calli could be produced from immature embryos of several maize genotypes. The scutellar region close to the coleorhizal area of immature embryos contains cells capable of producing somatic embryos under certain experimental conditions. They also indicated that factors influencing the expression of totipotency in cell culture are: genotype, composition of plant culture medium, growth regulators, and embryo size.

Medium N_6-Ag gave the highest significant regeneration frequency among all lines tested followed by MS-Ag, with the exception of line Gz613. This indicates that, addition of $AgNO_3$ together with 2,4-D to either N_6 or MS media significantly affected the quality and quantity of embryogenic calli formed, and consequently enhanced the regeneration frequency of most of the lines tested. These results agree with Armstrong and Green (1985) and Armstrong *et al.* (1991). In this context, Vain *et al.* (1989a&b), Songstad *et al.* (1991) and Bohorova *et al.* (1995) emphasized the effect of supplementing the

callus induction media with silver nitrate on increasing the number of embryogenic calli.

On the other hand, addition of chloramben (10 M) with 1mg/l $AgNO_3$ to MS medium in the absence of L-proline and 2,4-D did not enhance the callus type or regeneration frequency of most lines studied. However, the recalcitrant line Gz613 responded to this medium. This result is in contrary to the findings of Close and Ludeman (1987), who stated that chloramben induced a higher frequency of somatic embryogenesis than 2,4-D in the 14 inbreds examined.

Dicamba has been reported to increase somatic embryogenesis in some Gramineae such as maize (Duncan *et al.*, 1985; and Bohorova *et al.*, 1995), and wheat (Hunsinger and Schauz, 1987). In this study, the addition of 2 mg/l dicamba, together with 15 mg/l $AgNO_3$ to N6 medium gave a relatively higher frequency of callus induction overall the five lines tested, but the regeneration frequency of calli maintained on medium containing dicamba was significantly low compared to the other media. However, Bohorova *et al.* (1995) found that callus initiation and maintenance with dicamba was important for the induction and maintenance of embryogenic calli in tropical, subtropical, midaltitude, and highland maize lines.

The composition of macro and micro elements in modified N6 medium may also have an effect on embryogenic callus formation and plant regeneration. In this context, Bohorova *et al.* (1995) stated that nitrogen and the form of nitrogen must have a very crucial role in callus formation and regeneration. Basic N6 medium, which contains a lower nitrogen concentration than MS medium, proved to be excellent for embryogenic callus initiation and maintenance.

The genotype or genetic background also has a great effect on plant regeneration. Gz643 and Gz639 gave the highest regeneration frequency overall the media used. Therefore, these lines are recommended to be used in transformation experiments. In this context, Bingham *et al.* (1975) provided the first evidence that shoot regeneration in callus cultures was genetically controlled and could be manipulated using conventional breeding techniques. Different regeneration potentials among genotypes have been reported by Duncan *et al.* (1985), Tomes and Smith (1985), and Hodges *et al.* (1986).

It could be concluded that both medium N_6-Ag and MS-Ag could be used in the initial screening process of new lines to test their tissue culture ability and regeneration frequency.

Sd7 is one of the elite parental lines among commercially important Egyptian maize hybrids. However, this line has shown a poor *in vitro* response on different tissue culture media. Therefore, an attempt has been made to improve its culture ability through crossing between Sd7 to the highly regenerable American line A188. Immature embryos of the hybrid were excised and cultured on the N_6-Ag medium, which was shown to be the best medium in inducing embryogenic calli with Sd7 and the other lines. The *in vitro* response and the regeneration frequency of the hybrid Sd7 x A188 were compared with that of Sd7. The results presented in Table (3) and Fig.(2) revealed that there was significant improvement in the type of callus formed by Sd7 x A188, and this was reflected on its regeneration frequency, (62.33%). Therefore, It could be recommended to carry out transformation on the hybrid germplasm Sd7xA188 rather than the elite inbred line Sd7 and then the transgenes can be moved to Sd7 through backcross breeding.

Another approach worth considering would be the improvement of the *in vitro* response of this line through a selection and

backcross breeding program utilizing line A188 in its capacity of forming embryogenic calli **(Rosati *et al.*, 1994)**. In this context, **Armstrong *et al.* (1992)** reported that the response of the maize elite line B73, a notoriously recalcitrant genotype, has been dramatically increased through the use of a backcross breeding procedure. Moreover, Beckert and Qing (1984) reported that the frequency of regenerating calli proliferated from immature embryos increased from 5.3 to 14.4% after one cycle of selection in a recurrent selection program; Rosati *et al.* (1994) reported that two selection cycles led to almost a doubling of regeneration capacity of the base population and increased the number of families with a regeneration capacity comparable to A188.

Table (3): Mean number of embryogenic calli and regeneration frequency of Sd7 and Sd7xA188 on N_6-Ag medium.

Line	Average no. of Embryogenic Callus	Callus Type	Reg. Freq. (%)
Sd7	15.70b	I	22.33b
Sd7 x A188	29.20a	I/II	62.33a

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الملخص العربي

إنتاج الكالس و إعادة التمايزفى سلالات مصرية من الذرة الشامية

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تـم تقييم استجابة خمسة سلالات مصرية من الذرة الشامية (*Zea mays* L.) لإنتاج الكالس فى الأنبوب و مقدرتها على التمايز وذلـك باستخدام أربعة توليفات مختلفة من البيئة المغذية. وقد استخدمت بيئات MS ,N_6 مع تركيزات مختلفة من نترات الفضـة و الـداى كامبا (Dicamba (10 µM وكلورامبين (Chloramben (10 µM و 2,4-D وذلك لدراسة تأثير مكونات البيـئة المغذيـة و الخلفية الوراثية للسلالة على تكوين كالس من نوع type I أو type II و إعادة التمايز للنباتات. وقد أوضحت النـتائج أن السـلالتين جيزة–643 وجيزة–639 كانت استجابتهما عالية لتكوين الكالس على بيئة الأساسN_6 المضاف اليها نترات الفضـة (N_6-A_g) و أظهـرتا أعلى معدل لإعادة التمايز. كما استخدمت الأجنة غير الناضجة للهجين سدس A188 x 7 لمقارنة اسـتجابة الهجين بالسلالة سدس–7 ذات القيمة الإقتصادية العالية من حيث إنتاج الكالس وإعادة التمايز. وقد أوضحت النتائج زيادة معنوية فى معدل إعادة التمايز للهجين بالنسبة للسلالة سدس–7.