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IN VITRO REGENERATION OF INDIAN MAIZE (*ZEA MAYS* L.) INBRED LINES THROUGH IMMATURE EMBRYO DERIVED SOMATIC EMBRYOGENESIS

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ABSTRACT

The successful regeneration from immature embryos were tested in three inbred lines (UMI 29, UMI 54 and UMI 1007), MS and N6 were used. Among these, UMI 29 showed highest percentage of somatic embryogenesis and regeneration frequency. The embryos were cultured on N6 medium supplemented with different concentrations of 2, 4-Dichlorophenoxy acetic acid (2,4-D). The results showed that the highest percentage of somatic embryogenesis was obtained on N6 medium supplemented with 2,4-D (1 mg/l), proline (2.8 g/l), casein hydrolysate (200 mg/l), silver nitrate (10 mg/l) and 2% sucrose. The results showed that N6 (91.66%) medium was found better than MS (63.00%). Plants were regenerated successfully from embryogenic callus in hormone free MS medium. Regenerated plantlets were successfully acclimatized in soil and raised to maturity. The response to tissue culture proved to be highly genotype dependent.

KEYWORDS: Maize, Inbred Lines, Callus, Somatic Embryogenesis, Regeneration

INTRODUCTION

Maize (*Zea mays*) is one of the most important crops around the world because of its importance as food and feed in the past and present; thus, breeding technology in this crop has been the subject of intense efforts resulting in several biotechnology approaches. Molecular plant breeding is largely dependent on available *in vitro* regeneration protocols that are amenable for use in genetic transformation for improvement of the target traits. Although there are various reports on successful regeneration of maize, there are often limited to particular genotypes. The response of maize tissues in culture is genotype dependent. The success of regeneration procedures is affected predominantly by genotype, the type of explants material employed and media composition (Lindsay and Jones, 1989; Phillips *et al*., 1988). Therefore there is need to assess a wide range of genotypes to identify those with high regenerative response which can be used during genetic transformation.

Successful plant regeneration has also been reported from calli initiated from anthers (Ting *et al*., 1981), glumes (Suprasanna *et al*., 1986), immature inflorescences (Pareddy and Petolino, 1990), immature tassels (Rhodes *et al*., 1986; Songstad *et al*., 1992), leaf segments (Conger *et al*., 1987; Ray and Gosh, 1990), seedling segments (Santos *et al*., 1984), shoot tips (Zhong *et al*., 1992; O'Connor *et al*., 2002) and shoot apical meristems (Zhang *et al*., 2002). Since the first report on maize tissue culture by Green and Philips (1975), immature zygotic embryos have become the explant of choice in cereals including maize (El-Itriby *et al*., 2003; Ward and Jordan, 2001; Oduor *et al*., 2006; Vasil *et al*., 1984).

The objective of this study reported here was to evaluate the tissue culture responses of selected inbred lines popularly used in the maize breeding program of Tamil Nadu Agricultural University by using immature embryo as an explant. The developed information on tissue culture for the studied inbred lines eventually would be helpful for improvement

of maize using biotechnological tools.

MATERIAL AND METHODS

Plant Materials

The maize inbreds (UMI 29, UMI 54, and UMI 1007) used in this study was obtained from Department of Millets, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University. Immature embryos were used as explants for tissue culture experiments. The purity of inbred lines was maintained through selfing using hand pollination. The immature embryos were taken at the age of 8-10 day after controlled self pollination. The maize cobs were collected when embryos were at appropriate size (usually about 1.5 mm long). The husks were removed and the cobs were taken in a glass beaker for surface sterilization. The immature cobs were surface sterilized with 70% ethyl alcohol for 1 min followed by 2.5% sodium hypochlorite for 7 min. The cobs were then rinsed 3-4 times with sterile distilled water.

A forceps was stabbed to the bottom end of the cob to provide a makeshift handle. Using a #22 scalpel blade, the top half of the kernels in a row were sliced off in one pass. An arrow shaped dissection scalpel was run clockwise along the inside of the pericarp to make the embryo to slide out and stick to the scalpel. The embryo was collected and placed on the culture medium with the scutellar side up and the embryo axis side in contact with the media. This embryo orientation permits extensive proliferation in the scutellum and minimizes germination.

Callus Induction

For callus induction, N6 medium (Chu *et al.*, 1975) and MS medium supplemented with 20 g/l sucrose, 2.8 g/l proline, 200 mg/l casein hydrolysate, 10 mg/l silver nitrate and 2,4-D at different levels (0.5, 1.0, 1.5, 2.0, 2.5 mg/l) solidified with 4 g/l gelrite, adjusted to pH 5.8 with KOH was used. Media were autoclaved at 121°C, 15 psi for 20 min. Sterile silver nitrate was added to the medium after autoclaving. The embryos were cultured at 25±2°C in a culture room in dark for 2 week. The shoots of the germinating embryos were trimmed off at 7-8 day after inoculation. The primary calli produced at the end of two week were subcultured on to the same media composition periodically.

Embryogenic calli Formation

The primary calli, thus formed were subcultured into fresh callus induction medium and incubated at 25±2°C in dark condition for two subcultures at a two week interval. After four weeks of culture on subculture medium in darkness, the embryogenic calli were evaluated.

Plant Regeneration

The embryogenic callus induced from the immature embryos were transferred into regeneration medium containing MS medium supplemented with BAP (2, 4, 6, 8 and 10 mg/l), 2, 4-D (0.25 mg/l) and silver nitrate (0.85 mg/l) for shoot induction. The cultures were maintained at 26°C under 16 h photoperiod with cool white fluorescent lights.

Acclimatization

The regenerated shoots were transferred into rooting medium containing MS medium supplemented with silver nitrate (0.85 mg/l) for root development. Healthy shoots of 3-5 cm were transferred to basal MS medium for rooting. The rooted plants were transferred to paper cups containing sterile soil and vermicompost mixture in 1:1 ratio. The hardened plants were placed in the culture room under the light at 25°C for 10-15 days. After the plant produced 1-2 healthy leaves, they were transferred to larger pots with soil in the greenhouse and grown to maturity.

Statistical Analysis

Data analysis was done by AGRES statistical package. Mean values were separated by Fischer's Least Significant Difference (LSD) at 5% probability level. In case of percentage values obtained from count data, arcsine transformations of the variable was performed before analysis and were converted back to percentage for presentation.

RESULTS AND DISCUSSION

Callus Induction

In recent years *in vitro* technology has received considerable attention for further genetic improvement of maize. The success of any transformation work depends on culture duration (to avoid too many deleterious effects from somaclonal variation) and efficiency of the regeneration system utilized. So far almost all maize tissue culture and transformation involves the use of immature zygotic embryos as an explant source for regeneration (Armstrong and Green, 1985; Carvalho *et al.*, 1997; Danson *et al.*, 2006; Duncan *et al.*, 1985; El-Itriby *et al.*, 2003; Pareddy and Petolino, 1990). The production of compact embryogenic callus arises at low frequency and only for specific genotypes. Regeneration ability also influenced, to a great extent, by the media composition (Armstrong and Green, 1985).

The immature embryos larger than 2 mm often germinate without callus formation (Dolgykh, 1994; Armstrong, 1993 and Sellmer *et al*., 1993). Between 10 and 20 days after pollination, immature embryos provide several distinct developmental stages of meristematic tissue competent for transformation (Schlappi and Hohn, 1992). Among the temperate lines, the reported age of embryos is usually in the range of 8-13 DAP (Frame *et al*., 2002; Ishida *et al*., 1996; Lu and Vasil, 1983; Vain *et al*., 1989).

The immature embryos responded to culture on callus induction medium (CIM) by swelling to almost twice the initial size after 3-5 days. The colour of the embryos did not change during culture on CIM irrespective of MS and N6. Callus was induced in all the genotypes tested. After three to seven day of culture on CIM, callus was initiated with the swelling of the embryo on the middle portion of scutellum as well as the basal side. Later the callus proliferated and covered the entire dorsal surface. Similar observations were also recorded by Al-Abed *et al*. (2006), Huang and Wei (2004) and Vasil *et al*. (1985). Bimmineni and Jauhar (1997) suggested the use of pre-cultured immature embryos or isolated scutellar with competent cells for somatic embryogenesis. They also considered that these tissues are excellent target for microprojectile bombardment and for subsequent rapid recovery of transgenic plants. There were significant differences in percentage of callus formation observed among different media and 2,4-D levels tested.

Of the different levels of 2,4-D (0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) tested, N6 medium with 2,4-D level of 1.0 mg/l was found to be more effective for embryogenic callus induction with a frequency of 91.66% in UMI 29. The variation in embryogenic callus formation in different inbreds showed that callus induction frequency is genotype specific (Abe and Futsuhara, 1986; Hodges *et al*., 1986). Auxins, especially 2,4-D in the range of 1-3 mg/l are essential for the formation of embryogenic callus from cereal embryos (Bi *et al*., 2007; Bhaskaran and Smith, 1990; Danson *et al*., 2006; El-Itriby *et al*., 2003; Oduor *et al*., 2006). The findings on callus induction in the present study is in agreement with the results of Armstrong and Green (1985), who found that the nitrogen composition of the basal growth medium has a dramatic effect on embryoid and embryogenic callus formation in maize when L-proline and 2, 4-D were added to the medium, with N6 basal medium generating better results than MS medium.

The morphological variations exhibited by the developing calli were used to identify embryogenic calli from non-

embryogenic calli. Three types of callus morphotypes were observed in the present study. Calli which are regenerable and embryogenic in nature could be distinguished by their pearly structures on dorsal side, compactness, friability and pale or creamy colour and by their scutellar origin (Type II calli). Non regenerable calli were translucent, watery, white in colour and were of both scutellar and non-scutellar origin (Type I calli). Rhizogenic calli were formed hair-like structures on the scutella surface on CIM medium. The immature embryos of all the genotypes (except UMI 1007) formed a more friable, regenerable embryogenic callus on N6 medium in comparison to MS medium. These observations were supported by Vasil and Vasil (1982); Armstrong and Green (1985); Brettschneider *et al*. (1997) and Frame *et al*. (2006). The type of callus induced has also been shown to be dependent on the genotype (Carvalho *et al*., 1997).

There was no callus induction in medium devoid of 2, 4-D. Instead, embryos readily germinated to form shoots and roots after 4-6 day on induction medium. An increasing trend in callusing percentage with increasing levels of 2, 4-D was found both in N6 and MS medium. The callus induction frequency ranged from 64.00% to 99.66% depending on the 2, 4-D concentrations. Of the two different culture media (MS and N6) and different 2, 4-D levels (0.5, 1.0, 1.5, 2.0, 2.5 mg/l) tested, N6 medium with 2, 4-D level of 1.0 mg/l was found to be more effective for callus induction with highest frequency of 99.66% in UMI 29 followed by 98.33% in UMI 54 after 2 week of culture (Figure 1). The percentage of callus induction at different levels of 2, 4-D was significantly different among the inbreds and media (Table.1).

Somatic Embryogenesis

On N6 medium the embryos formed a more friable callus than on MS, and somatic embryos were produced both from the edge of the scutellum and from the callus. The proliferative, friable, fast growing calli were distinguished to be of regenerable nature by the presence of somatic embryos revealed by closer examination using light microscopy. These somatic embryos were initiated as spontaneous sectors growing from well formed friable calli and visible as globular protuberances. These cultures were characterized by its high regeneration capacity. These somatic embryos germinated rapidly when transferred to MS medium containing BAP. The present study documents the formation of somatic embryos on the surface of callus established from proliferating scutellum of immature embryos of maize as reported earlier by Freeling *et al*. (1976). The somatic embryos formed on top of the callus were globular but in some instances it appeared lobed and folded as reported by Vasil *et al*. (1984).

There were differences in percentage of embryogenic calli formation among the different levels of 2, 4-D were tested. Of the different levels of 2,4-D (0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) tested, N6 medium with 2, 4-D level of 1.0 mg/l found to be more effective for somatic embryogenic callus induction with a frequency of 91.66% in UMI 29 (Figure 2). The percentage of embryogenic calli at different levels of 2, 4-D was significantly different among the inbreds and media (Table 1).

Plant Regeneration

Mature calli from all the genotypes were placed on shoot induction media and cultured for 14 day in the light. After embryogenic calli were transferred to regeneration medium containing BAP (10 mg/l), calli turned green within a week and plantlet regeneration occurred within 14 days. When multiple shoots grown on regeneration medium were divided and transferred to rooting medium, thick white roots developed in about 2 week. However, a few abnormal plants were noticed among the regenerants irrespective of genotypes. Plantlets with a well-developed root system were transferred to greenhouse (Figure 3).

The experiments conducted to study the effect of different levels of BAP on regeneration indicated that the medium, MS+BAP-10 mg/l showed the highest percentage of shoot regeneration in all genotypes. The highest regeneration frequency of 88.33% and 60.33% was observed for UMI 29 and UMI 54, respectively (Figure 4).

The genotype UMI 29, that produced highest somatic embryogenic calli percentage exhibited highest shoot regeneration frequency of 88.33% and also highest mean number of shoots per explants (Figure 5). The regeneration frequency at different levels of BAP was significantly different among the inbreds and media (Table 1). Though shoot induction was observed in all the genotypes evaluated, frequency of embryogenic calli and regeneration were higher in UMI 29.

Some of the earlier reports indicate that the capacity of plantlet regeneration is correlated with the ability to form embryogenic callus but, not all calli recognized as embryogenic produced plants, and some recognised as non embryogenic produced plants, showing that this classification does not accurately predicts the regenerative capacity of the callus (Bohorova *et al*., 1995; Carvalho *et al*., 1997 and El-Itriby *et al*., 2003). MS medium was used as the regeneration medium as it was reported to be a better medium for plant regeneration (Armstrong and Green, 1985; Vasil *et al*., 1985; Emons and Does, 1993; Rosati *et al*., 1994; Das *et al*., 2001 and Shohael *et al*., 2003).

The proper ratio of cytokinin to auxin was reported to stimulate shoot formation (Skoog and Miller, 1957). Although a range of cytokinins are known to affect *in vitro* plant cultures (Bhaskaran and Smith, 1990), addition of BAP (10 mg/l) to regeneration medium had an effect on shoot induction in the present study. The experiments conducted to study the effect of different levels of BAP on regeneration indicated that the medium, MS + BAP-10 mg/l showed the higher percentage of shoot regeneration in all genotypes.

Most of the reports on maize shoot induction show regeneration in hormone free medium (Armstrong and Green, 1985; Lu *et al*., 1983; Bhaskaran and Smith, 1990; Huang and Wei, 2004). One possible explanation for this is that the somatic embryos capable of germination to give rise to new plantlets have already formed and their fate may be predetermined by the initiation media (Huang and Wei, 2004) which may again differ according to nature of the genotype, its region of origin i.e. tropical or temperate and culture conditions. The varying levels of regeneration of genotypes in different media showed that somatic embryogenesis and regeneration in maize is genotype specific and regeneration efficiency depends on media environment (Hodges *et al*., 1986 and Suprasanna *et al*., 1994). In conclusion, this study identified three maize inbred lines responding well to embryogenic callus induction and plant regeneration from immature embryos. The highest embryogenic callus and plant regeneration response was produced from inbred line UMI 29, hence this genotype can be used in genetic transformation.

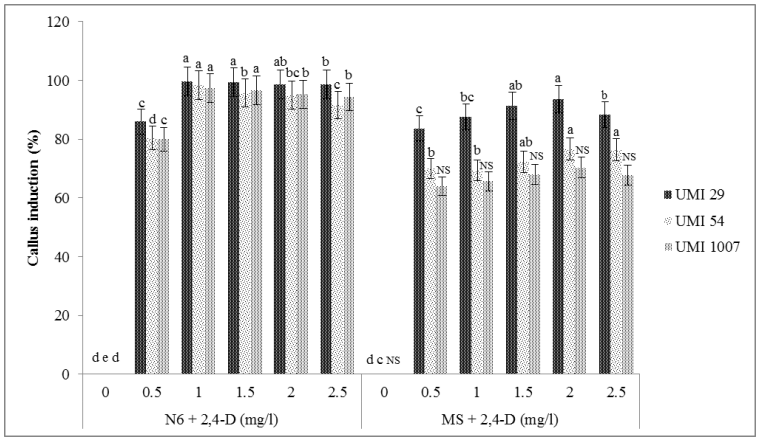

Figure 1: Calli Induction Response of Selected Maize Genotypes to Different Media Combinations. The Values Presented are Mean % Denoted by Alphabets to Imply Significant Difference (P≤0.05) and Values with Same Letters are not Significantly Different. Error Bars= Mean±SE

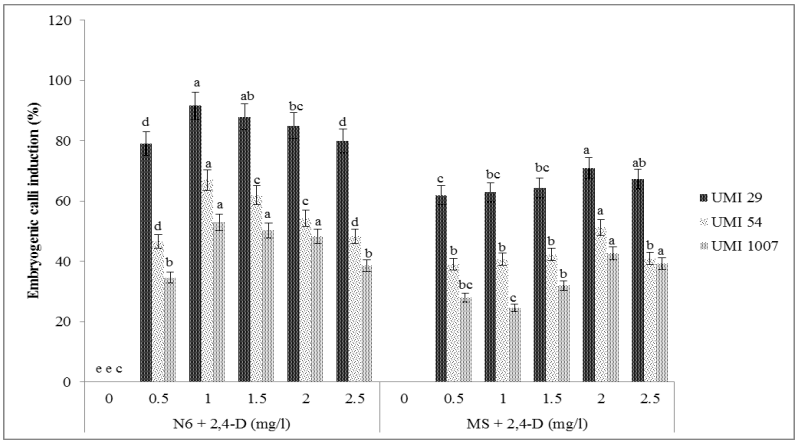

Figure 2: Embryogenic Calli Induction Response of Selected Maize Genotypes to Different Media Combinations. The Values Presented are Mean % Denoted by Alphabets to Imply Significant Difference (P≤0.05) and Values with Same Letters are Not Significantly Different. Error Bars= Mean±SE

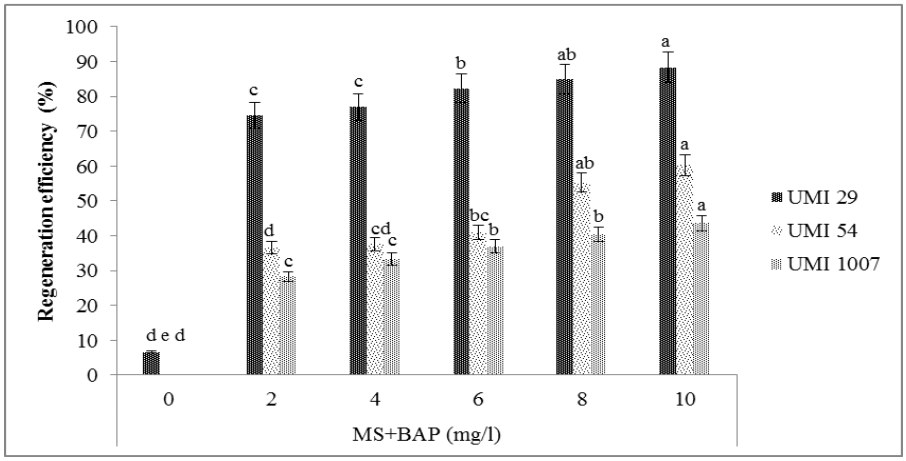

Figure 4: Regeneration Efficiency of Immature Embryo Derived Callus of Maize Genotypes at Different BAP Levels. The Values Presented are Mean % Denoted by Alphabets to Imply Significant Difference (P≤0.05) and Values with Same Letters Are Not Significantly Different. Error Bars= Mean±SE

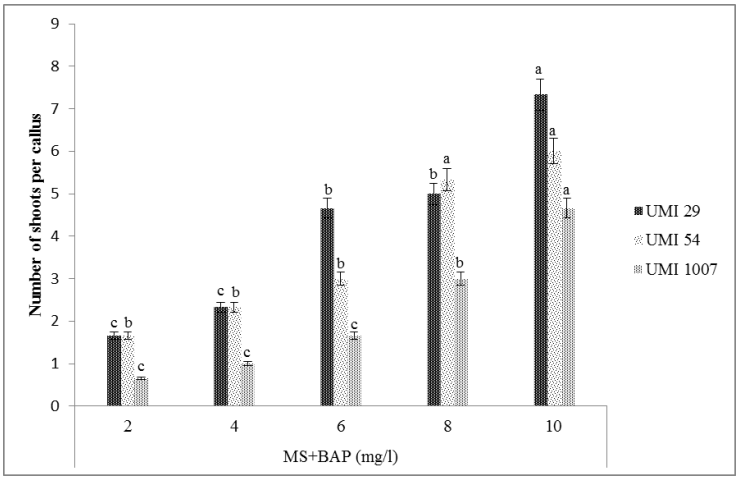

Figure 5: Effect of Different Levels of BAP on Mean Number of Shoots per Explants in Three Maize Inbreds. The Values Presented are Mean % Denoted by Alphabets to Imply Significant Difference (P≤0.05) and Values with Same Letters are not Significantly Different. Error Bars= Mean±SE

Table 1: ANOVA for the Callus Induction Percentage, Embryogenic Callus Percentage and Regeneration Frequency

Source of Variation	Callus Induction (%)			Embryogenic Callus (%)			Regeneration Frequency (%)	
	df	Mean Square		df	Mean Square		df	Mean Square
		N6	MS		N6	MS		
Inbreds	2	65.86 **	1912.20 **	2	6343.46 **	4103.75 **	2	8390.46**
Medium	4	396.94 **	80.72 **	4	471.24 **	231.91 **	4	485.47**
Inbreds X Medium	8	3.56 ^{NS}	7.83 ^{NS}	8	15.49 ^{NS}	25.47 *	8	3.38 *
Error	30	1.33	6.60	30	7.24	5.31	30	10.64

** - Significance at 0.01 levels; NS - Non-significant

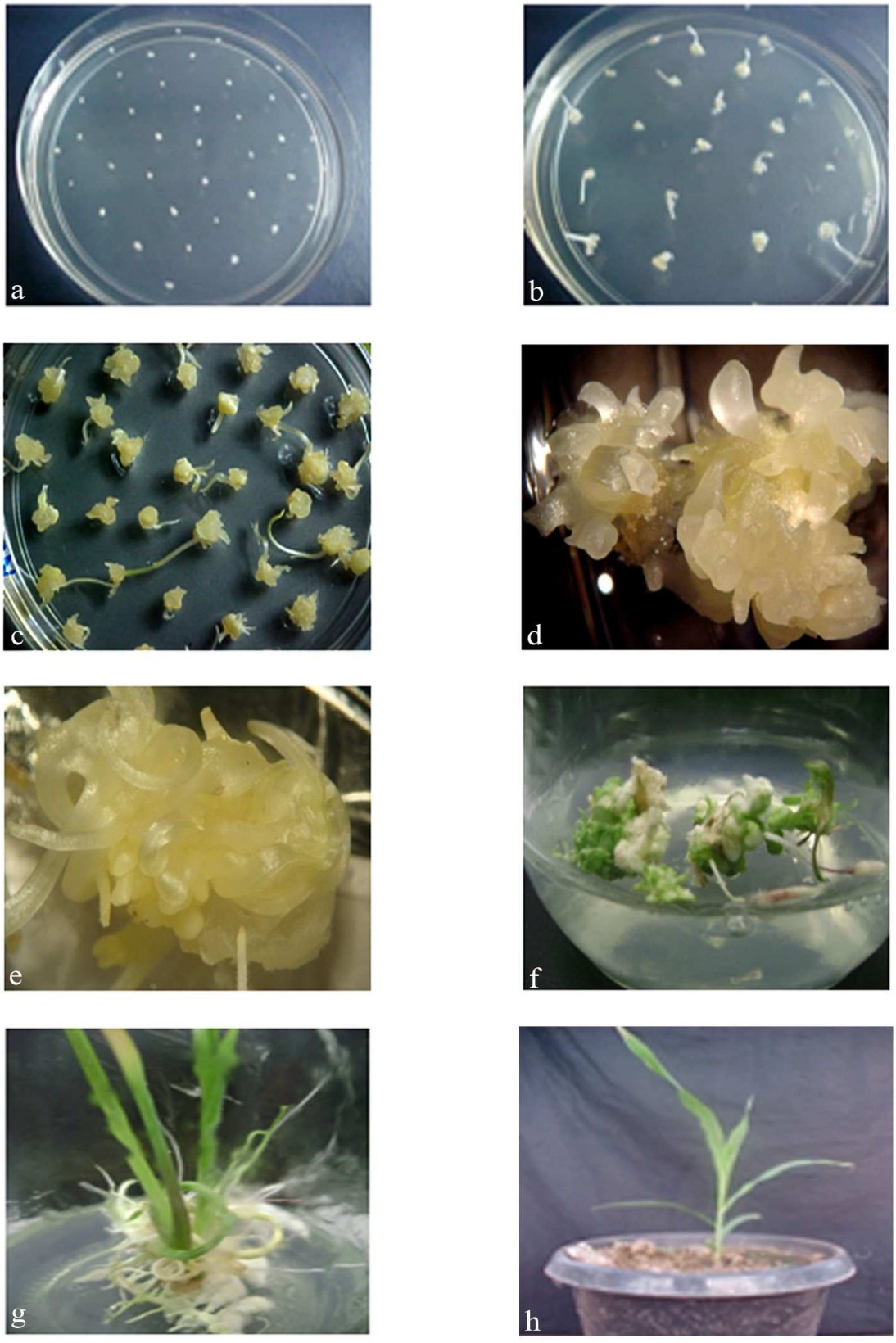

Figure 3: Callus Induction and Plant Regeneration from Immature Embryos of Maize Inbred Lines a. immature Embryos on N6 Media with 2,4-D, b. Immature Embryos (3 d Old), c. Immature Embryo Derived Calli, d. Somatic Embryos, e. Germination of Somatic Embryos, f. Calli on Regeneration Medium, g. Shoots on Rooting Medium, h. Rooted Plants

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