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Full Length Article

Establishment and Optimization of Callus-to-Plant Regeneration System using Mature and Immature Embryos of Maize (*Zea mays*)

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Abstract

Two experiments were conducted to develop an efficient maize regeneration system using mature and immature embryos of four maize elite lines (Agaiti-85, Golden, Soneri and Sultan). The comparative studies revealed that all maize lines were responsive to callus and plant regeneration from mature and immature embryos. The culture medium Chu's N6 was used in both experiments. Immature embryos efficiently produced callus and regenerated plants therefore, when cultured on medium supplemented with 2,4-D, kinetin, 6-BAP and IBA at concentrations of 2 mL L^{-1}, 0.2, 0.2 and 0.3 g L^{-1}, respectively. However, callus-to-plant regeneration system for mature embryos required these growth regulators at higher concentrations (5 mL L^{-1}, 0.4 g L^{-1}, 0.5 g L^{-1} and 0.6 g L^{-1}, respectively). The regeneration frequencies ranged from 40 to 75% using immature embryos, compared to 55 to 80% from immature embryos of all elite lines of maize under study. © 2014 Friends Science Publishers

Keywords: Maize (*Zea mays* L); Immature embryo; Mature embryo; Callus induction; Embryogenic callus; Shoot development; Plant regeneration
Abbreviations: 2,4-D (2,4-Diholorophenoxyacetic acid); BAP (6-benzylaminopurine); IBA (Indole-3-butyric acid); KT (Kinetin)

Introduction

Maize (*Z. mays* L.) is a third most important cereal food crop in the world (Almeida *et al*., 2004). Genetic transformation is an efficient tool to improve quality and quantity of maize (Wang *et al*., 2012; Ahmad *et al*., 2012). Genetically modified crops are the main source to solve the food security problems (Toft, 2007; Ahmad *et al*., 2012). Optimized protocols for callus formation (Rakshit *et al*., 2010) and recalcitrance of plantlets (Bedada *et al*., 2011) are the key basis to successful transformation in maize. Maize is particularly a challenging crop for callus induction and plant regeneration when compared with other cereal crops (Wu and Wang, 2006; Jia *et al*., 2008; Nedev *et al*., 2008; Zhao *et al*., 2008; Alatzas and Foundouli, 2009; Martinez and Wang, 2009; Niu *et al*., 2009; Anami *et al*., 2010; Chu *et al*., 2011). Plant regeneration through maize tissue culture was first reported by Green and Phillips (1975) using immature embryo. Later, transgenic maize was produced employing immature embryos via particle bombardment (Bohorova *et al*., 1999) and *agrobacterium*-mediated transformation methods (Ishida *et al*., 1996). Further studies revealed that immature embryos were mostly used to develop plantlets in maize (Armstrong *et al*., 1985; Rhodes *et al*., 1986; Keigley and Lamotte, 1987). Since then a lot of work was done on maize tissue culture (Rafiq *et al*., 2005; Danson *et al*., 2006; Oduor *et al*., 2006; Sandhu *et al*., 2009; Anami *et al*., 2010; Bedada *et al*., 2011; Chu *et al*., 2011; Asim, 2012). However, seasonal availability of immature embryos led to search for other explants sources like mature embryos of maize. Tissue culture of maize using mature embryos has several advantages being easy to handle and availability throughout the year. Various studies revealed successful callus-to-plant regeneration from mature seeds (Salavati *et al*., 2001; Jia *et al*., 2008; Bedada *et al*., 2011; Wang *et al*., 2012). Parasharami *et al.* (2006) used the mature maize kernals to induce callus and regenerated plantlets to carry on successful genetic transformation. Various studies indicated that callus formation from maize embryo is genotype dependent reported by Abdel-Latif *et al.* (2002.) and Bedada *et al*. (2011). Hence, present study employed four maize genotypes for the establishment of successful *in vitro* culture protocol. Reports are available on the effect of different media and their components for callus

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induction and plantlets regeneration in maize reported by Bronsema *et al*. (2001), Ceasar and Ignacimuthu (2010) Joshi *et al*. (2010). This study aims at the standardization of efficient callus-to-plant regeneration protocols using mature and immature embryos of maize and evaluation/comparison of different growth regulators involved in this process.

Materials and Methods

Plant Material

Four maize inbred lines (Soneri, Sultan, Agaiti-85 and Golden) were used for callus formation and recalcitrance of maize plantlets. Seeds of these genotypes were collected from the research area of University of Agriculture, Faisalabad-Pakistan at 14 days post anthesis (Jakubekova *et al*., 2011) in order to obtain immature healthy embryos for explants development. The mature embryo seeds were obtained after harvesting ears from the maize varieties.

Surface Sterilization

Immature seeds: Ear firstly cut into three pieces after husk and bract removal and sequential washing was done with 70% ethanol (v/v) and fresh bleach solution 10% (v/v) soaking time of up to 3 min (Almeida *et al*., 2004). This was followed by three times rinse in sterile distilled water**.** Immature embryos were aseptically excised from basal portion of seed putting a little pinch on the upper side with help of a sterilized forcep.

Mature seeds: Surface sterilization of mature seeds was done using 70% ethanol (v/v) for five minutes, two times with 10% (v/v) fresh bleach solution for 3 min and finally washed with double sterile distilled water (Wang *et al*., 2012). To make the mature seeds soften for mature embryo excision; seeds were soaked in ddH_2O and kept at -20^oC for four days (Martinez and Wang, 2009; Rakshit *et al*., 2010; Bedada *et al*., 2011). The plumules of mature maize seeds were sliced into two halves to cut out mature embryo on scutellar nodes.

Culture Media and Callus Induction

Media composition (macronutrients, micronutrients, light sensitive compounds and organics) to prepare 1 Liter Chu's N6 medium (50 mL, 1 mL, 20 mL and 1 mL, respectively) along with their basic ingredients is given in (Table 1).

For callus initiation, twenty mature/immature embryos were cultured treatment^{-1} petri plate^{-1}. Four replications for each genotype were used in completely randomized design (CRD) in both cases immature and mature seed embryo excision. Explants were incubated in dark at 28^oC ± 2^oC. The Petri plates were checked on daily basis to eradicate any contaminant from the plate and changed if required. After every sixth day, the explants were shifted on the fresh petri plates for proper nourishment of cultured embryos. After two weeks, the embryos developed the embryogenic callus; numbers of calli per genotype were counted and percent callus induction was calculated. To encourage callus initiation sub-culturing was done after every two weeks in both cases (Jia *et al*., 2008; Zhao *et al*., 2008; Jakubekova *et al*., 2011) during the 6-8 weeks incubation period at 28^oC ±2^oC in dark.

Table 1: Media composition to prepare 1 Liter N6 medium, the amounts per Liter are given; macronutrients, micronutrients, light sensitive compounds and organics (50 mL, 1 mL, 20 mL and 1 mL, respectively) along with their basic ingredients

Amount Liter^{-1} used to prepare 1 L Chu's N6 media	
Macronutrients	50 mL
Micronutrients	1 mL
Light sensitive compounds	20 mL
Organics	1 mL
Ingredients to prepare 1 L Macronutrients (20 X)	
NH_4NO_3	250 g L^{-1}
KNO_3	1.5 g L^{-1}
KH_2PO_4	20 mg L^{-1}
$MgSO_4$-$7H_2O$; Dissolve separately in water	350 g L^{-1}
$CaCl_2$-$2H_2O$; Dissolve separately in water	450 mg L^{-1}
Ingredients to prepare 1 L Micronutrients (1000 X)	
NH_4NO_3	5 g L^{-1}
KNO_3	30 g L^{-1}
KH_2PO_4	4 mg L^{-1}
$MgSO_4$-$7H_2O$; Dissolve separately in water	7 g L^{-1}
$CaCl_2$-$2H_2O$; Dissolve separately in water	9 g L^{-1}
Ingredients to prepare 1 L light sensitive compounds (50X) and cover it with aluminium foil	
Ferrous Sulphate.H_2O	0.5 g L^{-1}
Na_2.EDTA-$2H_2O$	0.7 g L^{-1}
Ingredients to prepare 1 L Organics (1000X)	
Glycerin	2 ml L^{-1} OR 2 g L^{-1}
Nicotinic acid	0.5 g L^{-1}
Pyridoxine HCL	0.5 g L^{-1}
Thymine HCL	1 g L^{-1}
Separately added compounds amount/L for N6 media	
Casein Hydrolysate	1 g L^{-1}
Proline	0.5 g L^{-1}
Sucrose	30 g L^{-1}
Supplementation 2,4-Dicholorophenoxyacetic acid (2 mL L^{-1}or 2 mg L^{-1}) in case of immature embryos but for mature embryos (5 mL L^{-1}or 5 mg L^{-1}) 2,4-D was used. Then add 4.8 g L^{-1} phytagel, same amount was used for solidification in both cases (mature and immature embryos). The pH of N6 medium was adjusted to 5.8 with 0.5 M NaOH and autoclaved for 20 minutes at 121^oC and at 110 kPa.	

Regeneration of Plantlets

The immature embryo-derived embryogenic calli were transferred onto N6 medium containing 0.3 mg L^{-1} of BAP and 0.2 mg L^{-1} of Kinetin. While shoots developed from mature embryo-derived calli when cultured to N6 medium supplemented with 0.5 mg L^{-1} of BAP and 0.4 mg L^{-1} of Kinetin. The cultures were incubated in 16 h photoperiod, 60-80 μE m^{-2} s^{-1} light intensity at 28^oC.

Root Induction

The regenerated shoots from immature embryo-derived calli of four maize genotypes (Agaiti-85, Golden, Soneri, Sultan) were separated and transferred onto half strength N6 rooting

medium containing IBA (0.3 mg L^{-1}). Rooting medium for mature embryo-derived shoots contained 0.6 mg L^{-1} of IBA. The concentrations of both BAP and Kinetin gradually decreased after every six day of sub-cultured media at the same concentration of IBA for efficient rooting (Zhao *et al*., 2008). After 6-8 weeks, plantlets with well-developed roots and shoots were transferred to pots containing peat moss and autoclaved soil then transferred to growth room.

Statistical Analysis

Mature and immature embryos of each maize genotype were replicated five times and a completely randomized design (CRD) was used. Analysis of variance (ANOVA) was carried out to evaluate the significant differences between mature and immature embryo-derived calli, Type I and Type II callus production, and regeneration potential, using Gen Stat Version 12 software (http://discovery.genstat.co.uk), at 5% significance level for LSD value.

Results

Callus Induction

After 5 days of culturing immature embryos, on callus induction medium, callus induction observed in all genotypes (Agaiti-85, Golden, Soneri, Sultan). First sub-culture on callus maintenance medium initiated the embryogenic callus. Immature and mature embryos gave rise to three types of calli: embryogenic, non-embryogenic and organogenic. The embryogenic callus further formed two types, type I and II callus. Type I callus was compact and white in colour. Type II callus was compact, friable and light yellow in colour. Non-embryogenic calli were also formed. This callus was so soft, watery and turned brown after some days of subsequent subculture. Age and size of immature embryo is a critical factor for callus initiation and proliferation. The immature embryos 1-2 mm long are effective for callus initiation.

In this experiment, optimum concentration of 2,4-D was standardized for immature and mature embryos (2 mL L^{-1} or 2 mg L^{-1} and 5 mL L^{-1} or 5 mg L^{-1}, respectively) for successful callus initiation and proliferation. It was observed that in case of immature maize embryo concentration of 2,4-D above 3 ml L^{-1} or 3 mg L^{-1} retarded the growth of callus. For successful callus induction and growth rate L-proline 0.5 mg L^{-1} and caesine hydrolysate 0.1 mg L^{-1} were used in N6 callus culture media.

In both experiments; from immature embryos Sultan performed best for callus initiation followed by Soneri, Golden and Agati-85 (80, 73, 67 and 60%, respectively) (Fig. 1), while from mature embryos the percentage performance for callus initiation was different as 69, 64, 58 and 50% incase of Sultan, Soneri, Golden and Agaito-85, respectively (Fig. 2). In this section it was found that there is no difference for callus initiation from mature and immature embryos (Fig. 9a, b, c and d).

Callus Proliferation

After every six day of culture, calli were transferred to subculture medium to enhance the growth of embryogenic callus. At this stage embryogenic calli (soft, friable and compact) obtained. This study showed that L-proline and casein hydrolysate had the same effect on callus proliferation developed from mature and immature embryos. The significant effect of L-proline is essential for embryogenic callus proliferation. In this study we used optimum concentration of L-proline and caesine hydrolysate (0.5 g L^{-1} and 1 g L^{-1}, respectively) for all genotypes (Agaiti-85, Soneri, Golden and Sultan). There is no difference found in growth rate of individual genotype regarding concentration of L-proline and caesine hydrolystae. The embryogenic calli are the determinants for further shoot formation. In both experiments; from immature embryos the Sultan performed best for callus proliferation followed by Soneri, Golden and Agaiti-85 (8%, 7%, 6% and 4%, respectively) as shown in (Fig. 3), while in case of mature embryos the percentage performance of all genotypes were 6%, 5%, 4% and 2% (Sultan, Soneri, Golden and Agaiti-85, respectively) (Fig. 4).

Shoot Regeneration

In this experiment; Kinetin and BAP (0.2 mg L^{-1} and 0.3 mg L^{-1}, respectively) along with 2 mg L^{-1} or 2 mL L^{-1} of 2,4-D used in regeneration media for efficient shoot regeneration in case of immature seed embryos but from mature seed embryos developed embryogenic calli Kinetin and BAP (0.4 mg L^{-1} and 0.5 mg L^{-1}, respectively) were used along with 5 mg L^{-1} or 5 mL L^{-1} of 2,4-D. Callus of all genotypes successfully transformed into plantlets within six to eight weeks. In case of immature embryos, Sultan performed best for shoot development followed by Soneri, Golden and Agaiti-85 (68%, 61%, 47% and 31%, respectively) (Fig. 5), while from mature embryos the performance percentage for shoot development remained different; 64%, 54%, 42% and 32% (Sultan, Soneri, Golden and Agaiti-85, respectively) (Fig. 6). The comparative results for shoot regeneration of both experiments; immature and mature embryos at earlier stages are shown in Figs. (7, 8, 10a, b, c and d), which explains the evidences that shoot development from mature and immature calli is same. Figs (11a,b,c,d) show the comparison of successfully regenerated plantlets in both experiments (immature and mature embryos) of studied genotypes Agaiti-85, Golden, Soneri and Sultan.

Discussion

Callus has been characterized as type I, II or embryogenic and non-embryogenic calli in the previous studies (Bronsema *et al*., 2001; Abdel-Latif *et al*., 2002; Rakshit *et al*., 2010; Rakshit *et al*., 2010). However, these organogenic

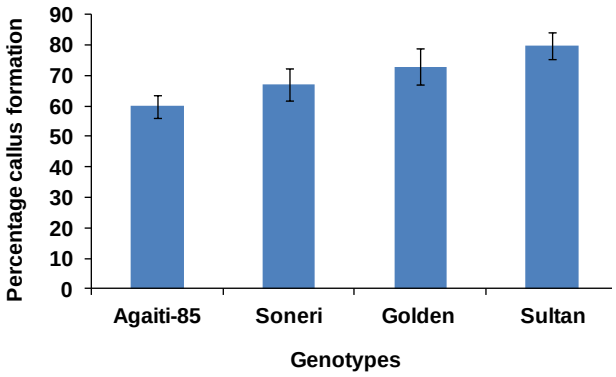

Fig. 1: Mean performance of genotypes for callus formation from immature seed excised embryo

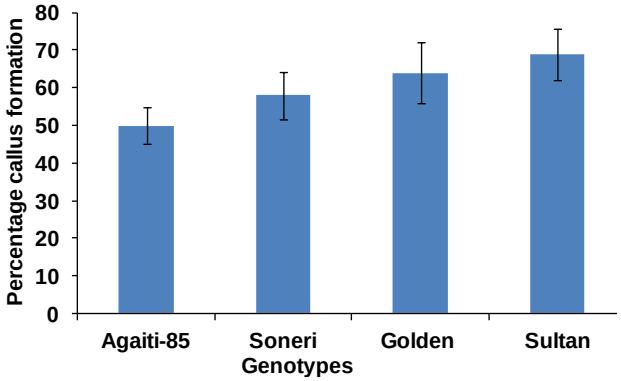

Fig. 2: Mean performance of genotypes for callus formation from mature seed excised embryo

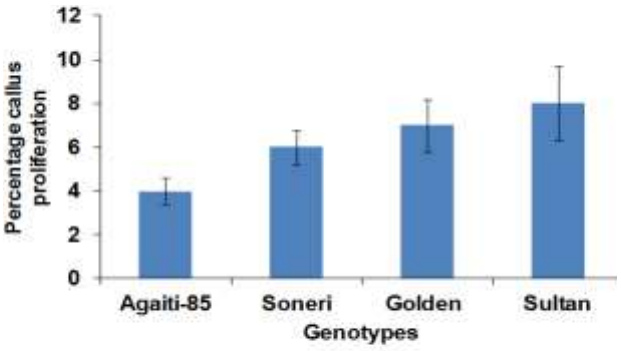

Fig. 3: Mean performance of genotypes for callus proliferation from immature seed excised embryo

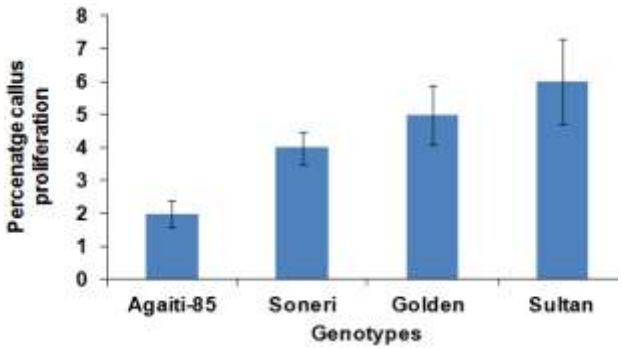

Fig. 4: Mean performance of genotypes for callus proliferation from mature seed excised embryo

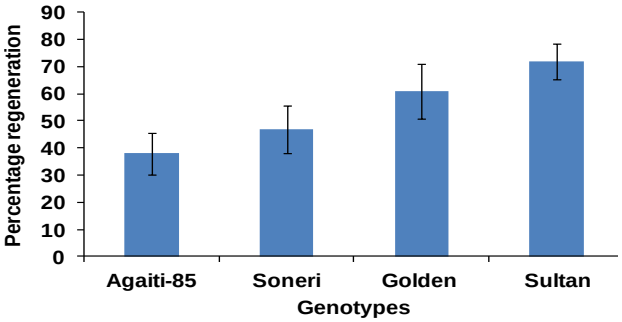

Fig. 5: Mean performance of genotypes for regeneration of plantlets from immature seed excised embryo callus

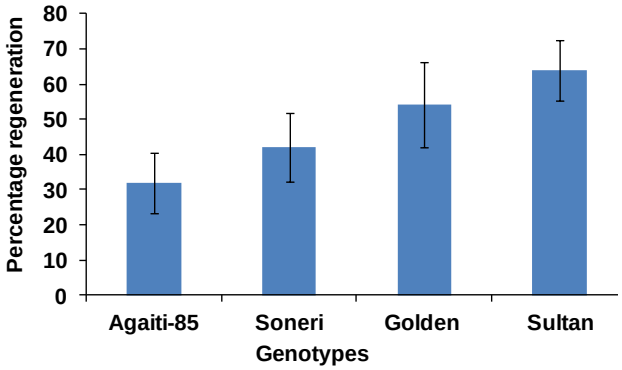

Fig. 6: Mean performance of genotypes for regeneration of plantlets from mature seed excised embryo callus

calli could lead to the regeneration of roots (Jakubekova *et al.*, 2011), which may be due to somatic mutations and type of explants from which callus was developed (Zhao *et al.*, 2008; Chu *et al.*, 2011). On the other hand, callus formation from imature embryo proliferated into regenerable callus. This may be due to response of explants to the critical concentration of 2,4-D i.e., 4 mL L^{-1} -5 mL L^{-1} during callus formation (Zhao *et al.*, 2008). Some other factors such as proline is a source of nitrogen supply for initially developed calli and provided the defensive mechanism against the metabolic changes during *in vitro* culture (Zhao *et al.*, 2008). The caesine hydrolyaste is another important amino acid and considered very important for initial development of callus (Wang *et al.*, 2012). Armstrong *et al.* (1985) working with maize inbred lines used optimum concentration of L-proline 0.3-0.5 g L^{-1} depending upon maize genotype. L-proline and caesinehydrolysate considered essential for callus formation but have no role in callus growth (Zhao *et al.*, 2008) and their high

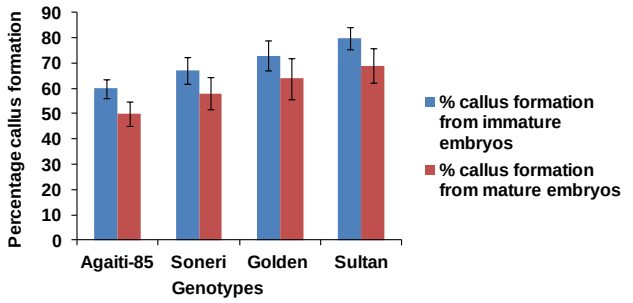

Fig. 7 : Comparison of mean performance for callus of four genotypes from mature and immature seed derived callus

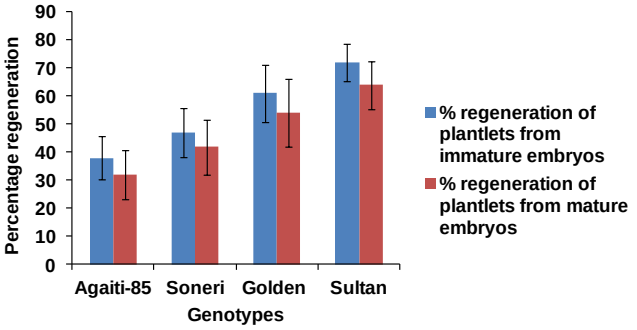

Fig. 8: Comparison of mean performance for regeneration of plantlets from mature and immature seed derived callus

concentration had adverse effects on callus proliferation (Joshi *et al*., 2010).

The regeneration of plantlets from embryogenic callus remained a problem for many years in maize (Vasil *et al*., 1985; Omwoyo *et al*., 2008). Plant growth regulators are important for efficient shoot regeneration of plantlets in case of mature and immature seed embryos (Zhao *et al*., 2008). In general somatic embryoids were transferred to harmone-free medium for shoot regeneration (Green and Phillips, 1975; Armstrong *et al*., 1985). The plant growth regulators such as cytokinins (BA and KT) are important for efficient shoot regeneration for successful development of plantlets (Keigley and Lamotte, 1987; Ceasar and Ignacimuthu, 2010; Wang *et al*., 2012). Initiation of somatic embryogenesis is dependent on cytokinins in case of cereals as reported by Zhang *et al*. (2008). The KT and BA are important along with 2,4-D in regeneration media for successful development of shoots from immature embryos (Ozcan, 2002) and mature embryos (Wu and Wang, 2006; Jia *et al*., 2008).

In this experiment; KT and BA (0.2 mg L^{-1} and 0.3 mg L^{-1}, respectively) along with 2 mg L^{-1} or 2 mL L^{-1} of 2,4-D used in regeneration media for efficient shoot regeneration in case of immature seed embryos but from mature seed embryos developed embryogenic calli KT and BA (0.4 mg L^{-1} and 0.5 mg L^{-1}, respectively) were used along with 5 mg L^{-1} or 5 mL L^{-1} of 2,4-D. The high concentration of KT and BA causes necrotic effects on newly developed cells as

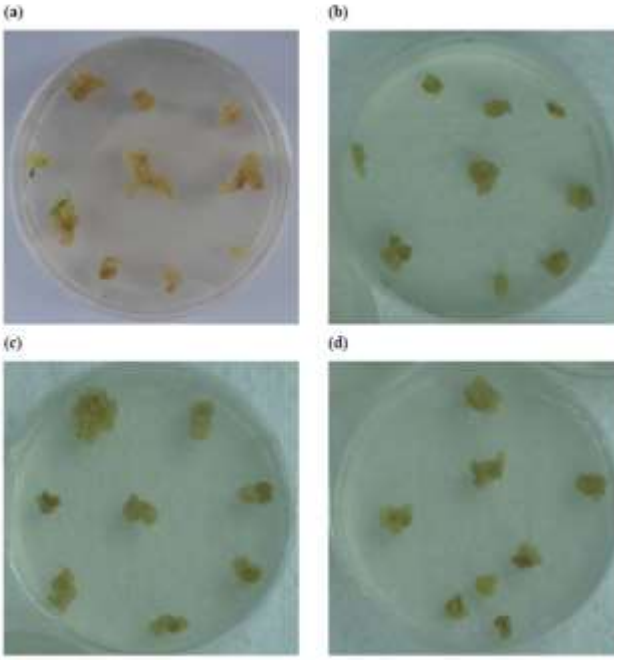

Fig. 9: Above figures shows the callus formation of different genotypes on N6 medium. (a) Agaiti-85, (b) Golden, (c) Soneri, (d) Sultan

earlier reported by Muoma *et al*. (2011).

The callus formation and regeneration of plantlets from the mature seed excised embryo callus remained a problem and it depends on many factors especially the genotype of the seed is very much responsible as earlier reported by Niu *et al*. (2009), Sandhu *et al*. (2009) and Muoma *et al*. (2011). The concentration of 2,4-D was kept double for successful callus formation from mature seed excised embryo was clearly explained by Bronsema *et al*. (2001) and Wu and Wang (2006). In this experiment all the genotypes performed better on regeneration media containing KT and BA. The comparison between these two experiments (mature and immature seed excised embryo) for callus formation and regeneration of plantlets concluded that callus formation and plantlets regeneration from mature seed is easy, manageable and cost effective as earlier discussed by Zhang *et al*. (2008) and Jia *et al*. (2008). The mature seed embryo excision method was more reliable and convenient, because these were available throughout the year. This method breaks the time barrier to wait; which is major problem during immature seed excised embryo. The results cleared a fact on us that seed of these genotypes could be used for maize tissue culture at both stages; immature stage 14-15 days post antheis period and after harvesting when seed is fully ripened. Callus growth and plantlets regeneration from mature seed is easy and less laborious. The percentage growth of calli and plantlets regeneration from the mature seed could be managed and handled by doubling the amount

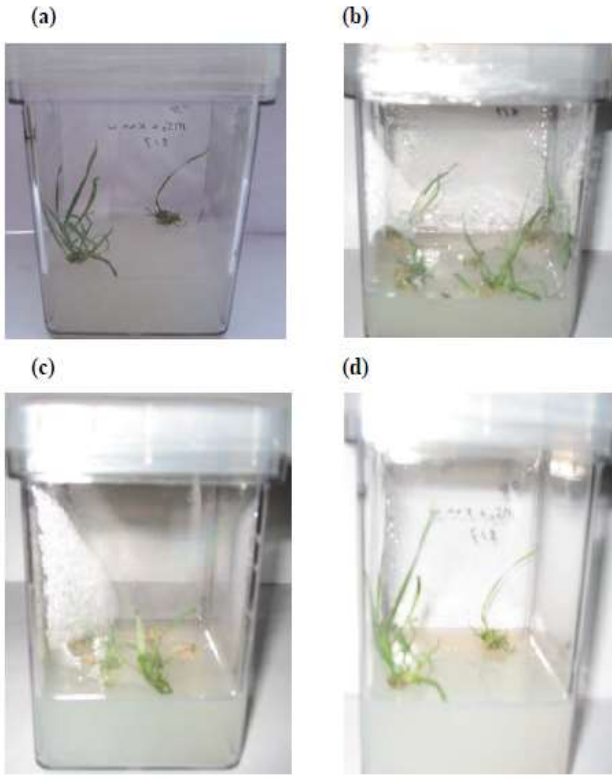

Fig. 11: Above figures shows the regeneration of plantlets from the callus of different genotypes on N6 medium. (a) Agaiti-85, (b) Golden, (c) Soneri, (d) Sultan

of 2,4-D, KT, BAP and IBA as early reported by Salavati *et al*. (2001), Abdel-Latif *et al*. (2002), Young and Reichert (2003), Danson *et al*. (2006), Jia *et al*. (2008) and Wang *et al*. (2012).

In conclusion, a comparative study on the same genotypes showed that percentage growth of callus from mature seed is in the acceptable range to develop embryogenic callus as compared to immature embryo. Similar results were found for plantlet regeneration from mature embryo in comparison to immature embryo. Therefore, callus growth and plantlet regeneration from mature seed significantly eases the process of tissue culture with no time limitation. This study provides a basis that maize tissue culture from mature seed is a useful tool to develop plantlets throughout the year. The main difference found regarding amount of 2,4-D, Kinetin, BAP and IBA, which kept double in case of mature embryo. The *in vitro* culture system standardized in the present study provides a baseline for the development of transgenic plants in the maize genotypes.

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