In vitro plant generation of tropical maize genotypes

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Abstract. Regeneration ability and callus induction of six elite maize inbred lines, B73, Oh28, Va35, Gss0966, Vog134 and Hi34 were investigated using 13-15-day-old immature embryos as explants. Among all the media tested, explants grown on N6 medium gave the highest frequency of organogenic callus.Medium, Genotype, source of auxin and their concentrations influenced induction of callus. N6 supplemented with 2 mg l−1 Dicamba induced the highest frequency of organogenic callus. Explants grown on Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid at 1 mg l−1 showed the highest frequency of callusing.Moreover, N6 supplemented with Dicamba promoted higher callus response in terms of both frequency of induction as well as quality, compared to N6 medium with 2,4-D. Among the six genotypes tested, Va35, Gss0966, Vag134 and Hi34 gave the best callus Though Va35 induced higher percentage of shoot formation than Vog134, the mean number of developed shoots per explant was higher for Vog134. The highest frequency of root formation was observed when shoots were grown on MS medium supplemented with 2 mg l−1 naphathalene acetic acid.Explants of both Va35 and Vog134 incubated on MS medium supplemented with 1 mg l−1 benzyladenine and 0.5 mg l−1 indole acetic acid promoted the highest frequency of shoot induction. Percentage of regenerated plants ranged from 53 to 67.

Keywords: Immature embryo - Regeneration - Shoot development - *Zea mays*

1. Introduction

Maize (Zea mays L.) is the third most planted cereal   crop after wheat and rice world wide.Globally it is top ranking cereal in terms of productivity and has worldwide significance as human food, animal feed and fodder as well as source of large number of industrial products. It is used as a raw material for manufacture of large number of industrial products like corn starch and starch-based products, and in fermentation and distillation industries. Due to uses of maize and maize-based products, demand for maize is increasing across the world, and more predominantly in Asia (Wada et al. 2008). Large collections of maize germplasm exist, such as the International Maize and Wheat Improvement Center (CIMMYT), which possesses inbreds, hybrids, synthetic cultivars, open-pollinating varieties, and improved landraces, and represent resources for genetic diversity to be used in breeding programmes and agriculture. Hence, it is time to embrace fast, effective and reliable techniques, like biotec hnology, in maize improvement program to ensure sufficient production (Machuka, 2001; Pingali and Pandy2001).So far almost all maize tissue culture and transformation involves the use of immature zygotic embryos as an explant source for regeneration (Danson et al., 2006; El-Itriby et al., 2003).However,immature embryos are seasonally available and have strictly limited suitable duration of culture,14-19 DAP(Odour et al.,2006).This imposes tedious routine tissue culture activities within the specified time frame and continuous planting for continuous supply of the immature embryos.In contrast,mature embryos are readily available throughout the year in large quantities.Furthermore despite few reports about the recalcitrance of tropical maize lines and mature embryos for tissue culture work(Bohorova et al.,1995;Hodages et al.,1986),successful regeneration of temperate maize lines and other cereal food crops from mature embryos has been reported by different authors(Akula et al.,1999;Green and Philips 1974; Ward and Jordan,2001). Green and Phillips (1974) first reported that mature embryos of maize could be used to induce callus but no plantlets

were regenerated. Wang(1987) successfully regenerated plants from mature embryos of two maize inbreeds, B73 and Mo17, but the regeneration was genotype dependent and the frequency was only 4 to 5%. Huang and Wei (2004) reported regeneration of temperate maize lines from mature embryos at a frequency ranging from 19.85 to 32.4%. Most recently Al-Abed et al. (2006) reported more efficient regeneration system for two hybrid and two inbred temperate maize lines using split mature seeds as an explant. Identification of genotypes that respond well to embryogenic callus induction and plant regeneration is a necessary initial step for their successful genetic transformation. Immature embryos have been the most widely used explants for initiation of regenerable tissue cultures (Armstrong and Green, 1985; Phillips et al., 1988). Immature embryos can initiate two types of callus cultures from their scutella surfaces: Type I and type II callus. Type I is compact and organogenic and easily obtained from immature embryos. On the other hand type II is friable and embryogenic and is initiated at a lower frequency than type I

(Carvalho et al., 1997). Only a few tropical genotypes have been shown to be capable of initiating type II callus (Oduor et al., 2006; Carvalho et al., 1997). Type II callus has been found to be more regenerable than type I (Armstrong andGreen, 1985). Maize plant regeneration can take place through two avenues, that is,organogenesis or somatic embryogenesis. Somatic embryogenesis is the most common avenue of plant regeneration (Odour et al.,2006). With the rapid development of tissue culture techniques, many types of explants,including gametic embryo and leaf tissue had been successfully regenerated into plants by tissue culture(Aulinger et al.,2003;Huang and Wei,2004;Ahamadabadi et al.,2007).But at present,the most popular is still immature zyotic embryo in maize transformation ,owing to simple inoculation operation and facile callus induction(Binott et al.,2008).Thus,screening of genotypes for in vitro plant regeneration is always a very important research task. Regarding the optimization of media composition, there have been many elite media compositions in literatures (Du et al., 2007; Binott et al., 2008; Zhang et al., 2008), but to be mentioned, any media containing specific reagents was only suitable for limited materials. Thus, to develop a new media for given materials is always necessary and significant. Tissue and cell culture systems are vital to many areas of plant science and crop improvement, particularly in mutant selection and plant transformation (Phillips,2004). The ability to regenerate shoots from callus and cells is essential for establishing a successful plant culture system. However, because of restrictions in genotype and culture conditions, not all plant species or varieties can be regenerated easily. The objectives in the present investigation were to establish a reproducible regeneration protocol for well-adapted maize inbred lines and to compare the efficiency of different sources of auxins on callus induction and regeneration in the inbred lines.To facilitate the application of biotechnology to maize improvement, strategies to improve shoot regeneration frequency have been emphasized and are steadily evolving (Huang and Wei 2004). Objectives were to(1) obtain an media composition used for callus induction,(2)evaluate different genotypes on their ttissue culture characteristics.

2.   Material and Methods

and exposing the embryo and placed with scutellar side up and flat surface down on the callus induction medium solidified with 0.8% agar. Callus induction media used included Murashige and Skoog (MS) (1962)and N6 medium (Chu et al.1975) supplemented with various levels of either 2,4-dichlorophenoxyacetic acid (2,4-D) or Dicamba . MS medium was supplemented with 1, 2, 3, 4 or 5 mg l^{-1} 2,4-D. N6 medium containing 3% sucrose, 2.305 g l^{-1} L-proline, 220 mg l^{-1} casein hydrolysate, 16 mg l^{-1} silver nitrate (AgNO$_3$) was supplemented with 1, 2, 3, 4 or 5 mg l^{-1} of either 2,4-D or Dicamba . PH of the different media was adjusted to 5.6 prior to autoclaving at 121°C (108 kPa) for 25 min. Ten explants per treatment/Petri plate were used. Five replications per treatment was used and arranged in a completely randomized block design. Explants were incubated in the dark for 24 h at 27°C. Then, these were transferred to 16 h photoperiod, 50–70 μE m^{-2} s^{-1} light intensity, and 27°C. After two weeks, number of explants (in percentage) producing primary callus was recorded. Calli were subcultured to fresh medium of the same composition for 15–20 days. The organogenic calli of the genotypes Va35 and Vog134, showing the hig In this study, the choice of maize genotyoes was based on their good agronomic traits such as high yields, drought tolerance, and resistance to maize streak virus, turcicum leaf blight resistance and Striga

weed. Six well-adapted maize genotypes, B73,Oh28,Va35,Gss0966,Vog134 and Hi34 were used in the study. Seeds of these genotypes were planted in the greenhouse at the Seed and plant Improvement Institute,Iran. Plants were self-pollinated and the whole ears were collected 13–15 days following pollination .The kernels were sterilized in 70% ethanol for 3 min and for 20 min in a 2.5% (v/v) sodium hypochlorite solution containing a drop of polyoxyethylene sorbitan monolaurate in an air-flow cabinet and rinsed four times with double-distilled water. The kernel crowns (1– 2 mm) were removed with a sterile scalpel blade and immature embryos (1–1.5 mm in size) were excised by inserting a spatula between the endosperm and the pericarp, releasing the endosperm from the seed coat hest callusing response on MS containing 2,4-D 1 mg l^{-1} and N6 with either 2 mg l^{-1} Dicamba or 2 mg l^{-1} 2,4-D, were transferred to regeneration medium. Two different regeneration media were tried in the investigation. According to Rooz (2002) in the first regeneration medium, referred to as regeneration medium 1 (RM1), calli were first incubated on MS medium supplemented with 0.1 mg l^{-1} 2,4-D, 0.1 mM abscisic acid (ABA), 0.25 mg l^{-1} Ca-pantothenate, 1.0 mM asparigine, and sucrose 2%. After 12 days, these were transferred to N6 medium with 7% sucrose and without any plant growth regulator (PGR). Developing shoots were subsequently placed for rooting on MS medium supplemented with 0.30 mg l^{-1} Ca-pantothenate and 1.0 mM asparigine. A total of 6 calli per plate were used, and these were replicated 8 times. In another set of regeneration media, referred to as regeneration media 2 (RM2), calli were transferred to MS medium supplemented with different combinations of 0, 0.5, and 1 mg l^{-1} indoleacetic acid (IAA)and 0, 1, and 2 mg l^{-1} 6-benzyladenine (BA). This experimental design was a four factorial randomized block design with induction medium and genotype at two levels and three levels each of BA and IAA. Shoot percentage and number of shoots per calli were calculated 3 weeks of culturing. Developing shoots were subsequently separated and transferred to rooting medium consisting of MS with NAA 0, 1, or 2 mg l^{-1}. A total of 10 shoots were used, with five replications per treatment. Cultures of RM1 and RM2 were maintained under 16 h photoperiod, 50–70 μE m^{-2} s^{-1} light intensity, and 27°C. Plantlets with well developed roots were transferred overnight to liquid 1/2 strength MS medium (pH 5.6) without sucrose and then transferred to Jiffy pots containing sterilized Soilrite mix for acclimatization under 16 h photoperiod for a period of 10 days. Following acclimatization, plants were moved to the greenhouse for further growth.All data were subjected to ANOVA. All percent data were subjected to arcsin transformation and shoot numbers were square root transformed before analysis of variance. Effects of various treatment combinations on callus induction were compared using contrast analysis using SPSS ver. 16.0 Mean comparisons were conducted using respective critical differences.Differences in embryogenic callus induction and regeneration frequency among the genotypes were analysed using the Genstat FOR WINDOS (DISCOVERY EDITION) STATISTICAL Software.Data were square root transformed before analysis.

3. Results

we made an effort to obtain regeneration using immature embryos as this has been most commonly reported as best explant in maize (Ahmadabadi et al.2007). Towards this endeavour we principally followed the protocols suggested by Bohorova et al. (1995), who reported that N6 medium containing dicamba and AgNO$_3$ give efficient callus induction and plant regeneration in maize germplasm. Thus, we made an effort to induce regeneration in maize using both MS and N6 media supplemented with various doses of 2,4-D and Dicamba . Based on analysis of variance, all the four parameters, including genotype, induction medium, type of auxin, and level of auxin influenced the frequency of callus induction.Frequency of callus induction in MS supplemented with 2,4-D ranged from 26.1 to 84.%. Callus initiation from cultured embryos was observed within 1 week following culture, with swelling of the scutellum and formation of mass on the surface of scutellum. Within 15 days of incubation, the swollen scutellum developed into irregular callus, which turned into organogenic callus after an additional 15 days (Fig. 1a).The highest frequency of callus induction was observed on the MS medium supplemented with 1 mg l^{-1} 2,4-D for all the six genotypes but the response for callusing reduced as the level of 2,4-D increased in the medium (Table 1). These findings were similar to those reported by Al-Abed et al. (2006) whereby elevated levels of 2,4-D decreased maize callus induction and resulted in browning of calli at levels ≥4 mg l^{-1} 2,4-D. Among the six maize genotypes investigated in this study, inbred line Gss0966 yielded the highest frequency (84.1%) of callus induction on

MS medium.The ability to induce embryogenic callus was assessed in immature embryos 14–16 days after fertilization, ranging from 1 to 1.5 mm in size.Contrast analysis for callus induction showed significant differences ($P = 0.022$) between N6 and MS media Frequency of callus induction for explants incubated in N6 medium in the presence of Dicamba, ranged between 0 and 100%; while, in the presence of 2,4-D, this ranged between 38.1 and 85% (Table 1). Quality of calli derived from N6 medium was better than the calli obtained upon incubation under MS medium. Among the 2,4-D levels on N6 medium, callus response was highest with medium containing 2 mg l^{-1} and this medium was designated as ND2. In ND2, B73 and Va35 showed maximum callusing percentage (84.9%). Dicamba beyond 3 mg l^{-1} had drastic effect in majority of cases, where the calli turned watery. In genotypes like Va35., Gss0966 and Vog134 no callus could be obtained at this concentration of Dicamba. N6 supplemented with Dicamba gave a better callusing response than 2,4-D supplemented N6, both in terms of callusing percentage and quality of the calli ($P = 0.011$).Significant genotypic differences were recorded among the inbred lines. Gss0966 was the most responsive, while Oh28 was the least responsive on MS media (Table 1). However, on N6 medium with 2,4-D Gss0966 was the least responsive. At the concentration of 2 mg l^{-1} of 2,4-D all the genotypes gave nearly similar callusing response. In case of Dicamba, Va35, Gss0966 and Vog134 produced nearly similar response, while B73 and Oh28 showed poor response. Comparison of means suggested that Gss0966 in N6 with 2 mg l^{-1} Dicamba as well as Vog134 in N6 with either 1 or 2 mg l^{-1} dicamba gave best callusing response. In terms of calli quality Va35 and Vog134 gave the best response in N6 supplemented with 2 mg l^{-1} Dicamba. We have found that N6 supplemented with 2 mg l^{-1} Dicamba (subsequently referred to as NDA2) was the most optimum in terms of quality and quantity of organogenic calli (Fig. 1a).However, when the organogenic calli developed on MS with 1 mg l^{-1} 2,4-D were transferred to regeneration medium, RM1, for plantlet formation, inbred line, Vog134 did not give any shoot development on this medium, while up to 12.2% shoot formation was recorded in case of Va35 with an average number of 1.26 shoots per callus. Due to low efficiency of the regeneration, RM1 medium was not tried subsequently. Organogenic calli obtained from NDA2 (Fig. 1a) and ND2, when induced for shoot development in MS supplemented with various combinations of IAA and BAP, gave rise to shoot initiation (Fig. 2). Analysis of variance suggested highly significant effect of induction media, IAA and BAP on both shoot percentage and shoot number per calus, while genotype had a significant effect only on shoot percentage. Genotype had no interaction with either of the hormones, while other second order interactions were significant or highly significant. Three factor interaction was highly significant only for induction media and both the hormones. MS medium with increasing concentration of BAP without IAA allowed the shoot induction in NDA2-derived calli (Fig. 2a–d). However, the efficiency was low. IAA alone (without BAP) did not give any response barring some sporadic shoot formation observed with Vog134. BAP beyond 1 mg l^{-1} had a diminishing effect on shoot initiation as well as on the number of shoots induced. Interaction between IAA and BAP was highly significant and was recognized as important for efficient shoot induction. BAP 1 mg l^{-1} and IAA 0.5 mg l^{-1} proved to be the best combination for shoot induction for both Va35 and Vog134 inbreds (Fig. 1 b, c). In terms of shoot percentage Va35 gave better response (81.7%) as compared to Vog134 (63.7%; Fig. 2 a, c). However, in terms of number of shoots per explant, Vog134 gave better response (4.4) than Va35 (2.4; Fig. 2 b, d). Organogenic calli obtained from ND2 were also tried for shoot initiation with similar hormonal combinations and the response obtained was similar to that obtained with NDA2-derived calli. The best response was obtained when 0.5 mg l^{-1} IAA and 1 mg l^{-1} BAP were tried. However, the overall shoot percentage and number of induced shoots were much lower in this case as compared to NDA2 derived calli. The only exception was that increased IAA alone without BAP also gave some limited shoot induction, particularly at 0.5 mg l^{-1} (Fig. 2e–h).Well developed multiple shoots (Fig. 1d) were separated and transferred to MS medium supplemented with 0, 1 or 2 mg l^{-1} NAA for root induction. MS medium without hormone also led to rooting but the rooting efficiencies were better at both 1 and 2 mg l^{-1} NAA. Quality of rooting was the best in MS supplemented with 2 mg l^{-1} NAA (Fig. 1e). In terms of root initiation and proliferation genotype or induction media had no effect. Regenerated plants were successfully established into complete plants. Establishment percentage for Va35 and Vog134 were 55 and 65, respectively. Among the established plants, 21% showed floral or structural abnormalities, which may be due to culture stresses induced during various stages of development. However, normal regenerated plants flowered and cobs were harvested (Fig. 1f). Seeds derived from the regenerated plants were established into normal plants in the field.

4. Discussion

Furini and Jewell (1994) also suggested that callus obtained from immature embryos in presence of dicamba developed into somatic embryos than the callus obtained with 2,4-D. Huang and Wei (2004) mentioned the role of 2,4-D with MS media in inducing highly regenerable calli from mature embryos.Many published reports are available in maize suggesting successful regeneration from mature embryos (Huang and Wei 2004), split seeds (Al-Abed et al. 2006) other than use of immature embryos as explant (Furini and Jewell 1994 ; Bohorova et al.1995). Genotypes are reported to play an important role in callusing response in various crop plants including maize (Bohorova et al.1995; Furini and Jewell 1994; Aguado-Santacruz et al.2007). Bohorova et al. (1995) suggested that N6 medium, which contained lower level of nitrogen than that of MS, showed better callus induction and maintenance.Rooz (2002) reported good regeneration in calli derived from MS with 2,4-D and regeneration in RM1 (refer materials and methods).This observation is in concurrence with that reported by Bohorova et al. (1995). Bohorova et al. (1995) reported genotype dependent regeneration response among tropical and sub-tropical maize lines. Genotype dependent regeneration response has also been reported by various authors (Wenbin et al.2002; Aguado-Santacruz et al. 2007). Genotypic differences in terms of regeneration response might be related to variations in endogenouse hormone levels (Bhaskaran and Smith 1990). However, because profound differences exist in the potential of maize lines for *in vitro* culture (Armstrong and Green,1985) with only a small number of maize genotypes possessing regenerative capacity , it is important to analyze the response of particular maize materials to *in vitro* culture to define the specific growth conditions required for generating the totipotent material across which to exploit the potential tools of the *in vitro* technology, such as genetic transformation, somaclonal variants recovery, somatic hybridization, and molecular farming, among others.We have found that N6 supplemented with 2 mg l^{-1} Dicamba (subsequently referred to as NDA2) was the most optimum in terms of quality and quantity of organogenic calli (Fig. 1 a).This supported the observation of Furini and Jewell (1994) who reported dicamba as superior to 2,4-D in promoting the callus induction. The success of regeneration procedures is affected oredominantly by genotype,the type of explants material employed and media composition(Lindsay and Jones.1989).Since the early tissue culture studies in maize were first reported by Green and Philips(1975), immature zygotic embryo have become the explant of choice in cereals(EI-ltriby et al.,2003; Ward and Jordan,2001;Oudor et al.,2006).Cavalho et al.(1997) reported that not all tropical genotypes that initiated embryogenic calli could regenerate plants and some genotypes classified as none embryogenic produced plants. They concluded that such a classification does not accurately predict the regenerative ability of a calli from a given genotype(Carvlho et al.,1997).This implies that plant regeneration is achievable for both embryogenic and non-embryogenic genotypes under appropriate tissue culture conditions.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;Danson et al.,2006;El-ltriby et al.,2003;Odour et al.,2006).Media composition is one of the most important factors affecting maize tissue culture(Frame et al.,2006;Binott et al.,2008).Somatic embryogenesis from immature embryos in temperate maize tissue cultures occurs from scutellum cells (Lu et al.1982).
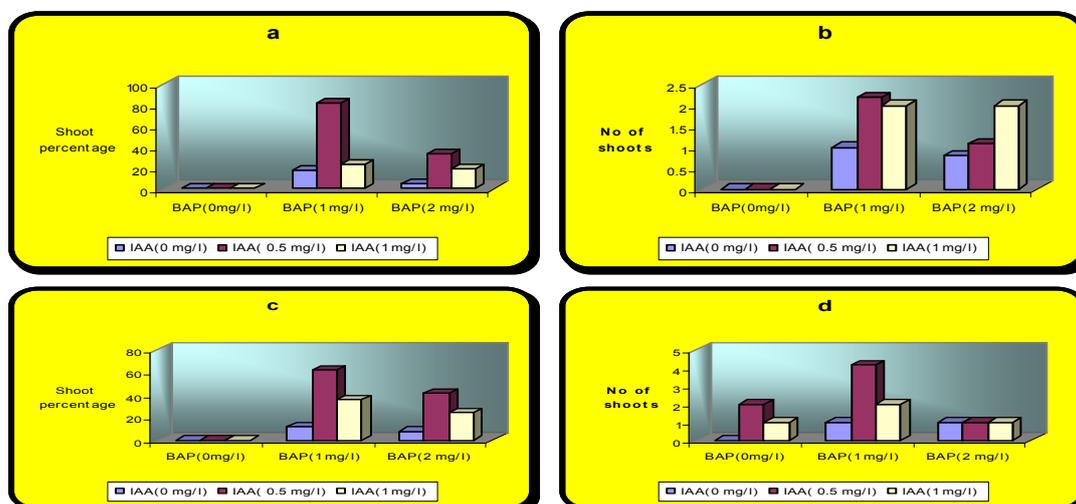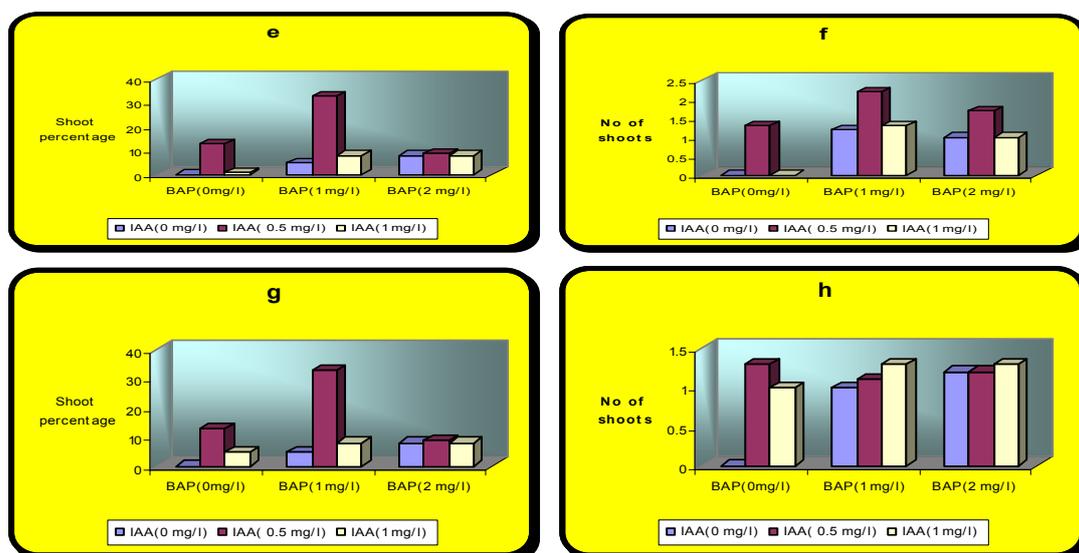
Fig. 2 Regeneration response of organogenic calli derived from ND2(**e. f . g. h**) and NDA2(**a . b. c. d**) under various combinations of BAP and IAA in Vog134(**c. d. g. h**) and Va35 (**a. b. e. f**). CDs for shooting percentage are 15.61 and 20.48 and for shoot number are 0.63 and 0.86 at 5 and 1% level of significance respectively.

5. References

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Table 1: Callus response of explants from six inbred Inbred lines of maize incubated on different media

			B73	Oh28	Va35	Gss0966	Vog134	Hi34
Frequency of callus Development (%)	N6 with Dicamba (mg/l)	1	1.0 ± 68.2 (57.2)	10.3 ± 26.4 (29.9)	13.7 ± 85.4 (67.6)	2.2 ± 91.6 (73.1)	2.3 ± 100.0 (89.2)	1.1 ± 98.2 (82.1)
		2	1.2 ± 50.1 (45.5)	1.5 ± 77.9 (62.3)	1.8 ± 90.2 (71.9)	1.6 ± 96.3 (81.6)	1.8 ± 96.7 (82.1)	1.7 ± 94.2 (67.2)
		3	5.2 ± 52.6 (46.4)	1.3 ± 33.3 (34.2)	1.2 ± 57.3 (49.1)	2.3 ± 91.2 (71.5)	1.2 ± 68.2 (54.6)	1.1 ± 67.2 (53.1)
		4	0.4 ± 31.5 (33.4)	1.4 ± 22.7 (26.9)	0 (0.7)	0 (0.7)	0 (0.7)	0 (0.7)
		5	0.3 ± 29.5 (28.1)	1.3 ± 20.1 (22.3)	0 (0.5)	0 (0.4)	0 (0.7)	0 (0.7)
	N6 with 2,4-D (mg/l)	1	4.2 ± 44.3 (41.7)	6.1 ± 68.7 (55.8)	2.0 ± 71.6 (57.5)	4.2 ± 54.7 (47.7)	0.6 ± 55.1 (47.8)	0.4 ± 53.2 (44.9)
		2	2.2 ± 84.6 (67.2)	1.4 ± 80.7 (64.1)	1.8 ± 85.0 (67.1)	1.0 ± 66.3 (54.5)	1.2 ± 82.2 (64.8)	1.1 ± 81.0 (63.8)
		3	2.1 ± 57.6 (49.7)	0.9 ± 71.6 (57.6)	0.3 ± 67.6 (55.2)	1.1 ± 59.6 (50.3)	1.2 ± 64.2 (53.2)	1.2 ± 63.9 (53.0)
		4	0.5 ± 50.7 (45.1)	1.7 ± 52.5 (46.4)	2.8 ± 51.7 (46.3)	2.8 ± 48.4 (44.2)	1.0 ± 40.5 (39.5)	1.0 ± 39.2 (39.1)
		5	1.1 ± 49.2 (44.4)	1.6 ± 50.4 (45.1)	2.4 ± 50.0 (45.2)	2.7 ± 46.3 (42.1)	1.0 ± 39.2 (38.7)	1.0 ± 38.1 (37.7)
	MS with 2,4-D (mg/l)	1	1.5 ± 73.2 (58.6)	1.7 ± 49.9 (44.8)	1.3 ± 82.2 (64.9)	2.2 ± 84.0 (66.3)	0.8 ± 72.9 (58.5)	0.7 ± 72.4 (58.4)
		2	1.2 ± 68.2 (55.9)	0.7 ± 42.3 (40.5)	1.3 ± 65.3 (53.8)	4.2 ± 75.7 (67.2)	0.6 ± 62.6 (67.2)	0.5 ± 62.5 (66.9)
		3	1.0 ± 51.6 (45.8)	2.4 ± 34.8 (36.1)	1.6 ± 52.4 (46.5)	1.2 ± 64.2 (53.2)	1.2 ± 47.3 (67.2)	1.0 ± 47.1 (66.5)
		4	0.5 ± 36.3 (37.0)	1.3 ± 26.6 (30.7)	0.7 ± 42.2 (40.6)	1.2 ± 44.8 (42.0)	1.0 ± 35.0 (36.2)	0.9 ± 34.5 (36.1)
		5	0.5 ± 35.2 (34.5)	1.4 ± 26.1 (30.2)	0.6 ± 41.2 (40.5)	1.1 ± 44.1 (41.8)	1.1 ± 33.1 (35.7)	1.0 ± 32.8 (35.1)
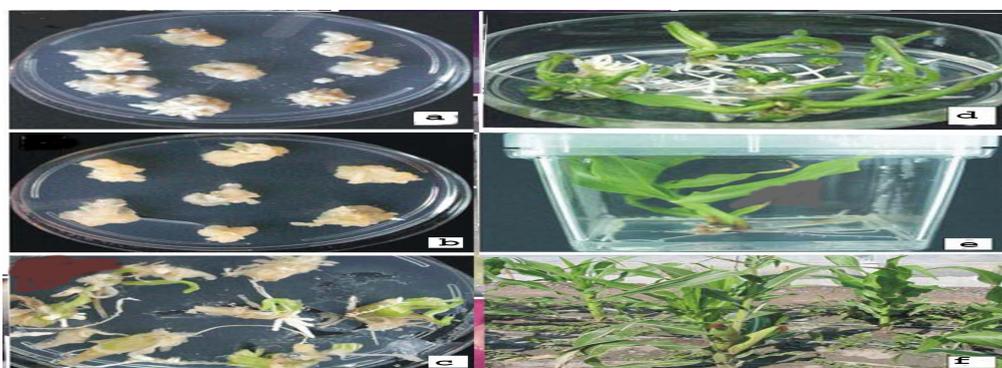
Fig.1 Callusing and regeneration of plants from immature emberyos of Vog134 **a** Organogenic calli in NDA2 medium 30 days after incubation. **b** intiation of shoots in shooting medium(MS with BAP 1 mg/l and IAA 0.5 mg/l). **c** Green shoots from organogenic calli in shooting medium. **d** Plant growth in shooting medium. **e** Plantlets with healthy roots in rooting medium(MS with NAA 1 mg/l). **f** Normal pant regenerated from immature embryos