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Callus induction and whole plant regeneration in sub-tropical maize (*Zea mays* L.) using mature embryos as explants

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Abstract

Thirteen sub-tropical maize genotypes were evaluated for *in vitro* callus induction and whole plant regeneration. Seeds were germinated on MS-based medium supplemented with 10 mg l^{-1} picloram and 3.0 mg l^{-1} BAP which induced the formation of nodes. The developed nodes were excised about 0.25 cm above and below and were split longitudinally into two halves. The split nodes were placed on the callusing medium supplemented with 2.2 mg l^{-1} picloram and 0.5 mg l^{-1} 2, 4-D, the cut surface facing down and touching the media. Among the thirteen genotypes, embryogenic calli were induced in seven genotypes ranging between 10.78-65.65%. Out of seven genotypes, only one genotype i.e., VQL 2 regenerated in to a whole plant with a frequency of 34% on MS basal medium under 80 µM m^{-2} s^{-1} of light intensity. Regeneration medium and light intensity played key role for the initiation of somatic embryogenesis and regeneration of whole plants. Survival efficiency of *in vitro* regenerated plantlets was found to be 95% out of which 85% of the plants grew normally without any morphological abnormality.

Key words: Maize, mature embryo, regeneration, VQL2

Introduction

Globally, maize occupies a predominant place among the food crops, ranking first with respect to total production and 2nd highest with respect to global area after wheat in 2013 (FAOSTAT, 2015). Together with other major staple food crops (rice and wheat), maize is a source of 30% of the food calories to billions of people in developing countries (Anonymous 2011-12). In India, maize is grown over a wide range of agro-climatic conditions. The total cultivated area of maize in India during the year 2013 was 9.50 million hectares with a production of 23.29 million tonnes (FAOSTAT 2015). However, despite gradual increase in the area, production and productivity, the potential of maize productivity has not being fully realized due to a range of biotic and abiotic stresses. To overcome these various stresses development of transgenic maize could be one of the viable strategies. One of the essential requirements for the development of transgenic maize is the availability of an efficient *in vitro* regeneration system.

Presently, immature embryos are mostly used as source of explants to establish regeneration-competent callus cultures in maize (Manivannan et al. 2010). Use of immature embryos as explants is laborious as well as time consuming (Pathi et al. 2013). For round-the-year availability of immature embryos, a growth chamber as well as staggered planting of the crop is essentially required. Alternatively, embryos from mature seeds can be an excellent source as explants which is easy to handle, available year-round and in bulk quantities (Huang and Wei 2004). Reports available for the induction of regenerable callus and development of whole plants from the genotypes adapted to sub-tropical conditions of Indian subcontinent are very meager. A reproducible plant regeneration system for the sub-tropical maize genotypes adapted to Indian condition is essential for the development and deployment of transgenic maize. The present investigation aimed to develop a robust regeneration system in sub-tropical maize genotypes adapted to the Indian condition using mature embryos as explants.

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Materials and methods

Plant materials and seed sterilization

Seeds of sub-tropical maize genotypes *viz*., VQL 1, VQL 2, V 338, V 340, V 351, V341, V 25, CM 145, CM 212, CML 189, CML 176, Vivek Maize hybrid 9, Vivek QPM 9 were collected from healthy plants grown at the Hawalbagh Experimental farm, VPKAS, Almora. Seeds were surface washed first by 1% bavistin for 20-30 minutes followed by washing with sterile double distilled H_2O for 4 to 5 times. The bavistin-treated seeds were again treated with 0.2% SDS and 0.1% $HgCl_2$ for 10 minutes for sterilization followed by washings with double distilled sterile H_2O for 4 times.

Induction of node

Sterilized seeds were dried on sterile tissue paper and were inoculated on seed germination medium (GM) [GM = MS salts and vitamins (Murashige and Skoog 1962) + 40 g l^{-1} maltose + 0.1 g l^{-1} casein hydrolysate + 1.95 g l^{-1} MES + 0.75 g l^{-1} magnesium chloride + 0.5 g l^{-1} glutamine + 0.1 g l^{-1} ascorbic acid + 10.0 mg l^{-1} picloram + 3 mg l^{-1} BAP]. The medium was free of thiamine HCL. 50 seeds of different genotypes were placed on Petri dishes (10 per 90 mm Petri dish) and were incubated under 16 hrs light (40 µmol s^{-1} m^{-2}) at 28°C ± 0.5°C. Seedlings of different genotypes were observed for the number of days taken for the development of conspicuous node (bulging) (Fig. 1a).

Induction of primary calli

The seedlings with developed nodes of different genotypes were selected and the nodes were excised about 0.25 cm above and below the node. The nodes were split longitudinally into two halves. The split nodes were placed on the callusing medium (CM), cut surface facing down and touching the medium [CM=MS salts and vitamins (Murashige and Skoog 1962) + 0.5 g l^{-1} casein hydrolysate + 30 g l^{-1} sucrose + 1.38 g l^{-1} L-proline + 3.4 mg l^{-1} $AgNO_3$ + 0.5 mg l^{-1} 2, 4-D + 2.2 mg l^{-1} picloram]. 150 split-nodes per genotype were cultured on CM medium contained 0.5 mg l^{-1} thiamine HCL (10 seeds per 90mm Petri dish) and incubated under 16 hrs light (40 µmol s^{-1} m^{-2}) at 28°C ± 0.5°C. The frequency of primary callus induction (%) was calculated as the ratio of calli induced to the total number of split node placed on the callusing medium.

Induction of embryogenic calli

The primary calli were separated from mucilegenous and non-embryogenic tissues and were sub-cultured onto the same medium (CM) for two cycles under the dark condition at 28 ± 0.5°C. The frequency of embryogenic callus induction (%) was calculated as the ratio of embryogenic calli induced to the total number of primary calli placed on medium after two subcultures, each of 15 days.

Plant regeneration

The embryogenic calli of VQL 2, V 338, V 340, CM 145, CM 212, CML 176, Vivek QPM 9 were placed on nine different regeneration medium *viz*., MS + kin (0.1), MS + kin (0.2), MS + BAP (0.1), MS + BAP (0.2), MS + BAP (3.5), MS + kin (3.5), MS + BAP (3.5) + NAA (0.5), MS + kin (3.5) + NAA (0.5) and MS basal under two light conditions (40 µmol s^{-1} m^{-2} and 80 µmol s^{-1} m^{-2}) at 28°C ± 0.5°C. Regeneration of whole plant from the embryogenic calli took 7-10 days from the day of placement on regeneration media. 10-15 day-old plantlets with one to two leaves were transferred to Magenta box containing ½ strength MS medium for vigorous growth of the roots. The plantlets possessing well developed roots were transferred to growth chamber for hardening.

Hardening of regenerated plants

Regenerated plants possessing well developed roots were selected and subsequently, the roots were washed with double distilled sterile H_2O to remove the clarigel adhered to roots. Roots were then treated with 1% bavistin for 1 minute followed by a brief exposure of 1 ppm IBA for 30 seconds. Following washing, plantlets were then transferred to small pots containing Soilrite mix (Karnataka Explosives, India) and subsequently, watered very lightly. Recommended dose of nitrogenous fertilizers were applied to the plants during planting, post-planting (15 days later), pre-flowering and mid-cob stage. The temperature range of green house was set at maximum of 35°C and minimum of 15°C.

Data analysis

Analysis of variance (ANOVA) of experiments laid out in Completely Randomized Design (CRD) was performed using Microsoft Excel (2007). All the data in percent were subjected to arcsin transformation and DMRT analysis was performed using SPSS ver. 10.0.

Results and discussion

Induction of nodes

A wide variability for number of days required for the induction of nodes was observed among the thirteen genotypes. The ANOVA revealed that genotypes

differed significantly for the induction of nodes (Table 1). Based on DMRT results, average number of days required for the induction of nodes varied significantly among the genotypes ranging from 7.31 ± 0.03 to 11.31 ± 0.03. However the genotypes V 340 and CML 189; V 25 and Vivek Maize Hybrid 9; CM 145 and Vivek QPM 9 did not differ significantly. Variability observed among the genotypes indicated that genotypes significantly affected the induction of nodes.

Induction of primary calli

Culturing of split nodes derived from 13 genotypes on the callusing medium led to the formation of primary calli. Primary calli were those which were induced directly from split-nodes placed on the callusing medium. During the present investigation two types of primary calli were noted. One type consisted of globular, compact and non-mucilaginous and other type consisted of mucilaginous, dark brown and green meristematic. The ANOVA revealed that genotypes differed significantly for the induction of primary calli (Table 1). As evidenced from the DMRT data, the induction percentage of primary calli among the different genotypes varied significantly, ranging from 7.11 ± 0.47 to 98.69 ± 0.29 (Table 2). VQL 1 possessed 92% of the genome of CM 212 whereas VQL 2 possessed 94.44% of the genome of CM145 (Gupta et al. 2009). The callus induction in case of CM 145 (11.44%) was lower than VQL 2 (30.84%). VQL 2 was developed by crossing CM 145 and CML 170. With all the possibility ~5.5% genome coming from CML 170 might be responsible for the callusing and regeneration potential of VQL 2.

Induction of embryogenic calli

Culturing of primary calli on the callusing medium derived from different genotypes led to the formation of embryogenic calli. Embryogenic calli were those which were induced from globular, compact and non-mucilaginous primary calli. The ANOVA revealed that genotypes differed significantly for the induction of embryogenic calli (Table 1). As evidenced from the DMRT data, the percentage of induction of embryogenic calli among the genotypes varied significantly, ranging from 0 to 65.65 ± 0.35 (Table 2). VQL 1 possessed 92% of the genome of CM 212 whereas VQL 2 possessed 94.44% of the genome of CM145 (Gupta et al. 2009). CM 212 showed 16.15% embryogenic calli development whereas VQL 1 did not show any embryogenic calli development. This indicates that during the process of conversion, genes responsible for embryogenic callus induction got lost. In case of VQL 2, it is quite opposite. The callus induction in case of CM 145 (11.44%) was lower than VQL 2 (30.84%). VQL 2 was developed by crossing CM 145 and CML 170. With all the possibility ~5.5% genome coming from CML 170 might be responsible for the callusing and regeneration potential of VQL 2. In addition, the background effect, allelic and non-allelic interaction together in Vivek Maize Hybrid 9 did not result in callus induction whereas there was callus induction in Vivek QPM 9. During the present investigation, the morphogenic characteristics of the embryogenic calli ranged from compact, globular, granular, slow growth, light yellow, pale yellow, brown and watery white. The formation of Type I calli (compact and slow growing) observed were in congruence with Vain et al. (1989). It was observed that none of the genotypes could induce Type II calli (fast growing and friable) which is in agreement to Carvalho et al. (1997) and Abhishek et al. (2014) who reported the induction of Type II calli to be genotype dependent. Among the genotypes which induced embryogenic calli, VQL 2 displayed typical Type I morphogenic characteristics such as compact organized, slow growth pattern which is in agreement with the findings of Armstrong and Green (1985) and Vain et al. (1989). Differences for the percentage of induction of embryogenic calli among the genotypes are in conformity with Manivannan et al. (2010) and Dhillon and Gosal (2013).

Table 1. ANOVA summary for the induction of node, primary calli and embryogenic calli

Source	Induction of node				Induction of primary calli				Induction of embryogenic calli			
	DF	SS	MSS	F value	DF	SS	MSS	F value	DF	SS	MSS	F value
G	12	50	4	1742*	12	15603	1300	2535*	12	202	2	7527*
E	26	0.1	0.002		26	13	0.51		26	0.006	0.0002	
CD (G)	0.1				1.2				0.03			

G=Genotypes, E=Error, DF=Degree of freedom, SS=Sum of square, MSS=Mean sum of square and *Significant at $p<0.05$

Table 2. DMRT analysis for the induction of primary and embryogenic calli among the genotypes

Geno-types	%age of primary calli development@,#	%age of embryogenic calli development$,++
VQL 1	14.07 ± 0.38 (22.03)^{i}	0(0.00)^{f}
VQL 2	30.74 ± 0.43 (33.69)^{e}	30.84 ± 0.42 (1.50)^{c}
V338	66.0 ± 0.46 (54.36)^{d}	51.72 ± 0.38 (1.72)^{b}
V340	82.97 ± 0.31 (65.66)^{b}	10.78 ± 0.39 (1.07)^{e}
V351	7.11 ± 0.47 (15.46)^{k}	0(0.00)^{f}
V341	28.75 ± 0.27 (32.44)^{f}	0(0.00)^{f}
V25	25.01 ± 0.57 (30.02)^{g}	0(0.00)^{f}
CM145	25.74 ± 0.79 (30.50)^{g}	11.44 ± 0.38 (1.09)^{e}
CM212	12.88 ± 0.5 (21.03)^{i}	16.15 ± 0.32 (1.23)^{d}
CML189	70.71 ± 0.41 (57.26)^{c}	0 (0.00)^{f}
CML176	98.69 ± 0.29 (83.57)^{a}	65.65 ± 0.35 (1.82)^{a}
Vivek Maize Hybrid 9	10.98 ± 0.49 (19.35)^{j}	0 (0.00)^{f}
Vivek QPM 9	18.67 ± 0.27 (25.61)^{h}	15.49 ± 0.68 (1.21)^{d}

@Data represents the means (± SE) of three replicates, each with 150 split-nodes; # = $^{a-k}$Values in parenthesis (represent transformed values) followed by different letters in a row are significantly different at $p<0.05$ according to DMRT; $ = Data represents the means (± SE) of three replicates, each with 100 primary callus and ++ = $^{a-f}$Values in parenthesis (represent transformed values) followed by different letters in a row are significantly different at $p<0.05$ according to DMRT

Plant regeneration

Embryogenic calli those were transferred to regeneration media did not differentiate presumably due to low light intensity (40 µM m^{-2} s^{-1}). However, when the light intensity was increased to two times (80 µM m^{-2} s^{-1}), one genotype *viz.*, L 2 showed a range of somatic embryos such as globular, heart and torpedo shapes on the surface of embryogenic calli those were transferred to MS basal medium. These embryoids later on regenerated into whole plant at a frequency of 34% (Fig. 1c, 1d, 1e). This suggests that the sub-tropical maize genotypes used during the present investigation required higher intensity of light for plant regeneration which is in agreement with the reports of Huang and Wei (2004) and Sidorov et al. (2006). The present investigation also revealed genotype-dependent regeneration in maize which is in agreement with other reports (Bedada et al. 2011; Pathi et al. 2013; Abhishek et al. 2014). Moreover, the present study revealing that the genotypes capable of initiating embryogenic calli did not regenerate in to whole plants is in agreement with Carvalho et al. (1997). Similar to the reports of Bedada et al. (2011), during the present investigation also all somatic embryos placed on regeneration medium did not produce plantlets revealing failure of some somatic embryos to form plantlets. Bedada et al. (2011) reported that genes controlling plant regeneration were down regulated during regeneration. During the present investigation, it was also observed that various hormonal combinations [MS + kin (0.1), MS + kin (0.2), MS + BAP (0.1), MS + BAP (0.2), MS + BAP (3.5), MS + kin (3.5), MS + BAP (3.5) + NAA (0.5), MS + kin (3.5) + NAA (0.5)] attempted in regeneration medium could not achieve whole plant regeneration in any of the genotypes both under 40 µM m^{-2} s^{-1} and 80 µM m^{-2} s^{-1} which emphasizes the importance of genotypes, source of explants and growth conditions of callus for robust plant regeneration system. Moreover, the results revealed that use of hormone(s) is not essential for plant regeneration which is in concurrence with the report of Armstrong and Green (1985). The possible explanation of this phenomenon was provided by Huang and Wei (2004) who assumed that somatic embryos competent to give rise to new plantlets were already formed and their fate may be predetermined by the initiation media. Moreover all plantlets produced roots on the medium without growth regulators. Hence their transfer to medium supplemented with rooting hormone was not necessary which is in agreement with Aguado-Santacruz et al. (2007).

Survival efficiency and morphological characterization of tissue culture derived plants

Seventy tissue culture derived plants of VQL 2 were transferred to soil and the whole plant survival efficiency observed after 15 days, ranged from 94.73 to 96.29%. Out of them 85% of the plants grew normally without any morphological abnormality (Fig. 1f to 1g) and 14.28% showed phenotypic abnormality like multiple ears, emergence of tassel and silk from the tassel stock and dwarf phenotype without tassel. Among the 10 abnormal plants, 40% of the plants showed 3 ears, 30% of the plants had 2 ears and remaining 30% of the plants had one ear per plant. The observed abnormalities in tissue culture raised plants are in congruence with reports of Anami et al. (2010) who reported that alternations of heterochromatic knobs or activation of transposable elements during culturing process might have played a role in causing phenotypic abnormalities in the maize regenerants.

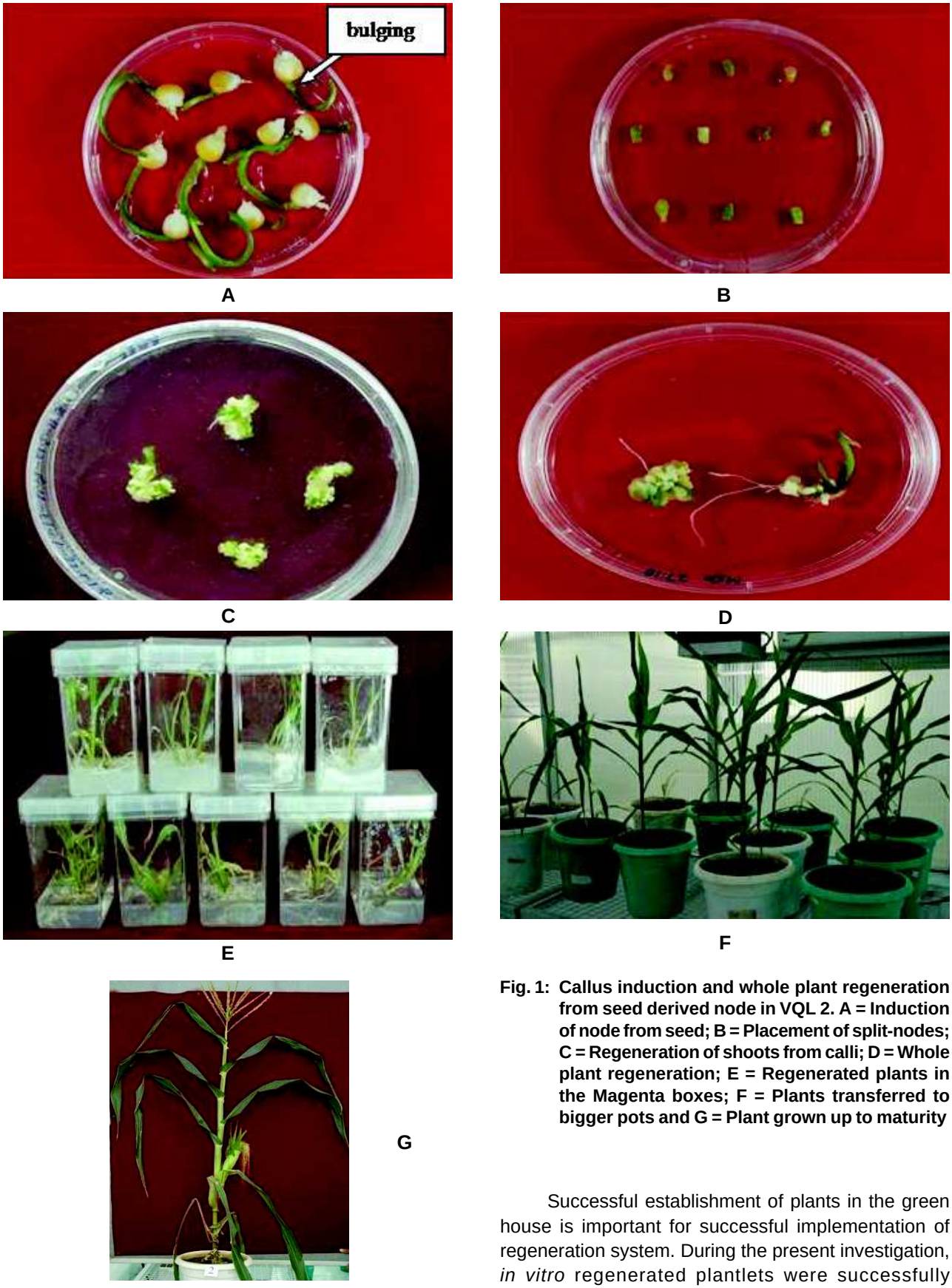

Fig. 1: Callus induction and whole plant regeneration from seed derived node in VQL 2. A = Induction of node from seed; B = Placement of split-nodes; C = Regeneration of shoots from calli; D = Whole plant regeneration; E = Regenerated plants in the Magenta boxes; F = Plants transferred to bigger pots and G = Plant grown up to maturity

Successful establishment of plants in the green house is important for successful implementation of regeneration system. During the present investigation, *in vitro* regenerated plantlets were successfully

established with a mean frequency of 95% which is higher than Gorji et al. (2011) who reported establishment of tropical maize genotypes, Va35 and Vog134 at a frequency of 55 and 65% respectively. Similarly, Anami et al. (2010) reported plantlet survival efficiency for tropical inbred lines (CML 216, CM 244) and their single-cross hybrids of maize (CML 216 × TL 26, CML 244 × TL 26) to be 24%, 24%, 83% and 29% respectively. Odour et al. (2006) reported 95% of the regenerants surviving the acclimatization process which is in agreement with that of the present investigation. The robust whole plant regeneration system reported during the present study with less percentage abnormal regenerants will pave way for developing maize transgenics and result in transgenic maize resistant to stem borer. The present investigation reports a whole-plant regeneration system in sub-tropical maize genotype through somatic embryogenesis. VQL 2 is an elite maize inbred and is a parent of QPM hybrid - Vivek QPM 9, a hybrid released for commercial cultivation in the hills and plains of India. The present investigation resulted in a robust callus induction whole-plant regeneration system in the sub-tropical maize genotype with less percentage of abnormal regenerants which will pave way for developing maize transgenics effective against biotic and abiotic stresses.

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