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Effect of Desiccation of Maize Calli on the Efficiency of Transformation Prior and Post to Infection by *Agrobacterium Tumefaciens*

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Abstract

MS media containing 6-benzyl-amino-purine (3mg/ L) and picloram (10mg/L) considered for seed germination, after 10 days conspicuous nodes were splitted into two longitudinal halves then inoculated on MS callusing media having 2-4 dicholorophenoxyacetic acid (0.5mg/L) with picloram (2.2 mg/L). Seedling derived callus of maize inbred line VQL2 was used for Agrobacterium transformation. Calluses desiccated for 1 hour prior to infection resulted in maximum greening 91.2%, whereas with 79.2% greening, calluses desiccated for 3 hours prior to infection showed highest percentage 10.4% of plant regeneration. Though greening was 6% and 12% in calluses desiccated for 2 and 3 hours respectively, prior to infection, no root and shoot regeneration was observed. Ascorbic acid was also having positive effects as 32.33% greening was observed in the calluses.

Keywords: Agrobacterium transformation, maize, Seedling derived callus, plant regeneration, callus desiccation.

Maize (*Zea mays* L) is considered an important crop as food and low cost quality feed for poultry, fishery, piggery and livestock. In India, with an annual production of 18.54 million tones in 2007-2008, maize ranked, third highest producing grains[1]. Limited land for agriculture, water resources, expanding population and environmental stresses, emphasised on the need to increase the production and productivity of maize. The losses due to biotic and abiotic stresses in maize are very significant in North-West Himalayan region like any other parts of India where maize is cultivated. It is desirable to engineer new genotypes for maize so as to obtain new hybrids, mapping of quantitative trait loci and characterization of linkage disequilibrium, as previously done for corn[2].

Over the past decade, substantial progress has been made in advancing maize transformation technologies, including *Agrobacterium mediated* transformation. In comparison to biolistic gun, this method resulted in much stable, low copy number transgenics[3,4] increasing the chances of cloning larger segments of DNA into the host cells[5]. Apart from plant genotype, *Agrobacterium* strains, components of media and factors affecting specific tissues, along with others, should be considered properly for recuperating transformation competence of plants.

Material and Methods

Seeds (maize inbred line VQL2) are available at Vivekananda Parvatiya Krishi Anusandhan Sansthan (V.P.K.A.S), (ICAR), Almora.

The *Agrobacterium* strain EHA105, carrying a binary vector pCAMBIA3301, is provided by V.P.K.A.S, Almora. The vector contains *Cry*1Ab gene and a reporter beta- glucoronidase (*gus*) gene driven by ubiquitin promoter of maize and nopaline synthase (NOS) terminator. Plant selection marker was bialaphos resistance (*bar*) gene.

Preparation of Antibiotics: Antibiotics Kanamycin and Rifampicin were dissolved in Water (200mg/ ml) and DMSO (20mg/ ml) respectively. The stock solutions were filter sterilized by passing them through sterile 0.22 mm filter units (Millipore) and were stored in refrigerator at 4^0C.

Plant Tissue Culture Medium: Murashige and Skoog (MS media) [Composition gm/l; Macronutrients: $NH_4NO_3$16.50; KNO_3 19.00; $MgSO_4$. $7H_2O$ 1.70; KH_2PO_4 1.70; $CaCl_2$ 4.40 Iron EDTA: Na_2 EDTA 1.8665; $FeSO_4$. $7H_20$ 1.39750; Micronutrients H_3BO_3 0.6; KI 0.083; $ZnSO_4$. $7H_2O$ 0.86; $MnSO_4.H_2O$ 2.25; Na_2MoO_4. $2H_2O$ 0.025; $CoCl_2$. $6H_2O$ 0.0025; CuSO4. 5H2O 0.0025 ; Vitamins and growth regulator: Thiamine 0.01; Pyridoxine 0.05; Nicotinic acid 0.05; Glycine 0.2 ; Myoinositol 0.1; Sucrose 30; Clarigel 3.

MS media +10mg / L picloran +3mg / L benzl-amino-purine was used for seed germination (MSG), MS media + 2.2 mg/l picloram + 0.5 mg/L 2-4 di-choloro-phenoxy-acetic acid was callusing media (MSC)[6] and basal MS was regeneration media . All the stock solutions of MS medium were stored at 4^0C in the refrigerator.

***In vitro* plant regeneration, Surface sterilization and seed Germination:** Seeds were surface sterilized for 10 minutes using SDS (0.4%) and $HgCl_2$ (0.2%), then washed with distilled water. Later, seeds were placed on MSG media and allowed to

germinate for 16 hours of light inside the culture room at 28 ^{0}C for 10-12 days.

Callus induction from split nodes: After 10-12 day germinated seeds were taken. The area about 0.5 cm above and below the node was aseptically spliced into two equal longitudinal halves. Split nodes were placed on MSC medium the cut surface facing the media under 16 hours of light inside the culture room at 28 ^{0}C. After 20-25 days of culturing in the light, primary calluses were selected and transferred to dark for embryogenic callus induction in the same medium for a month at 28^{0}C.

***Agrobacterium*- mediated transformation of callus induced from split-node, Yeast Extract Mannitol (YEM) medium:** It is considered as a rich source for growth of *Agrobacterium* [(Ph 7; composition gm gm l^{-1} $MgS0_4.7H_20$ 0.2, Mannitol 10.0, NaCl 0.1, KH_2P0_4 0.5, Yeast Extract 1.0; Agar 1.5% (w/v)] After autoclaving, the media was allowed to cool, then antibiotics *viz.* Rifampicin, 10mg l^{-1} and kanamycin, 50mg l^{-1} were added.

Infection and Co-cultivation: The bacterial culture was streaked on YEM solid medium and incubated at 28 ^{0}C for 24hr. Single isolated colony of *Agrobacterium* was inoculated in 5 ml YEM liquid medium at 28 ^{0}C and kept at 150 rpm for 24 hr. The culture was then added to 100 ml YEM medium in 250 ml Erlenmeyer flask and grown for another 12 hours. The bacterial culture was centrifuged at 10,000 rpm for 10 minute at 25^{0}C, pellet was resuspended into infection medium (Half strength MS salts + vitamins + 6.85% Sucrose + 3.6% Glucose + 200µM Acetosyringone) and the Optical Density of the *Agrobacterium* suspension solution was adjusted to 1.0 at 660 nm. Calluses were put in the conical flasks with *Agrobacterium* suspension solution. The infection was carried out with gentle shaking for 10 minutes at room temperature. After infection, extra bacterial suspension was pipette out and calluses were blot dried for 1 hour in laminar air flow bench. After that calluses were transferred to Co-cultivation media (MSC with 200 µM Acetosyringone) for 2 days at 23^{0}C. Calluses were then transferred to selection medium (MSC with 10mg/l Basta + 500mg l^{-1} Carbenicillin) for 1 month with a subculture after 15 days. Surviving calluses was regenerated (MSO media + 3mg l^{-1} Basta + 250 mg l^{-1} Carbenicillin) for 15 days and placed under 16 hours light inside the culture room at 28 ^{0}C. Transformation efficiency (%) is calculated as the number of Basta defiant calluses with root and shoot recovered per 100 embryos infected.

Experiments were conducted to test the effect of desiccation on calluses prior and post infection and to evaluate the effect of ascorbic acid in both infection and Co-cultivation media side by side along with above mentioned standard method of transformation. For evaluating the effect of ascorbic acid, it is added in the infection and Co-cultivation media after autoclaving them.

Results and Discussion

Embryogenic calluses were selected and desiccated in sterile filter paper in laminar air flow bench for 1 hour and 3 hour prior to infection and 2 hour and 3 hour post infection.

The effect of different parameters studied is depicted in table-1 while the explants growth in the medium is seen in the proceeding figures as follow: i. 300 numbers of calluses were infected with standard method of transformation, 42.8% greening observed with 0.8% shoot induction, 12.4% root induction and 2.8% both shoot and root. ii. 91.2% greening, 9.6 % shoot induction, 15.6% root induction and 6.4 % of shoot and root induction was observed when 310 number of calluses were desiccated for 1 hour prior to infection. iii. 79.2% greening, 5.6% shoot induction, 8.4% root induction and 10.4% of shoot and root induction was observed in calluses desiccated for 3 hours prior to infection. iv. 6.0 % and 12.5% greening observed in calluses desiccated for 2 hours and 3 hours post infection mediated by *Agrobacterium*. No shoot or root induction was observed in any of these treated calluses. v. When ascorbic acid was added in Infection and Co-cultivation media, 32.33% greening was observed.

Table-1
Different parameters studied for infection and plant regeneration

Parameters Considered	No. of calli infected	% Greening	% shoot induction	% Root induction	% Shoot and Root
Standard (S)	300	42.8	0.8	12.4	2.8
I	310	91.2	9.6	15.6	6.4
II	300	79.2	5.6	8.4	10.4
III	200	6.0	0.0	0.0	0.0
IV	200	12.5	0.0	0.0	0.0
V	300	32.33	0.0	0.0	0.0

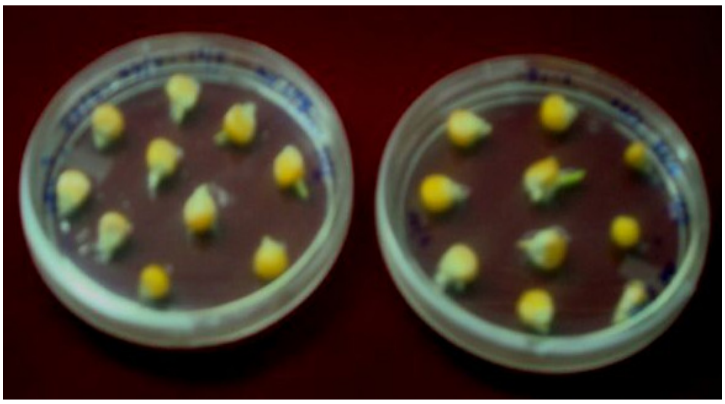

Figure-1
Germination of maize seeds on MSG medium

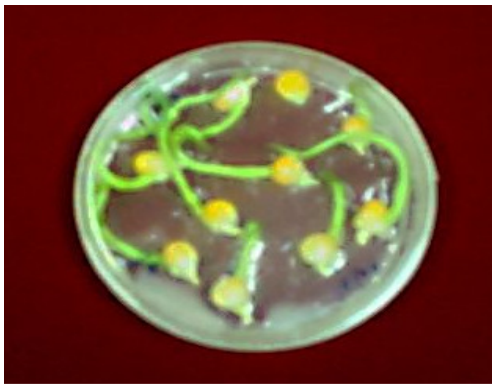

Figure-2
Germinated seeds (10 days) showing nodal region

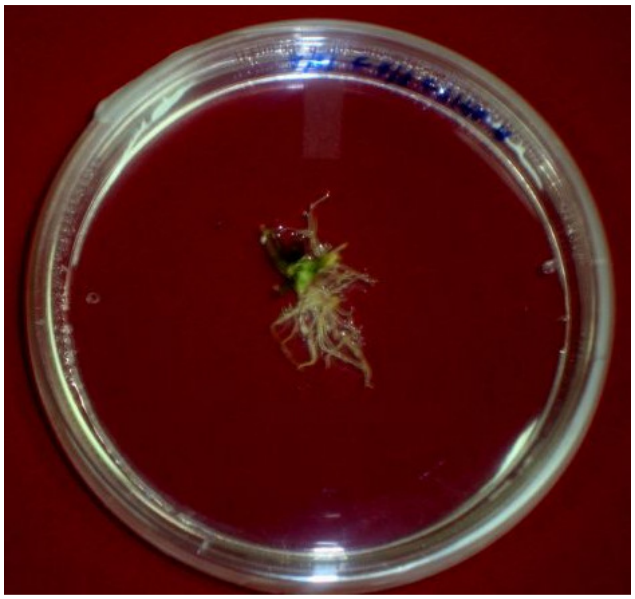

Figure-3
Root induction in callus

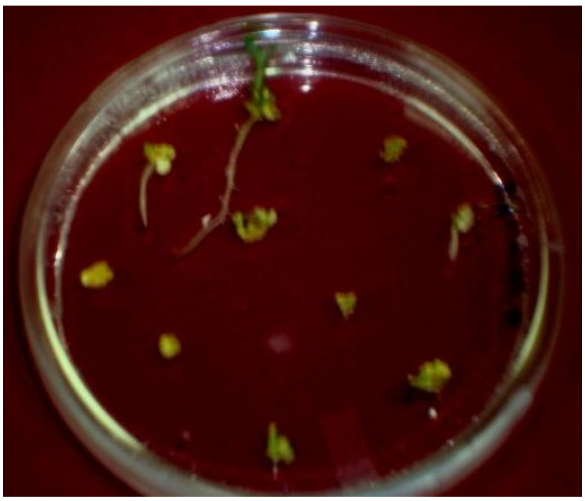

Figure-4
Root and shoot induction in calluses

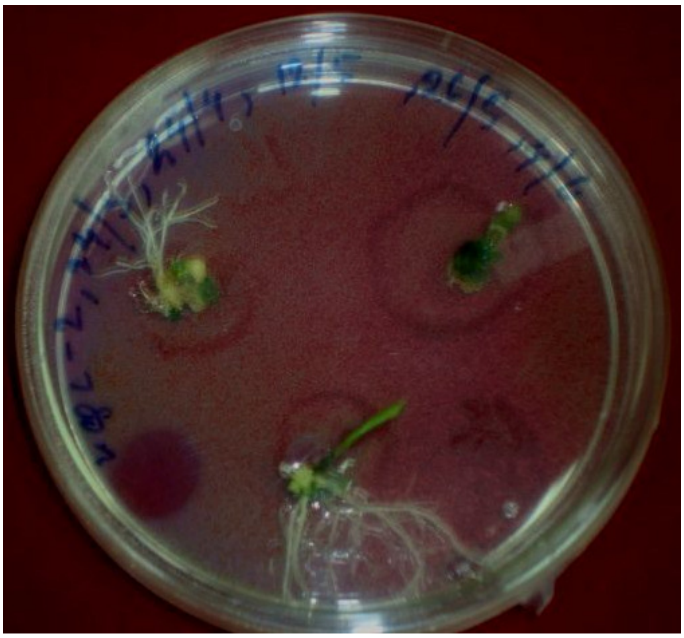

Figure-5
Calluses with root, shoot and greening

Discussion: The results are showing a visible effect of desiccation on calluses observed with 10.4% of shoot and root induction when calluses were desiccated for 3 hours prior infection. On comparing desiccation on calluses post and prior to infection, the regeneration frequency is higher for the later. Possible reasons like plasmolysis or wounding can be attributed to this observation which might be playing a role in increasing the stable transformation efficiency mediated by *Agrobacterium tumefaciens*.

Ascorbic acid, an important vitamin added in the infection media and Co-cultivation media, showed 32.33% greening which is near about to that found in control calluses.

Different influential factors that affect gene transformation in maize via *Agrobacterium tumifaciens* have been studied extensively. These factors mainly include *Agrobacterium* strain[3,4], binary vectors[4] , type of explants and plant genotype[7,8].

Plant cell or tissue desiccation post infection caused by *Agrobacterium tumifaciens*, was a new innovative physical parameter which was found to increase transformation efficiency via transfer -DNA delivery during co-culture[9]. It was found in studies that suppression of *Agrobacterium tumifaciens* growth have a positive impact on plant cell regeneration after desiccation of embryonic calluses in maize, the reason may be the enhanced transfer-DNA delivery after co-culture[10]. Although the molecular mechanism of desiccation is still unknown. Experiments have also showed that recovery of embryonic calluses in maize after desiccation is better than non desiccated conditions. When compared to the control, 10-15 min of calluses air drying increased the transformation efficiency up to 10 fold or more[11].

Moderate and rapid desiccation varies in terms of their results. Moderate desiccation bettered shot regeneration yield in maize where as rapid regeneration enhanced shoot activity but the efficiency is very much relied on time duration of desiccation but in both cases regeneration percentage increased during initial stage of induction.

Conclusion

The findings of our experiments clearly suggest the positive effect of desiccation on calli prior to infection enhance gene transformation frequency mediated by *Agrobacterium tumifaciens*. Also ascorbic acid when added to infection and co cultivation medium resulted in higher percentage of greening. Thus we can conclude that desiccation of calluses speed up regeneration course in maize.

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