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Screening for Water Deficit Tolerance, Relative Growth Analysis and *Agrobacterium*-Infectivity in Tropical Maize [*Zea Mays* L.] Inbred Lines in Nairobi, Kenya

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Abstract*: The gap between maize demand and regional supply is increasing as small-holder farmers grapple with many challenges, key among them drought. Research in identifying maize lines that are tolerant to water deficit and that are amenable to *A. tumefaciens*-mediated transformation is a step towards enhancing food security. The objectives of this study were, to assess the physiological response of tropical maize inbred lines to water deficit, to determine whether *A. tumefaciens* elicits host resistance when in contact with zygotic embryos and determine if YEP is an effective infection medium compared to MS medium in immature zygotic embryo infection. Tropical maize lines CML 395, CML 216, CML 144, TL 21, A 04, E 04 and T 04 and *Agrobacterium* strain EHA101 habouring pTF102 vector containing *GUS* reporter gene were used. Physiological response of tropical maize genotypes to drought stress was evaluated by measuring plant height, leaf length, leaf width and fresh weight. T 04 and CML 216 seedlings exhibited the fastest growth rates of 4.33 cm and 4.28 cm respectively between the 7th and 8th day post leaf four emergence while TL 21 and CML 395 seedlings had the lowest rates of 2.93 cm and 3.59 cm respectively under normal growth conditions. A 04 seedlings exhibited the highest differences in fresh weight between stressed and unstressed plants [56.13 gm] while CML 216 seedlings had the lowest [24.57 gm]. Upon salt stress, leaf discs of CML 216 seedlings, exhibited low chlorophyll a: b ratios of 0.84, 0.78, 0.74, 0.75 and 0.70 in 100 mM, 125 mM, 150 mM, 175 mM and 200 mM NaCl concentrations respectively, while leaf discs of CML 144 seedlings exhibited higher chlorophyll a: b ratios of 1.51, 1.19, 1.24, 1.26, 1.45 and 1.39 under the same concentrations. The use of YEP medium in contrast to MS medium led to an improvement in transient *GUS* expression observed in immature embryos and significant increase in transformation frequency. The transformation protocol using YEP infection media as used in this study should be optimized and used in transformation of tropical maize inbreds.

***Keywords*: Transformation, *A. tumefaciens*, zygotic embryo, vector, water deficit, senescence, genotype, phytohormones, transgene, recalcitrant.**

I. INTRODUCTION

The wide gap in maize production between developing countries and developed countries could be due to disparities in farming technologies where agriculture in the west is characterized by high levels of inputs such as quality seed stock, fertilizers, herbicides and pesticides and a high degree of mechanization [Gallup and Sachs, 2000]. Increased maize productivity and production are key in ensuring food security in this region. Conventional breeding methods have been used by maize breeders to develop and improve populations and gene pools that can serve as sources of superior open pollinated varieties (OPVs), inbred lines, and hybrids [Pandey and Gardner, 1992].

Drought is ranked among the most important constraints to maize productivity in the East and Central African region, contributing to production losses of about 17%, equivalent to US$ 280 million [Diallo *et al.*, 2005]. Despite the fact that there are alternative plant transformation tools, *A. tumefaciens* is the most preferred vehicle of gene delivery because of its simplicity, cost-effectiveness and frequent single copy gene integration into the host plant genome [Franklin *et al*., 2008]. *A. tumefaciens* possess the unique ability of inter-kingdom gene transfer by introducing a defined piece of DNA, from its tumour or hairy root inducing plasmid to the host plant cells by conjugal transfer. This study aimed at evaluating the physiological response of seven selected tropical maize genotypes to water deficit to distinguish those that can grow under water limited environments, assess the suitability of their zygotic immature embryos to *A. tumefaciens* infectivity and to determine if Yeast Extract Peptone (YEP) medium is effective in the infection of immature zygotic embryos (IZE) medium compared to Murashige and Skoog (MS) medium.

II. MATERIALS AND METHODS

Establishment of the plants and Plant growth analysis

Seven inbred maize lines CML 395, CML 216, CML 144 , TL 21, A 04, E 04, and T 04 were used in this research. Twelve seeds from each inbred line were planted individually in 5 liter plastic pots containing soil outside the research field. Measurements of leaf four commenced immediate leaf four emerged and continued until no further growth of the leaf was noted. The same inbred lines were planted in a research farm at the Kenya Agricultural Research Institute (KARI) Kabete with controlled pollination and were used in the provision of immature zygotic embryos for *A. tumefaciens* infection. Leaf elongation rate was determined by getting the difference between growths of leaf four on daily intervals.

Leaf disc assays measurements

Leaf discs of about 0.3 cm in diameter were excised from the flag leaf of three plants per genotype using a paper punch. An average of ten leaf discs were floated in 15 ml solution of NaCl at different concentrations (100, 150, 175, 200 mM) and mannitol (100, 200, 400, 600, 800 mM) in 90 ml petri plates and kept in the dark over night. The next day, the discs were placed under 16/8 hour photoperiod at room temperature. Distilled water was used as a control. The effects of these treatments on the leaf discs were observed by checking the leaf-disc senescence and morphological effects on cells in response to the treatments.

Water deficit assays

Twelve seeds per genotype were sown in each pot in a completely randomized design. The plants were watered daily with 250 ml of tap water. On the emergence of the 8^{th} leaf, a cycle of drought was induced by not watering for 10 days. A control set was watered daily with 250 ml water. The length and width of the 8^{th} leaf, as well as plant height were measured and recorded. After 10 days of water deficit, the plants were watered with 250 ml water for 3 days for recovery. On the third day, plant height, leaf length and leaf width of the 8^{th} leaf were measured. The plants were then cut at the base of the stem and the fresh weight of each plant was measured using a Mettler Toledo PB 1502- S/fact analytical scale [Goindustry Dovebid European instruments, Switzerland].

Immature zygotic embryo induced inhibition of *A. tumefaciens* growth

Embryos were excised from the kernels using a pointed spatula in aseptic conditions according to [Ombori *et al.*, 2008]. 100 % (v/v) *A. tumefaciens* concentration was serially diluted to 75 %, 50 %, and 25 % (v/v) using fresh YEP medium. One ml of each dilution was spread on sterile petri plates containing solid YEP, YEP A (2,4-D 2 mg/ml), YEP B (dicamba 3 mg/ml), YEP C (picloram 15 mg/ml), MS A (2,4-D 2 mg/ml), MS B (dicamba 3 mg/ml) and MS C (picloram 15 mg/ml) media regimes. Ten embryos from each genotype were placed on the petri plates according to the concentrations, with the scutellum side up and the embryo axis in contact with the medium plus *A. tumefaciens* cells. Factorial design (2x4x5) was used in this experiment and three replicates set. The plates were incubated in the dark at a temperature of 28° C for 24 hours and observations of *A. tumefaciens* growth around the embryos were made. The inhibition rings (halos) were measured under an optical microscope with a millimeter ruler and the readings averaged for each genotype. The activity of the β-glucuronidase enzyme was determined by counting the number of embryos with blue staining.

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Data management, analysis and presentation

Growth parameters (leaf length, leaf width, plant height and fresh weight) of stressed and unstressed plants of the selected genotypes were analyzed using ANOVA at 95 % confidence interval with MINITAB statistical computer software (version 23.22). Mean separation was carried out using Tukey's pairwise comparison test at 5 % probability level. Transformation frequencies were calculated as the number of embryos that showed *GUS* expression over the total number of embryos infected as a percentage.

III. RESULTS

Plant growth analysis

Analysis of variance [ANOVA] revealed no significant differences in the lengths of the 4th leaf among all the genotypes at P>0.05, on the first day up to the fourth day after leaf four emergence. On the 5th day, leaf lengths of TL 21and E 04 seedlings were significantly shorter compared to leaf four of all other seedlings from other genotypes (P<0.05). On the 14th day of the experiment there was significant difference in length of the 4th leaf among the genotypes except inbred lines A 04, CML 395 and CML 144 (P<0.05). Seedlings of T 04 recorded the longest leaf length with a mean of 44.54 cm. This was closely followed by leaves of CML 216 and A 04 seedlings with mean leaf lengths of 43.29 cm and 41.95 cm respectively. Seedlings of TL 21 had the shortest leaf length with a mean of 33.0 cm.

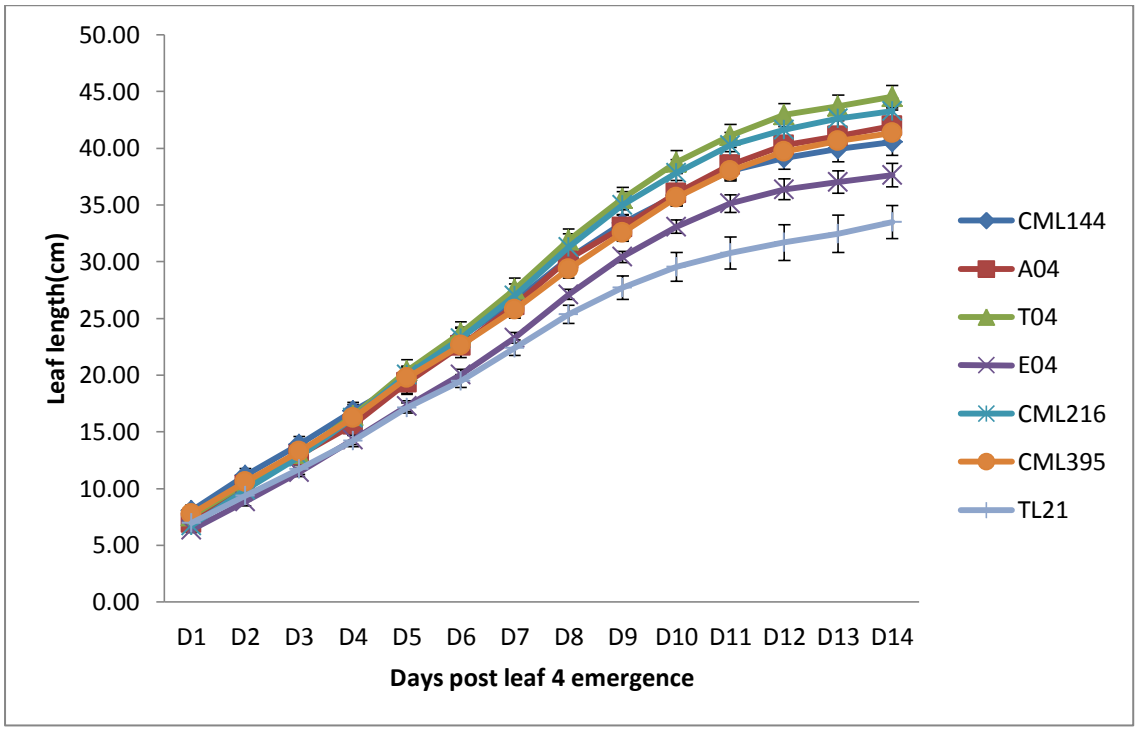

Figure 1: Lengths of leaf four of selected inbred lines under optimal conditions

Leaf disc senescence assays and chlorophyll estimation

Genotypes T 04, A04 and CML 144 had 17 % , 17% (highest) and 7 % (lowest) disc senescence respectively in 100 mM NaCl. Inbred lines CML 395 and T 04 had 33 % (highest) senescence in 200 mM NaCl treatment, while E 04 had 17 % [lowest] in 200 mM NaCl. Leaf discs of CML 144 showed less senescence in both NaCl and mannitol treatments (7 %) taking place at 100 mM compared to the other genotypes. Genotypes TL 21, T 04 and CML 144 had 3 % senescence in 100 mM mannitol concentration, which was the lowest. Overall, there was more senescence in leaf discs floated in NaCl than in mannitol across all the genotypes as shown in **table 1** below.

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Table 1: Senescence of leaf discs of inbred maize lines subjected to NaCl and mannitol stress after 24 hours expressed as a percentage (%).

Genotype	NaCl treatment					Mannitol treatment				
	100 mM	125 mM	150 mM	175 mM	200 Mm	100 mM	200 mM	400 mM	600 mM	800 mM
CML 144	7	10	13	17	20	3	7	13	17	17
CML 216	13	10	13	20	23	10	13	23	27	20
CML 395	13	20	23	27	33	7	10	13	13	17
A 04	17	20	20	27	30	7	13	13	13	17
E 04	10	10	13	13	17	7	10	10	10	10
T 04	17	20	23	27	33	3	13	17	17	20
TL 21	10	17	20	23	27	3	7	13	13	17

Impact of water deficit on length of the 8th leaf of maize seedlings

Seedlings of genotype CML 144 and A 04 showed significant differences in the mean length of the 8th leaf before and after water deficit with differences of 44.59 cm and 45.80 cm respectively. There was no significant difference in the mean length difference of the 8th leaf before and after water deficit in genotypes E 04, CML 395, T 04 and TL 21 with mean difference lengths of 28.97 cm, 35.66 cm, 36.83 cm and 38.04 cm respectively (Table 2).

Table 2: Effects of water deficit on 8th leaf length (cm) of maize seedlings

GENOTYPE	TREATMENT		Difference in length (cm)
	STRESSED	UNSTRESSED	
A 04	$40.83\pm3.173^{a**}$	86.63 ± 1.889^{b}	45.80^{a***}
CML 144	49.67 ± 3.756^{a}	94.26 ± 0.897^{c}	44.59^{a}
CML 216	45.70 ± 1.193^{a}	67.00 ± 2.082^{d}	21.30^{b}
CML 395	44.00 ± 3.055^{a}	79.66 ± 1.453^{b}	35.66^{ab}
E 04	48.53 ± 1.484^{a}	77.50 ± 1.835^{b}	28.97^{ab}
T 04	41.33 ± 2.333^{a}	78.16 ± 2.619^{b}	36.83^{ab}
TL 21	50.63 ± 3.376^{a}	88.67 ± 1.202^{bc}	38.04^{ab}
Mean for stress levels	45.81	81.12	

The data represents 4 plants per experiment replicated thrice Values are means (cm) ± SE

***Means followed by the same letter within columns are not significantly different (Tukey's pairwise comparison) at 5 % probability level.

**Values followed by the same letter within rows are not significantly different at 5 % probability level.

Immature zygotic embryo induced inhibition of *A. tumefaciens* growth

Growth of *A. tumefaciens* on all MS plates was not observed. All YEP plates had *A. tumefaciens* growth and when viewed under a light microscope, visible halos were seen around some embryos across all genotypes (Plate 1)

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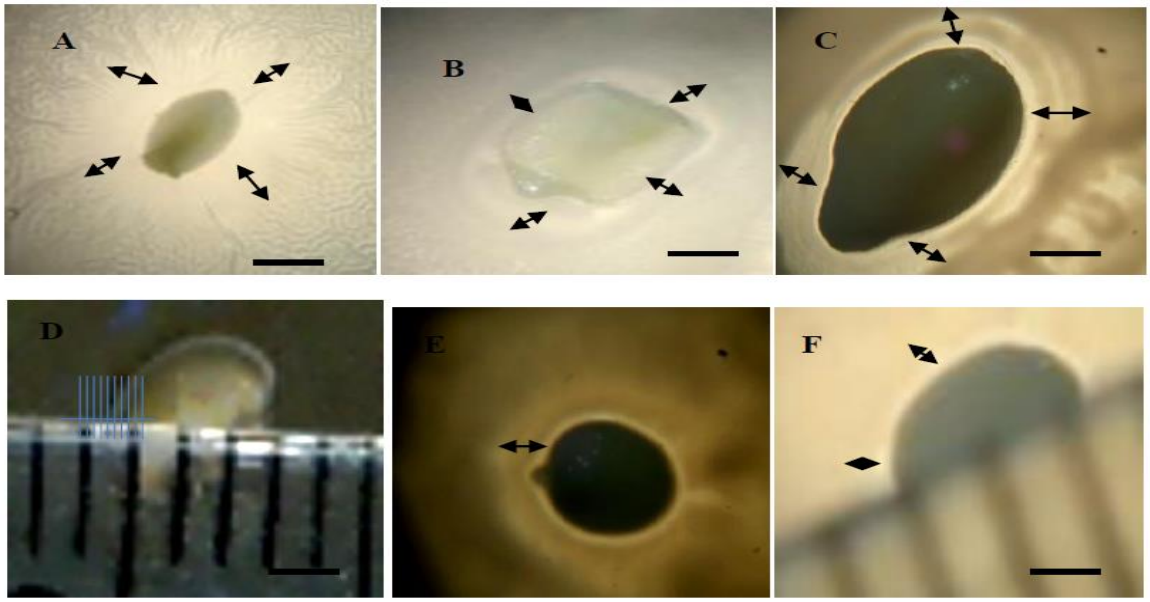

Plate 1: Immature zygotic embryos from selected inbreds showing different inhibitions of *A. tumefaciens* growth on different YEP media regimes

(A) CML 395 embryo on YEP + 2,4-D and 25 % *A. tumefaciens* dilution. **(B)** T 04 embryo on YEP and 50 % *A. tumefaciens* dilution. **(C)** A 04 embryo on YEP + dicamba and 50 % *A. tumefaciens* dilution. **(D)** CML 216 embryo on YEP+ picloram and 75 % *A. tumefaciens* dilution. **(E)** TL 21 embryo on YEP + picloram and 75 % *A. tumefaciens* dilution. **(F)** E 04 embryo on YEP + 2,4-D and 100 % *A. tumefaciens* concentration **(a)** Zone of inhibition Bar ≈ 1 mm. **(a)** Arrow showing zone of inhibition.

Histochemical X-Gluc assay

No *GUS* activity was noted across all genotypes on MS media regardless of *A. tumefaciens* density or augmentation. Notable *GUS* activity was seen in embryos infected on YEP media, YEP+2,4-D, YEP+dicamba and YEP+picloram media regimes indicating successful transformation.

Immature embryos of genotype E 04 showed significantly higher transformation frequencies ($P<0.05$) of 30.7 % (YEP), 33.3 % (YEP+2,4-D), 28 % (YEP+dicamba), 28.7 % (YEP+picloram) compared to immature embryos of the other genotypes when infected on YEP based media **(Table 3).**

Table 3: Transformation frequencies [%] of selected inbred lines in all treatments

	YEP	YEP+2,4-D	YEP+DIC	YEP+PIC	Genotype MEAN
CML 395	6.0^{a**}	0.0^{a}	0.0^{a}	0.0^{a}	1.5^{a***}
CML 216	22.0^{bc}	22.0^{bc}	20.0^{b}	21.3^{bc}	21.33^{bc}
CML 144	16.0^{b}	12.7^{b}	14.0^{b}	16.0^{b}	14.68^{b}
A 04	14.0^{b}	13.3^{b}	14.7^{b}	16.0^{b}	14.5^{b}
E 04	30.7^{d}	33.3^{d}	28.0^{c}	28.7^{c}	30.18^{c}
T 04	8.7^{ab}	6.7^{a}	8.7^{ab}	6.0^{a}	7.5^{ab}
TL 21	3.3^{a}	0.0^{a}	6.0^{a}	5.3^{a}	3.65^{a}
Treatment MEAN	14.385	12.571	13.5	13.328	

The data represents 50 immature zygotic embryos per experiment replicated thrice.

The means include transformation frequencies from all bacteria dilutions.

***Means followed by the same letter within column are not significantly different (Tukey's pairwise comparison) at 5 % probability level.

**Values followed by the same letter within rows are not significantly different at 5 % probability level.

IV. DISCUSSION

When plants are grown under optimum conditions, the relative primary and secondary growth rate vary from genotype to genotype. Seedlings of inbred line TL 21 showed a significantly slow growth rate as compared to the other genotypes under the same environmental conditions. The rate of growth can influence the response of a plant to adverse environmental conditions such that plants with slow growth rates are more adversely affected as they are not capable of fully utilizing resources while plants with a fast growth rate escape the harsh environmental conditions [Badu-Apraku *et al*. 2003]. The drought tolerance of each of the selected maize inbred lines was assessed as the difference in growth parameters between the stressed and the well watered plants. The results in this study indicate that inbred lines A 04 and CML 144 were the genotypes whose fresh weight most affected by water deficit conditions while inbred lines CML 216 and T 04 were the most tolerant. It is known that as water availability becomes limiting, plant growth is decreased due to two factors. One is due to turgor loss in expanding cells inhibiting cell division and expansion or due to metabolic regulation serving an adaptive role by restricting the development of transpiring leaf area in drought stressed plants [Bajji *et al*., 2002].

The choice of phytohormones is an important factor affecting transient *GUS* expression. Saini and Jaiwal [2007]; Nandakumar *et al.* [2004]; Wu *et al.* [2003] and Trifonova *et al.* [2001], reported that explants of black gram, wheat ,*Typha latifolia* and barley respectively, became susceptible to *A. tumefaciens* infection when pre-cultured on media containing phytohormones. In this study, infection media supplemented with different phytohormones (2,4-D 2 mg/ml, dicamba 3 mg/ml, picloram 15 mg/ml) enhanced *A. tumefaciens* infection and transient expression of *GUS* activity in immature embryos of three inbred lines used as compared to infection medium without phytohormones. However the phytohormones did not significantly increase transformation frequency (*GUS* expression) in the immature embryos of the CIMMYT maize inbred lines (CML 395, CML 216 and CML 144). Contrary to reports that minimum medium (MS) is more conducive to the induction and infection process than the rich YEP medium [Kunik *et al*., 2001] MS medium without an inducer (Acetosyringone) did not lead to infection thus no *GUS* expression was observed on all MS infected immature zygotic embryos, suggesting that minimum medium in this case MS requires an inducer to aid in *vir*-gene expression. Inbred lines E 04 and CML 216 had higher mean transformation frequencies of 30.18 % and 21.33 % respectively than the other genotypes indicating that they had stronger T-DNA transfer (*GUS* expression).

Success in maize transformation is primarily dependent on response of immature embryos in tissue culture, quality of immature embryos and immature embryo development stage [Ishida *et al.*, 2007]. Tropical maize genotypes have been reported to have low transformation frequencies and are also known to be recalcitrant to *A. tumefaciens* mediated transformation as reported by [Gelvin, 2003] due to inherent limitations. Contrary to expectations, the inbred maize lines with zygotic embryos that induced the largest inhibition for *A. tumefaciens* growth were not necessarily having the lowest transformation frequencies. Immature embryos of inbred line E 04 had large inhibition distances yet recorded the highest transformation frequencies while inbred line A 04 which had low transformation frequencies across all media had very low zygotic embryo induced inhibition for *A. tumefaciens* growth.

V. CONCLUSION AND RECOMMENDATIONS

It was evident that all selected genotypes had reduced plant growth after water deficit with some genotypes responding faster to stress than other genotypes. CML 216 responded better to water deficit compared to A 04 and CML 144 which were adversely affected by drought. The difference in fresh weights, leaf lengths and plant height upon water deficit clearly indicate genetic variability in theses selected lines.This study was also undertaken to identify an infection medium regime for improved transformation frequency of tropical maize inbred lines using *A. tumefaciens* EHA101 strain containing pTF102 vector. The lines were tested for transformation frequency on MS and YEP media augmented with phytohormones 2,4-D, dicamba and picloram. However, no transformation of zygotic embryos was observed on MS media. Transformation frequencies of inbred lines CML 216 and E 04 were significantly higher compared to those of other genotypes on YEP media as well as on YEP augmented with phytohormones. This suggests that these two genotypes are the most transformable using YEP as an alternative infection media.

Contrary to expectations, the inbred maize lines with zygotic embryos that induced the largest inhibition for *A. tumefaciens* growth were not necessarily having the lowest transformation frequencies. Immature embryos of inbred line E 04 had large inhibition distances yet recorded the highest transformation frequencies while inbred line A 04 which had low transformation frequencies across all media had very low zygotic embryo induced inhibition for *A. tumefaciens*

growth. This suggests that host defense to infection could occur after delivery of T-DNA into the host, and just before its integration.

There was however no significant difference in inhibition distance across all media regimes but significant difference in transformation frequencies across the 4 media regimes. This suggests that phytohormones are important in enhancing transformation of immature zygotic embryos of Kenyan inbred maize lines. Overall E 04 and CML 216 are the most transformable lines; however, CML 216 exhibited tolerance to water deficit so its transformation would not add much value. Maize inbred line E 04 would be a good line to transform with an actual gene that confers drought tolerance as it has proved to be highly transformable.

Selected maize inbred lines are hence recommended for molecular breeding programs by maize breeders and researchers, and the findings made available to policy makers for formulation of policies on germplasm improvement. Also, the transformation protocol using YEP infection media as used in this study should be optimized and used in transformation of tropical maize inbreds by researchers

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REFERENCES

[1] Badu-Apraku B., Menkir A., Fakorede M. A. B., Fontem Lum A. and Obeng-Antwi K. (2006). Multivariate analyses of the genetic diversity of forty-seven Striga resistant tropical early maturing maize inbred lines. Maydica, 51:551-559.

[2] Bajji M., Kinet J. and Lutts S. (2002). The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. Plant Growth Regulation, 36:61-70.

[3] Diallo A. O., Kanampiu F., Mugo H. and Mbogo P. [2005]. "Herbicide resistant maize. A novel method to control striga in Africa" Paper presented at the 5th West and Central Africa Biennial Regional Maize Workshop, 2-6th May 2005, IITA Cotonou, Benin Republic, pp 120.

[4] Franklin G., Conceicao F. R., Kombrink E. and Dias A. C. P. [2008]. Hypericum perforatum plant cells reduce Agrobacterium viability during co-cultivation. Planta, 227[6]:1401-1408.

[5] Gallup J. L. and Sachs J. D. [2000]. Agriculture, climate and technology; Why are the tropics falling behind? Available online [http://www.ajae.oxfordjournals.org]. [Accessed on 13th February 2013].

[6] Gelvin S. B. (2003). *Agrobacterium*-mediated plant transformation: The biology behind the "gene-jockeying" tool. Microbiology and Molecular Biology Review, 67: 16–37.

[7] Ishida Y., Hiei Y. and Komari T. [2007]. Agrobacterium-mediated transformation of maize. Nature Protocols, 2[7]:1614-1621.

[8] Kunik T., Tzfira T., Kapulnik Y., Gafni Y., Dingwall C. and Citovsky V. [2001]. Genetic transformation of HeLa cells by Agrobacterium. Proceedings of the National Academy of Science of the United States of America, 98[4]:1871-1876.

[9] Nandakumar R., Chen L. and Rogers S. M. D. [2004]. Factors affecting the Agrobacterium-mediated transient transformation of the wetland monocot, Typha latifolia. Plant Cell Tissue Culture, 79:31-38.

[10] Pandey S. and Gardner C. O. [1992]. Recurrent selection for population, variety and hybrid improvement in Tropical maize. Advances in Agronomy, 48:1-87.

[11] Saini R. and Jaiwal P. K. [2007]. Agrobacterium tumefaciences-mediated transformation of blackgram: an assessment of factors influencing the efficiency of uidA gene transfer. Plant Biology, 51:75-79.

[12] Trifonova A., Madsen S. and Olesen A. [2001]. Agrobacterium-mediated transgene delivery and integration into barley under a range of in vitro culture conditions. Plant Science, 161:871-880.

[13] Wu H., Sparks C., Amoah B. and Jones H. D. [2003]. Factors influencing successful Agrobacterium-mediated genetic transformation of wheat. Plant Cell, 21:659-668.