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AN EFFICIENT RECOVERY OF TRANSGENIC PLANTS FROM A TROPICAL INDIAN MAIZE INBRED LINE

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ABSTRACT

Tropical Indian maize inbreds are known for their recalcitrance in tissue culture which is a prerequisite for development of transgenic lines from such cultivars. In the present study, particle bombardment-mediated transformation of a tropical Indian maize inbred line, UMI29 was attempted. Parameters that infulence transforamation efficiency such as size of embryo (1.5 mm and 2.0 mm), micro-carrier flying distance (6 cm and 9 cm) and post-bombardment resting period (2, 7 and 10 days) on transient and stable expression of transgene were investigated. The greatest transformation efficiency of 2.67% was obtained using immature embryos of 1.5 mm at 6 cm of microcarrier flying distance with a resting period of 2 days. Stable inheritance of transgenes, *viz., gusA* and *bar* was confirmed in T_1 lines.

Keywords: Maize, particle bombardment, *gusA*, *bar*, transgenic plants

INTRODUCTION

Maize is one of the most important food staples in the world. In 2011-2012, the worldwide maize production was about 877 million tonnes and accounted for about 24% of the total cereal production (**www.igc.int;** retrieved on 10.4.2014). Though conventional breeding tools are most widely used in crop improvement, biotechnological tools widened the scope of crop improvement by way of helping scientists to understand the roles of different genes and engender foreign genes in the plant with a view to imparting novel and beneficial traits. Several genetic engineering studies have been focused on maize since 1990 after achieving regeneration of complete fertile plant through Biolistic® method (**Gordon-Kamm *et al.*, 1990**). Later, *Agrobacterium*-mediated maize transformation was also demonstrated using super binary vector (*Agrobacterium* strains harbouring extra copies of *virB*, *virC* and *virG*) (**Ishida *et al.*, 1996; Negrotto *et al.*, 2000; Frame *et al.*, 2002 and Miller *et al.*, 2002**). Success of either method of gene delivery chiefly depends on genotypes which are capable of producing highly competent embryogenic calli with regeneration potential. Somatic embryogenesis in maize is controlled by one or a group of several genes and the QTL associated were identified (**Krakowsky *et al.*, 2006**). Many temperate maize genotypes are able to regenerate plants from tissue culture (**Vasil *et al.*, 1985; Duncan *et al.*, 1985; Tomes and Smith, 1985; Hodges *et al.*, 1986**), while on the other hand, a fewer genotypes adapted to tropical areas have exhibited the capacity to produce Type II callus and plant regeneration from tissue culture (**Bohorova *et al.*, 1995; Danson *et al.*, 2006**). Maize transformation was successfully achieved using the temperate maize such as hybrid HiII (A188 X B73), harbouring genes for somatic embryogenesis from the A188 genotype (**González *et al.*, 2012**). However, such transformation system is not available for tropical maize and they are considered recalcitrant for genetic transformation. In this study, with a view to developing a high frequency transformation protocol, we optimised different parameters which influence generation of transgenic plants in maize. We studied the effect of size of the immature embryos, microcarrier flying distance and resting period on transient and stable expression of transgene.

MATERIAL AND METHODS

Plant material and immature embryo culture

A relatively more tissue culture-responsive Indian tropical maize inbred line, UMI29 was used in this study. Cobs were harvested 8-12 DAP and used for isolation of immature embryos. The husk was removed and the cobs surface sterilised using 2.5% sodium hypochlorite for 5 min followed by three washes with sterile distilled water. Immature embryos of 1.5 and 2.0 mm-long were excised aseptically from sterilized immature cobs using a sterile scalpel as described by **Frame *et al.* (2002)**. Isolated immature embryos were cultured *in vitro* with their scutella facing upward on the callus induction medium (N6 medium supplemented with 1 mg/l 2,4–D, 15 µM DICAMBA,10 mg/l $AgNO_3$ and 2.88 g/l proline) placed for 4 days in the dark at 25±2 °C prior to bombardment.

Plasmid

The immature embryos were bombarded with pAHC25 (**Christensen & Quail, 1996**) which contains *bar* gene, the selectable marker gene conferring resistance to the herbicide, phosphionthricin and β-glucuronidase (*gus*) gene, the reporter gene, either of them placed under the control of a maize ubiquitin 1 (*ubi-1*) promoter (Figure 1).

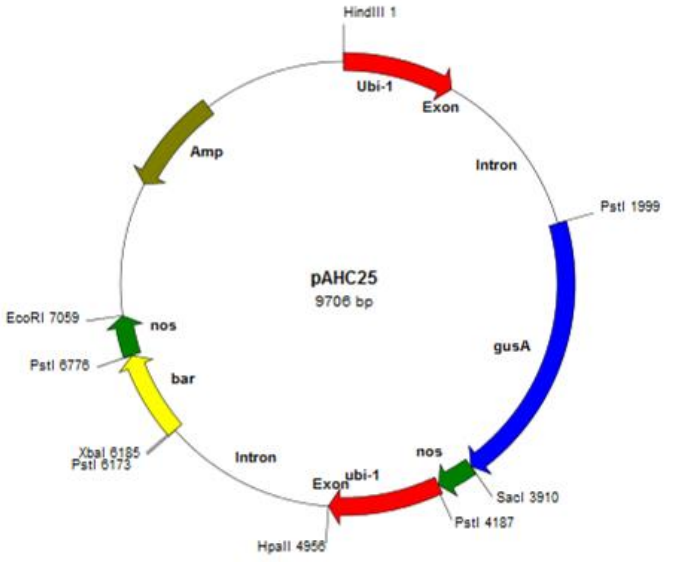

Figure 1 Physical map of pAHC25 vector

Particle bombardment

The pre-cultured immature embryos (1.5 or 2.0 mm long) were arranged at the centre of the plate containing osmoticum medium (callus induction medium containing 36.4 g/l mannitol and 36.4 g/l sorbitol) with scutella facing upward 4 hours prior to bombardment. The embryos were bombarded twice at a 6 or 9 cm microcarrier flying distance with the DNA coated onto 0.9 µm dia gold particles (prepared as described by the manufacturer) using the PDS 1000/He device (Biorad, USA) with a 4 hour interval in between. The bombarded embryos were transferred onto a fresh callus induction medium 16 hour post bombardment treatment on osmoticum medium.

Selection

On the lapse of 2, 7 or 10 days of resting period meant for facilitating an easy recovery from the bombardment shock, the embryos were transferred onto a selection medium (callus induction medium containing 3 mg/l phosphinothricin) and incubated in the dark at 25±2 °C. The cultures were sub-cultured onto a fresh selection medium at a 15-day interval and maintained in the dark at 25±2 °C.

Shoot initiation and regeneration

The embryogenic calli that withstood three rounds of phosphinothricin selection were transferred onto a regeneration medium (MS medium supplemented with 1 mg/l kinetin and 1 mg/l BAP) and incubated under 16 hours of light and 8 hours of dark at 25±2 °C in a plant growth chamber. The matured somatic embryos germinated on the regeneration medium produced roots and shoots.

Shoot elongation and root proliferation

The shoots of 3-5 cm in length with primary roots were transferred to MS medium and incubated at 25±2 °C with a photoperiod of 16 hours light and 8 hours dark in a plant growth chamber for elongation of shoots and induction of secondary roots. The plantlets with 2-3 well developed leaves were forwarded for hardening.

Hardening

The plants with well developed root and shoot system were transferred to sterile pots containing sterile soil, sand and vermicompost mixture in 1:1:1 ratio maintained in a culture room at 25±2 °C under 16 hours of light for 10-15 days. After the plant had produced 1-2 healthy leaves, they were transferred to transgenic greenhouse.

PCR analysis

The presence of *gusA* gene in the transformed plants was confirmed by a PCR amplification of 878 bp of an internal fragment of *gusA* gene using a set of primers, GUS1F (5'CAACGAACTGAACTGGCAGA3') and GUS1R (5'TTTTTGTCACGCGCTATCAG3').

GUS assay

GUS analysis was carried out as described by **Jefferson (1987)**. Transient GUS expression assay was carried out 48 hours after second bombardment in the bombarded and control embryos and the number of blue spots per embryo was scored. In the stable GUS assay, GUS expression was studied in different plant parts (leaf bit, seed and bract) of the putative transgenic (T_0) and control plants.

Basta leaf painting assay

The Basta leaf painting assay was carried out on a photosynthetically active third leaf. At the centre of the third leaf, 5 cm area was marked and was swabbed with cotton soaked in 1.0% Basta solution containing 0.1% tween 20. Seven days after Basta painting, the leaves were scored for herbicide tolerance. A control was maintained by using the same procedure in the wild type UMI29 plants.

Statistical analysis

The data were analysed using AgRes Statistical Software, Version 3.01 (**Pascal International Software Solutions, 1994**). ANOVA was worked out on the data transformed by arcsine or square root transformation of the percentage or count data, followed by least significant difference (LSD) test to select the best treatment. Mean and standard error were performed in worksheet format using the data analysis tool pack feature available in MS Office Excel 2007 software.

RESULTS AND DISCUSSION

Several factors are known to influence genetic transformation efficiency of particle bombardment-mediated method and optimization of these parameters is critical to recover transgenic plants with high transformation efficiency. Immature embryos of UMI29, an Indian tropical maize inbred were used as explants in particle bombardment experiments. The immature embryos are sporophytic tissue capable of producing somatic embryos which eventually give rise to plants. Due to their amenability for tissue culture and regeneration, immature embryos are the most widely used explants in maize *in vitro* culture (**Green and Phillips, 1975; Armstrong and Green, 1985; Rafiq *et al.*, 2005; Jakubeková *et al.*, 2011; Manivannan *et al.*, 2010, González *et al.*, 2012**).

Size of the immature embryo

The size of the immature embryo used is one of the biological factors that affect the culture response and transformation efficiency. When two different sizes of embryos (1.5 and 2.0 mm) were bombarded, these bombarded immature embryos produced four different types of calli namely, Type I, Type II, non embryogenic and rhizogenic calli on selection medium. Among them, Type I and Type II calli which were capable of regeneration were forwarded to further rounds of selection. On the other hand, the embryos that could not withstand selection either remained as such and died or formed watery calli that later turned brown and died. The embryogenic calli originated six days after culture in 1.5 mm embryos and 10 days after in 2.0 mm sized embryos. The embryo of 1.5 mm long produced callus predominantly of Type II nature (74%) while 2.0 mm embryos produced callus mostly of Type I nature (31%; Tab 1). The use of Type I and Type II calli in producing regenerable plants after bombardment were reported by earlier workers (**Brettschneider *et al.*, 1997; Frame *et al.*, 2000**). The 1.5 mm long embryos were tender and young enough to undergo complete dedifferentiation while 2.0 mm long embryos produced large nodular embryogenic calli from the scutellar region (Figure 2). Since the 1–2 layer thick mantle of embryogenic units were capable of regeneration, genetic transformation targeting this region in both the sizes of embryos produced stable GUS expressing transformed plants (1.5 mm embryos produced 7 plants and 2.0 mm embryos produced 3 plants; Tab 1). Similar reports on recovery of transgenic maize plants by targeting the surface cell layers of scutellum were reported by **Vasil *et al.* (1985), Kausch *et al.* (1995) and Kemper *et al.* (1996)**.

Table 1 Effect of embryo size on type I and type II callus induction frequency and particle bombardment-mediated transformation of maize.

Embryo size	Type I (%)	Type II (%)	Number of PCR positive and GUS expressing plants obtained
1.5 mm	18.00 ± 3.39^{b}	74.00 ± 6.18^{a}	1.4 ± 0.50^{a} (7)
2.0 mm	31.00 ± 3.14^{a}	53.00 ± 7.89^{b}	0.6 ± 0.40^{b} (3)

Legend: Each treatment was replicated 5 times with 60 explants per replication. The values presented are as mean ± SE followed by alphabets to imply significant difference ($p \leq 0.05$) after grouping of treatment means after ANOVA by LSD and values with same letter are not significantly different. Percentage data has been transformed by the arc-sine transformation and count data by square root transformation prior to analysis. Value within the parenthesis is the total number of plants obtained in each treatment.

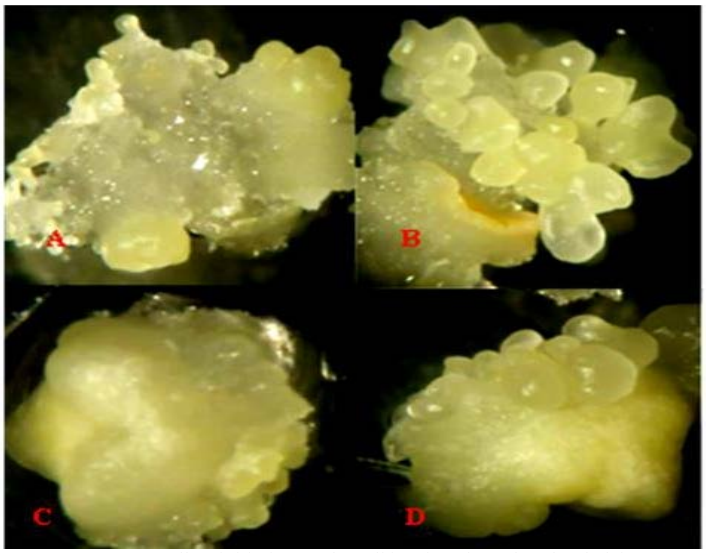

Figure 2 (A-D) Callus initiation and proliferation from bombarded immature maize embryos of UMI29: 1.5 mm long embryos (A,B); 2.0 mm long embryos (C,D)

Resting period

Different periods of resting (2, 7 and 10 days) were given to immature embryos post-bombardment in order for allowing the proliferation of transformed calli before subjecting them to selection pressure. With an increase in resting period, the number of calli that survived in selection got increased (Tab 2). However, a two-day resting period generated the greatest number of GUS expressing transgenic plants (4 plants), while that associated with 7 and 10 day resting periods produced 3 plants each (Tab 2). Resting period of two days was found to be sufficient for recovering the greatest number of transformants. Besides it was found that the number of escapes was the least in two-day resting. A two-day resting period was adopted by **Vain *et al.* (1993)** while 10 and 14 days of resting were adopted in maize biolistic transformation by **El-Itriby *et al.* (2003); Oneto *et al.* (2010); Frame *et al.* (2000) and Petrillo *et al.* (2008)**.

Table 2 Effect of resting period on particle bombardment-mediated maize transformation

Resting period	No. of calli selected	No. of plants regenerated	No. of PCR positive and GUS expressing plants
2d	14.00 ± 0.48^{c} (70)	7.00 ± 0.54^{b} (35)	0.80 ± 0.37^{NS} (4)
7d	19.20 ± 0.43^{b} (96)	8.00 ± 0.47^{b} (40)	0.60 ± 0.24^{NS} (3)
10d	25.60 ± 0.48^{a} (128)	10.20 ± 0.48^{a} (51)	0.60 ± 0.24^{NS} (3)

Legend: Each treatment was replicated 5 times with 40 explants per replication. The values presented are as mean ± SE followed by alphabets to imply significant difference ($p \leq 0.05$) after grouping of treatment means after ANOVA by LSD and values with same letter are not significantly different. Count data has been transformed by the by square root transformation prior to analysis. NS – not significant. Value within the parenthesis is the total number of plants obtained in each treatment.

Microcarrier flying distance

The microcarrrier flying distance (the distance from the microprojectile launch site to the biological target) is one of the critical physical factors that affect the transformation efficiency. **Sanford *et al.* (1993)** observed that the microcarrier flying distance was critical when working with small microcarriers and had to be minimised to maintain adequate velocity. With the increase in distance of flying distace the velocity of microprojectiles gets reduced, resulting in less penetration of microprojectiles into the callus. A higher number of transient GUS expression spots were detected when explants were placed at 6 cm (71.6 spots) microcarrier flying distance compared to 9 cm (53.4) distance (Tab 3; Figure 3). The number of stable transformants generated was also maximum at 6 cm microcarrier flying distance (Tab 3). **Tadesse *et al.* (2003)** and **Petrillo *et al.* (2008)** reported similar results of higher number of transient GUS expression as well as stable transformation events at a microcarrier flying distance of 6 cm compared to 12 or 13 cm.

Table 3 Effect of microcarrier flying distance on transient and stable GUS expression and transformation efficiency.

Micro carrier flying distance	Transient GUS spots (Mean ± SE)	Stable GUS expressing plants	Transformation efficiency (%)
6 cm	71.60 ± 12.49^{a}	1.4 ± 0.50^{a} (7)	2.33
9 cm	53.40 ± 7.90^{b}	0.6 ± 0.40^{b} (3)	1.00

Legend: Each treatment was replicated 5 times with 60 explants per replication. The values presented are as mean ± SE followed by alphabets to imply significant difference ($p \leq 0.05$) after grouping of treatment means after ANOVA by LSD and values with same letter are not significantly different. Count data has been transformed by square root transformation prior to analysis.

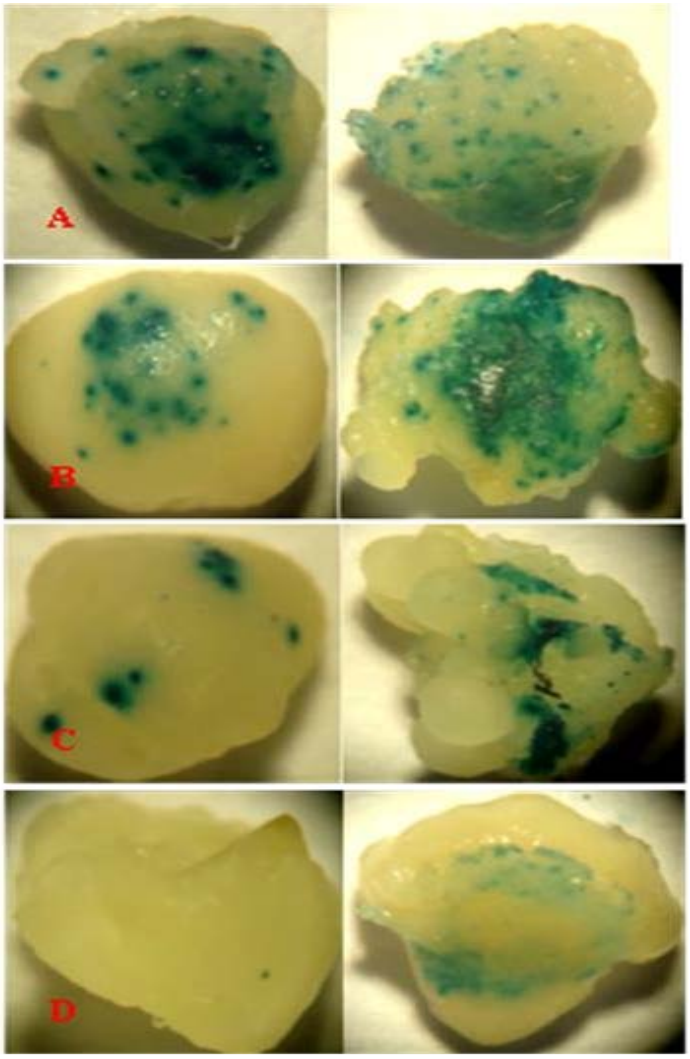

Figure 3 (A-D) Transient GUS expression in bombarded immature maize embryos at different microcarrier flying distance: 1.5 mm size at 6 cm distance (A); 2.0 mm size at 6 cm distance (B); 1.5 mm size at 9 cm distance (C); 2.0 mm size at 9 cm distance (D)

Transgenic plant recovery and stability of the transformants

Thirty four putative transformants were generated and 10 plants showed the presence of *gus* gene in PCR analysis (Figure 4). Stable GUS expression analysis in different plant parts of PCR positive plants revealed a strong GUS expression in seeds followed by bracts (Figure 5). The intensity of GUS gene expression was varying in different events. This may be due to the methylation of the cytosine residues of the introduced DNA (**Southgate *et al.*, 1995**). **Klein *et al.* (1990)** also observed the variation in GUS expression between the events derived from suspension culture by particle bombardment due to the variation in DNA methylation of introduced gene.

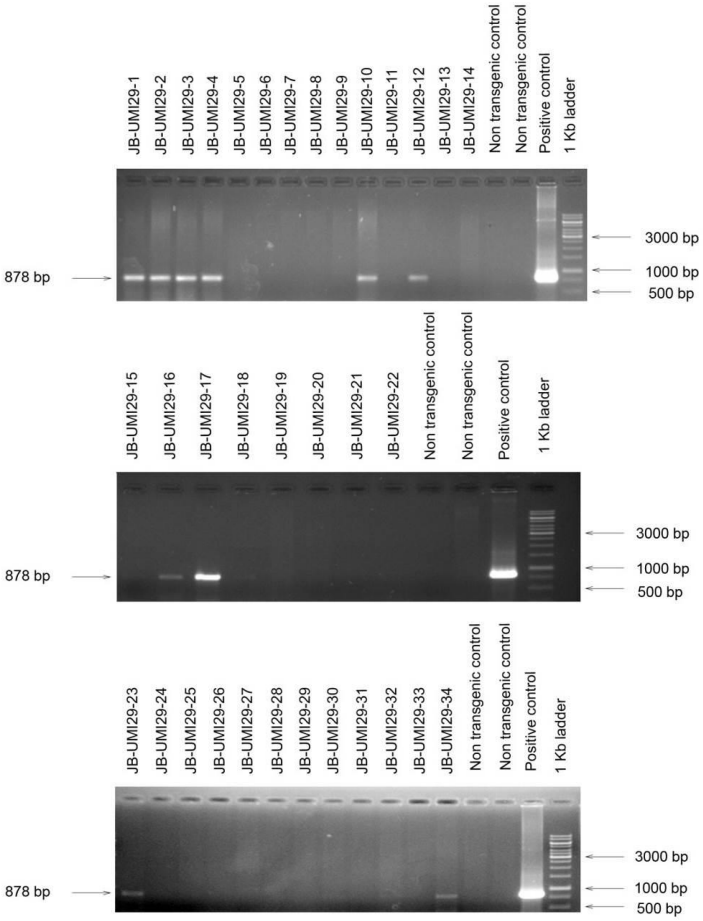

Figure 4 PCR amplification of *gusA* gene in putative transgenic maize lines transformed with pAHC25 vector

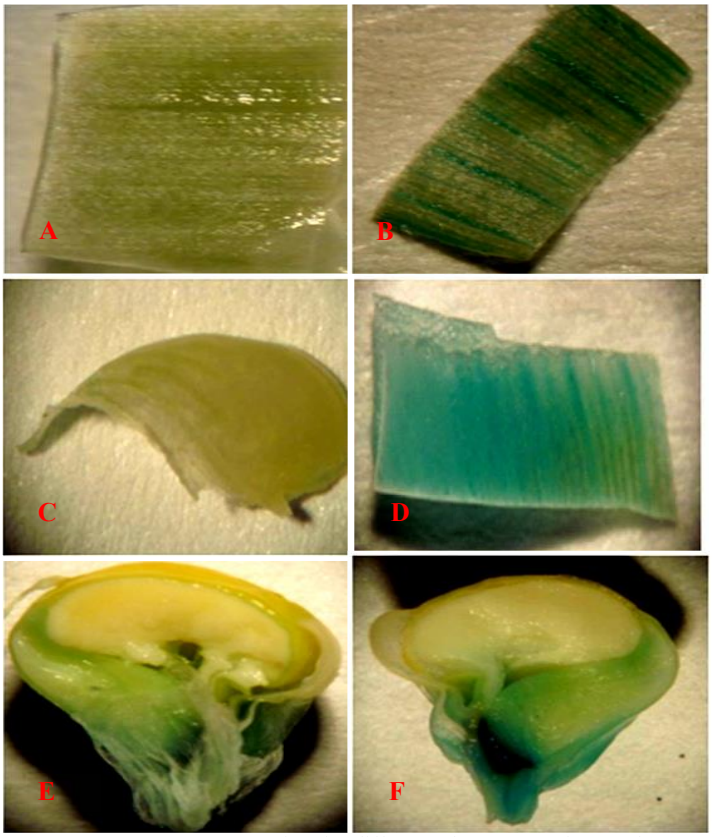

Figure 5 (A-F) GUS expression in different plant parts of control (non-transformed UMI29) and transgenic (transformed UMI29) plants: Leaf of control plant (A); Leaf of transgenic plant (B); Bract of control plant (C); Bract of transgenic plant (D); Seeds of control plant (E); Seeds of transgenic plant (F)

The most commonly used selectable marker gene in maize transformation is the *bar* or the *pat* gene (bialaphos or ammonium glufosinate resistance gene) (**Gordon-Kamm *et al.*, 1990; Valdez *et al.*, 2004; Yan *et al.*, 2010**). Several earlier workers (**Huang and Wei, 2005; Petrillo *et al.*, 2008; Zhu *et al.*, 2011 and Oneto *et al.*, 2010**) have demonstrated the expression of herbicide tolerance of transgenic maize harbouring *bar* gene by basta paining or spray. The positive transgenic plants which were generated by us in the present study were analysed for the expression of herbicide tolerance by Basta painting. The Basta swabbed area and the portion below the area turned yellowish and dried completely with the upper whorls of leaf showing chlorosis in control while in the transformed plants a slight yellowing was seen at the swabbed portion and other parts remained green (Figure 6).

Figure 6 (A, B) Basta painting assay: Transformed T_0 maize plant (A); Non-transformed (Control) UMI29 maize plant (B)

The interaction effect between the embryo size and microcarrier flying distance was significant and a maximum number of stable GUS expressing transgenic plants (4 plants) were generated from 1.5 mm long immature embryos bombarded at 6 cm. Since the embryogenic calli developed from 1.5 mm long immature embryos were soft and fragile, the penetration of microprojectiles and subsequent integration of foreign DNA into the nuclear genome was efficient even at 9 cm distance, where the velocity of microprojectiles was low compared to 6 cm distance to penetrate harder tissue (3 plants; Tab 4). In contrast, deeper layers of scutellar tissue had to be targeted in 2.0 mm embryos. **Kemper *et al.* (1996)** reported that the 3rd or deeper layers of scutellar tissue can be targeted by using heavy bombardment procedures like 1800 psi and 4.8 cm microcarrier flying distance. But the tissue damage must to be minimised by optimising the pre and post osmoticum treatments.

Table 4 Effect of microcarrier flying distance and embryo size on the production of stable GUS expressing plants and transformation. efficiency

Microcarrier flying distance	Embryo size	Stable gus expressing plants (Nos)	Transformation efficiency (%)
6 cm	1.5 mm	4	2.67
	2.0 mm	2	1.33
9 cm	1.5 mm	3	2.00
	2.0 mm	1	0.6

Legend: Each treatment was replicated 5 times with 30 embryos per replication.

We recorded a **maximum** transformation efficiency of 2.67% at 6 cm microcarrier flying distance using 1.5 mm long-immature embryos compared to 9

cm microcarrier flying distance (1.33% for 1.5 mm and 0.6% for 2.0 mm–long immature embryos; Tab 4). A similar report of decrease in transformation efficiency with increase in microcarrier flying distance was reported by **Klein *et al.* (1988)** and **Aragao *et al.* (1993)** but **Taylor and Vasil (1991)** reported visible damage to cells when the microcarrier flying distance was reduced, due to the cells being bombarded by large aggregates of gold.

The stability of the transgene in T_1 generation was studied in JB-UMI29–2 and JB-UMI29-8 events. Thirteen out of nineteen and twenty two out of forty eight plants were found to be positive for GUS expression in JB-UMI29–2 and JB-UMI29–8 events respectively (Tab 5). This demonstrated a stable inheritance of transgenes.

Table 5 Analysis of transgene segregation in T_1 plants.

Event No	Number of seeds		GUS expressing plants
	Sown	Germinated	
JB-UMI29–2	30	19	13
JB-UMI29–8	50	48	22

Though the transformation efficiency of temperate genotypes was as high as 17-30% (**Wang *et al.*, 2009**), tropical genotypes have recorded a maximum transformation frequency from 0.6 to 2.31% (**Petrillo *et al.*, 2008**). Here, we report the highest transformation efficiency of 2.67% from an Indian tropical inbred, UMI29 for the first time using 1.5 mm long immature embryos at 6 cm of microcarrier flying distance.

CONCLUSION

Genetic transformation is one of the tools in crop improvement programs. To utilize these tools, transformation protocol for cultivars of regional importance needs to be standardized. In an attempt to develope a high frequency transformation protocol for Indian tropical inbred UMI29, we investigated different parameters which influence generation of transgenic plants. We achieved a transformation efficiency of 2.67% in an Indian tropical inbred, UMI29 using 1.5 mm long immature embryos at 6 cm of microcarrier flying distance. In future, the protocol developed by us can be followed in gentic transformation experiments to transfer various other useful genes.

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EFFECT OF THE SINGLE DOSES OF CURCUMIN AND KAINIC ACID ON CHANGES IN THE AMOUNT OF REDUCED GLUTATHIONE IN SELECTED ORGANS OF MICE

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ABSTRACT

In the present study the effect of various doses administration of curcumin prior to the injection of kainic acid (KA) on the changes in the content of reduced glutathione (GSH) in selected organs of the male white mice were investigated. Animals were conducted in five groups: one control group, four experimental groups. The first experimental group received a single intraperitoneal injection of KA (12 mg/kg b. w.). The second, third and the fourth experimental group were administered intraperitoneally curcumin at doses of 50, 100 and 200 mg/kg b. w. 30 minutes before the injection of KA. In the brain, liver, kidneys, pancreas and spleen of the mice GSH was determined. Administration of KA resulted in a significant change in the amount of GSH in all the examined organs of mice. Injections of curcumin and then KA, caused an increase in GSH compared to a group of animals which received only KA. This increase was not significant only in the group which received curcumin in the lowest dose. Results suggest that curcumin has antioxidant properties because advantageously affected changes in the amount of GSH, and thus the size of the oxidative stress induced by KA.

Keywords: Curcumin, kainic acid, mouse, reduced glutathione

INTRODUCTION

Curcumin (lE, 6E)-l,7-bis (4-hydroxy-3-methoxyphenyl)-l,6-heptadiene-3,5-dione, also known as diferuloilmetan is a polyphenol present in turmeric rhizomes (*Curcuma longa* L.), with the content of 5-10% of dry mass. In the Eastern medicine it has been used for centuries for medical purposes. Only recent research begun to investigate its medicinal properties (**Sreejayan and Rao, 1997**). Many studies have succeeded in the following process of curcumin biotransformation in the body and suggested therapeutic effectiveness against many diseases, including diabetes, asthma, allergies, arthritis, atherosclerosis, neurodegenerative diseases and other chronic diseases such as cancer (**Ireson *et al*., 2002; Duvoix *et al*., 2005; Anand *et al*. 2007; Darvesh *et al*.,2012; Kapakos *et al*., 2012**). Due to the potential applicability of curcumin for therapeutic purposes, many research studies focus on understanding of the mechanisms of antioxidant curcumin activity of after the kainic acid (KA) administration induced excitotoxicity (**Sumanont *et al*., 2006; Shin *et al*., 2007; Sumanont *et al*., 2007; Gupta *et al*. 2009**).
Kainic acid (KA) is a specific agonist of ionotropic glutamate receptors (iGluRs) and a strong neurotoxin (**Vincent and Mulle, 2009**). KA acts on kainate receptors (KARs) in the central nervous system (CNS) and imitates the excitotoxic action of glutamate in models of neurodegenerative disorders. The KA binding to KARs receptors causes a number of cellular events, including the influx of Ca^{2+} into cells, the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which leads to the mitochondrial dysfunction, apoptosis of neurons and necrosis (**Wang *et al*., 2005**).
Excitotoxicity is considered to be an important mechanism involved in various neurodegenerative diseases in the CNS, such as, for example, Alzheimer's disease (AD), Parkinson's (PD), amyotrophic lateral sclerosis (ALS) and epilepsy (**Frantseva *et al*., 2000; Johnson *et al*., 2009; Zheng *et al*., 2011; Mitra *et al*., 2013**). However, the mechanism via which excitotoxicity is involved in the induction of the disease remains unclear (**Zheng *et al*., 2011**). Accordingly, many researchers are interested in the question of whether it is possible to reduce excitotoxicity and its effects, not only in the CNS, but also in other animal organs.
Reduced glutathione (GSH) is the basic non-protein thiol in a cell displaying multiple properties (**Dringer *et al*., 2000; Meister, 1981**). It is considered that it modulates the level of ROS and thus, participates in the cellular response to oxidative stress (**Gorrini *et al*., 2013; Presnell, 2013**). Therefore, the content of GSH in the cell, besides the physiological values, may be a useful indicator of the level of oxidative stress (**Pastore *et al*., 2003**). Hence the question of whether curcumin, which is ascribed antioxidant properties, will affect the changes in the amount of GSH, and, at the same time, in the size of the oxidative stress induced by KA. In the present experiment, we evaluate the amount of GSH in the brain, liver, kidney, pancreas and spleen of mice after the injection of KA alone, as well as after the administration of various doses of curcumin and additional KA after 30 minutes.

MATERIAL AND METHODS

In the experiment consisting of one control group and four experimental groups the total of 40 four-month male white mouse of Swiss strain with an average body weight of 27 g was used. All animals were obtained from the experimental research laboratory of the Department of Animal Physiology and Toxicology, Institute of Biology, Pedagogical University of Cracow. All mice of the control group and the experimental groups included 8 individuals each; animals were kept in suitable cages with full access to the standard food and water throughout the experiment. The lighting was regulated in LD 12:12 cycle (light phase starting at 8^{00} till 20^{00}). The breeding room was soundproof, the average temperature was 20°C ± 2°C and the relative humidity equaled 55% ± 5%.
Animals of the control group received at 8^{00} (at the start of the light phase of LD 12:12) a 0.9 % solution of NaCl (Polfa) in the amount of 0.3 ml as an intraperitoneal injection. In the first group of experimental mice kainic acid (KA) (Sigma, St. Louis, MO) was also intraperitoneally administered at the same time of day, i.e. at 8^{00} at a dose of 12 mg/kg body weight (b.w.) dissolved in normal saline (pH adjusted to 7.4 with sodium hydroxide). In turn, the second group of experimental animals received at 8^{00}, curcumin (Sigma, St Louis, MO) dissolved in diamethyl sulfoxide (Sigma, St. Louis, MO) at a dose of 50 mg/kg b.w. as an intraperitoneal injection and after 30 mins – KA, also as an intraperitoneal injection at a dose of 12 mg/kg b.w. Mice of the third experimental group received at 8^{00}, an intraperitoneal injection of curcumin at 100 mg/kg b.w. and after 30 mins KA was added in the amount of 12 mg/kg b.w. Animals of the fourth experimental group received at 8^{00} intraperitoneally curcumin at a dose of 200 mg/kg b.w. and, after 30 mins, KA – at a dose of 12 mg/kg b.w. Mice of the control group were decapitated 4 hrs after the administration of physiological

saline, and all the experimental groups – four hours after the injection of kainic acid. Initially, the animals were put into a state of deep anesthesia by intramuscular administration of vetbutal (Biowet, Poland) in the amount of 35 mg/kg b.w. diluted solution of NaCl 0.9% (Polfa).
Weighed organs were homogenized in a homogenizer with a Teflon plunger in 6 ml of, cooled up to 4°C, 0.1 M phosphate buffer, pH 7.4, containing 10 mM EDTA. The homogenates were centrifuged in a centrifuge MPW-365 at 4°C for 15 minutes at 15 000 g. Thus obtained supernatants of homogenates of brain, liver, kidney, pancreas and spleen were then deproteinized by adding 500 µl of supernatant into 500 µl TCA and 500 µl EDTA. This mixture was put in the fridge at 4°C for 10 minutes, then centrifuged for 5 min at 5 000 g at 4°C. In the obtained post-centrifugation deproteinized supernatants of brain, liver, kidney, pancreas and spleen GSH concentration was determined using the Ellman's method (**Ellman, 1959**).
From the results of GSH content in the brain, liver, kidney, pancreas and spleen in the control and experimental groups, arithmetic means and standard deviations were calculated. Percentage changes in the amount of GSH in the experimental groups as compared to the control group were calculated. The results were verified with the use of the statistical test t "Student – Gosset". All statistical calculations of the data obtained during experiments were done with the computer program STATISTICA 9 (StatSoft, Cracow, Poland).

RESULTS AND DISCUSSION

Results

The obtained results of the experiment on GSH determination in the brain, liver, kidney, pancreas and spleen after 4 hours of kainic acid (KA) intraperitoneal administration to the four-week-old male mice, in the amount of 12 mg/kg, of different doses of curcumin (50, 100, and 200 mg/kg. b.w.), and, after 30 minutes, of additional KA are shown in Figure 1-5.
In the mouse brain, in relation to the control values 4 hours after the KA injection, a statistically significant decrease in the GSH content was found ($p < 0.001$; 54.92 %) (Fig. 1). After the intraperitoneal injection of curcumin at a dose of 50 mg/kg b.w. and KA after 30 mins a slight increase in the content of GSH (6.38%) relative to the group after KA injection but not statistically significant (Fig. 1) was observed. In the animals that received intraperitoneal curcumin at the dose of 100 and 200 mg/kg b.w. and KA after 30 mins statistically significant ($p < 0.001$) increase in the concentration of GSH in the brain, 30.39% and 63.87%, respectively were found (Fig. 1).

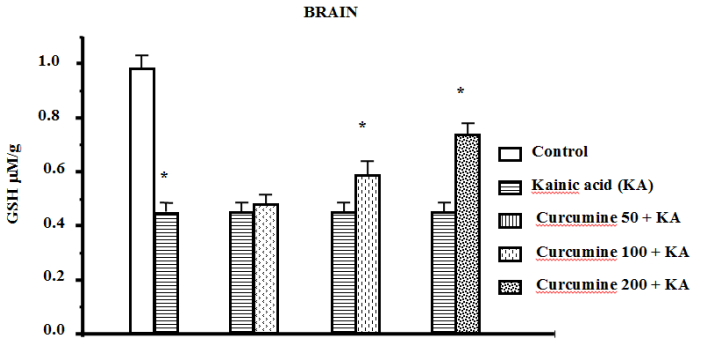

Figure 1 The level of reduced glutathione (GSH) in the brain of control and experimental male mice 4 hours after the intraperitoneal administration of kainic acid (KA) at a dose of 12 mg/kg b.w., (I experimental group), curcumin at a dose of 50 mg/kg b.w., and after 30 min. KA (experimental group II), curcumin at a dose of 100 mg/kg b.w., and KA (III experimental group), curcumin at a dose of 200 mg/kg b.w., and KA (IV experimental group). * - Indicates statistically significant at $p < 0.001$

In the liver, as compared to the control values, 4 hours after the injection of KA, statistically significant ($p < 0.001$) decrease in GSH concentration was observed (Fig. 2). This decrease equalled 40.93%. In the group of animals that received curcumin at a dose of 50 mg/kg b.w. followed by additional KA after 30 mins, an increase in GSH level reached 6.14% which was statistically insignificant (Fig. 2). In turn, the mice after the injection of curcumin in the amount of 100 and 200 mg/kg b.w., followed by KA administration after 30 mins, showed a significant increase in the concentration of this tripeptide in the liver; 19.03% and 48.24%, respectively.

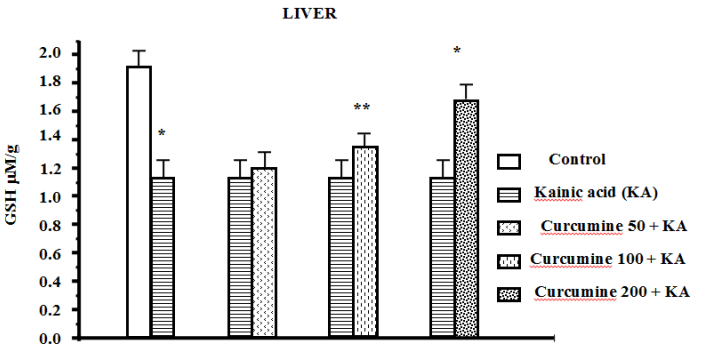

Figure 2 GSH level in the liver as in the experiment in Fig.1. * indicates statistically significant at $p<0.001$ and ** indicates $p<0.01$

In kidneys of animals which received the KA, after 4 hours, the concentration of the tested tripeptide was statistically significant ($p < 0.001$), reduced as compared to the control by 54.62% (Fig. 3). After the intraperitoneal injection of curcumin at 50 mg/kg, and post additional KA implementation after 30 mins, slightly increased GSH concentration occurred as compared to that obtained from animals receiving only KA. This increase was 8.83% and was statistically insignificant. In mice treated with curcumin intraperitoneally at 100 and 200 mg/kg, followed by additional KA administration after 30 mins a statistically significant ($p < 0.001$) increase in GSH levels, respectively 31.73% and 69.01% (Fig. 3) was detected.

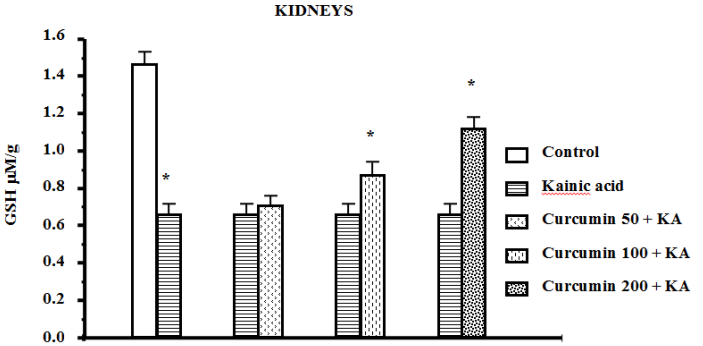

Figure 3 GSH level in the kidneys as in the experiment in Fig.1. * indicates statistically significant at $p<0.001$ and ** indicates $p<0.01$

In male mice, 4 hours after the KA injection, statistically significant ($p < 0.001$) decrease of GSH level was found in the pancreas. This reduction amounted to 31.35% (Fig. 4). The animals treated with curcumin (50 mg/kg b.m.) followed by additional KA administration after 30 mins, showed an increase in the GSH levels of 6.14% as compared to animals that received KA only. This increase was statistically insignificant (Fig. 4). Conversely, a statistically significant ($p<0.001$) increase in the concentration of GSH in the pancreas by 33.09% and 58.88%, respectively was recorded in mice that received curcumin at higher doses and KA after 30 mins (Fig. 4).

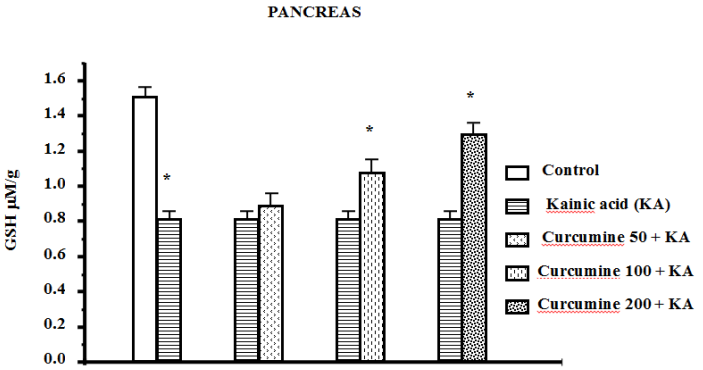

Figure 4 GSH level in the pancreas as in the experiment in Fig.1. * indicates statistically significant at $p<0.001$ and ** indicates $p<0.01$

In the spleen, compared to the values in the control animals, 4 hours after KA injection, a statistically significant ($p < 0.001$) decrease in GSH concentration was found (Fig. 5). This decrease was 43.43%. After the intraperitoneal injection of curcumin at 50 mg/kg b.w. and KA after 30 mins, statistically insignificant slight increase of GSH concentration (7.63%) was observed as compared to the animals

treated with KA alone. In mice, after the injection of curcumin in the amount of 100 and 200 mg/kg b.w., followed by additional KA administration after 30 mins, significant increase ($p < 0.001$) of this tripeptide level, 19.03% and 48.24% respectively was detected (Fig. 5).

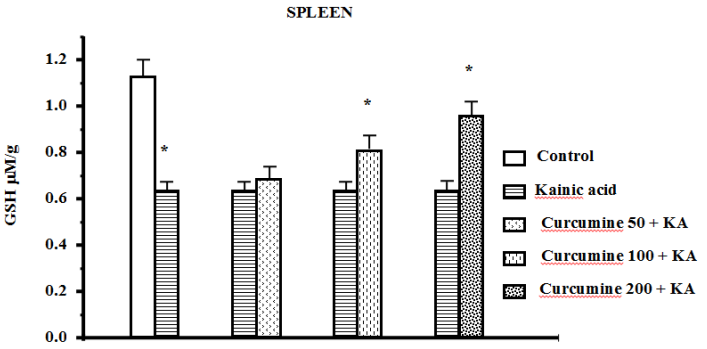

Figure 5 GSH level in the spleen as in the experiment in Fig.1. * indicates statistically significant at $p<0.001$ and ** indicates $p<0.01$

DISCUSSION

The obtained results show that the administration of the KA caused statistically significant reduction in the amount of GSH in all organs of the examined mice. After the administration of curcumin, followed by additional KA administration after 30 mins, there was a smaller decrease in the amount of GSH in relation to the group of animals which received KA. The decrease in the amount of GSH was respectively smaller when before the KA application higher doses of curcumin were used.

After KA administration, it binds to KARs receptors that are present on neurons and microglia. This leads to membrane depolarization, rapid influx of Ca^{2+} into cells (**Vincent and Mulle, 2009**), activation of Ca^{2+}-dependent enzymes and the production of ROS and RNS (**Kew and Kemp, 2005**). Excess of Ca^{2+}, ROS and RNS results in mitochondrial dysfunction and fragmentation of nuclear DNA (**Collingridge *et al*., 2009**). Alternatively, intense Ca^{2+} overload can directly cause mitochondrial swelling and damage, resulting in cell death (**Wang *et al*., 2005**). Following the KA administration, an increased amount of substances reacting with thiobarbituric acid and a reduction of the total antioxidant status (TAS) in the brain of rats were observed. This indicates the presence of oxidative stress in excitotoxicity (**Swamy *et al*., 2009**). The presence of such stress after KA is confirmed, also in the present experiment, changes in the amount of GSH, not only in the brain but also in the liver, spleen and kidney of mice. Similar changes in the amount of GSH in the organs of mice post KA administration were also observed in earlier experiments (**Szaroma *et al*., 2012**).

GSH is synthesized in its reduced form and then converted to the oxidized form by the formation of intramolecular disulfide bond. The basic function of glutathione is to maintain redox balance of the cells, reducing the oxidized particles and the detoxification of ROS, xenobiotics and heavy metals (**Yu and Zhou, 2007**). GSH by a free thiol group, directly reduces the oxidized molecules. It is a coenzyme of certain antioxidant enzymes such as glutathione peroxidases and glutathione transferases (**Avery and Avery, 2001; Garcerá *et al*., 2006; Lillig *et al*., 2008**). Undoubtedly, GSH is the most important non-enzymatic antioxidant in the body. This peptide is present in every cell of the body, particularly rich in its resources are kidneys, liver, brain and spleen.

GSH directly reacts with radicals in non-enzymatic reactions and is an electron donor in the reduction of superoxide by glutathione peroxidase (**Wefers and Sies, 1983**). The product of oxidation is glutathione disulfide (GSSG) which is re-reduced by glutathione reductase to GSH (**Dringen *et al*., 2000**). Several studies have shown that the amount of GSH significantly decreased in ischemia (**Cooper *et al*., 1980**), after the supply of ethanol (**Calabrese *et al*., 1998**) or compounds with very strong oxidizing properties. Thus, it suggested that the amount of GSH may be one of the markers of oxidative stress and damage to organs (**Bukowska, 2004; Bukowska and Kowalska, 2004**).

The reduction of GSH post KA administration, found in the current experiment, confirms the data cited by many authors that GSH plays a key role in the adaptation of the cells to cell stress induced by ROS and RNS. The decrease in GSH concentration after the administration of KA can induce not only a reduction in GSH synthesis, but also increase its consumption in the removal of ROS and RNS (**Swamy *et al*., 2009**).

The doses of curcumin used in the present experiment inhibited the decrease in the amount of GSH caused by the administration of KA. As already mentioned, curcumin is one of the polyphenolic compounds with antioxidant properties (**Giovannini *et al*., 2006**). Curcumin in micro and millimolar concentration is an effective scavenger of free radicals, such as superoxide anion, hydrogen peroxide and nitric oxide. This compound limits lipid peroxidation and reduced levels of oxidized proteins. Curcumin is capable of maintaining cell status of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and increases the concentration of GSH. Besides antioxidative properties, it also possess anti-inflammatory ones (**Rahman, 2006**). It is suggested that curcumin increases the level of cellular GSH and induces the *de novo* synthesis of GSH, by stimulating the activity and gene expression ligase γ-glutamyl-cysteine (GCL). GCL is an important step of adjusting the speed of the process (**Zheng *et al*., 2007**). Moreover, it has been shown to expression of the gene besides ligase γ-glutamyl-cysteine and increased GSH synthesis that curcumin, in appropriate dose, is cytoprotective because of its strong antioxidant activity and the ability to engage with GSH (**Donatus *et al*., 1990**).

CONCLUSION

Results of this study describe that curcumin is a potent antioxidant and may protect the organs of the tested mice against oxidative stress induced by the administration of KA. It can be concluded that all doses of curcumin increase the percentage of GSH level in selected tissues to protect against the harmful effects of KA. Supplementing with turmeric may therefore be important research for more research into comprehensive action of the substance and its use in treating diseases.

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ASSESSMENT OF RAPD POLYMORPHISM IN RICIN GENOTYPES

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ABSTRACT

The aim of this work was to detect genetic variability among the set of 30 castor genotypes using 6 RAPD markers. Amplification of genomic DNA of 30 genotypes using RAPD analysis yielded 50 polymorphic fragments with an average of 8.33 fragments per primer. Number of amplified fragments varied from 5 (RLZ7) to 11 (RLZ8) and the amplicon size ranged from 330 to 1200 bp. All 50 amplified bands were polymorphic. The polymorphic information content (PIC) values ranged from 0.774 (RLZ7) to 0.870 (RLZ8) with an average of 0.825 and index diversity (DI) value ranged from 0.786 (RLZ7) to 0.872 (RLZ8) with an average of 0.831. The dendrogram based on hierarchical cluster analysis using UPGMA algorithm was prepared. Dendrogram separated ricin genotypes into three main clusters. Two genotypes (RM-72 and RM-73) were genetically the closest. Knowledge on the genetic diversity of castor can be used for future breeding programs for increased oil production to meet the ever increasing demand of castor oil for industrial uses as well as for biodiesel production.

Keywords: Castor, genetic variability, molecular markers, RAPD technique

INTRODUCTION

Castor (*Ricinus communis* L., 2n = 2x = 20, Euphorbiaceae), is industrially important non-edible oilseed crop widely cultivated in the arid and semi-arid regions of the world. The seed of castor contains more than 45% of oil and this oil is rich (80–90%) in an unusual hydroxyl fatty acid, ricinoleic acid. Castor oil is the only vegetable oil soluble in alcohol, presenting high viscosity, and requiring less heating than others oils during the production of biodiesel (**Jeong and Park, 2009**). Due to its unique chemical and physical properties, the oil from castor seed is used as raw material for numerous and varied industrial applications, such as: manufacture of polymers, coatings, lubricants for aircrafts, cosmetics, etc, and for the production of biodiesel (**Jeong and Park, 2009**) with more than 95% of the world's castor production concentrated in limited parts of India, China, and Brazil (**Sailaja *et al.*, 2008**). Because of the ever increasing world-wide demand of castor for industrial use, there is a pressing need to increase the hectarage and productivity of castor (**Gajeraa *et al*., 2010**).
Castor is a cross pollinated crop and is usually cultivated as a hybrid in India, as hybrids give significantly greater yields than pure lines or varieties (**Birchler *et al*., 2003; Reif *et al.*, 2007**). Higher magnitude of heterosis and genetically superior hybrids can be obtained by combining diverse parents in hybrid development. Conventional diversity analysis methods, in the field, are time consuming, laborious, resource intensive and drastically affected by environmental factors, therefore, a technique that is rapid and not affected by environment is needed for assessment of genetic diversity and selection of parental lines for use in hybrid development programmes (**Santalla et al., 1998**). Genetic diversity assessment prior to developing hybrids can aid in better exploitation of heterosis. Assessment of genetic variation using molecular markers appears to be an attractive alternative to the conventional diversity analyses and can also aid in management and conservation of biodiversity. A large number of polymorphic markers are required to measure genetic relationships and genetic diversity in a reliable manner (**Santalla *et al.*, 1998**). DNA-based molecular analysis tools are ideal for germplasm characterization and phylogenetic studies. Among the various DNA-based markers, amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) have been used to study genetic diversity. These markers elucidate the phylogenetic relationships among various lines, for their efficient use in breeding and genetic resource management. These methods, however, involve the use of expensive enzymes, radioactive labeling which are cumbersome and hence they appear unsuitable (**Gajeraa *et al*., 2010**). Random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers on the other hand, require only small amounts of DNA sample without involving radioactive labels and are simpler as well as faster. RAPD has been also proven to be simple and quite efficient in detecting genetic variations used for diversity assessment and for identifying germplasm in a number of plant species (**Welsh and McClelland, 1990, Gwanama *et al.*, 2000, Kapteyn and Simon, 2002, Gajeraa *et al.*, 2010**). ISSR has been shown to provide a powerful, rapid, simple, reproducible and inexpensive means to assess genetic diversity and identify differences between closely related cultivars in many species (**Gonzalez *et al.*, 2002, Labajová *et al.*, 2011**). Limited studies have been carried out on the genetic diversity and phylogenetics of castor using molecular markers. Recently, studies have been initiated on assessment of genetic variation in castor germplasm using AFLP and SSR markers (**Allan *et al.*, 2008**).
The aim of this study was to detect genetic variability among the set of 30 castor genotypes using 6 RAPD markers.

MATERIAL AND METHODS

Plant material and DNA extraction

Ricin lines (30) were obtained from the breeding station Zeainvent Trnava Ltd. (Slovakia). DNA of 30 castor genotypes was extracted from 10 day old leaves using the Gene JET Plant Genomic DNA Purification Mini Kit (Thermo Scientific).

RAPD amplification

Amplification of RAPD fragments was performed according to **Gajeraa *et al.* (2010)** (Table 1) using 6 decamer arbitrary primers. Amplifications were performed in a 25 µl reaction volume containing 5 µl DNA (100 ng), 12.5 µl Master Mix (Promega, USA) and 1 µl of 10 pmol of primer. Amplification was performed in a thermocycler (Biometra, Germany) with initial denaturation at 94 °C for 5 min, 42 cycles of denaturation at 94 °C for 1 min, primer annealing at 38 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. Amplified products were separated in 1.5% agarose in 1× TBE buffer. The gels were stained with ethidium bromide and documented using gel documentation system UVP PhotoDoc-t®. The size of alleles was determined by comparing with 100 bp standard lenght marker (ThermoFisher Scientific).

Table 1 List of used RAPD primers

Primers	Primer sequence (5´-3´)	Expecting molecular weight range (bp)	Localization
RLZ3	5´TGTCCAGCTT 3´	1200	2RL
RLZ6	5´GTGATCGCAG 3´	330	7RL
RLZ7	5´GTCCACACGG 3´	750	2RL
RLZ8	5´GTCCCGACGA 3´	350	7RL
RLZ9	5´TGCGGCTGAG 3´	650	2RS
RLZ10	5´ACGCGCATGT 3´	1100	4RL

Data analysis

The RAPD bands were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity. The binary data generated were used to estimate levels of polymorphism by dividing the polymorphic bands by the total number of scored bands and to prepare a dendrogram. A dendrogram based on hierarchical cluster analysis using the unweighted pair group method with arithmetic average (UPGMA) with the SPSS professional statistics version 17 software package was constructed.
For the assessment of the polymorphism between ricin genotypes and usability of RAPD markers for differentiation we used diversity index (DI) **(Weir, 1990**), the probability of identity (PI) (**Paetkau et al., 1995**) and polymorphic information content (PIC) (**Weber, 1990**).
They were calculated according to formulas:

Diversity index (DI)

$$DI = 1 - \sum p_i^2$$

Probability of identity (PI)

$$PI = \sum p_i^4 + \sum_{i=1}^{i=n-1} \sum_{j=i+1}^{n} \left(2 p_i p_j\right)^2$$

Polymorphic information content (PIC)

$$PIC = 1 - \left(\sum_{i=1}^{n} p_i^2\right) - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 p_i^2 \cdot p_j^2$$

where pi and pj are frequencies of ith and jth fragment of given genotype.

RESULTS AND DISCUSSION

PCR amplifications using 6 RAPD primers produced 50 DNA fragments that could be scored in all genotypes. The selected primers amplified DNA fragments across the 30 genotypes studied with the number of amplified fragments varying from 5 (RLZ7) to 11 (RLZ8) and the amplicon size varied from 330 to 1200 bp. Of the 50 amplified bands, all 50 were polymorphic with an average of 8.33 fragments per primer (Table 2). The polymorphic information content (PIC) values varied from 0.774 (RLZ7) to 0.870 (RLZ8) with an average of 0.825 and index diversity (DI) value ranged from 0.786 (RLZ7) to 0.872 (RLZ8) with an average of 0.831 (Table 2). Similar values of DI and the PIC were detected by other authors (**Gajeraa *et al*., 2010; Machado *et al.*, 2013; Tomar Rukam *et al.*, 2014; Vivodík *et al*., 2014; Kallamadi *et al*., 2015**). Dendrogram based on hierarchical cluster analysis using UPGMA algorithm was prepared. In constructed dendrogram genotypes were divided into three main clusters (1, 2, 3). Cluster 1 contained unique genotype (RM-76), cluster 2 included two genotypes (RM-62, RM-68) and cluster 3 contained 27 genotypes of ricin. Cluster 3 subdivided 27 genotypes of ricin into two subclusters, subcluster 3a with unique genotype RM-70 and subcluster 3b with 26 genotypes of ricin. Two genotypes of subcluster 3b (RM-72 and RM-73) were genetically the closest (Figure 1). Using more polymorphic RAPD markers genetically close genotypes can be distinguished.
In our study we used 6 RAPD primers to study genetic polymorphism of 30 ricin lines and similar PIC values we obtained as **Gajeraa *et al.* (2010)** who used 30 RAPD polymorphic primers for the analysis of 22 castor bean genotypes. We can consider used RAPD primers as sufficiently polymorphic. RAPD analysis of **Gajeraa *et al.* (2010)** yielded 256 fragments, of which 205 were polymorphic, with an average of 6.83 polymorphic fragments per primer. Number of amplified fragments with RAPD primers ranged from 6 to 12, with the size of amplicons ranging from 160 to 3000 bp in size. The polymorphism ranged from 27.2 to 100.0, with an average of 80.2%. Genetic diversity of 37 ricin genotypes grown in China using RAPD markers was studied by **Li et al. (2012).** Using RAPD markers, together they detected 122 fragments, of which 71 were polymorphic, representing the percentage of polymorphism fragments 58.20%. Dendrogram constructed using UPGMA algorithm divided 37 analyzed ricin genotypes into 4 main clusters. In the study **Machado *et al.* (2013**) used 58 RAPD primers for the analysis of 15 castor bean cultivars. The genetic dissimilarity between cultivars was calculated by Jaccard's index, using the unweighted pair-group method with arithmetic mean (UPGMA). Authors identified 552 fragments, of which 311 were polymorphic (56.3%). The cultivars were clustered in five groups, evidence that there is genetic difference among them. Authors of their work confirmed, that RAPD markers are efficient in the study of genetic dissimilarity in castor bean.

Table 2 The statistical characteristics of the RAPD markers used in castor

RAPD primer	number of fragments	DI	PIC	PI
RLZ3	9	0.862	0.859	0.003
RLZ6	9	0.792	0.792	0.013
RLZ7	5	0.786	0.774	0.036
RLZ8	11	0.872	0.870	0.002
RLZ9	7	0.828	0.821	0.006
RLZ10	9	0.842	0.837	0.005
average	8.33	0.831	0.825	0.011

DI- diversity index
PIC- polymorphic information content
PI- probability of identity

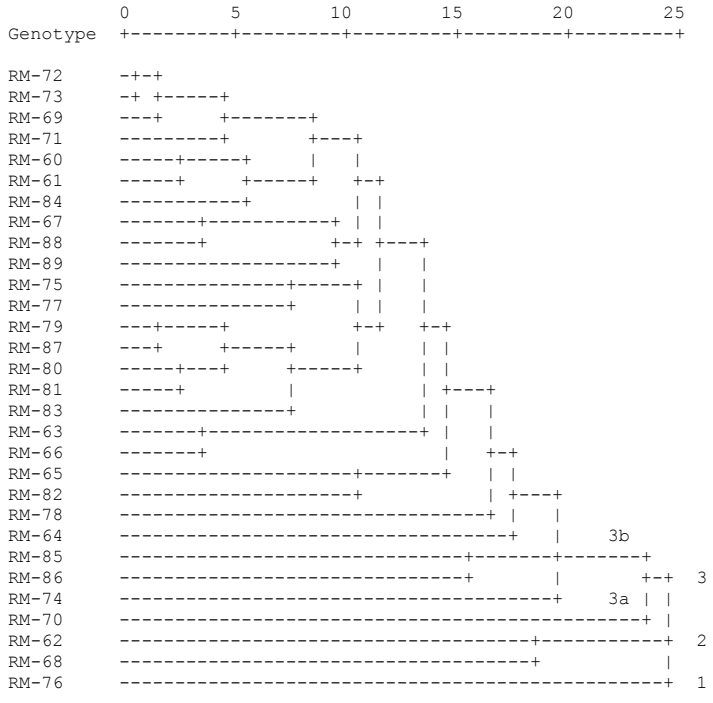

Figure 1 Dendrogram of 30 castor genotypes prepared based on 6 RAPD markers

Tomar Rukam *et al.* (2014) investigated the fingerprinting and phenotyping of 25 castor genotypes available in Gujarat and other States of India using RAPD and ISSR markers. One hundred thirty decamer RAPD primers from Operon series (OPA to OPZ – five from each series) were screened with the DNA of the two castor genotypes. Only fifty-seven primers generated reproducible and scorable RAPD profiles. These produced multiple band profiles with a number of amplified DNA fragments ranging from 4 to 13 with an average of 7.70 fragments per primer. The total number of fragments produced by the fifty seven primers was 439. Maximum number of 13 amplicons was amplified with primer OPG-04 while the minimum number of fragments (4) was amplified with primer OPQ-01. The number of polymorphic fragments ranged from 0 to 7. The total number of polymorphic amplicons obtained by the fifty-seven studied primers was 122. The UPGMA cluster analysis was carried out to represent graphically the genetic distances among the 25 castor genotypes. The obtained dendrogram was divided into three main clusters; cluster one included 19 genotypes while cluster II and III included 5 and 1 genotype, respectively. The main cluster (cluster I) included two subclusters A and B. Subcluster B contained only two

genotypes i.e. SKI332 and SKI271 while subcluster A contained 17 genotypes which were further divided into subclusters C and D.

RAPD molecular markers have been used in population genetic studies (**Parsons *et al.*, 1997, Esselman *et al.*, 1999**). Some researchers have considered RAPD markers to represent segments of DNA with noncoding regions (**Landergott *et al.*, 2001**), and some studies have shown that RAPD markers are distributed throughout the genome and may be associated with functionally important loci (**Penner, 1996**).

CONCLUSION

The analysis showed that the RAPD markers are very effective molecular markers for the assessment of the genetic diversity in castor bean. The dendrogram prepared based on UPGMA algorithm divided 30 analyzed genotypes into three main clusters. Using 6 RAPD markers only two castor bean genotypes have not been distinguished (RM-72 and RM-73). For better discrimination of analyzed genotypes of ricin, it is necessary to use a higher number of RAPD markers. Our analysis proved utilization of RAPD markers for differentiation of used set of castor genotypes. We can consider used RAPD primers as sufficiently polymorphic. RAPD markers are useful in the assessment of castor bean diversity, for the detection of duplicate sample in genotype collection and the selection of a core collection to enhance the efficiency of genotype management for use in castor bean breeding and conservation.

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