*Indian Journal of Weed Science* 40 (3&4) : 162-165, 2008

# Evaluation of advanced generation transgenic groundnut lines resistant to herbicide-glyphosate

**S.B. Manjunatha, T.C. Suma, Rohini Sreevathsa, R. Devendra, M. Udaya Kumar and T.G. Prasad**
*Department of Crop Physiology, University of Agricultural Sciences, Bangalore (Karnataka)*
*E-mail : sbmanju.acharya@gmail.com*

## ABSTRACT

**In groundnut, glyphosate resistant plants (cul. TMV-2) were developed by over expressing pEGAD-EPSPS with altered kinetics of enzyme, which do not bind to glyphosate. *Agrobacterium* mediated *in planta* transformation adopted to develop transgenic groundnut lines expressing EPSPS. Single leaflet glyphosate induced chlorosis bioassay was standardized and used to assess the glyphosate resistance in groundnut transgenic lines of $T_1$ and $T_2$ generations. The $T_1$ generation plants grown under transgenic housing facilities along with wild type and their relative tolerance analyzed by the leaf swabbing technique indicated the integration of the transgene in tolerant plants by PCR. The $T_2$ generation plants screened for glyphosate resistance by swabbing 3000 ppm of glyphosate at 45 DAS observed that 30% of transgenic plants showed some degree of yellowing and leaf mortality and resistance confirmed by PCR. The chlorophyll degradation was less in transgenic and also maintained higher membrane integrity compared to wild type plant.**

**Key words:** Groundnut, EPSPS, Glyphosate, Gene transfer

Glyphosate is eco-friendly herbicide, due to its degradation in soil within 24 hrs by soil microbes. Glyphosate is broad-spectrum and translocative herbicide. It kills plant by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which catalyzes the reaction of shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to form 5-enolpyruvyl-shikimate-3-phosphate (EPSP). EPSP is subsequently dephosphorylated to chorismate, an essential precursor in plants for the aromatic amino acids: phenylalanine, tyrosine and tryptophan. These amino acids are used as building blocks in peptides and to produce secondary metabolites such as folates, ubiquinones and naphthaquinone. This basic available information on achieving herbicide tolerance in crop plants clearly indicates that transgenic crop varieties highly tolerant to glyphosate can be developed either by altering the structure of EPSPS enzyme for low affinity to glyphosate or by increasing the ability to detoxify the herbicide. Recent advances in genetic engineering have provided the requisite tools to transform plants to contain foreign genes. Certainly, one such advantageous trait is more cost effective, environmentally compatible weed control via herbicide tolerance.

The present investigation has been to develop glyphosate tolerant groundnut transgenics expressing a modified EPSPS with altered kinetics to minimize binding of glyphosate. The hypothesis is that transgenic plants over-expressing modified *EPSPS* have better herbicide tolerance capacity than normal plants. Since groundnut is a recalcitrant species a non tissue culture based transformation method (Rohini and Rao, 2000) was used to generate transgenic plants.

## MATERIALS AND METHODS

The modified EPSPS gene was codon optimized for maximum expression in tomato, potato, soybean and tobacco and custom synthesized at Bionexus Inc., USA. The EPSPS gene was cloned into the pEGAD binary vector and mobilized into *Agrobacterium* strain LBA4404. *Agrobacterium*-mediated *in-planta* transformation method was followed to develop transgenic groundnut (variety TMV-2) plants. The groundnut variety TMV-2 was obtained from NSP (National seed project), UAS, GKVK campus, Bangalore-65.

The pods from $T_0$ plants were collected and seeds were separated by shelling and separated seeds were dried completely. $T_1$ seeds from $T_0$ plants were used to develop $T_1$ generation and $T_2$ seeds collected and $T_2$ generation was raised.

In $T_1$ generation, groundnut leaves were swabbed with glyphosate and these plants were observed for resistance. The transformants showing tolerance to

glyphosate by leaf swabbing technique at 3000 ppm were further analyzed by Polymerase Chain Reaction (PCR). Genomic DNA was extracted from the tissues of transgenic and non-transgenic plants following the CTAB method (Dellaporta et al.1983). PCR was performed in a total reaction volume of 50 µl consisting of 200 ng DNA, 4 µl milliQ water, 5 µl 10X buffer, 3 µl of dNTPs, 5 ng of primer set and 1 U of taq polymerase. The PCR cycles were, 1 min at $95^0$C, 1 min 30 sec at $58^0$C for the amplification of 35S-EPSPS gene fragment and 2 min at $72^0$C for 30 cycles. The PCR cycles were later analysed on 1% agarose gel.

In the analysis of the $T_2$ generation plants, 45 days old putative transformants and wild type plants were sprayed with 1000 ppm of glyphosate and allowed to recover for a period of 18 days and periodically chloratic visual observations were recorded. The plants which showed resistance were subjected to a repeated screening of 3000 ppm.The stability of the transgene were checked by PCR with promoter-gene specific primers. Resistant plants were further analyzed for physiological parameter comparing with untransformed control with respect to chlorophyll content (Hiscox and Israestam, 1979) and membrane integrity (Sullivan and Ross 1979).

## RESULTS AND DISCUSSION

Herbicide resistance is the major trait that has been engineered into crops and herbicide-resistant crops (HRCs) occupy the largest area under transgenic crops in the world. One of the viable approaches for crop improvement towards herbicide tolerance is by genetic engineering. In this study the transgenic groundnut plants over expressing EPSPS were screened for their glyphosate tolerance capacity using molecular and physiological techniques.

With the existing approaches, the gene integration with any of the transformation technique is random and hence the main requirement would be to develop large number of the primary transformants (independent gene integration events). The conventional tissue culture approach has a limitation in this direction. Therefore, a novel non-tissue culture based *in-planta* transformation approach was used to develop transgenics. This technique has provided the leads to generate alternate technique for transformation without involving tissue culture procedures. In this method basically, *Agrobacterium* infection is directed towards the differentiated embryo and the T-DNA is transferred not only to the cells of apical meristem but also to other cells of the differentiated embryo. In view of this, the transformants at the $T_0$ stage are chimeric and analysis is carried out in the $T_1$ generation (Rohini and Sankara Rao 2002, Keshamma *et al.* 2008). In the present study, 49 $T_0$ plants were raised in the transgenic facility. They flowered and set seeds normally. The $T_1$ seeds were harvested and taken for analysis.

Since the *in planta* transformation gives rise to a large number of primary transformants, a high throughput primary screening is necessary for the selection of primary transformants. With this objective a leaf swabbing bioassay (visual chloratic rating single leaflet bioassay system) was developed. The $T_1$ generation plants were grown under green house conditions along with wild type and their relative tolerance was subsequently analyzed by the leaf swabbing technique using concentration of 3000 ppm. To check consistency of resistant plants, the bioassay carried out for three times at different level of age groups. Since the objective was to identify high expressing transgenic plants, 24 plants that showed tolerance to 3000 ppm after repeated swabbing (Fig. 1) were selected for molecular analysis. PCR analysis of these plants revealed amplification of a 750 bp 35S-EPSPS gene fragment (Fig. 2).

$T_2$ seeds of transgenic glyphosate resistant lines were sown in the soil in containment facility. The plants were sprayed with 1000 ppm glyphosate and allowed to recover for a period of 18 days. More than 30% of the transgenic plants were highly resistant, whereas the other 30% were moderately resistant and the rest were susceptible. The resulting 48 plants that showed tolerance to a spray of 1000 ppm glyphosate and these 48 resistant plants were subsequently screened at 3000 ppm adapting leaflet swabbing bioassay. Out of 48 plants, 24 plants showed tolerance at 3000 ppm (Fig. 1) and they were also characterized for the stability of the transgene by PCR. The amplification of a 750 bp 35S-EPSPS gene specific fragment in the selected transgenics (Fig. 2) confirmed the stable inheritance of the gene in the groundnut plant genome.

The chlorophyll degradation and loss of membrane integrity are the two effects of glyphosate. A secondary mode of action by glyphosate occurs in the aminolevulinic acid pathway or the porphyrin synthesis pathway. In this pathway, glyphosate inhibits conversion of succinyl CoA (from the TCA cycle) to aminolevulinic acid by interfering with activity of aminolevulinate synthase. By blocking this step in the pathway, synthesis of compounds containing porphyrin ceases. This affects production of chlorophyll, cytochromes, and peroxidases, *etc*. (Devine 1993). This is a secondary mode of action and in most cases, plant death occurs before results from the secondary mode of action are expressed.

Inadequate use of excitation energy can also lead to generation of ROS (Reactive oxygen species). These radicals are extremely reactive and readily destroy

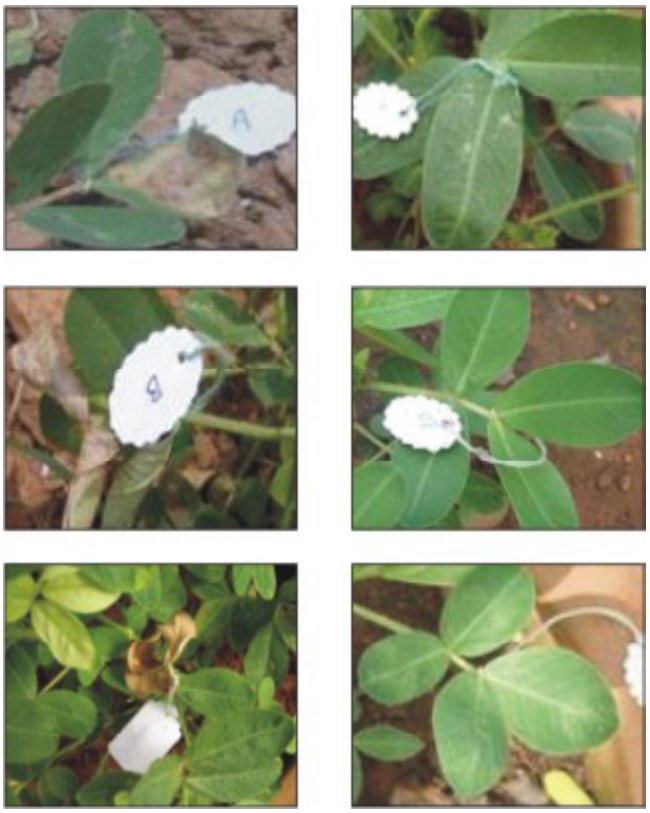

**Fig. 1.** Glyphosate swabbed leaves of both transgenic ($T_1$ and $T_2$ generation) and wildtype plants of groundnut.

$T_1$: Response of the wildtype (A-B) and putative transgenics (C-D) to 3000 ppm glyphosate

$T_2$: Response of the wildtype (E) and putative transgenics (F) to 3000 ppm glyphosate

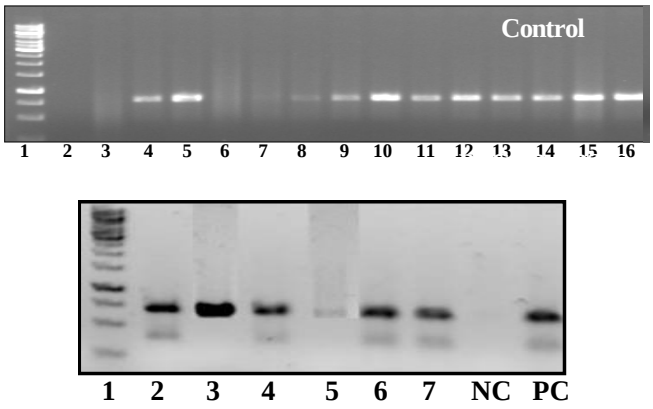


**Fig. 2.** PCR analysis of $T_1$ and $T_2$ generation plants for the amplification of a 750bp 35S promoter-EPSPS gene fragment.

T1: Lane 1: marker Lane 2: DNA from untransformed plant, Lanes 3-15: DNA from putative transformants, Lane 16: positive control.

T2 : Lane 1: marker Lane 2-7: DNA from transformants, Lane NC: DNA from untransformed plant, Lane PC: positive control

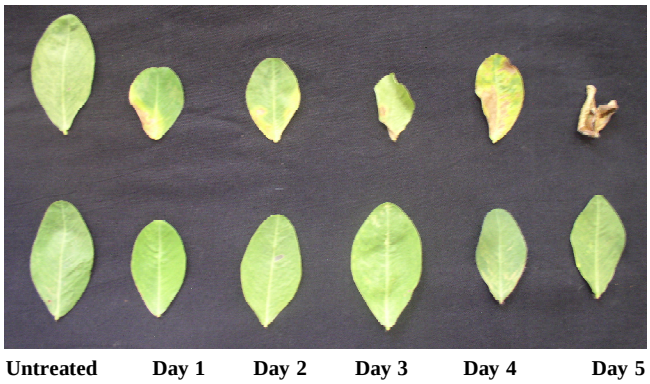


**Fig. 3.** Transgenic groundnut leaves and untransformed groundnut leaves (control) swabbed with 3000 ppm of glyphosate taken at 5 consecutive days for chlorophyll estimation

unsaturated lipids, including membrane fatty acids and chlorophyll. This in turn can affect lipid peroxidation and hence membrane damage occurs. This destroys cell membrane integrity, so that cells and organelles "leak", leading to rapid leaf wilting and desiccation, and eventually to plant death (WSSA 1994) (Fig. 3).

The putative transformants retained fairly high levels of chlorophyll when the leaves were swabbed with 3000 ppm glyphosate and in case of wild type plants huge reduction in chlorophyll content was observed because glyphosate spray leads to chlorosis, drying and leaf fall (Fig.3). Since the leaves collected for five consecutive days, gradual decrease of chlorophyll level was scene in wild type plant but chlorophyll level maintained in transgenics (Fig.4).The damage (% leakage) was more in untransformed control plants 48 hours after swabbing the leaves 3000 ppm of glyphosate (76.3%). It was still high (90.2%) 96 hours after swabbing because the leakage was more when compared to transgenic lines (23.5% and 26%, respectively) swabbed with 3000 ppm of glyphosate. This clearly indicates that the loss in turgor is preceded by loss of membrane integrity (Fig. 5). The above observation from experiment gives the clear-cut difference between transformed and untransformed plants regarding level of tolerance for glyphosate.

Since the segregation of population occurs till $T_7$-$T_8$ generation, yield parameters were not recorded. However the visual observation of $T_1$ and $T_2$ generations suggests that, with glyphosate treatment transgenic material were normal on pod formation.

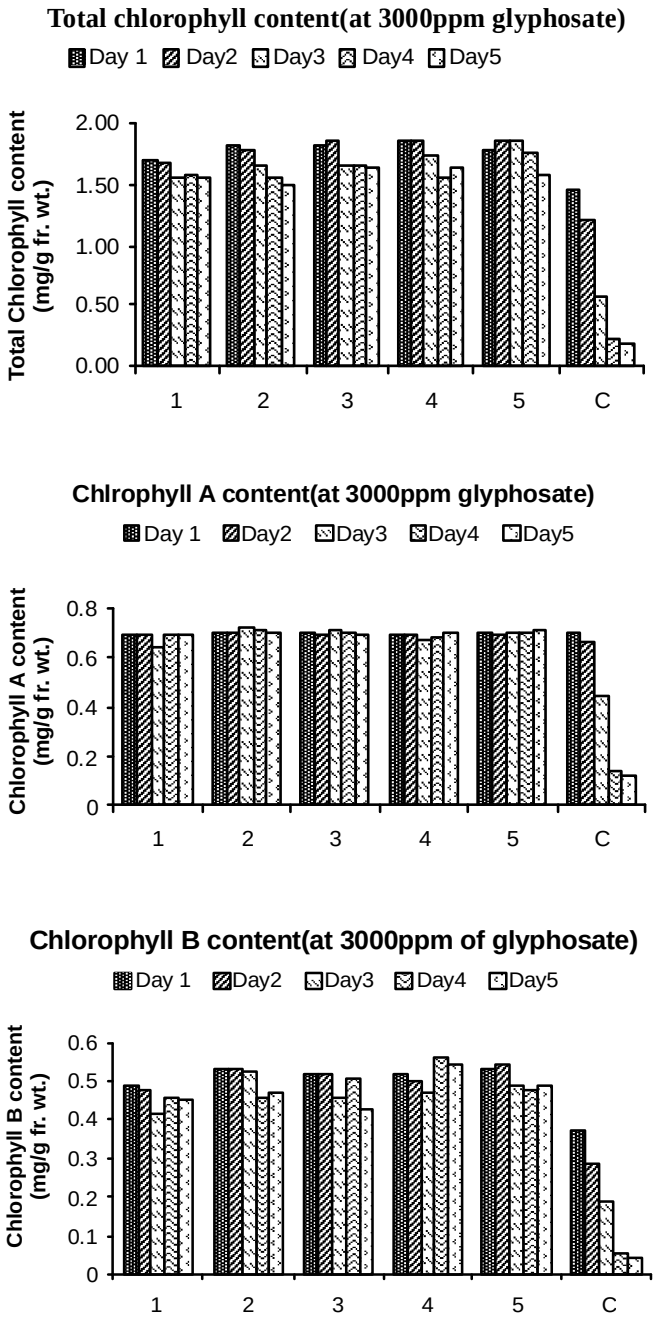


**Fig. 4.** Chlorophyll content of pEGAD-EPSPS $T_2$ groundnut transgenic lines and untransformed control plants swabbed with 3000 ppm of glyphosate estimated for 5 consecutive days.{1-5…. Transgenic plants C-control or wild type}

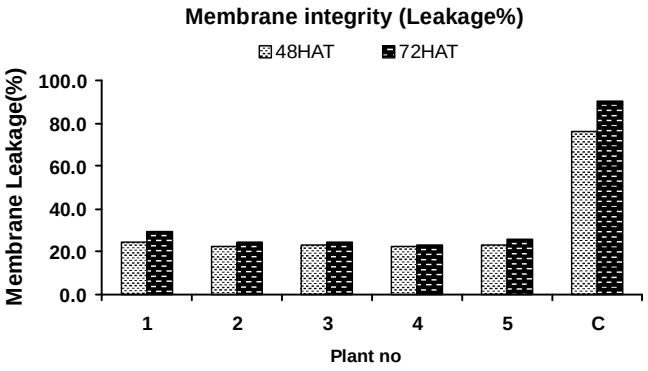


**Fig. 5.** Variation in the extent of cell membrane damage by glyphosate (3000 ppm) in pEGAD EPSPS $T_2$ transgenics and untransformed control groundnut plants. {1-5…. Transgenic plants C-control or wild type HAT (hrs after treatment)}

## REFERENCES

Dellaporta SL, Wood J and Hicks JB. 1983. A plant DNA minipreperation: version II. *Plant Molecular Biology Reporter* **1** : 19-21.

Devine MD, Duke SO, and Fedtke C. 993 *Physiology of Herbicide Action*. 1. Prentice Hall NJ.

Feldmann K and Marks MD. 1987. *Agrobacterium*-mediated transformation of germinating seeds of *Arabidopsis thaliana*: a non-tissue culture approach. *Molecular and General Genetics* **208** : 1-9.

Hiscox JD and Israelstam GF. 1979. A method for the extraction of chlorophyll from leaf tissue without maceration using dimethyl sulphoxide. *Canadian Journal of Bot*any **57** : 1332–1334.

Keshamma E, Rohini Sreevathsa, Manoj Kumar A, Ananda Kumar P, Kumar ARV, Madhusudhan B and Udayakumar M. 2008. A chimeric gene imparts resistance to *Spodoptera litura* and *Helicoverpa armigera* in the transgenic groundnut. *Eurasian Journal of Biosciences* **2** : 53-65.

Rohini VK and Rao KS. 2000. Transformation of peanut (*Arachis hypogeae* L.): a non-tissue culture based approach for generating transgenic plants. *Plant Science* **150** : 41-49.

Sullivan CY and Ro WM.1979. *Selecting for drought and heat resistance in grain sorghum.* p. 263–281.

WSSA. 1994. *Herbicide Handbook.* Weed Science Society of America. Champaign, Illinois. 352pp.