



Molecular approach to evaluate the genotoxicity of glyphosate (roundup) using mosquito genome

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Abstract: Glyphosate, an active ingredient in Roundup is a broad spectrum, systemic and non -selective herbicide which is commonly used for eliminating weeds in agriculture and forest landscapes. The present studies deal with the evaluation of the genotoxic potential of Glyphosate with two different dose concentration of LD_{20} and LD_{40} on a mosquito Culex quinquefasciatus taken as an experimental model. For this, polymerase chain reaction technique (PCR) was used for detecting DNA damage by amplifying ribosomal DNA internal transcribed spacer 2 (ITS 2) region. The amplified products were sequenced and the results of treated and non-treated controls were compared by using Clustal W software programme. The results were studied in the form of transitions, transversions, deletions and additions of bases. The DNA band amplified from control stocks consisted of 440 bases while those from LD_{20} and LD_{40} treated individuals were comprised of 423 and 468 bases respectively. The total number of mutations caused in LD_{20} treated stock was 205 out of which 68 were transitions, 90 transversions, 32 deletions and 15 additions. In case of LD_{40} treated individuals, as many as 221 bases had suffered mutations, out of which 66 were transitions, 90 transversions , 12 deletions and 41 additions. In both the cases the rate of transversions was higher than transitions. From these results it was evident that glyphosate has a potential to promote gene mutations in the individuals exposed to its semilethal doses.

Keywords: Glyphosate, PCR, ITS 2, Culex quinquefasciatus

INTRODUCTION

Glyphosate is a non-selective broad spectrum herbicide commonly sold as a commercial formulation named Roundup. Since its introduction in 1970s, it has been widely used for killing unwanted plants both in agriculture and non - agriculture landscapes (Williams et al., 2000). It is a combination of the active ingredients glyphosate and various adjuvants in different concentrations. One of the major adjuvants is a surfactant polyethoxylated tallowamine (POEA) along with minor components including antifoaming and colouring agents, biocides and inorganic ions for pH adjustment. The POEA itself causes ocular burns, redness, swelling and blisters, short term nausea and diarrhoea. In combination with these components glyphosate becomes more effective in its action as a pesticide due to increased stability and bioaccumulation (Cox 1998; Richard et al., 2005; Benachour et al., 2007). Its action starts with penetration through plasmatic membranes followed by inhibition of the enzyme 5- enolpyruvoyl – shikimate 3- phosphatesynthase, which is essential for the synthesis of aromatic amino acids in plants. This ultimately leads to the inhibition of nucleic acid metabolism and protein synthesis that are required for its growth and survival (Steinrucken and Amrhein 1980; Malik et al., 1989). A

variety of toxic effects of glyphosate have also been observed on various stages of reproduction and genetic material of the animals exposed to it (Bolognesi et al., 1997; Peluso et al., 1998; Walsh et al., 2000; Daruich et al., 2001; El Demerdash $et\ al.$, 2001). There are a number of techniques to assess the genotoxicity of pesticides on genetic material which involves the use of a number of tests or protocols (Gillet 1970; Sobels 1974; Evans 1977; Gaulden and Liang 1982; Menzer 1987; Zaman et al., 1994; Chaudhry and Anand 2004 2005). In the last few years the development of new assays, such as comet assay (McKelvey et al., 1993; Pandrangi et al., 1996), automatic scoring techniques for micronuclei (OCDE, 1998) and ³²P- post labeling assay for the detection of DNA adducts (Phillips 1997). Some of the recent advances in the field of molecular biology, like gene amplification and DNA fingerprinting with PCR technique, offer new possibilities for detecting DNA damage even at the level of single nucleotide. Jones and Kortenkamp (2000) demonstrated that the genomic alterations in the nucleotide sequence can be detected with PCR assay even if 2% of the cells are affected by the mutagens. In the present study rDNA internal transcribed spacer 2 (ITS 2) sequence was selected to assess the genotoxic effect of glyphosate. This spacer lies between

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Fig. 1. Chemical structure of glyphosate.

5.8s and 28.5s rRNA coding sequence. It is a phylogenetic marker which is highly conserved within all eukaryotes and carry some of the unique nucleotide sequences of rDNA, therefore any change occurring in them in the form of deletions, additions, transitions and transversions are considered significant. The present set of investigations is a first ever attempt in recording the glyphosate induced sequence alterations in rDNA domain of $Culex\ quinquefasciatus$ taken as an experimental insect. In relevance to this, two different concentrations LD_{20} and LD_{40} of glyphosate were used in evaluating the mutagenic consequences in the genome of $Culex\ quinquefasciatus$.

MATERIALS AND METHODS

Glyphosate [N- (phosphonomethyl)glycine]) is commonly sold in the form of a formulation named Roundup (Monsanto Company, St. Louis, MO) under CAS no. 1071-83-6 , with a molecular formula $\rm C_3H_8NO_5P$ (Fig. 1) and molecular weight of 169.08. For the present purpose, $\rm LD_{20}$ and $\rm LD_{40}$ were calculated by probit analysis (Finney 1971) had the values of 0.064 $\mu l/$ ml and 0.275 $\mu l/$ ml respectively, (Figs. 2 and 3). The gravid females of Culex quinquefasciatus were collected from inhabitation in the village Nadasahib along a rivulet, 20 kms East of

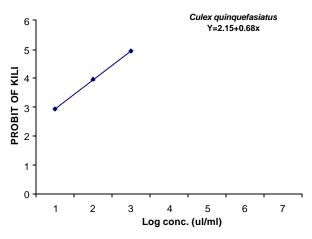


Fig. 2. Relationship between the probit of kill and LD_{20} of Glyphosate showing the regression line represented by the equation Y = a + bx.

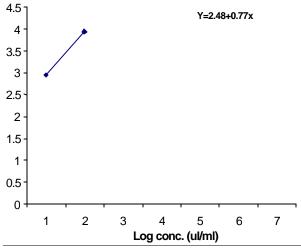


Fig. 3. Relationship between the probit of kill and LD_{40} of Glyphosate showing the regression line represented by the equation Y = a + bx

Chandigarh. They were allowed to lay eggs in water filled petridishes placed in the breeding cages. The egg rafts obtained in this way were allowed to hatch and the larvae were reared on a protein rich diet consisting of a mixture of finely powdered dog biscuits and yeast powder in the ratio of 6: 4 respectively. A colony was raised under suitable conditions of temperature and humidity in mosquito rearing laboratory (Krishnan 1964; Singh*etal.*, 1975, Clements 1994). Fixed number of freshly hatched healthy fourth instar larvae were treated with selected doses of the pesticide by rearing them in glyphosate containing rearing medium for 24 hours after which they were transferred to pesticide free water and allowed to grow upto adult stages. The desired number of control

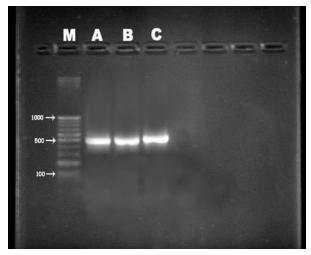


Fig. 4. PCR amplification of rDNA ITS 2 of treated and nontreated individuals of Culex quinquefasciatus. Lane M: Gene ruler (DNA ladder), Lane A: DNA band from non-treated individual, Lane B: DNA band from LD $_{20}$ treated individual, Lane C: DNA band from LD $_{40}$ treated individual.

| CONTROL_ | CCGGGGGGGTACGGGCCTCTCGT- CACGACAGTAGGGGAAGACAATTTTTGAGTGGCCT 59 |
|----------|---|
| TREATED_ | CGGGTTAACCAAACATGTCACAGATGGTACACA-TTTTTGAGTG-CCT 46 |
| | **** |
| | |
| CONTROL_ | ATATGTTATCTATTCA- CTGTGCACGC- GCGGCTGTAAAGTGGA TTACTGCCTCCTT 114 |
| TREATED_ | ATAT-TTATCTATTCAACTGTGCACGCAGTGCCCCCAAAATGGTGTTTTGCTGCCTTCGG 105 |
| | **** ******* ****** * * * * * * * * * * |
| | |
| CONTROL_ | TTTATTTGAGAACATTTTGGAAGTACAACGTCCTTGGGAGATAGCTCTTGGAAAGTAGCC 174 |
| TREATED_ | TGGCTGGCAAAACATTCAAGACGCTCAGCGCTTCGGGGGTTTTCGTTCG |
| | * |
| | |
| CONTROL_ | CCTGGCGGGCCTCCTTGGATGTTTTGCTTATTCTAACCGCTGTCTAAATACAATCCGC-C 233 |
| TREATED_ | TTGGGGGGGACGCCCGGGAATGAACGGACGAAGACGAGAAAAAAAAATCCCCAC 219 |
| | ** *** * ** ** ** * * * * * * * * * * * |
| | |
| CONTROL_ | AAGCGGTTTGTAGGGGGGGCGTATCTTCTAACAAGAAAAGCCGGCCG |
| TREATED_ | AAACACCCTGGGTGGGGGGGGATGAAGAATCCTTCCCGCCCG |
| | ** * ** **** * * * ***** ** ** |
| | |
| CONTROL_ | TTTTTGTCGACCAG CCGGCGGGGGGGGCCCCTCTACATGAAAAAACC ACCCCCC 348 |
| TREATED_ | GTTCGGTCATCCGGGGTCGTGGGGGGGGGGGCCCCGCAAAAAAAA |
| | ** *** ** * * * **** *** * * * ****** **** |
| | |
| CONTROL_ | GGGAGGAGGTAGAAAAAACCCCCCTGCGGCGGAAAGAAGAGGAGTCCCCC 405 |
| TREATED_ | ACCAAGGGAGAGAAAAAAAAAACCCCCCCCGGGGGCCCAAAAAA |
| | * * * ** ** ********* *** ** *** ** *** |
| COMEDO | CITETA DININITA A CAA A CATECITIC CO CA CAA A A A A A A A A A A A A A A |
| CONTROL_ | CTTATTTTAAGAAAGATGTGGCAGAAAAAAAAA 440 |
| TREATED_ | CCCCTTTAAAAAAA AAAAAAA 423 |
| | * **** ** * * ***** |

Fig. 5. Analysis of multiple sequence alignment in the rDNA ITS 2 of control and LD_{20} treated individual of Culex quinquef asciatus (* complementary bases, - missing bases).

and treated specimens were processed immediately for DNA extraction while the remaining were preserved in ethanol at -20° C for future use.

DNA extraction and amplification: The DNA extraction was carried out as per the standard protocol of Ausubel *et al.*, (1999) with minor modifications for mosquito genome by Chaudhry *et al.*, (2004) and Chaudhry and Sharma. (2006). The integrity of the DNA sample was tested by following the procedure of Sambrook *et al.*, (1989) while the concentration and purity were determined by ultraviolet absorption spectroscopy. The two specific primers *viz:* forward primer (FP) 5'-

TGTGAACTGCAGGACACAT-3', and reverse primer (RP): 5'—TATGCTTAAATTCAGGGGGT-3' were used for amplifying the ITS 2 region of the control and treated stocks of *Culex quinquefasciatus*. The amplification reactions were carried out according to the procedure of Williams *et al.*, (1990) according to which the reaction mixture was prepared by mixing 16.8 µl of distilled water, 3 µl Taq buffer, 3 µl DNTP's, 1.2 µl forward primer, 1.2 µl reverse primer, 1.2 µl Taq polymerase, 1.2 µl MgCl₂ and 2.4µl genomic DNA. After loading this reaction mixture in the thermocycler, the reaction was programmed for initial denaturation at 94°C for 5m, followed by 37 cycles

| CONTROL_ | CCGGGGGGGGTACGGCCTCTCGT- CACGACAGTAGGGGAAGACAATTTTTGAGTGGCCT 59 |
|----------|--|
| TREATED_ | |
| | **** |
| CONTROL_ | ATATGTTATCTATTCA- CTGTGCACGC- GCGGCTGTAAAGTGGA TTACTGCCTCCTT 114 |
| TREATED_ | ATAT-TTATCTATTCAACTGTGCACGCAGTGCCCCCAAAATGGTGTTTTGCTGCCTTCGG 105 |
| | **** ******* ****** * * * * * * * * * * |
| CONTROL_ | TTTATTTGAGAACATTTTGGAAGTACAACGTCCTTGGGAGATAGCTCTTGGAAAGTAGCC 174 |
| TREATED_ | TGGCTGGCAAAACATTCAAGACGCTCAGCGCTTCGGGGTTTTCGTTCG |
| | * * * ***** ** ** ** * * * * * * * * |
| CONTROL_ | CCTGGCGGGCCTCCTTGGATGTTTTGCTTATTCTAACCGCTGTCTAAATACAATCCGC-C 233 |
| TREATED_ | TTGGGGGGGACGCCCGGGAATGAACGGACGAAGACGAGAAAAAAAATCCCCAC 219 |
| | ** *** * ** ** * * * * * * * * * * * * * |
| CONTROL_ | AAGCGGTTTGTAGGGGGGGCGTATCTTCTAACAAGAAAAGCCGGCCG |
| TREATED_ | AAACACCCTGGGTGGGGGGGGATGAAGAATCCTTCCCGCCCG |
| | ** * ** *** * * * * * * * * * * * * * * |
| CONTROL_ | TTTTTGTCGACCAG CCGGCGGGGGGGGCCCCTCTACATGAAAAAACC ACCCCC 348 |
| TREATED_ | GTTCGGTCATCCGGGGTCGTGGGGGGGGGGCCCCGCAAAAAAAA |
| | ** *** ** * * * * **** *** * * * ***** |
| CONTROL_ | GGGAGGAGGTAGAAAAAAACCCCCCTGCGGCGGAAAGAAGAGAGGAGTCCCCC 405 |
| TREATED_ | ACCAAGGGAGAGAAAAAAAAAAAACCCCCCCCGGGGGCCCAAAAAA |
| | * * * ** ** ** ******* *** *** *** *** |
| CONTROL_ | CTTATTTTAAGAAAGATGTGGCAGAAAAAAAAAA 440 |
| TREATED_ | CCCCCTTTAAAAAAAA 423 |
| | * **** * * * ***** |

Fig. 6. Analysis of multiple sequence alignment in the rDNA ITS 2 of control and LD_{40} treated individual of Culex quinquefasciatus (* complementary bases, - missing bases).

of denaturation, annealing and extension at 94°C for 1m, 59°C for 1m, 72°C for 1m respectively followed by one cycle of final extention at 72°C for 5m. The end products of PCR were resolved on 2% agarose gel containing ethedium bromide dye using 1X TAE buffer at a constant voltage of 75V. The gel was visualized over long wave UV transilluminator and photographed using Polaroid camera. A 100 bp DNA ladder (gene ruler) was also run along with all the amplification reactions for calculating the number of base pairs in each DNA band.

RESULTS AND DISCUSSION

In figure 4, lane M shows the production of bands of standard DNA gene ruler while lanes A, B and C show the bands of ITS 2 region of the control, $\rm LD_{20}$ and $\rm LD_{40}$ treated stocks respectively. The bands were sequenced and analysed by using Clustal W software programme. The sequence amplified from the DNA of control stocks consisted of 440 bases while those from $\rm LD_{20}$ and $\rm LD_{40}$ treated individuals were comprised of 423 and 468 bases respectively. In the sequence alignment of treated and control individuals of $\it Culex quinque fasciatus$ the loci

marked with asterisk (*) are the regions where bases were identical in the normal and treated mosquitoes while dashes (-) indicate the loci differing due to deletion and addition of bases (Fig. 5, 6). In addition to the places marked with asterisk and dashes, there were some regions which showed differences in the complementary bases in the sequence of the treated mosquitoes. These were the regions where transitions and transversions had taken place. In LD_{20} treated sequences, 205 bases had suffered these mutations in which 68 were transitions, 90transversions , 32 deletions and 15 additions (Table 1). Similarly, in case of LD₄₀ treated sequences a total of 221 bases had suffered such point mutations, out of which 66 were transitions, 90 transversions, 12 deletions and 41 additions (Table 2). In both the cases the rate of transversions was higher than transitions. Traditionally, pesticide induced mutations in the integrity of DNA have been studies in the form of numerical and structural changes in the chromosomes, production of micronuclei, errors in the organization and functioning of spindle apparatus, substitutions by base analogues, DNA adducts and dislodging of phosphodiester bonds. While

Table 1. Different types of aberrations in the ITS-2 sequence of the LD₂₀ treated Culex quinquefasciatus.

Deletions - total 32

1 -12 (12), 45, 56, 64, 195, 196, 197, 198, 211, 212, 422-427 (6), 436, 330 (5).

Additions - total 15

4-5 (1), 75-76 (1), 85-86 (1), 101-102(3), 232-233(1), 307-308 (3), 341-342 (2), 387-388 (3).

Transitions - total 68

A? G (13) 34, 104, 168, 204, 245, 272, 306, 321, 355, 361, 363, 395, 396.

G? A (19) 23, 35, 97, 133, 172, 236, 238, 302, 317, 333, 349, 353, 357, 362, 366, 391, 416, 419, 429.

C? T (11) 17, 18, 27, 87, 111, 146, 147, 159, 175, 176, 292.

T? C (25) 21, 91, 93, 131, 138, 145, 148, 170, 189, 203, 208, 215, 240, 241,255, 290, 293, 297, 326, 358, 380, 400, 407, 408, 410.

Transversions - total 90

A? T (**9**) 29, 39, 101, 139, 153, 155, 264, 267, 303.

T? A (16) 19, 24, 33, 132, 194, 202, 206, 217, 219, 223, 259, 260, 262, 328, 332, 367.

T? G (16) 113, 114, 116, 117, 120, 121, 149,162, 163, 177, 186, 190, 205, 244, 294, 298.

G? T (**6**) 36, 154, 246, 268, 281, 310.

C? G (9) 89, 180, 218, 253, 258, 261, 309, 312, 385.

G? C (16) 32, 41, 92, 122, 136, 142, 157, 167, 171, 231, 239, 252, 273, 350, 351, 381.

A? C (8) 118, 266, 269, 270, 271, 284, 286, 409.

C? A (10) 20, 124, 174, 184, 201, 214, 225, 285, 330, 348.

studying the effect of glyphosate Bolognesi et al., (1997) reported an elevation in the frequency of sister chromaid exchanges in human lymphocytes while Lioi et al., (1998) observed different types of chromosomal aberrations. In the same way Peluso et al., (1998) demonstrated dose dependent formation of DNA adducts in the cells of kidney and liver of mice. Atienzer et al. (1999) while working on Dephnia magna concluded that DNA damage and mutations were the main causes which influenced that the RAPD pattern variations between benzo{a}pyrene exposed and non-exposed individuals, provided sufficient number of cells got affected due to genotoxicity of the agents. In some of the related studies Rank et al., (1993) and Grisolia (2002) found that the commercial formulations of glyphosate were more toxic than its pure form due to various adjuvants present in it. The present results of the limited scope tend to raise a point of caution about the use of glyphosate as exposure to such directly acting pesticides can also prove deleterious to the genome of other living systems including man and animals of economic importance.

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