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HERBICIDE RESISTANCE IN SOYBEAN CELL SUSPENSION CULTURES

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Glyphosate (N-phophonomethyl glycine) is a highly effective broad spectrum herbicide whose target site is 5-Enolpyruvyl shikmate 3-phosphate synthase (EPSPS) an enzyme in the shikmic acid pathway, which synthesizes aromatic aminoacids. Cell suspension cultures of *Glycine max* were subjected to stepwise selection with increasing glyphosate concentrations (0.1 to 55 mMG) for selection of resistant cell lines. The wild type of suspensions showed 50% growth inhibition at 0.13 mMG, which is most sensitive. The cell lines were less adaptive upto 2.0 mMG. In a stepwise selection from 2.0 mMG onwards the cell lines showed greater efficiency and tolerance for selection pressure when compared to other concentrations applied. Selected cell lines showed a 16 fold increase in enzyme activity and 285 fold increase in the I_{50} value than that of non-selected cell lines. The increased EPSPS activity in selected cell lines is due to the herbicide of resistance and amplificaton or over expression of the corresponding gene.

Keywords : Cell line selection Jack and wild type; Cell Suspension Cultures; EPSP Synthase; *Glycine max*; Glyphosate.

Introduction

Globally soybeans are the most inportant source of vegetable oil and are an exreemly important source of vegetable protein. Recent break through in varietal development for the tropics and in small-scale processing make soybeans an extremely promising crop to improve human and livestock nutrition, reduce poverty throgh establishment of appropriate rural processing technologies and for the enhancement of sustainable croping system. It ranks high among the Legume crops in its nutritional value owing to its high protein content as high as 42 percent. The cells of soybean grow readily when placed under *in vitro* culture conditions as suspension cultues. Recent developments and new approaches were developed to produce cultures which are capable of regenerating in to fertile plants either through organogenesis or embryogenesis. These cultlure systems usually consist of relatively large tissue masses, which are ideal as single or small clumps of cells for *in vitro* simple and complicated selection experiments. The tissue culture techniques facilitates the experimental approaches with a large variety of objectives and applications in developmental biology. The theory and goals of mutant and variant selection from tissue is reviewed by several people[1]. Due to presence of a large population of totipotent cells in plants, the tissue culture techniques are considered as an ideal system for genetic manipulation of crop plants.

The use of herbicides to reduce loss in crop yields has become an integral part of modern agricultural practices. There is a continuous demand for new herbicides that are highly effective and safe for human and animal consumption. Most of the herbicides do not distinguish between weeds and crop plants[2]. A new group of herbicides has emerged and this fulfils these need by inhibiting specific aminoacid biosynthesis pathway in plants. Modified plants which became resistant to broad-spectrum herbicides would allow their selective use for crop protection[3,4]. Glyphosate (N-phosphonomethyl glycine) is a highly effective braod spectrum herbicide, a competitive inhibitor with regard to the other substrate, S-3-P in the EPSP reaction. This herbicde lacking specificity between weeds and crops has been used as selective agent for microorganisms and higher plant cells[5].

Material and Methods

Germplasm of *Glycine max* (Cv.Jack) was obtained from Illinois experimental Station at Urbana-Champaign, Illinois. Embryogenic suspension cultures were initiated from

immature pods of field grown Jack cultivar of soybean plants on MSD-40 medium. The selection and growth studies were carried by inoculation of 0.5 - 1.0 g of fresh weight of cell suspension into liquid MX medium, a modified Murashige and Skoog[6] with 1.18μM/L 2,4-D (2,4, dichlrophenoxyacetic acid), the only growth regulator incorporated in to the medium. Glyphosate can be autoclaved in a liquid medium. For determination of I_{50} value, different concentrations of Glyphosate were incorporated into liquid medium and three replicates were maintained for each concentration. The optimum growth period for suspension culture is 14-16 days and then the cultures were maintained under continuous photoperiod with 120 rpm on a rotary shaker. Stepwise selection were made depending on tolerance and growth of cell line against the herbicide. In the final phase of selection, the resistant cell line selected on 35 mMG which was made by several subcultures on the same medium.

EPSPS Enzyme Assay : EPSP synthase enzyme extracts were pepared by powdering the cells in liquid nitrogen and resuspending in 2mg/L 50mM hepes-KOH, 10% glycol (v/v) 2mM DTT. 0.1 mM EDTA, 0.1 mM Ammonium molybdate (VI) tetrahydrate. pH 7 was adjusted with 1% polyvenyle pyrrolidine (w/v). All the reactions were carried out at 0-4°C. The homogenate was centrifuged at 27000g for 10 min and the pellet was discarded. After adding 2ml of saturated ammonium sulphate the extract was held on ice for 210 min then certrifuged as above. The pellet was resuspended in extraction buffer and EPSP synthase activity was measured by determining inorganic phosphate release using the technique of Forlani[7] malachite green dye assay method.

Results and Discussion

The wild type embryogenic cell suspension cultures of *Glycine max* showed 50 percent growth inhibition at 0.13 mMG which is most sensitive. Growth experiments were conducted with different concentrations ranging from 0.1 to 35 mM of Glyphosate. Stepwise selections were made depending upon the I_{50} value and growth plotted with log phase cells of Jack suspension. The intial selection experiments 33.85% of growth inhibition was observed at 0.1 mM of Glyphosate in wild type of cell lines (Table 1). The results of inhibitory level of selection in certain food legumes are in conformity with earlier reports[8]. During step wise selection on Glyphosate medium, cell suspensions were adaptive upto 2.0 mMG. Considerable time was taken for achieving optimum growth in wild type cell suspensions. From 2.0 mMG concentration onwards, cell lines showed greater, efficiency of resistance against the selection pressure. Gradual increase in concentration of glyphosate was applied in intial selection experiments and optimum growth was obtained at higher concentration of glyphosate (2.0 mMG). The tolerance of cell suspension to the herbicide is greater in efficiency in induction of resistance. Increasing fresh weight values and corresponding with high growth index was observed at 2.0 mM of 19 days period and also at 17 mM of 18 days growth period. When the concentration of glyphosate was doubled (35mMG) cells were more efficiently adapted and tolerant cell lines yielded good growth with cell proliferation (Table 2). The enzyme activity in wild type of cell lines showed 149 pkat mg^{-1} and selected cell lines (35mMG) showed 14 folds increased enzyme activity and enhancement of gene copy number were recorded as 2385 pkat mg^{-1} (Table 3). Increased enzyme activity and enhancement of gene copy number were reported in certain Legumes while selection against the Glyphosate. The cell lines selection on 35 mMG the I_{50} values observed at 37 mMG, which has increased 285 folds over the unselected control cell lines. This clearly indicated that the tolerance to herbicide in a adaptive cell line is stable and consistent in selected cell lines on 35 mMG. The time period 259s days for the selection the soybean cell lines for efficient tolerant to glyphosate after 10 subcultures progressively (Table 4). Biotechnological methods were

Table 1. Influence of Glyphosate on Embryogenic cell suspension culture of soybean Jack cultivar.

Type of cell Line	Conc. of Glyphosate in mM	Fresh Weight in g.	Percentage of gropwth
Jack Wild	0	6.5 ± 0.30	100
	0.1	4.3 ± 0.45	66.15
	0.3	4.5 ± 0.21	69.23
	0.5	4.0 ± 0.08	61.53
	1.0	1.25 ± 0.78	19.23
	3.0	1.10 ± 0.01	16.92
	10	0.80 ± 0.06	12.30
	35	0.60 ± 0.02	9.23
Jack 35 mMG	0	4.30 ± 0.50	100
	3	3.60 ± 0.60	83.72
	10	4.0 ± 0.21	93.02
	35	3.1 ± 0.39	72.09
	50	1.80 ± 0.41	41.80
	55	0.90 ± 0.32	20.93

Table 2. Increasing concentration of Herbicide for stepwise selection of soybean CV Jack cell suspension on MX medium.

Sl. No.	Conc. of Glyphosate in mM	No. of days for optimum growth	No. of days for optimum growth	Grpwth index value
1	0	14	6.5	12.0
2	0.1	17	7.5	14.0
3	0.3	15	7.3	13.60
4	0.5	18	6.8	12.60
5	1.0	16	4.9	8.80
6	2.0	19	8.6	16.20
7	4.0	23	7.6	14.2
8	6.0	20	5.1	9.2
9	10	60	2.6	4.2
10	15	23	4.8	8.60
11	17	18	8.7	16.40
12	30	20	5.2	9.40
13	34	16	6.9	12.80
14	35	18	6.4	11.8

Table 3. Enzyme EPSP Synthase activity in Soybean cell suspension cultures.

Name of the cell line	EPSP Enzyme activity in Pkat mg/L	No. of folds increased
Jack 0mMG	149	1
Jack 35 mMG	2385	16

Table 4. Growth inhibition Value of Soybean cell lines on MX medium.

Nme of the cell line	I_{50} Values (Con. in mM)	No. of Days	No. of Subcultures
Jack 0mMG	0.13	12	1
Jack 35 mMG	37	150	9

very effective in crop modification to understand the DNA amplification of EPSP synthase gene which confers the glyphosate resistance[10]. Gene amplification with glyphosate resistance in tobacco cell suspension cultures was reported where the enzyme activity increases several folds[11]. Stepwise increase in the concentration of herbicide (Glyphosate) resulted in the over production of the target enzyme, EPSP synthase due to gene amplification. Amplification of EPSP synthase gene and increases enzyme activity in several folds are well documented in several species of Alfalfa, Nicotine and carrot[12]. Stepwise selection of *Daucus carota*(L.) cells against Chlrosulfuran showed over production of fragment of DNA which increased in 10 copies[13]. The increased enzyme activity is due to over expression of EPSP synthase gene by production of more mRNA. Stepwise selection for glyphosate resistance in *Cordialis sempervirens* suspension cultures produced high EPSPS activity due to post transcriptional changes associated with mRNA stability[14].

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