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SOYBEAN CELL LINE SELECTION FOR AMPLIFICATION OF EPSP SYNTHASE GENE

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Plant biotechnology offers a greater stimulus for crop modifications by using r-DNA technology, genetic transformations and gene amplification for the herbicide resistance. Glyphosate (N-Phosphonomethyl glycine) is a broad spectrum herbicide and its target enzyme is 5-Enlophyruvyl shikmate 3 phosphate synthase (EPSPS) which play key role in synthesizing the aromatic amino acids in the shikimic acid path way. Embryogenic cell suspension cultures of *Glycine max* were subjected to stepwise selection with increasing Glyphosate concentrations (0.01 to 50 mMG) for induction of tolerance and gene amplification studies. The wild type of suspension cultures showed 50% growth inhibition at 0.06 mMG, which is considered to be highly sensitive. The cell lines were less adoptive in terms of herbicide resistance upto concentration 2.0 mMG. In a stepwise selection from 2.0 mMG onward the cell lines showed greater efficiency and tolerance for selection pressure when compared to other concentrations W-82/35G cell lines showed 14 fold increase in the enzyme activity and 650 folds increase in the I_{50} value than that of unselected wild type of embryogenic soybean cell lines. Enhanced EPSPS enzyme activity is due to over expression of corresponding target gene or amplification of DNA.

Keywords : Cell suspension cultures; Gene amplification; Glyphosate; Soybean.

Introduction

Recent advances in genetics and plant biotechnology have made tremendous progress in crop improvement programmes by manipulation of genetic information at cellular and molecular level. The cell line selection for crop modifications has made tremendous progress in plant biotechnology by manipulation of genetic material at cellular and molecular level. Soybean is the most important legume crop in terms of total production and international trade. Presently it ranks high among the legume crops in its nutritional value owing to its high protein content as high as 42 percent. Soybean cells grow readily when placed under culture conditions and have been studied as undifferentiated friable callus or suspension cultures. In the last decade new approaches were developed to produce cultures capable of regeneration in fertile plants via either organogenesis or embryogenesis. These culture 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. Plant tissue culture comprises the *In Vitro* cultures of various kinds of explants ranging from cells to tissues and organs. It facilitates experimental approaches with a large variety of objectives in developmental biology. The theory and goals of mutant (or) variant selection from tissue is reviewed by several people. Due to the presence of a large population of totipotent cells under aseptic conditions, the plant tissue culture system is considered as ideal for genetic manipulations of crop plants.

The use of herbicide to reduce loss in crop yield has become an integral part of modern agricultural practices. There is a continuous demand for new herbicide that are highly effective and safe for both animals and the environments. Most of the herbicides do not distinguish between weeds and crop plants[1]. A new group of herbicides has emerged and this fulfills these needs by inhibiting specific amino acid biosynthesis pathway in plant. Modifying plants to become resistant to broad-spectrum herbicides would allow their selective use for crop protection[2,3]. Glyphosate (N-phosphonomethyl glycine) is highly effective broad-spectrum herbicides, a competitive inhibitor with respect to PEP and an uncompetitive inhibitor with regard to the other substrate, S-3-P, in the EPSPS reaction. This Glyphosate is lacking specificity between weeds and crops has been used as a selective agents for micro-organisms and higher plants cells[4,5].

This paper reports the study of selection for amplification of EPSPS gene product in soybean W-82 cell lines as suspension cultures on modified MS liquid medium.

Materials and Methods

Germplasm of *Glycine max* (CV Jack) was obtained from Illinois Experimental Station at Urbana-Champaign, Illinois.

Callus cultures were initiated and grown from hypocotyl explants on B5[6] medium. The selection and growth studies were carried by inoculation of 0.5-1 of fresh weight of cell suspension into liquid MX medium, modified from Murashige and Skoog[7] with 0.4 mg/L (1.18 µM/L) 2,4-D (Dichlorphenoxyacetic acid), the only growth hormone in 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 the cultures were maintained under continuous photoperiod with 120 rpm on a rotary shaker. Depending on the tolerance of the cell line and growth response and further step wise selection were made after 15-30 days to select for highest tolerance level on Glyphosate at 35 mMG. Finally resistance cell lines W-82 at 35 mMG were selected and several sub-cultures were also made on the same concentration.

Measurement of EPSP-synthase Activity: EPSP-synthase extracts were prepared by powdering cells in liquid nitrogen and re-suspending in 2 ml g^{-1} 50 mm Hepes – KOH, 10% glycerol (v/v), 2 mM DTT, 0.1 mM EDTA, 0.01 mM $(NH_4)_6Mo_7O_{24}$ $4H_2O$, pH 7.0 with 1% polyvinylpolypyrrolidone (w/v). All subsequent operations were carried out at 0-4 ^{0}C. The homogenate was centrifuged at 27,000g for 10 min and the pellet discarded. After adding 2 ml of saturated ammonium sulfate per ml supernatant, the extract was held on ice for 10 min. then centrifuged as above. The pellet was re-suspended in the extraction buffer, 1 ml g^{-1} cells. EPSP-synthase activity was measured by determining inorganic phosphate release using with minor modifications a malachite green dye assay described by Forlani *et al.*[8]. All assays were performed in the presence of 0.5 mM $(NH_4)_6Mo_7O_{24}$ $4H_2O$ to inhibit phosphatase activity. EPSP0-synthase extracts were diluted with extraction buffer or added directly to the assay solution and incubated from 1-20 min at 30 ^{0}C and compared to 0 min. values. Controls contained 10 mM glyphosate or only one substrate, S-3-P or PEP. Release of inorganic added was generally less than 10% of the rate with both substrates present. The molar absorption coefficient of the phosphomolybdate complex was determined to be 79,000 M^{-1} cm^{-1}.

Results and Discussion

The wild type cell suspension cultures of *Glycine max* showed 50 percent growth inhibition at 0.06 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 W-82 cell suspension. The initial selection experiment with wild type cell line was made with 0.1 mM concentration of Glyphosate 40% of growth inhibition (Table-1). The results of inhibitory level of selection in certain food legumes are in conformity with reports of Ramulu[9].

Table 1. Growth of cell suspension culture of soybean W-82 on MX medium with various concentration of Glyphosate.

Type of Cell line	Conc. of glyphosate in mM	Fresh Weight in Grams	Percentage of Inhibition
Wild	0	5.5±0.30	100
	0.1	2.2±0.45	40
	0.3	1.5±0.21	26.2
	0.5	1.0±0.08	19.3
	1	0.8±0.78	14
	3	0.8±0.01	14
	10	0.8±0.06	7.7
	35	0.3±0.02	4.7
W-82 mMG	0	9.3±0.50	100
	3	9.2±0.60	98
	10	7.9±0.21	87
	35	6.8±0.39	73
	50	1.6±0.41	17.2

Table 2. EPSP Synthase Enzyme activity in soybean W-82 cell lines.

Name of the cell line	EPSP Enzyme activity in Pka mg/L	No. folds increased
Soybean (W-82) 0mMG	169	1
Soybean (W-82) 35 mMG	2366	14

During stepwise selection on Glyphosate medium, embryogenic cell suspension was adoptive up to 2.0 m MG. Considerable time has taken for achieving optimum growth as that of wild type cell lines. From 2.0 mMG concentration onwards, cell lines showed greater efficiency of resistance against the selection pressure. Gradual increase in concentration of glyphosate is applied in initial selection experiments and optimum growth was obtained at high concentration of glyhosate (2.0 mMG). The tolerance of W-82 cell line to herbicides is more efficient. Increasing fresh weight values and also corresponding high growth index value was observed at 16 mMG period of 32 days. When the concentration of glyphosate was doubled (35 mMG), cells were more efficiently adapted and tolerant cell lines yield good growth with cell proliferation (Table 3).

The enzyme activity in wild type of cell lines showed 169 pka mol^{-1} and selected cell lines (35 mMG) showed 14-folds increased enzyme activity and was recorded as 2366 pka mol^{-1} (Table 2). Increased enzyme activity and enhancement of gene copy number were reported in certain legumes while selecting against the Glyphosate[3]. Cell lines selection on (35 mMG) I_{50} value at 39 mMG, which has increased 650-folds over the unselected

control cell lines. This clearly indicates that the tolerance to herbicide in an adaptive cell line is stable and consistent in selected cell lines on (35 mMG). The time period 297 days required for the selection of the soybean cell lines for efficient tolerance to glyphosate after 10 subcultures progressively (Table 4). Biotechnological methods were very effective in crop modification to understand the DNA amplification of EPSP synthase gene which confers the glyphosate resistance in tobacco cell suspension cultures was reported where the enzyme activity increases several folds[10].

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 increased enzyme activity in several folds are well documented in several species of Alfalfa, Nicotina and Carrot. Stepwise selection of *Daucus carota* (L) cells against chlorosufuran showed over production of fragment of DNA, which increased in 10 copies[11]. The increased enzyme activity is due to over expression of ESPS synthase gene by production of more mRNA. Stepwise selection for glyphosate resistance in *Cordialis sempervirens* suspension cultures produced high EPSP activity due to post-transcriptional changes associated with mRNA stability[12].

Table 3. Step wise selection of soybean CV W-82 cell suspension on MX medium with different concentration of herbicide.

Sl. No.	Conc. of Glyphosate mM	No. of days for optimum growth	Suspension F.W. (gm)	Growth index value
1	0	16	5.5	111
2	0.1	19	13.0	25
3	0.3	16	3.5	6
4	0.5	20	9.3	17.6
5	1.0	16	2.9	4.8
6	2.0	19	12.7	24.4
7	4.0	41	7.6	14.2
8	6.0	38	2.1	3.2
9	10	60	2.6	4.2
10	16	32	13.3	25.6
11	35	36	11.5	22

Table 4. Growth inhibition value of soybean cell lines.

Name of the cell line	150 values (Con. in mM)	No. of days	No. of subcultures
Soybean (W-82) 0 mMG	0.06	15	1
Soybean (W-82) 35 mMG	39	297	10

The possible explanations for this may be increase in target due to either in gene expression or gene amplification. The over production of target enzyme EPSP synthase in soybean embryogenic cell lines may be due to the amplification of gene (DNA) encoding corresponding EPSP synthase[13].

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