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Selection of Acifluorfen-tolerant Eastern Black Nightshade (*Solanum ptycanthum* Dun) and the Expression of This Tolerance in Regenerated Plants and Their Progeny

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제초제 Acifluorfen 저항성 세포주 선발 및 분화된 식물체와 그 후대에서의 저항성 발현

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Acifluorfen-tolerant cell lines of *S. ptycanthum* were isolated by stepwise selection using suspension culture. Growth of unselected line was completely inhibited at 0.5 μM, while some selected lines grew at 8 μM acifluorfen. After subculturing on acifluorfen-free medium for 4 passages, six of the eleven cell lines screened and maintained their tolerance to 2 μM acifluorfen. The regeneration capacity of selected cell lines in *Solanum ptycanthum* differed depending on the cell line. The acifluorfen tolerance of the somaclones regenerated from acifluorfen-tolerant cell lines differed depending on the somaclone. When plants were treated with 16 mM acifluorfen, unselected control plants had over 75% phytotoxicity. Many selected cell lines had less phytotoxicity than the seed-grown control plants. Tolerance to acifluorfen was inherited to the self-pollinated progenies. The inheritance patterns differed depending on the clone. Acifluorfen tolerance was inherited as a semidominant trait. Other segregation patterns were also observed. Acifluorfen tolerance was recessive and acifluorfen sensitivity was dominant.

Key words: herbicide tolerance, in vitro selection

Plant cell culture techniques have been used to develop the herbicide-resistance and transfer its trait to agronomic important crops (Feulkner, 1982). In vitro techniques offer a number of advantages for studying the potential of herbicide resistance and developing approaches to prevent herbicide resistant weeds (Camper and McDonald, 1989). Cell cultures provide both uniform growth environment and uniform exposure to the herbicide. Tissue cultures allow large populations of cells to be exposed to a herbicide (Chaleff, 1983).

Many herbicide-resistant cell lines have been selected to a wide range of herbicide classes using in vitro culture systems. For example, cell lines resistant to glyphosate (Dyer et al. 1988), paraquat (Thomas and Pratt, 1982), sulfonylurea (Saxena and King, 1987), amitrole (Singer and McDaniel, 1984), and picloram (Chaleff, 1981) have been isolated. In many studies, both resistant and non-resistant plants occurred after regeneration from selected cell lines (Miller and Hughes, 1980; Singer and MacDaniel, 1985). Singer and MacDaniel (1985) reported that tolerance in plants regenerated from six

amitrole-tolerant tobacco cell lines was expressed in only some plants from each cell line.

Differential tolerance to acifluorfen has been identified in *Lycopersicon* (Masiunas, 1989). If this tolerance could be enhanced through use of in vitro techniques, acifluorfen use could be expanded to tomatoes and other Solanaceous crops. Herbicide resistance for minor crops such as industrial crops is important because of the high liability and increasing costs of research and development *Solanum ptycanthum* used in this study is one of the medicinal plants containing solasodine in leaf and fruit. This solasodine is useful in isolating steroidal alkaloid (Bhatt et al., 1983). This study was conducted to select the resistant cell lines to acifluorfen *in vitro* and to characterize the acifluorfen resistance in somaclones and their progeny level.

MATERIALS AND METHODS

In Vitro Culture and Selection

The cotyledons of *Solanum ptycanthum* were excised and cultured on Murashige and Skoog medium with 2 mg/L 2,4-D and 1 mg/L BA to initiate callus. Friable callus was subcultured on liquid Murashige and Skoog salts with the B5 vitamins and 2,4-D 1 mg/L until a fine suspension of cells was obtained. Suspension cultures were maintained in a orbit shaker at 140 rpm with 24 h light. In order to obtain acifluorfen-tolerant cell lines, suspension cultured cells were transferred to filter paper on petridishes with 2 μM acifluorfen. For subculture, the filter paper with the suspension cultured cells was transferred to a petri dishes with fresh selective medium every two weeks. After subcultured for 3 months, growing colonies were considered tolerant cell lines and each colony was assumed to be a clone. One half of the growing colonies was transferred to proliferation medium with and without acifluorfen to determine the level of tolerance.

In Vitro Tolerance and Stability

The stability of the tolerance was determined by growing the colonies for 4 growth cycles (over 4 months) without acifluorfen and then transferred them to solid medium with acifluorfen. Each concentrations had a minimum of three replicates and each experiment was repeated two times.

Regeneration from Acifluorfen-tolerant Cell Lines

Calli proliferated from tolerant lines were transferred to MS B5 medium containing 0.2 mg/L IAA and 2 mg/L BA for regeneration. Two types of regeneration media, without acifluorfen or with acifluorfen (0.01 to 5 μM) were used.

Acifluorfen Tolerance of Whole Plants

Shoots regenerated from tolerant cell lines of *Solanum ptycanthum* were rooted in vitro and transferred to plastic pots and transferred to a greenhouse. Five healthy somaclones from each regenerated cell line were randomly chosen. Somaclones were cutted and rooted. Rooted cutting plants were allowed to grow for 5 to 6 weeks. 16 mM acifluorfen was applied using a compressed air sprayer with a mobile 8002 flat fan nozzle tip at 50 MPH. Plants were returned to the greenhouse and arranged in a completely random design with 3 replicates and 3 untreated controls for each somaclone from each cell line. The experiment was repeated. Ten days after herbicide treatment, plants were harvested and fresh weight and visual injury were determined. Plants were dried in a dryingoven for 3 days and dry weights determined.

Acifluorfen Tolerance of R_1 Progeny

In order to investigate the heritability of acifluorfen-tolerant traits, somaclones regenerated from tolerant *Solanum ptycanthum* cell lines were selfed. From fertile R_0 plants, berries were collected to obtain seeds. R_1 seeds and seeds of noncultured, wild type *Solanum ptycanthum* were planted in plastic pots (700 cm^3). sixteen mM acifluorfen sprayed and plants were harvested as above described.

RESULTS AND DISCUSSION

In Vitro Tolerance and Stability

Acifluorfen-tolerant cell lines of *Solanum ptycanthum* were selected (Table 1). Growth of the unselected and selected cell lines differed on medium containing acifluorfen. The unselected line of *S. ptycanthum* was completely inhibited at 0.5 μM acifluorfen. Growth of the selected cell lines varied, ranging from 0.23 to 0.63 g per calli at 8 μM, depending on the line. Three selected cell lines (EBN2, EBN8, and EBN21) of *S. ptycanthum* grew better at 0.5 μM than on medium containing no acifluorfen. Cell lines of tomato selected for salt tolerance have been reported to grow better at 100 and 200

Table 1. Fresh weights for acifluorfen-tolerant and unselected *Solanum ptycanthum* cell lines after 30 day culture on media with different acifiuorfen concentrations.

Cell Lines	Acifluorfen Concentration (μM) 0	0.5	2	8
	– g/calli –	..... % fresh weight control		
EBN[a]	0.75 ± 0.04[b]	18.7 ± 1.3[c]	18.7 ± 4.7	16.0 ± 5.4
EBN-2	0.85 ± 0.02	107.7 ± 1.8	64.7 ± 1.2	31.2 ± 1.8
EBN-3	0.90 ± 0.01	87.8 ± 7.8	86.7 ± 5.6	32.8 ± 3.9
EBN-4	0.61 ± 0.03	92.4 ± 5.3	65.5 ± 11.5	45.9 ± 0.2
EBN-5	0.85 ± 0.01	92.4 ± 5.3	68.8 ± 1.8	74.1 ± 7.1
EBN-6	0.95 ± 0.01	89.5 ±3.2	66.3 ± 2.1	24.7 ± 0.5
EBN-7	0.81 ± 0.01	87.0 ± 3.1	67.9 ± 4.9	37.7 ± 1.9
EBN-8	0.84 ± 0.14	114.3 ± 22.6	75.0 ± 45.2	57.1 ± 23.8
EBN-9	0.71 ± 0.06	59.2 ± 32.4	69.2 ± 30.3	32.4 ± 12.7
EBN-10	0.77 ± 0.18	42.9 ± 22.1	70.1 ± 15.6	36.4 ± 3.9
EBN-11	1.03 ± 0.21	61.2 ± 19.4	29.1 ± 9.7	24.3 ± 4.9
EBN-12	0.69 ± 0.03	81.9 ± 18.1	56.5 ± 10.2	37.0 ± 9.4
EBN-13	0.95 ± 0.01	83.7 ± 4.7	32.6 ± 2.1	25.8 ± 1.6
EBN-16	0.70 ± 0.02	52.9 ± 7.1	28.6 ± 3.3	35.0 ± 6.4
EBN-17	0.46 ± 0.10	97.8 ± 0.1	63.00 ± 2.2	41.3 ± 4.3
EBN-18	0.76 ± 0.04	77.6 ± 4.0	50.0 ± 10.5	30.9 ± 0.7
EBN-21	0.72 ± 0.10	125 ± 20.8	67.4 ± 3.5	33.3 ± 0.01
EBN-25	1.10 ± 0.28	43.2 ± 10.4	61.4 ± 29.5	27.7 ± 9.6
EBN-26	1.18 ± 3.8	48.7 ± 3.8	93.7 ± 2.1	42.8 ± 0.4
EBN-27	0.72 ± 0.04	83.3 ± 7.0	67.4 ± 13.2	41.0 ± 0.7

[a]Unselected cell line.

[b]Mean of the callus fresh Weight (g) ± SE.

[c]Percent control fresh weight (%) of callud ± SE.

than at 0 μM sodium chloride (Hassan and Wilkins, 1988).

Acifluorfen-tolerant lines also differed in whether they were still tolerant after subculturing on medium without acifluorfen for 4 passages (referred to as stability). Eleven cell lines of *S. ptycanthum* were tested for their stability. Only six callus lines maintained their tolerance to 2 μM acifluorfen ranged from 20.8 to 70.8 depending on the cell line. In general, when the selected cell lines were subcultured on acifluorfen-free media and then tested on acifluorfen, growth was lower than when the lines were continually grown on acifluorfen and tested

Many studies have reported varying stability of herbicide tolerance in selected lines (Chaleff and Parson, 1978; Camper and McDonald, 1989). Tolerance was lost because the cell lines were a mixed population of sensitive and tolerant cells. Plant cells grow in aggregates. Wild-type cells could be protected from a herbicide by nearby tolerant cells. Then in the absence of selection pressure, wild type cells could outgrow variant cells (Meredith and Carlson, 1982).

Regeneration from Acifluorfen-tolerant Cell Lines

The regeneration capacity of selected cell lines in *S. ptycanthum* differed depending on the cell line. The regeneration rate varied, ranging from 0.3 to 37.3 shoots per calli. Six selected cell lines had better shoot regeneration and eleven cell lines had less regeneration than the unselected line. Selected cell lines were more effective than the unselected line in regeneration on medium containing acifluorfen (Figure 1). The unselected line of *S. ptycanthum* did not regenerate. In general, increased levels of herbicide reduced shoot regeneration. The regeneration capacity of selected cell lines was greater than that of unselected line on medium with acifluorfen. The unselected cell line did not regenerate on media containing acifluorfen above 1 μM, while some selected lines regenerated in the presence of up to 5 μM acifluorfen.

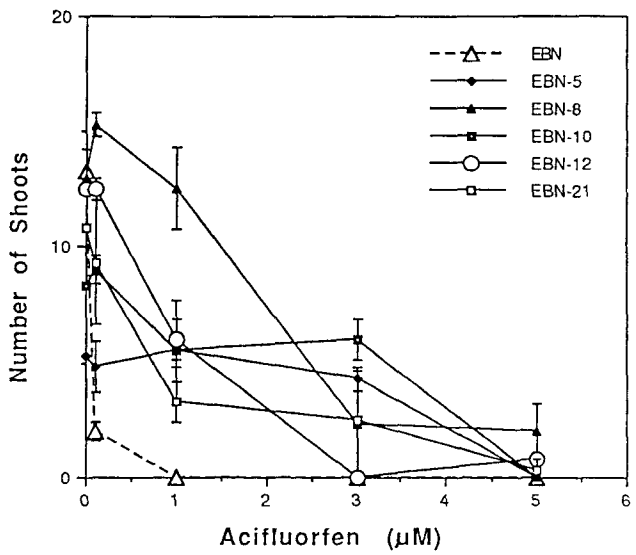

Figure 1. Shoot regeneration on acifluorfen media of *Solanum ptycanthum* cell lines, either unselected (EBN) or selected for acifluorfen-tolerance (EBN-5, EBN-8, EBN-10, EBN-12, and EBN-21).

Acifluorfen Tolerance in Whole Plants

Acifluorfen tolerance of whole plants regenerated from acifluorfen tolerant *S. ptycanthum* cell lines differed depending

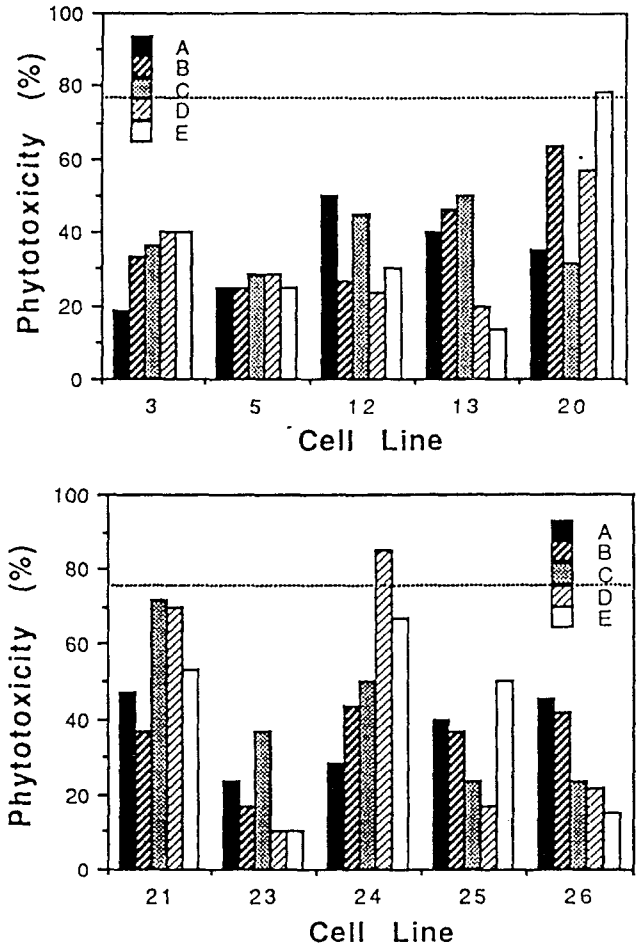

Figure 2. Tolerance of *Solanum ptycanthum* somaclones to a spray application of 16 mM acifluorfen. Each bar (A, B, C, D, and E) represents a somaclone regenerated from a selected callus line. The dotted lines represent the phytotoxicity (%) of the seed grown-Plants.

on the cell lines and somaclones within each cell line (Figures 2). When 16 mM acifluorfen were sprayed on the whole plants, the unselected cell line showed over 75% phytotoxicity after 10 days. Selected cell lines had less phytotoxicity than unselected cell line. Even though somaclones were regenerated in the same cell line, tolerance differed depending on the somaclone. Somaclone A in EBN 3 line, E in EBN 13 line, and D or E in EBN 23 line had the least phytotoxicity, indicating their high herbicide tolerance. Soamclone E in EBN 20 line and D in EBN 24 cell line had the highest phytotoxicity and these somaclones in each cell line showed less tolerance than the unselected cell line having approximately over 75% phytotoxicity.

Other researchers also reported differences in tolerance between somaclones (Miller and Hughes, 1980: Singer and McDaniel, 1984). Somaclones differing in tolerance could result from chimera calli, comprising of both resistant and sensitive cells. Without selection pressure, sensitive cells could outgrow resistant variant cells, resulting somaclones sensitive to the herbicide (Singer and McDaniel, 1984: Hughes, 1983). Continued subculture on selective medium may eliminate the sensitive cells (Hughes, 1983: Wersuhn et al. 1987). The regeneration in the presence of the herbicide reduce the chances of losing the resistant trait if sensitive cells outgrow the resistant cells during plant development (Hughes, 1983).

Differences in gene expression can also occur. Miller and Hughes (1980) reported that calli derived from both resistant and sensitive regenerated plants were herbicide resistant in most cases, indicating that the resistance trait was transmitted but not expressed in the whole plant. The genes for herbicide resistance are differently activated, i.e., there may be genes that are expressed only in the callus state but not in differentiated plant tissues. The resistance trait may be expressed in plant tissues but the metabolic change conferring herbicide resistance at callus level may not be sufficient to protect the plants (Hughes, 1983: Meredith and Carlson, 1982). Cultured cells and whole plants represent different developmental states with different patterns of gene expression (Meredith and Carlson, 1982).

Acifluorfen Tolerance in R_1 Progeny Plants

Fertile tolerant plants regenerated from tolerant cell lines were selfed and the collected seeds were planted into the pot. When plants were grown, 16 mM acifluorfen sprayed on R_1 progeny plants. Ten day after treatment, fresh weights were measured. EBN-12E line had a 1:2:1 segregation of tolerant, intermediate tolerant, and sensitive individuals (Table 2). This cell line also had the normal phenotype similar to unselected control plant in leaf shape and plant type. These results indicate that acifluorfen tolerance was inherited as a semidominant trait and the original regenerated plant was a heterozygote. In other progeny plants from five lines including EBN-13B, EBN-13E, EBN-26B, EBN-26D, and EBN-26E, a 3:1 segeregation of sensitive and intermediate tolerant individuals was observed, indicating that tolerance was inherited as recessive trait and acifluorfen sensitivity was dominant trait. In two progeny plants from EBN-20B and EBN-3B, a 15:1 segreagation of sensitive and intermediate tolerant individuals was observed. This ratio fits the segregation expected if there was independent assortment of two gene pairs, with intermediate tolerance a double recessive.

Table 2. Segregation of acifluorfen-tolerance in selfed progenies of acifluorfen-tolerant *Solanum ptycanthum* cell lines.

cell Lines	Segregation						
	Observed			Expected			X^2
	Tol.[a]	Inter.	Sens.	Toi.	Inter.	Sens	
EBN-12E	8	20	7	8.8	17.5	8.8	0.77[b]
EBN-3B		4	31		2.2	32.8	1.57
EBN-13A		0	23		0	23.0	-
EBN-13B		6	37		10.8	32.3	2.37
EBN-20B		4	54		3.6	54.4	0.16
EBN-26B		9	28		9.3	27.8	0.008
EBN-26D		9	26		8.8	26.3	0.008
EBN-26E		3	24		6.8	20.3	2.78

[a]Tol.=Tolerance, Inter.=Intermediate tolerance, Sens.=Sensitive.Only a shoot tips of tolerant plsnts were injured. Intermediate tolerant plants had injury to both the shoot tips and lower leaves. Susceptible plants were completely killed by the herbicide.

[b]Segeregation ratios do not differ significanty from expected values ($p>0.05$).

EBN-13A cell line produced all twenty-three sensitive individuals, and there were no tolerant or intermediate tolerant plants.

In many studies, herbicide-tolerant mutants can be selected by culturing cells in the presence of a herbicide and was transmitted to progeny as a dominant or semidominant nuclear allele (Chaleff and Ray, 1984: Thomas and Pratt, 1982: Chaleff and Parson, 1978). Chaleff (1980) reported that self-fertilization of a tobacco plant regenerated from picloram-resistant cell line 85 produced 114 resistant, 252 intermediate, and 137 sensitive progeny, indicating that tolerant for picloram is conferred by a single semidominant nuclear mutation. Several mutants resistant to the herbicides chlorsulfuron and sulfometuronmethyl were isolated from cultured cells of *Nicotiana tabacum*. Resistance was inherited as a single dominant or semidominant mutation (Chaleff and Ray, 1984). Amitrole tolerant plant was a dominant trait since it was expressed in heterozygotes. However, simple mendalian inheritance patterns in crosses were not observed (Singer and McDaniel, 1984). Non-mendelian segregation ratios, and the loss of tolerance in Progeny generations may be explained by aneuploidy and irregular chromosome transmission through meiosis (Singer and McDaniel, 1984).

적 요

야생약초이며 미국에 자생하는 까마중의 일종인 *Solanum ptycanthum*을 현탁배양에 의하여 제초제 acifluorfen 저항성인 세포주를 선발하였으며 감수성인 세포주는 0.5 μM 제초제에서 callus 생장이 멈추었으나 저항성 세포주는 10 μM이 첨가된 배지에서도 callus 생장을 계속하였다. 저항성 세포주를 제초제가 첨가 안된 배지에서 4개월 이상 계대배양 후 저항성 정도를 조사하였으며, 저항성 정도는 세포주에 따라 상이하였다. 저항성 세포주로부터 식물체 분화 능력도 세포주에 따라 상이하였으며 저항성인 세포주는 제초제가 첨가된 배지에서도 식물체가 분화되었다. 선발된 세포주로부터 분화된 식물체를 온실에서 5-6주 키운 후 16 mM acifluorfen을 살포한 후 식물체와 자식후대에서의 제초제 저항성을 조사하였다. 내성인 세포주로부터 분화된 식물체가 감수성인 선발이 안된 종자로부터 얻은 식물체보다 저항성을 나타내었다. 저항성 정도는 cell line과 somaclone에 따라 차이를 보였다. 자식후 자식후대에서 저항성분리비를 조사하였는데 EBN-12E 세포주는 저항성, 중간 저항성, 감수성이 1:2:1로 분리가 일어나서 저항성이 불완전우성을 보였다. EBN-13B는 감수성과 저항성이 3:1로 분리가 일어나 저항성이 열성이고 감수성이 우성인 것으로 나타났다.

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