Korean J. Medicinal Crop Sci. 10(4) : 263-268(2002)

# *In vitro* Selection of Acifluorfen-tolerant *Solanum ptycanthum* and Phenotypic Variation in Regenerated Plants

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**ABSTRACT** : Aciflùorfen-tolerant callus lines of ***Solanum ptycanthum*** **were isolated by stepwise selection. Growth of the unselected line was completely inhibited at 0.5 uM. while some selected lines grew at 8 uM acifluorfen. Twenty-two of twenty-five acifluorfen-tolerant callus lines regenerated shoots. Many of the regenerated somaclones were variants, differing in leaf shape, leaf color, number of flower parts, flower color, and fertility. The acifluorfen tolerant *S. ptycanthum* callus lines differed.**

***Key words*** : Acifluorfen-tolerant, Suspension culture , Phenotype variation

## INTRODUCTION

Plant cell culture techniques have been used to develop herbicide-resistance and transfer it to agronomicly important crops (Feulkner, 1982). In vitro culture provides both a uniform growth environment and uniform exposure to the herbicide. Tissue cultures allow large populations of cells to be exposed to a herbicide (Chaleff, 1983). They provide a source of genetic variation and may allow the expression of different mechanisms of resistance (Hughes, 1983 ; Singer and McDaniel, 1984). Herbicide-resistant cell lines have been selected to a wide range of herbicide classes using in vitro culture systems. Cell lines resistant to glyphosate (Dyer et al., 1988; Smith et al., 1986). paraquat (Thomas and Pratt, 1982). sulfonylureas (Saxena and king, 1987 ; Chaleff and Bascomb, 1987; Swanson et al. 1988). amitrole (Singer and McDaniel, 1984). picloram (Chaleff, 1981 ; Chaleff and Parson, 1978), and phpsphinothricin (Donn et al., 1984) have been isolated. In many studies, both resistant and non-resistant plants have occurred after regeneration from selected cell lines (Chaleff and Parson, 1978; Miller and Hughes, 198 ; Singer and McDaniel, 1984 and 1985). For example, Singer and McDaniel (1984) reported that twenty-four plants were regenerated from six amitrole-tolerant tobacco cell lines and tolerance was expressed in only five plants from three cell lines U16, X2, and Y2.

The development of herbicide-tolerant plants has the potential to extend the number of herbicides available for use in crops. plants differing in herbicide tolerance can be used to elucidate mechanisms of action and means of selectivity which are not fully understood for many herbicides (Ashton and Crafts, 1981; Hughes, 1983). Herbicide-resistant plants can reduce the amounts of herbicides released into the environment, by allowing use of more effective and environmentally acceptable products (Gasser and Fraley, 1989).

*In vitro* techniques can be used to study the potential of weed resistance to herbicides and develop approaches to prevent herbicide resistance (Camper and McDonald, 1989). Developing herbicide resistance in minor crops such as vegetable is important because

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Received 30 August, 2002 / Accepted 30 October, 2002

of the high liability if these crops are injured and the increasing costs of developing new herbicides (Souza Machado et al., 1982).

Differential tolerance to acifluorfen has been identified in *Lycopersion* (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. Furthermore, Lebaron and McFarland (1990) predicted that there is a high potential for weeds developing resistance to diphenylether herbicides. In vitro system for studying tolerance in *Solanaceous* species may prevent weed resistance occurring in the field.

The objectives of this study were to select acifluorfen tolerant callus lines of *Solanum ptycanthum* and to regenerate resistant plants. This study also determined phenotypic variation regenerated somaclones.

## MATERIALS AND METHODS

### *In vitro* Culture and Selection

Callus, originally established from cotyledonary explants, of *Solanum ptycanthum* Dun. was subcultured on liquid Murashige and Skoog salts (Murashige and Skoog, 1962) with B5 vitamins (Gamborg et al., 1968) and 2,4-D (2,4-dichlorophenoxyacetic acid). Suspension culture were maintained on a orbital shaker at 140 rpm with 24 h light. The selection of increasing acifluorfen concentrations (0.01 to 2uM) every 7-10 days through decanting half of the medium and replacing it with fresh selection medium.

To obtain acifluorfen-tolerant callus, suspension cultured cells were transferred to filter paper placed on a solid MG medium containing 2uM acifluorfen. The filter paper with colonies was transferred to fresh selection medium every two weeks. After subculturing for 3 months, growing colonies were assumed to be clones and potentially tolerant to acifluorfen. The colonies were subdivided with part of a colony transferred to proliferation medium with acifluorfen and another part transferred to medium without the herbicide.

### *In vitro* tolerance and stability

After 3 months, tolerance was determined by transferring callus-lines to petridishes (100 ×15mm) containing solid MG medium with various concentrations of acifluorfen (0.01 to 10uM). The stability of the tolerance was determined by growing the colonis for 4 growth cycles (over 4 months) without acifluorfen and then transferred them to solid medium with acifluorfen and then transferred them to solid medium with acifluorfen (0.01 to 10uM). Each concentration had a minimum of three replicates and each experiment was repeated. Fresh weight was recorded after 30 days.

### Reqeneration and Phenotypic Variantion From Acifluorfen-Tolerant Cell Lines.

Callus proliferated from tolerant lines was transferred to MG medium containing IAA (0.2 mg/$l$) and BA (2 mg/$l$) for regeneration. Two types of regeneration media, without acifluorfen or with acifluorfen (0.01 to 5 uM), were used. The number of shoots and shoot height were recorded after culturing for 30 days.

Shoots regenerated from tolerant callus lines of *Solanum ptycanthum* were rooted in vitro on MS medium with 0.5 mg/$l$ IAA. The rooted plantlets were transferred to plastic pots (700 $cm^3$) containing a greenhouse soil mixture (1 : 1 : 1 v/v/v loam soil : peat : perlite). The plantlets were acclimated in a growth room for 2 weeks before transferring to a greenhouse. The plantlets were grown to flowering. Phenotypic observations including shoot height, leaf color, leaf shape, flower color, petal number, and fertility of somaclones were made at flowering.

## RESULTS AND DISCUSSIONS

### *In Vitro* Tolerance and Stability

Twenty-five acifluorfen-tolerant callus lines of *Solanum ptycanthum* were selected. Growth of the unselected and selected callus lines differed on medium containing acifluorfen. The unselected lines of *S. ptycanthum* was completely inhibited at 0.5 uM acifluorfen. Callus lines of tomato selected for salt tolerance have been reported to grow better at 100 and 200 than at 0uM 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. Eleven callus lines of *S. ptycanthum* were tested for their stability. Six callus lines maintained their tolerance to 2uM acifluorfen (Table 1). The percentage control fresh weight at 2uM acifluorfen ranged from 20.8 to 70.8 depending on the callus line. In general, when the selected callus 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. But their growth was still greater than the unselected line.

Many studies have reported selected cell lines that vary in the stability of their herbicide tolerance (Chaleff and Parson, 1978; Thomas and Pratt, 1982; Singer and McDaniel, 1984 and 1985; Camper and McDonald, 1989). Singer and McDaniel (1985) reported that 18 of 51 glyphosate-tolerant tobacco cell lines were still tolerant even when cultured on selective medium for 3 years. They hypothesized that tolerance was lost because the cell lines were a mixed population of sensitive and tolerant cells. Meredith and Carlson (1982) reported that even if the population originally contained only tolerant cells, it could become mixed as a result of reversions (back mutation). Also, 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 (Singer and McDaniel, 1984; Hassan and Wilkins, 1988).

Table 1. Fresh weights of acifluorfen-tolerant and unselected *S. ptycanthum* cell lines grown on acifluorfen-free medium for four growth cycles.

| Cell lines | Acifluorfen Concentration (uM) | | | |
|---|---|---|---|---|
| | 0 | 0.5 | 2.0 | 8.0 |
| | (g/Calli) | | | |
| EBN [x] | 0.83±0.17[z] | 0.16±0.02 | 0.16±0.02 | 0.11±0.01 |
| EBN-5 | 0.89±0.08 | 0.84±0.08 | 0.63±0.07 | 0.15±0.02 |
| EBN-6 | 0.89±0.12 | 0.64±0.04 | 0.20±0.03 | 0.19±0.02 |
| EBN-8 | 1.05±0.19 | 0.48±0.03 | 0.26±0.04 | 0.14±0.03 |
| EBN-9 | 0.91±0.21 | 0.76±0.21 | 0.43±0.08 | 0.27±0.01 |
| EBN-10 | 0.98±0.08 | 0.37±0.08 | 0.36±0.07 | 0.17±0.02 |
| EBN-12 | 0.51±0.07 | 0.31±0.05 | 0.16±0.02 | 0.18±0.02 |
| EBN-13 | 0.68±0.07 | 0.47±0.03 | 0.16±0.02 | 0.19±0.02 |
| EBN-14 | 1.0±0.03 | 0.54±0.06 | 0.52±0.01 | 0.18±0.01 |
| EBN-16 | 1.15±0.24 | 0.66±0.04 | 0.67±0.08 | 0.20±0.02 |
| EBN-17 | 0.88±0.02 | 1.04±0.21 | 0.17±0.03 | 0.15±0.01 |
| EBN-18 | 0.72±0.01 | 0.15±0.03 | 0.15±0.01 | 0.12±0.01 |

[x] Unselected cell line.
[z] Each growth value represents the mean of three replicates of repeated experiments ± SE (standard error).

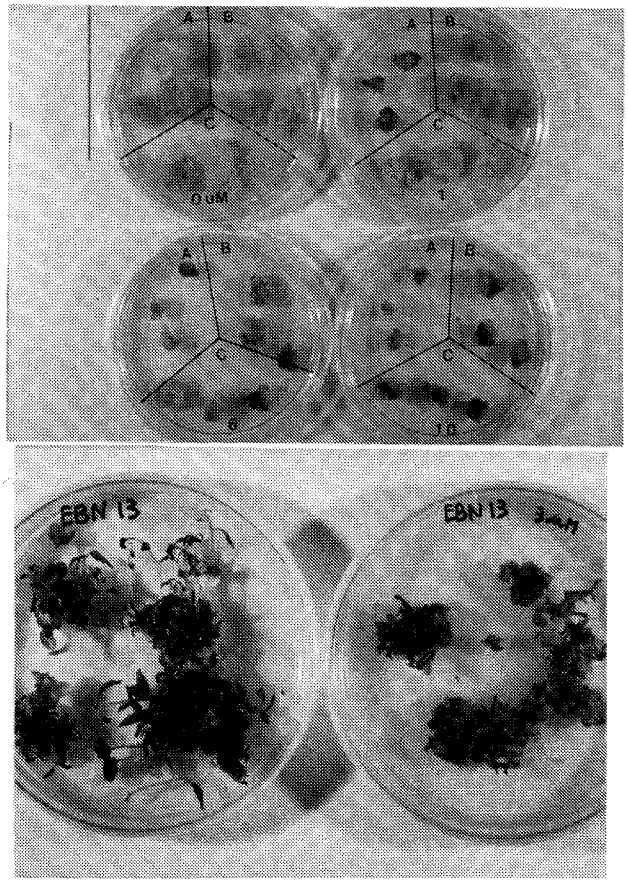


Fig. 1. Variation in somaclones regenerated from acifluorfen tolerant cell lines of *S. ptycanthum*.

## Reqeneration From Acifluorfen-Tolerant Cell Lines

The regeneration capacity of selected cell lines in *S. ptycanthum* differed depending on the callus line. Twenty two of twenty-five acifluorfen-tolerant callus lines regenerated shoots (Table 2). The regeneration rate varied, ranging from 0.3 to 37.3 shoot per calli. Six selected callus lines had better shoot regeneration and eleven callus lines had poorer regeneration than

the unselected line. Shoot growth also varied, averaging from 0.2 to 3.7 cm depending on the callus line. Fifteen calli lines had better shoot growth than the unselected calli line. Overall, *S. ptycanthum* callus lines regenerated relatively easily and rapidly.

Selected callus lines were more effective than the unselected line in regenerating on medium containing acifluorfen (Table 2). The unselected line of *S. ptycanthum* did not regenerate shoots on medium containing 3uM aciflourfen. but 22 selected callus lines regenerated. In general. increased levels of the herbicide decreased shoot regeneration.

The regeneration capacity of unselected line on medium with acifluorfen. The unselected callus line did not regenerate above 1uM acifluorfen, while some selected lines regenerated up to 5uM. Wersuhn et al., (1987) reported that increased concentrations of sodium 2,2-dicholropropionate decreased the regeneration of tolerant potato lines. But, inclusion of high concentrations of herbicide in regeneration media would be useful for obtaining somaclones with stable tolerance because it would suppress the regeneration of sensitive plants.

**Table 2.** Regeneration of acifluorfen-tolerant and unselected *S. ptycanthum* Cell lines.

| Cell lines | O uM Acifluorfen | | O uM Acifluorfen | |
|---|---|---|---|---|
| | No. of Shoot | Shoot height | No. of Shoot | Shoots height |
| | (#/Calli) | (cm) | (#/Calli) | (cm) |
| EBN [x] | 13.3±2.6 [z] | 0.9 | 0.0 | 0.0 |
| EBN-1 | 0.0 | 0.0 | 0.7±0.7 | 0.2 |
| EBN-2 | 11.0±1.5 | 2.1 | 6.0±1.5 | 0.4 |
| EBN-3 | 12.0±1.2 | 2.9 | 3.3±2.9 | 0.5 |
| EBN-4 | 37.3±1.8 | 1.4 | 7.0±0.6 | 0.5 |
| EBN-5 | 6.7±0.3 | 3.0 | 4.0±0.6 | 0.2 |
| EBN-6 | 25.7±1.2 | 0.7 | 5.7±1.2 | 1.3 |
| EBN-7 | 1.3±0.3 | 0.2 | 0.3±0.3 | 0.1 |
| EBN-8 | 2.7±0.3 | 1.4 | 2.3±0.3 | 0.1 |
| EBN-10 | 12.0±1.5 | 1.7 | 6.0±0.6 | 0.4 |
| EBN-11 | 0.3±0.3 | 0.4 | 2.0±1.2 | 0.3 |
| EBN-12 | 16.7±2.7 | 3.7 | 0.3±0.3 | 0.1 |
| EBN-13 | 16.0±2.3 | 3.0 | 11.0±1.2 | 1.3 |
| EBN-14 | 0.0 | 0.0 | 0.0 | 0.0 |
| EBN-15 | 3.7±0.3 | 1.7 | 3.0±0.6 | 0.9 |
| EBN-16 | 12.7±2.7 | 0.9 | 11.3±1.8 | 0.5 |
| EBN-17 | 1.7±0.9 | 0.2 | 0.0 | 0.0 |
| EBN-18 | 0.0 | 0.0 | 1.3±1.3 | 0.1 |
| EBN-19 | 8.0±2.1 | 1.6 | 3.0±0.6 | 0.6 |
| EBN-20 | 15.0±1.2 | 2.8 | 5.0±0.6 | 1.6 |
| EBN-21 | 7.0±2.5 | 2.2 | 0.0 | 0.0 |
| EBN-22 | 7.7±1.2 | 1.5 | 2.3±0.3 | 0.8 |
| EBN-23 | 7.3±1.9 | 2.4 | 1.0±0.6 | 0.8 |
| EBN-24 | 3.3±0.3 | 1.1 | 0.7±0.3 | 0.6 |
| EBN-25 | 11.0±1.5 | 2.1 | 3.0±0.6 | 1.6 |
| EBN-26 | 27.7±2.2 | 3.4 | 8.3±1.5 | 1.6 |

[x] Unselected cell line.
[z] Each values represent the mean of three replicates of repeated experiments ± SE.

### Phenotypic Variation in Regenerated Plants

Many of the somaclones were variants, differing in leaf shape, leaf color, number of flower parts, flower color, and fertility (Table 3). The rate of phenotypic variants differed depending on the callus

**Table 3.** Phenotypic variation of plants regenerated from acifluorfen-tolerant *Solanum ptycanthum* cell lines

| Cell lines | Shoot Height (cm) | | | Leaf variants (% plsny) | Flower variants (% plant) |
|---|---|---|---|---|---|
| | X[x] | SD[y] | CV[z] | | |
| EBN-2 | 24.5 | 12.1 | 49.8 | 75.0 | 75.0 |
| EBN-3 | 35.2 | 11.9 | 33.8 | 94.4 | 78.0 |
| EBN-5 | 34.7 | 6.3 | 18.3 | 82.4 | 76.5 |
| EBN-6 | 41.8 | 9.0 | 21.6 | 66.7 | 33.3 |
| EBN-8 | 33.0 | 12.8 | 38.6 | 100 | 100 |
| EBN-10 | 4.8 | 0.4 | 7.4 | 100 | 100 |
| EBN-12 | 42.9 | 18.3 | 42.7 | 12.1 | 63.0 |
| EBN-13 | 40.9 | 9.6 | 23.4 | 78.6 | 80.0 |
| EBN-15 | 24.8 | 2.9 | 11.6 | 100 | 80.0 |
| EBN-19 | 18.7 | 11.2 | 60.2 | 100 | 100 |
| EBN-20 | 34.5 | 13.3 | 38.7 | 50.0 | 46.4 |
| EBN-21 | 31.9 | 4.6 | 14.2 | 76.5 | 58.8 |
| EBN-24 | 32.0 | 9.3 | 28.9 | 89.7 | 41.4 |
| EBN-25 | 38.5 | 7.6 | 19.7 | 75.0 | 87.5 |
| EBN-26 | 34.9 | 7.3 | 20.9 | 60.0 | 66.7 |

[x] Mean
[y] Standard deviation
[z] Coefficient of variant

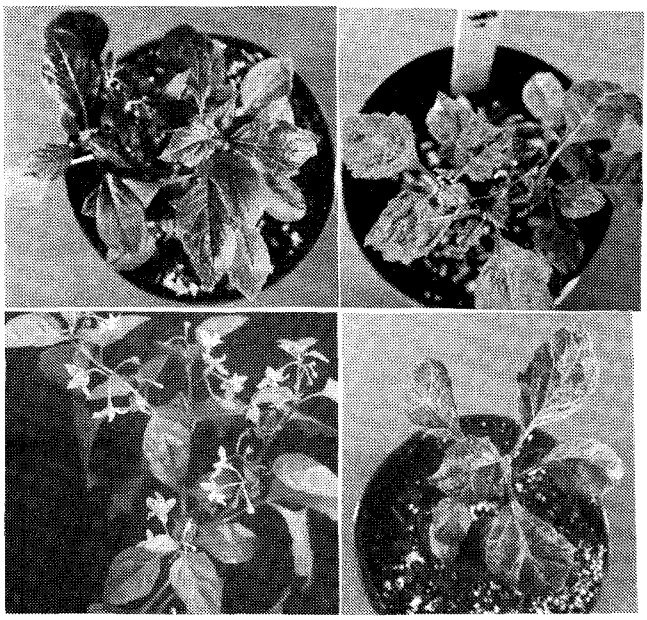

Fig. 2. Variation in somaclones regenerated from acifluorfen tolerant cell lines of *S. ptycanthum*.

line. For example, variation in leaf morphology ranged from 12.1% to 100%, and within individual callus lines, there were a large standard deviation (SD) and coefficient of variation (CV).

There were also differences in the type of variant phenotypes which occurred. Many variants had deformed leaves, which long petioles, leaf rolling variegation, and entire leaves. Leaves with long petiole and rolling had not been observed in previous studies. The growth habit of the someclones also differed, ranging from broad and prostrate to upright and compact. Fertility varied : some plants never flowered, while other plants produced flowers which either aborted or had seedless berries. There were changes in flower morphology. Many variant flowers had purple coloration on the abaxial surface of the petals (wild-type plants have completely white flower). Other variants had petal numbers from 4 to 6 (the wild type have 5).

Thomas and Pratt (1982) reported that morphology, vigor and fertility of plants regenerated from paraquat-tolerant tomato cell lines varied widely depending on the clone. The plant with the greatest paraquat-tolerance was the most abnormal clone, having small leaves, a reduced growth rate and complete sterility. In addition to extensive morphological abnormalities, plants regenerated from selected NaCl-tolerant cell lines were cytological variants with numerous genetic alteration (McCoy, 1987) . In this study, there were also differences in plant growth characteristics depending on the callus lines.

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