

Soybean Plants and Tissue Culture Selection for Glyphosate Tolerance

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Abstract

Selection of soybean (*Glycine max* (L.) Merr.) plants for glyphosate tolerance was conducted in the greenhouse. A rate of glyphosate higher than 0.5 kg ai/ha caused severe phytotoxicity to all cultivars and breeding lines. Cultivars Doikham and S.J.5 were highly tolerant. Breeding lines 8416-7 and 8418-4 were moderately tolerant. Cultivars Chiangmai 60 and S.J.4 were susceptible to glyphosate.

Selection by cell suspension was similar to the whole plant selection. The maximum glyphosate concentration that cells of all cultivars and breeding lines could tolerate was 10^{-2} M. When a suspension of cells previously cultured in medium containing glyphosate were transferred a second time into the same medium, cells of all cultivars and breeding lines could slightly adapt for tolerance at the same concentration of glyphosate. Moreover, cells of cultivars Chiangmai 60 and S.J.4 were better adapted than others.

Keywords : soybean, tissue culture, glyphosate

Soybean production in Thailand is presently insufficient for local consumption. The low productivity of soybean is due to various factors. A major factor is the improper control of weeds. Manual weeding is limited by its cost and the unavailability of labor. Herbicide offers another approach for weed control but users have to know the application technique and have to select for proper herbicides.

At present, a single herbicide cannot control all kind of weeds. In order to solve this problem, new compounds have been synthesised and developed. The development process for such compounds requires a large amount of investment. In addition, the newly developed compound might cause harmful effects toward environments. The

development of nonselective herbicides which are less hazardous to the environment for weed control for soybean production should be investigated.

Glyphosate, a nonselective and systemic herbicide belongs to the class phosphonate used as postemergence for control of grassy and broadleaf weeds. This herbicide can control serious weeds such as *Cyperus rotundus* and *Imperata cylindrica*. Glyphosate has low toxicity to experimental animals (LD_{50} (rat) = 5,600 mg/kg) including fishes and wildlife. Furthermore glyphosate is strongly bound to soil colloids (Anonymous, 1983) thus preventing contamination of ground water. However low rate of glyphosate causes phytotoxicity to soybean. Improvement of soybean for glyphosate tolerance is

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very interesting since the serious weeds in soybean like *C. rotundus* can be controlled by glyphosate.

When glyphosate is uptaken by plants, it will inhibit synthesis of aromatic amino acids such as phenylalanine, tyrosine, and tryptophan which are the precursors of proteins and phenols. The site of glyphosate inhibition is in the shikimic acid pathway especially on EPSP (5-enolpyruvylshikimic acid-3-phosphate) synthase (Nafziger *et al.*, 1984).

Resistance or tolerance to herbicides differ among plants that depend on their inheritance (Chu, 1983; Hughes, 1983). However, the effect of environment before, during and after herbicides application on their activity can not be controlled. Many soybean varieties would appeared to have tolerance to glyphosate when it was applied under water stress conditions. However, the same varieties were susceptible to glyphosate when herbicide is applied to soybean grown under high soil moisture condition (Hartwig, 1987). Therefore, testing crops for herbicide tolerance should be done under controlled environmental condition. Otherwise, the results will be variable.

To select for herbicide tolerance either the whole plant or the cell suspensions can be used. Crops reported to tolerant to herbicide by cell selection method include carrot to bensulfuronmethyl, glyphosate, and glufosinate (Suwanwong and Ishizuka, 1988) and glyphosate (Nafziger *et al.*, 1984); *Lotus corniculatus* to 2,4-D (Suanson and Tomes, 1980 a, b); sugarcane to ametryn and dalapon (Crocomo *et al.*, 1984); corn to imidazolinones (Shaner and Malefyt, 1985); and tobacco to paraquat (Miller and Hughes, 1980).

The objectives of these experiments were to select soybean cultivars for glyphosate tolerance under greenhouse and by tissue culture method, study the adaptation of soybean cells for glyphosate tolerance.

Materials and Methods

Greenhouse experiments

The seeds of six cultivars and breeding lines of soybeans listed in Table 1 were planted in 6 × 32 × 42 cm trays containing Pakchong clay loam soil. Seven days after planting the plants were thinned to one plant per hill. There are five cultivars per tray and one cultivar with four plants per row. At 2 weeks after germination glyphosate at concentrations given in Table 1 was applied to plants by laboratory sprayer with 8004 flat fan T-jet at a pressure of 2.1 kg/cm² and spray volume of 500 l/ha. At two weeks after herbicide application, plant height, and dry weight were recorded.

A preliminary study indicated that S.J.5 and Doikham represented the group of tolerance, 8418-4 and 8416-7 breeding line represented the group of moderately tolerance and S.J.4 and Chiangmai 60 represented the group of susceptible (Table 1).

Table 1 Effect of glyphosate on plant height of soybean 4 cultivars and 2 breeding lines.

Cultivars and breeding lines	Glyphosate (kg ai/ha)			
	0	0.125	0.250	0.500
	cm			
S.J.5	30.2 a	26.9 bc	24.7 def	23.2 efgh
Doikham	31.5 a	26.4 bcd	23.8 efg	21.5 ghijk
8418-4	27.8 b	23.5 efgh	22.1 ghij	21.7 ghij
8416-7	25.4 cde	23.8 efg	21.7 ghij	20.6 ijk
S.J.4	30.7 a	22.4 fghi	19.9 jk	16.2 m
Chiangmai 60	27.6 b	21.2 hijk	19.4 kl	17.6 lm
Average	28.9 X	24.0 Y	21.9 Z	20.1 Z
C.V.	6.01%			

Means within a column or row followed by the same letter are not significantly different at 5% level by DMRT.

The experiment was conducted in the greenhouse at Dept. of Agronomy, Kasetsart University, Bangkok Campus, from April 1988 to April 1989. The temperature was $30 \pm 3^{\circ}\text{C}$ and the plants were watered every day and soil moisture was at field capacity $\pm 5\%$. The experiment was factorial with a randomized complete block design and 4 replications.

Laboratory experiments

Tissue culture experiments were conducted in the laboratory at Dept. of Agronomy, Kasetsart University, Kampaengsaen Campus, July 1987 to April 1989. The experiments were factorial in an RCB with 4 replications.

The callus inducing medium was prepared following the procedure of Murashige and Skoog (1962). The prepared stock solution was mixed with 20 gm/l of sucrose, and 10 mg/l of 2,4-D as callus inducing agent then the pH was adjusted to 5.8 by 1.0 N KOH or 1.0 N HCl. Agar at 6.5 g/l was added and mixed to solution and boiled.

The liquid medium for selection of soybean cells for glyphosate tolerance was prepared in the same manner of callus inducing medium. Glyphosate (isopropyl amine salt of phosphonomethyl glycine) was filtered through the membrane with $0.22 \mu\text{m}$ pore size to eliminate bacteria (Susan and Carl, 1985).

After soybean seeds were cleaned, they were germinated on MS medium with 2% sucrose under sterile conditions. After germination for 5 to 7 days the tissue at the tip, stem, and leaves of the seedling were cut into pieces of 0.5 to 1.0 cm and cultured in MS medium containing 4 mg/L of 2,4-D to produce callus.

After the callus was formed, it was transferred into MS liquid medium with 5 mg/l of 2,4-D and shaken at 120 rpm (round per minute) for 14 days to

yield the suspension cells. The suspended cells were then transferred into MS liquid medium with 5 mg/l of 2,4-D and 0, 10^{-5} , 10^{-4} , 10^{-3} and 10^{-2} M glyphosate, shaken at 120 rpm for 21 days. The surviving cells were transferred into MS liquid medium without glyphosate for 14 days to increase cell population. After that the cells were transferred again into medium with 0, 10^{-4} , 10^{-3} , 10^{-2} , 10^{-1} M glyphosate and shaken at 120 rpm for 21 days. After cells were cultured in medium for 21 days, they were stained by Feulgen reagent for 2 h which was the method modified from previous report (Hosaka *et al.*, 1984) and living cells counted by hemacytometer.

Results and Discussion

Greenhouse experiments

Only 4 cultivars and 2 breeding lines with difference in their toleration were used as a representative group for selection in the greenhouse experiment. By using plant height reduction as the indicator it was found that cultivars Doikham and S.J.5 were tolerate to glyphosate at 0.5 kg ai/ha. Breeding lines 8418-4 were moderately tolerate while cultivars Chiangmai 60 and S.J.4 and breeding lines 8416-7 were susceptible to glyphosate (Table 1). There was the interaction between soybean cultivars and rates of herbicide, without glyphosate S.J.5, Doikham, and S.J.4 were tallest, while 8418-4 and Chiangmai 60 were medium, and 8416-7 was shortest (Table 1). When glyphosate was applied at any rate, S.J.5 and Doikham were tallest while 8418-4 and 8416-7 were medium tall but S.J.4 and Chiangmai 60 were shortest (Table 1). The experiment was conducted under greenhouse condition where soil moisture was controlled. Since the tolerance of soybean to glyphosate was variable under field condition (Hartwig, 1987).

Certain soybean cultivars are tolerant to glyphosate in the field with dry conditions, but they become susceptible under high moisture condition in the following year (Hartwig, 1987).

Laboratory experiments

It was found that at the beginning the number of suspension cells were equal after that the number of cells were highest in MS medium without glyphosate and the number of cells were decreased when the concentration of glyphosate increased (Table 2). The

Table 2. the percentage of soybean suspension cells after first culture in liquid MS medium containing 5 mg/l of 2,4-D and different concentration of glyphosate for 21 days. (Number of cells at 0 days were equivalent to 100%).

Cultivars and breeding lines	Glyphosate (M)				
	0	10^{-5}	10^{-4}	10^{-3}	10^{-2}
	%				
Doikham	144.9 a	128.6 c	95.5 ef	56.6 hi	23.6 jk
S.J.5	140.9 ab	113.8 d	84.1 g	50.0 i	23.1 jkl
8416-7	140.4 ab	108.5 de	69.5 h	22.7 jkl	9.3 mn
8418-4	138.9 ab	88.5 fg	57.6 hi	26.4 jk	12.5 mn
Chiangmai 60	136.4 ab	65.8 h	27.5 jk	18.9 klm	4.4 n
S.J.4	143.6 b	63.6 h	33.0 j	14.4 lmn	4.6 n
Average	140.8 V	94.8 W	61.2 X	31.5 Y	12.9 Z
C.V. (%)	9.17				

Means within a column or row followed by the same letter are not significantly different at 5% level by DMRT.

least number of cells were found in medium with 10^{-2} M glyphosate. The growth of cells from different cultivars and breeding lines shown different response to glyphosate. The number of suspended cells of Doikham and S.J.5 soybean were highest while breeding line 8416-7 and 8418-4 and cultivar Chiangmai 60 and S.J.4 were of

medium and low levels, respectively. This result suggested that the genetics have an influence on growth, development, and tolerance of soybean to glyphosate. Chu (1983) and Hughes (1983) reported that tolerance or/and resistance of various crops were depend on their genetics. There have been reports that callus from different cultivars of sugarcane are tolerant to different concentrations of ametryn and dalapon (Crocomo *et al.*, 1984).

The second transferral of cells into the medium with 5 mg/l of 2,4-D and 0, 10^{-4} , 10^{-3} , 10^{-2} and 10^{-1} M of glyphosate was made for 21 days (Table 3). At the beginning the number of cells of all the cultivars were equal. The number of cells were highest in MS medium with 5 mg/l of 2,4-D without glyphosate (Table 3).

Table 3. The percentage of soybean suspension cells after second culture in liquid MS medium containing 5 mg/l of 2,4-D and different concentration of glyphosate for 21 days. (Numbers of cells at 0 day were equivalent to 100%).

Cultivars and breeding lines	Glyphosate (M)				
	0	10^{-4}	10^{-3}	10^{-2}	10^{-1}
	%				
Doikham	140.0 a	106.5 ⁺ b	65.1 ⁺ c	31.2 ⁺ def	0.0 j
S.J.5	140.5 a	112.4 ⁺ b	55.6 ⁺ c	23.9 ⁻ fg	0.0 j
8416-7	137.0 a	62.5 ⁺ a	26.4 ⁺ efg	12.9 ⁺ hij	0.0 j
8418-4	137.6 a	55.0 ⁻ c	28.1 ⁺ defg	13.2 ⁺ hi	0.0 j
Chiangmai 60	139.8 a	37.0 ⁺ de	17.9 ⁻ gh	7.6 ⁺ ij	0.0 j
S.J.4	139.2 a	38.5 ⁺ d	12.4 ⁻ hij	5.4 ⁺ ij	0.0 j
Average	139.0 V	68.7 W	34.2 X	15.7 Y	0.0 Z
C.V. (%)	13.72				

Means within a column or row followed by the same letter are not significantly different at 5% level by DMRT.

+ = The percentage of cells were higher than the first culture at the same concentration of glyphosate (Table 2).

- = The percentage of cells were lower than the first culture.

The number of cells of all cultivars were decreased when the concentration of glyphosate increased. The cells of all cultivars could survive in medium with 10^{-2} M of glyphosate. This result was similar to the report of Suwanwong and Ishizuka (1988). They reported that cells of carrot could tolerate to glyphosate at 10^{-3} M. Furthermore there was a report that 50% of corn suspension cells were reduced when they were cultured in medium with 10 and 30 μ M of imazapyr (Anderson and Hibberd, 1985). The growth of corn callus were 50% reduced in medium with 0.03 mg/l of imidazolinone (Shaner and Malefyt, 1985.)

The percentage of number of cells of cultivars Doikham and S.J.5 were highest at any concentrations of glyphosate indicated that these two cultivars were tolerant to glyphosate (Table 3). The percentage of the number of cells of breeding lines 8416-7 and 8418-4 had medium performance while those of cultivar Chiangmai 60 and S.J.4 were the least. These results suggested that breeding lines 8416-7 and 8418-4 and cultivar Chiangmai 60 and S.J.4 were of medium tolerance and susceptible to glyphosate respectively.

The basic tolerance of different cultivars and breeding lines of soybean to glyphosate was might be due to a different rate of glyphosate metabolism or different reaction of herbicide on EPSP synthase inside the cells. However absorption and translocation of glyphosate in soybean plants could not be counted in this study. The tolerant soybean species could metabolize glyphosate more quickly while the susceptible species could not or metabolize more slower. The other reason is the activity and quantity of EPSP synthase in the tolerant cultivar might be higher than the susceptible one and therefore synthesis more aromatic amino acids. Furthermore the tolerant cultivars might synthesis aromatic amino acids by other pathway without EPSP

synthase. Nafziger *et al.*, (1984) reported that the tolerance of carrot cells to 25 mM of glyphosate can be achieved by 15 transferings of cells to medium with higher herbicide concentration. They found that the tolerance was 52 times increased. Furthermore, the activity of EPSP synthase in tolerant cells was 12 times increased. Anderson and Hibberd (1985) found that the tolerance of corn cells to imazapyr contributed to alternation of molecular structure or the higher quantity of AHAS (acetohydroxy acid synthase). Therefore this enzyme was not inhibited by the herbicide.

There was a similar result between the tolerance of the whole plant (in the greenhouse experiment) and suspension cell (in laboratory) of soybean cultivars and breeding lines in this study. Which were similar to the reports of Swanson and Tones (1980 a, b). They stressed that there was a positive correlation between the whole plants and tissue culture for selection of *Lotus corniculatus* L. for 2,4-D tolerant.

Chiangmai 60 and S.J.4 soybean were susceptible to glyphosate when cells were first cultured in glyphosate containing medium for 21 days (Table 2). However when the survive cells were the second time transferred to the glyphosate containing medium, the percentage of suspension cells were slightly increased and did not differ from the moderately tolerant breeding lines (8416-7 and 8418-4, Table 3). When the percentage of suspension cells from all cultures in the medium with 10^{-4} , 10^{-3} and 10^{-2} M of glyphosate at 21 days (Table 2) were compared to the second sample cultured at the same period (Table 3), it was found that the tolerance of cells from all cultivars and breeding lines were 5-10% increased except breeding line 8416-7 and 8418-4. Because of the adaptation of cells from cultivars and breeding lines, for glyphosate tolerance showed differences.

Only two transferrings of cells to medium with glyphosate were done in this study. The tolerance of cells to glyphosate might be increased when the number of transferring increased which was reported by Nafziger et al. (1984). Furthermore X-ray or other chemicals can be used to induce mutation of plant cells. Miller and Hughes (1980) reported that the suspension cells of the carrot could be induced for resistance to 10^{-5} M of paraquat by X-ray. When they become plants, 15 out of 43 plants were resistant to paraquat.

The selection of soybean for glyphosate tolerance in this study followed the technique of diploid cell selection which was reported by Chu (1983). However the development from cells to be plants was not successful and the study should be further developed.

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