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Selection of Herbicide-Tolerant Rice (*Oryza sativa* L.) Callus by Tissue Culture

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組織培養을 통한 水稻耐性 카루스 選拔

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ABSTRACT

The response of callus growth of rice (*Oryza sativa* L.) cultivars which showed different responses to herbicides as seedlings was investigated to select resistant or tolerant calli. Callus growth of IR28 which was susceptible to thiobencarb (*S*-[(4-chlorophenyl)methyl]diethylcarbamothioate) during callus induction was not inhibited by 10^{-5} M and 10^{-6} M thiobencarb, indicating that there was a difference in tolerance among callus induction and growth, and the intact plant level. A similar result was obtained with IR31917-45-3-2-2 to butachlor [*N*-(buthoxymethyl)-2-chloro-N-(2, 6-diethylphenyl)acetamide]. The fresh weight of IR28 callus transferred into 10^{-5} M thiobencarb after treatment at 10^{-6} M for 30 days was not affected by the herbicide, indicating that transferring callus into gradually higher herbicide concentrations can be a useful method for selection of herbicide-tolerant cell lines.

Key words : Herbicide, rice, tolerance or resistance, tissue culture.

INTRODUCTION

In recent years, plant cell and tissue culture techniques have been employed in research involving herbicide selectivity and resistance in plants (1). Selection in cell cultures has been used as a routine means of obtaining tobacco (*Nicotiana tabacum* L.) mutants which are resistant to herbicides. Tobacco cell lines resistant to 2, 4-D (2, 4-dichlorophenoxyacetic acid) (14), chlorsulfuron (2-chloro-*N*-[[(4-methoxy-6-methyl-1, 3, 5-triazin-2-yl)amino]carbonyl]benzene sulfonamide) and sulfometuron (2-[[[[(4, 6-dimethyl-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]benzoic acid)-methyl (2), amitrole (1*H*-1, 2, 4-triazol-3-amine) (12), vernolate (*S*-propyldipropyl-carbamothioate) (3), picloram (4-amino-3, 5, 6-trichloro-2-pyridine-carboxylic acid) (1), bentazon[3-(1-methylethyl)-(1*H*)-2, 1, 3-benzothiadiazin-4(3*H*)-one 2, 2-dioxide] and phenmedipham (3-[(methoxycarbonyl) amino]phenyl(3-methylphenyl)carbamate) (10), and paraquat (1, 1'-dimethyl-4, 4'-bipyridinium ion) (4) have been isolated. Isolation of carrot (*Daucus carota*) cell lines resistant to glyphosate [*N*-(phosphonomethyl)glycine] (9) and tomato (*Lycopersicon esculentum* L.) cell lines resistant to paraquat (13) have been reported.

Populations of cultured plant cells generally contain spontaneous variants which differ from the rest of the cell populations in any of a number of characteristics which may or may not be the result of mutation (6). Furthermore, a number of cases have now been reported in which traits, such as herbicide resistance, selected from cell culture have been retained in regenerated plants and transmitted genetically to the progeny (7).

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This experiment was conducted to determine the response of callus growth of rice cultivars which showed different response to herbicides and to select calli which were resistant or tolerant to different herbicides.

MATERIALS AND METHODS

Experiment 1. Screening for Tolerance to Butachlor and Thiobencarb

Seventeen cultivars were screened for tolerance to butachlor or thiobencarb using the screening method described in Shin et al. (11). Butachlor and thiobencarb were applied at a concentration of 2×10^{-5} M. There were three replications. Plant height and dry weight were measured 10 days after treatment.

Experiment 2. Herbicide Tolerance through Tissue Culture

Cell culture. Dehulled mature seeds of rice which were sterilized with 70% ethanol for 1 min. followed by 0.1% mercuric chloride solution for 15 min. were washed three times with sterilized water.

Callus was induced in the dark at 25 ± 1℃ from these seeds and their scutellar tissues on modified Murashige Skoog (MS_3) (8) medium supplemented with 2.0 mg/l of 2, 4-D and 0.2 mg/l of BAP (benzylamino purine) and on modified Gamborg's B5 (A) (5) medium supplemented with 0.5 mg/l of 2, 4-D. The callus was subcultured in the same modified MS medium. The shoot regeneration medium consisted of the MS medium supplemented with 2.0 mg/l of BAP and 0.2 mg/l of NAA (α-naphthalence acetic acid) (MSB). Shoots were regenerated in the light at 25 ± 1℃.

Media containing various concentrations of herbicides were prepared by adding the appropriate amounts of herbicide sterilized through a millipore filter from stock solutions.

Experiment 2a. Callus Induction from Rice Tissues

Callus was induced from mature seeds and scutellar tissues of each cultivar on MS_3 and A medium. Callus formation was determined 50 days after incubation for mature seed and 30 days after incubation for scutellar tissue and expressed as follow :

$$\text{Callus formation}(\%) = \frac{\text{Number of seeds with callus}}{\text{Total seeds inoculated}} \times 100$$

The callus was removed from seed and weighed to determine its fresh weight. The treatments were replicated three times.

Experiment 2b. Cultivar Growth Response of Calli to Herbicides

The objective of this experiment was to investigate the growth response of rice callus from different cultivars to herbicides of different concentrations.

Scutellar callus obtained from mature seeds of IR43 and Taipei 309 were subcultured every 2 weeks. Fifty milligram pieces of healthy callus were transferred to A medium which was a modified B5 medium (5) containing 10^{-6} M, 10^{-5} M, and 10^{-4} M of butachlor or thiobencarb. Each concentration had a minimum of five replications. Callus cell growth was determined by measurement of initial fresh weight and fresh weight after 30 days.

Experiment 2c. Selection of Herbicide-Tolerant Cell Lines

This experiment was conducted to select calli tolerant to herbicides and to regenerate plants from selected calli.

Callus obtained from mature seeds of IR43 and Taipei 309 was used. Calli which were subcultured for 8 weeks were divided into small pieces of approximately 50 mg and transferred to A medium containing 10^{-5} M of butachlor or thiobencarb. After 1 month, vigorously growing callus were selected and transferred to fresh medium containing the same herbicide concentration. The surviving or growing callus lines were transferred to a regeneration medium after four consecutive selections and the plants grown to maturity.

RESULTS AND DISCUSSION

Responses of Cultivars to Herbicide and Callus Formation

Cultivars which were screened for tolerance to the herbicides were divided into the two groups. Those with plant heights greater than 50% of the untreated control were regared as being tolerant to the herbicide and those that had greater than 80% inhibition in plant height were considered to be susceptible to the herbicides (Tables 1 and 2).

Differences in frequency of callus formation between cultivars which were tolerant and those which were susceptible to herbicides were observed. Cultivars tolerant to butachlor such as New Sabarmati(BAS), IR29738-48-2-3-4, IR8866-30-3-1-4-2, and IR27095-20-18 had higher callus formation and fresh weight than susceptible cultivars such as IET3257, IR17494-32-3-1-1-3, IR31917-45-3-2-2, 5207, and Cheriviriuppu (Table 1). A similar trend was observed with thiobencarb (Table 2).

Table 1. Effect of butachlor on plant growth and callus formation of different rice cultivars.

Cultivar	Tolerance to butachlor		Callus formation (%)	Fresh weight of callus (mg/seed)
	Plant height	Dry weight		
	% of control			
Tolerant group				
New Sabarmati (BAS)	71.3	50.7	82.4	37.4
IR29738-48-2-3-4	54.9	37.8	43.8	25.7
IR8866-30-3-1-4-2	69.5	41.0	44.8	17.9
IR27095-20-18	61.4	63.2	72.2	45.7
Average	64.3	48.2	60.8	31.7
Susceptible group				
IET3257	16.6	13.7	36.1	14.7
IR17494-32-3-1-1-3	9.0	8.6	20.0	12.8
IR31917-45-3-2-2	15.0	18.1	29.0	21.8
5207	18.0	21.1	30.0	13.2
Cheriviruppu	14.8	14.0	16.7	9.5
Average	14.7	15.1	26.4	14.4

Table 2. Effect of thiobencarb on plant growth and callus formation of different rice cultivars.

Cultivar	Tolerance to thiobencarb		Callus formation (%)	Fresh weight of callus (mg/seed)
	Plant height	Dry weight		
	% of control			
Tolerant group				
IR9660-50-3-1	74.0	82.2	92.1	41.8
IR10198-66-2	75.1	85.9	33.3	14.5
Nona Bokra	76.6	74.0	84.6	30.4
Kami-Iku 382	80.0	70.4	75.0	34.3
Average	76.4	78.1	71.3	30.3
Susceptible group				
IR5	1.7	2.5	31.6	12.1
IR28	4.8	2.5	46.9	13.7
IR28150-84-3-3-2	4.7	7.6	38.7	13.7
NR10041-66-3-1	2.0	4.9	66.7	45.6
Average	3.3	4.4	46.0	21.3

Effect of Herbicides on Callus Formation and Fresh Weight from Mature Seeds

The frequency of callus formation from Taipei 309 was higher than that of IR20 and IR43 with both butachlor and thiobencarb, regardless of the herbicide concentration used when mature seeds were cultured for callus induction in modified MS medium containing herbicide (Tables 3 and 4). With a herbicide concentration of 10^{-5} M, no callus was induced from IR20 and IR43 but callus formation equivalent to 87% of the untreated control was obtained with Taipei 309 which had been found to be more tolerant to both herbicides in a seedling test (11). The same trend was observed for callus fresh weight (Tables 3 and 4).

Effect of Herbicides on Callus Formation and Fresh weight from Scutellar Tissue

Differential responses in callus induction and fresh weight were observed with different concentrations of herbicides on MS_3 and A medium. A concentration of 10^{-4} M butachlor and thiobencarb completely inhibited the induction of callus from scutellar tissue of Taipei 309 regardless of the media. No significant inhibition in callus formation was obtained with 10^{-6} M butachlor and thiobencarb at 30 days after incubation on MS_3 medium, and 10^{-6} M butachlor, thiobencarb and glyphosate and 10^{-5} M glyphosate on A medium (Tables 5 and 6).

When 10^{-5} M and 10^{-6} M glyphosate were applied to A and MS_3 medium no inhibition in callus formation occurred on A medium but on MS_3 medium significant inhibition was observed. These results indicate that there are different responses in callus formation between media which contain different nutritional components when herbicides are incorporated into the media.

Table 3. Effect of butachlor on callus formation from mature seeds.[1)]

Cultivar	Herbicide conc. (M)	Seeds inoculated (no.)	Callus formation (%)	Fresh weight of callus (mg/seed)	% of control	
					Callus formation	Fresh weight
IR20	10^{-5}	48	0.0 d	0.0 f	0.0	0.0
	10^{-6}	48	46.3 c	5.4 e	68.3	66.7
	Control	48	67.8 b	8.1 de	-	-
IR43	10^{-5}	48	0.0 d	0.0 f	0.0	0.0
	10^{-6}	41	67.0 b	7.7 de	99.4	30.6
	Control	44	67.4 b	25.2 c	-	-
Taipei 309	10^{-5}	32	84.7 a	12.3 d	87.1	14.7
	10^{-6}	40	97.2 a	54.4 b	100.0	65.2
	Control	39	97.2 a	83.4 a	-	-

[1)] In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Table 4. Effect of thiobencarb on callus formation from mature seeds.[1)]

Cultivar	Herbicide conc. (M)	Seeds inoculated (no.)	Callus formation (%)	Fresh weight of callus (mg/seed)	% of control	
					Callus formation	Fresh weight
IR20	10^{-5}	48	0.0 d	0.0 e	0.0	0.0
	10^{-6}	48	51.2 c	6.2 de	76.0	76.8
	Control	48	67.8 b	8.1 d	-	-
IR43	10^{-5}	48	0.0 d	0.0 e	0.0	0.0
	10^{-6}	39	66.4 b	7.7 d	97.8	31.0
	Control	44	67.4 b	25.2 b	-	-
Taipei 309	10^{-5}	43	86.6 a	16.4 c	88.3	19.3
	10^{-6}	44	97.9 a	80.3 a	100.3	97.7
	Control	39	97.2 a	83.4 a	-	-

[1)] In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Table 5. Effect of different herbicides on callus induction and fresh weight from scutellar tissue of Taipei 309 on A medium.[2]

Herbicide	conc. (M)	Scutellar tissues inoculated (No.)	Frequency of callus induction (%)		Fresh weight of callus (mg/callus)	% of control	
			15 DAI[1]	30 DAI[1]		Callus induction	Fresh weight of callus (30 DAI)
Butachlor	10^{-4}	60	0.0 e	0.0 f	0.0 g	0.0	0.0
	10^{-5}	45	6.7 e	13.0 e	2.0 f	19.8	10.4
	10^{-6}	75	44.5 bc	66.7 bc	14.4 c	99.3	75.0
Thiobencarb	10^{-4}	60	0.0 e	0.0 f	0.0 g	0.0	0.0
	10^{-5}	60	15.5 d	15.5 de	4.7 e	27.2	24.5
	10^{-6}	88	37.8 c	57.8 c	16.0 b	87.9	83.3
Glyphosate	10^{-4}	45	2.2 e	24.5 d	2.8 f	36.3	14.6
	10^{-5}	60	55.5 a	75.5 ab	12.7 d	114.1	66.1
	10^{-6}	60	55.5 a	84.4 a	12.3 d	124.0	64.1
Control		58	48.9 ab	66.7 bc	19.2 a	-	-

[1] DAI ; days after incubation.

[2] In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Table 6. Effect of different herbicides on callus induction and fresh weight from scutellar tissue of Taipei 309 on MS_3 medium.[2]

Herbicide	conc. (M)	Scutellar tissues inoculated (No.)	Frequency of callus induction (%)		Fresh weight of callus (mg/callus)	% of control	
			15 DAI[1]	30 DAI[1]		Callus induction	Fresh weight of callus (30 DAI)
Butachlor	10^{-4}	60	0.0 d	0.0 g	0.0 e	0.0	0.0
	10^{-5}	60	8.9 d	15.5 f	1.6 e	18.2	6.3
	10^{-6}	45	80.0 a	93.3 a	24.5 a	113.5	96.8
Thiobencarb	10^{-4}	60	0.0 d	0.0 g	0.0 e	0.0	0.0
	10^{-5}	75	35.5 c	42.2 d	8.9 d	51.9	35.2
	10^{-6}	45	75.6 a	82.2 b	20.5 b	100.0	81.0
Glyphosate	10^{-4}	60	26.7 c	28.9 e	3.1 e	36.5	12.3
	10^{-5}	45	60.0 b	64.5 c	14.5 c	78.3	57.3
	10^{-6}	45	55.5 b	57.8 c	11.9 cd	70.3	47.0
Control		45	77.8 a	82.2 b	25.3 a	-	-

[1] DAI ; days after incubation.

[2] In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

The fresh weight of Taipei 309 callus induced from scutellar tissue was decreased significantly by all concentrations of butachlor, thiobencarb, and glyphosate on both media except when 10^{-6} M butachlor was used on MS_3 (Tables 5 and 6).

Growth Response of Callus to Herbicides

Inhibition of callus growth was greater as the herbicide concentration increased from 10^{-6} M to 10^{-4} M. Butachlor at 10^{-5} M and 10^{-6} M inhibited callus growth of IR43 and IR31917-45-3-2-2 more than that of Taipei 309. This trend was not observed at the 10^{-4} M concentration (Table 7). IR28 callus growth was not inhibited by 10^{-5} M and 10^{-6} M thiobencarb. This differs from the results found in the seedling test indicating a difference in herbicide tolerance between the callus level and the whole plant level (Table 8). However, Zilkah et al. (15) reported that except for some photosynthetic inhibitors there were fairly good correlations in phytotoxicity between seedlings and callus of *Rumex obtusifolius* L.

Differential cultivar response to herbicides can be

Table 7. Growth response of 8-week old rice callus of different cultivars to different butachlor concentrations.[2]

Cultivar	Herbicide conc. (M)	Calli inoculated (No.)	Fresh weight of callus (mg/piece)		Increase in fresh weight (mg/piece)	% of control
			0 DAT[1]	30 DAT[1]		
Taipei 309	10^{-4}	35	53.4	57.0	3.6 g	5.1
	10^{-5}	70	53.4	109.4	56.0 d	79.7
	10^{-6}	63	55.7	131.0	75.3 c	107.0
	Control	56	57.5	127.9	70.4 c	-
IR43	10^{-4}	35	55.2	65.9	10.7 fg	15.6
	10^{-5}	70	52.3	96.6	44.3 e	65.2
	10^{-6}	42	59.8	106.5	46.7 de	68.9
	Control	56	55.2	123.1	67.9 c	-
IR31917-45-3-2-2	10^{-4}	63	49.5	66.5	17.0 f	15.0
	10^{-5}	63	57.5	108.2	50.7 de	44.6
	10^{-6}	63	47.3	139.9	92.6 b	81.4
	Control	35	56.8	170.4	113.6 a	-

[1] DAT ; days after treatment

[2] In a column, means followed by a common letter are not significantly at the 5% level by DMRT.

Table 8. Growth response of 8-week old rice callus of different cultivars to different thiobencarb concentrations.[2]

Cultivar	Herbicide conc. (M)	Calli inoculated (No.)	Fresh weight of callus (mg/piece)		Increase in fresh weight (mg/piece)	% of control
			0 DAT[1]	30 DAT[1]		
Taipei 309	10^{-4}	49	53.7	65.5	11.8 g	16.8
	10^{-5}	70	53.3	103.4	50.1 f	71.2
	10^{-6}	42	54.6	111.4	56.8 ef	80.7
	Control	56	57.5	127.9	70.4 e	-
IR43	10^{-4}	35	55.6	75.5	19.9 g	29.3
	10^{-5}	63	53.2	100.5	47.3 f	69.7
	10^{-6}	35	56.3	113.0	56.7 ef	83.5
	Control	56	55.2	123.1	67.9 e	-
IR28	10^{-4}	35	54.0	101.8	47.8 f	44.9
	10^{-5}	105	51.7	165.1	113.4 cd	106.4
	10^{-6}	70	50.8	176.8	126.0 bc	118.4
	Control	35	46.2	152.7	106.5 d	-
IR9660-50-3-1	10^{-4}	35	44.5	109.4	64.9 e	44.1
	10^{-5}	48	56.7	183.9	127.2 b	86.3
	10^{-6}	35	47.8	185.4	137.6 ab	93.4
	Control	35	54.6	201.9	147.3 a	-

[1] DAT ; days after treatment

[2] In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

attributed to inherent morphological or physiological characteristics, biochemical differences among cultivars, or to differential rates of uptake and translocation of the herbicide. Thus, the lack of specific target sites for phytotoxic action in highly undifferentiated callus may be the reason for differential responses to herbicides between callus and whole plants.

Selection of Herbicide Tolerant Calli through Cell Culture

Callus growth of IR31917-45-3-2-2 was promoted on media containing 10^{-5} M and 10^{-6} M butachlor as incubation time was increased from 30 days to 60 days (Fig. 1) indicating that cell culture selection for butachlor-tolerant cell lines is possible.

Growth of IR28 callus treated with 10^{-4} M and 10^{-6}

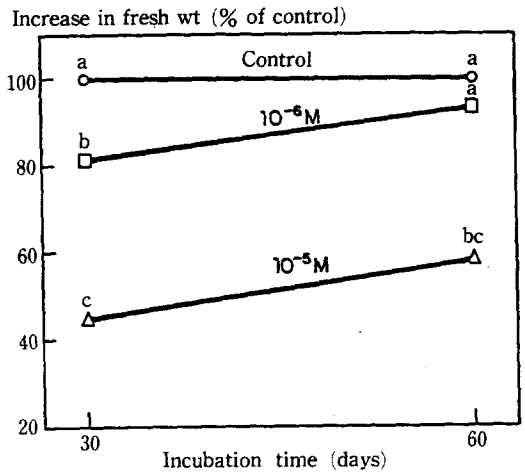

Fig. 1. Fresh weight of IR31917-45-3-3-2 calli as affected by different butachlor concentrations and incubation times. Means followed by a common letter are not significantly different at the 5% level by DMRT.

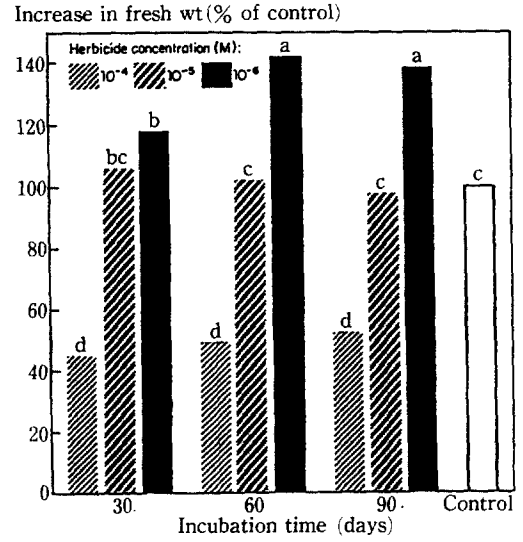

Fig. 2. Fresh weight of IR28 calli as affected by different thiobencarb concentrations and incubation times. Data bars having common letters are not significantly different at the 5% level by DMRT.

M thiobencarb increased slightly as the incubation time was increased from 30 days to 90 days (Fig. 2).

In order to determine herbicide concentrations for selection of herbicide-tolerant cell lines, IR28 calli treated with 10^{-6} M and 10^{-5} M thiobencarb for 30 days were transferred into 10^{-5} M thiobencarb and incubated for an additional 30 days. After incubation, the fresh weight of callus pretreated with 10^{-6} M increased, while that of the callus pretreated with 10^{-5} M decreased. This means that the callus cells adapted themselves to the low herbicide concentration but damaged by the higher concentration. Thus, transferring calli into progressively higher herbicide concentrations is a useful method for selection of herbicide-tolerant cell lines.

Nafziger et al. (9) selected carrot cell lines to grow in 24 mM glyphosate by transferring them into progressively higher concentrations of the herbicide. Tolerance was increased 52-fold, and the adaptation was stable in the absence of glyphosate. Development of herbicide-tolerant cell lines and plants in crops has been reported by a number of scientists (2, 3, 4, 12, 13).

Plant Regeneration from the Calli Affected by Herbicides

The calli which were incubated on the A and MS_3 media supplemented with 10^{-4} M butachlor, 10^{-5} M thiobencarb, and 10^{-6} M glyphosate were transferred into the regeneration medium (MSB) and incubated for 60 days. The rate of plant regeneration was markedly higher on the MS_3 medium than on the A medium regardless of the herbicide and the concentrations used. The rate decreased with the highest concentration of herbicides on MS_3 medium. Seven plantlets were regenerated from 12 calli which were incubated on the MS_3 medium containing 10^{-5} M thiobencarb and 4 plantlets from 6 calli on the MS_3 medium containing 10^{-4} M glyphosate (Table 9). These results indicate that plant regeneration from calli stressed under herbicide treatment is possible and selection of herbicide-tolerant cell lines and plant regeneration from the selected cell lines through tissue culture can be used for development of herbicide resistant rice cultivars. The response of seeds harvested from the regenerated plants to herbicides is being investigated.

Table 9. Plant regeneration from Taipei 309 callus affected by different herbicides and different media.

Medium	Herbicide	Concentration (M)	No. of calli inoculated	No. of regenerated green plants
A	Butachlor	10^{-6}	45	0
		10^{-5}	3	0
		10^{-4}	–	–
	Thiobencarb	10^{-6}	80	1
		10^{-5}	8	0
		10^{-4}	–	–
	Glyphosate	10^{-6}	30	0
		10^{-5}	41	0
		10^{-4}	3	0
	Control	–	30	1
MS_3	Butachlor	10^{-6}	18	17
		10^{-5}	–	–
		10^{-4}	–	–
	Thiobencarb	10^{-6}	20	52
		10^{-5}	12	7
		10^{-4}	–	–
	Glyphosate	10^{-6}	18	51
		10^{-5}	14	–
		10^{-4}	6	4
	Control	–	18	17

摘 要

除草劑에 耐性 또는 抵抗性을 지닌 水稻 카루스를 選拔하기 위하여 幼苗期에 除草劑에 反應을 달리하는 品種을 대상으로 카루스의 生長 反應을 調査한 結果는 다음과 같다.

카루스 誘導時에 thiobencarb에 感受性을 보였던 IR28의 카루스 生長은 thiobencarb 10^{-5} M 및 10^{-6} M에서도 抑制 되지 않아 特定 除草劑에 대한 水稻 品種의 反應은 카루스의 誘導期 및 生長期, 成植物體에 따라 差異가 있음을 나타내었다. Butachlor에 대한 IR31917-45-3-2-2의 反應도 IR28과 類似한 結果를 보였다.

Thiobencarb에 耐性을 지닌 카루스를 選拔키 위하여 IR28의 카루스를 thiobencarb 10^{-6} M에 30日間 處理한 後 高濃度인 10^{-5} M에 옮겨 置床시킨 結果 카루스의 生體重은 전혀 抑制 되지 않아서 除草劑 耐性 細胞를 選拔키 위해서는 除草劑의 濃度를 漸進的으로 높이는 것이 有用한 選拔方法이 될 것으로 思料된다.

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