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Callus Induction and Plant Regeneration from Stolon in Zoysiagrass

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한국잔디류에서 포복경 배양을 통한 캘러스 유기와 재분화에 관한 연구

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ABSTRACT

This study was carried out to induce and maintain callus from 59 zoysiagrass lines, to know the effective disinfestation method for zoysiagrass stolon as explant and the difference in the response of callus induction among 59 lines, and to investigate the effect of medium, growth regulators, light, temperature, stolon part and internode position on callus induction and embryogenic callus(E.C.) formation. The treatment of 0.1mg/L $HgCl_2$ for 15 min resulted in no contamination and the highest callus induction(46.6%). Callus was induced from the 59 zoysiagrass lines. The callus growth of *Z. japonica* and *Z. sinica* was generally better than *Z. matrella*. Ten cell lines whose callus and stolon grow fast in culture and in field, respectively were selected to be used for breeding. Callus induction was the most effective at 2.0mg/L of both 2, 4-D and picloram in MS medium. MS medium was the best for callus induction and growth while LS medium was the best for embryogenic callus and shoot formation. Callus induction and growth was better at 28, 31℃ than 25℃, and dark condition was better than light condition in MS medium containing 2mg/L 2,4-D. While callus induction was better with node part as explant than with internode part, callus growth and embryogenic callus formation was better with internode part. In 'Japonica 1', the first internode was the most effective in callus induction, but third internode was the best in '$M_2 \times S_2$'.

Key words: Zoysiagrass, Callus induction, Plant regeneration, 2,4-D, *Z. japonica, Z. sinica.*

INTRODUCTION

Zoysiagrass is a warm season grass commonly used for home lawn, parks and golf

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courses, and requires low maintenance. Althoughy it has many desirable characteristics such as tolerance to drought, low and high temperature, salinity, etc, breeding work on this turfgrass has not been done much and only few improved cultivars have been developed(Engelke and Murray, 1989). Tissue culture is a valuable tool for propagation, selection and genetic transformation of plants and the practical use of this technique depends on medium composition, culture conditions, genotype, explant, and plant growth regulators(Jain *et al.*, 1988). Monocot plants such as grass and cereal crops are generally difficult to use tissue culture technique compared to dicot plants, but so much progress with monocot plants has been made recently(Vasil, 1987). Therefore, somaclonal variation and gene transformation can be readily used for the improvement of the monocot plants.

Plant regeneration from callus in turfgrass has been reported in many grasses such as bermudagrass(Ahn *et al.*, 1985, 1987), creeping bentgrass(Blanche *et al.*, 1986; Krans *et al.*, 1982), Kentucky bluegrass(Mcdonnel and Conger, 1984), red fescue(Torello *et al.*, 1984), switchgrass(Denchev *et al.*, 1994), and Italian ryegrass(Dale *et al.*, 1981; Jackson *et al.*, 1988). However, the lack of regeneration potential in zoysiagrass limits the application of biotechnology techniques for the genetic improvement. The application of tissue culture techniques for zoysiagrass improvement requires screening within specific cultivars in order to identify genotypes capable of regenerating entire plants(Varga and Badea, 1992). The objectives of this study were i) to evaluate 59 zoysiagrass lines as explant source; ii) to identify the best method for disinfestation of stolon; iii) to investigate 2,4-D and picloram as source of growth regulator, and iv) to determine the effect of medium, temperature, light, stolon part and internode order on callus induction and embryogenic callus formation.

MATERIALS AND METHODS

The stolons of 42 and 17 zoysiagrass lines from the field were collected in fall and summer, respectively. They were washed in tap water, and soaked in 70% ethanol for 3 min and then 0.1% $HgCl_2$ for 15 min with agitation. The stolons were then rinsed in five changes of sterile distilled water, and cut into segments each containing one node. In order to determine the best disinfestation method, stolons of the 'Japonica 1' were surface sterilized with 2% NaOCl for 15 or 30 min, 2% NaOCl for 15 or 30 min followed by 3% H_2O_2 for 5 min, or 0.1% $HgCl_2$ for 10, 15 or 20 min. The contamination rate was recorded five or six days later and callus induction rate was recorded one month later. Stolon segments were placed in a petridish(60 × 15mm) containing MS media with 2mg/L 2,4-D. All cultures were incubated in dark at 28℃ for five weeks. The pH of medium was adjusted to 5.8 with 1N NaOH and 1N HCl. The midium was autoclaved at 121℃ for 15 min and then solidified with 8.0g/L purified agar(Junsei Chemical Co. Ltd., Japan). Vitamin and growth regulators were sterilized with a 0.22㎛ filter and added after au toclaving. Medium was dispensed into steril disposal petridishes(60 × 15mm) in 10ml

aliquots. Callus was maintained by transferring to the same medium monthly. The stolons of 'Japonica 1' and '$C_2 \times S_5$' were used to investigate the effect of growth regulators on callus induction. MS medium used in this experiment was supplemented with 2,4-D or picloram at 1.0, 2.0, 3.0, 4.0, 5.0 mg/L. The MS medium containing 1.0 mg/L 2,4-D was used for maintenance. The presence of embryogenic callus was evaluated every week by dissectmicroscope.

MS(Murashige-Skoog, 1962), LS(Linsmaier-Skoog, 1965) and UM(Uchimiya-Murashige, 1974) media were compared to identify the effect on callus induction and growth and regeneration ability in stolons of zoysiagrass. The culture was done at 25, 28, 31℃ and in light or dark. Stolons of 'Japonica 1' and '$M_2 \times S_2$' were cut into 5 mm segments in the order of position and inoculated on MS medium above mentioned.

Plant regeneration was stimulated by transferring embryogenic callus clump of 1.5~3 mm size to the MS or half strength MS medium without growth regulators and supplemented with 3% sucrose. Cultures were incubated at 28℃ under 16 hr photoperiod. Plantlets regenerated from embryogenic callus sectors appeared within 2 to 3 week of incubation. Plants were acclimatized in a culture vessel for one week and then transplanted to soil in 15 × 15 cm pots after two months of incubation.

RESULTS AND DISCUSSION

1. Disinfestation method

It is very important to establish a suitable disinfestation method for callus induction from stolon explant. In the pretest, The contamination rate for the zoysiagrass stolons collected in summer is higher than those in spring and fall. With stolons collected in fall, 2% NaOCl treatment showed high contamination rate up to 46.6%, but 0.1% $HgCl_2$ treatments for 10, 15, 20 min had no contamination. The treatment of 0.1% $HgCl_2$ for 15 min showed the highest callus induction rate(46.3%), whereas 2% NaOCl for 30 min resulted in the

Table 1. Effect of disinfestation methods on contamination and callus induction with 'Japonica 1' stolons collected in fall

Treatment			No. of	No. of	No. of callus
2%NaOCl (min)	3% H_2O_2 (min)	0.1%$HgCl_2$ (min)	inoculated stolons	contaminants (%)	induction (%)
15	−*	−	87	35(40.2)	26(29.9)
30	−	−	68	31(46.6)	15(22.1)
15	5	−	69	17(24.6)	20(28.9)
30	5	−	78	13(16.7)	22(28.2)
−	−	10	68	0(0.0)	27(39.7)
−	−	15	67	0(0.0)	31(46.3)
−	−	20	65	0(0.0)	25(38.5)

* − : Not treated

lowest(22.1%)(Table 1). The mixed combination of 2% NaOCl and 3% H_2O_2 had a little positive effect in comparison with 2% NaOCl only. Chai(1993) also used 0.1% $HgCl_2$ for disinfestation of zoysiagrass stolons and had a good result.

2. Evaluation of 59 zoysiagrass lines for callus induction and E.C. formation

The first evidence of callus initiation from cultured zoysiagrass stolon was noted 5 to 10 days after incubation. The stolon segments, that were placed on MS medium containing 2.0mg/L 2,4-D in dark at 28℃, produced white to yellowish callus a month later. Two types of calli were obtained; embryogenic callus(E.C.) with yellowish or sometimes white-opaque, nodular, compact and somatic embryo-like appearance(Fig. 4b), and non-embryogenic callus with white, transculent and friable appearance. In general, non-embryogenic callus grows better than embryogenic callus and embryogenic callus sometimes changes into non-embryogenic callus. Induction of embryogenic callus in zoysiagrass was also reported in mature caryopsis(Al-Khayri, 1989; Asano, 1989; Inokuma, 1993; Chai, 1993) and immature influorescence(Noh, 1995). The callus growth of *Z. japonica* and *Z. sinica* were generally better than that of *Z. matrella*. In test of the embryogenic callus formation in zoysiagrass lines, 'J 85-10', 'S_5', 'S_6', '$M_2 \times M_1$', 'Che-Ju', 'Japonica 1', '$M_1 \times S_8$', '$C_2 \times M_1$1' showed the good result in embryogenic callus formation. Out of 59 lines, Che-Ju, 'S_5', 'S_6', '$M_2 \times M_1$', 'Japonica 1', '$M_1 \times S_8$', 'FL-19', 'Koreana', '$J \times S_1$', and '$M_1 \times J$' showed

Table 2. Callus induction and embryogenic callus formation of 17 zoysiagrass lines using stolons collected in summer

Line	No. of inoculated explants	No. of callus induction(%)	E.C. formation**
52-22(24)	62	12(19.4)	+++
Che-ju*	42	24(57.1)	+++
M_1	16	3(18.8)	++
Japonica 1*	27	16(59.2)	++++
Japonica 2	39	16(40.0)	++
Ks	35	13(37.1)	++
Japonica 3	70	25(35.7)	++
$J \times S_1$*	20	12(60.0)	+++
$M_1 \times J$*	41	10(24.4)	++
$M_1 \times S_8$*	87	32(36.8)	+++
$S_1 \times J$	14	7(50.0)	+++
$s_4 \times M_2$	19	5(26.0)	+
$C_2 \times M_1$	32	17(53.1)	+++
S_5*	112	47(42.0)	+++
S_6*	51	25(49.0)	++++
$M_2 \times S_5$	41	6(14.6)	++
$J \times M_2$	10	2(20.0)	++

* Seven cell lines selected for breeding.

** ++++ : Very good , +++ : Good , ++ : Normal, + : Poor.

Table 3. Callus induction and embryogenic callus formation of 42 zoysiagrass lines using stolons collected in fall

Line	No. of inoculated explants	No. of callus induction(%)	E.C formation**
Japonica	12	1(8.3)	-
41-21(3)	9	2(22.2)	++
Sunbust	51	13(25.4)	++
Belt ZNF	23	9(39.1)	++
FL-19*	9	3(33.3)	-
J 85-10	47	30(63.8)	+++
K 184	15	4(26.6)	+
KSL 17	17	3(17.6)	+
S1 석모도	20	2(10.0)	+
Z. T	39	6(15.4)	++
14-21(6)F	7	3(42.0)	-
52-22(6)S	5	4(80.0)	-
Midwest	40	14(35.0)	++
FL-41	55	19(34.5)	++
JS 9-10	37	18(48.6)	+
Ms	67	43(64.2)	++++
Matrella	26	10(38.5)	++
Koreana*	15	7(46.7)	++
Sunrise	18	4(22.2)	+++
243	37	6(16.2)	++
245	45	12(26.6)	+++
$J \times M_1$	26	11(42.3)	+++
$M_1 \times S_1$	18	12(66.6)	++
$M_2 \times S_2$	21	8(38.1)	++
$M \times C_2$	5	2(40.0)	-
$M_2 \times M_1$*	115	35(30.4)	+++
$S_1 \times M_1$	60	18(30.0)	++
$S_5 \times M_1$	36	11(30.6)	+++
$C_2 \times M_2$	38	13(34.2)	++
$C_2 \times J$	54	21(38.8)	++
S_4	69	39(56.5)	+++
S_7	29	11(37.9)	+++
S_8	7	3(42.8)	-
S_9	50	34(68.0)	+++
$M_2 \times S_4$	34	10(29.4)	+++
$S_8 \times C_2$	127	24(18.9)	+
$J \times S_2$	64	24(37.5)	++
$J \times S_5$	19	5(26.3)	+++
$S_8 \times M_2$	26	8(30.7)	+
$S_5 \times M_2$	44	20(45.4)	++
S_3	33	8(24.2)	++
$J \times S_6$	103	35(33.9)	+++

* Three cell line selected for breeding.

** ++++ : Very good, +++ : Good, ++ : Normal, + : Poor, - : Data not shown.

good callus induction and embryogenic callus formation in culture, and fast stolon growth in field. Therefore, those ten lines were selected for the future breeding(Table 2, 3).

The callus induction with 2,4-D was better than with picloram in both 'Japonica 1' and '$C_2 \times S_5$'. The percentage of callus induction was the highest at 2mg /L for both 2,4-D and picloram(Fig. 1, 2). For most of cereal and grass species, 2,4-D was known necessary for initiating and maintaing callus(Al-Khayri *et al.*, 1989). Although kinetin supplements have been used to induce callus in grass species, it has not been shown to be a requirement (Green, 1978). Picloram is water soluble and more effective at low concentration than 2, 4-D. Therefore, picloram offers the potential for direct regeneration of plants from callus (Collins, Vian, and Phillips, 1978). While 2,4-D is normally used to induce callus in grasses, picloram can be used for the formation of embryogenic callus.

3. Effect of medium, temperature, light, stolon part, and internode position on callus induction

MS medium was the best for callus induction and growth in 'Japonica 1' while LS medium was the best for embryogenic callus and shoot formation(Table 4). The choice of a

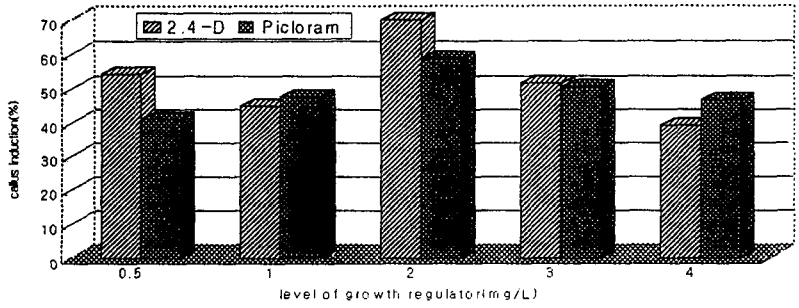

Fig. 1. Effects of growth regulators on callus induction of 'Japonica 1' stolons.

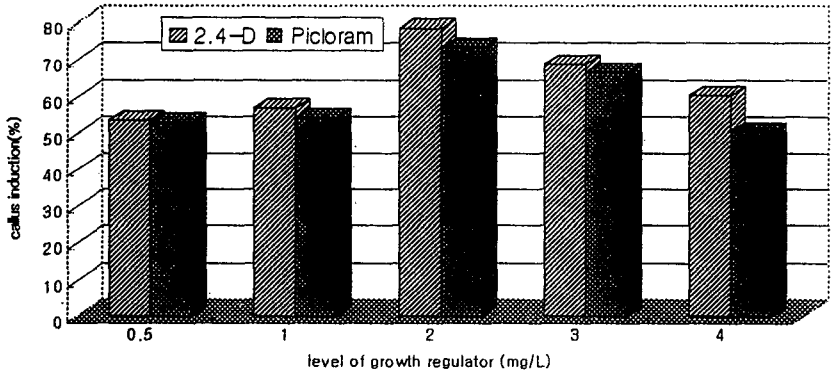

Fig. 2. Effects of growth regulators on callus induction of '$C_2 \times S_5$' stolons.

Table 4. Effect of medium on embryogenic callus formation from mature seeds in 'Japonica 1'

	Medium**		
	MS2D	LS2D	UM2D
No. of seeds	135	146	135
No. of callus(%)	49(36.3)	40(27.4)	34(25.2)
No. of green spot(%)	42(85.7)	37(92.5)	27(79.4)
No. of shoots(%)	29(59.2)	30(75.0)	23(67.6)
Callus length(cm)	4.24 ± 0.38*	3.81 ± 0.09	3.79 ± 0.24

* Data represent the mean values(±SE) of six replicates containing six explants each.

** MS2D : MS basal salt + 2,4-D 2mg /L
LS2D : LS basal salt + 2,4-D 2mg /L
UM2D : UM basal salt + 2,4-D 2mg /L

Table 5. Effect of light and stolon part on callus induction and E.C. formation in stolons of 'Japonica 1'

Light condition	Stolon part	No. of inoculated explants	No. of callus induction (%)	Callus size (cm)	No. of E.C. (%)	E.C. formation*
Light	Node	23	12(52.2)	0.16§	1(8.3)	+
	Internode	138	15(10.9)	0.25	4(26.6)	++
Dark	Node	29	19(65.5)	0.27	4(21.1)	++
	Internode	145	33(22.8)	0.43	14(42.4)	+++

* ++++ : Very good, +++ : Good, ++ : Normal, + : Poor, § Mean of 10 calli

Table 6. Effect of temperature on callus induction, E.C. formation and callus growth in 'Japonica 1'

Temperature	No. of inoculated explants	No. of callus induction(%)	E.C. formation	Callus size (cm)
25℃	63	14(22.2)	++	0.83 ± 0.09*
28℃	64	24(37.6)	+++	1.15 ± 0.16
31℃	61	23(37.8)	+++	1.05 ± 0.11

* Data represent the mean values(±SE) of four replicates containing 10 calli each.

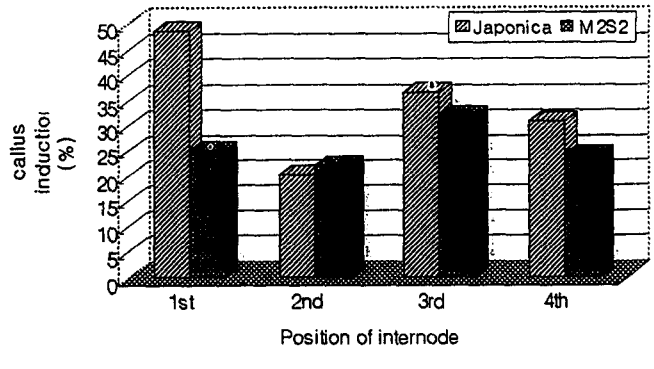

Fig. 3. Effect of the internode position on callus induction in 'Japonica 1' and '$M_2 \times S_2$'.

certain medium depends on the species of the plants, the tissue or organ to be cultured, and the purpose of experiment. One characteristic of MS medium is its relatively high concentration of nitrate, potassium, and ammonium ions in comparison with other media. While the node part as explant was better for callus inductiont than the internode in 'Japonica 1', the internode part was better for callus growth and embryogenic callus formation. Dark condition gave better callus induction and E.C. formation than light condition (Table 5). The same results were reported in many other grasses(Ahn *et al.*, 1987; Asano, 1989; Chai *et al.*, 1993; Denchev and Conger, 1994; Cardona and Duncan, 1997). Callus in-

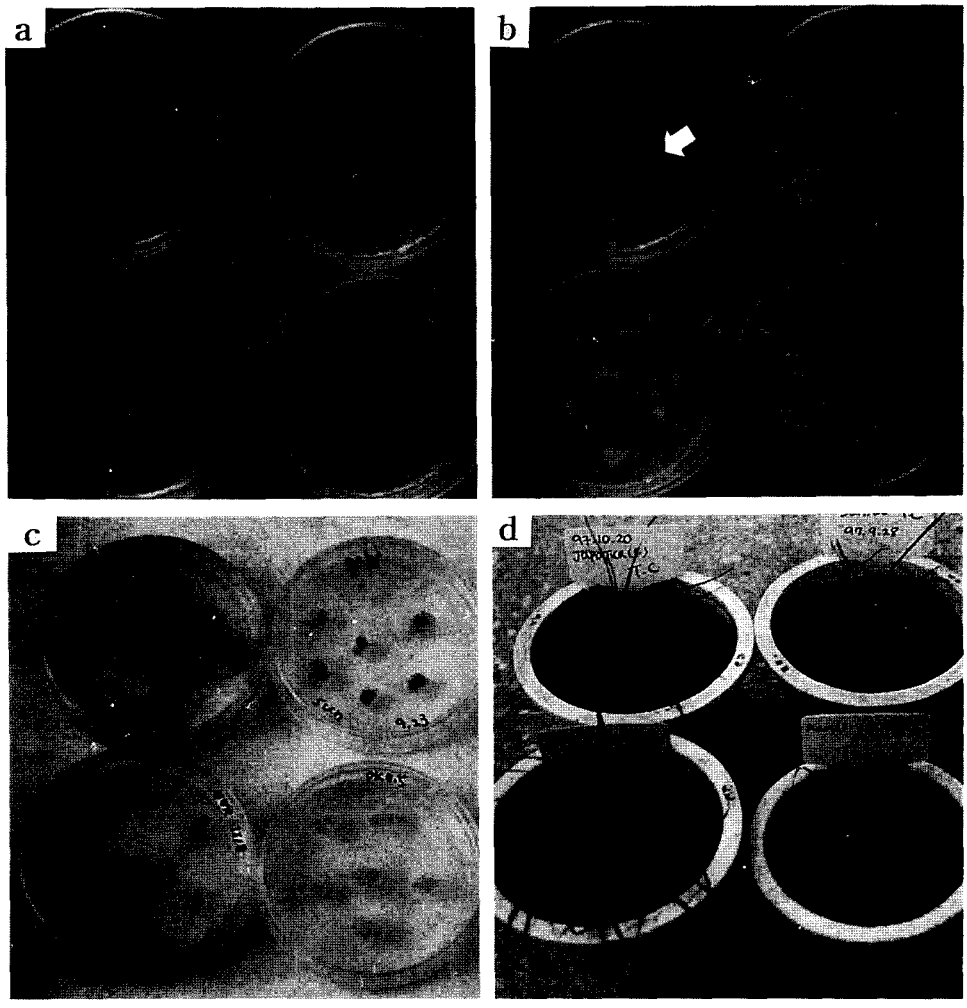

Fig. 4. Plant regeneration of zoysiagarss lines.
a) Callus-derived from stolon explant
b) Embryogenic callus proliferation and somatic embryo producing(arrow)
c) Shoot development from green spots
d) Plants grown one month in pots

duction and growth were better at 28, 31℃ than 25℃ in dark condition, and so did E.C. formation(Table 6). The first internode was the most effective in callus induction in 'Japonica 1', but the third internode was the best in '$M_2 \times S_2$'(Fig. 3).

적 요

본 실험은 한국잔디류(zoysiagrass) 59개 계통의 포복경을 통한 조직배양 반응성 차이와 절편체의 효과적 소독법 그리고 캘러스 유기 및 배발생 캘러스 형성에 미치는 배지, 식물생장조절물질, 광, 온도, 포복경 부위와 순서 등의 영향을 구명하여 한국잔디류의 육종을 위한 기초 자료로 삼고자 수행하였으며 그 결과는 다음과 같다.

1. 포복경 표면 소독에 0.1% 염화수은 처리가 가장 효율적이었다. 특히 0.1% 염화수은 15분간 처리에서 오염율 0%, 캘러스 유기율 46.6%로 가장 좋은 결과를 나타내었다.
2. Zoysiagrass 59개 계통에서 배발생 캘러스가 형성되었으며 *Z. japonica*와 *Z. sinica* 계통의 캘러스 유기율 및 배발생 캘러스 형성이 *matrella* 계통보다 더 높은 경향을 보였다. 특히 'J85-10', 'S_5', 'S_6', '$M_2 \times M_1$', 'Che-ju', 'Japonica 1', '$M_1 \times S_8$', '$C_2 \times M_1$' 등의 배발생 캘러스 형성이 매우 좋았으며 조직배양을 이용한 육종에 활용하기 위하여 조직배양 반응성과 포장에서의 포복경생육이 양호한 9계통의 세포주를 선발하였다.
3. 캘러스 유기를 위하여 2,4-D와 picloram 모두 2mg/L 농도에서 가장 좋았으며, 28, 31℃가 25℃보다 그리고 암조건이 명조건보다 더 적합한 것으로 나타났다.
4. 절편체는 node부위가 internode보다 캘러스 유기율이 더 높았으나 캘러스 생장 및 배발생 캘러스 형성을 위하여는 internode부위가 더 좋은 경향을 보였다. 특히 'Japonica 1'의 경우 첫 번째 internode에서 48.8%, '$M_2 \times S_2$'의 경우 3번째 internode에서 32.0%의 가장 높은 캘러스 유기율을 보였다.

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