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Influence of Antibiotics on Shoot Regeneration and *Agrobacteium* Suppression Using Cotyledonary Node in Korean Soybean Cultivars

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ABSTRACT Mature dry seeds of Korean cultivars, Daepungkong, Muhankong, Myeongjunamulkong, Somyeongkong, Sowonkong, Jinpumkong, and Pungsannamulkong were used. The influence of antibiotics on elimination of *Agrobacterium* growth and shoot regeneration was estimated with cotyledonary node. Cefotaxime and timentin at the concentration of 250 and 500 mg/l suppressed *Agrobacterium*, especially cefotaxime was an efficient antibiotic to suppress *Agrobacterium* in all cultivars. While carbenicillin and timentin at the concentration of 50 and 100 mg/l were not sufficient to control the development of *Agrobacterium*, respectively. Cefotaxime and timentin represented high shoot formation rates compared with carbenicillin. Carbenicillin at low concentrations did not effectively suppress *Agrobacterium* and also had no effect on shoot development. Cefotaxime at the concentration of 250 mg/l showed maximum frequency of shoot regeneration in cvs. Somyeongkong and Sowonkong. Furthermore, on medium containing cefotaxime, shoot was more quickly formed than the other antibiotics. The use of cefotaxime was very useful for elimination of *Agrobacterium* growth with cotyledonary node of Korean soybean cultivars.

Keywords : antibiotics, soybean, cotyledonary node, regeneration, *Agrobacterium* suppression

Cotyledonary node was reported as a useful explant in soybean regeneration (Cheng *et al*., 1980). So far various explants have used for plant regeneration in soybean, such as immature cotyledon embryos (Barwale *et al*., 1986), cotyledonary node (Cheng *et al*., 1980; Wright *et al*., 1986; Barwale *et al*., 1986; Margie *et al*., 2004; Margie *et al*., 2006), seedling shoot tip (Kartha *et al*., 1981), embryo axes (McCabe *et al*., 1988; Liu *et al*., 2004), cotyledons (Franklin *et al*., 2004), primary leaf tissue (Wright *et al*., 1987), and whole cotyledonary node (Ma & Wu, 2008). Later, researchers tried to produce new breeding materials by using two transformation methods. The first method was the use of particle bombardment with somatic embryogenic tissue (Sato *et al*., 1993; Droste *et al*., 2002) and the second was *Agrobacterium*-mediated transformation with cotyledonary node tissue (Trick & Finer, 1998; Santarem *et al*., 1998; Olhoft & Somers, 2001; Olhoft *et al*., 2003). The first transgenic soybean plants using *Agrobacterium*-mediated transformation were obtained from cotyledonary node tissue (Hinchee *et al*., 1988). Olhoft & Somers (2001) achieved that utilization of thiol compounds (L-cysteine, dithiothreitol, and sodium thiosulfate) during co-cultivation results in a significantly increased transformation rate.

The improvement of an effective *Agrobacterium* transformation depends on several factors including plant genotype, explants vigor, *Agrobacterium* strain, vector, and co-cultivation length. After co-cultivation, the bacteria suppression is necessary, because microbial contaminants in cultured plants can meddle with multiplication and rooting rates or induce a plant death (Mayolo *et al*., 2003). For eliminating them, plant explants are transferred to medium containing some antibiotics. Carbenicillin, cefotaxime, and timentin are widely used as antibiotics. The effects of these antibiotics have been studied on different plant species. Carbenicillin (500 mg/l) suppressed shoot formation and improved callus induction (Yepes & Aldwinckle, 1994). Pollock *et al*. (1983) reported that carbenicillin was relatively non toxic to protoplast-derived cells of *Nicotiana* and rather more resistant to betalactamases but less stable in acid. Meanwhile, carbenicillin reduced tissue growth by 50% of Skita spruce (Sarma *et al*., 1995). Cefotaxime exerted a stimulatory action

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on wheat tissues (Mathias & Boyd, 1986; Borelli *et al*., 1992) and barley culture (Mathias & Mukasa, 1987). In cotton embryo axes, cefotaxime stimulated shoot formation depending on the cultivars (Agrawal *et al*., 1998). Cheng *et al*. (1998) and Ling *et al*. (1998) have suggested timentin is an effective antibiotic in suppressing *Agrobacterium tumefaciens* in tobacco and tomato transformation. Timentin is also more economical than cefotaxime because it is sufficient a small amount due to its high activity against *Agrobacterium* and high efficiency of shoot regeneration in wheat (Han *et al*., 2007). In this paper, we compared the effects of various antibiotics on eliminating *Agrobacterium tumefaciens* and shoot regeneration using the cotyledonary node of Korean soybean cultivars.

MATERIALS AND METHODS

Plant materials and explants preparation

Mature dry seeds of Korean cultivars, Daepungkong, Muhankong, Myeongjunamulkong, Somyeongkong, Sowonkong, Jinpumkong, and Pungsannamulkong were used. Soybean seeds were surface sterilized with 70% ethanol for 1 min, 70% Clorox containing Tween 20 for 30 min and then rinsed in sterile distilled water three times. The sterilized seeds were germinated on germination medium (GM, Table 1) for 7 days at 25℃ in the 16 h light / 8 h dark condition, or until the cotyledons turned green but before the first leaves were visible. Cotyledonary nodes were collected from seedlings. The roots and the hypocotyls were removed approximately 5 mm below the cotyledonary node by cutting the hypocotyls with a sterile surgical blade. Two explants were obtained by separating the cotyledons and removing the epicotyls and axillary buds 1 mm above the cotyledonary node. Then the fragment between the cotyledon and the hypocotyls was wounded by cutting several times with the blade vertical to the hypocotyls.

Agrobacterium strain and infection

Agrobacterium tumefaciens, KYRT1 containing the plasmid pCAMBIA1305.1 (CAMBIA, Australia) was used. This plasmid contains the coding sequence of the hygromycin resistance gene (*hpt*Ⅱ) and the *β*-glucuronidase (*gus*A) gene. Liquid LB medium containing 100 mg/l of kanamycin, 100 mg/l of rifampicin, and 400 mg/l of L-cysteine was inoculated with a single colony of bacteria and shaken at 28℃ (240 rpm) until the OD_{600} reached 0.8~1.0. The 200 μM of

Table 1. Media components for mature cotyledonary nodes culture in Korean soybean

Component	Germination medium (/L) (GM)	Co-cultivation medium (/L) (CCM)	Shoot induction medium (/L) (SIM)	Shoot elongation medium (/L) (SEM)	Rooting medium (/L) (RM)
MS (Murashige and Skoog 1962) salts & B5 vitamins (Gamborg *et al*., 1968)	4.3 g	4.3 g	4.3 g	4.3 g	2.15 g
Sucrose	30 g	30 g	30 g	30 g	20 g
MES	-	3.9 g	0.59 g	0.59 g	0.59 g
Asparagine	-	-	-	50 mg	-
Glutamine	-	-	-	100 mg	-
pH adjusted to	5.8	5.4	5.6	5.6	5.6
Phytoagar	7 g	7 g	7 g	7 g	7 g
			pH adjusted then autoclaved		
BAP	-	1.67 mg	1.67 mg	1.67 mg	-
GA3	-	0.25 mg	-	0.5 mg	-
IAA	-	-	-	0.1 mg	-
Zeatin	-	-	-	1 mg	-
IBA	-	-	-	-	1 mg
Acetosyringone	-	200 μM	-	-	-
Timentin	-	-	0, 50, 100, 250 & 500 mg	-	-

acetosyringone was added to the cell suspension at 2 h before inoculation. Cotyledonary nodes were immersed in the *Agrobacterium* suspension for 30 min and then co-cultured on co-cultivation medium (CCM, Table 1). Surplus bacteria were cleared with sterilized filter paper. The explants were placed adaxial side down on the co-cultivation medium at 25℃ for 5 days in the dark. And the non-inoculated explants were immersed into liquid CCM for 30 min, cultured on CCM for 5 days.

Antibiotics on shoot formation

Three antibiotics, carbenicillin, cefotaxime, and timentin (ticarcillin / clavulanate potassium = 15:1) were used. To determine the optimum levels of antibiotics to eliminate *Agrobacterium* cells on shoot induction medium (SIM, Table 1), different concentration of antibiotics was tested. Concentration at 0, 50, 100, 250, and 500 mg/l of carbenicillin, cefotaxime, and timentin were used. All antibiotics were dissolved in sterile distilled water, filter-sterilized through a 0.20 μm membrane and added to the shoot induction medium after autoclaving. After co-cultivation, the explants were transferred to each shoot induction medium containing different concentrations of antibiotics (Table 1) and cultured for 4 weeks. The explants were subcultured to fresh SIM. Some of the cotyledonary nodes were coated with Agrobacteria and dead. For suppression rate, the percentage of surviving green cotyledonary nodes per tested cotyledonary nodes was estimated. Following 4 weeks on SIM, green cotyledonary nodes were transferred to shoot elongation medium (SEM) without antibiotics (Table 1). After shoots elongated, the shoots clusters were separated and cultured on rooting medium (RM, Table 1).

Statistical analysis

Data were analyzed by ANOVA (analysis of variance). The means were compared using the Duncan's Multiple Range Test at $P<0.05$.

RESULTS AND DISCUSSION

Effect of antibiotics on suppression of *Agrobacterium*

The three antibiotics, carbenicillin, cefotaxime, and timentin were used to examine the effect on suppression of *Agrobacterium tumefaciens*. At a concentration of 50 and 100 mg/l, carbenicillin and timentin were not sufficient to control the growth of the *Agrobacterium*, whereas cefotaxime and timentin at 250 and 500 mg/l suppressed *Agrobacterium* growth, especially cefotaxime was an efficient antibiotic to suppress *Agrobacterium* in all cultivars except cv. Sowonkong (Table 2). In cv. Sowonkong, timentin at 250 and 500 mg/l

Table 2. Suppression rate of *Agrobacterium tumefaciens* by antibiotic treatments in Korean soybean cultivars

Antibiotics	Concentration (mg/l)	Daepungkong		Muhankong		Myeongjunamulkong		Somyeongkong		Sowonkong		Jinpumkong		Pungsannamulkong	
		No infection	Infection	No infection	Infection	No infection	Infection	No infection	Infection	No infection	Infection	No infection	Infection	No infection	Infection
Free antibiotic	0	50.0ab†	39.3f	52.8a	39.4g	55.6a	30.6f	52.9a	35.3g	57.6a	36.1f	47.2a	31.4g	60.6a	31.1g
Carbenicillin	50	38.9def	40.9f	40.0de	44.4f	42.4c	47.2e	39.0def	41.2f	41.2cd	42.3e	40.4cd	42.3f	41.8de	39.3f
	100	38.5def	47.8ef	39.0de	56.5e	39.4def	55.6d	35.0f	51.4e	39.4cd	46.3de	39.0cd	55.3d	40.6de	47.5e
	250	31.9f	68.2de	34.8e	76.9d	34.3f	62.7d	36.2ef	68.1c	32.9d	68.0b	38.3cd	60.3c	33.9f	70.3d
	500	30.4g	82.1bc	29.8f	84.8c	29.4g	73.2c	29.2g	71.4c	30.8e	76.3a	28.3e	62.4c	29.8g	82.6b
Cefotaxime	50	52.8a	80.0c	50.0ab	54.5e	45.8b	33.3f	50.0ab	50.0e	51.7a	50.0de	45.2ab	42.9ef	46.9b	48.5e
	100	46.2bc	83.8bc	48.6bc	54.3e	41.7de	75.0c	46.9bc	62.5d	50.8ab	51.6de	41.5cd	68.3b	46.3bc	73.1d
	250	42.1cde	92.1a	45.9bcd	89.2b	42.3cd	84.6b	41.5def	81.3a	41.4cd	72.3b	33.3d	92.9a	42.6cde	95.0a
	500	36.8ef	94.7a	37.8de	97.3a	36.7def	96.2a	39.1def	90.6a	38.8cd	78.7a	35.7d	95.2a	37.0f	91.0a
Timentin	50	42.6cd	40.9f	42.2cd	48.5f	38.9def	45.8e	42.9cd	59.3de	43.3bc	53.3d	42.9bc	44.8e	44.4cd	67.9d
	100	41.7def	55.0de	41.4de	57.1e	34.3f	66.7cd	41.9de	64.3d	39.7cd	58.3c	39.1cd	58.3c	43.9cde	78.1c
	250	37.5def	77.3c	36.2e	87.5b	32.4f	81.2b	37.8def	76.2b	34.3d	89.2a	30.4e	87.5a	40.4de	90.2a
	500	33.3f	89.3a	32.6e	80.2c	30.3g	84.0b	31.9f	86.7a	32.7d	85.7a	27.1e	89.1a	34.5f	88.3a

†Different letters within a column indicate significant differences for antibiotics at P<0.05 using Duncan' s Multiple Range Test

had the highest frequency of growth suppression. Timentin was previously reported as efficiently eliminating *Agrobacterium* at 50~250 mg/l in tobacco, tomato, and wheat (Nauerby *et al*., 1997; Tang *et al*., 2000; Han *et al*., 2007). Overall, *Agrobacterium* did not grow on medium with cefotaxime and timentin at 250 and 500 mg/l (Fig. 1).

Effect of antibiotics on regeneration

Cefotaxime and timentin showed high shoot regeneration rates compared with carbenicillin (Fig. 2). Cefotaxime at a concentration of 250 mg/l showed the maximum frequency of shoot regeneration (53.1% and 60.5%) in cvs. Somyeongkong and Sowonkong. Shoot development was promoted in

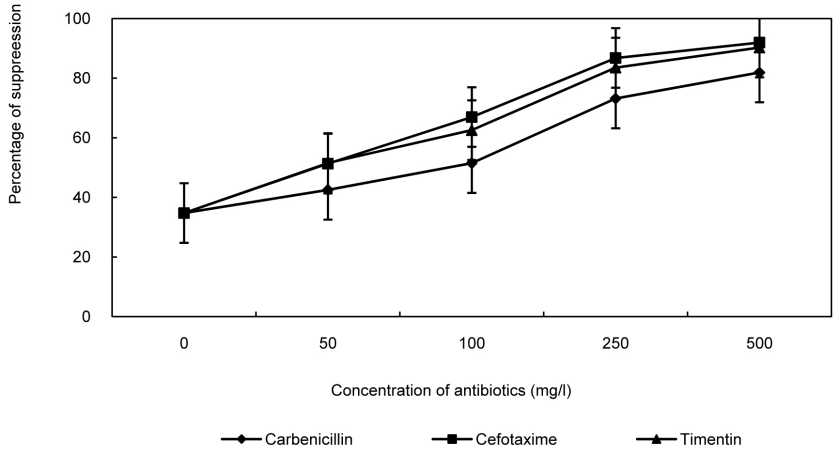

Fig. 1. Suppression of *Agrobacterium tumefaciens* on cotyledonary node by different antibiotic treatments. The suppression of *A. tumefaciens* was screened after 4 weeks on the SIM (Shoot Induction Medium). The percentage of surviving green cotyledonary nodes per tested cotyledonary nodes was evaluated.

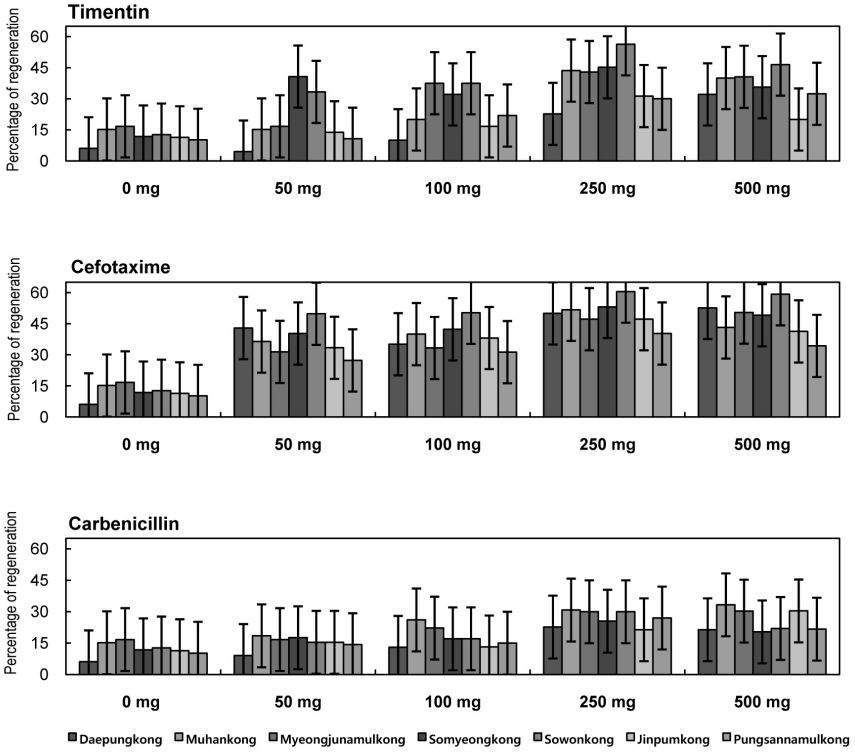

Fig. 2. Regeneration rate with various concentrations of antibiotics in Korean soybean cultivars.

medium containing cefotaxime, especially above the concentration of 250 and 500 mg/l. This result indicated that the shoot development rate at high concentration of cefotaxime was higher than in low concentration of cefotaxime in this experiment. Meanwhile low concentration (60~100 mg/l) of cefotaxime enhanced callus and shoot formation in wheat (Mathias & Boyd 1986). Danilova & Dolgikh (2004) reported that cefotaxime did not affect the production of embryogenic callus but vigorously stimulated the development of regenerated plants. They grant a commendation to treat embryogenic maize callus with the cefotaxime in order to improve plant regeneration.

Timentin showed a high shoot formation rate at a concentration of 250 mg/l compared to other concentrations (Fig. 2). Han *et al*. (2007) reported that timentin is an efficient antibiotic for elimination of *Agrobacterium* growth at a concentration 50 mg/l and for shoot regeneration at a concentration of 500 mg/l in wheat embryos. And they also reported that timentin accelerated more green spots per callus. Timentin, which is ticarcillin coupled with a specific β-lactamase inhibitor, clavulanic acid, was reported efficient in suppressing *Agrobacterium* from tobacco leaf explants (Cheng *et al*. 1998). A similar effect of timentin (150 mg/l) on cotyledon explants in tomato was shown enough to suppress *Agrobacterium tumefaciens* and shoot formation was significantly prompted on medium with timentin (Ling *et al*., 1998). And Tang *et al*. (2000) also showed that timentin was most effective in eliminating the *Agrobacterium*. They indicated that timentin was better than carbenicillin and ampicillin for reducing *Agrobacterium* from walnut somatic embryos.

Cefotaxime showed the multiple shoots compared with timentin (Fig. 3). Almost all Korean soybean cultivars developed multiple shoots at 250 mg/l of cefotaxime. Shacklford & Chlan (1996) reported that cefotaxime was the most effective antibiotic at eliminating LBA4404 *Agrobacterium* strain from tobacco explants. Additionally, cefotaxime was reported as effectively promoting somatic embryogenesis in some *Dianthus* cultivars at 100~500 mg/l (Nakano & Mii 1993).

Carbenicillin at low concentrations did not effectively suppress *Agrobacterium* and also lower concentration (lower than 250 mg/l) had not effect on shoot regeneration. Similar result in soybean transformation using immature cotyledonary node was reported by Wiebke *et al*. (2006). Cefotaxime and carbenicillin belong to antibiotics, cephalosporins and penicillins, repectively. These are known as β-lactams antibiotics, prevent bacteria proliferation by inhibiting cell wall synthesis during its division (Pollock *et al*., 1983). Nauerby *et al*. (1997) reported that antibiotic binding prevents cell wall synthesis and provokes the death of the bacteria by cell wall lysis. They showed that cefotaxime and carbencillin are active antibiotics against many kinds of bacteria. And cefotaxime is highly resistant while carbenicillin is sensitive to β-lactamases produced by *Agrobacterium*.

In conclusion, cefotaxime at the concentration of 250 and 500 mg/l was more effective than timentin and carbenicillin for eliminating *Agrobacterium* growth from mature cotyledonary node explants in Korean soybean cultivars. This antibiotic showed that multiple shoots formation was higher in the treatment with cefotaxime (250 mg/l) than with timentin and carbenicillin. In addition, shoot on medium with cefotaxime was formed more quickly than the other antibiotics.

Fig. 3. Shoot formation using *Agrobacterium*-mediated transformation of mature cotyledonary node explants from Korean soybean cultivars. **A**; Shoot elongation at 250 mg/l of cefotaxime for 2 weeks in cv. Sowonkong, **B**; Shoot development at cefotaxime of 250 mg/l for 4 weeks in cv. Somyeongkong, **C**; Shoot formation in medium containing 250 mg/l of timentin for 4 weeks in cv. Somyeongkong.

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