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Effects of carbenicillin and cefotaxime on callus growth and somatic embryogenesis from adventitious roots of papaya

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Abstract. Carbenicillin and cefotaxime, two antibiotics commonly used for excluding *Agrobacterium tumefaciens* during plant transformation, were tested for their bacteriostatic effects as well as for their effects on plant regeneration in adventitious root explants of papaya following co-culture with *Agrobacterium.* A washing step with sterilized distilled water two days after co-culture enhanced the bacteria-suppressing effects of antibiotics. Proliferation of *Agrobacterium* was completely suppressed in the medium containing 125 mgl^{-1} carbenicillin or cefotaxime. Callus fresh weight increase was apparently enhanced in the media with higher concentrations of carbenicillin (250-500 mgl^{-1}), but was extremely inhibited in media with the same concentrations of cefotaxime. Higher percentages of somatic embryos were found in the medium with 125 mgl^{-1} carbenicillin or 250 mgl^{-1} cefotaxime; however larger numbers of somatic embryos from the individual callus were obtained in the medium with 125 mgl^{-1} carbenicillin than in the medium with 250 mgl^{-1} cefotaxime. Percentages of abnormal somatic embryos were lower in the medium with lower concentrations of carbenicillin (125-250 mgl^{-1}). Favorable conditions for use of the two antibiotics for suppressing bacteria growth and enhancing regeneration of papaya plantlets from adventitious roots are discussed.

Keywords: Antibiotics; Bacteriostatic effect; Somatic embryogenesis.

Introduction

Considerable progress has been made in plant transformation during the last decade, using in many instances *Agrobacterium* mediation (Nauerby et al., 1997). For *Agrobacterium*-mediated DNA transfer, antibiotic use in plant tissue culture has become routine. Antibiotics are added to culture media to control *Agrobacterium* that may affect the plant regeneration process and to select transformants with an antibiotic-resistance that is cotransferred with the gene of interest (Shaw et al., 1983). Although many antibiotics have been described for effective control of *Agrobacterium* cells, carbenicillin and cefotaxime, both belonging to the β-lactam group, have minimal toxicity on most plant tissues (Mathias and Boyd, 1986) and thus have become most widely accepted in *Agrobacterium*-mediated transformation. However, both antibiotics have been known to have plant hormone-like effects on cultured plant tissues and could affect somatic embryogenesis in many plant species (Nauerby et al., 1997).

For *Agrobacterium* mediated gene transfer in papaya, carbenicillin (Fitch et al., 1990; 1993; Yang et al., 1996; Cheng et al., 1996) and cefotaxime (Fitch et al., 1993; Cabrera-Ponce et al., 1996) are often added to the medium during plant regeneration to control *Agrobacterium* growth, but information about their effects on development of callus and somatic embrys of papaya has not been considered.

Immature embryos are commonly used for papaya transformation (Fitch et al., 1990; Cheng et al., 1996). However, their use is time consuming and hindered by a seasonal factor. Moreover, the desired sex and other cultivar traits of transgenic lines can only be determined several months after planting. Recently, we established an efficient system for inducing adventitious roots from in vitro shoots, derived from selected hermaphrodite plants with good horticultural qualities (Yu et al., 2000). The adventitious roots thus obtained can regenerate somatic embryos within 4 months. Regeneration from adventitious roots avoids the disadvantages of immature embryos. However the parameters for *Agrobacterium*-mediated transformation using adventitious roots have remained unestablished. In this investigation, root segments were co-cultured with *Agrobacterium*, then treated with carbenicillin and cefotaxime to investigate the antibiotic effects. Also, the effects of carbenicillin and cefotaxime on induction of callus, initiation of somatic embryos, and germination of somatic embryos during the plant regeneration process from papaya root segments were investigated.

Materials and Methods

Plant Materials and Culture Conditions

Source of adventitious roots: Individual shoots (>5 mm) with 2-3 leaves from multiple shoots of papaya (*Carica papaya* L. cv. Tainung No. 2) that arose in vitro

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were placed in root induction medium for 1 wk in darkness, then transferred to sterile perlite with 1/2 MS solution for two wk for root growth (Yu et al., 2000). The adventitious roots that emerged were cut into 5 mm segments and employed as explants.

Culture media: The basal medium consisted of MS salts (Murashige and Skoog, 1962), B_5 vitamins (Gamborg et al., 1968), 3% sucrose, and 0.8% agar. The root induction medium contained 0.5 mgl^{-1} indole-3-butyric acid (IBA). The medium for callus formation and somatic embryogenesis (CSM) included 0.25 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mgl^{-1} 6-bezyladenine (BA). The medium for germination of somatic embryos (SGM) was supplemented with 0.02 mgl^{-1} α-naphthaleneacetic acid (NAA) and 0.2 mgl^{-1} BA (Yang and Ye, 1992). The pH of all the media was adjusted to 5.7 ± 0.1 with 1 N KOH before autoclaving at 1.1 kg cm^{-2} (121°C) for 20 min. Prescribed volumes of concentrated solutions of filter-sterilized (0.22 μm membrane filter) carbenicillin and cefotaxime (Sigma Chemical Co, St. Louis. MO) were added to autoclaved media.

Culture conditions: All explants were cultured in a growth chamber at 28 ± 1°C, under darkness, for induction of callus and development of somatic embryos. For germination of embryos, cool white florescent lamps were provided 14 h daily at an intensity of 53 $\mu Em^{-2}s^{-1}$.

Bacteriostatic Effects of Antibiotics on Agrobacterium tumefaciens

Bacteriostatic effects of the antibiotics were tested on *Agrobacterium tumefaciens* LBA 4404 that had been cultured in LB medium (10 gl^{-1} tryptone, 5 gl^{-1} yeast extracts and 10 gl^{-1} NaCl) containing 100 mgl^{-1} streptomycin, at 28°C overnight. Root segments were dipped into the *Agrobacterium* suspension and co-cultured on CSM medium for two days. To reduce the bacterial density, segments were washed with autoclaved distilled water (SDW) or a 500 mgl^{-1} carbenicillin or cefotaxime solution under gentle shaking for 3 min, blotted with filter paper, then transferred to CSM medium with 0, 62.5, 125, 375 or 500 mgl^{-1} carbenicillin or cefotaxime for 4 wk. They were evaluated by counting the development of bacterial colonies around the root segments. Bacterial growth was recorded either as negative or positive, based on presence or absence of colonies. The bacteriostatic effects were determined as percentages of root segments without bacterial colonies.

Effects on Callus Formation and Somatic Embryogenesis

To investigate the effects of carbenicillin and cefotaxime on the formation of callus and somatic embryos in root segments, segments of the roots were cultured on CSM medium containing 0, 125, 375, 500 mgl^{-1} carbenicillin or cefotaxime. Percentages of callus forming segments were determined. Fresh weights of callus were also measured 4 wk after incubation. To compare the effects of carbenicillin and cefotaxime on somatic embryogenesis, the percentages of explants that produced somatic embryos were determined after 8, 12, and 16 wk of culturing in CSM medium. They were based on the numbers of callus with at least one embryo.

Effects on Germination of Somatic Embryos

The effects of carbenicillin and cefotaxime on germination were evaluated by transferring the embryos that emerged in CSM medium, with different concentrations of antibiotics, to SGM medium containing the same concentrations of carbenicillin or cefotaxime. Percentages of somatic embryos that germinated were determined after 4 wk.

Results and Discussion

Bacteriostatic Effects of Antibiotics on Agrobacterium tumefaciens

The antibacterial effects of carbenicillin or cefotaxime on adventitious root segments of papaya two days after co-culture with *A. tumefaciens* are shown in Figure 1. Explants were transferred to CSM medium with antibiotics after washing with SDW or a 500 mgl^{-1} carbenicillin or

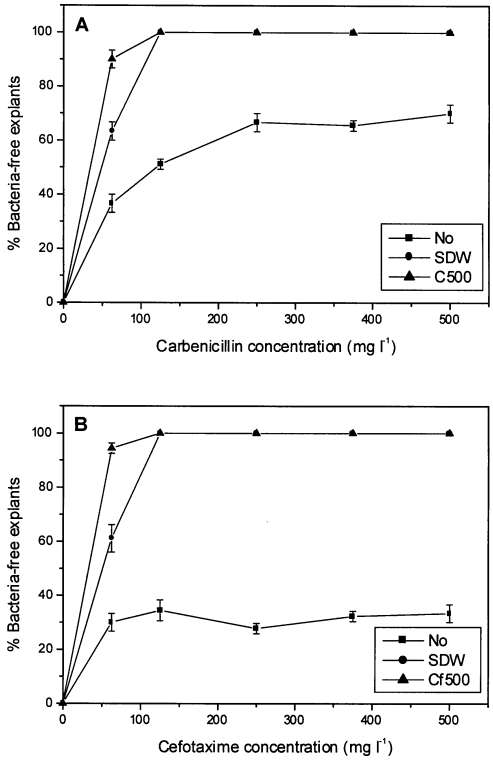

Figure 1. Antibacterial effects of carbenicillin (A) and cefotaxime (B) on adventitious roots of papaya after co-culture with *Agrobacterium tumefaciens*. No: root segments were not washed two days after co-culture and directly transferred to the medium. SDW: washed with sterile distilled water for 3 min; C500 and Cf500: washed with a solution of 500 mgl^{-1} carbenicillin and cefotaxime, respectively. Values represent means from 3 replicates with a total of 90 explants for each treatment; bar = ± S.D.

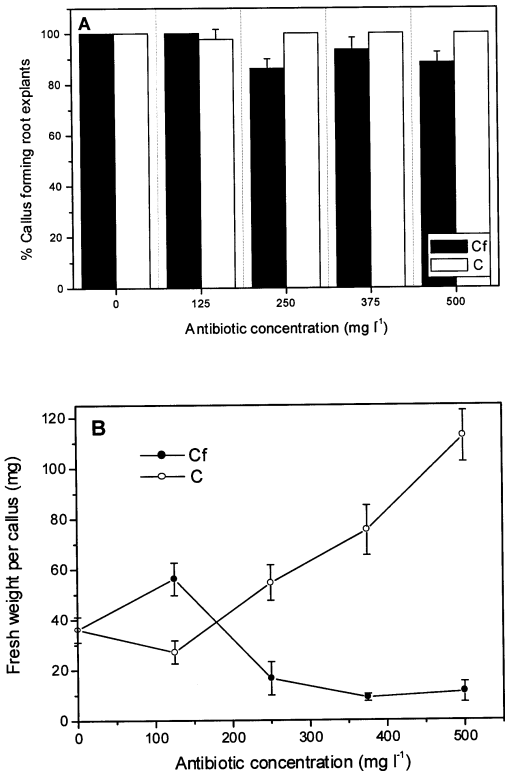

Figure 2. Effects of different concentrations of antibiotics on the percentage of callus forming root explants (A) and fresh weight (B) of adventitious root segments of papaya after culturing on CSM medium for 4 wk. C: carbenicillin; Cf: cefotaxime. Values represent means from 3 replicates with a total of 90 explants for each treatment; bar = ± S.D.

cefotaxime solution. Bacteria were completely eliminated by carbenicillin or cefotaxime when included in concentrations of 125 mgl^{-1} and higher. When co-cultured tissues were unwashed, exclusion of bacteria was not complete in any concentration of carbenicillin or cefotaxime.

Agrobacterium tumefaciens LBA 4404 has been the preferred strain as transformation vector since it is easy to control, with complete inhibition requiring only 10 mgl^{-1} carbenicillin or 5 mgl^{-1} cefotaxime in suspension cultures (Lin et al., 1995). However, higher concentrations of carbenicillin or cefotaxime, 250-500 mgl^{-1}, have been more widely adapted for many plant tissue cultures, e.g., *Arabidopsis thaliana* (Akama et al., 1992) and *C. papaya* (Fitch et al., 1993; Cabrera-Ponce et al., 1996; Cheng et al., 1996; Yang et al., 1996). Our results indicate that washing with antibiotic solutions or SDW two days after co-culture enhances bacteria elimination significantly. *Agrobacterium* cells were completely inhibited by 125 mgl^{-1} carbenicillin or cefotaxime when preceded by washing. This is apparently due to the reduction of the bacterial density by the washing process. Since the effects of washing with 500 mgl^{-1} of carbenicillin or cefotaxime were not significantly different from that using only SDW, the use of the antibiotic solutions for washing seems unnecessary.

Effects on Callus Formation

The rates of callus formation 4 wk after culturing root segments on CSM medium containing antibiotics are summarized in Figure 2. Percentages of callus forming explant were not significantly different among media with varying concentrations of carbenicillin. Values in all treatments approached 100% (Figure 2A). In cefotaxime treatments, a slight suppression of callus formation occurred in media with 250 mgl^{-1} and higher cefotaxime concentrations (Figure 2A).

As evident in Figure 2B, the fresh weight of callus was increased by carbenicillin concentrations of 250 mgl^{-1} and higher. The fresh weights increased 0.5-2.1 times (54.5-112.5 mg per callus) between 250 mgl^{-1} and 500 mgl^{-1}. In media containing cefotaxime, fresh weight of callus (56.1 mg per callus) increased slighly in the medium with 125 mgl^{-1} of the antibiotic, then decreased considerably in all higher concentrations (16.5-8.9 mg per callus).

Cefotaxime in a concentration range of 60-100 mgl^{-1} has been previously reported to enhance the callus growth, embryogenesis, and regeneration of wheat (Mathias and Boyd, 1986). In *Antirrhinum majus* culture, carbenicillin (250-500 mgl^{-1}) stimulates callus growth and has little impact on shoot production, while cefotaxime has no effctect on callus formation but reduces shoot and root formation

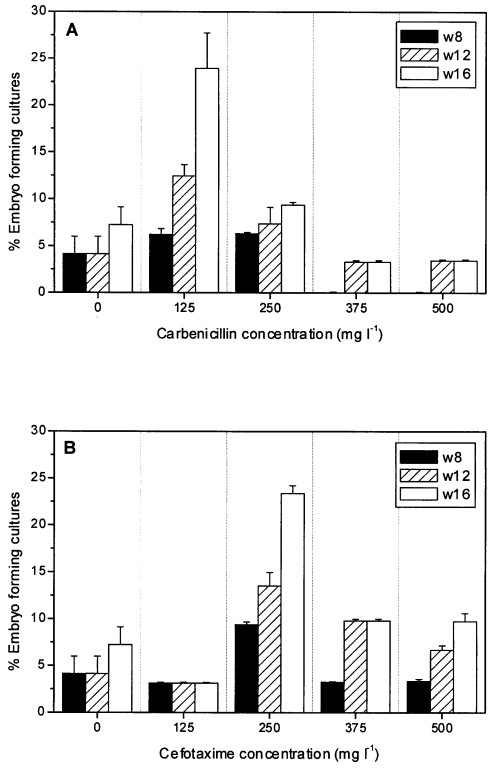

Figure 3. Effects of carbenicillin (A) and cefotaxime (B) concentrations on formation of somatic embryos in adventitious root segments of papaya after varying periods of culturing on CSM medium (w8: 8 wk culture, w12: 12 wk culture, w16: 16 wk culture). Values represent means from 3 replicates with a total of 90 explants for each treatment; bar = ± S.D.

(Holford and Newbury, 1992). Our findings indicate that growth of papaya root-derived callus is enhanced by carbenicillin in concentrations above 125 mgl^{-1} and is inhibited by cefotaxime in the same concentration range.

Effects on Somatic Embryogenesis

Percentages of somatic embryo-forming callus in CSM media, supplemented with carbenicillin or cefotaxime, are summarized in Figure 3. In carbenicillin media, the highest frequency of embryogenic callus occurred at 125 mgl^{-1} carbenicillin, with 6.2, 12.4 and 23.9% of callus forming somatic embryos after 8, 12 and 16 wk, respectively. No significant differences were observed between the medium with 250 mgl^{-1} and without carbenicillin after 12 or 16 wk of culturing. On the other hand, the percentages of somatic embryo formed were significantly reduced in media containing 375-500 mgl^{-1} carbenicillin (Figure 3A).

Embryogenesis was apparently promoted when root segments were cultured in the medium containing 250 mgl^{-1} cefotaxime for 8 wk (9.4%), 12 wk (13.5%), and 16 wk (23.4%). No significant differences emerged among the 0, 375, and 500 mgl^{-1}cefotaxime treatments (7.3-10.7%) after 16 wk, although somatic embryogenesis was lower in the medium containing 125 mgl^{-1} cefotaxime (3.1%) (Figure 3B).

With either carbenicillin or cefotaxime, the first somatic embryo was observed after culturing for 8 wk, and numbers of somatic embryos increased with further culture. Most somatic embryos were separated and detached from callus in the medium containing 125 mgl^{-1} carbenicillin (Figure 4A). Somatic embryos were fewer in the medium with 250 mgl^{-1} cefotaxime than in the medium with 125 mgl^{-1} carbenicillin, and they were aggregated and not easily detachable (Figure 4B).

Cefotaxime (100-500 mgl^{-1}) has been reported as most effectively promoting somatic embryogenesis of *Dianthus* cultivars, and carbenicillin (250-500 mgl^{-1}) has been less effective (Nakano and Mii, 1993). Yepes and Aldwinckle (1994) observed an enhancement of regeneration and shoot development in apple tissue culture by a 250 mgl^{-1} concentration of cefotaxime, whereas carbenicillin at a dose of 500 mgl^{-1} stimulated callus development and inhibited regeneration. Lin et al. (1995) proposed that carbenicillin has auxin-like functional structures, i.e., like 2,4-D or NAA, and attributed the toxic effects of combinations of carbenicillin and 2,4-D to excessive auxin activity.

In this study we observed an enhancement of callus growth, and repression of somatic embryo formation by high concentrations of carbenicillin (375-500 mgl^{-1}). Cal-

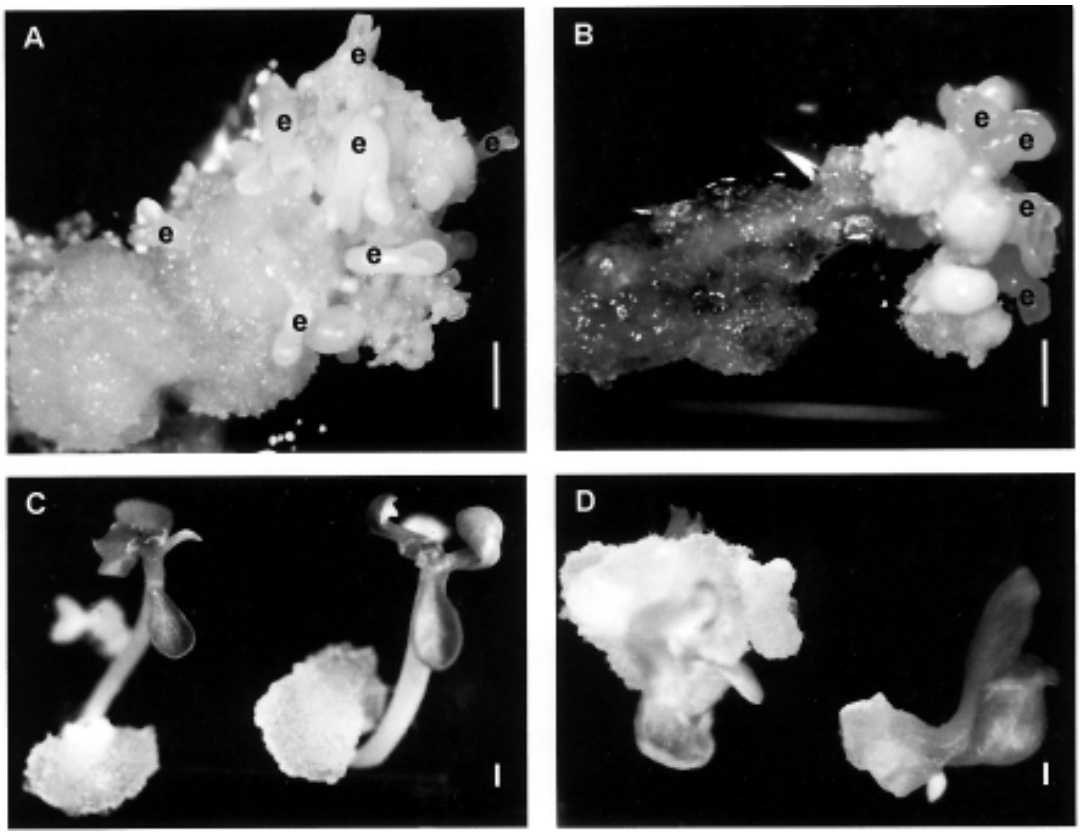

Figure 4. Somatic embryos derived from callus in the medium with 125 mgl^{-1} carbenicillin (A) or 250 mgl^{-1} cefotaxime (B) after culturing 16 wk. When the normal somatic embryo germinated, straightening of hypocotyl raised cotyledons and shoot apex above the medium and lengthening of epicotyl led shoot apex away from cotyledons (C). When the abnormal somatic embryos germinated, only aberrant cotyledons were noticed, and shoot apex disappeared or became stunted (D). e: somatic embryos; bar = 1 mm.

Table 1. Effects of media containing carbenicillin or cefotaxime on germination of somatic embryos after 4 wk in SGM medium.

Treatments	Germination rates		Germinated embryos with abnormal phenotype	
	(No. germinated embryos/Total No. embryos[1])	(%)	(No. abnormal embryos/Total No. germinated embryos)	(%)
Control	62/67	92.54	6/62	9.68
C_{125}	96/105	91.43	10/96	10.42
C_{250}	68/74	91.89	10/68	14.71
C_{375}	41/47	85.10	7/41	17.07
C_{500}	42/49	85.71	8/42	19.05
Cf_{125}	8/10	80.00	2/8	25.00
Cf_{250}	89/103	86.41	22/89	24.72
Cf_{375}	20/25	80.00	5/20	25.00
Cf_{500}	4/8	50.00	2/4	50.00

[1]Somatic embryos collected individually from CSM media with different concentrations of carbenicillin or cefotaxime, then transferred to SGM media with same antibiotics as those for CSM medium. Control: medium without antibiotics.

lus growth was also reduced, but somatic embryo formation was unaffected, by high concentrations of cefotaxime (375-500 mgl^{-1}), although fewer somatic embryos (1-2) were formed per callus. The medium with 125 mgl^{-1} carbenicillin or 250 mgl^{-1} cefotaxime clearly enhanced somatic embryogenesis, but greater numbers of somatic embryos were observed among individual callus in the medium with 125 mgl^{-1} carbenicillin, than among those in 250 mgl^{-1} cefotaxime. Furthermore, callus in these media was smaller and light brown in color.

Effects on Germination of Somatic Embryos

Somatic embryos that developed in antibiotic-containing CSM medium were placed on GSM medium containing the same concentrations of antibiotics as those in CSM. Germination percentages of embryos after 4 wk are shown in Table 1. The data revealed that germination was slightly reduced. More than 90% germinated in the control (0 antibiotic) and low carbenicillin concentration (125, 250 mgl^{-1}) media, whereas only about 85% germinated in the medium with 375 or 500 mgl^{-1} carbenicillin. Germination percentages of somatic embryos were below 86.4% in media with all concentrations of cefotaxime. During germination of a normal somatic embryo, the hypocotyl straightened and raised the cotyledons and the shoot apex to emerge above medium, and the epicotyl lengthened to lead the shoot apex away from the cotyledons (Figure 4C). On the other hand, abnormal somatic embryos germinated by producing only aberrant cotyledons, and shoot apices disappeared or were stunted (Figure 4D). Abnormal somatic embryos were found in all treatments, but at a higher rate in all cefotaxime-containing media. Studying protoplast-derived cells of *Nicotiana* spp, Pollock et al. (1983) discovered that carbenicillin was the least toxic of the antibiotics tested. In wheat cultures, Mathias and Boyd (1986) found cefotaxime to be less toxic than carbenicillin. When transforming papaya with *Agrobacterium*, high concentrations of carbenicillin (500 mgl^{-1}) or cefotaxime (200-500 mgl^{-1}) were used to control bacterial growth, and many abnormal transgenic somatic embryos often resulted during regeneration (Fitch et al., 1993; Cabrera-Ponce et al., 1996; Yang et al., 1996). In this study, we found that the frequency of abnormal embryos decreased when lower concentrations of carbenicillin (125-250 mgl^{-1}) were employed.

This investigation disclosed that carbenicillin and cefotaxime were not only antibacterial, but also affected the regeneration process. A washing step with sterile distilled water after co-culture with *Agrobacterium* greatly enhances the antibacterial effects. Complete exclusion of bacteria was possible by employing a medium with a concentration as low as 125 mgl^{-1} of either antibiotic. Higher carbenicillin (375-500 mgl^{-1}) concentrations promoted callus growth, but inhibited somatic embryo formation. Cefotaxime in high concentrations (375-500 mgl^{-1}) seriously inhibited callus growth. Although embryogenesis was not significantly affected, fewer numbers of somatic embryos were observed to emerge from callus cultured in cefotaxime-containing medium. The optimum antibiotic addendum for somatic embryo formation was either 125 mgl^{-1} of carbenicillin or 250 mgl^{-1} of cefotaxime. More embryos, as well as fewer abnormal embryos, resulted in the medium with 125 mgl^{-1} carbenicillin.

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卡苯尼西林（carbenicillin）和西弗士林（cefotaxime）兩種抗生素對於木瓜不定根的癒合組織與體胚形成之影響

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卡苯尼西林（carbenicillin）和西弗士林（cefotaxime）為兩種最常加入培養基中，用來控制農桿菌生長的抗生素。本研究中，在無菌的狀況下利用由組織培養苗所產生的不定根片段為材料。根片段經農桿菌共同培養感染後，利用不同濃度之抗生素去探討抗生素的抑菌作用以及對根片段的植物再生之影響。由結果得知，與農桿菌共同培養兩天後，事先以無菌水清洗培殖體後，有促進抑菌的作用，只要在培養基中含125 mgl^{-1} 抗生素濃度，即可有效的控制農桿菌之生長。在癒合組織生長方面，在含有較高濃度的carbenicillin 培養基（250-500 mgl^{-1}）中，即可增加癒合組織的鮮重。但在 cefotaxime 方面，同樣的濃度卻明顯的降低癒合組織的鮮重。在體胚形成方面，在 125 mgl^{-1} 的 carbenicillin 和 250 mgl^{-1} 的 cefotaxime 濃度之培養基中，有較高的體胚形成率，但就單一癒合組織形成體胚而言，在含 125 mgl^{-1} 的 carbenicillin 濃度的培養基中，有較多的體胚數目形成。在體胚的發芽狀況方面，在含較低濃度的 carbenicillin 培養基（125-250 mgl^{-1}）中，異常體胚的形成率有明顯下降的現象。在本研究中，有關此兩種抗生素控制農桿菌及影響木瓜不定根再生的有利條件均加以說明。

關鍵詞：抗生素；靜菌作用；體胚。