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Effect of Carbenicillin on Callus Induction and Regeneration Efficiency of Tissues of Horseradish(*Armoracia rusticana*)

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ABSTRACT

The effect of carbenicillin on the dedifferentiation and the regeneration efficiency of plant tissues of horseradish(*Armoracia rusticana*) was evaluated. Inhibition effect for callus initiation was observed when leaf blade, root and petiole segments were grown on MS medium containing 500 mg/L to 2000 mg/L carbenicillin and 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). The regeneration of horseradish shoots from leaf blade, root and petiole explants were decreased as the addition of carbenicillin increased from 1000 mg/L to 2000 mg/L in MS medium containing 0.5 mg/L of 6-benzylaminopurine (BAP) or kinetin. Especially, 500 mg/L carbenicillin treatment significantly inhibited shoot induction when leaf blade explants were grown on hormone-free MS medium. It was suggested that the toxic effects of combinations of carbenicillin and 2,4-D may be due to high auxin activity levels.

Key Words : Auxin, Cytokinin, Leaf Blade Segment, Petiole, Root segment

INTRODUCTION

Horseradish plant(*Armoracia rusticana*) is a important spice crop. And the plant produce large amounts of peroxidase(EC1.11.1.7), which is widely used as a reagent for clinical diagnosis and microanalytical immunoassays(Mano and Matsuhashi, 1995; Saitou et al., 1991). Effective propagation systems for horseradish plant has been developed because the plants scarcely bear seeds(Araki et al., 1995). Therefore, this plant has become a useful tissue culture system for plant regeneration and transformation (Araki et al., 1995; Bae, 1995; Bae et al., 1994; Mano and Matsuhashi, 1995; Martin et al., 1977; Noda et al., 1987; Saitou et al., 1991).

Successful transformation using *Agrobacterium* depends not only on the efficiency of the plant regeneration systems but also on the subsequent elimination of this bacterium from transformed cells. The elimination of *Agrobacterium* is quite important because the continued presence of *Agrobacterium* can present a problem for identifying transformants or interfere with the growth and development of the transformed plant cells or cause the death of the cultures. Antibiotics have been used in the regeneration medium in order to eliminate the Agrobacteria, when *Agrobacterium tumefaciens* vector system was adopted in the process of insertion of foreign genes in higher plants(Lin et al., 1995; Pollock et al., 1983; Okkels and Pedersen 1988; Tang et al., 2000). Until now carbenicillin and cefotaxime have become the most

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Table 1. Effect of carbenicillin on shoot formation and fresh weight of horseradish leaf blade segments cultured on MS medium containing 0.5 mg/L of BAP.

Carbenicillin (mg/L)	No. of shoots per explant[z]	Fresh weight per explant(mg)[z]
0	17.6a[y]	734a
250	14.4b	552b
500	10.8c	440c
1000	9.2c	414c
2000	5.6d	418c

[z]Shoot number and fresh weight per explant was obtained as the average of explants from 10 tobacco leaf discs incubated on MS medium containing BAP and different concentrations of carbenicillin for 4 weeks.

[y]Means followed by the same letters within a column are not significantly different at 5% probability level.

Table 2. Effect of carbenicillin on shoot formation and fresh weight of horseradish petiole segments cultured on MS medium containing 0.5 mg/L of BAP.

Carbenicillin (mg/L)	No. of shoots per explant[z]	Fresh weight per explant(mg)[z]
0	14.2b[y]	322b
250	21.0a	418a
500	11.8b	496a
1000	5.6c	334b
2000	0.2d	162c

[z]Shoot number and fresh weight per explant was obtained as the average of explants from 10 tobacco leaf discs incubated on MS medium containing BAP and different concentrations of carbenicillin for 4 weeks.

[y]Means followed by the same letters within a column are not significantly different at 5% probability level.

widely accepted antibiotics to eliminate the Agrobacteria efficiently from the plant material for performing *Agrobacterium*-mediated transformation (Lin et al., 1995; Pollock et al., 1983; Okkels and Pedersen 1988). However, both antibiotics showed minimal toxicity and plant hormone-like effects on plant tissues, such as inhibiting shoot formation and promoting callus induction in the tissue cultured explants(Lin et al. 1995; Mathias and Boyd, 1986; Nauerby et al., 1997; Okkels and Pedersen, 1988; Pollock et al., 1983). Also, the plant hormone-like effect of the antibiotics may complicate the hormone ratio in the culture media and be different from plant species. Because the plant tissue in culture is affected by the different components in the culture media, it is important to clarified effects of antibiotics on plant regeneration.

In this paper, we investigated the toxicity and hormone effects of carbenicillin on plant tissue grown on the media with and without exogenous plant growth regulators.

MATERIALS AND METHODS

Plant Materials

Explants were prepared from *Armoracia rusticana* at 4 to 5 leaf stages described in Bae et al. (1997). The explants were placed on MS medium (Murashige and Skoog, 1962) containing 30 g/L sucrose in 100 *ml* flasks. And the *in vitro* yielded plantlets cultured for 8 weeks were used for subsequent experiments. The culture media were adjusted to pH 5.8 prior to autoclaving and were solidified with 0.8% agar(w/v). The cultures were kept at 25±1℃ with continuous fluorescent light at 30μmol · m^{-2} · s^{-1} light intensity.

Effect of Carbenicillin on Callus Induction

In order to investigate effects of carbenicillin on callus induction, leaf blade(disks of 5mm in diameter), petiole and root(about 10mm in length) segments were cultured on MS medium containing 0.5 mg/L 2,4-D and 0, 250, 500, 1000, 2000 mg/L carbenicillin. Explants were cultured in irradiated 9cm Petri dishes sealed with micro pore tape. All experiments were performed with twelve explants per Petri dish, for three independent replicates.

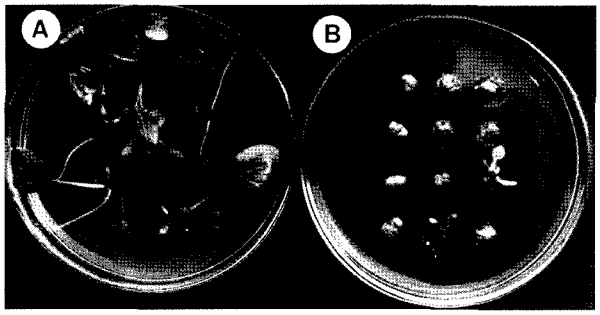

Fig. 1. Morphogenesis from leaf blade segments cultured on hormone-free MS medium without carbenicillin(A) and with 500 mg/L carbenicillin(B) for 3 weeks.

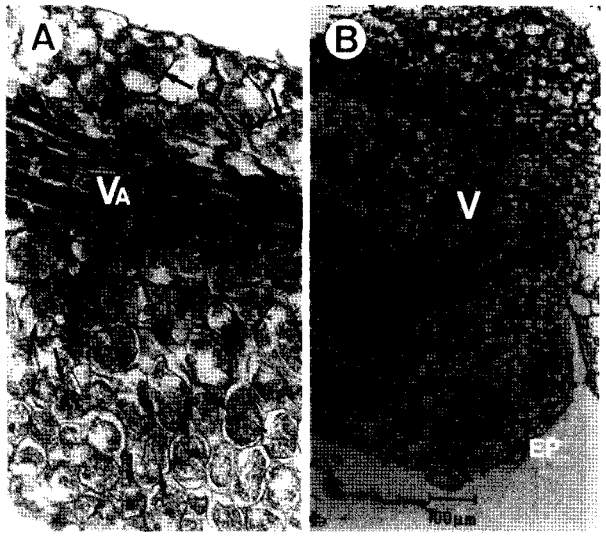

Fig. 2. Histological observation of cells around vascular bundle(A) and callus growth(B) from leaf blade segments. A, Cells(arrows) around vascular bundle cultured on MS medium containing 0.5 mg/L BAP for 3 days; B, Callus showing active growth cultured on MS medium containing 0.5 mg/L BAP for 12 days. EP, Epidermis; V_A, Vascular bundle; V, Wounding vessel element.

Effect of Carbenicillin on Shoot Regeneration

In order to investigate effects of carbenicillin on shoot induction, the same size of explants mentioned above were cultured on MS medium containing 0.5 mg /L BAP or kinetin and 0, 250, 500, 1000, 2000 mg/L carbenicillin. Explants were cultured in irradiated 9cm Petri dishes sealed with micro pore tape. All experiments were performed with twelve explants per *Petri dish, for three independent replicates.*

Histological Observation of Callus Induction

Callus formation from leaf blade segments was observed histologically. Samples of callus were fixed in Carnoy's solution(acetic acid, ethanol 1:3), dehydrated with an ethanol series, followed by paraffin embedding. Embedded tissue was cut with a rotary microtome into 10㎛ thick sections, stained with 0.5% hematoxylin and 0.5% orcein, and observed under a light microscope.

RESULTS AND DISCUSSION

Morphogenesis of Leaf Blade Segments

Multiple shoots were induced from leaf blade explants on MS medium containing 0.5 to 2 mg/L BAP in the previous experiments (Araki et al., 1995; Bae, 1995; Bae et al., 1997). Using hormone-free MS medium, plant regeneration from leaf blade explants was examined in medium with or without carbenicillin. Shoots and roots were differentiated directly from leaf blade explants of horseradish in MS medium without exogenous plant growth regulators in 3 weeks culture(Fig. 1A). When the explants were cultured on MS medium containing 500 mg/L of carbenicillin, however, most of leaf blade explants did not produce shoots and roots(Fig. 1B). These results are consistent with other reports that the carbenicillin inhibits shoot and root formation(Bae et al., 2000; Nauerby et al., 1997). Also, the carbenicillin effect for shoot formation was very similar to auxins, such as 2,4-D and NAA.

As for didifferentiation from leaf blade explants, no adventitious buds were observed but calli were developed in the medium containing 0.5 mg/L 2,4-D. In 3-day culture the leaf blade explants were expanded and activated cells were observed around vascular bundle(Fig. 2A). In 12-day culture, callus derived-from

the cut end of a leaf showed wounding vessel element in the middle of callus cluster(Fig. 2B). This vessel element has been reported to be a path of nutrient(Kim et al., 1993).

Interestingly, this plant has an efficient regenerability in the hormone-free medium as shown in Fig. 1A. This result may be attributable to the changes of an endogenous auxin/cytokinin ratio after cutting organ segments from whole plant. Similar result was reported in *Catasetum*(Orchidaceae) which produces shoots on hormone-free medium and changes endogenous auxin-to-cytokinin ratio favoring cytokinins during tissue culture(Peres and Kerbauy, 1999).

Effect of Carbenicillin on Callus Induction

A large number of reports showed that carbenicillin promoted callus induction and inhibited root formation in various plant species(Bae et al., 2000; Lin et al., 1995; Mathias and Boyd, 1986; Nauerby et al., 1997; Okkels and Pedersen, 1988; Pollock et al., 1983). To test the potential hormone effects of carbenicillin, horseradish organ explants were placed on MS media containing 0.5 mg/L 2,4-D and different concentrations of carbenicillin. Interestingly, the formation of callus

Table 3. Effect of carbenicillin on shoot formation and fresh weight of horseradish root segments cultured on MS medium containing 0.5 mg/L of BAP.

Carbenicillin (mg/L)	No. of shoots per explant[z]	Fresh weight per explant(mg)[z]
0	13.8b[y]	116c
250	26.6a	162b
500	8.0c	206a
1000	0.4d	180ab
2000	0d	110c

[z]Shoot number and fresh weight per explant was obtained as the average of explants from 10 tobacco leaf discs incubated on MS medium containing BAP and different concentrations of carbenicillin for 4 weeks.

[y]Means followed by the same letters within a column are not significantly different at 5% probability level.

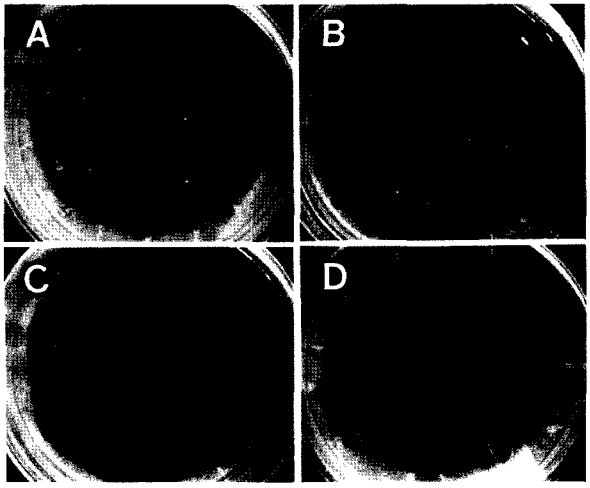

Fig. 3. Effect of different concentrations of carbenicillin on callus induction from root segments of horseradish cultured on MS medium containing 0.5 mg/L 2,4-D for 4 weeks. A, 0 mg /L carbenicillin; B, 250 mg /L carbenicillin; C, 500 mg/L carbenicillin; D, 1000 mg/L carbenicillin.

from root segments was significantly decreased as the concentration of carbenicillin increased from 250 mg/L to 2000 mg/L(Fig. 3A, B, C, D). Whereas, the explans grown on MS medium containing 2,4-D alone did not showed any toxic effect in the callus formation. Also, the callus formation from leaf blade explants and petiole explants gradually decreased as the concentration of carbenicillin increased from 500 mg/L to 2000 mg/L(data not shown). The results suggest that the inhibition effect of combination of carbenicillin and 2,4-D may be caused by the addition of an auxin-like activity supplied by carbenicillin(Lin et al., 1995). Thus, the combination of carbenicillin resulted in the inhibition of callus initiation. Similar results were reported when explants were grown in MS medium containing both carbenicillin and 2,4-D(Bae et al., 2000; Lin et al., 1995).

Effect of Carbenicillin on Shoot Induction

To determine the effect of carbenicillin on shoot formation, horseradish organ explants were placed on MS media containing BAP and different concentration

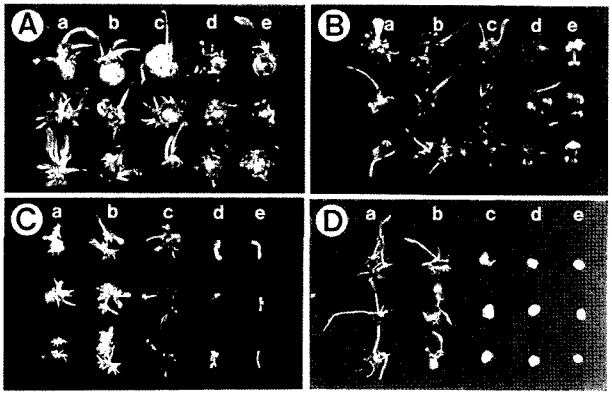

Fig. 4. Effect of different concentrations of carbenicillin(a: 0 mg/L, b: 250 mg/L, c: 500 mg/L, d: 1000 mg/L, e: 2000 mg/L) on shoot induction of tissues of horseradish cultured on MS media containing BAP(A, B, C) and kinetin(D) for 4 weeks. A and D, Leaf blade segments; B, petiole segments; C, Root segments.

of carbenicillin(Fig. 4A, B, C). The regeneration of shoots from horseradish leaf blade explants decreased from 17 shoots per leaf explant to lessen than 6 shoots per leaf explant as carbenicillin increased from 250 mg /L to 2000 mg/L on the MS medium containing 0.5 mg/L BAP(Fig. 4A, Table 1). Fresh weight per explant of leaf blade explants decreased from 734 mg to 418 mg as carbenicillin increased from 250 mg/L to 2000 mg/L. The regeneration of shoots from petiole explants decreased from 14 shoots per leaf explant to lessen than 1 shoot per leaf explant as carbenicillin increased from 250 mg /L to 2000 mg/L on the MS medium containing 0.5 mg/L BAP(Fig. 4B, Table 2). Fresh weight per explant of petiole explants decreased from 322 mg to 162 mg as carbenicillin increased from 250 mg/L to 2000 mg/L. The regeneration of shoots from root explants decreased from 13 shoots per leaf explant to zero per leaf explant as carbenicillin increased from 250 mg/L to 2000 mg/L on the MS medium containing 0.5 mg/L BAP(Fig. 4C, Table 3). While, fresh weight per explant of leaf blade explants approximately similar as carbenicillin increased from 250 mg/L to 2000 mg/L. In MS medium containing 0.5 mg/L kinetin, the regeneration of shoots from horseradish leaf blade explants decreased as carbenicillin increased from 250 mg/L to 2000 mg/L(Fig. 4D). These results were compatible with many other reports that carbenicillin inhibited shoot formation in various plant species(Bae et al., 2000; Lin et al., 1995; Nauerby et al., 1997; Okkels and Pedersen, 1988; Pollock et al., 1983).

However, the formation of callus was gradually increased as the concentration of carbenicillin increased to 2000 mg/L in the all kinds of explants(Fig. 4A, C, D). Especially in petiole explants, callus formation was significantly increased in MS medium containing 0.5 mg /L BAP and 2000 mg/L carbenicillin(Fig. 4B). The combination effect of carbenicillin and 0.5 mg/L BAP was similar to the addition of a higher concentration of 2,4-D or NAA to MS medium. These results indicate that carbenicillin may be has an auxin-like activity. The carbenicillin effects are correlated with the chemical structure. Because the chemical structure of auxins, such as 2,4-D and NAA contains either a phenolic or benzyl group connecting to a side chain of an acetic group. And carbenicillin also has an auxin related structure(Lin et al., 1995).

In the present work, effect of carbenicillin on shoot, root and callus formation was demonstrated in the cultured horseradish explants. Although, shoot formation was severely inhibited by the treatment of carbenicillin in hormone-free medium, it was not significantly decreased in a combination of a higher concentration of cytokinin and carbenicillin in horseradish tissue culture. Thus, optimal concentration of the combination of phytohormone and carbenicillin be able to lead an efficient regeneration in the process of transformation.

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