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BRIEF COMMUNICATION

Influences of cefotaxime and carbenicillin on plant regeneration from wheat mature embryos

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Abstract

The influences of cefotaxime and carbenicillin on regeneration potential of wheat (*Triticum aestivum* L.) mature embryos were investigated. Filter-sterilized cefotaxime enhanced regeneration capacity although it did not affect the average number of shoots per explant. The highest regeneration capacity of 55.4 % was obtained on regeneration medium supplemented with 100 mg dm^{-3} cefotaxime. Filter-sterilized carbenicillin did not stimulate plant regeneration. However, higher concentration (100 mg dm^{-3}) accelerated callus browning and inhibited the following regeneration. Autoclaved antibiotics at all tested concentrations showed detrimental effects on callus morphogenesis and plant regeneration.

Additional key words: *Triticum aestivum*, antibiotics, embryogenic callus, plant tissue culture.

Wheat, one of the most important staple food crops in the world, has been extensively investigated with respect to plant regeneration and genetic transformation. However, wheat transformation has remained to be the bottleneck in cereal biotechnology and transformation frequency is still very low (0.6 - 4.3 %; Cheng *et al*. 1997). A prerequisite to conduct successful transformation is the development of efficient callus induction and plant regeneration systems. Immature embryos are the most frequently used explant sources for tissue culture and transformation (Redway *et al*. 1990, Wu *et al*. 2003). But it is usually unpractical to obtain immature embryos year round, and their suitable stage for culture is strictly limited. Mature embryos, which can be stored in the form of dry seeds and be available at all times, are a feasible alternative to immature embryos. However, low regeneration capacity is the limiting factor in the use of mature embryos as explants (Ozgen *et al*. 1998, Delporte *et al*. 2001, Zale *et al*. 2004).

Antibiotics, in particular cefotaxime and carbenicillin, have been used extensively to eliminate *Agrobacterium* from the culture medium after transforming the explants. However, the influences of these antibiotics on callus morphogenesis and plant regeneration were also noted for some plant species (Leifert *et al*. 1992, Park *et al*. 1995). As to wheat, Mathias and Boyd (1986) firstly reported that cefotaxime and carbenicillin promoted callus growth and enhanced organogenesis in cultured tissues. Similar results were obtained for cultured durum wheat and barley tissues (Borrelli *et al*. 1992, Mathias *et al*. 1987). Whereas explants involved in these studies are all immature embryos and the influences of cefotaxime and carbenicilloin on cultured wheat mature embryos have not been investigated up to now. Therefore, present research has been undertaken to demonstrate the effects of these antibiotics on wheat mature embryos culture and provide constructive suggestions for plant regeneration and transformation research in wheat.

Mature dry seeds of five wheat (*Triticum aestivum* L.) elite genotypes, Yang158, Yan22, G8901, Yu34 and J177, were used in the current study. Mature seeds were surface-sterilized with 70 % ethanol for 1 min, followed

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; BA - N^6-benzyladenine; NAA - α-naphthaleneacetic acid; MS - Murashige and Skoog (1962) medium.
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by 0.1 % $HgCl_2$ with 1 - 2 drops of *Tween 20* for 2 min. Sterilized seeds were rinsed five times with sterile distilled water then soaked in 8 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) for 5 h at 33 °C. The swollen mature embryos were aseptically dissected from the caryopsis, longitudinally sliced into halves, then planted cut-side down on induction medium. The medium used to induce primary calli was based on Murashige and Skoog (1962; MS) medium supplemented with 134 mg dm^{-3} L-aspartic acid, 115 mg dm^{-3} L-proline, 100 mg dm^{-3} casein hydrolysate, 2 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D), 30 mg dm^{-3} sucrose, and were solidified by the addition of 7 g dm^{-3} agar. The pH was adjusted to 5.8 before autoclaving at 121 °C for 15 min. Unless indicated otherwise, the chemicals used in this research were purchased from *Duchefa* (Haarlem, The Netherlands).

After four weeks (25 °C, in darkness), the primary calli were transferred to subculture medium for embryogenic callus induction. Subculture medium was similar to the induction medium, with the exception of phytohormones [2 mg dm^{-3} 2,4-D, 0.5 mg dm^{-3} N^6-benzyladenine (BA) and 0.1 mg dm^{-3} α-naphthaleneacetic acid (NAA)]. After three bi-weekly subcultures (irradiance of 10 μmol m^{-2} s^{-1}, temperature of 22 °C, 16-h photoperiod), the induced embryogenic calli were then transferred to regeneration medium. Various concentrations of cefotaxime or carbenicillin were included to investigate their effects on plant regeneration. Antibiotics were filter-sterilized and added to the autoclaved medium after autoclaving and cooling. In the case of autoclaved antibiotics treatments, the antibiotics were added into the medium before autoclaving.

Four weeks later (30 μmol m^{-2} s^{-1}, 25 °C, 16-h photoperiod), regenerated plantlets were designated for rooting (80 μmol m^{-2} s^{-1}, 25 °C, 16-h photoperiod). Half-strength MS medium devoid of phytohormones were used for rooting. Finally, the well rooted plantlets were washed thoroughly under running tap water and transplanted to a mixture of vermiculite, perlite and moss (1:1:1) and grown in the greenhouse. After 3 - 4 weeks, plants were vernalized for additional 3 weeks at 4 °C then grown to maturity.

Each Petri dish was considered an experimental unit and two Petri dishes were used for each treatment. Each experiment was repeated three times. Analysis of variance was used to compare the means. *SPSS/PC* statistical program was used for all computations.

The appearance of primary calli from explants was observed 3 - 4 d after inoculation as translucent, rough and some-what watery structures. After 4 weeks, these calli were transferred to subculture medium to induce embryogenic calli. Following three subcultures under low irradiance, embryogenic calli became visible as compact, friable, light-yellow or creamy structures. Green shoots were readily noticeable within 2 weeks after embryogenic calli were transferred to regeneration medium. We found that the promotive effect of filter-sterilized cefotaxime on plant regeneration resulted from the higher regeneration capacity, not from higher average number of plantlets per explants (Table 1). Moreover plantlets induced on medium supplemented with filter-sterilized cefotaxime showed more vigorous growth compared with control (Fig. 1*A*,*B*). Cefotaxime at 100 mg dm^{-3} appears to be the optimal concentration, giving the highest regeneration capacity (55.4 % for Yang158) (Table 1). However, autoclaved cefotaxime at all tested concentrations

Table 1. Effects of filter-sterilized or autoclaved antibiotics on plant regeneration from wheat (genotype Yang 158) mature embryos. Means ± SE. Values within a column followed by different letters are significantly different at the $P < 0.05$ (Duncan's multiple range test).

Antibiotics	[mg dm^{-3}]	Number of explants	Regeneration capacity [%]	Number of shoots [explant^{-1}]
MS basal	0	40	39.2 ± 1.6cd	2.21 ± 1.0a
Filter-	50	38	46.7 ± 2.3bc	1.76 ± 0.9ab
sterilized	75	35	51.9 ± 3.0b	2.02 ± 1.2a
cefotaxime	100	40	55.4 ± 2.1a	2.37 ± 0.8a
	125	41	49.8 ± 0.9b	2.16 ± 0.9a
Autoclaved	50	37	37.7 ± 3.1d	1.67 ± 0.6b
cefotaxime	75	40	34.1 ± 4.1e	1.21 ± 0.7b
	100	40	30.5 ± 2.2ef	1.34 ± 0.8b
Filter-	40	38	38.4 ± 2.9cd	1.17 ± 0.5b
sterilized	60	40	36.4 ± 3.8cd	1.29 ± 0.8b
carbenicillin	80	40	33.9 ± 1.6de	1.08 ± 0.4d
	100	40	31.4 ± 2.5e	1.19 ± 0.7c
Autoclaved	40	34	28.7 ± 3.5ef	0.94 ± 0.5e
carbenicillin	60	40	25.4 ± 1.5f	1.03 ± 0.4de
	80	38	26.3 ± 0.6f	0.81 ± 0.3e

Table 2. The effect of 100 mg dm^{-3} filter-sterilized cefotaxime on regeneration capacity from mature embryos of five elite wheat genotypes. Means ± SE, $n = 3$. Values within a column followed by different letters are significantly different at the $P < 0.05$ (Duncan's multiple range test).

Genotype	[mg dm^{-3}]	Regeneration capacity [%]	Number of shoots [explant^{-1}]
Yang158	0	39.2 ± 1.6c	2.21 ± 1.0a
	100	55.4 ± 2.1a	2.37 ± 0.8a
Yan22	0	31.2 ± 3.3de	1.45 ± 1.2e
	100	45.3 ± 3.9b	1.87 ± 1.0d
G8901	0	28.4 ± 2.8e	1.89 ± 0.9d
	100	36.9 ± 4.6d	2.02 ± 0.9b
Yu34	0	34.0 ± 1.9d	1.77 ± 1.3de
	100	47.3 ± 2.7b	1.98 ± 1.1bc
JI77	0	30.0 ± 3.7e	1.63 ± 0.6e
	100	40.5 ± 3.1c	1.93 ± 0.7c

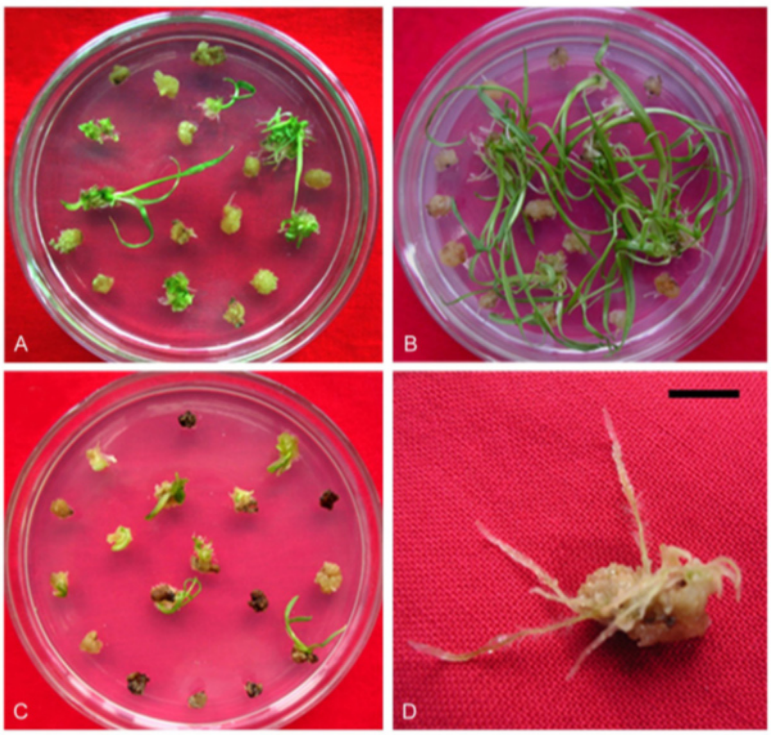

Fig. 1. Effects of cefotaxime and carbenicillin on plant regeneration from wheat mature embryos. *A* - Shoots induction on MS devoid of phytohormones and antibiotics (as control). *B* - Shoots induction on MS supplemented with filter-sterilized cefotaxime. *C* - Shoots induction on MS supplemented with filter-sterilized carbenicillin (60 mg dm^{-3}). *D* - Root hair-like structure induced from the explant on MS supplemented with autoclaved cefotaxime (*bar* = 5 mm).

significantly inhibited plant regeneration. The explants gradually turned brown and differentiated into root hair-like structure and died (Fig. 1*D*).

Compared to cefotaxime, no significant differences were detected among 40, 60 and 80 mg dm^{-3} filter-sterilized carbenicillin for regeneration capacity and regeneration capacity decreased gradually compared with control. But 100 mg dm^{-3} carbenicillin strongly inhibited plant regeneration, with regeneration capacity decreasing to 31.4 % (Table 1). Many of the regenerated plantlets started browning and exhibited teratogenesis (Fig. 1*C*). As to average number of shoots per explant, it was significantly affected by filter-sterilized carbenicillin at all tested concentrations. Clearly the most detrimental influence on plant regeneration was that in which autoclaved carbenicillin was present in regeneration medium. Both regeneration capacity and average number of shoots per explant dropped to the lowest level (25.4 % and 0.81 respectively) (Table 1). We also noted that the explants became smaller, darker and readily regenerated roots compared with shoots.

Genotype has been reported extensively as important factor for tissue culture in all major cereal species, including wheat (Ozgen *et al*. 1998, Zale *et al*. 2004, Haliloglu 2006). So a set of five elite wheat genotypes was screened for plant regeneration (Table 2). A fluctuation of regeneration capacity was observed among genotypes, varying from 55.4 % (Yang158) to 36.9 % (G8901). Hence different wheat genotypes appeared to have quantitatively different responses to cefotaxime, although they were all significantly enhanced compared with control.

The results of the present investigation demonstrated that filter-sterilized cefotaxime strongly promotes regeneration capacity in wheat mature embryos culture, although it does not affect average number of shoots per explants. The stimulative effect of cefotaxime on immature embryos culture of cereal species has been well documented (Mathias *et al*. 1987, Borrelli *et al*. 1992, Pius *et al*. 1993). According to Mathias and Boyd (1986), the use of filter-sterilized cefotaxime (60 mg dm^{-3}) was beneficial to shoot regeneration, similar to the result in the current study, albeit at different concentration. We also noted that the active cefotaxime is required to exert stimulative effect on plant regeneration because autoclaved treatment exhibited detrimental influence, identical to the former reports (Mathias and Boyd 1986). A possible explanation for the activity of cefotaxime in culture is that it is converted by cell metabolism to an unknown compound with phytohormone activity. But there have not been convincing results up to now. Cefotaxime has also been reported to interfere with ethylene (Pius *et al*. 1993). Whether it acts at the biosynthetic pathway of ethylene or participates in other

metabolic processes is under investigation.

It has been demonstrated that carbenicillin breaks down to give physiologically active auxin phenylacetic acid (Holford *et al*. 1992). As a result, the normal endogenous auxin/cytokinin balance in the explants would be changed. This could be an explanation for the reduced plant regeneration observed in our research, results which are not consistent with former reports (Mathias and Boyd 1986). As we know, callus induction, callus morphogenesis and plant regeneration are influenced by a complicated relationship between the explant used for culture, the components of the medium and the culture conditions. All these may account for the discrepancies. Moreover, to our knowledge, there are no references in the literature to the thermal degradation products of carbenicillin. So related investigations based on HPLC and other chemical analyses are in progress in our laboratory.

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