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Original Article

Evaluation of Effect of β-Lactam Antibiotics on Suppression of Different Strains of *Agrobacterium tumefaciens* and on Wheat Mature Embryo Culture

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Abstract

The efficiency of four β-lactam antibiotics meropenem, imipenem, ceftazidime and ceftriaxone were tested on three strains of *Agrobacterium*, *tumefacience* LBA4404, AGL0 and C58 and were compared with the commonly used antibiotic, cefotaxime. Meropenem exhibited the most effective antibacterial activity against *Agrobacterium tumefacience* LBA4404, AGL0 and C58. In addition to the high levels of activity of the antibiotics meropenem and imipenem on *Agrobacterium* inhibition (MIC less than 10 mg.l-l and MBC less than 150 mg.l-l), they allowed a high rate of shoot formation and had no significant negative effects on shoot and root weights of wheat explants. Therefore, they can be recommended for *Agrobacterium*-mediated transformation projects for wheat.

Keywords: Agrobacterium tumefacience; Genetic; Impenem; Meropenem, Transformation; Wheat.
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1. Introduction

Agrobacterium-mediated transformation is an effective and widely used method to introduce DNA into dicotyledonous plants. The method has been used successfully for many monocotyledonous plants [1]. This method of transformation involves the suppression and elimination of agrobacteria in plant tissue that is necessary to reduce any detrimental effect on growth and regeneration of the transforming plant tissue and to minimize any risk during the release of genetically modified microorganisms [2].

For this purpose β-lactam antibiotics, such as carbenicillin and cefotaxime that have a broad spectrum of activity against bacteria, have been widely used for the process of transformation [3]. They kill bacteria by specifically interfering with biosynthesis of the peptidoglycan component of the bacterial cell wall by binding to Penicillin-Binding Proteins (PBPs) and there appears to be little or no detrimental effect on eukaryotic plant cells. Plant tissue is affected by various components in culture media during plant transformation and antibiotics are added to the culture media

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to control *Agrobacterium*, however, they may have a negative effect on plant tissue and regeneration [4]. Studies have shown that when antibiotics were used in high concentration, they reduced callus growth and stimulated shoot regeneration and transformation efficiency [5]. Thus, it is necessary to find an alternative antibiotic against *Agrobacterium*, which is more active and has a negligible effect on plant regeneration. However, few studies exist regarding antibiotic activity on *Agrobacterium*.

Before using antibiotics in plant tissue culture, it is necessary to determine their effective concentrations based on quantitative bactericidal activity measured by Minimum Bactericidal Concentration (MBC), and bacteriostatic activity (Minimum Inhibitory Concentration (MIC). Among crop plants, wheat (*Triticum aestivum* L.) is the most important human food supply to be studied extensively regarding plant regeneration and genetic transformation [6]. For plant genetic transformation, there are two common methods, those of particle bombardment and *Agrobacterium*- mediated, in which various explants have been used [1, 7]. Immature embryos have been frequently used as a source of explantin tissue culture and genetic transformation studies [7]. Because it is difficult to obtain immature embryos over the years, and their suitable stage for culture is strictly limited, mature embryos that can be stored in the form of dry seeds and be available at all times are a suitable alternative to using immature embryos [8]. However, low regeneration capacity is a factor that limits the use of mature embryos as explants. Regeneration is important for gene transformation but it is affected by many factors. This study was done to evaluate the effects of five β-lactam antibiotics on suppression of three different *Agrobacterium* strains and on wheat mature embryo culture for providing profitable suggestions for plant transformation research in wheat. The effects of three antibiotics under different concentrations on shoot regeneration and growth suppression of *Agrobacterium* in tissue culture of wheat were evaluated.

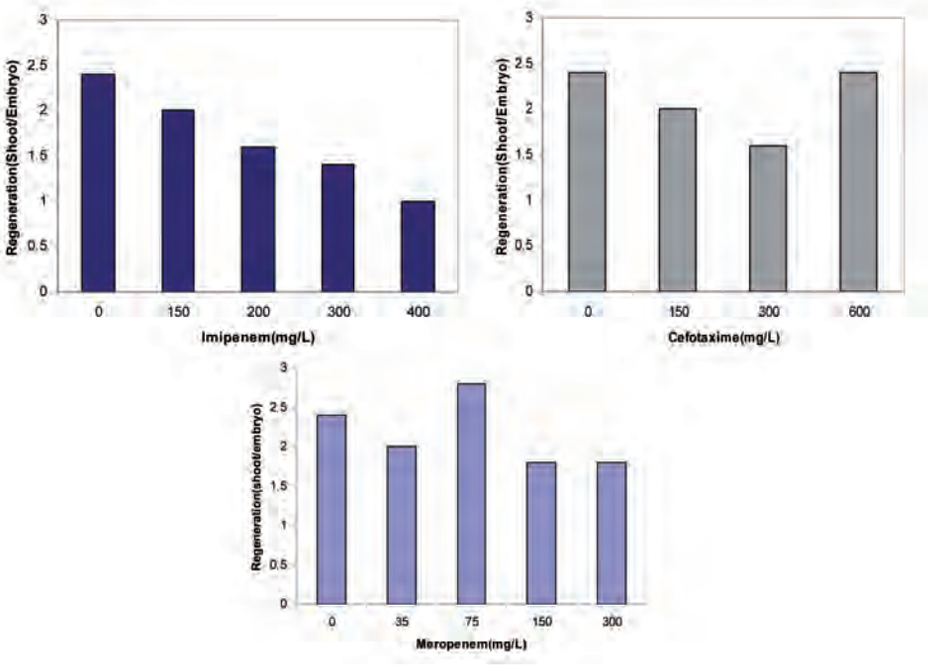

Figure 1. Effect of different concentrations of meropenem, imipenem and cefotaxime on mean number of regenerated shoots per embryo.

Table 1. MIC values ($mg.l^{-1}$) for different antibiotics against three strains of *A. tumefuciens* in LB medium.

Antibiotics	Meropenem	Imipenem	Ceftriaxone	Ceftazidime
Bacteria				
LBA4404	5.12	8	40.96	110
C58	5.12	8.5	44.53	95
AGL0	6	8.5	44.53	100

2. Materials and Methods

2.1. Plant materials

In this study, mature embryos of alamoot were used as explant. Mature seeds were surface-sterilized with 70% ethanol for 90 sec, 2% sodium hypochlorite containing 0.1% Tween-20 for 30 min and rinsed five times with sterile distilled water under aseptic condition, then placed on wet sterile paper for 24 h in room conditions. Embryos were isolated from seeds in an aseptic condition using scalpel and forceps. Isolated embryos were cultured in hormone free MS [9] (Duchefa, Netherlands) medium containing 100 mg.l-1 myo-inositol, 200 mg.l-l casein hydrolyzate and different concentrations of β-lactam antibiotics. This medium was solidified with 6 g/l plant agar (Duchefa, Netherlands).

2.2. Agrobacterium tumefaciens strains and plasmid

Disarmed *Agrobacterium tumefaciensstrains* LBA4404, C58 and AGL0 that harbor binary vector pBI121 and neomycin phosphotransfrase resistance gene (*npt*II) were used in this study. A single colony of bacteria was grown in Luria Bertani (LB) broth medium (10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl, pH 7) containing 50 $mg.l^{-1}$ of Kanamaycin and 50 $mg.l^{-1}$ of Rifampicin at 28 °C with shaking (220 rpm) for 24 h.

2.3. Antibiotics and preparation of stock solutions

The β-lactam antibiotics; meropenem, imipenem, cefotaxime, ceftazidime and

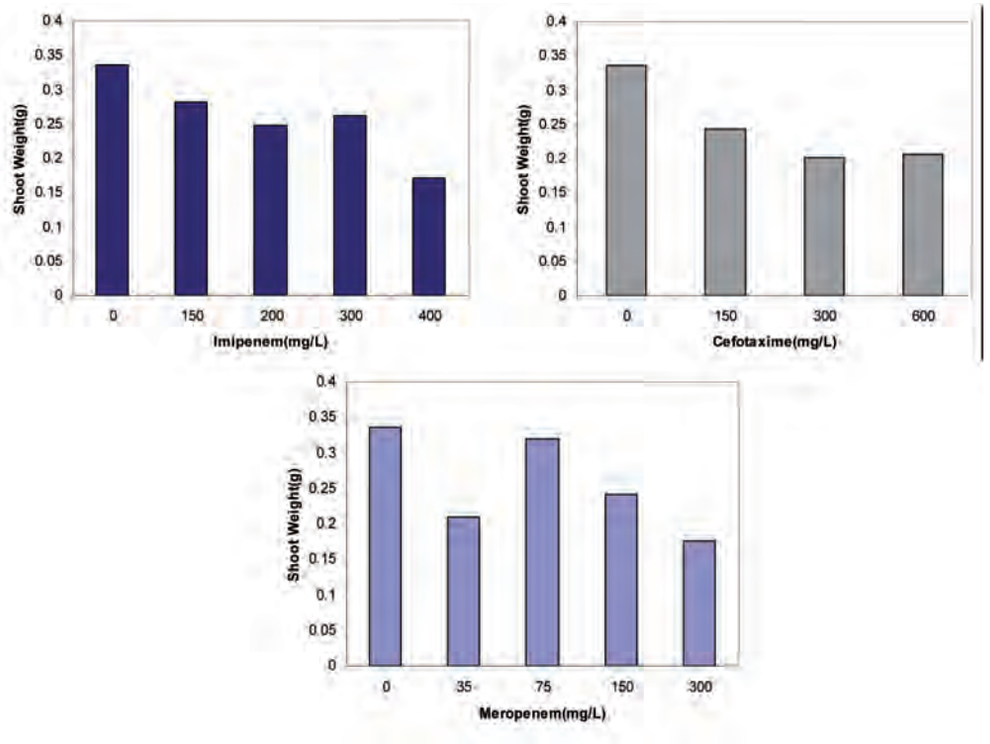

Figure 2. Effect of different concentrations of meropenem, imipenem and cefotaxime on shoot weight of wheat embryo explants.

Table 2. MBC values (mg.l^{-l}) for different antibiotics against three strains of *A. tumefuciens* in LB medium.

Antibiotics	Meropenem	Imipenem	Ceftriaxone	Ceftazidime
Bacteria				
LBA4404	30.72	120	1638.4	3850
C58	30.72	136	2048.38	2755
AGL0	36	136	2048.38	3300

ceftriaxone were used. They were dissolved in water, filter-sterilized and then stored at -20 °C. In this experiment, 100, 1000 and 10000 mg.l-l concentrations were used. Stock solutions were prepared using the following formula:

$$\frac{1000}{P} \times V \times C = W$$

Where P=potency given by the manufacturer in relation to the base, V= volume in ml required, C=final concentration of solution (multiplied by 1000), W= weight of the antimicrobial to be dissolved in the volume V.

2.4. Determinations of minimum inhibitory concentrations (MIC)

Minimum inhibitory concentrations of the β-lactam antibiotics against three strains of A. tumefaciens were determined by the agar dilution method [10]. Two-fold serially diluted of each antibiotic was prepared in Luria Bertani (LB) medium. Agrobacteria strains were grown overnight and diluted with LB medium to give final concentrations of ca. 5×10^5 colony-forming unit (CFU) ml^{-1}. Samples of 100 µl were inoculated onto LB plates containing antibiotic and incubated at 28 °C for 24 h. MIC was defined as the lowest drug concentration effecting no visible growth of agrobacteria.

2.5. Determinations of minimum bactericidal concentrations (MBC)

The bactericidal activities of the β-lactam antibiotics against three strains of A. tumefaciens were tested by the agar dilution method as described above. After 24 h of incubation at 28 °C with multiple of MIC of

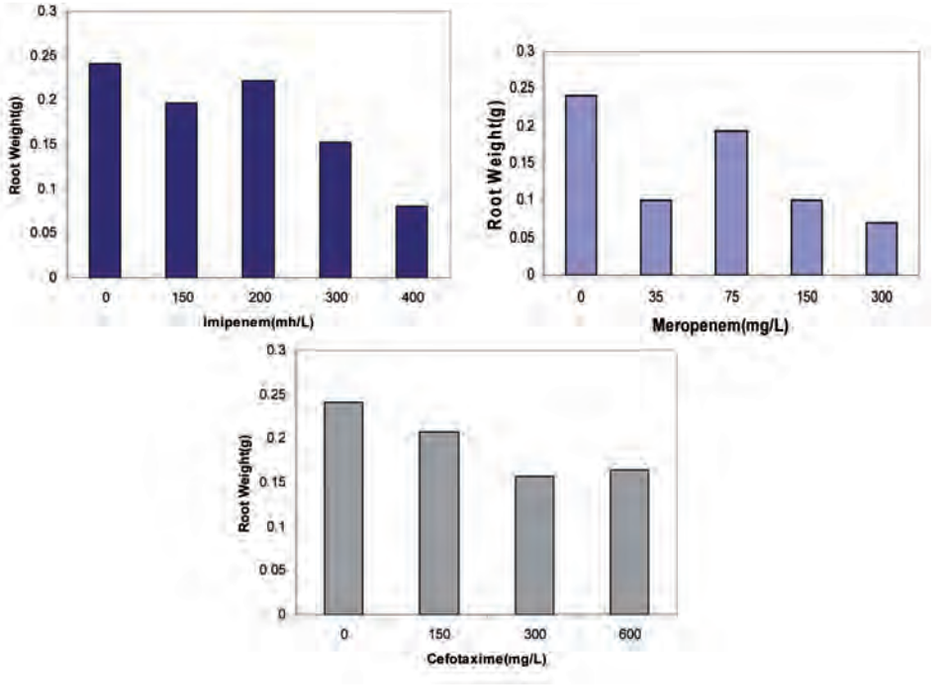

Figure 3. Effect of different concentrations of meropenem, imipenem and cefotaxime on root weight of wheat embryo explants.

the β-lactams in LB broth, 100 µl samples of cultures were transferred onto antibiotic-free LB agar plates, which were then incubated at 28 °C. After incubation for 3 days, the number of colonies recovered was counted. MBC was defined as the lowest drug concentration producing no visible growth of agrobacteria.

2.6. Effects of antibiotics on shoot regeneration and mature embryo growth

To determine the effect of antibiotics on shoot regeneration and mature embryo growth, after removing the mature embryo from the seed with a scalpel they were transferred to an MS medium (Duchefa, Netherlands) supplemented with different concentrations of antibiotics. Meropenem (Jaberebnhayan, Iran) imipenem (Jaberebnhayan, Iran) and cefotaxime (Dena, Iran) were used for this part of the experiment because they had the minimum MBC and higher level of activity against strains of *Agrobacterium*. In the following days, growth of embryos was monitored every 4 days and the totals of regeneration were recorded. Each experiment was repeated three times. Analysis of variance was used to compare the means. The SAS statistical program was used for all computations.

3. Results and discussion

3.1. MIC and MBC determination

In this study, MIC and MBC were determined for four different antibiotics against three strains of *Agrobacterium tumefacience* that are usually used in plant gene transformation projects (Tables 1 and 2).

Although a group of scientists believed that genetic transformation method based on *Agrobacterium* is not a suitable approach for transformation of monocotyledonous plants such as wheat, there are studies that have successfully utilized this method in these plants. Therefore, this study was done to assess the use of *Agrobacterium* in genetic transformation and to identify appropriate antibiotics for this process, including determination of the minimum effective quantity of antibiotics for effective elimination of *Agrobacterium* and any other influence they may have on wheat growth.

In the first step, appropriate evaluations were made for MIC and MBC of antibiotics to eliminate three strains of *Agrobacterium*. Meropenem demonstrated the highest activity in suppressing all A. tumefaciens strains (MIC 5.2 $mg.l^{-1}$ for LBA4404 and C58, and MIC 6 $mg.l^{-1}$ for AGL0) compared with other antibiotics (imipenem, cefotaxime, ceftazidime and ceftriaxone), results are shown in Table 1. These results confirm meropenem as a promising antibiotic because of its economic viability and less probable negative side effects from excessive use in antibiotic in plant tissue culture medium. The same results were observed for imipenem (MIC 8 $mg.l^{-1}$ for LBA4404, and MIC 8.5 $mg.l^{-1}$ for C58 and AGL0. The above mentioned advantages of meropenem can also be applied to imipenem.

Meropenem and imipenem are β-lactam antibiotics and belong to the subgroup of carbapenem. They both showed strong activity in the suppression of all *Agrobacterium* strains and results were similarly reported by different studies [11-14].

The strong anti-agrobacterial activity of meropenem may be explained by its high stability to a wide range of β-lactamases as well as by its inhibitory activity against β-lactamase [15].

MIC of ceftriaxone was 40.96 $mg.l^{-1}$ for LBA4404 and 44.53 $mg.l^{-1}$ for C58 and AGL0. MIC of ceftazidime was 110 $mg.l^{-1}$ for LBA4404, 95 $mg.l^{-1}$ for C58 and 100 $mg.l^{-1}$ for AGL0. It was clearly observed that MICs of two recent antibiotics as members of 3rd generation cephalosporins were more than meropenem and imipenem. This lower antibacterial effect is related to their different structure and resistance to β-lactamase.

MBCs were determined for all four antibiotics against different strains of *Agrobacterium* and the results are shown in Table 2. As for the MIC results, the MBC results showed that meropenem had the highest level of bactericidal activity against all three *Agrobacterium* strains (MBC 30.72 $mg.l^{-1}$ for LBA4404 and C58 and 36 $mg.l^{-1}$for AGL0).

The results for imipenem were also interesting because MBC for LBA4404 was 120 $mg.l^{-1}$ for C58 and AGL0 was 136 $mg.l^{-1}$. Meropenem and imipenem exhibited similar levels of bacteriostatic and bactericidal activities (MICs less than 50 $mg.l^{-1}$ and MBCs less than 250 $mg.l^{-1}$), therefore, these high antibacterial activities appeared to be suitable options for suppressing serious infections caused by LBA4404, C58 and AGL0.

MBCs of ceftazidime and ceftriaxone against all strains of *Agrobacterium* were greater than 1600 $mg.l^{-1}$ and even raised to 3850 $mg.l^{-1}$ for ceftazidime in elimination of LBA4404, so they showed low bacteriostatic and bactericidal activities against *Agrobacterium*, consequently these two antibiotics were ignored and only effects of meropenem and imipenem were compared with the commonly used cefotaxime.

Strain LBA4404 was more susceptible to all classes of tested antimicrobial agents compared to the strains C58 and AGL0 except ceftazidime. Since LBA4404 is a non-β-lactamase producing strain in contrast to AGL0 and C58, which are β-lactamase-producing strains [11, 13, 14]. Thus, strain LBA4404 carrying a chromosomal background of strain Ach5 might be more easily eliminated by β-lactams than disarmed strains carrying a chromosomal background of strain C58 [11].

According to these results, meropenem and imipenem might be effectively used as an alternative antibiotic in genetic transformation projects in wheat with both non-β-lactamase producing and β-lactamase producing *Agrobacterium* because of MICs less than 50 $mg.l^{-1}$ and MBCs less than 250 $mg.l^{-1}$ against all *Agrobacterium* strains.

Despite other studies that have classified LBA4404 as a non β-lactamase producing *Agrobacterium*, Ogawa has suggested that LBA4404 produces either inducible β-lactamase, which is an important mechanism of β-lactam resistance found in a wide range of bacteria [16] or constitutive but minimal amounts of β-lactamase because they found that sulbactam (β-lactamase inhibitor) intensified the susceptibility of this strain against ampicillin [11]. Although it was found that LBA4404 was more sensitive to meropenem and imipenem, there was little difference between MIC or MBC of imipenem against this strain and other strains; also, the equal MIC and MBC of meropenem to this strain and C58 confirms the hypothesis of Ogawa et al.

3.2. Effects of antibiotics on plant growth

Effects of five different antibiotics from β-lactams were investigated on growth factors of wheat such as regeneration, shoot and root weight.

3.2.1. Regeneration

Shoot formation rate (mean number of shoots per explant) for all antibiotics compared with the control showed that there was no antibiotic in the culture medium of plants, shown in Figure 1. Shoots appeared 6 days after transferring mature embryos to the MS medium. Records were taken after 4 weeks for rate of regeneration, shoot and root weights.

Imipenem slightly decreased shoot regeneration of non-transformed wheat embryos compared with control plants, in which there were no antibiotics in the MS medium. Statistically, there was not significant difference between regeneration capacity of imipenem treatment and the control embryos.

By an increase in imipenem concentration from 0 to 400 mg.l^{-1} the average regenerated shoots per embryo decreased from 2.4 to 1 shoot per explant. Because this decrease is not significantly different at $p \leq 0.05$, there was no negative effect from application of imipenem in culture medium of the wheat embryos.

Meropenem decreased regeneration from 2.4 to 1.8 shoots per explant with one notable exception in 75 mg.l^{-1}, in that regeneration was 2.8 shoots per explant. High regeneration rate was observed at 75 mg.l^{-1} of meropenem compared with other antibiotics and the control. Again, although meropenem increased regeneration at 75 mg.l^{-1}, these differences were not statistically significant, thus, it was demonstrated that meropenem did not affect the regeneration rate of wheat mature embryos.

Because cefotaxime is commonly used in the genetic transformation of plants and its optimum concentration has been determined in several projects, in this study 3 concentration levels of this antibiotic were used to assess its influence on plant growth and to compare utilizing this antibiotic to other probably new alternative antibiotics. At 150 and 300 mg.l^{-1} of cefotaxime, the mean number of shoots decreased to 2 and 1.6 compared with 2.4 in the control but at 600 mg.l^{-1} it was equal to 2.4.

Several studies have confirmed the negative effect of cefotaxime on organogenesis and embryogenesis and shoot regeneration for a number of plant species [13, 17-21]. For example, addition of a high concentration of cefotaxime inhibited shoot formation in many plants, such as in Antirrhinum majus [17] Malus [18] and Nicotiana tabacum [19]. In contrast, some groups have introduced cefotaxime with little or no detrimental effect on eukaryotic plant cells [22, 23] and with a positive effect of low concentration of cefotaxime on shoot formation in wheat [24, 25] and barley [26, 27]. Yu found that filter-sterilized cefotaxime strongly promoted regeneration capacity in wheat mature embryos culture without any effect on average number of shoots per explant. 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 so far, research has not produced any convincing resultsto confirm the assertion [3].

3.2.2. Shoot weight

Shoot weights of wheat explants under different concentrations of all antibiotics are shown in Figure 2. An increased imipenem concentration from 150 to 400 mg.l^{-1} caused a slight decrease in shoot weight 0.28 g to 0.17 g in wheat plants after 4 weeks; it was 0.34 g for the control. A meropenem addition of 35 mg.l^{-1} to the culture medium notably decreased shoot weight to 0.21 g, with an increase in meropenem concentration to 75 and 150 mg.l^{-1} shoot weight decreased to 0.32 and 0.24 g, respectively. Then in 300 mg.l^{-1} it was decreased to 0.18 g. At 75 mg.l^{-1} shoot weight was approximately the same as the control (0.34 g).

Although at higher concentrations of meropenem, shoot weight was decreased, this decrease was not statistically different. Additionally, according to MIC and MBC, a low concentration of meropenem can effectively inhibit *Agrobacterium*, and it is not necessary to add extra quantities of antibiotic to the medium increasing cost and probably having a negative effect on plants.

An increase in cefotaxime from 150 to 600 mg.l^{-1} caused a decrease in shoot weight 0.24 to 0.20 g compared to the control 0.34 g. No detrimental effect of antibiotics meropenem, imipenem and cefotaxime was observed on shoot regeneration frequencies of embryo explants of wheat.

3.2.3. Root weight

Non-transgenic shoots rooted at a rate of 100% in media supplemented with meropenem, imipenem and cefotaxime but results for root weight varied at different concentrations of each antibiotic (Figure 3). As the concentration of meropenem increased (0 to 300 $mg.l^{-1}$), there was a gradual decrease in the root weight of shoots. Root weight decreased slightly from 0.24 to 0.07 g. Interestingly, root weight was 0.19 g at 75 $mg.l^{-1}$ of meropenem, a result close to that of the control. An increase in imipenem concentration showed a similar effect to that of meropenem, and root weight decreased from 0.24 to 0.08 g.

An increase in cefotaxime concentration from 150 to 600 $mg.l^{-1}$, declined root weight from 0.24 to 0.16 g. Cefotaxime showed a lower negative effect on root weight compared with the other two antibiotics, although the negative effect of all antibiotics were not significant compared to the control.

There are different reports on antibiotic effect on root growth. Cefotaxime at 500 mg.dm-3 significantly inhibited the mean number of roots per shoot, whereas meropenem (6.25 and 12.5 $mg.dm^{-3}$) significantly improved it in pineapple [21]. Cefotaxime inhibited root growth in both transgenic and non-transgenic shoots [2], but the results of this study showed that antibiotics did not influence the rate of rooting of non-transgenic shoots.

Shoot regeneration, shoot weight and root weight of non-transgenic plants on a medium containing meropenem, imipenem and cefotaxime irrespective of their concentration, did not differ from that of the control.

Recent studies showed that meropenem had little inhibitory effect on the shoot regeneration of tobacco and Phaluenopsis in Agrobucterium-mediated transformation [13].

Meropenem and imipenem were effective in suppressing all three strains of A. tumefaciens at low concentrations and had little effect on shoot and root growth and shoot regeneration of wheat, so, they can feasibly replace cefotaxime in *Agrobacterium*-mediated transformation of wheat. However, it should be mentioned that cefotaxime is commonly used in *Agrobacterium*-mediated transformation, but it has less activity against *Agrobacterium* strains and is more phytotoxic than some other antibiotics. It is very important to find more effective antibiotics against *Agrobacterium* tumefaciens [14].

Meropenem is a carbapenem antibiotic which is highly resistant to degradation by β-lactamases; additionally it has been classified as a moderately phytotoxic antibiotic because of the low doses required for inhibition of *Agrobacterium*. The results of a study by Mendes et al. [21] have demonstrated that imipenem has all of the advantages of meropenem application against the three strains of *Agrobacterium* tumefaciens that were tested, therefore, both of these antibiotics could be useful for transformation of wheat using *Agrobacterium* strains including β-lactamaseproducers (C58 and AGL0) and non-β-lactamaseproducers (LBA4404).

According to the results of this study, meropenem and imipenem might be effectively used as alternative drugs for genetic transformation of wheat with *Agrobacterium* strains LBA4404, AGL0 and C58. While meropenem and imipenem are more expensive than conventionally used β-lactams, the low dose requirement of both of these antibiotics balances the cost. Therefore, if meropenem or imipenem are antibiotics of choice, it is suggested that doses of 75 and 150 $mg.l^{-1}$ are used to inhibit the strains of *Agrobacterium* tested in this study. Further investigation is needed to study the interaction of antibiotic, bacteria and plant in transformation projects.

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