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The effects of β-lactam antibiotics and hygromycin B on *de novo* shoot organogenesis in apple cv. Golden Delicious

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Abstract: Since the genetic transformation of the apple is strongly genotype-dependent and generally inefficient, the evaluation of factors affecting shoot regeneration are crucial for the establishment of a successful transformation process. In this report, we evaluated the effects of the β-lactam antibiotics meropenem and timentin on *in vitro* regeneration via *de novo* shoot organogenesis from leaf explants of apple cv. Golden Delicious, as well as on the growth of the *Agrobacterium tumefaciens* strain EHA 105, and compared them with the commonly used β-lactam cefotaxime. Also, we report for the first time the effect of hygromycin B as a selective agent in the domesticated apple, as regards shoot regeneration and shoot multiplication efficiency. We observed that cefotaxime and timentin at concentrations higher than 100 mg L^{-1} were sufficient to prevent *Agrobacterium* growth during a two-week period, while meropenem exhibited an inhibitory effect on bacterial growth at all tested concentrations (25-150 mg L^{-1}). Cefotaxime at a concentration of 300 mg L^{-1} increased the number of regenerated shoots per explant (9.39) in comparison with the control (7.67). In contrast to cefotaxime, meropenem and timentin caused a decrease in shoot regeneration efficiency, but larger and more developed shoots were obtained on meropenem (25-125 mg L^{-1}) after the same period of cultivation. Hygromycin B at a concentration of 5 mg L^{-1} or higher completely inhibited shoot regeneration and induced explant tissue necrosis. Therefore, the selection procedure with a final concentration of 4 mg L^{-1} throughout organogenesis and 10 mg L^{-1} for further shoot growth and multiplication is recommended for an efficient transformation process in apple cv. Golden Delicious.

Key words: cefotaxime; meropenem; *Malus* × *domestica* Borkh.; regeneration; timentin

Abbreviations: BAP – N^6-benzilaminopurin; BM – basal medium; IBA – indole-3-butyric acid; MIC - minimal inhibitory concentration; MS – Murashige and Skoog mineral solution; NAA – α-naphthaleneacetic acid; HPT – hygromycin B phosphotransferase; SFC – the index of shoot-forming capacity; SMM – shoot multiplication medium; SRM – shoot regeneration medium; TDZ – thidiazuron

INTRODUCTION

Golden Delicious is one of the main apple (*Malus* × *domestica* Borkh.) cultivars that is widely grown, both as a commercial variety known for its sweet, tasty and juicy pommes, as well as a breeding stock for a number of other varieties. Primarily because of its economic importance, Golden Delicious was chosen for the apple genome sequencing project [1] and it is becoming a model for the understanding of important traits in apple, including the production of phenolic compounds with health benefit properties [2-4]. Thus, gene function analyses via genetic engineering/transformation methods, such as silencing or overexpression of genes included in biosynthetic pathways, are nowadays at the focus of apple molecular research.

Unfortunately, the genetic transformation of apple still poses a challenge; it has been shown to be strongly genotype-dependent [5-7] and of generally low efficiency [6-10]. Maximova et al. [6] suggested that during the most commonly applied *Agrobacterium*-mediated transformation practice, bacterial and plant cell interactions and T-DNA transfer were not the key factors responsible for the low transformation efficiency in apple. It is considered that the factors

affecting shoot regeneration from transgenic calli are probably crucial to the success of the transformation process [11]. Since the agrobacteria remaining in plant tissue and growth media after gene transfer negatively affect plant regeneration and growth, their efficient elimination could greatly improve final transformation efficiency. β-lactam antibiotics are commonly used to eliminate the residual agrobacteria in subsequent steps. This group of antibiotics act by interfering with penicillin-binding proteins and by inhibiting the biosynthesis of the peptidoglycan network of the cell wall that provokes cell wall lysis and consequently bacterial death [12]. However, it has been shown that the application of β-lactam antibiotics frequently affects plant growth and development, leading to either the inhibition [13,14] or the promotion [15-18] of regeneration ability. They may differently affect callus induction [14], shoot formation [19], somatic embryogenesis [20] or rooting [13]. It is known that plant sensitivity to antibiotics is species-, genotype- or even tissue-specific [21]. For this reason, the correct selection of the antibiotic to be used in the transformation protocol for the elimination of residual agrobacteria is vital for every cultivar *per se*, especially in recalcitrant-to-transformation species such as apple. To the best of our knowledge, cefotaxime and carbenicillin are the only two β-lactams for which some data on *in vitro* organogenesis in apple cv. Golden Delicious has been obtained [22]. With the appearance of novel β-lactam antibiotics, such as meropenem and timentin, there is a need to expand this knowledge with more detailed studies.

The selection of transformed cells is another key factor in developing a successful method for genetic transformation. An optimum choice of a selective agent can make the process of selection of transformed cells more efficient. Hygromycin B, an aminoglycoside antibiotic produced by *Streptomyces hygroscopicus* [23] has been frequently used in the selection process in a large number of plant species [24-26]. It effectively inhibits the translocation of mRNA and tRNAs on the ribosome in both bacteria and eukaryotes, and has a subtle effect on decoding fidelity [27,28]. Since it is more toxic than kanamycin, it eliminates sensitive cells more quickly, reducing the selection time and frequency of generating chimeric plants [29]. Although hygromycin B has good characteristics as a selective agent, there are no reports about its use in transformation of domesticated apple, except in apple rootstocks [30,31]. It is possible that the use of *hpt*, coding for hygromycin B phosphotransferase (HPT) as a selectable marker gene, may contribute to higher transformation frequency and reduced duration of the selection process over standard kanamycin selection in apple. The sensitivity to hygromycin may vary within the same plant species among different cultivars [32], tissues and organs [33,34]. A suboptimal dose may result in the high frequency of escapes and chimeras, while unnecessarily high doses kill untransformed tissues and inhibit the growth of transformed cells, leading to a delay in the regeneration process [35]. Therefore, careful optimization of dose concentration is the key factor for successful selection during the transformation process.

With this in mind, the aim of this work was to evaluate the effect of the β-lactam antibiotics, timentin and meropenem, on *in vitro* regeneration via *de novo* shoot organogenesis in recalcitrant-to-transformation apple cv. Golden Delicious and compare it to the widely used cefotaxime. Also, we tested the susceptibility of *Agrobacterium* strain EHA 105, which we plan to use for genetic transformation of apple in our future experiments, to these antibiotics. For further optimization of the regeneration procedure, the determination of the minimal inhibitory concentration (MIC) of hygromycin B suitable for the selection of transgenic cells was carried out by testing the effect of a range of concentrations on the leaf explant regeneration potential, as well as on the survival and multiplication rate of regenerated shoots.

MATERIALS AND METHODS

Antibiotics

Three types of β-lactam antibiotics, cefotaxime (Tolycar; Jugomedija AD, Zrenjanin, Serbia), meropenem (Merocid; PharmaSwiss, Belgrade, Serbia) and timentin (ticarcillin disodium and potassium clavulanate mixture, 15:1; Duchefa Biochemie, Haarlem, Netherlands), were tested for their influence on *de novo* shoot regeneration in apple cv. Golden Delicious, as well as on the growth of *Agrobacterium tumefaciens*. Additionally, the aminoglycoside antibiotic hygromycin B (Sigma, St. Louis, MO, USA) was used in the

shoot regeneration and subsequent shoot multiplication experiments. The antibiotics were dissolved in deionized water, filter-sterilized and added to media after autoclaving and cooling. Antibiotics were applied individually into the respective growing media at increasing concentrations: 100, 200, 300, 400, 500 and 600 mg L^{-1} for cefotaxime and timentin, 25, 50, 75, 100, 125 and 150 mg L^{-1} for meropenem, and 2, 4, 5, 6, 8 and 10 mg L^{-1} for hygromycin B.

Bacterial strain, growth medium and culture conditions

Agrobacterium tumefaciens strain EHA 105 [36] carrying the binary vector pCAMBIA1301 planned to be used for genetic transformation of apple, was used to test the susceptibility to the β-lactam antibiotics cefotaxime, meropenem and timentin. Strain EHA 105 has a chromosomal background of a succinamopine strain C58 and carries a disarmed helper plasmid pEHA105. *Agrobacterium* was cultivated for 5 days in the dark at 25±2°C in Petri dishes on agar solidified YEB medium (25 ml per dish) consisting of 5 g L^{-1} beef extract, 1 g L^{-1} yeast extract, 5 g L^{-1} peptone, 5% (w/v) sucrose, 1.4% (w/v) agar (Torlak, Belgrade, Serbia for all) and 0.5 g L^{-1} $MgSO_4x7H_2O$ (Lachner, Czech Republics), pH 7.2, and supplemented with 100 mg L^{-1}ampicilin (Panfarma, Belgrade, Serbia) and 50 mg L^{-1} kanamycin (Sigma-Aldrich). To obtain the bacterial suspension, three full loops (Ø 3 mm) of cold-stored *Agrobacterium* colonies were resuspended into 10 mL of liquid YEB medium containing ampicillin and kanamycin at the abovementioned concentrations, in 50-mL sterile falcon tubes that were placed horizontally on an orbital shaker at 95 rpm for 24 h in the dark at 25±2°C.

Effect of β-lactam antibiotics on *Agrobacterium* growth

Fifty μL of bacterial suspension (OD_{600}=1.09) were diluted into 5 mL of sterile liquid YEB medium containing ampicillin and kanamycin in 30-mL glass tubes (18 x 150 mm). The β-lactam antibiotics were added individually to the bacterial suspension at the concentrations given above. Every treatment was done in three replicates. Glass tubes were incubated on an orbital shaker at 95 rpm in the dark at 25±2°C. OD_{600} was measured spectrophotometrically (Agilent 8453, Santa Clara, CA, USA) 1, 3, 7 and 14 days after inoculation of agrobacteria.

Plant material, nutrition media and culture conditions

The *in vitro* shoot cultures of apple cv. Golden Delicious used were established from vegetative buds of 1-year-old increments taken from a 15-year-old tree, as described in Mitić et al. [37]. Briefly, the shoot apex with one pair of primordial leaves was excised aseptically from the buds and cultured on basal medium (BM) consisting of Murashige and Skoog [38] mineral salts and Linsmaier and Skoog [39] vitamins, 3% (w/v) sucrose, 100 mg L^{-1} myo-inositol and 0.7% (w/v) agar (Torlak, Belgrade, Serbia). The BM was supplemented with 0.125 mg L^{-1} N^6-benzylaminopurine (BA) and 0.09 mg L^{-1} α-naphthaleneacetic acid (NAA) (both from Sigma-Aldrich, St. Louis, MO, USA). For propagation, regenerated shoots were transferred to BM supplemented with 0.5 mg L^{-1} BA and 0.05 mg L^{-1} NAA, and designated as the shoot multiplication medium (SMM). Shoots were subcultured at 4-week intervals.

Shoot regeneration parameters were tested on shoot regeneration medium (SRM), consisting of BM supplemented with 5 mg L^{-1} thidiazuron (TDZ), and 0.3 mg L^{-1} indole-3-butyric acid (IBA) (both from Sigma-Aldrich), as presented in Mitić et al. (2012) [37]. The pH of all media was adjusted to 5.8 before autoclaving at 114°C for 25 min. The cultures were maintained for 3 weeks in the dark and additionally 5 weeks under light (16-h photoperiod) provided by cool-white fluorescent tubes (45 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in a growth chamber at 25±2°C.

Effect of antibiotics on *de novo* shoot regeneration and multiplication

To test the influence of antibiotics on *de novo* shoot regeneration, healthy and fully expanded young leaves (5 to 8 mm in length) were excised from the upper third of shoots grown on SMM for 2 weeks. Leaves were cut transversely across the midrib into three pieces (proximal, middle, and distal) that were placed in Petri dishes (90 x 10 mm, 25 ml medium) with the abaxial surface in contact with SRM supplemented with antibiotics at increasing concentrations (see sec-

tion Antibiotics). Control explants were grown on antibiotic-free (0 mg L^{-1}) SRM. The number of regenerated shoots per explant was counted after 3 weeks of culturing in the dark and additionally after 5 weeks under light, using a stereomicroscope (Carl Zeiss, Jena, Germany). The results were presented as the regeneration frequency, the mean number of shoots per explant and the index of shoot-forming capacity (SFC). Regeneration frequency was expressed as the percentage of explants that produced at least one shoot. The mean number of shoots per explant was calculated as the total number of regenerated shoots divided by the number of regenerating explants. The index of the SFC was used to evaluate the cumulative effect of the two aforementioned variables and was calculated as follows:

SFC = (the mean number of shoots per explant × % of regenerating explants)/100.

The experiment with cefotaxime, meropenem and timentin was performed in five replicates (Petri dishes) with 12 explants (n=60) per treatment. In experiments with hygromycin B, four replicates with 18 explants (n=72) per treatment were appointed.

To evaluate the influence of a selective antibiotic on subsequent shoot multiplication, shoots (≈ 5 to 8 mm long) regenerated from leaf segments on antibiotic-free SRM were separated from shoot bunches and transferred into Petri dishes containing 25 mL of SMM supplemented with 6, 8 and 10 mg L^{-1} hygromycin B. Control explants were grown on antibiotic-free (0 mg L^{-1}) SMM. The percentage of viable, partially necrotic and fully necrotic shoot clusters (each originating from one initial shoot), and the mean number of multiplied shoots per shoot cluster, were recorded after four weeks of cultivation. Four replicates with seven shoots in each (n=28) were evaluated for the hygromycin B treatment.

Statistical analysis

In all treatments, the cultures were placed in a completely randomized design. Percentage data were subjected to angular transformation, the mean shoot number and SFC data to the square root transformation before analysis, followed by inverse transformation for presentation. The data were subjected to standard analysis of variance (ANOVA), and the means were separated using Fisher's least significant difference (LSD) test at $P \leq 0.05$.

RESULTS AND DISCUSSION

The effect of β-lactam antibiotics on *Agrobacterium* growth

Different concentrations of β-lactam antibiotics were tested for their influence on the growth of *A. tumefaciens* strain EHA 105. All tested concentrations of cefotaxime, meropenem and timentin inhibited the growth of EHA 105 during the first 3 days after inoculation, in contrast to the antibiotic-free medium where bacterial growth was observed after the first day of inoculation (Fig. 1). However, after 7 days of incubation on antibiotics, vigorous bacterial growth was observed in the presence of both cefotaxime and timentin at a concentration of 100 mg L^{-1}. Obviously, the lowest applied concentration of these two antibiotics was not sufficient to inhibit the growth of bacteria for a period longer than 3 days, in contrast to the higher concentrations of cefotaxime and timentin, although it delayed the beginning of log phase in comparison to the control. In the next 7 days (from day 7-14), a mild increase in the optical density of bacterial suspensions (cultivated in the presence of 100 mg L^{-1} cefotaxime or timentin) was observed in comparison to the optical density recorded at the 7^{th} day (Fig.1). This mild increase, which was very similar to that observed in the control, could be explained by less intensive divisions, as bacteria were approaching the stationary phase due to the deficiency of nutrients and the accumulation of waste materials, toxic metabolites and inhibitory compounds in the growth medium [40]. From the presented results, it could be concluded that cefotaxime and timentin at concentrations of 200 to 600 mg L^{-1} were stable in YEB medium during the 2-week period and sufficient to prevent *Agrobacterium* growth.

On the other hand, meropenem exhibited an inhibitory effect on bacterial growth at all tested concentrations (25-150 mg L^{-1}, Fig. 1). Da Silva Mendes et al. [13] observed that meropenem at 6.25 mg L^{-1} was insufficient to completely inhibit the growth of strains EHA 101 and EHA 105 16 h after bacterial inoculation, and suggested the use of a higher concentration

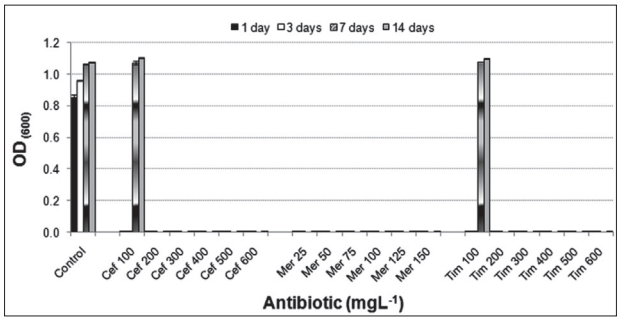

Fig. 1. The effect of β-lactam antibiotics cefotaxime (Cef), meropenem (Mer) and timentin (Tim) on *Agrobacterium tumefaciens* EHA 105 growth. The data present the optical density at 600nm (OD_{600}), measured 1, 3, 7 and 14 days after inoculation. The values are means (±SE) of three replicates per treatment.

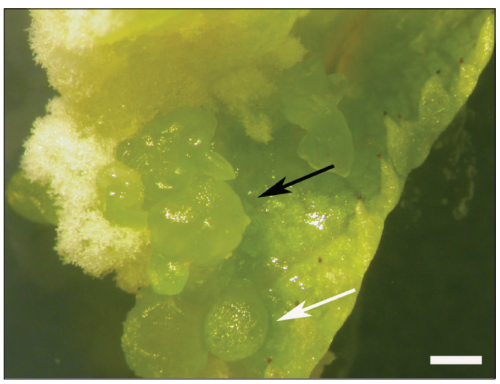

Fig. 2. Small globular shoots formed on the calli (black arrow) and directly from leaf tissue (white arrow) after 3 weeks of cultivation in the dark and additionally after one week under light on antibiotic-free SRM. The bar is 1 mm. SRM – shoot regeneration medium.

(12.5 mg L^{-1}) for the control of β-lactamase-producing strains. Considering the results from the presented study, it is important to be aware of the observation that the low concentrations of meropenem could inhibit bacterial growth for a limited period of time only, as can be observed in the case of cefotaxime or timentin, at a concentration of 100 mg L^{-1}. Accordingly, the testing of different meropenem concentrations by measuring the optical density of bacterial suspensions after a prolonged period of cultivation (longer than 3 days) is recommended. In line with this, Ogawa and Mii [41] highlighted that persisting *Agrobacterium* was frequently detected in the leaves of putative transformed shoots up to 8 weeks of culturing on 12.5 mg L^{-1} of meropenem, but with no visible bacterial overgrowth. The authors suggested the introduction of meropenem at a concentration of 25 mg L^{-1} to *Agrobacterium*-mediated transformation as a simple and efficient strategy to improve the transformation efficiency in tobacco, tomato and rice [41]. Results from the present study point to the effectiveness of the lowest antibiotic concentrations in preventing *A. tumefaciens* strain EHA 105 growth for a prolonged period of cultivation: meropenem at a concentration of 25 mg L^{-1} and cefotaxime and timentin at 200 mg L^{-1}. The final decision on the selection of β-lactam antibiotic for use in the transformation procedure must take into consideration not only its effect on bacterial growth but also the antibiotic effect on *in vitro* regeneration from desired plant tissue.

De novo shoot regeneration from leaf explants cultured on β-lactam antibiotic-containing medium

After two to three weeks of incubation in the dark, most leaf explants of cv. Golden Delicious cultured on antibiotic-free SRM produced calli that were formed mainly on cut edges or along the midribs. The calli even started to regenerate buds during the induction period in the dark. A week after transfer to the light, small globular or leafy shoots were clearly visible on the calli, whereas some were formed directly from leaf tissue (Fig. 2). Neither of the three applied β-lactams, cefotaxime, meropenem and timentin, affected the consequent steps in morphogenesis, nor altered the morphology of regenerated shoots. Their application resulted in the appearance of healthy and green shoots that were morphologically identical with those produced on antibiotic-free SRM, indicating no phytotoxic effect of the tested concentrations. However, after eight weeks of cultivation, exposure to β-lactam antibiotics influenced the regeneration potential of Golden Delicious explants to a degree that varied, depending on the type and concentration of antibiotics used, while plant regeneration frequency of control explants grown on antibiotic-free media (0 mg L^{-1}) was very high (94.9%), with an average of 7.67 shoots per explant (Fig. 3).

The effect of cefotaxime on the regeneration potential was strongly dose-dependent (Fig. 3a-c). Treatment with 300 mg L^{-1} of cefotaxime induced the highest number of regenerated shoots per explant (9.39) compared to the control (7.67) and other applied

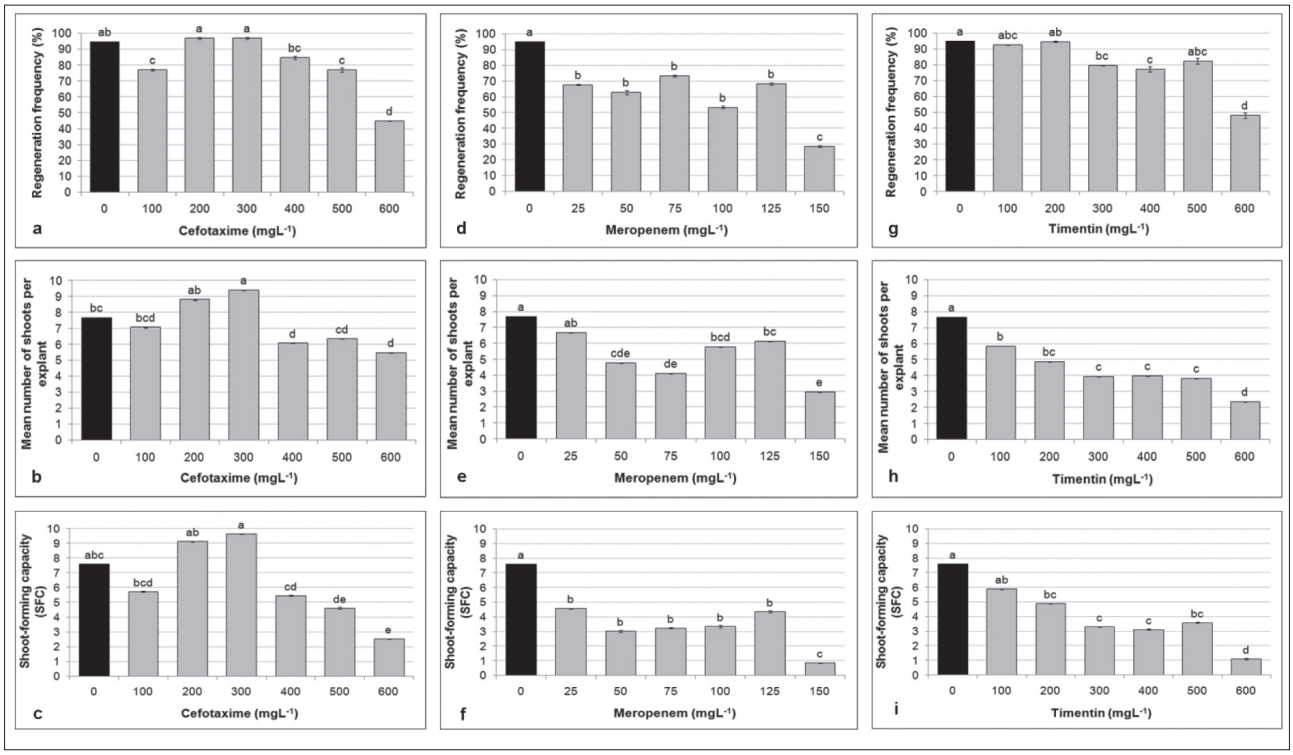

Fig. 3. The effect of β-lactam antibiotics cefotaxime, meropenem and timentin on the regeneration frequency, the mean number of shoots per explants and shoot forming capacity (SFC) of apple cultivar Golden Delicious leaf explants, after 3 weeks of cultivation in the dark and after 5 weeks under light. The values are presented as means (±SE) of five replicates with 12 explants (n=60) per treatment. Treatments denoted by the same letter are not significantly different (P≤0.05) according to Fisher's LSD test.

concentrations (Fig. 3b). There were no significant differences in the frequency of regeneration between treatments with 200 and 300 mg L^{-1} of cefotaxime (97.2% both) compared to the antibiotic-free control (94.9%) (Fig. 3a). On the other hand, concentrations of 100, 500 and 600 mg L^{-1} lowered the regeneration frequency, reaching more than a 2-fold decline in the case of the 600 mg L^{-1} cefotaxime treatment (44.7%). Overall, the treatment with 300 mg L^{-1} of cefotaxime was the most stimulating for the process of regeneration, with an SFC of 9.62 (Fig. 3c). In contrast, treatment with 600 mg L^{-1} of cefotaxime restrained shoot regeneration, with SFC being almost 3-fold lower (2.52) than for the antibiotic-free treatment (7.6). The stimulatory effect of cefotaxime at 250 mg L^{-1} on apple shoot regeneration was reported in the work of Yepes and Aldwinckle [22] for several cultivars (Empire, Freedom, Golden Delicious, Liberty, McIntosh, Mutsu) and rootstock M7A. In rootstock MM106, a similar concentration of cefotaxime (200 mg L^{-1}) promoted regeneration by increasing the frequency of regeneration as well as the number of regenerated shoots per explant [31]. This concentration-response phenomenon, characterized by low-dose stimulation and high-dose inhibition for numerous plant traits, is well known in plant biology [42], with renewed attention on herbicide-related hormesis [43]. In the present study, the minimal tested concentration of cefotaxime (100 mg L^{-1}) exhibited an inhibitory effect on regeneration frequency when compared to the antibiotic-free control and 200 mg L^{-1} and 300 mg L^{-1} cefotaxime treatments. This could be explained by the fact that the effects of antibiotics on plant regeneration or growth in some cases were not dose-dependent. Thus, timentin at 200 mg L^{-1} significantly inhibited regeneration efficiency in carrot protoplast cultures, while lower or higher concentrations did not influence plant production [44]. Cefotaxime and carbenicillin considerably inhibited chrysanthemum plant height, the number of leaves per plant and plant weight at a concentration of 100 mg L^{-1}, while the higher dose of 200 mg L^{-1} did not have a significant inhibitory effect [45]. The mechanism that underlies this phenomenon has not yet been elucidated. The effect of cefotaxime

may be attributed to its chemical properties resembling growth regulators. Cefotaxime is a semisynthetic cephalosporin consisting of an acetyl side chain and an aminothiazoyl ring and α-syn-methoxy-imino group [46]. It is assumed that plant esterases can break down cefotaxime to new compounds that might have growth-regulating properties [47]. Moreover, it was proposed that cefotaxime might inhibit ethylene production in cultures, which is positively correlated with plantlet differentiation from the callus mass [48]. Grewal et al. [49] reported that Basmati rice plants regenerated from calli grown on cefotaxime-containing medium were greener than those from control calli, and assumed that cefotaxime can act at the level of chlorophyll synthesis. In addition to this, the authors speculated that the stimulatory effects of cefotaxime might be the consequence of its ability to eliminate endophytic bacteria, which would finally result in disease-free and vigorously growing plant cultures. The last explanation is questionable because the higher concentrations of cefotaxime used in the present work (500 and 600 mg L^{-1}), which are expected to be more efficient in eliminating endophytic bacteria and would result in better growth of plants, significantly reduced both the regeneration frequency and the mean number of regenerating explants. The application of cefotaxime at 500 mg L^{-1} in the regeneration protocol of apple rootstock MM106 slightly decreased the percentage of regenerating leaf explants as well as the number of regenerated shoots per explant [31]. A decrease in regeneration frequency was also observed in a 500 mg L^{-1} cefotaxime treatment in seven apple cultivars and M7A rootstock [22]. For the Golden Delicious cultivar, these authors reported that the inhibitory effect of the 500 mg L^{-1} cefotaxime treatment induced a decline in regeneration frequency (from 95% in the control to 68% in the treatment) and the mean number of shoots per explants (from 5.6 in the control to 3.1 in the treatment group). The present study reported a less pronounced inhibitory effect of the treatment with 500 mg L^{-1} cefotaxime (from 94.9% to 77.1% for frequency, and from 7.67 to 6.34 for the mean number of shoots per explant), as compared to the previously mentioned work of Yepes and Aldwinckle [22], confirming not only the contribution of cultivar but also of individual variability to the strong modulating effect of cefotaxime on organogenesis in apple.

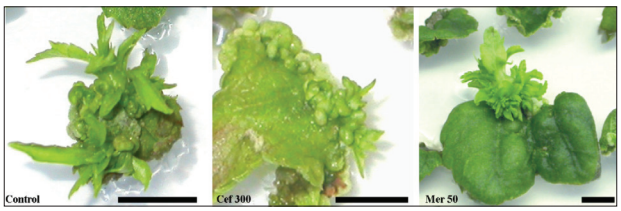

Fig. 4. Shoots regenerated on antibiotic-free SRM (Control), SRM supplemented with 300 mgL^{-1} cefotaxime (Cef 300) or 50 mgL^{-1} meropenem (Mer 50) after 3 weeks of cultivation in the dark and after 5 weeks under light. SRM – shoot regeneration medium. The bar is 0.5 cm.

The other two antibiotics, meropenem and timentin, at all tested concentrations decreased the regeneration potential of Golden Delicious leaf explants to a higher degree than cefotaxime (Fig. 3d-i). Thus, meropenem applied at concentrations ranging from 25 to 125 mg L^{-1} induced minimal fluctuations in the inhibition of regeneration frequency, while the concentration of 150 mg L^{-1} had the most pronounced inhibitory effect (28.5%) on regeneration frequency (Fig. 3d), and the mean number of shoots per explant (2.95) (Fig. 3e), with an SFC index of only 0.87 (Fig. 3f).

Although meropenem affected organogenesis in Golden Delicious more than cefotaxime, shoots regenerated from leaf explants cultivated on meropenem (25 to 125 mg L^{-1}) containing SRM grew vigorously and became larger and stronger, with leaves more developed than shoots derived from explants treated with cefotaxime after the same period of cultivation (Fig. 4). Since Ogawa and Mii [41] reported that meropenem (25 mg L^{-1}) was the best option for transformation of tobacco, tomato and rice compared to carbenicillin, cefotaxime and moxalactam, we can assume that its application in Golden Delicious transformation could significantly improve transformation efficiency.

Timentin is a β-lactam antibiotic composed of ticarcillin coupled with clavulanic acid, the β-lactamase inhibitor that inactivates some β-lactamase enzymes commonly found in bacteria resistant to penicillins and cephalosporins [50]. Although ticarcillin has been successfully used in apple transformation procedures at concentrations of 150 or 400 mg L^{-1} [51,52], the effect of timentin on apple morphogenesis in wide range of concentrations has not been evaluated until now. Since ticarcillin is metabolized to phenylacetic acid, a naturally occurring weak auxin [53], an increase in

timentin concentration might result in an improved regeneration potential of apple leaf explants, as has already been reported for several other plant species [12,13,15,54]. However, the results of the present study indicated a moderate negative influence of this antibiotic on apple organogenesis that was dose-dependent. Application of timentin had a more pronounced inhibitory effect on the number of regenerated shoots per explant than on the regeneration frequency. The regeneration frequencies obtained at concentrations of 100, 200 and 500 mg L^{-1} of timentin (92.6, 94.7 and 82.4%, respectively) were in the control range (94.9%; Fig. 3g). The remaining tested concentrations of timentin exhibited a weak inhibitory effect on regeneration frequency, culminating in the case of 600 mg L^{-1}. All timentin treatments resulted in a lower mean number of shoots per explant than the control (Fig. 3h). Timentin at 600 mg L^{-1} induced the strongest inhibition with a 3-fold decrease. The cumulative effects of reduction in the mean shoot number and frequency of regeneration resulted in a decrease in the SFC of leaf explants cultivated on media with different timentin concentrations (Fig. 3i). This negative effect of timentin might be the consequence of a disruption of hormonal balance in apple tissue due to the presence of ticarcillin, clavulanic acid or their breakdown products. The inhibitory effect of timentin was also reported in somatic embryogenesis of walnut [55] and cocoa [56].

Since the results obtained in the presented study showed that the regeneration potential of explants was enhanced by the application of 300 mg L^{-1} cefotaxime, which was also highly effective in preventing bacterial growth, this concentration could be recommended for use in genetic transformation protocols in recalcitrant-to-transformation apple cv. Golden Delicious. Also, meropenem at 25-125 mg L^{-1} could be a good alternative if more vigorous and rapid growth of regenerated shoots is a priority over obtaining shoots in large numbers.

Effect of hygromycin B on shoot regeneration from leaf explants

To determine the MICs suitable for use in the selection procedure during transformation of the Golden Delicious cultivar, we tested a range of hygromycin B concentrations (0, 2, 4, 6, 8, 10 mg L^{-1}) in order to evaluate its effect on leaf explant regeneration efficiency, as well as on the survival and multiplication rate of the regenerated shoots.

As was expected, the application of hygromycin B induced necrosis of apple leaf tissue. Areas of necrotic degradation became wider and more numerous with the increase in hygromycin B concentration (Fig. 5). *De novo* shoot organogenesis occurred only after treatments with 2 and 4 mg L^{-1} hygromycin B. The percentage of regeneration, the mean number of regenerated shoots per explant and the SFC were significantly lower at 4 mg L^{-1} than at 2 mg L^{-1} of the applied antibiotic (Fig. 5 and 6). The hygromycin B applied at concentrations of 5-10 mg L^{-1} induced necrotic degradation of leaf tissue, inhibiting regeneration in Golden Delicious explants. Necrotic segments were visible on explants after approximately two weeks of cultivation. After exposure to light, the area of necrotic degradation became larger with time, and finally it expanded over the whole explant at the moment of recording. Norelli and Aldwinckle [57] reported that high doses of kanamycin caused apple leaf tissue to turn pale yellow or white, whereas high doses of aminoglycosides, such as geneticin, paromomycin and neomycin, resulted in necrosis. Our results revealed that the effect of hygromycin B was similar to that of geneticin, paromomycin and neomycin, despite differences in the mechanisms of inhibition of protein synthesis.

As shown in the potato [32], sensitivity toward hygromycin B may vary among cultivars. In the case of apple, due to its high sensitivity to hygromycin B, even minor variations in hygromycin B concentration can have a dramatic impact on plant regeneration. Modgil and Sharma [31] found that increasing hygromycin B concentration resulted in the gradual decline of regeneration frequency and the number of regenerating leaf explants in apple rootstock MM106, while 5 and 5.5 mg L^{-1} of hygromycin B completely inhibited the process of regeneration, with 83% and 96% frequencies of necrosis, respectively. Since hygromycin B at 6 mg L^{-1} caused complete necrosis of leaf explants, the concentration of 5 mg L^{-1} was selected as optimal for the selection of putative transformed shoots. The same concentration (5 mg L^{-1}) was successfully used for selection of apple rootstock N545 [30]. Our results indicated that Golden Delicious was slightly more sensitive to hygromycin B than MM106 and N545 rootstocks and that the concentration of 4

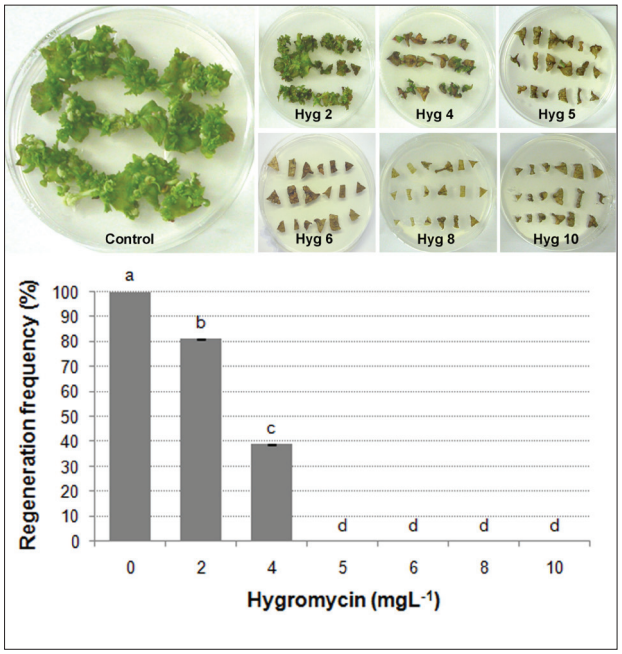

Fig. 5. Regeneration frequency of apple cultivar Golden Delicious leaf explants on antibiotic-free SRM (0 mgL^{-1}) and SRM supplemented with: 2, 4, 5, 6, 8 and 10 mgL^{-1} of hygromycin B after 3 weeks of cultivation in the dark and 5 weeks under light. The values are presented as means (±SE) of four replicates with 18 explants (n=72) per treatment. Treatments denoted by the same letter are not significantly different (P≤0.05) according to Fisher's LSD test. SRM – shoot regeneration medium.

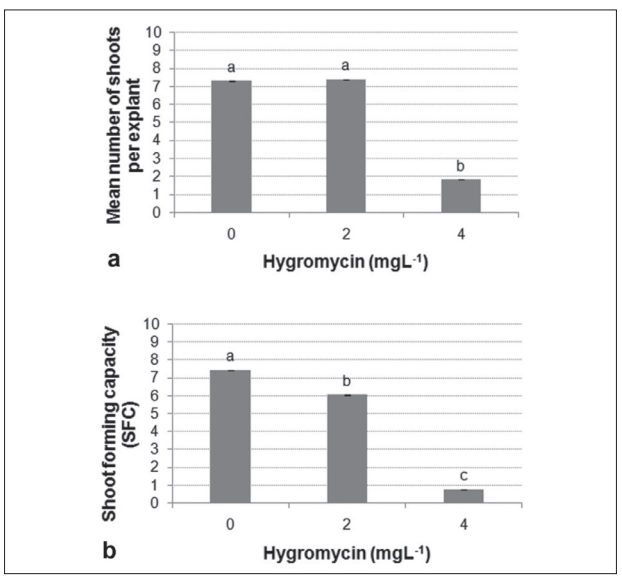

Fig. 6. The effect of hygromycin B on the mean number of shoots per explants (**a**) and shoot forming capacity (SFC) (**b**) of apple cultivar Golden Delicious leaf explants after 3 weeks of cultivation in the dark and 5 weeks under light. The values are means (±SE) of four replicates with 18 explants (n=72) per each treatment. Treatments denoted by the same letter are not significantly different (P≤0.05) according to Fisher's LSD test.

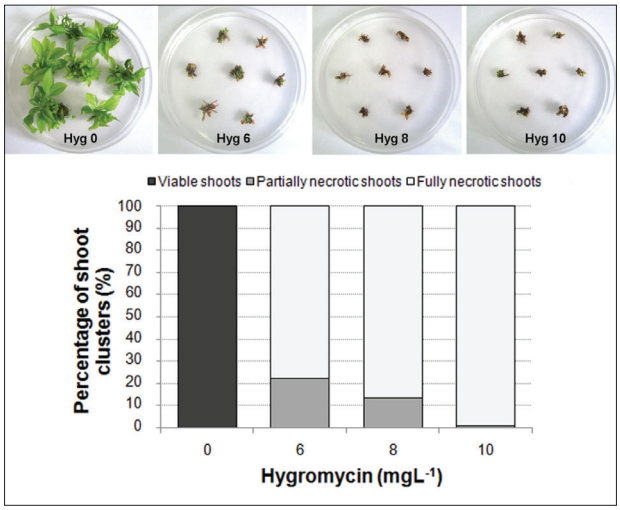

Fig. 7. The effect of hygromycin B on growth and multiplication of Golden Delicious shoots on SMM, expressed as percentage of viable shoot clusters, partially necrotic shoots and fully necrotic shoots. The values are means (±SE) of four replicates with seven explants (n=28) per treatment. Treatments denoted by the same letter are not significantly different (P≤0.05) according to Fisher's LSD test. SMM – shoot multiplication medium.

mg L^{-1} could be chosen as the MIC for the selection of transformed cells.

The use of low doses of hygromycin B in the selection process of Golden Delicious cultivar gives hygromycin B selection an advantage over kanamycin, which in doses from 25 to100 mg L^{-1} provides sufficient selection of transformed apple cells [51,58,59]. The use of minimal concentrations of the selection agent renders the transformation system more economical since selection agents count for almost half of transformation process costs.

Effect of hygromycin B on shoot growth and multiplication

A further effect of hygromycin selection at the level of growth and multiplication of regenerated Golden Delicious shoots was evaluated in experiments conducted on SMM without antibiotic or supplemented with 6, 8 and 10 mg L^{-1} hygromycin B (Fig. 7). Multiplication of shoots was observed only in the hygromycin B-free treatment, the outcome of which were green and healthy shoot clusters (designated as viable shoot clusters) with 3.64 shoots per shoot cluster. Brownish shoots with portions of green, nonnecrotic tissue

(designated as partially necrotic shoots) were found mostly at 6 mg L^{-1} (22%) and 8 mg L^{-1} of hygromycin B (12%), while the highest concentration (10 mg L^{-1}) induced necrosis in 99.1% of the tested shoots (Fig. 7). Thus, this concentration could be recommended for further selection of putative transformed shoots of Golden Delicious in order to avoid the production of chimeras. The effect of hygromycin B on growth and multiplication of apple shoots has been reported for the first time in the present work, since previous works only investigated the effect of hygromycin on shoot regeneration [30,31].

CONCLUSIONS

This study presents an evaluation of the effects of β-lactam antibiotics (cefotaxime, meropenem and timentin) and the aminoglycoside antibiotic hygromycin B on *in vitro* regeneration via *de novo* shoot organogenesis in recalcitrant-to-transformation apple cv. Golden Delicious. The obtained results indicate that cefotaxime at 300 mg L^{-1} provided both a reliable control of growth of *A. tumefaciens* strain EHA 105 and an increase in the regeneration potential of leaf explants. However, if the priority is to obtain larger and more developed shoots rather than more numerous but smaller shoots after a shorter period of time, the use of meropenem at concentrations ranging from 25 to 125 mg L^{-1} is more appropriate. Golden Delicious leaf explants displayed high sensitivity to hygromycin B. Even low doses of hygromycin B impaired the process of organogenesis, while concentrations of 5 mg L^{-1} or higher completely inhibited shoot regeneration and induced explant tissue necrosis. Therefore, a one-step or stepwise selection procedure, with a final hygromycin B concentration of 4 mg L^{-1} throughout organogenesis and 10 mg L^{-1} for further shoot growth and multiplication can be recommended for an efficient transformation process in apple cv. Golden Delicious.

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Author contributions: MS, NM and SN designed the experiments and prepared the manuscript. MS provided the plant material, collected the data and carried out the morphological and statistical analysis. JS and TĆ assisted with the data analysis and manuscript preparation.

Conflict of interest disclosure: We declare that there is no conflict of interest. We confirm that this is an original manuscript that has not been previously published, neither in part nor whole, in any other scientific journal.

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