JOURNAL OF FOREST SCIENCE, *55*, 2009 (4): 156–161

# Tolerance of Norway spruce (*Picea abies* [L.] Karst.) embryogenic tissue to penicillin, carbapenem and aminoglycoside antibiotics

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**ABSTRACT**: Somatic embryogenesis is conveniently utilized for the preparation of Norway spruce (*Picea abies* [L.] Karst.) transgenic clones by means of *Agrobacterium*. The establishment of successful transformation protocol requires to determine the tolerance of growing embryogenic tissue to antibiotics in culture and selective media. In 5 Norway spruce lines (genotypes) differences in the tolerance of embryogenic tissues to penicillin antibiotics (amoxicillin, carbenicillin, and ticarcillin), carbapenem antibiotic (meropenem) used for the *Agrobacterium* growth prevention, and aminoglycoside antibiotic (kanamycin) used in selective media were determined. Of the penicillin derivatives, amoxicillin was optimally tolerated in all lines and, in addition, its highest concentration accelerated growth in more rapidly growing lines. Ticarcillin was similarly tolerated but no growth acceleration was observed in any line. As regards carbenicillin, only the lowest concentration was observed to be well tolerated by all lines whereas all concentrations of meropenem were well tolerated in all lines except for slowly growing line 28, the growth of which was retarded by the concentration of 20 mg/l. The aminoglycoside antibiotic kanamycin was well tolerated by the embryonic tissue of all lines in the concentration of 10 mg/l and less in the concentration of 25 mg/l. The concentrations of 50 mg/l and 100 mg/l appeared as intolerable in all lines. Toxicity of kanamycin manifested at first in the browning and later in the growth cessation of embryogenic tissue.

**Keywords**: somatic embryogenesis; transformation; penicillin antibiotics; carbapenem antibiotics; aminoglycoside antibiotics; Norway spruce; *Agrobacterium tumefaciens*

**Abbreviations**: GD – Gupta-Durzan medium, BAP – 6-benzylaminopurin, 2,4D – 2,4-dichlorophenoxyacetic acid, Kin – N6-furfuryladenine

Somatic embryogenesis represents an efficient regeneration system conveniently used in forest improvement and breeding for resistance programs (Hasnain, Cheliak 1986; Attree, Fowke 1993). The successful clonal propagation of conifers by somatic embryogenesis depends on the selection, elaboration, and standardization of suitable procedures involving the induction of embryogenetic processes in somatic cells of the primary explant (e.g. immature zygotic embryo) and the development of embryogenic tissue into a complete somatic embryo capable of subsequent conversion into a viable plantlet. All differentiation processes during somatic embryogenesis are regulated by extrinsic and intrinsic factors and can be influenced by the chemical composition of culture media (Quiroz-Figueroa et al. 2006). Somatic embryogenesis was already exploited for the preparation of *Picea* transgenic clones by means of

Supported by the Ministry of Agriculture of the Czech Republic, Projects No. QH71290, No. 0002070203 and No. AV0Z50510513.

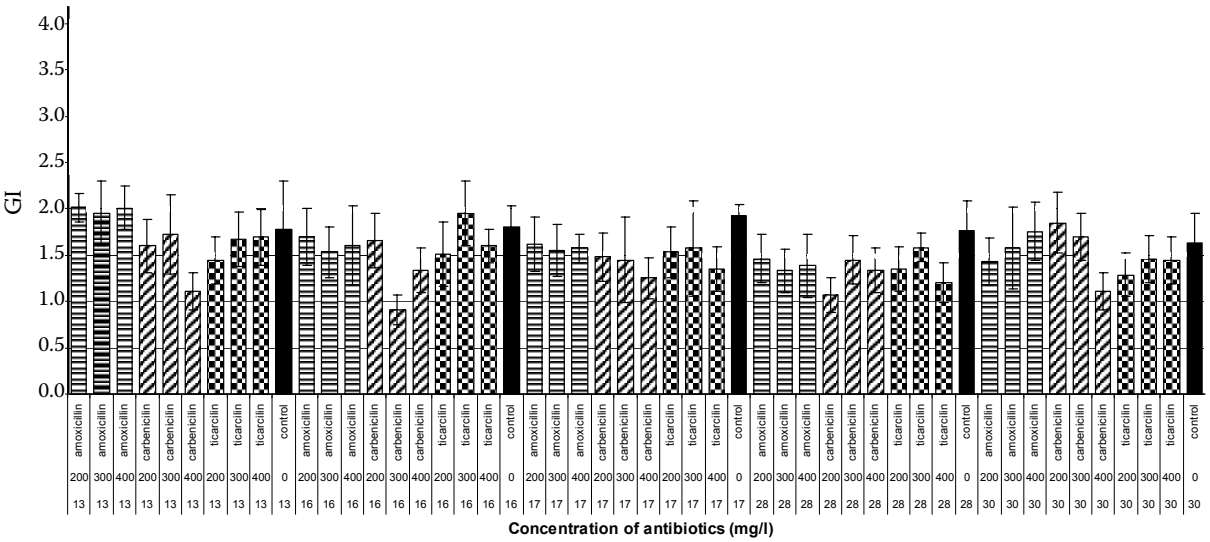


Fig. 1. Mean growth indices (GI ± SE) of Norway spruce embryogenic lines 13, 16, 17, 28 and 30 cultured in 200, 300, 400 mg per l of antibiotics (2 weeks of cultivation; GI = $m/m_0$, $m_0$ – inoculum fresh weight, m – sample fresh weight)

the *Agrobacterium* gene transfer (Klimaszewska et al. 2001, 2005; Cyr, Klimaszewska 2002). Nevertheless, the transformation of Norway spruce is far from being a routine. The search for the most effective antibiotics preventing bacterial overgrowth and also antibiotics suitable for selective media having minimal damaging effects on the growing embryonic tissue during somatic embryogenesis is an essential prerequisite for successful transformation.

The objective of the present study was to determine the tolerance capability of embryogenic tissues of 5 Norway spruce lines (genotypes) to different concentrations of the penicillin antibiotics (amoxicillin, carbenicillin, and ticarcillin) and the carbapenem antibiotic (meropenem) which are used for *Agrobacterium* growth prevention, and to the aminoglycoside antibiotic kanamycin used for killing of non-transformed cells.

## PLANT MATERIAL AND METHODS

### Embryogenic tissue preparation

Open-pollinated immature cones of a 140-years-old plus tree of Norway spruce (*Picea abies* [L.] Karst.) growing in the Habitat Conservation Area Labské Pískovce (Northern Bohemia, Czech Republic) were collected in late July 2006. The extracted seeds were sterilized with 1% NaClO (Savo, Bochemie, Czech Republic) and stored at 4°C. Immature embryos were extirpated and placed onto inductive solid E medium consisting of modified (Malá et al. 1995) GD medium supplemented with 0.2 mg/l Gelrite, 0.5 mg/l of BAP, 1.0 mg/l of 2,4D, and 0.5 mg/l of Kin (all Sigma–Aldrich, Seinheim, Germany). Five embryogenic lines (genotypes) were selected for our experiments.

### Testing of antibiotics

The embryogenic tissue of each line was cultivated in three parallels (initial inoculum 200 mg, dark, 24°C) on the solid E medium supplied with amoxicillin (Augmentin™, GlaxoSmithKline, Worthing, UK), carbenicillin (Carbenicillin disodium, Duchefa Biochemie, B.V., Haarlem, the Netherlands), ticarcillin (Timentin™, GlaxoSmithKline, Worthing, UK), each in concentrations 200, 300, and 400 mg/l, meropenem (Meronem®, AstraZeneca SpA, Caponago, Italy) in concentrations 10, 20, 30, 40, and 50 mg/l or kanamycin (Kanamycin monosulphate, Duchefa Biochemie, B.V., Haarlem, the Netherlands) in concentrations 25, 50, and 100 mg/l. The control embryogenic tissues (three parallels) of each line were cultured on the same media without antibiotics. After 2 weeks, the growing embryogenic cultures were weighed and transferred onto the fresh E media with corresponding concentrations of antibiotics or without antibiotics (controls), and cultured for another 2 weeks, when the experiment was finished and all embryogenic tissues were weighed again. Mean growth indices (GI) ± SE were calculated for all parallel cultures weighed after 2 and 4 weeks of cultivation: GI = $m/m_0$; $m_0$ – weight of the inoculum, m – sample weight.

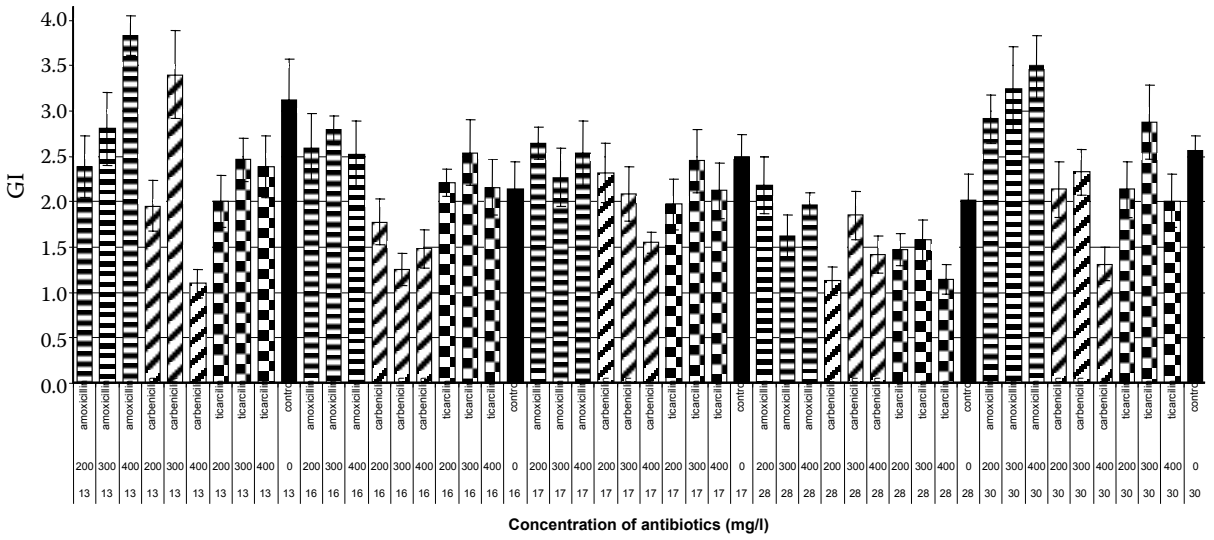


Fig. 2. Mean growth indices (GI ± SE) of Norway spruce embryogenic lines 13, 16, 17, 28 and 30 cultured in 200, 300, 400 mg per l of antibiotics (4 weeks of cultivation; GI = $m/m_0$, $m_0$ – inoculum fresh weight, m – sample fresh weight)

## RESULTS

### Evaluation of growth capability of control embryogenic tissues

After 2 weeks, the GI of embryogenic tissues of all control lines increased on average to 1.78 ± 0.05 (Fig. 1). After 4 weeks, the GI of all controls increased on average to 2.30 ± 0.27 with the exception of line 13, the GI of which increased to 3.13 ± 0.45 (Fig. 2).

### Evaluation of tolerance to penicillin antibiotics

#### *Amoxicillin*

Embryogenic tissues of all lines optimally tolerated all concentrations of amoxicillin during the whole experiment. In lines 13 and 30, 400 mg/l amoxicillin appeared as growth supporting after 4 weeks of cultivation because the average GI of line 13 increased to 3.83 ± 0.22 (the average control GI 3.13 ± 0.45) and the average GI of line 30 to 3.49 ± 0.34 (the average control GI 2.57 ± 0.17) (Fig. 2).

#### *Carbenicillin*

Embryogenic tissues of all lines tolerated the 200 mg/l concentration of carbenicillin during the whole experiment with the exception of line 28, the average GI of which decreased to 1.13 ± 0.16 (the average control GI 2.02 ± 0.29). Carbenicillin in the concentration of 300 mg/l decreased the average GI of line 16 to 0.90 ± 0.16 (the average control GI 1.81 ± 0.23) after 2 weeks (Fig. 1) and to 1.25 ± 0.18 (the average control GI 2.13 ± 0.31) after 4 weeks of cultivation. After this time, the concentration of 400 mg/l carbenicillin was well tolerated only by the embryogenic tissue of line 28 but the average GI of other lines notably decreased: GI of line 13 to 1.10 ± 0.16 (the average control GI 3.13 ± 0.45), GI of line 16 to 1.48 ± 0.21 (the average control GI 2.13 ± 0.31), GI of line 17 to 1.55 ± 0.11 (the average control GI 2.49 ± 0.24), and GI of line 30 to 1.31 ± 0.19 (the average control GI 2.57 ± 0.17) (Fig. 2).

#### *Ticarcillin*

Embryogenic tissues of all lines optimally tolerated all concentrations of ticarcillin during the whole experiment with the exception of line 28, where the concentration of 400 mg/l decreased the average GI to 1.15 ± 0.16 (the average control GI 2.02 ± 0.29) after 4 weeks of cultivation (Fig. 2).

### Evaluation of tolerance to carbapenem antibiotic meropenem

Embryogenic tissues of all lines well tolerated all concentrations of meropenem during the whole experiment with the exception of line 28, where the concentration of 20 mg/l substantially decreased the average GI to 6.17 ± 2.00 (the average control GI 11.94 ± 1.18) after 4 weeks of cultivation (not shown).

### Evaluation of tolerance to aminoglycoside antibiotic kanamycin

Embryogenic tissues of all lines tolerated kanamycin in the concentration of 10 mg/l during the whole experiment. The concentration of 25 mg/l decreased

Fig. 3. The growth of embryogenic tissue cultured on control medium (left) and kanamycin medium in the concentration of 50 mg/l (right)

the average GI to 0.5 ± 0.04 of all lines (the control average GI 2.30 ± 0.21, not shown). Kanamycin in concentrations of 50 mg/l and 100 mg/l appeared as intolerable in all lines. The toxicity of kanamycin manifested at first in the browning and then in the growth cessation of embryogenic tissue (Fig. 3).

## DISCUSSION

The assignment of effective genetic transformation protocols successfully applicable to the construction of plants resistant to negative biotic and abiotic factors needs the study of the capability of plant tissue to tolerate antibiotics used for elimination of bacterial vectors and for selection of transformed cells (Cheng et al. 1998; Tang et al. 2000; Van Quy Le et al. 2001; Alsheikh et al. 2002; Taniguchi et al. 2005). In this study, the tolerance of embryogenic tissues of 5 differently growing Norway spruce lines (genotypes) to various concentrations of amoxicillin, carbenicillin, ticarcillin, meropenem, and kanamycin was determined during somatic embryogenesis. The tolerance capability was expressed as growth indices (GI) after 2 and 4 weeks of cultivation (Figs. 1 and 2). These intervals were selected according to the established protocol of somatic embryogenesis induced in Norway spruce (Malá 1991; Malá et al. 1995). The embryogenic tissues of lines 13 and 30 were shown as rapidly growing: in line 13 the fresh weight increased from 200 mg of initial inoculum to approximately 625 mg (average GI 3.13) and in line 20 to approximately 514 mg (average GI 2.57) (Fig. 2).

The best tolerance of embryogenic tissues of all lines was proved to amoxicillin and ticarcillin. Amoxicillin in the concentration of 400 mg/l even stimulated the embryogenic tissue growth of rapidly growing lines 13 and 30. Similar effects of amoxicillin on root growth during transformation of taxonomically different recalcitrant plants already in the concentration of 250 mg/l (with still preserved antibacterial efficiency) were demonstrated (ur Rahman et al. 2004). Ticarcillin and carbenicillin were far less tolerated and their higher concentrations even reduced the embryogenic tissue growth in all lines. Similar retarding effects of carbenicillin during somatic embryogenesis of Sitka spruce (*Picea sitchensis* [Bong.] Carr.) were described by Sarma et al. (1995). Meropenem was practically well tolerated except for the concentration of 20 mg/l in slowly growing line 28. Similar effects of meropenem were described on somatic embryogenesis of orchids (Cao et al. 2006; Ogava, Mii 2007). Kanamycin was tolerated by the embryogenic tissue of all lines only in the concentration of 10 mg/l. Concentrations of 50 mg/l and 100 mg/l were intolerable and even toxic. Chun et al. (1989) reported kanamycin toxicity already in the concentration of 10 mg/l in somatic embryogenesis of hybrid poplar (*Populus alba* × *P. glandulosa*). Similarly to our findings, Taniguchi et al. (2005) reported tolerability of 10 mg/l of kanamy-

cin during somatic embryogenesis of *Chamaecyparis obtusa* Sieb. et Zucc., whereas the concentration of 25 mg/l was toxic. Generally, the effects of antibiotics on growth and simultaneously on elimination of *Agrobacterium* are clearly pleiotropic. Here we report the screening of antibiotics suitable for the establishment of transformation protocol using somatic embryogenesis in Norway spruce. Among the tested antibiotics, amoxicillin and meropenem were optimally tolerated by the growing embryogenic tissues of all Norway spruce lines. Kanamycin was intolerable at 50 mg/l already after 2 weeks of cultivation by all lines. In conclusion, amoxicillin and meropenem could be recommended for the elimination of bacteria from explant cultures whereas kanamycin for the killing of non-transformed cells.

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Received for publication September 21, 2008
Accepted after corrections November 4, 2008

# Tolerance embryogenního pletiva smrku ztepilého (*Picea abies* [L.] Karst.) k antibiotikům penicilinového, karbapenemového a aminoglykosidového typu

**ABSTRAKT**: Pro reprodukci transgenních linií smrku ztepilého (*Picea abies* [L.] Karst.) získaných metodou nepřímé transformace pomocí *Agrobacterium* se obvykle používá somatická embryogeneze. Vypracování úspěšného transformačního postupu je mimo jiné podmíněno určením tolerantnosti embryogenního pletiva k antibiotikům, obsaženým v kultivačních a selekčních médiích. Na citlivost k antibiotikům bylo testováno pět embryogenních linií smrku ztepilého. Testovala se antibiotika penicilinového typu (amoxicillin, carbenicillin a ticarcillin), antibiotika karbapenemového typu (meropenem), která se používají pro omezení růstu *Agrobacterium,* a aminoglykosidového typu (kanamycin), používaná jako selekční látky. Z penicilinových derivátů byl jako nejvhodnější vyhodnocen amoxicillin, který byl tolerován všemi liniemi a u nejvyšší koncentrace (400 mg/l) se projevilo pozitivní ovlivnění růstu nejrychleji se multiplikujících linií. Aplikace ticarcillinu byla také dobře snášena, ale stimulující efekt na růst linií nebyl pozorován. Carbenicillin byl tolerován všemi liniemi pouze v nejnižší koncentraci a meroponem byl velmi dobře snášen všemi liniemi s výjimkou pomalu rostoucí linie 28, u které došlo k omezení růstu již při koncentraci 20 mg/l. Aminoglykosidové antibiotikum kanamycin bylo tolerováno embryogenním pletivem všech linií v koncentraci 10 mg/l, méně v koncentraci 25 mg/l a koncentrace 50 a 100 mg/l se projevovaly pro všechny linie jako toxické. Toxicita kanamycinu se po aplikaci projevila nejdříve zhnědnutím, později úplným zastavením růstu embryogenního pletiva.

**Klíčová slova**: somatická embryogeneze; transformace; penicilinová antibiotika; karbapenemová antibiotika; aminoglykosidová antibiotika; smrk ztepilý; *Agrobacterium tumefaciens*

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