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Effect of heavy metal ions on Norway spruce embryos

Diploma thesis

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Abstract

Effect of heavy metal ions on Norway spruce embryos

Aim of this work was to evaluate effect of copper and arsenic on Norway spruce embryogenic cell masses (ECMs) in different concentrations during 14 days proliferation period but as well during maturation and germination stages. Copper in lower concentrations increased the growth of ECMs while in higher concentrations growth was inhibited. Arsenic in concentrations 50, 250 and 500 µM showed very high toxicity and after 14 days proliferation period all ECMs were dead. In experiments with lower concentrations (10 and 25 μM), control had highest proliferation ratio while arsenic had insignificant difference on growth. Cell line I – 1 – 3 had higher proliferation ratio and produced more somatic embryos during maturation in comparison with cell line III – 3 - 3, respectively. After maturation certain number of abnormally developed cotyledonary somatic embryos was obtained. Norway spruce as a fast growing conifer tree is one of the most widely planted spruces and one of the most economically important species in Europe. The results of this thesis can help to understand effect of two tested metals on somatic embryogenesis but as well can be used for investigation of two tested clones in polluted soils.

Keywords: copper, arsenic, somatic embryogenesis, proliferation, maturation, germination

Abstrakt

Vliv iontů těžkých kovů na embryích smrku ztepilého

Cílem této práce je hodnocení vlivu mědi a arzenu na raná somatická embrya (RSE) smrku ztepilého. Byly použity různé koncentrace v průběhu 14ti dnů proliferační periody. Vliv nižších koncentrací mědi zvyšovala růst RSE na rozdíl od vyšších koncentrací, kde byl růst zpomalen. Arzen v koncentracích 50, 250 a 500 µM ukázal vysokou toxicitu a po 14 dnech veškerá embrya odumřela. V experimentech s nižšími koncentracemi (10 a 25 µM), kontrola vykazovala nejvyšší proliferační poměr, zatímco RSE měla nevýznamný rozdíl růstu. Buněčná linie I-1-3 měla vyšší poměr proliferace a vytvářena vice somatických embryi v průběhu maturace v porovnání s buněčnou linií III-3-3. Po maturaci bylo pozorováno určité množství abnormalit u kotelydonárních embryí. Smrk ztepilý jako jehličnan patří k nejvíce rozšířeným smrkům v Evropě s vysokým ekonomickým významem. Znečištění životního prostředí je velmi důležitým tématem, které nesmí být opomíjeno v žádných diskuzích. Pochopení vztahu dvou těžkých kovů a jejich vlivu na somatickou embryogenezi je základním nástrojem při testování a hledání nových odolných buněčných linií smrku ztepilého.

Klíčová slova: měď, arzen, somatická embryogeneze, proliferace, maturace, germinace

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1 INTRODUCTION

Nowadays, it can be seen that degradation of the environment significantly disturbs the ecological balance. From the beginning of the industrial revolution, offensive technological development, uncontrolled population growth and insufficiently developed environmental awareness have led to the rapid depletion of natural resources and environmental degradation. The biggest influence on the environment was made by humans since they change it to suit their needs much more than the other species. Most of the pollution originates from human-made sources, including mobile sources (e.g., cars, trucks, buses) and stationary sources (e.g., factories, refineries, power plants) (EPA, 2015). Pollutions are characterized as any unwanted change in air, water and soil which can have negative impact on health and survival of human beings or other living organisms (Pojman et al. 2012). Pollutants can be found into the water, air or in the ground and can change natural balance in the environment. Water resources are constantly polluted by waste from industries, sewage systems, from pesticides and herbicides used in agriculture. Billions of tons of waste from different sources are moved into nature while the biggest problem is that majority of waste is not biodegradable which means that microorganisms cannot decompose it. The biggest pollutants of air are car engines and power plants which emit substances that can destroy ozone layer while subsequently contribute to global warming by inducing the planet's natural greenhouse effect. Moreover, acid rain has destroyable effect on forest and lake habitats. Billions of tons of carbon dioxide (CO_2) and other greenhouse gases are discharged into the atmosphere every year. The more greenhouse gases humans emit, climate changes in the future will be stronger. Greenhouse gases in the atmosphere and the warming of the planet are responsible for fluctuations in the temperatures and precipitations, increase of ocean temperatures, melting of glaciers and sea ice, changes on concentration and period of extreme weather events. The greenhouse effect is the process by which radiation from a planet's atmosphere warms the planet's surface to a temperature above what it would be in the absence of its atmosphere. Global warming is the gradual heating of Earth. Scientists have documented the rise in average temperatures worldwide since the late 1800's. Earth's average temperature has risen 0.8 ºC degrees over the past century. Temperatures are projected to rise another 1.133 to 6.42 ºC degrees over the next 100 years (IPCC, 2007).

Forest vegetation's absorb large amounts of carbon dioxide, which is the most important in the group of greenhouse gas emissions. Thus, forests are a kind of natural defense system from climate change. Forests are also very important for providing shelters and food for many different types of plants and animals. Moreover, forests around the world are under threat due to deforestation. Deforestation, clearance or clearing is the removal of forest or stand of trees where the land is converted to a non-forest use. According to WWF (2016) deforestation is a particular concern in tropical rainforests since these forests are home for most of the world's biodiversity.

For a long time humans did not pay attention to the influence of substances which are produced and their effect on ecosystems, but today it is clear that with such an approach people will not be able much longer to enjoy in the natural resources. Among different pollutants the most dangerous are heavy metals. In the small concentrations, many metals are essential to life and ecosystems, but chronic low exposures to metals can lead to severe environmental and human health effects. The main metal threats are associated with heavy metals such as lead, arsenic, cadmium and mercury (Järup, 2003). Metals in the environment are present in small quantities, and are classified in the group of microelements. Microelements are essential for normal functioning of the metabolism of living organisms, but in large amounts can be harmful and dangerous. Heavy metals are significant environmental pollutants, and their toxicity is a problem of increasing significance for ecological, evolutionary, nutritional and environmental reasons (Jaishankar et al. 2014; Nagajyoti et al. 2010). Effect of heavy metals on plant growth and soil pollution is not easy to estimate. The presence of one compound in the particular amount should not cause a disruption in plant production in one type of soil, but its presence in the second type of soil, can reduce the quality and quantity of yield.

In vitro culture system can provide standard conditions in order to test genotypes for various types of stresses, including effect of heavy metal ions. Both *in vitro* methods, organogenesis and somatic embryogenesis proved to be efficient methods for regeneration of plantlets but somatic embryogenesis is more preferable for regeneration of conifers. Somatic embryogenesis (SE) is a cloning technique which has very important role in production of trees particularly, because unlimited number of copies can be produced from a single seed. One of the advantages of SE is that with introduction of elite clones it can advance forest productivity much more than conventional production techniques.

2 AIM OF THE RESEARCH

The aim of this work was to test the effect of arsenic and copper in different concentrations on the growth and development of *Picea abies* somatic embryos. It was hypothesized that due to toxicity of both metals in higher concentrations, further development of somatic embryos will be strongly affected. In order to test this hypothesis, the evaluation of proliferation rate was carried out. Experiments were performed with observations during different sampling days. In addition, the effect of heavy metals applied during proliferation stage was observed during maturation and germination stages. Moreover, during maturation stage abnormal cotyledons development was recorded and documented.

3 LITERATURE REVIEW

3.1 *Picea abies* – Norway spruce

Norway spruce (Fig.1) is a coniferous tree, originated from montane and boreal European forests with the distribution area from Alps to the Balkan and the Carpathians with the extension in the north of Scandinavia and northern Russia (Barnes and Wagner, 2004). As fast growing tree it is one of the most widely planted spruces and economically important coniferous species in Europe and Scandinavia (Skrøppa, 2003).

Fig. 1 Norway spruce (http://www.norwayspruce.com/)

It has shown good yield and quality performance on very different site conditions which favored the species over a long period. The species has a long history of cultivation in Central and Eastern Europe and has been planted very intensely since the middle of the 19th century. This has changed natural forests into artificial forests and has led to the introduction of the species far outside its natural range, both in countries where it occurs naturally e.g. in Germany and Norway but as well in new countries such as Denmark, Belgium, Ireland and North America.

It is widely used as an ornamental tree in parks and gardens, mostly planted for use as a Christmas tree. Its wood is used for construction paper (pulpwood), lumber, millwork, crates and musical instrument (soundboards). The Norway spruce can grow up to 0.6-0.9 meters per year in the first 25 years, under good conditions, but in heavy or poor

soils the trees decline their growth with an average of 0.30 meters per year. Conditions such as soil, moisture and adequate insolation are determinant to a plant's growing rate.

3.2 Sources and causes of natural pollution by heavy metals

Heavy metals (HM) are characterized as metallic elements with high atomic weight and a density (Tchounwou et al. 2012). In traces, heavy metals can be included in food chain trough water pipes (copper) and/or through food which was grown on polluted soils or treated with herbicides and pesticides. The major risk appears if uptake of metals is on everyday level which can cause chronic health disorders, mutations and different abnormalities. As well, heavy metals toxicity depends on concentrations, time and route of exposure, species and organ exposed. Since some heavy metals are non-essential and does not have any biological function (e.g. cadmium, lead, mercury, arsenic) it can be assumed that toxicity can induce multiple organ damage, even at lower levels of exposure (Duffus, 2002).

Due to industrial development but as well being a part of Earth's crust myriad of heavy metals are widespread in the environment. Multiple applications of metals in industry, agriculture, medicine and technology have raised the concerns for increasing toxic effect on human health and environmental degradation (Bradl, 2002). Major sources of pollution are results from anthropogenic activities such as mining and smelting, industrial production and use, but as well can occur through metal corrosion, atmospheric deposition and metal evaporation from water resources to soil and ground water (Nriagu, 1989). Furthermore, industrial sources can be also metal refineries, coal combustion in power plants, oil combustion, nuclear and high-voltage lines, plastics, textiles, microelectronics, preservation of wood and paper processing plant (Arruti et al. 2010; Sträter et al. 2010). Volcanic eruptions could have a very significant contribution to heavy metal pollution (Fergusson, 1990; He et al. 2005). The low amount of certain metals such as cobalt (Co), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se), zinc (Zn) and copper (Cu) may have a beneficial role in the metabolic function of the human being which is necessary for various biochemical and physiological functions. Although, microelements play a crucial role for functioning of biological systems in excess can become very toxic and cause different abnormalities and disorders (Bååth, 1989).

Moreover, it was reported that HM affects cellular organelles, some enzymes involved in metabolism and detoxification but as well cell components such as DNA and nuclear proteins, causing DNA damage and conformational changes that may lead to cell cycle modulation, carcinogenesis or apoptosis (Wang and Shi, 2001; Beyersmann and Hartwig, 2008).

3.3 Heavy metals as pollutants

Pollutants referred as toxic heavy metals are cadmium (Cd), lead (Pb), mercury (Hg) and arsenic (As) but elements such as chromium (Cr), zinc (Zn), selenium (Se), fluorine (F), nickel (Ni) and copper (Cu) are included in the list of possibly toxic elements (NORD, 2015). Majority of toxic elements can be found in nature because they are frequently used in various industrial activities. The highest application of cadmium in industry is for the production of alloys, pigments and batteries but as well sources of pollution can be mining and smelting activities (ATSDR, 2008; Wilson, 1989). From the other hand, lead is dispersed more widely in the environment, particularly from car exhausts while the industrial use of lead has been significantly reduced from lead-based paints and ceramic products, caulking and pipe solder (Tong et al. 2000). Mercury in the nature can be found in three forms elemental, inorganic and organic, where each has its own level of toxicity (Clarkson et al. 2003). It is used for production in electrical industry and numerous industrial processes such as production of caustic soda in nuclear reactors, as antifungal agents for wood processing, as a solvent for reactive and precious metal and as a preservative of pharmaceutical products (Tchounwou et al. 2003). The presence of cadmium and lead in vegetation may arise from the deposition either directly on plant surfaces or by absorption through roots. Plants vary in their tolerance to cadmium and lead in soil and in the amounts they are able to accumulate (Kuzovkina et al. 2004). Lead can contaminate food through atmospheric fallout or from water used for cooking (Naja and Volesky, 2009). Mercury is a widespread environmental toxicant and pollutant and since it is ubiquitous in the environment plants are unable to avoid exposure (Holmes et al. 2009).

Chromium has wide application in myriad of industrial processes (tanning agents, paint pigments and catalysts to impregnation solution for wood or photography) and it is possible contaminant in many environmental systems (Cohen et al. 1993). Moreover,

chromium concentration in natural waters is very limited by the low solubility of Cr (III) oxides. Main contaminations are generated by industrial waste-waters where heavily polluted agricultural land would lead to chromium accumulation in the food chain via plants (Naja and Volesky, 2009). Zinc is the fourth most common metal in use with an annual production of about 13 million tons. Soils can be contaminated with zinc through the mining, processing of metal or where zinc-containing sludge is used as fertilizer. The metal is most commonly used as an anti-corrosion agent. Selenium is a naturally occurring chemical element that is toxic at high concentrations but is also a nutritionally essential element with different requirements according to plant species. It is used in the electronics industry, the glass industry, in pigments used in plastics, paints, enamels, inks and rubber, as a catalyst in the preparation of pharmaceuticals and as a constituent of fungicides, pesticides (EPA, 2000a). Nickel is fifth most widespread element on (and in) our planet. It plays important functions in the biology of microorganisms and plants (Sydor and Zamble, 2013). The major source of nickel exposure is oral consumption through food and water but as well through breathing of polluted air from nickel metal refining, fossil fuel combustion and tobacco smoking. Fluorinated gases have a huge impact on global warming since they do not damage the atmospheric ozone layer but they are often used as substitutes for ozone-depleting substances.

3.4 Arsenic

Arsenic (Fig.2) is a chemical element with symbol As and atomic number 33. From both the biological and the toxicological points of view, arsenic compounds can be classified in three groups: inorganic forms (trivalent and pentavalent arsenate), organic forms - methylated metabolites (monomethylarsonic acid (MMA), dimethylarsinic acid (DMA) and trimethylarsine oxide) and arsine gas. Arsenic appears in nature primarily in the form of sulfides in association with the sulfides of ores of silver, lead, copper, nickel, antimony, cobalt and iron.

It can be found in soil but it is mainly transported in the environment by water. In oxygenated water, arsenic usually occurs as arsenate, but under reducing conditions in deep well-waters, arsenites predominate (Bhattacharya et al. 2002). In water, the methylation of inorganic arsenic to methyl - and dimethylarsenic acids is associated with biological activity. Environmental pollution by arsenic occurs as a result of natural

phenomena such as volcanic eruptions, soil erosion and anthropogenic activities. Arsenic contamination has been reported in different parts of the world but still in countries such as Bangladesh, China and India it is high priority problem (Naja and Volesky, 2009). Therefore, drinking-water, crops irrigated with contaminated water and food prepared with contaminated water are the major sources of exposure.

Several arsenic compounds are produced industrially and have been used to manufacture products with agricultural applications such as insecticides, herbicides, fungicides, algicides, wood preservatives and dye-stuffs (Tchounwou et al. 1999).

Fig. 2 Arsenic (http://goo.gl/BKF3XW)

Concentrations of arsenic in the air may vary depending if they are away from human exposures e.g. in remote locations (1 to 3 ng/m^3) or in cities (20 to 100 ng/m^3). However, levels of 100–2500 mg/kg have been found in the vicinity of copper smelters. Natural levels of arsenic in soil usually range from 1 to 40 mg/kg, but pesticide application or waste disposal can produce much higher values (Tchounwou, 2004).

3.5 Copper

Copper (Fig. 3) is a chemical element with symbol Cu and atomic number 29. It was one of the first metals ever manipulated by humans and it stayed an important metal in the industry today. Alloy of copper-arsenic has been found in the 3400-3200 BC. Moreover, it is important to emphasize that in copper ores small amounts of arsenic can be found since arsenic appear as a by-product. Most copper occurs in ores and have to be smelted for purity before it can be used. About two-thirds of the copper on Earth is found in igneous (volcanic) rocks while a quarter occurs in sedimentary rocks. The

metal is ductile and malleable and conducts heat and electricity well, explaining its use in electronics and wiring. Copper is also relatively corrosion resistant, although it does oxidize slowly in air. It is present in the Earth's crust at a concentration of about 50 ppm where it occurs as native copper or in minerals such as the copper sulfides chalcopyrite and chalcocite, the copper carbonates azurite and malachite, and the copper (I) oxide mineral cuprite. Moreover, it can be found in the water due to copper plumbing.

Fig. 3 Native copper (https://en.wikipedia.org/wiki/Copper)

Copper is essential to all living organisms as a trace dietary mineral because it is a key constituent of the respiratory enzyme complex cytochrome c oxidase. This metal is example of a heavy metal that is a nutrient in a low concentrations but extremely toxic at higher levels. Copper in excess is prone to creating free radical activity and damaging many cells when in a free form. Trace levels of copper are essential to all living organisms. An important consideration is that only soluble copper is bio-available. Copper complexes with organic matter, or copper oxides and other insoluble compounds are not accessible to living organisms. Insufficiency of copper in plants is manifested as chlorosis between the nerve tissue of the young leaves, while the edges and tops are of normal green color; nerves chlorosis is followed by rapid necrosis of the entire leaf surface. The major copper-producing countries are Chile, Peru and China.

3.6 Effect of heavy metals on generative propagation

Generative propagation is natural way of propagation in many plant species. Seed germination is the first and most important step in a growth and development of a new plant. It could be affected by numerous abiotic and biotic factors. The factors which have highest influence are water, temperature, oxygen, light and substrate characteristics (Gorai et al. 2011). Being a critical stage in life cycle of plants, determination of seed germination in extreme conditions is of significant importance. Moreover, seed germination is one of the most sensitive processes to metal pollution because of lack of defense mechanisms and hence is an important consideration while studying effects of heavy metals on seedling growth (Liu et al. 2005).

Since studying the effect of HM on seed germination is not an easy assignment there are not so many researches published on this topic (Sethy and Ghosh, 2013). Palowski (2000) examined the number of seed scales, filled and empty seeds for two populations of *Pinus sylvestris* grown in polluted areas. It was noticed that there was no significant difference among tested variants and control. Moreover, it was concluded that seeds are probably protected against the poisonous impacts of heavy metals though protecting barrier. Muszyńska et al. (2013) tested seed germination ability of native calamine plant species e.g. *Alyssum montanum*, *Biscutella laevigata* and *Dianthus carthusianorum* on different substrates. Results show that calamine substrate used in the experiment was very strongly polluted with zinc, lead and cadmium, but its alkaline reaction lowered the solubility of these metals and their amount directly available to plants. It was shown that *A. montanum* and *B. laevigata* seeds demonstrated a high ability to germinate on calamine substrate, which was characterized by large contents of soluble forms of zinc (115.1 mg·kg-1), lead (0.91 mg·kg-1) and cadmium (3.12 mg·kg-1) and low water capacity (18.95% g/g). Moreover, the seed germination ability of *Dianthus carthusianorum* ecotype was comparable on both studied substrate types. Słomka et al. (2011) analyzed effect of metalliferous (Zn, Pb, Cd, Cu) and nonmetalliferous sites on *Viola tricolor* morphological, anatomical features and also on sexual reproduction. It was seen that on metalliferous sites, heavy metals inhibited embryological processes in ovules and anthers – microsporogenesis was disturbed, sustainability of pollen was lower, degeneration in ovules was higher. Moreover, it was

proven that reproductive processes are sensitive to elevated heavy metals in soil and therefore can be viewed as an expense of metal tolerance. Metals in higher concentration hamper the plant germination, slower further growth and development which are mainly associated with the physiological, biochemical and genetic changes of the plant system (Sethy and Ghosh, 2013). Nanda and Agrawal (2016) investigated effect of zinc and copper during seed germination in *Cassia angustifolia*. Seeds were germinated on Knop's medium containing Zn and Cu individually in various concentrations (0, 1, 10, 50, 100 and 200 mg/l). Decline in seed germination initiated above 1 mg/l and maximum inhibition was seen at 200 mg/l where it was 40 and 25.0%, respectively under Zn and Cu over control (67.67%). Extensive DNA damage was observed under higher concentrations of zinc and copper but as well protein analysis showed various low molecular weight proteins (20–14 kDa) at higher concentration. Lopes Júnior (2016) reported that cadmium in higher concentrations interferes with the seed germination of sunflower, by increasing the dormancy time (ca. 50% higher) and by decreasing the germination rate (ca. 60% lower) compared with the control group. A comprehensive analysis of the responses of *Nigella sativa* L. to elevated zinc concentrations was assessed (Marichali et al. 2016). Zn excess supply did not affect the germination but drastically reduced radicle elongation. A concentration dependent reduction in all growth parameters, yield, and yield components was observed. Wahid and Khaliq (2015) tested the influence of cadmium on germination, morphological, biochemical and histological characteristics of developing embryonic tissue of maize. The highest amount of Cd accumulated was found in the coleorhiza and radicle. Cd stress reduced cortical cell size and vascular tissues and deformed xylem and phloem parenchyma in all plant parts. Oxidative stress and physiological changes caused by influence of Cd in coleorhiza and coleoptile were the main reason for reduced germination. Moreover, He et al. (2008) recorded that cadmium influenced a substantial reduction in germination strenght and index as well as elongation of the radicle and plumule in rice, which was due to a reduced mitotic index and amylase activity.

3.7 Effect of heavy metals on plant development

Being a sessile organisms, plants cannot escape unwanted changes in the environment. Expositions to heavy metals cause a wide range of physiological and biochemical alterations and plants have to develop and/or adopt a series of strategies that allow them to cope with the negative influences of heavy metal toxicity (Singh et al. 2016).

Heavy metals as Cd, Hg, As, and Pb are not fundamental for plants growth, since they do not perform any known physiological function in plants. Others Co, Cu, Fe, Mn, Mo, Ni and Zn are essential elements needed for normal growth and metabolism of plants, but these elements can without difficulties lead to poisoning when their concentration is greater than optimal values. Accumulation and absorption of heavy metals in tissue of plant depend from many factors which include moisture, organic matter, temperature, pH and nutrient availability. The heavy metals that are accessible for plant uptake are those that are present as soluble components in the soil or those that are easily solubilized by root exudates (Blaylock and Huang, 2000). Although plants demand particular heavy metals for their growth and uptake, exceeding amounts of these metals can become toxic to plants. The capability of plants to accumulate essential metals evenly enables them to acquire other non-essential metals (Djingova and Kuleff, 2000). Non-essential and essential heavy metals commonly produce toxic effects on plants, such as chlorosis, inhibition of growth, low biomass accumulation, inhibition of photosynthesis, altered water balance and nutrient assimilation, senescence which ultimately cause plant death (Singh et al. 2016). Some of the direct toxic effects caused by high metal concentration include inhibition of cytoplasmic enzymes and damage to cell structures due to oxidative stress and production of reactive oxygen species (ROS) (van Assche and Clijsters 1990; Jadia and Fulekar, 2009). An example of indirect toxic effect is the substitution of essential nutrients at cation exchange sites of plants (Taiz and Zeiger, 2002). These toxic effects (both direct and indirect) lead to a decrement in plant growth which sometimes results in the death of plant. The effect of heavy metal toxicity on the growth of plants can be distinguished according to the particular heavy metal involved in the process. For metals such as Pb, Cd, Hg and As adverse effects have been recorded at very low concentrations of these metals in the growth medium (Kibria, 2008; Hayat et al. 2012; Gill et al. 2013). Kibria (2008) observed significant reduction in height of rice plants growing on a soil contaminated with mercury. For other metals which are beneficial to plants, low concentrations of these metals in the soil could actually improve plant growth and development. Chen et al. (2015) studied accumulation and physiological responses of heavy metals on *Medicago sativa* growing on acidic copper mine tailings in arid lands. Seedling growth, cell membrane and photosynthesis were detrimentally affected when the plants were grown in soils with high proportions of tailings. Five woody species (*Amorpha fruticosa*, *Vitex trifolia var. simplicifolia*, *Glochidion puberum*, *Broussonetia papyrifera*

and *Styrax tonkinensis*) and one herbaceous species (*Sesbania cannabina*) were planted in Cu and Pb/Zn tailings to assess their growth, root morphology, nutrition uptake, metal accumulation and translocation in plants (Shi et al. 2011). *Amorpha fruticosa* maintained normal growth, while the other species demonstrated stress related growth and root development. Moreover, a decrease in mitotic activity has been reported in several plant species after exposure to heavy metals, which consequently results into a suppressed root growth (Sundaramoorthy et al. 2010; Thounaojam et al. 2012).

Plants growing on heavy metal-rich soils suffer from both decreased growth and yield (Keunen et al. 2011), indicating an implication of heavy metal toxicity in hampering the overall growth performance of the stressed plants (Hayat et al. 2012; Silva, 2012; Anjum et al. 2014). Jayakumar et al. (2013) reported that there was an increase in nutrient content of tomato plants grown on lower cobalt concentrations in comparison with the control. Conversely, at higher concentrations of cobalt, reductions in plant nutrient content were recorded. Improvements in growth and physiology of cluster beans have also been reported at Zn concentration of 25 mg/L of the soil solution (Manivasagaperumal et al. 2011). On the other hand, growth reduction and harmful effect on the plant's physiology started when the soil solution contained 50 mg/l of zinc. Assimilation of Cd, Pb and Zn by plants from soil is highly dependent on the pH of soil reaction. Many researches have confirmed that Cd, Pb and Zn contents in soil solution evidently increased with drop in soil pH (Tlustoš et al. 2006; Blake and Goulding, 2002; Hinsinger et al. 2003). Soils around non-ferrous metallurgical industries can be highly polluted by metals such as Cd, Pb, Zn, Cu etc., due to the large emissions resulting from past pyrometallurgical production processes. Extremely high contamination, in combination with specific soil conditions (e.g. infertile and acid sandy soils) can result in a complete disappearance of the natural vegetation (Vangronsveld et al. 1995). Athar and Ahmad (2002) conducted the research in order to evaluate toxic effects of certain heavy metals on the plant growth and grain yield of wheat (*Triticum aestivum*). The results revealed that heavy metals significantly reduced both parameters. Moreover, the presence of Cd in the soil resulted in the maximum inhibition in the number of free living *Azotobacter chroococcum* cells over the control.

It is important to point out that certain plants are able to tolerate high concentration of heavy metals in their environment. Plants are able to tolerate these metals via three mechanisms: (1) exclusion: restriction of metal transport and maintenance of a constant

metal concentration in the shoot over a wide range of soil concentrations; (2) inclusion: metal concentrations in the shoot reflecting those in the soil solution through a linear relationship; and (3) bioaccumulation: accumulation of metals in the shoot and roots of plants at both low and high soil concentrations (Baker, 1981).

3.8 Phytoremediation techniques

Recently, public concerns related to ecological threats caused by heavy metal pollution have led to intensive research of new economical plants which can be used in remediation technologies (Sarma, 2011). Conventional methods used for re-cultivation of contaminated soils namely physical, chemical and microbiological methods, are costly to install and not easy to operate. The prompt increase in population connected to fast industrialization growth trigger serious environmental problems, including the production and discharge of considerable amounts of toxic waste materials into environment. Soil and water pollution is the major problem in the world. Soil pollution is result of highly toxic compounds, radioactive materials chemical compounds which were moved to nature (Sharma and Pathak, 2014).

Heavy metals that have been identified in the polluted soils include As, Cu, Cd, Pb, Cr, Ni, Hg and Zn. The presence of any metal may differ from site to site, depending upon the source of individual pollutant. High concentrations of heavy metals in soil can negatively affect crop growth, as these metals interfere with metabolic functions in plants, including physiological and biochemical processes, inhibition of photosynthesis, respiration and degeneration of main cell organelles, even leading to death of plants (Garbisu and Alkorta, 2001; Schmidt, 2003; Schwartz et al. 2003). Soil contamination with heavy metals may also cause changes in the composition of soil microbial community, negatively affecting soil characteristics (Giller et al. 1998; Kozdrój and van Elsas, 2001; Kurek and Bollag, 2004). Bioremediation is the use of organisms (microorganisms and/or plants) for the treatment of polluted soils. It is a broadly accepted method of soil remediation because it occurs via natural processes. Although bioremediation is a no disruptive method of soil remediation, it is generally time consuming and its use for the treatment of heavy metal polluted soils is sometimes affected by the climatic and geological conditions of the site which has to be remediated (Schmoger, 2000). Phytoremediation is an aspect of bioremediation that uses plants for

the treatment of polluted soils. It is suitable when the pollutants cover a wide area and when they are within the root zone of the plant (Garbisu and Alkorta, 2001). According to EPA (2000b) phytoremediation is the direct use of living green plants for *in situ*, removal, degradation or containment of contaminants in soils, sludge, sediments, surface water and groundwater. Systems of phytoremediation are:

- Phytodegradation
- Phytostimulation or rhizodegradation
- Phytovolatilisation
- Phytoextraction
- Phytostabilisation

Phytodegradation (phytotransformation) is the breakdown of contaminants taken up by plants through metabolic processes within the plant or the breakdown of contaminants external to the plant through the effect of compounds (such as enzymes) produced by the plants (Sharma and Pathak, 2014).

Rhizodegradation is the breakdown of an organic contaminant in the soil through microbial activity that is enhanced by the presence of the root zone. Root exudates are compounds produced by plants and released from plant roots. They include sugars, amino acids, organic acids, fatty acids, sterols, growth factors, nucleotides, flavanones, enzymes and other compounds.

Phytovolatilisation is the uptake of pollutants by plants and its transpiration to the atmosphere in same or in the modified form. Formation of less toxic or no toxic compounds occurs in processes of uptake, metabolism and transpiration which take place in plants. Simultaneously with this process, rhizodegradation and phytodegradation can be carried out. This method is used for treatment of groundwater, sediments, soil and sludge. Climatic conditions, temperatures, rainfalls, insolation and wind greatly influence the amount of transpired pollutant. Disadvantage of this method is possibility of accumulation of HM in plants and fruits. Genetic engineered plants are mainly used in phytovolatilization. Examples of transgenic plants which have been used for phytovolatilization of Hg polluted soils are *Nicotiana tabacum*, *Arabidopsis thaliana*, and *Liriodendron tulipifera* (Rugh et al. 1998). Phytovolatilization can also be employed for the remediation of soils polluted with selenium (Marques et al. 2009).

Plants which have successfully been used for phytovolatilization of soils polluted with Se are *Brassica juncea* and *Brassica napus* (Bañuelos, 1997).

Phytoextraction is the process of pollutants uptake by means of plant root and its translocation in plant. Removing pollutants from polluted soils is done by removing plants from the surface which is easier than removing the upper layer of soil. This technology is used for land, tailings and sludge. Disadvantage of this method is reflected in reducing growth of plant, but at the same time reducing the biomass of the root system due to the negative impact of heavy metals and phytotoxicity (Nanda Kumar et al. 1995). Plants used for phytoextraction usually have the following characteristics: rapid growth, high biomass, extensive root system and ability to tolerate high amounts of heavy metals.

Phytostabilisation is the process of pollutants immobilization in soil by means of absorption and accumulation in root system, adsorption on root or deposition in the root zone of plants, as usage of plants and/or roots for prevention of pollutants migration by wind, water erosion, washing or dispersing in soil. Phytostabilisation process is carried out in root zone via microbiological and chemical mechanisms of the zone or by changing chemical reaction of soil and/or pollutant. Changes in pH of soil occur due to secretion of root exudates or due to formation of CO_2. Soil under the influence of plants can transfer metal from soluble to insoluble oxidation state (Salt et al. 1995). By this way soils, sediments and sludge's with pollutants which are placed in the root zone can transport exudates in lower soil parts. Advantages of these systems are: economical values due to unnecessary removal of land, the return of vegetation and unnecessary disposal of hazardous materials or biomass. Disadvantages are that pollutant stay in the soil and vegetation should be supplied with fertilizers. Organic materials are mostly used as soil amendments in phytostabilization. Marques et al. (2009) showed that Zn percolation through the soil reduced by 80% after application of manure or compost to polluted soils on which *Solanum nigrum* was grown. The best soil amendments are those that are easy to handle, safe for workers who apply them, easy to produce, and inexpensive and most importantly are not toxic to plants.

3.9 Somatic embryogenesis of coniferous trees

The quality of human life has been maintained and improved for generations by the use of trees and their products. Population growth, environmental pollution and deforestation put an enormous pressure on development of new technologies and/or improvement of the old ones. Combination of biotechnology with conventional methods such as plant propagation and breeding could help to produce large number of trees with superior genotype characteristics. Moreover, forestry is on the threshold of the widespread introduction of biotechnology into its operational practices – mainly thanks the progress with the biotechnological methods of vegetative propagation - micropropagation (organogenesis and somatic embryogenesis).

Somatic embryogenesis (SE) is the developmental process by which somatic cells, undergo restructuring through the embryogenic pathway to generate embryogenic cells (Vondráková et al. 2016). These cells then go through a series of morphological and biochemical changes that result in the formation of a somatic embryo and the generation of new plants (Yang and Zhang, 2010; Smertenko and Bozhkov, 2014). SE is defined as a non-sexual developmental process that produces a bipolar embryo from somatic tissue without having the vascular connection with the original tissue (Attree and Fowke, 1993). Somatic embryos morphologically resemble zygotic embryos and undergo almost the same developmental stages (Dodeman et al. 1997). They are bipolar and bear typical embryonic organs, the radicle, hypocotyls and cotyledons (von Arnold et al. 2002).

SE is a very powerful tool for cloning trees and it is considered to be the *in vitro* regeneration system of choice in woody plants (Gupta et al. 1991). Most reports on somatic embryogenesis in woody species described "embryo cloning" where an unlimited number of genetically identical copies of trees can be produced from a single seed (Merkle et al. 1997). The most important practical application of SE is in tree improvement and clonal forestry with introducing genetically superior and high-value trees in order to improve forest productivity (Park, 2002; Klimaszewska et al. 2009). Important advantage of cloning conifers by SE is that the embryogenic tissue can be cryopreserved without changing its genetic make-up and without loss of juvenility while field testing is still in progress. This offers an opportunity to develop high-value clonal varieties by defrosting and repropagating cryopreserved clones after genetic testing has shown which clones are the best performers (Park et al. 1998). Moreover, propagation

through SE allows formation of multiple, genetically identical embryos and avoids waiting for the following reproductive season (Vondráková et al. 2016).

First reports about somatic embryogenesis of coniferous species was more than 30 years ago and since then it was eruption of researches with aims for developing and optimizing protocols for efficient regeneration of plantlets (Stasolla and Yeung, 2003).

The first reports on conifer SE induced from immature embryos were published for *Picea abies* (L.) Karst. (Chalupa, 1985; Hakman et al. 1985) and since then for many other tree species including *P. glauca × engelmannii* (Webster et al. 1990; Sutton et al. 1993), *P. sitchensis* (Krogstrup, 1990), *P. marianna* (Adams et al. 1994), *Pinus sylvestris* (Häggman et al. 2009; Aronen et al. 2009), *P. pinaster* (Klimazewska et al. 2009), *P. radiata* (Minocha et al. 1999), *P. nigra* (Salajova and Salaj, 1992), *Pseudotsuga menziesii* (Gupta and Durzan 1987), *Larix × leptoeuropaea* (Lelu et al. 1994), *Abies cephalonica* (Krajňáková et al. 2008), *A. alba* (Hristoforoglu et al. 1995), *A. balsamea* (Guevin et al. 1994), *A. nordmanniana* (Nørgaard ,1997), *A. numidica* and *A. cilicica* (Vooková and Kormuťák 2002; 2003) etc. Although, a lot of researches was published on inducing SE in conifers not so many was able to undergone whole process and obtain plantlets. Compared to other conifers, SE of spruce species has been the most successful and most advanced commercially. Out of the 11 species reported to undergo SE process, five species are being evaluated in clonal trials and in large-scale propagation programs. These species are *P. glauca× engelmannii* (Webster et al. 1990; Sutton et al. 1993), *P. sitchensis* (Krogstrup, 1990; Cyr et al. 2001), *P. mariana* (Adams et al. 1994), *P. glauca* (Lamhamedi et al. 2000) and *P. abies* (Högberg et al. 2001).

Moreover, SE in Norway spruce has been used as a model for the study of morphological, physiological, molecular, and biochemical events occurring during the development of embryogenesis in higher plants (von Arnold et al. 2016; Yang and Zhang, 2010; Elhiti et al. 2013; Smertenko and Bozhkov, 2014).

3.10 Stages of somatic embryogenesis

Somatic embryogenesis in conifers is a multistage regeneration process. Each stage represents different challenges, and these are often dependent on the outcome of the previous stage.

SE process is divided into following stages:

1. Initiation
2. Proliferation
3. Maturation of somatic embryos
4. Post-maturation treatment of somatic embryos
5. Germination and conversion to plants
6. Early growth ex vitrum

3.10.1 Initiation/induction

Induction is the stage of SE in which embryogenic tissue is derived from primary explant on induction medium. Choice of explants has a very important role in initiation of embryogenic tissue in coniferous species. As reported by Atree and Fowke (1993), for the conifers, immature zygotic embryos are more used explants for producing somatic embryos in comparison with mature zygotic embryos. The media used for initiation strongly depends on the species. Usually, media is enriched with cytokinins and auxins in different concentrations.

The end of this stage is marked by the appearance of whitish translucent ECMs (Fig. 4).

Fig. 4 Initiation of embryogenic cell masses on immature megagametophytes of
Picea abies (Čermáková, 2012)

ECMs are composed of early somatic embryos, single cells and cell aggregates each of them consist of densely cytoplasmic embryonal head and vacuolated elongated suspensor cells. The frequency of SE initiation from immature embryos, in addition to other factors (i.e., genetic), strongly depends on the genotype, type of plant age and the developmental stage of an explants, physiological state of an explants - donor plant, and the external environment which includes composition of media and physical culture

conditions (light, temperature). Interaction between all these factors leads to the induction and expression of a specific mode of cell differentiation and development. This stage of SE does not require light and the cultures are typically placed in darkness at approximately 22 – 25°C (Klimazsewska and Cyr, 2002).

3.10.2 Proliferation

Proliferation (maintenance) represents establishment of embryogenic cultures and continuous growth (increase in fresh mass) upon periodical subcultures onto a fresh semi-solid medium, usually of the same composition as the one used for initiation (Fig.5). At this stage, the vigorously growing embryogenic culture may be cryopreserved. If not cryopreserved, the embryogenic tissue must be subculture onto a fresh medium every 12 to 21 days. This stage of SE does not require light (Klimazsewska and Cyr, 2002).
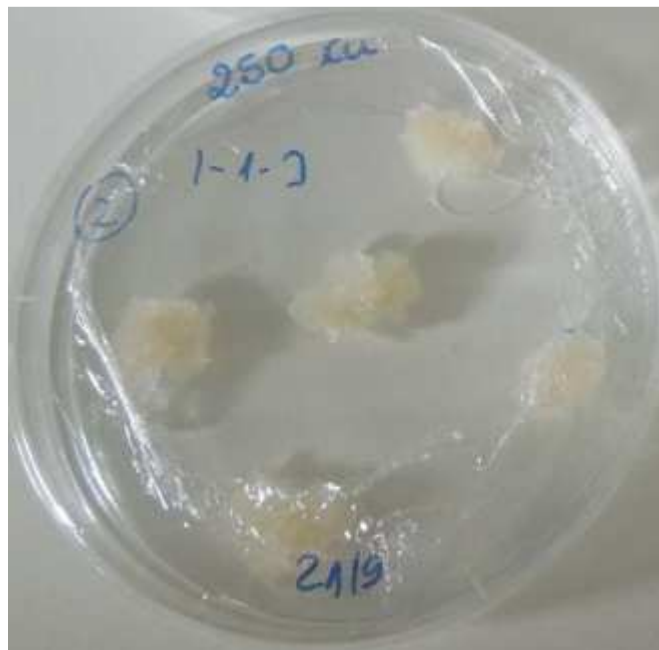

Fig. 5 Proliferation of embryogenic tissue

Cryopreservation is preservation of embryogenic tissue in liquid nitrogen at a temperature ranging from -130 to -196°C without loss of viability or change in genetic makeup. It is based on the reduction and subsequent standstill of metabolitic activities, as well as cell division in the explants. The general aim is to aid the gradual removal of free water from the embryogenic cells and to minimize the formation of intracellular ice by using slow cooling. Storage of conifer embryogenic tissue in liquid nitrogen was first reported for *Picea glauca* by Kartha et al. (1988). This protocol, with minor

modifications, has been extended to numerous genera and species (Cyr, 1999). For regeneration, vials are rapidly thawed for 1 to 2 minutes at 37°C, the storage solution is removed via draining and the cultures are transferred onto a fresh semi-solid medium while growth of cultures typically occurs within 1 to 2 weeks after thawing (Klimazsewska and Cyr, 2002).

3.10.3 Maturation

The maturation of somatic embryos represents development of immature (early) somatic embryos (Fig. 6). For completion of this process, the embryos must achieve both 'morphological' and 'physiological' maturity (Stasolla and Yeung, 2003). Maturation represents histodifferentiation of ECMs into cotyledonary embryos on a nutrient medium that typically contains abscisic acid (ABA) and provides reduced water availability to the developing cultures. Development of embryo in conifers is initiated by arresting cell proliferation through the removal of auxins and cytokinins and is continued by application of ABA (reviewed by Stasolla et al. 2002, von Arnold, 2002). These changes in plant growth regulators (PGR) represent an important developmental switch critical for successful competition of embryogenic process (Bozkov et al. 2002).
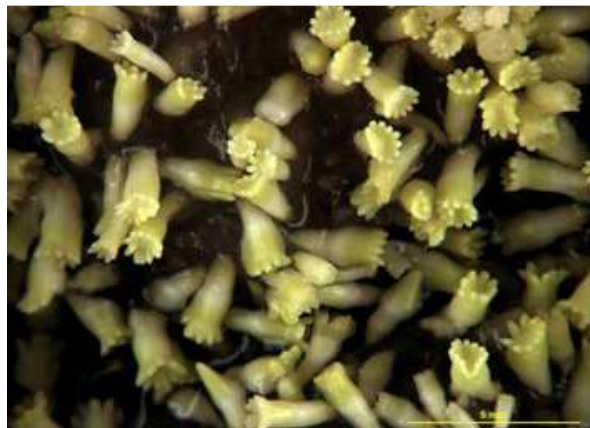

Fig. 6 Maturation (http://goo.gl/xYyLLg)

The modulation of water availability is performed either by decreasing osmotic potential of the medium (addition of osmotically active solutes) or by increasing the medium's gel strength (physical means) or by a combination of both. A developed somatic embryo that morphologically and physiologically resembles a zygotic embryo marks the end of this stage, which lasts 6 to 12 weeks. Some protocols apply a pre-maturation step, which involves a brief (3 to 7 days) culture of embryogenic tissue on a

medium where application of PGR is omitted and containing activated charcoal prior to the transfer onto a maturation medium.

Post-maturation treatment of somatic embryos - the mature somatic embryos can be either partially desiccated at a high relative humidity 98 % prior to germination or dried to low water content at a low relative humidity for short or long - term storage, respectively. The aim of partial desiccation of somatic embryos prior to germination is to reduce the water content and/or to complete the maturation process.

3.10.4 Germination

Germination and conversion to plants - somatic embryos are usually germinated *in vitro* on a semi-solid nutrient medium that contains sucrose and may or may not contain a source of organic nitrogen and activated charcoal. This stage is completed after the elongation of an epicotyl and the development of needles occur, most frequently after 12 to 16 weeks, depending on the species. If the somatic embryo maturation medium contains a gelling agent concentration that is higher than the one routinely used, then the germination medium should also have an elevated level of this compound (but lower than in the maturation medium) to prevent hyperhydricity. The light intensity is low for the first two weeks of germination (5 mol m-^{2} s 16 h photoperiod) and then it is gradually increased during the growth of plantlets (up to 40 mol m-^{2} s, 16 h photoperiod).

Early growth ex vitrum represents establishment of *in vitro* grown somatic seedlings in a substrate under greenhouse conditions. Typically, during the first 2 to 3 weeks of growth, a high relative humidity is provided to facilitate the plants' acclimatization to ambient conditions (Klimazsewska and Cyr, 2002).

4 MATERIALS AND METHOD

4.1 Plant material

Plant material was obtained from Department of Plant Biology, Faculty of Agronomy. Immature cones of Norway spruce were collected during summer 2011, following the procedure as described by Krajňáková et al. (2013). Briefly, immature cones were collected from selected open-pollinated mother trees according to their growing and healthy characteristics. The origin of mother trees was Černá Hora belonging to the district Blansko in the South Moravian region. Seeds were separated, surface sterilized with 70% ethanol for 2 minutes and rinsed three times with sterile distilled water. Furthermore, 0.2% of mercury chloride for 13 minutes was used for additional surface sterilization and seeds were rinsed 3 times with sterile distilled water. Zygotic embryos were excise and placed on the induction medium. Embryogenic cultures of *P. abies* III-3-3 and I-1-3 were used for experiments with heavy metals.

4.2 Cultivation media composition and preparation

Embryogenic cultures of *P. abies* were cultivated on LP medium during proliferation stage (Bozhkov and von Arnold, 1998). The composition of proliferation media is given in Tab.1. The pH value was adjusted to 5.7–5.9 before autoclaving (121°C, 100 kPa, 20 min). Thermolabile components of the media e.g. glutamine, growth regulators - benzyl adenine (BA) and 2,4-dinitrophenylhydrazine (2.4-D) were filter sterilized and added separately to autoclaved medium. The cultures were maintained in a cultivation room in the dark at 23±2°C. Embryogenic cultures were sub-cultured and transfer on fresh solid medium every two weeks for maintenance and multiplication. For first set of experiments during proliferation stage, standard cultivation media (LP) were supplemented with an addition of arsenic and copper at 50 µM, 250 µM and 500 µM concentration. A stock solution of As and Cu were prepared by mixing $AsHNa_2O_4$ x $7H_20$ and $CuSo_4$ x $5 H_2O$ (Fig.7). During preparation of copper stock solution, precipitation was observed and it was mixed with ethylene diamine tetra-acetic acid (EDTA) in a 1:1 molar ratio. The filter-sterilized arsenic and copper-EDTA complex was added to the previously autoclaved culture medium.

Fig. 7 Preparation of $AsHNa_2O_4$ x $7H_2O$ and $CuSo_4$ x $5 H_2O$ stock solutions

Tab. 1 Composition of LP proliferation media

Proliferation media	
	Picea abies (LP)
Inorganic macroelements	[mg/l]
NH_4NO_3	600
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	340
Inorganic microelements	[mg/l]
H_3BO_3	0.63
KI	0.75
$MnSO_4.4H_2O$	2.23
$Na_2MoO_4.2H_2O$	0.025
$CuSO_4.5H_2O$	0.0025
$CoCl_2.6H_2O$	0.0025
$FeSO_4.7H_2O$	13.90
Zn.EDTA	18.70
Vitamins	[mg/l]
Thiamine	5.0
Nicotinic acid	2.0
Pyridoxine hydrochloride	1.0
Myo-inositol	100
Glycine	5.0
Organic compounds	
Glutamine	450
Casein hydrolysate	500
Growth regulators	[µM/l]
Benzyladenine (BA)	4.44
2.4-Dichlorophenoxyacetic acid	9.0
Sucrose	20g/l
Gelrite	3.5g/l
pH	**5.7- 5.9**

After autoclaving, 25 ml of media was put in sterile petri plates in advance cleaned flow box. After first set of experiments with copper and arsenic during proliferation stage, it was observed that concentrations of arsenic were very high and all ECMs died. In second set of experiments, new proliferation media were prepared with concentrations 10 µM, 25 µM and 50 µM. Growth and development of ECMs was monitored during two weeks interval with observation at 3rd, 7th, 10th and 14th day.

ECMs treated with heavy metals were subjected to maturation experiment (Fig.8). Maturation medium of *Picea abies* was enriched with 0.1% casein hydrolysate and 3.4 mM l-glutamine. The media was solidified with 0.35% Phytagel, supplemented with 78.8 mM sucrose and 32 µM ABA (Tab.2). pH was adapted to 5.7 before autoclaving (121°C, 100 kPa, 20 min). At the beginning of the maturation experiment, 3 g of fresh embryogenic tissue was transferred to sterile Falcon flasks with 20 ml of liquid pre-maturation media without plant growth regulators (Tab.3). The suspension was gently mixed by vortex and allowed to settle. After removal of supernatant, 1 ml of suspension containing approximately 250 mg embryogenic tissue (fresh weight) was plated onto sterile Whatman filter paper on maturation media. Sub-culturing was performed every 2 weeks during maturation for up to 6 weeks. After 6 weeks on maturation media number of somatic embryos formed was calculated.

Fig. 8 Set up of maturation experiment

Tab. 2 Composition of maturation media

Maturation media - *Picea abies* (LP)	
	[mg/l]
MS basal salts - macronutrients and micronutrients	2150
Vitamins	1
Casein hydrolysate	1000
Inositol	100
L-Glutamine	500
	[µM/l]
Abscisic acid	32
	[g/l]
Sucrose	27
Phytagel	3.5
pH	**5.7-5.8**

Tab. 3 Composition of liquid pre-maturation media (growth regulators and gelling agent are omitted)

Pre-maturation media - *Picea abies* (LP)	
	[ml/l]
Macronutrients	50
Micronutrients	0.5
Fe-EDTA	2.5
	[mg/l]
Inositol	100
Vitamins	1
Casein hydrolysate	500
Sucrose	200000
pH	**5.7-5.8**

Mature somatic embryos were subjected to partial desiccation under high humidity for 3 weeks at 24°C degree in the dark. Embryos were transferred onto sterile Whatman filter paper in a 60 mm Petri dish which was placed inside a 90 mm Petri dish containing 1 ml of water and the whole set up was sealed with Parafilm (Fig.9).
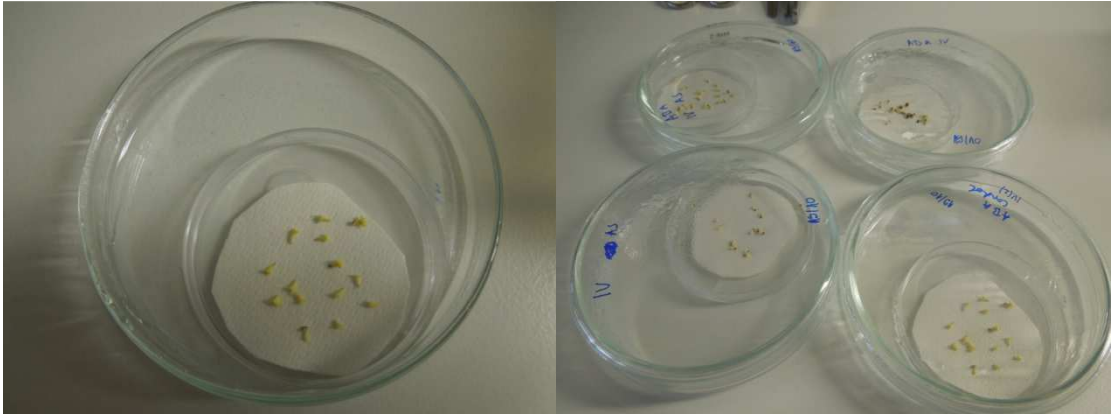
Fig. 9 Desiccation of somatic embryos

After 3 weeks of desiccation in the dark, embryos were then transferred to M1 medium for germination (Tab.4) (Fig.10). The pH was adjusted to 5.5 - 5.8 before autoclaving (121°C, 100 kPa, 20 min).

Tab. 4 Composition of germination media

Germination media	
	Picea abies (M1)
	[g/l]
MS basal salts - macronutrients and micronutrients	2.15
Sucrose	15
Agar	7
Vitamins	0.001
Active charcoal	1
pH	5.5-5.8
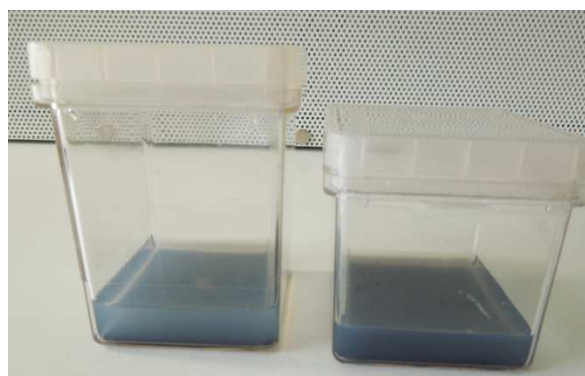
Fig.10 Germination media in Magenta vessels

After one month on germination media all plantlets were thoroughly washed in lukewarm water. Before add to perlite substrate plantlets were submerged also in solution of Previcur Energy fungicide. Acclimatization substrate (perlite) was submerged in water to keep humidity. For acclimatization plastic bottles and boxes filled with perlite were used and covered with foil to keep humidity at high level (Fig.11).

Fig. 11 Box filled with perlite substrate prepared for plantlets

4.3 Experimental design and statistical analysis of results

4.3.1 Determination of proliferation rate

To determine the rate of ECMs proliferation, five pieces of ECMs were weighted (200±20 mg, fresh weight, FW0) and placed equidistant from each other on a Petri plate. For each sampling day 3, 7, 10 and 14, three Petri plates (i.e. 15 numbered pieces of ECMs) were used. On each sampling day ECMs were detached from the medium and weighted (fresh weight at the i-th day FWi). The proliferation rate was recorded as the ratio between the ECMs fresh weight at the sampling day and the ECMs initial weight (FWi/FW0; Krajňáková et al. 2013).

4.3.2 Observation of cotyledon abnormalities

All formed somatic embryos were divided into three categories: early-precotyledonary, precotyledonary and cotyledonary. Moreover, since toxicity of metals used had effect on morphology of somatic embryos different cotyledon abnormalities were observed and photo documented. Microscope Olympus SZH10 was used for somatic embryos

development observation which was coupled with Olympus digital camera E 450 and QuickPHOTO MICRO 3.0 software.

4.3.3 Experimental design and data analysis

The effect of two metal ions As^{2+} and Cu^{2+} were tested during consecutive stages of SE (proliferation, maturation and germination). In the first set of experiments effect of Cu^{2+} and As^{2+} were tested in three different concentrations (50, 250, 500 µM) whereas in second set of experiments effect of As^{2+} was tested in concentrations (10, 25 and 50 µM). Altogether, three independent experiments were established. During proliferation stage, two embryogenic cell lines of *P. abies*, III – 3 - 3 and I – 1 – 3 were tested (four sampling days: 3, 7, 10 and 14). Proliferation rate (FW_i/FW_0) was recorded individually for each cell line and heavy metal ion combination using a three-way analysis of variance (ANOVA; effects of cell line, concentration of heavy metal ions and sampling day were considered fixed). For studying the effect of metal ions during maturation of somatic embryos it was assayed after 6 weeks of maturation period. At the end of the maturation period the presence of developing somatic embryos in the ECMs was documented. Somatic embryos at different developmental stages (early-precotyledonary, precotyledonary and cotyledonary) were counted and recalculated per 1 g of fresh weight (FW) of ECMs. Differences in the average numbers of somatic embryos per 1 g FW were analyzed using a two-way ANOVA (effects of cell line and concentration of heavy metal ions were considered fixed). For statistical evaluation STATISTICA 12.0 software was used.

5 RESULTS

5.1 Proliferation ratio

First analyzed parameter in experiments with arsenic and copper was proliferation ratio. As explained before, proliferation ratio was recorded as the ratio between the ECMs fresh weight at the sampling day and the ECMs initial weight. Analysis of variance confirmed that in case of Cu^{2+} application to proliferation medium in different concentrations (0, 50, 250 and 500 μM), proliferation rate was significantly affected (Tab.5). In the first set of experiments with As^{2+} tested in three different concentrations (50, 250, 500 μM) in the end of proliferation period all ECMs were dead (data were not statistically evaluated). Moreover, in second set of experiments with As^{2+} in concentrations (0, 10, 25 and 50 μM) analysis of variance was statistically significant for almost all the interactions (Tab.6). Significant differences was not observed in cell line response to arsenic but as well in interaction cell line * heavy metal. Embryogenic cell masses grown on media enriched with higher copper concentrations show necrosis after one week of growth (Fig.12). On Fig. 13 it can be seen that highest concentration of As^{2+} (50 μM) stop the growth of ECMs.

Tab. 5 Analysis of variance (significance of *F*-tests) of the effects of Cu^{2+} on the proliferation rate of III – 3 – 3 and I – 1 – 3 during 14 days lasting proliferation period, concentrations 0, 50, 250 and 500 μM

Significance labels (P) (used also in subsequent tables: *** *P < 0.001*, ** *P < 0.01*, * P < 0.05, NS P > 0.05 (non-significant); DF – degree of freedom

Source	DF	P
Cell line	1	***
Sampling day	3	***
Concentrations	3	***
Cell line*Sampling day	3	**
Cell line*Concentrations	3	***
Sampling day* Concentrations	9	***
Cell line*Sampling day* Concentrations	9	**
Error	448	

Tab. 6 Analysis of variance (significance of *F*-tests) of the effects of As^{2+} on the proliferation rate of III – 3 – 3 and I – 1 – 3 during 14 days lasting proliferation period, concentrations 0, 10, 25 and 50 µM

Source	DF	P
Cell line	1	NS
Sampling day	3	***
Concentrations	3	***
Cell line*Sampling day	3	NS
Cell line*Concentrations	3	***
Sampling day* Concentrations	9	***
Cell line*Sampling day* Concentrations	9	*
Error	448	
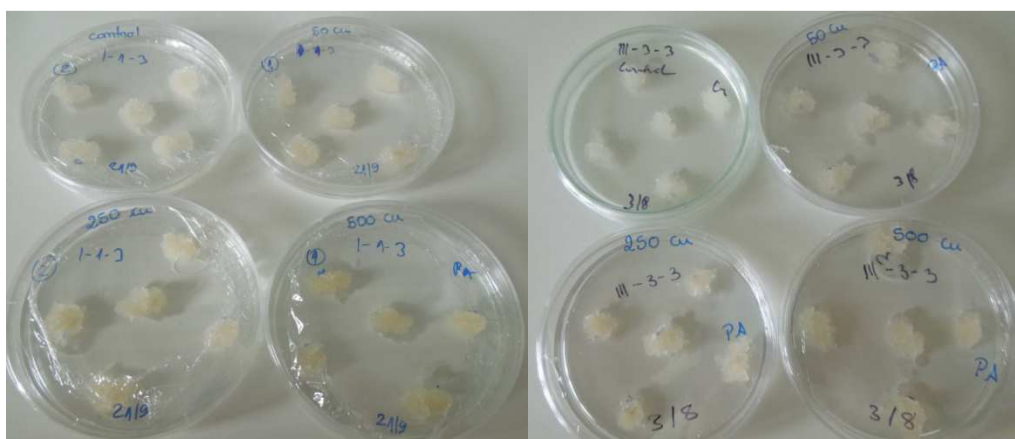
Fig. 12 Effect of Cu^{2+} in different concentrations (control, 50, 250 and 500 µM) on proliferation ratio of I – 1 – 3 and III – 3 – 3 embryogenic cell lines
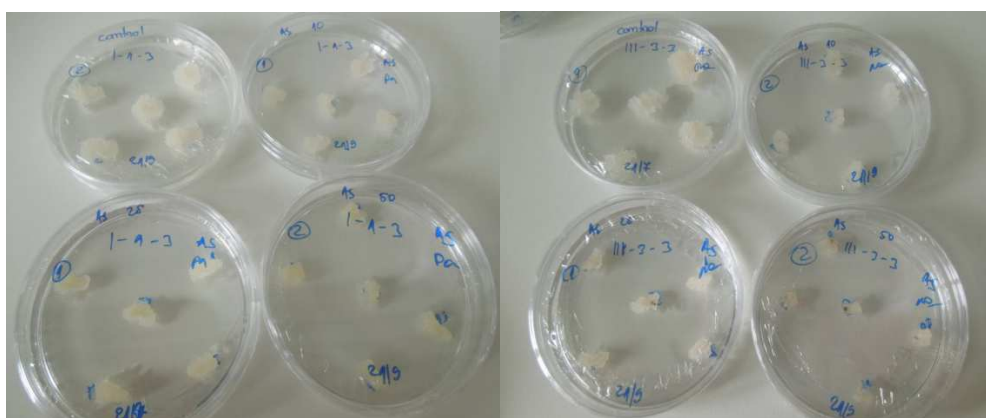

Fig. 13 Effect of As^{2+} in different concentrations (control, 10, 25 and 50 µM) on proliferation ratio of III – 3 – 3 and I – 1 – 3 embryogenic cell lines
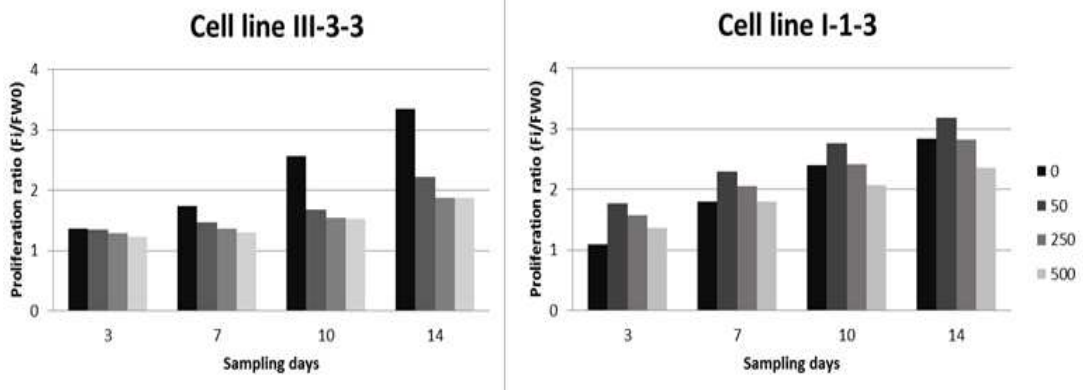
Fig.14 Effect of Cu^{2+} in different concentrations (control, 50, 250 and 500 μM) on proliferation ratio

In case of cell line III – 3 – 3, control had the highest proliferation ratio in comparison with other variants. Lowest concentration of copper (50 μM) applied was a bit higher while no difference was observed in two other concentration (250 and 500 μM) (Fig.14). Growth of ECMs was proportional to period of proliferation (14 days). On the other hand, in cell line I – 1 – 3, copper ions in concentrations of 50 μM had highest proliferation ratio, followed by control and 250 μM. The highest concentration 500 μM, at certain point stop further growth of ECMs (Fig.14).
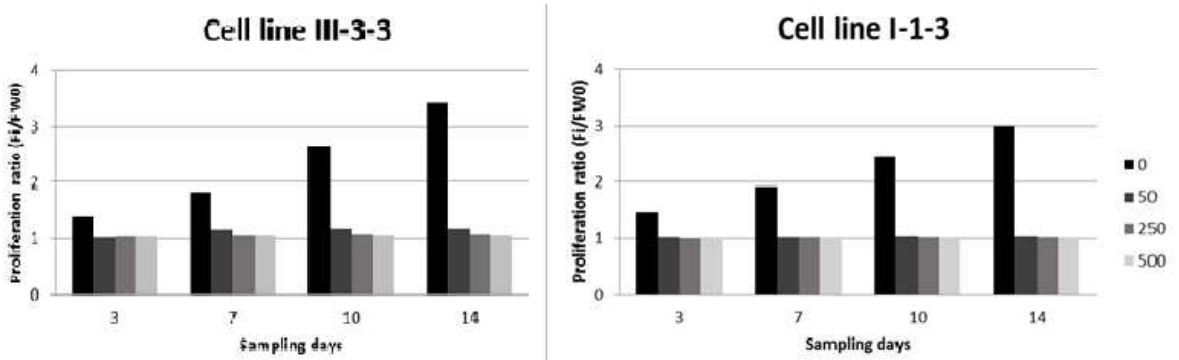

Fig. 15 Effect of As^{2+} in different concentrations (control, 50, 250 and 500 μM) on proliferation ratio

In the preliminary experiment with As^{2+} all concentrations applied showed to be very toxic for growth and development of ECMs in both cell lines (Fig. 15). In case of cell line III – 3 – 3 negligible growths were noticed with concentration 50 μM. It was

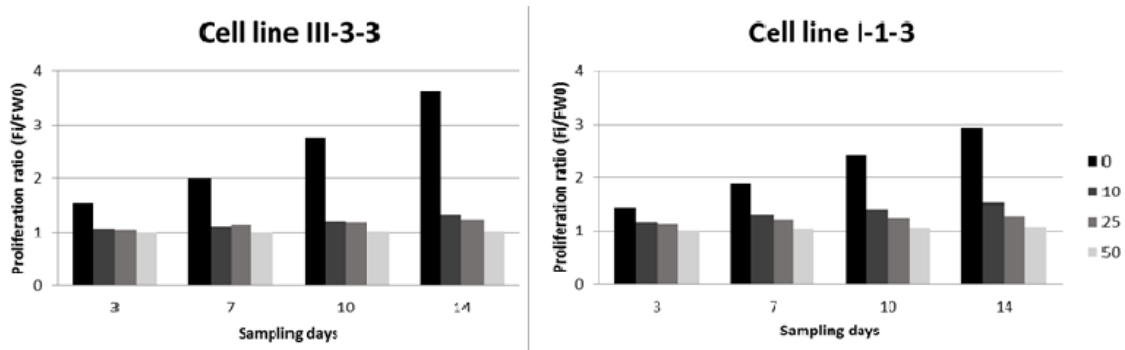decided that in next set of experiments, 50 μM should be the highest tested concentration.

Fig. 16 Effect of As^{2+} in different concentrations (control, 10, 25 and 50 μM) on proliferation ratio

Considering the toxicity of As^{2+}, in the second set of experiments lowest concentration applied was 10 μM. As in preliminary experiment, control had highest proliferation ratio, while ECMs had insignificant growth on 10 and 25 μM concentrations (Fig. 16).
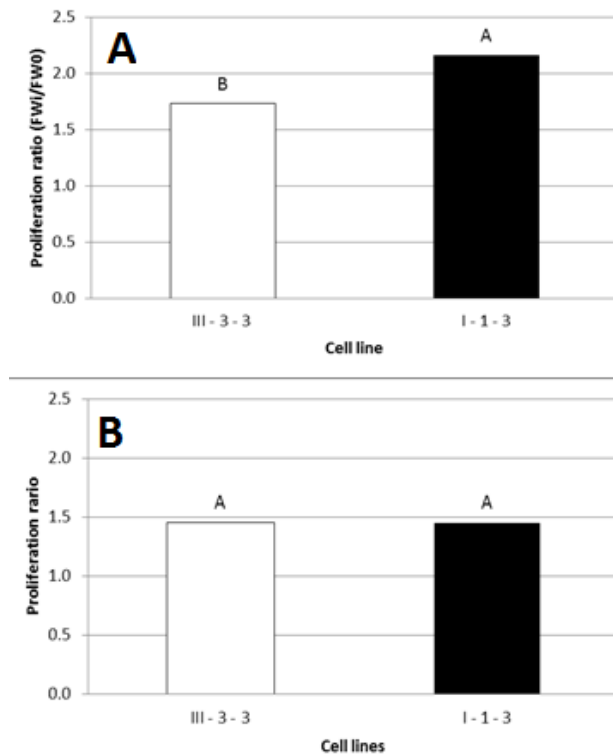
Fig. 17 Proliferation ratios of cell lines according to tested heavy metal ions A (Cu^{2+}) and B (As^{2+})

Statistical evaluation showed that cell line I – 1 – 3 had higher proliferation ratio in comparison with cell line III – 3 – 3 in treatment with copper. Unlike, in case of arsenic treatment both cell lines could not survive very toxic effect and no statistical difference was recorded (Fig. 17).
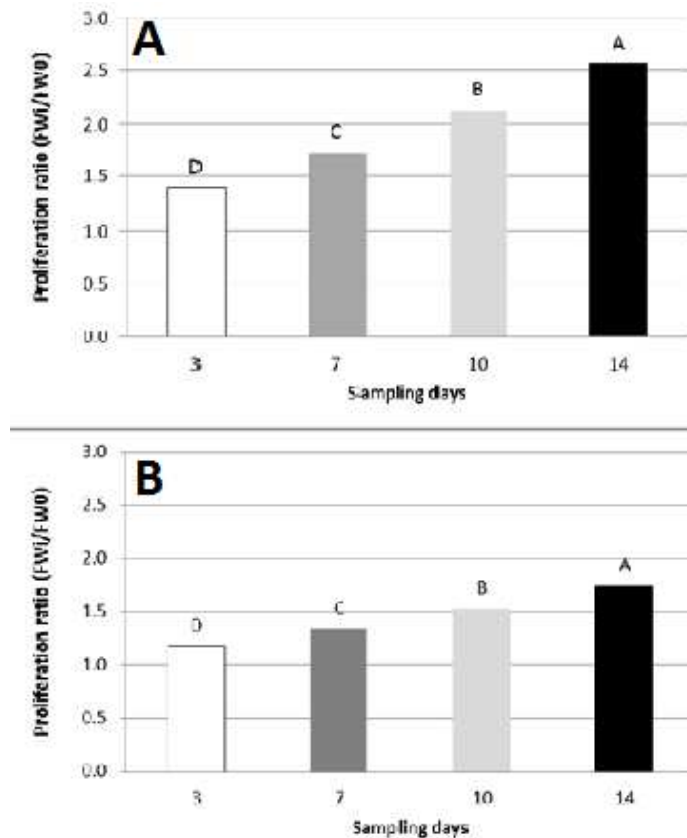

Fig. 18 Proliferation ratios during different sampling days (3, 7, 10 and 14) according to tested heavy metal ions A (Cu^{2+}) and B (As^{2+})

On Fig. 18 it can be seen that proliferation ratio was proportional to length of experiment duration. The highest ratio was recorded at last sampling day in case of both metals. Despite copper had 10 fold higher concentration, growth of ECMs was higher in comparison with lower arsenic concentration.
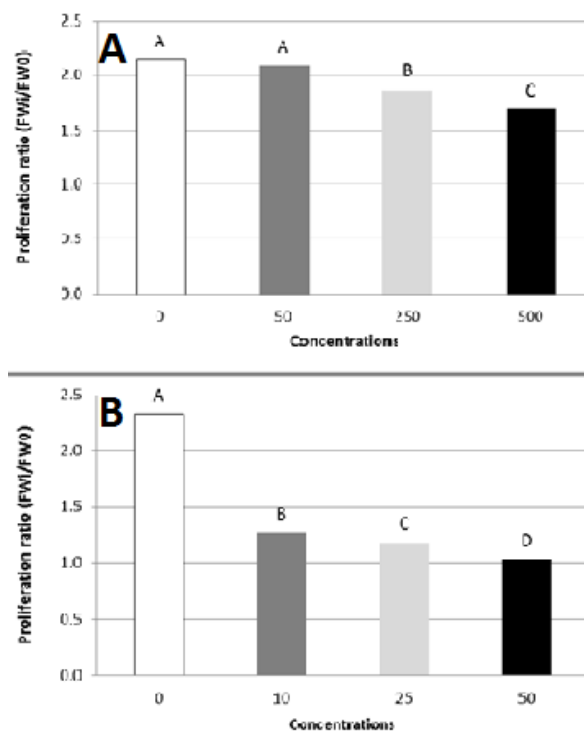
Fig. 19 Proliferation ratios under different concentrations A (Cu^{2+} - control, 50, 250 and 500 μM) B (As^{2+} - control, 10, 25 and 50 μM)

In case of copper, statistical evaluation showed that there was not any significant difference among control and 50 μM concentration whereas in case of arsenic all concentrations tested were statistically significant (Fig. 19).

5.2 Maturation

In order to calculate number of somatic embryos formed, all embryos were divided in three categories: early – precotyledonary, (B) precotyledonary, (C) cotyledonary somatic embryos (Fig. 20).

Fig. 20 Different developmental stages of *Picea abies* somatic embryos observed during the maturation process (A) early – precotyledonary, (B) precotyledonary, (C) cotyledonary somatic embryos

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The number of somatic embryos varied considerably among cell lines and metals tested. In treatment with copper, cell line I – 1 – 3 had higher amounts of embryos in comparison with cell line III – 3 – 3 (Tab. 7; Fig. 21).
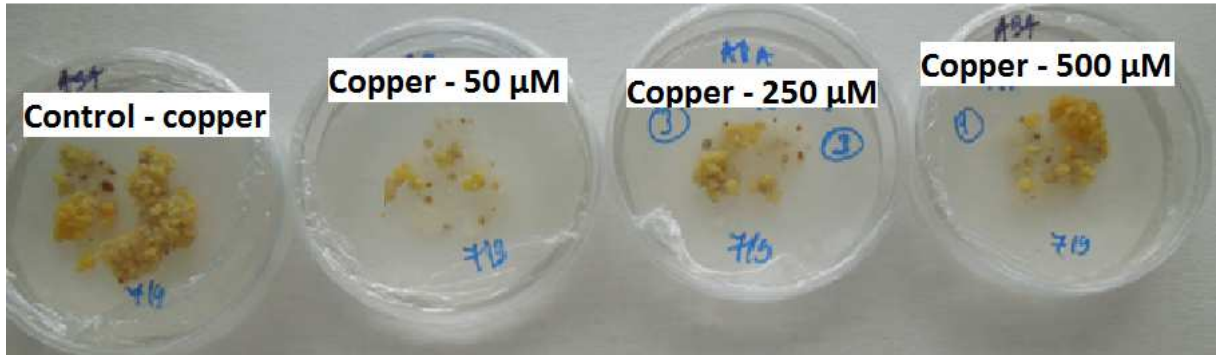

Fig. 21 Maturation of somatic embryos treated with Cu^{2+} during proliferation stage, cell line III – 3 – 3

Furthermore, in cell line I – 1 – 3 the highest number of embryos matured was from category early-precotyledonary whereas in cell line III – 3 – 3 prevails early-precotyledonary and precotyledonary.

Tab. 7 Effect of Cu^{2+} in different concentrations on maturation of III – 3 – 3 and I – 1 – 3 embryogenic cell lines (E-P – early precotyledonary; P – precotyledonary; C-cotyledonary somatic embryos)

I-1-3	Control				50 µM				250 µM				500 µM			
Petri plates	E – P	P	C	Total	E – P	P	C	Total	E – P	P	C	Total	E – P	P	C	Total
1.	19	10	5	**34**	12	8	8	**28**	15	12	4	**31**	3	2	3	**8**
2.	32	18	6	**56**	11	5	7	**23**	14	10	3	**27**	5	1	2	**8**
3.	41	12	7	**60**	14	3	7	**24**	13	18	7	**38**	8	4	2	**14**

III-3-3	Control				50 µM				250 µM				500 µM			
Petri plates	E – P	P	C	Total	E – P	P	C	Total	E – P	P	C	Total	E – P	P	C	Total
1.	17	24	5	**46**	7	8	2	**17**	10	5	6	**21**	4	3	1	**8**
2.	12	9	6	**27**	14	5	1	**22**	12	2	3	**17**	3	8	1	**12**
3.	25	14	4	**43**	10	7	3	**18**	14	4	1	**19**	1	7	5	**13**

From the other hand in treatment with arsenic, two highest concentrations did not mature and produce no somatic embryos (Fig. 22; Tab. 8). In concentrations 10 µM,

higher number of somatic embryos was formed in cell line I – 1 – 3. Like in treatment with copper, the lowest number of somatic embryos observed was in cotyledonary stage of development.
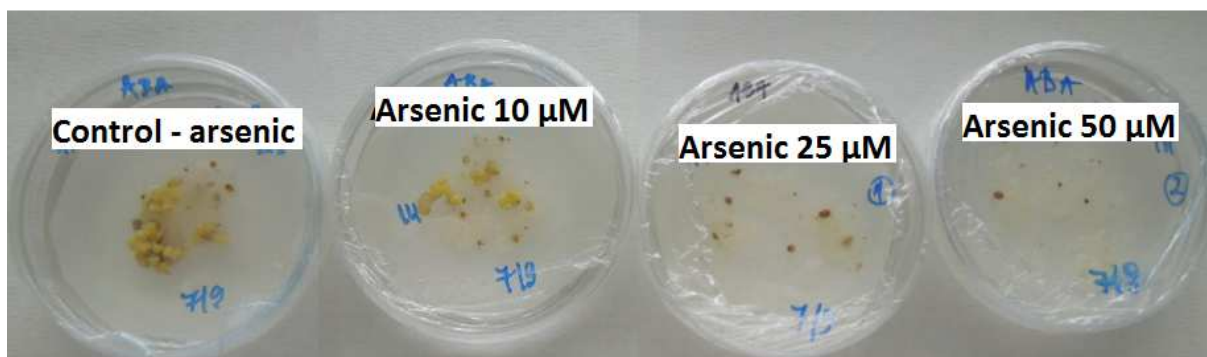

Fig. 22 Maturation of somatic embryos treated with As^{2+} during proliferation stage, cell line I – 1 – 3

Tab. 8 Effect of As^{2+} in different concentrations on maturation of III – 3 – 3 and I – 1 – 3 embryogenic cell lines (E-P – early precotyledonary; P – precotyledonary; C-cotyledonary somatic embryos)

I-1-3	Control				10 µM				25 µM				50 µM			
Petri plates	E – P	P	C	Total	E – P	P	C	Total	E – P	P	C	Total	E – P	P	C	Total
1.	24	25	14	**63**	10	15	1	**26**	0	0	0	**0**	0	0	0	**0**
2.	32	10	11	**53**	17	5	3	**25**	0	0	0	**0**	0	0	0	**0**
3.	38	12	5	**55**	9	7	2	**18**	0	0	0	**0**	0	0	0	**0**

III-3-3	Control				10 µM				25 µM				50 µM			
Petri plates	E – P	P	C	Total	E – P	P	C	Total	E – P	P	C	Total	E – P	P	C	Total
1.	37	48	10	**95**	8	10	3	**21**	0	0	0	**0**	0	0	0	**0**
2.	45	53	19	**117**	16	7	5	**28**	0	0	0	**0**	0	0	0	**0**
3.	29	42	17	**88**	5	9	2	**16**	0	0	0	**0**	0	0	0	**0**

5.3 Observation of cotyledon abnormalities

In treatments with arsenic (10 µM) concentrations, different abnormal developed embryos were observed e.g. higher and lower number of cotyledons, meristemless, embryos with single cotyledon (Fig. 23).
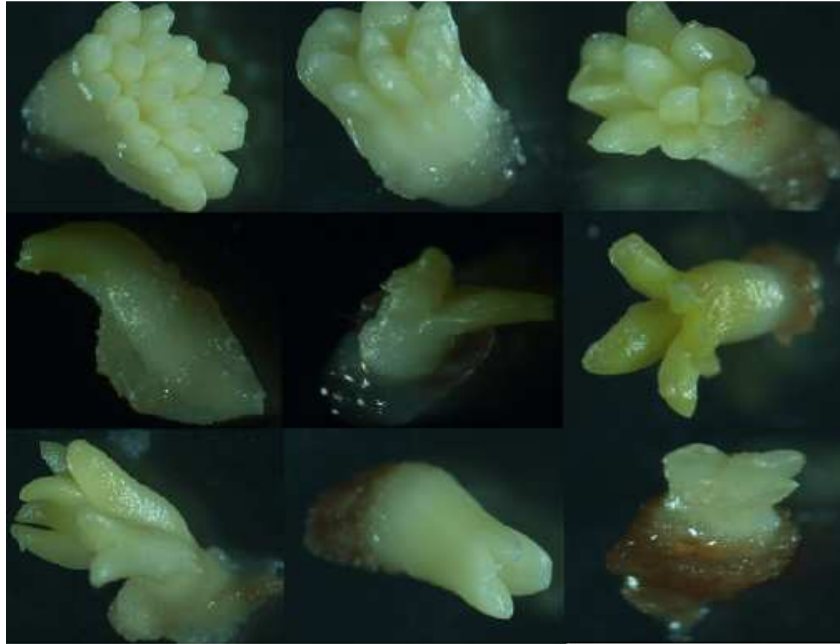

Fig. 23 Observation of cotyledon abnormalities

5.4 Desiccation, germination and conversion

Once, it was obtained sufficient number of somatic embryos, partial desiccation treatment was performed for 3 weeks (as explained in Section 4). After desiccation, embryos were put on medium for germination for further development (Fig. 24).
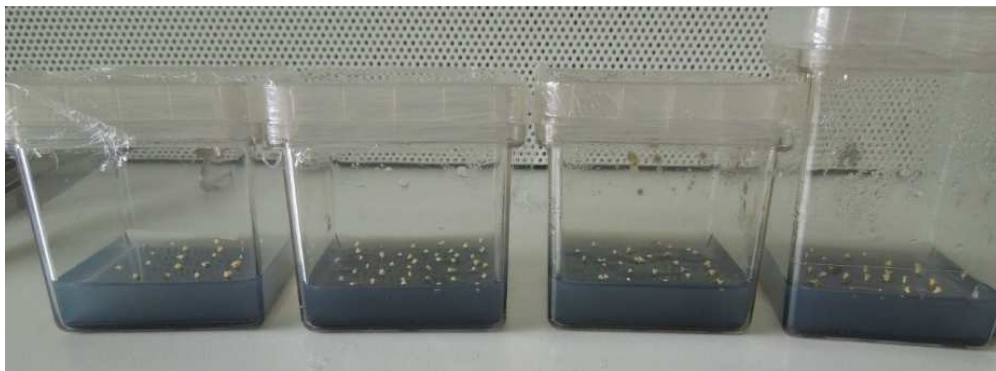

Fig. 24 Somatic embryos on germination medium

After one month on germination medium, development of roots and cotyledons was observed (Fig.25 A and C). Abnormally developed embryos were also included for germination experiments but further growth and development was not recorded (Fig. 25 B and D). Moreover, there was not protruding of radicle, cotyledons ceased to develop and vitrification was observed.
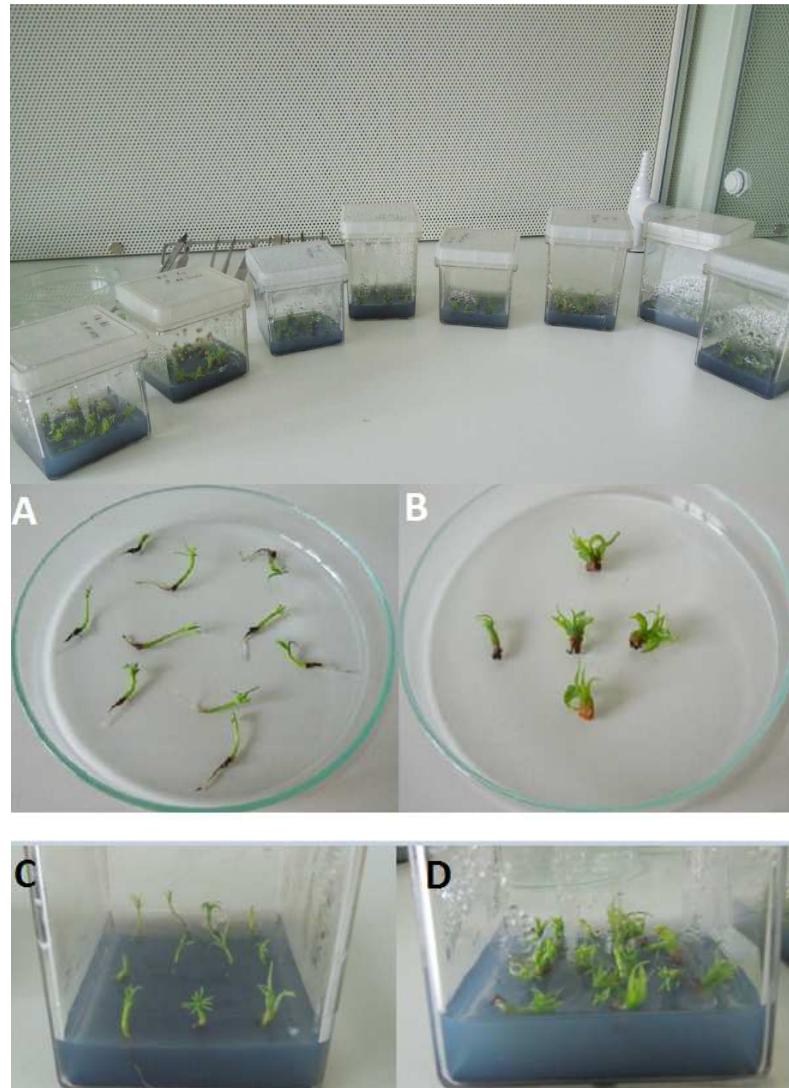

Fig. 25 Somatic embryos grown on germination medium for one month

After one month on germination medium, embryos which developed by abnormal way were discarded and only properly developed embryos were used for further experiments (Fig.26).

Fig. 26 Conversion of plantlets on perlite substrate

Plantlets were put in perlite substrate in order to keep high humidity. Due to lacking of proper container for acclimatization purposes after two weeks on perlite, all plantlets died.

6 DISCUSSION

In this work effect of copper and arsenic in different concentrations on subsequent stages of Norway spruce somatic embryogenic was evaluated. The question which may arise is why to use excess of copper since it is well know that copper in lower concentrations is microelement which is essential constituent of several enzymes (Cu/Zn superoxide dismutase (SOD), cytochrome C oxidase, amino oxidase, laccase, plastocyanin and polyphenol oxidase) mainly involved in electron transfer chain in mitochondria and chloroplasts. From the other hand, arsenic is a heavy metal which does not have any biological function in plant organism. Both, copper and arsenic in excess could be very toxic for growth and development of plants. Moreover, these two metals are interconnected since arsenic is a by-product of some copper ores. Today, all over the world is known pollution of water and poisoning mainly by these two metals since copper is found in surface water, groundwater, seawater and drinking-water through corrosion of interior copper plumbing while arsenic is found in groundwater and tube wells (ATSDR, 2002). Plants can be polluted by various ways with excess of copper and arsenic through water flows e.g. rainfalls, drainage, discharge of sewage, irrigation etc. It is very difficult to eliminate arsenic contamination in the environment. Arsenic contamination of soil, streams and underground water causes a major environmental and human health risk.

Excess of copper can cause disorders in plant growth and development by adversely affecting important physiological processes in plants. Plants grown in the presence of high levels of Cu show reduced biomass and chlorosis symptoms (Demirevska-Kepova et al. 2004). A lower content of chlorophyll and alterations of chloroplast structure and thylakoid membrane composition was found in leaves under such growth conditions (Ciscato et al. 1997). Cu toxicity is related to the binding of Cu to sulfhydryl groups of the plasma membrane (Yruela, 2005). Furthermore, it was observed that excess of Cu in plants led to oxidative stress inducing changes in the enzyme activity and content of some components of the antioxidative pathways (Gupta et al. 1999). From the other hand, arsenic can severely inhibit plant growth by slowing or arresting expansion and biomass accumulation, as well as compromising plant reproductive capacity through losses in fertility, yield and fruit production (Garg and Singla, 2011). At sufficiently high concentrations, As interferes with critical metabolic processes, which can lead to plant death.

Plant tissue culture is a technique used for fast multiplication of many genetically identical plants in controlled conditions. It relies on the totipotency which means that plant cells have ability to regenerate whole plant. Plant tissue culture techniques have enormous potential for various applications, including testing of effect of heavy metals. Maróti and Bognár (1988) tested effect of $ZnSO_4$, $NiSO_4$ and $CuSO_4$ on the growth of the secondary callus tissue of *Nicotiana tabacum* and *Ruta graveolens*. The increase in fresh weight of the secondary callus tissue was inhibited by the metal compounds applied with both plant species (to 75-87% by zinc, 7-97% by nickel, 5-98% by copper with tobacco; to 47-69% by zinc, 5-88% by nickel, 57-90% by copper with rue). The cell number and dry weight per g of callus tissue partly increased, partly decreased compared to the control in response to the heavy metal treatment. Moreover, in treatments with various concentrations of the heavy metals, growth values were different in the two plant species due to differences in metabolism and organization potential between them. In cell suspension cultures of *Catharanthus roseus* the effect of Cd (II), Cr (III), Cr (VI), Cu (II), Hg (II), Pb (II) and Zn (II) was tested (Lizhong and Cullen, 1995). It was observed that toxicities of HM are interrelated to their oxidation states, species and pH of culture media. *In vivo* and *in vitro* studies on heavy metal tolerance in *Sesbania grandiflora* were conducted (Ibrahim and Yousir, 2009). Heavy metals (Cd, Co, Cu, Cr or Zn) were added to the culture medium at different concentrations as contamination agents. In order to assess the effect of these heavy metals on seed germination; seeds were sown in soil contaminated with different concentrations of heavy metals for 3 weeks. Results showed that callus fresh weight decreased with increasing heavy metal concentration in cultural medium. Germination percentages and plant heights increased over time. However, a reduction occurred in these parameters with increasing heavy metal concentrations. These results are in agreement with our results where under effect of higher metal concentration the callus growth decreased.

In this work cell line I – 1 – 3 had higher proliferation ratio in comparison with cell line III – 3 – 3 in treatments with both metals. Screening and selection at the plant cell level could establish plant clones with increased tolerance or resistance in plants to various environmental stresses like salt, heat, cold, drought, disease, insects, heavy metals and herbicides. Cell lines tolerant to elevated levels of salt in the medium have been selected in *Brassica juncea* (Jain et al. 1991). Furthermore, cell lines resistant to elevated

concentrations of aluminum have been chosen in *Nicotiana plumbaginifolia* (Conner and Meredith, 1988).

In our experiments it was observed that ECMs treated with lower concentrations of copper were grown better than control while highest concentration (500 µM) inhibited growth. Nassar (2004) examinated the effect of different concentrations of cupric sulfate, cupric chloride or cupric acetate on rhizogenesis of banana micropropagated shoots. The results showed that 1 µM $CuSO_4$ stimulated root induction, elongation and shoot growth compared with the control (0.1 µM $CuSO_4$ present in Murashige and Skoog (1962) medium). Higher level of $CuSO_4$ (100 µM) had toxic effect on banana leaves and completely inhibited root formation. Cupric chloride proved to be more convenient in the culture medium than $CuSO_4$ and stimulated good quality of roots, enhanced shoot growth and showed no toxicity symptoms at higher concentrations. Cupric acetate was very toxic even at low concentration. Ouzounidou et al. (1992) reported that increasing Cu concentration in nutrient medium reduced the uptake of nutrient elements such as Ca, Mg, K and Fe. Reboredo (1994) also indicated that Cu exposure induced changes in mineral metabolism, especially Fe and Zn. AL-Mayahi (2014) reported effect of copper sulphate and cobalt chloride on growth of the *in vitro* culture tissues of date palm (*Phoenix dacrylifera*). As a result the rate of callus proliferation was significantly higher in the medium supplemented with 2 µM copper sulphate and 2 µM cobalt chloride together. Kowalska et al. (2012) tested effect of $CuSO_4 \times 5H_2O$ in concentrations 1, 10 and 100 µM on growth of androgenetic embryos of carrot. In the very beginning of experiments copper has positive effect on rooted rosette formation and secondary embryos, but after longer exposures (9-15 weeks on media) negative effect such as deformation of rosettes appear. Prolonged exposure to media containing elevated concentrations of $CuSO_4$ caused a reduction in the accumulation of phenolic compounds in the rosettes. Gori et al. (1998) observed in the tobacco variety Bel W3 that 50 µM $CuSO_4$ considerably inhibited the growth of callus and the regeneration of shoots after one month of culture. In the presence of 100 µM and 150 µM $CuSO_4$, the fresh matter content decreased substantially while 200 µM $CuSO_4$ almost completely inhibited the growth of callus.

Many researches were done regarding arsenic accumulation, uptake, distribution, binding forms and content in plants (Del Río Celestino et al. 2002; Alam et al. 2003;

Patra et al. 2004). However, only few studies exist on effect of seed germination treated with arsenic or on plants grown in conditions of tissue culture.

Shri et al. (2009) investigated the effect of arsenic on growth, oxidative stress and antioxidant system in germinated rice seedlings. A marked decrease in germination percentage, shoot and root elongation as well as plant biomass was observed with arsenic treatments as compared to control. Li et al. (2007) investigate the effects of arsenic on seed germination and physiological activities of wheat seedlings. At lower concentrations of As (0–0.5 mg/kg) it was seen that germination index increased. However, in higher concentrations germination percentage, germination index, vitality index, length and biomass of root and shoot all displayed decreasing trend with increasing concentrations of As. Abedin and Meharg (2002) reported that germination and early seedling growth of rice decreased significantly with increasing concentrations of As. Talukdar (2011), evaluated effect of arsenic-induced toxicity on morphological traits of *Trigonella foenum-graecum* and *Lathyrus sativus* during germination and early seedling growth. Mean value of germination percentage, germination index and relative germination rate decreased with concomitant increase in arsenic-induced injury level in increasing concentration of arsenic in both plants and the effect was significant at 30 and 40 mg/L treatments. Speer (1973) was investigated effect of arsenic using both intact and punched seeds of lettuce (*Lactuca sativa* L.). The inhibition of germination in punched seeds by arsenate given in conjunction with phosphate compared with the lack of inhibition of arsenate plus phosphate on the growing seedling, suggest a distinct metabolic change in the germinating embryo at some time between the onset of germination and subsequent seedling growth. It was seen that plant growth is stimulated at low As concentrations (Miteva, 2002; Garg and Singla, 2011). The fact that this phenomena occurs under arsenic conditions in cultured plants, such as *Arabidopsis thaliana* (Chen et al. 2010), indicates that the trait is not based on As disrupting plant-biotic interactions. Instead, it results either from a direct interaction of As with plant metabolism, or from an interaction of As with plant nutrients. Arsenate is chemically similar to phosphate and it is probably taken up into many plants via phosphate transporters (Pigna et al. 2009). In wheat seeds, for example, germination is considerably affected by both arsenite and arsenate, probably reflecting the inhibition of both α- and β-amylase (Liu et al. 2005).

Moreover, in our work it was observed abnormal cotyledonary development in higher copper and both lower and higher arsenic concentrations during maturation process.

Sethy and Ghosh (2013) reported that the main effects of HM on seeds are manifested by overall abnormalities and decrease in germination rate.

From our results it can be seen that abnormal developed somatic embryos which were transferred on germination medium ceased further development and that root was not formed. The symptoms of As toxicity include poor seed germination and profound growth inhibition (Smith et al. 2010). Maize plants treated with toxic concentration of As(V) and As(III) produced stunted roots that were thicker and stiffer than normal, and that had a significantly lower mitotic index; micronuclei and chromosome aberrations were also observed in the root meristems (Duquesnoy et al. 2010). In some species, the effect of As on root growth depends on its concentrations. For example, root growth in *Artemisia annua* is stimulated at low As concentrations but inhibited at higher concentrations (Rai et al. 2011).

Although, we were able to produce plantlets of Norway spruce in conditions *in vitro* without appropriate equipment where high humidity needs to be maintained plantlets ceased further growth and development and experiments in greenhouse were not performed. The last stage of SE to *ex vitro* conditions is known to be a critical step (Stasolla and Yeung, 2003; Tompson, 2015). Montablán et al. (2010) reported conversion of *Pinus radiata* somatic embryos to plantlets as a major bottleneck while Krajňáková and Häggman, (2016) emphasized the low conversion rates in *Abies cephalonica*. In Norway spruce low conversion rate was also reported (Becwar et al. 1989).

Since plants are sessile organisms and have only limited mechanisms for stress avoidance, they need flexible means for acclimation to changing environmental conditions. In order to improve a plant's protection, it is important to understand the mechanisms contributing to stress tolerance (Schützendübel and Polle, 2001). In the field, plants are exposed to additional abiotic and biotic factors, which complicate further plant response while *in vitro* selected species are grown under standard conditions. Results obtained from *in vitro* plant tissue cultures and whole plant hydroponic experiments, indicate on the phytoremediation potential of different plant species and the biochemical mechanisms involved in plant tolerance.

7 CONCLUSIONS

Aim of this work was to evaluate effect of copper and arsenic on Norway spruce ECMs in different concentrations during 14 days proliferation period. The other goal was related to maturation and germination stages. Based on performed experiments it can be concluded that:

- ✓ Copper in lower concentrations increased the growth of ECMs while in higher concentrations (250 and 500 µM) growth was inhibited. Cell line I – 1 – 3 had better response in comparison with cell line III – 3 – 3, where concentrations of 50 µM had highest proliferation ratio.

- ✓ In preliminary experiments with arsenic concentrations 50, 250 and 500 µM showed very high toxicity and after 14 days proliferation period all ECMs were dead. New set of experiments was set up with lower concentrations (10, 25 and 50 µM). Control had highest proliferation ratio while ECMs had insignificant growth on 10 and 25 µM concentrations. Again, cell line I – 1 – 3 was proliferating better than cell line III – 3 - 3.

- ✓ In treatment with copper, cell line I – 1 – 3 had higher amounts of embryos in comparison with cell line III – 3 – 3. Furthermore, in cell line I – 1 – 3 the highest number of embryos matured was from category early-precotyledonary whereas in cell line III – 3 – 3 prevails early-precotyledonary and precotyledonary. From the other hand in treatment with arsenic, two highest concentrations did not mature and did not produce cotyledonary somatic embryos. In concentrations 10 µM, higher number of somatic embryos was formed in cell line I – 1 – 3. Nevertheless, certain number of abnormally developed cotyledonary somatic embryos was obtained.

- ✓ Cotyledonary somatic embryos from both treatments were subjected to germination, but ceased further development during acclimatization stage in perlite substrate.

- ✓ From this research can be seen that in treatments with both metals cell line I – 1 – 3 had higher resistance.

- ✓ Norway spruce as a fast growing evergreen tree is one of the most widely planted spruces and one of the most economically important species in Europe. Since, nowadays pollution is widespread the results of this thesis can help to

understand effect of two tested metals on somatic embryogenesis but as well can be used for investigation of two tested clones in polluted soils.

✓ For further research is necessary to test as many different clones as possible that clone which show higher resistance in *in vitro* conditions can be tested in the field on the polluted sites.

✓ It is necessary to work on the protocol improvement for acclimatization of somatic embryos to viable plantlets in greenhouse conditions.

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9 LIST OF ABBREVIATIONS

ECMs - embryogenic cell masses

SE - somatic embryogenesis

Cu – cooper

As – arsenic

HM - heavy metals

MMA - monomethylarsonic acid

DMA - dimethylarsinic acid

ABA - abscisic acid

PGR - plant growth regulators

2,4 – D - 2,4-Dinitrophenylhydrazine

BA - benzyl adenine

EDTA - ethylene diamine tetra-acetic acid

MS – Murashige Skoog medium

FW - fresh weight

E-P – early precotyledonary somatic embryos

P – precotyledonary somatic embryos

C- cotyledonary somatic embryos

SOD - superoxide dismutase

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Tab. 3 Composition of liquid pre-maturation media (growth regulators and gelling agent are omitted)

Tab. 4 Composition of germination media

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Tab. 6 Analysis of variance (significance of F-tests) of the effects of As2+ on the proliferation rate of III – 3 – 3 and I – 1 – 3 during 14 days lasting proliferation period, concentrations 0, 10, 25 and 50 μM

Tab. 7 Effect of Cu^{2+} in different concentrations on maturation of III – 3 – 3 and I – 1 – 3 embryogenic cell lines (E-P – early precotyledonary; P – precotyledonary; C-cotyledonary somatic embryos)

Tab. 8 Effect of As^{2+} in different concentrations on maturation of III – 3 – 3 and I – 1 – 3 embryogenic cell lines (E-P – early precotyledonary; P – precotyledonary; C-cotyledonary somatic embryos)

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Fig. 24 Somatic embryos on germination medium

Fig. 25 Somatic embryos grown on germination medium for one month

Fig. 26 Conversion of plantlets on perlite substrate