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Physiologie des plantes

EFFECTS OF MINERAL NUTRIENT CONCENTRATION ON THE BIOMASS ACCUMULATION AND NITRATE ASSIMILATION RATE OF MILK THISTLE (*SYLIBUM MARIANUM* L.)

I. Stancheva, M. Geneva, G. Georgiev

(*Submitted by Academician E. Karanov on March 24, 2004*)

Abstract

Milk thistle plants (*Sylibum marianum* L., Asteraceae family), an important medicinal plant, source of sylimarin, a pharmaceutical product of great importance, have been studied. The aim of this experiment was to determine the nitrogen use efficiency of young plants (21-days old), grown in solution culture and controlled environment. The conditions of growth were: phytotron chamber with a 12 h photoperiod, day/night temperature 25/20 °C and photon flux density equal to 95 μmol $m^{-1}s^{-1}$. The nutrient solutions supplied represented 0.125; 0.25; 0.5; 1 and 1.5 strength of the Hellriegel's solution [1] containing trace elements according to Hoagland-Arnon recipe [2]. Strong positive correlation was established between accumulated fresh and dry biomass and nitrate reductase (NR) activity of leaves when the solution strength was up to one half of its maximal value. The following increase of nutrient solution concentration up to 1 and 1.5 strength inhibited the plant growth and leaf nitrate NR activity. However, NR inhibition did not correlate with the higher nitrate supply in the solution. The leaf nitrates increased proportionally to the rate of nitrate supply to the solution which was in contrast to inhibited NR of leaves in these treatments. Chlorophyll "*a*" and "*b*" of leaves accumulates increasingly in concentration-dependent manner in all studied treatments. The inhibition of NR can be regarded as result of metabolic deregulation of nitrate assimilation pathways of young seedlings by the higher solute concentration. The optimal nutrient concentration that correlates with the maximum nutrient use efficiency of *S. marianum* at this stage of growth was 0.5 strength of the full solution concentration.

Key words: milk thistle (*Sylibum marianum* L.), fresh and dry biomass, nitrate reductase activity, nitrate content, pigments

Introduction. Milk thistle is a Mediterranean plant of great pharmaceutical importance [3]. Now it is widely spread all over the world. Milk thistle seeds are important source of sylimarin – a common name of mix (usually 4 of them) of different flavonolignans with content varying between 1–3%. They derived biosynthetically from taxipholin and coniferic alcohol in phenyl propanoid metabolic pathway [3]. The sylimarin substance possesses important antioxidant characteristics for free radicals scavenging, lipid peroxidation inhibition and membrane stabilization of liver cells. In pharmacy it is used as medicine for curing liver toxic and inflammatory diseases.

Little is known about milk thistle nutrient use requirements and growth conditions. In several field and pot experiments the effect of heavy metals accumulation on milk thistle productivity and quality was studied [4]. Since there is a close relationship

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GRIGOR ZEHIROV, IRA STANCHEVA, MARIA GENEVA, GEORGY GEORGIEV
Acad. M. Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Sofia
"Acad. G. Bonchev" Str., Bl. 21, 1113 Sofia, Bulgaria

Comparison of the Effects of Different Site of Supply (root or foliar) of Phosphate and Nitrate on the Growth and Nitrate Assimilation Enzymes Activity in Milk thistle (*Silybum marianum L.*) Plants

Abstract

The Milk thistle plants (*Silybum marianum L.*) were grown for 21 days under glasshouse conditions in a 0.8 L plastic pot (4 plants/pot) contained Hellriegel's solution at the following variants: 1) control - ½ strength solution in the growth medium; 2) ¼ strength solution with the exception of NO_3 (reduced to ¼ strength solution - 1.5 mM) and $PO4^{-3}$ (increased to full strength solution - 1.0 mM) in the growth medium; 3) ¼ strength solution in the growth medium with adding 0.3 % of foliar fertilizer Agroleaf in order to make level equal to the control variant nutrients.

Increased P supply did not influence leaf number towards the control, but resulted in higher values of rosette diameter, which corresponded to the higher shoot dry weight. The plants with foliar fertilization had the lower leaf number towards the control but bigger rosette diameter, which suggests its larger leaf area. The plants grown with addition of foliar fertilizer showed the highest activities of the enzymes connected with primary N assimilation - nitrate reductase (NR: NADH, EC 1.6.6.2) and glutamine synthetase (GS: EC 6.3.1.2). A correspondence between root N content and S:R dry weight ratio was found.

Keywords: foliar fertilizer, nitrate reductase, glutamine synthetase, shoot and root dry weight, shoot and root total P and total N content

Introduction

The seeds, flower heads and leaves of milk thistle (*Silybum marianum* L.), a plant native to Europe, have been used since the ancient times as a liver tonic. The key pharmaceutical constituent in milk thistle seeds is silymarin a flavonoid composed of four isomers: silybin, silychristin, silydianin, isosilybin. There is a shortage of information about milk thistle nutrient and growth conditions requirements, because the plants grow naturally in lands without management. In several pot and field experiments Zheljazkov and Nikolov (1996) have established the effects of some heavy metals on milk thistle productivity and silymarin content. In our previous study low nitrogen requirements of milk thistle seedlings was shown (Stancheva et al., 2004a).

Regularly supply of all macronutrients required by plants (N, P, S, K, Mg and Ca) was limiting factor for the rate of plant growth. Nitrogen is required for the synthesis of proteins, protoplasm, chlorophyll and enzymes. Nitrates also serves as an important signal for growth as plants respond to nitrate by altering their metabolism and by including genes in the nitrate assimilation pathway (Crawford and Glass, 1998). P is involved in the transfer of energy from the leaves to the active growing points. P is also essential for the development of cell nuclei, cell membranes and cell subdivision, all vital functions in the early stages of plant and root growth. There is general agreement that shoot to root dry weight ratio (S:R) decreases when growth is limited by N supply (Andrews, 1993), or P supply (Ingestad and Agren, 1991). There are reports for several higher plant species that S:R changes with growth development independently of nutrient supply (Gedroc et al., 1996).

Assimilation of N is altered when NO_3^- grown plants are deprived of adequate P. At least three types of effects have been identified: 1) decreases uptake of nitrate by roots (Rufty et al., 1990); 2) decreases NO_3^- translocation from the root to

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EFFECTS OF FOLIAR FERTILIZER CONCENTRATION ON THE BIOMASS ACCUMULATION AND NITRATE ASSIMILATION RATE OF MILK THISTLE (*SILYBUM MARIANUM* L)

Ira Stancheva, Maria Geneva, Grigor Zehirov, Georgy Georgiev*

Department of Mineral Nutrition and Water Regime, Acad M.Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Block 21, Sofia 1113, Bulgaria.
Corresponding author:
E-mail: ira_stancheva@abv.bg

ABSTRACT . The milk thistle plants (Silybum marianum L.) were grown for 21 days under glasshouse conditions in a 0,8 plastic pot (4 plants/pot) contained ½ strength Hellriegel's solution at the following variants: (1) control plants, without application of foliar fertilizer; (2) plants, grown with application of 0.3% foliar fertilizer; (3) plants, grown with application of 0.5% foliar fertilizer. The plants grown with addition of foliar nutrition have shown increased leaf number and dry biomass accumulation, reduced rosette diameter, enhanced rates of nitrogen assimilatory enzymes. A favorable effect of foliar feeding on the protein content, tissue nitrogen and potassium concentration have been also established.

KEY WORDS: Milk thistle (*Silybum marianum* L.), foliar fertilizer, dry biomass, nitrate reductase activity, glutamine synthetase activity.

INTRODUCTION

During development, the milk thistle (*Silybum marianum*) seeds accumulate flavonolignans (often referred to as silymarin complex). Therapeutic efficacy of silymarin in treating liver disorders is well known. However little is known about milk thistle nutrient and growth conditions requirement. In our previous study low nitrogen requirements of milk thistle seedlings was reported (Stancheva et al., 2004a). There is some evidence indicating that elevated P concentration in the growth medium positively influenced some of the morphological parameters of the 21 day

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Effect of Soil Fertilizer, Foliar Fertilizer, and Growth Regulator Application on Milk Thistle Development, Seed Yield, and Silymarin Content

Maria Geneva, Grigor Zehirov, Ira Stancheva, Lubomir Iliev, and Georgi Georgiev

Acad. M. Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

Abstract: An important consideration for milk thistle (*Silybum marianum L.*) cultivation is regulating development to lengthen the reproductive stage and increase seed yield with high silymarin content. The treatment of milk thistle with foliar fertilizers and growth regulators—thidiazuron (Dropp®), 2,3,5-triiodobenzoic acid (Tiba®), mepiquat chloride (Pix®), and prohexadione-Ca (Regalis®)—resulted in an increase in the proportion of mature flower heads. Highest seed yield was observed in plants treated with Pix® and mineral soil fertilization, whereas in plants treated with foliar fertilizers, highest yields were observed with Pix® and Regalis®. The highest content of silymarin was found in plants treated with Dropp® and foliar fertilizer. Generally, treatment of milk thistle with plant-growth regulators in combination with soil or foliar mineral fertilizers increased the total amount of silymarin by increasing seed yield per hectare.

Keywords: Dropp®, Pix®, Regalis®, *Silybum marianum L.* silymarin, Tiba®

INTRODUCTION

Milk thistle plants produce seeds that are an important source of silymarin, which is used in modern pharmaceutical industries. Silymarin is a group of

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Address correspondence to Maria Geneva, Acad. M. Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Block 21, Sofia 1113, Bulgaria. E-mail: boykova2@yahoo.com

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Regulation of Milk Thistle (*Silybum marianum* L.) Growth, Seed Yield and Silymarin Content with Fertilization and Thidiazuron Application

Ira Stancheva[1*] · Abd el Ghany Youssef[2] · Maria Geneva[1] · Lubomir Iliev[1] · Georgi Georgiev[1]

[1] Department of Mineral Nutrition and Water Relations of Plants, Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev str., bl. 21, Sofia 1113, Bulgaria

[2] Department of Cultivation and Production of Medicinal and Aromatic Plants, National Research Centre, Cairo Dokki, Egypt

Corresponding author: * ira_stancheva@abv.bg

ABSTRACT

This study looks into the effect of foliar or soil fertilization and growth regulator thidiazuron (TDZ) treatment on the vegetative and reproductive growth, some physiological parameters, seed yield and silymarin content of field grown milk thistle (*Silybum marianum* L.) plants. Foliar fertilizer Agroleaf® of different NPK proportions was applied at different plant developmental stages. Combined application of the fertilizers with TDZ affected the growth, accumulation of nutrients (N, P, K), nitrate reductase activity, reducing sugars and free amino acids content positively. These changes were associated with altered flowering rate, enhanced seed ripening and increased yield. Treatment of milk thistle plants with TDZ in combination with foliar fertilizer increased seed yield due to an increase in the number of lateral stems, the number of flower heads and the seed fresh weight per flower head. Silymarin accumulation in the seeds was also positively influenced by the combined application of foliar fertilizer and TDZ.

Keywords: dry biomass, flowering dynamics, foliar and soil fertilization, lateral shoots, TDZ
Abbreviations: FF, foliar fertilization; **FF+TDZ**, foliar fertilization plus thidiazuron, **NRA**, nitrate reductase activity; **SF** soil fertilization; **SF+TDZ**, soil fertilization plus thidiazuron; **TDZ**, thidiazuron

INTRODUCTION

Milk thistle (*Silybum marianum* L., *Asteraceae* family) is a medicinal plant, cultivated for seed production. Seeds contain important substances used in the pharmaceutical industry. These substances, commonly known as silymarin, are powerful antioxidants (Dewick 1998). Silymarin primarily consists of an isomeric mixture of six phenolic compounds: silydianin, silychristin, diastereoisomers of silybin (silybin A and B), and diastereoisomers of isosilybin (isosilybin A and B) (Gus and Stermitz 2000; Lee *et al.* 2007). These compounds are flavonolignans, products of the phenyl-propanoid metabolic pathway in plants. Regulation of development in order to lengthen the reproductive stage length and to increase the yield of the seeds, with high silymarin content is an important problem for milk thistle cultivation. Enhanced milk thistle seed quantity and quality is dependent on the control of growth, flowering rate and transport of assimilates to seeds during the maturation process. Internal control of flowering includes perception of some external (day length, temperature) or internal (circadian rhythms, plant phase change, hormones) signals (Taiz and Zeiger 2002). The interaction between these factors is responsible for the synchronization of the plant reproductive process with environ-mental conditions. Among the external factors that can be successfully used for control of reproductive development of milk thistle are fertilization and growth regulators (Moor 1989). The control of flowering includes the action of some plant hormones such as gibberellins, cytokinins or auxins (Daphne *et al.* 2005).

Fertilization is a beneficial approach to regulate the reproductive development of plants. Foliar feeding with mineral nutrients has proved to be an effective regulator of nutritional disorders in plants. It would be conceivable to suggest that foliar fertilization cannot entirely replace the effect of soil fertilizers, but it may enhance the efficiency of soil nutrients assimilation (Eddy 2000; Wojcik 2004). The effects of foliar fertilization on the improvement of fruit quantity and quality have been reported by Eddy (2000) and Stancheva *et al.* (2004).

It could be expected that the appropriate fertilizer application and growth regulator can be effective tools to regulate milk thistle flowering rate, seed yield and quality. Among the group of plant growth regulators successfully used for control of flowering rate or seed ripening are cytokinins (Capelle *et al.* 1983). Thidiazuron (TDZ) (Dropp®) is a powerful plant growth regulator with high cytokinin activity, successfully used in tissue culture propagation or for regulation of plant growth or flowering (Cappelle *et al.* 1983; Alexieva *et al.* 1997).

The effect of foliar or soil fertilization and exogenously-applied TDZ during milk thistle cultivation on dry biomass, leaf content of the main macroelements, reducing sugars and amino acid accumulation, activity of leaf nitrate reductase, flowering dynamics, seed yield and silymarin accumulation are compared in this study.

MATERIALS AND METHODS

The study was conducted over three years (2004-2006) at an experimental field, on a leached cinnamonic meadow soil (Chromic Luvisols, according to FAO- Unesco-Isric legend). Milk thistle

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IMPROVEMENT OF MILK THISTLE (*SILYBUM MARIANUM* L.) SEED YIELD AND QUALITY WITH FOLIAR FERTILIZATION AND GROWTH EFFECTOR MD 148/II

M. Geneva[1], I. Stancheva[1], M. Sichanova[1], M. Boychinova[1], G. Georgiev[1], M. Dolezal[2]*

[1]Department of Plant Mineral Nutrition and Water Relations, Acad M.Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev str., bl. 21, Sofia 1113, Bulgaria
[2]Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, Heyrovskeho 1203, Hradec Kralove, Czech Republic

Summary. The effects of foliar or soil fertilization and MD 48/II (3-methylphenylamide 5-tert-butylpyrazine-2-carboxylic acid) on the vegetative and reproductive growth, some physiological parameters, seed yield and silymarin content of field grown milk thistle (*Silybum marianum* L. Gaertn.) plants were studied. Foliar fertilizer Agroleaf® was applied at different plant developmental stages, with different proportions of N, P, K. Combined application of the foliar fertilizer with MD 148/II at a concentration of 1.10^{-3} M affected most positively growth, number of plant lateral shoots and flower heads per plant. These changes were associated with altered flowering rate, enhanced seed ripening and increased yield. The accumulation of flavonoids and silymarin compounds in the seeds was also positively influenced.

***Key words*:** 5-tert-butyl-*N*-m-tolylpyrazine-2-carboxamide, foliar fertilization, milk thistle (*Silybum marianum* L. Gaertn.), seed yield, silymarin.

*Corresponding author, e-mail: boykova2@yahoo.com

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Influence of foliar fertilization and growth effector 5-tert-butyl-*N*-m-tolylpyrazine-2-carboxamide on the milk thistle (*Silybum marianum* L.) seed yield and quality

Ira Stancheva[1], Georgi Georgiev[1], Maria Geneva[1], Albena Ivanova[1], Martin Dolezal[2], Lenka Tumova[2]

[1] *Acad. M. Popov Institute of Plant Physiology, Bulgarian Academy of Sciences*
[2] *Faculty of Pharmacy in Hradec Kralove, Charles University in Prague*

ABSTRACT

The aim of this study was to evaluate the effects of foliar fertilization and a growth effector 5-tert-butyl-*N*-m-tolylpyrazine-2-carboxamide (MD148/II) on the growth, seed yield and silymarin content of field grown milk thistle (*Silybum marianum* L. Gaertn.) plants. Foliar fertilizer Agroleaf^{R} was applied at different plant developmental stages, with different proportions of N, P, K. Combined application of foliar fertilizer with MD148/II in concentration 1.10^{-3} M affected most positively growth, number of plant lateral shoots and flower heads per plant. Treatments with foliar fertilizer and MD148/II resulted in improvement of whole plant biomass, and seed yield and quality. The increase of seed yield per unit area was a result of the flowering rate improvement and flower head setting per plant. Dry seeds contained more total flavonoids and silymarin compounds. Analysis of the fatty acid composition of milk thistle seed oil showed some increase of the fraction of saturated over the polyunsaturated fatty acids and the content of high molecular fatty acids declined.

Keywords: milk thistle (*Silybum marianum* L. Gaertn.), 5-tert-butyl-*N*-m-tolylpyrazine-2-carboxamide, foliar fertilization, seed yield, silymarin, fatty acids

INTRODUCTION

Silymarin, derived from the seeds of milk thistle plant (*Silybum marianum* L. Gaertn.) has been used widely for the treatment of toxic liver damage (Dewick 1998). The dried seeds contain 1- 4% of silymarin flavonoids. Silymarin primarily consists of an isomeric mixture of six phenolic compounds: silydianin, silychristin, diastereoisomers of silybin (silybin A and B), and diastereoisomers of isosilybin (isosilybin A and B) (Lee et al., 2007). In our previous field experiments (Geneva et al. 2008) it was found that the treatment of milk thistle plants with thidiazuron and foliar fertilizer resulted in higher silymarin content in the seeds. Although the effects of growth regulators on the growth and development of plants are well stated, their applicability as plant secondary metabolite effectors is still unknown. Recently Dolezal et al., (2002, 2007), synthesized and tested biological activity of amides derived from substituted pyrazinecarboxylic acid. Additionaly, Tumova et al, (2005) found that the newly synthesized compound 5-tert-butyl-*N*-m-tolylpyrazine-2-carboxamide (MD148/II, Figure 1) increased flavonolignan production milk thistle suspension culture.

On the basis of the results of the previous studies regarding biological activity of the substituted pyrazine -2- carboxamides in a milk thistle callus culture we carried out experiments aiming to evaluate the combined effects of abiotic elicitor MD 148/II (3-methylphenylamide 5-tert-butylpyrazine-2-carboxylic acid) and foliar fertilization on seed yield, flowering rate, silymarin content and composition of fat in the seeds of milk thistle grown in field experiments.

ESSENTIAL OIL VARIATION OF *SALVIA OFFICINALIS* (L.), GROWN ON HEAVY METALS POLLUTED SOIL

I. Stancheva[1], M. Geneva[1], M. Hristozkova[1], M. Boychinova[1], Y. Markovska
[1]"Acad. M.Popov" Institute of Plant Physiology – Bulgarian Academy of Sciences
[2]Sofia University "St. Kliment Ohridski"Faculty of Biology
Correspondence to: Ira Stancheva
E-mail: ira_stancheva@abv.bg

ABSTRACT

Two months-old sage seedlings were grown during 10 weeks on the soil/sand substrate in the ratio 3:1. The soil was collected from the vicinities of a Non-Ferrous Metals Combine near Plovdiv with pH (H_2O) – 7.35 and the following content of heavy metals (μg g^{-1}DW): Cd - 9.02±0.98, Cu- 82.10±3.69, Pb- 301.75±21.03, Zn- 641.60±35.89. For the control non-polluted leached cinnamonic forest soil (Chromic Luvisols – FAO) was used. Heavy metal pollution with Cd, Zn and Pb resulted in 15% root dry biomass inhibition and 10% shoot dry biomass inhibition. Cd, Pb and Cu were accumulated previously in the sage roots, while Zn was accumulated both in the roots and shoots. In total, 11 constituents of the sage essential oil were identified and quantified. Because of the shoot dry biomass inhibition percentage of the essential oil yield towards dry biomass increased under conditions of heavy metals pollution. The main compounds in the essential oil that decreased as a result of heavy metals pollution are α-thujon, β-thujon β- cariophyllene and viridoflorol, while camphor, borneol, 1,8-cineole and bornylacetate significantly increased. Observed decrease of the levels of α- and β-thujones and elevated camphor level in the leaves of sage grown on heavy metals polluted soil indicated a deterioration of the essential oil quality.

Keywords: essential oil, heavy metals, *Salvia officinalis*

Introduction

Garden sage (*Salvia officinalis* L.) essential oil is applied in the treatment of a large range of diseases such as those of the nervous system, heart and blood circulation and respiratory (8, 9). Heavy metal content in medicinal plants is a subject of great interest, published in many papers (1, 3, 4, 14, 15). Some essential aromatic and medicinal plants are capable to accumulate heavy metals from contaminated soils (17). All .heavy metals, both essential (Cu, Zn) and non-essential (Cd, Pb) can cause toxic effects to plants and humans if found in high concentrations (2). In Bulgaria large areas of agricultural soils in the vicinity of the Non-Ferrous Metals Combines near Plovdiv, Kirdzalli, Pirdop, and Kremikovci are contaminated with heavy metals, resulting in a serious environmental problem. In the field experiments conducted in the vicinities of the Non-Ferrous Metals Combine near Plovdiv Zheljazkov et al. (18) reported about the reduction of essential oil yields of several medicinal plants and distribution of heavy metal accumulation in the plant organs. This study demonstrated that high concentrations of heavy metals in soil did not result in metal transfer into essential oil.

Cleansing the soil of heavy metals via conventional methodologies is expensive and in most cases not feasible. Phytoremediation is low-cost alternative to the traditional remediation technologies. The objectives of our study were to evaluate the effects of heavy metals polluted soil on the growth, essential oil composition and accumulation of Cd, Pb,Cu and Zn in *Salvia officinalis*.

Materials and methods

Garden sage plants were grown starting from seeds under conditions of climatic chamber at 12 h photoperiod, day/night temperature 25/18 °C and photon flux density of 95 μmol m^{-1} s^{-1}. Two months-old seedlings were transferred to the 5 kg plastic pots in glass house (1 plant per pot) and were grown 10 weeks on the soil/sand substrate in the ratio 3:1. Water was added to make up about 60% of water holding capacity. The soil was collected from the vicinities (1 km) of a Non-Ferrous Metals Combine near Plovdiv with pH(H_2O) – 7.35 and the following content of heavy metals (μg/gDW): Cd - 9.02±0.98, Cu- 82.10±3.69, Pb- 301.75±21.03, Zn- 641.60±35.89. Because the Bulgarian permissible limit

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RESEARCH PAPERS

Antioxidant Capacity of Sage Grown on Heavy Metal-Polluted Soil[1]

I. Stancheva[a], M. Geneva[a], M. Hristozkova[a], Y. Markovska[b], and I. Salamon[c]

[a] *Acad. M. Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev str., Bl. 21, 1113 Sofia, Bulgaria; fax: +359-2-873-9952; e-mail: ira_stancheva@abv.bg*

[b] *Sofia University "St. Kliment Ohridski", Faculty of Biology, 8 D. Tzankov Blvd., 1164 Sofia, Bulgaria*

[c] *Presov University, Faculty of Humanities and Natural Science, 17 November str., 080 01, Presov, Slovakia*

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Abstract—Oxidative stress response and essential oil composition of sage (*Salvia officinalis* L.), grown on industrially polluted soil were studied. Sage plants were grown on the soil polluted with Cd, Cu, Pb, Zn, and non-polluted control soil. One-year-old sage possessed a high potential for heavy metal accumulation mainly in the roots. Heavy metal pollution resulted in root and shoot dry biomass inhibition. The increased levels of hydrogen peroxide and MDA showed that the heavy metal uptake caused oxidative stress. The increase towards the control was observed in the levels of glutathione, ascorbate, dehydroascorbate, catalase, dehydroascorbate reductase, and glutathione peroxidase. Weak activities of the most enzymes of the ascorbate–glutathione cycle allowed to suppose that H_2O_2 neutralization is rather non-enzymatic than enzymatic process. Observed decline in α- and β-thujones and elevated camphor content in the sage leaves did not indicate a deterioration of the essential oil quality. Sage grown on heavy metal-polluted soil successfully accumulated cadmium, lead, and zinc, which is resulted in plant biomass inhibition, but essential oil yield and quality was not declined.

Keywords: Salvia officinalis, heavy metals, antioxidants metabolites, antioxidant enzymes, essential oils.

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INTRODUCTION

Medicinal properties of the Lamiacea plants have been used since antique times for their healing properties. Garden sage (*Salvia officinalis* L.) essential oil is applied in the treatment of a large range of diseases, such as those of the nervous system, heart, blood circulation, and respiratory [1].

Some essential aromatic and medicinal plants are capable to accumulate heavy metals from contaminated soils [2]. All heavy metals, both essential (Cu, Zn) and non-essential (Cd, Pb) can cause toxic effects to plants and humans if found in high concentrations. In the field experiments conducted in the vicinities of the Non-Ferrous Metals Combine near Plovdiv. Zheljazkov et al. [2] reported about the reduction of essential oil yields of several medicinal plants and distribution of heavy metal accumulation in the plant organs. This study demonstrated that high concentrations of heavy metals in soil did not result in metal transfer into essential oil.

To combat the metal toxicity of several free radicals, there is a mobilization of the antioxidant reserves in the plant, which react both enzymatically and non-enzymatically with these toxic molecular species, making them less harmful. Non-enzymatic antioxidants include ascorbate (ASC) and glutathione (GSH), and enzymatic antioxidants are superoxide dismutase (SOD), various specific peroxidases, catalase (CAT) and enzymes of the ascorbate–glutathione cycle, as reviewed by Foyer [3]. It is well known that the ascorbate–glutathione cycle plays a vital role in ROS detoxification.

Several reports have indicated the development of oxidative stress under Cd toxicity [4, 5], Cu and Zn toxicity [6], and Pb toxicity [7]. The development of oxidative stress symptoms was judged by the accumulation of H_2O_2 and the oxidative damage to lipids.

In order to improve knowledge regarding the response generated by heavy metal producing oxidative stress, we investigated specific antioxidant protection and essential oil composition of one-year-old

[1] This text was submitted by the authors in English.

Abbreviations: APX—ascorbate peroxidase; ASC—ascorbate; CAT—catalase; DHAR—dehydroascorbate reductase; DHASC—dehydroascorbate; GPO—guaiacol peroxidase; GPX—glutathione peroxidase; GR—glutathione reductase; GSH—reduced gluthatione; GSSG—oxidized gluthatione; GST—glutathione S-transferase; MDHAR—monodehydroascorbate reductase.

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Essential Oil Variation of *Salvia officinalis* Leaves during Vegetation after Treatment with Foliar Fertilizer and Thidiazuron

IRA STANCHEVA,[1] MARIA GENEVA,[1] GEORGI GEORGIEV,[1] MILKA TODOROVA,[2] AND LYUBA EVSTATIEVA[3]

[1]Academecian M. Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Sofia, Bulgaria
[2]Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria
[3]Institute of Botany, Bulgarian Academy of Sciences, Sofia, Bulgaria

The essential oil yield and chemical composition of Salvia officinalis *L. (*Dalmatica *origin) have been analyzed. Leaf samples for essential oil analysis were harvested at different developmental stages after treatment with foliar fertilizer Agroleaf® and foliar fertilizer + thidiazuron. In total, 10 constituents were identified and quantified. The main compounds in the essential oil that increased during the vegetative to the fruiting-set stage are α-thujon and camphor, whereas borneol, viridoflorol, and manool decreased. The effect of thidiazuron applied together with foliar fertilizer was established mainly at the flowering stage, increased essential oil yield by 16% over the control, and positively affected the percentage of β-caryiophylene, α-humulene, viridoflorol, and manool. Application of foliar fertilizer resulted in a greater increase of essential oil yield at the flowering stage in the combined foliar and thidiazuron application over the control. Both treatments decreased camphor at flowering and fruiting stages.*

Keywords Chemical composition, essential oil, *Salvia officinalis* L., thidiazuron

Introduction

Garden sage (*Salvia officinalis* L.) of the family *Lamiaceae* is well known as a common medicinal and aromatic plant and is widely used in food and herbal products (Hohmann et al. 2003). *Salvia officinalis* essential oil is applied in the treatment of a large range of diseases, such as those of the nervous system, heart and blood circulation, and respiratory system (Duke 2001). Good-quality sage oil contains a high percentage (>50%) of the α- and β-thujones and low proportion (<20%) of camphor (Putievsky, Ravid, and Sanderovich 1992). It seemed likely that different developmental stages of *S. officinalis* would have different oil compositions because Mirjalili et al. (2006) reported the essential oil variation of sage aerial parts during its phenological

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Address correspondence to Ira Stancheva, Acad. M. Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev, block 21, Sofia 1113, Bulgaria. E-mail: ira_stancheva@abv.bg

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INFLUENCE OF FOLIAR FERTILIZATION AND GROWTH REGULATOR ON MILK THISTLE SEED YIELD AND QUALITY

Ira Stancheva,[1] Georgi Georgiev,[1] Maria Geneva,[1] Albena Ivanova,[1] Martin Dolezal,[2] and Lenka Tumova[2]

[1]*Academy M. Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Sofia, Bulgaria*
[2]*Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, Hradec Kralove, Czech Republic*

□ *The effects of foliar fertilization and a growth regulator 5-tert-butyl-N-m-tolylpyrazine-2-carboxamide (MD148/II) on the growth, seed yield, and silymarin content of milk thistle (*Silybum marianum *Gaertn.) plants were evaluated. The study was conducted over two years at an experimental field on a slightly acid-leached cinnamonic meadow soil. The MD148/II was applied in the beginning of milk thistle flowering stage. Foliar fertilizer was applied at different plant developmental stages with different proportions of nitrogen (N), phosphorus (P), and potassium (K). Treatments with foliar fertilizer and MD148/II resulted in improvement of plant biomass, number of plant lateral shoots, flowering rate, and seed yield and the content of some active substances in milk thistle seeds. A reduction of high molecular fatty acids was observed. The increase of seed yield was a result of the flower head setting enhancement. Therefore the combined treatment of foliar fertilizer and MD148/II was efficient in elicitation milk thistle production under field conditions.*

Keywords: 5-tert-butyl-*N*-m-tolylpyrazine-2-carboxamide, silymarin, fatty acids

INTRODUCTION

Silymarin, derived from the seeds of milk thistle plant (*Silybum marianum* Gaertn.) has been used widely for the treatment of toxic liver damage (Dewick, 1997). The dried seeds contain 1% to 4% of silymarin flavonoids. Silymarin consist primarily of an isomeric mixture of six phenolic compounds: silydianin, silychristin, diastereoisomers of silybin (silybin A and B), and diastereoisomers of isosilybin (isosilybin A and B) (Guz and Stermitz, 2000; Lee et al., 2007). In field experiments Geneva at al. (2008) reported

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Address correspondence to Ira Stancheva, Academy M. Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev str., Bl.21, 1113 Sofia, Bulgaria. E-mail: ira_stancheva@abv.bg

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Research Article

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Effects of foliar fertilization and arbuscular mycorrhizal colonization on *Salvia officinalis* L. growth, antioxidant capacity, and essential oil composition

Maria P Geneva,* Ira V Stancheva, Madlen M Boychinova, Nadezhda H Mincheva and Petranka A Yonova

Abstract

BACKGROUND: The effect of foliar fertilization and *Glomus intraradices* inoculation on the growth, qualitative and quantitative pattern of essential oil in *Salvia officinalis* was determined. Sage plants were grown in a glass house on a soil/sand mixture (w/w = 3 : 1). Agroleaf® total, N : P : K = 20 : 20 : 20 + microelements, was used at the whole vegetative growth stage as a 0.3% solution. Inoculation with *Glomus intraradices* was done at the sowing stage.

RESULTS: Application of foliar fertilization and/or mycorrhizal colonization improved dry biomass accumulation and increased the content of antioxidant metabolites (ascorbate and reduced glutathione). Applied treatments lowered the activities of the antioxidants enzymes catalase, ascorbate peroxidase and superoxide dismutase, while guaiacol peroxidase increased. The relative quantity of essential oil pattern was also altered as a result of the applied treatments. Combined application (FF+Gi) significantly promoted 1,8-cineole and α-thujone, mycorrhizal colonization enhanced bornyl acetate, 1,8-cineole, α- and β-thujones, while foliar fertilization increased bornyl acetate and camphor. The favorable effect of root colonization by *Glomus intraradices* was determined both on quantitative and qualitative pattern of sage essential oil.

CONCLUSION: We conclude that inoculation with *Glomus intraradices* resulted in improved essential oil yield and quality, while combined application of foliar fertilizer and mycorrhizal fungi predominantly enhanced shoot biomass accumulation.

Keywords: sage (*Salvia officinalis* L.); foliar fertilization; *Glomus intraradices*; antioxidant capacity; essential oil

INTRODUCTION

Essential oils of the plants belonging to the Lamiaceae family possess high biological activities and show antioxidant, antibacterial, antimicrobial and anti-inflammatory activities.[1,2] It is generally accepted that the medicinal properties of this family are due to the secondary metabolites as total phenols (including flavonoids and phenylpropanoids) as well as anthocyanins. There is increasing interest in the use of antioxidants in the food, pharmaceutical, and cosmetic industries. Usage of natural antioxidants as food preservatives is a very appropriate policy for many countries. Among these natural antioxidants, ascorbate, glutathione and vitamin E (tocopherols and tocotrienols) are of significant importance and have been extracted from a number of medicinal plant species.[3] Antioxidants and antioxidant enzymes function to interrupt the cascades of uncontrolled oxidation.[4]

Many species belonging to the Lamiaceae form arbuscular mycorrhizal (AM) associations. In addition to increasing uptake of poorly accessible nutrients[5] or conferring protection against pathogens,[6] AM fungi can also induce changes in the accumulation of secondary metabolites in host plant roots.[7] Relatively little is known about the effects of AM colonization on the accumulation of active compounds in shoots of medicinal plants, which are often the harvest products. However, it was recently reported that *Glomus mosseae* directly increases the essential oil content in shoots of different species of Lamiaceae medicinal plants.[8,9] Endomycorrhizal colonization of *Salvia officinalis* roots can provide an efficient and natural way of improving the growth of these medicinal herbs and increase essential oil yield. To the best of our knowledge there are no reports on variations of the antioxidant capacity and essential oil composition as a result of the foliar feeding with fertilizers containing balanced nutrients.

The aim of the present study was to establish the effects of foliar fertilization and AM fungus (*Glomus intraradices*) inoculation applied separately and in combination on *Salvia officinalis* growth, essential oil composition, level of secondary metabolites and antioxidant capacity.

* *Correspondence to: Maria P Geneva, Acad. M Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Block 21, Sofia 1113, Bulgaria. E-mail: boykova2@yahoo.com*

Acad. M Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria

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Antioxidant Potential of Marigold (*Calendula officinalis* Linn.) Flowers Grown in Slovakia and Bulgaria

Andrea Plackova[1] • Maria Geneva[2] • ~~Maria Geneva~~[3] • Ivan Salamon[1] • ~~Ivan Salamon~~[2*]

[1] Presov University, Faculty of Humanities and Natural Science, 17 November str., 080 01, Presov, Slovakia
[2] Acad. M. Popov" Institute of Plant Physiology – Bulgarian Academy of Sciences, Acad. G. Bonchev str., Bl. 21,1113 Sofia, Bulgaria
[3] Sofia University "St. Kliment Ohridski", Faculty of Biology, 8 D. Tzankov Blvd., 1164 Sofia, Bulgaria

Corresponding author: * ira_stancheva@abv.bg

ABSTRACT

Marigold plants (*Calendula officinalis* Linn) var. 'Plamen Plus' were grown under field conditions during the 2009 season in Trebišov, Slovakia and in Sofia, Bulgaria. The antioxidant potential in Slovak marigold flowers was due to the content of glutathione and ascorbate, two peroxidases (glutathione peroxidase and guaiacol peroxidase) and monodehydroascorbate reductase. The antioxidant capacity in Bulgarian marigold flowers was determined by higher levels of flavonoids, catalase, ascorbate peroxidase, glutathione-*S*-tansferase and dehydroascorbate reductase. Marigold grown in Slovakia had a higher dry biomass of flower heads with a higher carotenoid content. Different soil-climatic conditions mobilize different compounds of the antioxidant defense system, and accumulate biomass and carotenoids in marigold plants grown in both countries.

Keywords: antioxidants, carotenoids, enzymes, marigold (*Calendula officinalis* Linn.), secondary metabolites
Abbreviations: **APX**, ascorbate peroxidase; **ASC**, ascorbate; **CAT**, catalase; **DHAR**, dehydroascorbate reductase; **DHASC**, dehydroascorbate; **GPO**, guaiacol peroxidase; **GPX**, glutathione peroxidase; **GR**, glutathione reductase; **GSH**, reduced gluthatione; **GST**, glutathione-*S*-transferase; **MDHAR**, monodehydroascorbate reductase; **MDA**, malondialdehyde; **ROS**, reactive oxygen species

INTRODUCTION

The species *Calendula officinalis* or pot marigold has been cultivated for many years as an attractive garden plant (Duke *et al.* 2002). This plant has a long history of medicinal use; it is mentioned in many ancient herbals for use in the treatment of headaches, toothache, swellings and for strengthening the heart. Marigold petals are used to make eyewash. This medicinal plant species is also used in both homeopathic and conventional medicine as a healing ointment for cuts and grazes. Marigold has anti-septic, stimulant and anti-fungal properties (Muley *et al.* 2009). Plants produced bioactive compounds with several uses. These include the essential oils and pigments from its flowers, especially carotenoids. Carotenoids are known as biologically active compounds with multiple applications in therapy. The very important attribute of these natural components is their antioxidative activity, which protects organisms against reactive oxygenic radicals. Compounds that might contribute to the total lipid antioxidant capacity include carotenoids, flavonoids and cinnamic aid derivates (Wu *et al.* 1984). Vitamin E (tocopherol) is a major contributor to overall lipid-soluble extracts (Schwants *et al.* 1996). Antioxidant potential is a mobilization of the antioxidant reserves in the plant that reacts both enzymatically and non-enzymatically with these toxic free radicals making them less harmful. Non-enzymatic antioxidants include ascorbate (ASC) and glutathione (GSH) and enzymatic antioxidants are superoxide dismutase (SOD), different specific peroxidases, catalase (CAT) and enzymes of ascorbate-glutathione cycle, as reviewed by Ahmad *et al.* (2008). It is well known that ascorbate-glutathione cycle plays a vital role in the detoxification of reactive oxygen species (ROS). The development of oxidative stress symptoms is judged by the accumulation of H_2O_2 and the oxidative damage to lipids. There is an increasing interest in the use and measurement of antioxidants in the food, pharmaceutical and cosmetic industries.

The aim of the present study was to evaluate carotenoid contents, non-enzymatic antioxidants and antioxidant enzymes in flowers of marigold grown in Bulgaria and Slovakia.

MATERIALS AND METHODS

Plant material and soil-climatic conditions

Field experiments were carried out in Slovakia (Trebišov) and Bulgaria (Sofia). Trebišov is an area of the Easter Slovakian Lowland. The altitude is about 107 m above sea level, a warm and dry climatic region during summer and colder winter. The average annual temperature ranges from 9 to 10°C and annual precipitation is 550–600 mm. The experimental site is situated on private plots with neutral soil (pH = 7.1–7.2), which has a higher content of phosphorus (320 mg kg^{-1}), magnesium (730 mg kg^{-1}) and potassium (285 mg kg^{-1}) (Kobza *et al.* 1999). The relief is formed by an undulating plain on fluvio-eolian substrate.

The study in Bulgaria was conducted at an experimental field, on a leached cinnamonic meadow soil (Chromic Luvisols, according to FAO - Unesco-Isric legend). The experimental field of the Institute of Plant Physiology is situated in a mild and rainy climatic region of sub-mountain areas in West-Central Bulgaria. The altitude is about 564 m above sea level. The average annual temperature ranges from 8 to 10°C and annual precipitation is 224–304 mm. The slightly acid soil is characterized by the following agrochemical characteristics: pH (H_2O) = 6.2, 8 mg kg^{-1} soil total mobile nitrogen ($N\text{-}NO_3^-$ + $N\text{-}NH_4^+$), 30 mg kg^{-1} soil P_2O_5, 120 mg kg^{-1}soil K_2O and 1.88% of organic matter (Geneva *et al.* 2006).

Marigold seeds were sown in lines with an inter-row spacing of 0.5 m. Flower heads of var. 'Plamen Plus' were picked during the 2009 season in Trebišov (Slovakia) and in Sofia (Bulgaria).

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Original Research Paper

Metal uptake by Saint John's wort (*Hypericum perforatum* l.) grown on industrially polluted soil

M. Geneva[1*], Yu. Markovska[2], I. Stancheva[1]

[1]Department of Plant Mineral Nutritional and water relations, "Acad. M.Popov" Institute of Plant Physiology, Bulgarian Academy of Sciences, Sofia, Bulgaria
[2]Sofia University "St. Kliment Ohridski", Faculty of Biology, Sofia, Bulgaria

*Corresponding author. Tel: +359 2 979 26 89; fax: +
E-mail address: boykova2@yahoo.com

Abstract

Possibilities for phytoaccumulation of heavy metals, antioxidant capacity, essential oil quality and quantity of Saint John's worth (*Hypericum perforatum* L.), grown on industrially polluted with Cd, Pb and Zn soil were studied. Content of Cd, Pb Zn in the polluted soil exceeded permissible concentrations 3, 4 and 2 times respectively. Saint John's worth plants were grown under glasshouse conditions on polluted and non-polluted control soils. Saint John's worth possessed high potential for phytoaccumulation of Cd and Pb, because its contents in the shoots were 66 times and 11.9 times more than the in the control respectively. Soil pollution with Cd, Pb and Zn did not cause oxidative stress response in the plants because the main antioxidants (ascorbate, gluthatione, vitamin E and hypericin) did not increase. Essential oil yield and quality of *Hypericum perforatum* L. was not influenced by the increased content of Cd, Pb and Zn in the soil.

Key words: Hypericum perforatum, heavy metals, antioxidants capacity, essential oils

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ORIGINAL ARTICLE

Accumulation of Cd, Pb and Zn in *Tribulus terrestris* L. Grown on Industrially Polluted Soil and Plant Antioxidant Response

[1]I. Stancheva, [1]M.Geneva, [1]P.Yonova and [2]Yu. Markovska

[1]*"Acad. M.Popov" Institute of Plant Physiology – Bulgarian Academy of Sciences, Acad. G. Bonchev str., Bl. 21,1113 Sofia, Bulgaria, fax: +359 2 873 99 52*
[2]*Sofia University "St. Kliment Ohridski", Faculty of Biology, 8 D. Tzankov Blvd., 1164 Sofia, Bulgaria*

I. Stancheva, M.Geneva, P.Yonova and Yu. Markovska: Accumulation of Cd, Pb and Zn in *Tribulus terrestris* L. Grown on Industrially Polluted Soil and Plant Antioxidant Response

ABSTRACT

Possibilities for phytoaccumulation of heavy metals and antioxidant capacity of puncture vine, grown on industrially polluted with Cd, Pb and Zn soil were studied. Content of Cd, Pb and Zn in the polluted soil exceeded permissible concentrations 3, 4 and 2 times respectively. Puncture vine plants (*Tribulus terrestris* L.), were grown under glasshouse conditions on polluted and non-polluted control soil. Plants grown on heavy metal polluted soil accumulated in the aboveground parts 3.3 times more Cd, 4.3 times more Pb and 2.3 times more Zn, in comparison with the control plants. Heavy metals concentration in plant and soil samples were determined on the inductively – coupled Plasma Mass Spectrometer. Spectrophotometric quantification of ascorbate, reduced glutathione and vitamin E was performed through the formation of phosphomolybdenum complex. Total antioxidant capacity (free radicals scavenging activity) was measured from the bleaching of the purple-colored methanol solution of free stable radical (diphenylpycril-hydrazyl, DPPH˙) inhibition. All antioxidant enzymes (ascorbate peroxidase, catalase, dehydroascorbate reductase, guaiacol peroxidase, glutathione peroxidase, glutathione reductase, glutathione-*S*-transferase and monodehydroascorbate reductase) were assayed spectrophotometrically. Puncture vine plants possess good ability to accumulate heavy metals. Plants grown on heavy metal polluted soil accumulated heavy metals in both the shoots and roots. Cd and Pb accumulated more in the roots than in the shoots of plants both from the non - polluted and polluted soil. The observed levels of main contaminates in aboveground parts were 3.3 times more Cd, 4.3 times more Pb and 2.3 times more Zn, in comparison with the control plants. Heavy metals content in the roots of treated plants was 2.5, 2.8 and 1.4 times more than in the controls for Cd, Pb and Zn respectively. The levels of heavy metals accumulation in aboveground parts allowed supposing that *Tribulus terrestris* is a plant that could be used for phytoremediation, more over that higher Cd and Zn levels were found in the plants than in the soil. Absence of biomass reduction indicated that puncture vine plants tolerate the existing concentration level of Cd, Pb and Zn. The antioxidant potential of the puncture vine plants is defined by the content of antioxidant metabolites vitamin E, ascorbate, glutathione and total phenols and antioxidant enzyme activities of glutathione peroxidase, glutathione reductase and dehydroascorbate reductase. From the results we can conclude that soil Cd, Pb and Zn in concentrations far exceeded permissible limit concentrations influenced only a part of antioxidant capacity of *Tribulus terrestris* plants.

Key words: *Tribulus terrestris* L. - heavy metals - antioxidants metabolites – antioxidant enzymes

Corresponding Author

I. Stancheva, Acad. M.Popov" Institute of Plant Physiology – Bulgarian Academy of Sciences, Acad. G. Bonchev str., Bl. 21,1113 Sofia, Bulgaria, fax: +359 2 873 99 52;
E-mail: ira_stancheva@abv.bg

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SPECIAL ISSUE (PART II) – PROCEEDINGS OF THE XI NATIONAL CONFERENCE ON PLANT PHYSIOLOGY
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EFFECT OF ENDOMYCORRHIZAL COLONIZATION WITH *GLOMUS INTRARADICES* ON GROWTH AND ANTIOXIDANT CAPACITY OF *SIDERITIS SCARDICA* GRISEB

Geneva M.*, M. Hristozkova, P. Yonova, M. Boychinova, I. Stancheva

Acad. M. Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Street, Bldg. 21, 1113 Sofia, Bulgaria

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Summary. The effect of *Glomus intraradices* inoculation on the growth and antioxidant activity of mountain tea (*Sideritis scardica* Griseb.) was determined. Two-months-old mountain tea seedlings were grown during 10 weeks on the soil/sand (w/w=3:1) substrate in a glass house. Mycorrhizal colonization improved shoot and root dry biomass accumulation and increased total phenols and flavonoid content. During the period of vegetative growth the level of antioxidant metabolites (ascorbate acid and reduced glutathione) and the antioxidant enzymes guaiacol peroxidase and catalase decreased as a result of the mycorrhizal colonization. A favorable effect of root colonization with G*lomus intraradices* was observed regarding the levels of ascorbate peroxidase and super oxide dismutase. We conclude that inoculation of *Sideritis scardica* Griseb. with *Glomus intraradices* resulted in enhanced plant dry biomass accumulation, but the antioxidant defense was not efficient enough during the period of vegetative growth.

Key words: Mountain tea (*Sideritis scardica* Griseb); *Glomus intraradices;* antioxidant capacity.

INTRODUCTION

Sideritis scardica belonging to the *Lamiaceae*, also known as mountain tea, is a perennial herb that grows in the mountainous regions between Southern Europe and Eastern Mediterranean. These plants are known to have antispasmodic, anti-feedant, carminative, analgesic, nervous system stimulant, sedative, antitussive, stomachic, anticonvulsant, antibacterial, antiinflammatory, antimicrobial and antioxidant activities (Ozturk et al., 1996; Navarro et al., 2001; Ozkan at al., 2005). Studies on the biological and ecological properties of four populations *S. scardica*, factors determining the optimal plant development and the growth of the species under cultivation

*Corresponding author: boykova2@yahoo.com

RESPONSE OF *TRIBULUS TERRESTRIS* L. TO ELEVATED LEVELS OF HEAVY METALS

M. Geneva[1*], Yu. Markovska[2], M. Sichanova[1], I. Stancheva[1]

[1]*Institute of Plant Physiology and Genetics – Bulgarian Academy of Sciences, Acad. G. Bonchev str., Bl. 21,1113 Sofia, Bulgaria, fax: +359 2 873 99 52; *E-mail: boykova2@yahoo.com*

[2]*Sofia University "St. Kliment Ohridski", Faculty of Biology, 8 D. Tzankov Blvd., 1164 Sofia, Bulgaria*

Abstract

Response to heavy metals and possibilities for their phytoaccumulation in puncture vine, grown on industrially polluted with Cd and Pb soil were studied. Content of Cd and Pb in the polluted soil exceeded permissible concentrations 4.6 and 2 times respectively. *Tribulus terrestris* L plants were grown under field conditions on two (control and polluted) experimental fields. The polluted field is near waste depository of ferrous metallurgical combine "Kremikovci".

Elevated soil levels of Cd and Pb resulted in reduction of biomass accumulation, number of lateral stems and number of fruits of puncture vine plants. In case of plant growth on heavy metal industrially polluted soil higher levels of main contaminates in aboveground parts were observed – 3.0 times more Cd, 4.4 times more Pb and 2.2 times more Zn, in comparison with the control plants. *Tribulus terrestris* showed good ability for Cd, Pb and Zn accumulation. The values of bioaccumulation factor of puncture vine, concerning heavy metals accumulation in the plant tissues, allowed supposing that *Tribulus terrestris* is a plant that could be used for phytoremediation of soils polluted with Cd and Zn.

Key words: *Tribulus terrestris* L., phytoremediation, heavy metals, bioaccumulation factor.

Introduction

The contamination of heavy metals to the environment, i.e., soil, water, plant and air is of great concern due to its potential impact on human and animal health. Cheaper and effective technologies are needed to protect the precious natural resources and biological lives. It has already been mentioned that metal accumulation by plants is the fundamental feature for phytoremediation. Phytoremediation (phytoextractoin) is a low-cost alternative to the traditional remediation technologies.

Recently, for classification of plants as accumulators or hyperaccumulators is used bioaccumulation factor (BF) (Ma et al., 2001; Masarovičova et al., 2010). The term BF, defined as the ratio of metal concentration in plant dry mass (μg gDW^{-1}) to those in soils (μg gDW^{-1}), has been used to determine the effectiveness of plants in removing metals from soils (Tu and Ma, 2002). In general, it is accepted that for excluders, accumulators and hyperaccumulators the BF values are as follows: <1, >1, >10 (Zhao et al., 2003; Wei and Chen, 2006).

Aromatic plants, used for production of essential oil as opposed to food or feed, may be suitable alternative crops in heavy metal contaminated agricultural soils. It was established that some medicinal plants extract and accumulate heavy metals from soil and do not change the quantity and quality of essential oils into the drug (Zheljazkov et al., 2008). Therefore they can be used both for phytoremediation and for essential oil producer for pharmaceutical and cosmetic industry needs. *Tribulus terrestris* commonly known as puncture vine belongs to the Zygophyllaceae family and is widely used in folk medicine (Ody, 2000). The main constituents of *Tribulus terrestris* are saponins (furostanol, spirostanol), diosgenins, alkaloids and amides (Wang et al, 1997; Cai et al., 2001). The saponins fraction of this plant finds application in the contemporary medicine as a component of drugs effective in treatment impotence (Tribestan in Bulgaria and Libillov in USA).

Application of foliar fertilizers on plants usually results in fast accumulation of nutrients in leaves, although in some cases they can influence nutrient mobilization to roots as well (Wojcik, 2004). According to Stancheva et al. (2010) sage grown on heavy metals polluted soil successfully accumulated cadmium, lead and zinc, which is resulted in plant biomass inhibition but essential oil yield and quality did not decline.

The objectives of the present study were to investigate the ability of the accumulation of heavy metals in *Tribulus terrestris* plants growing in industrially polluted soil.

ACCUMULATION OF Cd, Pb AND Zn IN *MATRICARIA RECUTITA* L. GROWN ON INDUSTRIALLY POLLUTED SOIL

I. Stancheva, M. Geneva, M. Boychinova, P. Yonova

Institute of Plant Physiology and Genetics – Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 21, 1113 Sofia, Bulgaria, fax: +359 2 873 99 52

E-mail: ira_stancheva@abv.bg

Abstract

This study evaluates the extent of accumulation of Cd, Pb and Zn in *Matricaria recutita* and the effect of heavy metals uptake on the plant biomass. Content of Cd and Pb in the polluted soil exceeded permissible concentrations 4.6 and 2 times respectively. *Matricaria recutita* L plants were grown under field conditions on two (control and polluted) experimental fields. The polluted field is near waste depository of ferrous metallurgical combine "Kremikovci".

The content of heavy metals in soil decreased after plant harvest in comparison with their initial levels which indicates their accumulation in the plant tissues. The presence of high levels of heavy metals in the soil resulted in retained plant growth. In *Matricaria recutita* grown on industrially polluted soil a decrease of root and shoot dry biomass and reduction of number of lateral steams was observed. Otherwise the number and biomass of flowers per plant increase. Chamomile possesses good ability to accumulate heavy metals. The levels of bioaccumulation factor determine *Matricaria recutita* as accumulator for Cd, Cu and Zn. Therefore Matricaria recutita can be used for cleaning up the heavy metal contaminated soils.

Key words: *Matricaria recutita* L., phytoremediation, heavy metals, bioaccumulation factor.

Introduction

Chamomile (*Matricaria recutita* L.) is one of the most important and most frequently cultivated medicinal herbs all over the world. Chamomile anthodia (*Matricaria flos* drug) contain a range of pharmacologically effective secondary metabolites with anti-inflamatory and spasmolitic action. The unique medicinal effect of chamomile results from combined action of all inherent substances: sesquiterpenes: [(-)-α-bisabolol, matricin chamazulene], flavonoids (apigenin glucosides), coumarins (herniarin and umbelliferone), mucilages etc. (Schilcher 1987).

Some medicinal plants are capable to accumulate heavy metals from contaminated soils (Zheljazkov *et al.*, 2008). Accumulation and hyper-accumulation of heavy metals is very important assumption for clearing up of contaminated substrates by plants – phytoremediation. Kovačik et al. (2006) reported that metabolism of chamomile was altered slightly under high Cd stress, indicating that chamomile is tolerant to this metal, but plant species could not be classified as a hyperaccumulator.

However, detailed physiological studies on the combaine impact of heavy metals on this medicinal plant are not available. The main aim of our study was to evaluate the extent of accumulation of Cd, Pb and Zn in *Matricaria recutita* L. and the effect of heavy metals uptake on the plant growth. Heavy metal accumulation in the leaves, flowers and roots was determined in order to indicate whether this medicinal plant is heavy metal accumulator or metal excluder.

Materials and methods

Matricaria recutita L plants were grown under field conditions on two (control and polluted) experimental fields near Sofia. Control field with non-polluted leached cinnamonic forest soil (Chromic Luvisols – FAO) possess the following agrochemical characteristics: $pH(H_2O) = 6.2$, 8 mg kg^{-1} soil total mobile nitrogen ($N\text{-}NO_3^- + N\text{-}NH_4^+$), 30 mg kg^{-1} soil P_2O_5, 120 mg kg^{-1}soil K_2O. The following content of studied heavy metals (μg g^{-1}DW) was measured: Cd – 0.25, Cu – 22.83, Pb – 16.00, Zn – 46.03.

The polluted field near waste depository of ferrous metallurgical combine "Kremikovci" with leached cinnamonic forest soil have the following agrochemical characteristics: $pH(H_2O) = 7.94$, 10 mg kg^{-1} soil total mobile nitrogen ($N\text{-}NO_3^- + N\text{-}NH_4^+$), 36.8 mg kg^{-1} soil P_2O_5, 308 mg kg^{-1}soil K_2O. The following content of studied heavy metals (μg g^{-1}DW) was measured: Cd – 14 Cu -27, Pb – 142 and Zn 207. Because the Bulgarian permissible limit concentrations (PLC) at $pH(H_2O)$ – 7.94 are Cd – 3.0, Cu < 260, Pb < 80 and Zn < 340 μg g^{-1}DW the soils are heavily polluted with Cd, and Pb. The content of Cd and Pb exceeded PLC 4.6 and 1.8 times respectively. All treatments were arranged in randomized complete block design with four replications. The experimental plot area was 6 m^2 with density 350 plants m^{-2}.

Fertilization: Two formulations of foliar fertilizers (Agroleaf™, Scotts Company, Wooster, Ohio, USA) were applied at different developmental stages: (1) Agroleaf® total – N:P:K=20:20:20 + microelements (N –

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1113 СОФИЯ, ИНСТИТУТ ПО
ФИЗИОЛОГИЯ НА РАСТЕНИЯТА
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(54) МЕТОД ЗА РЕГУЛИРАНЕ ДОБИВА И КАЧЕСТВОТО НА СЕМЕНА ОТНОСНО СЪДЪРЖАНИЕТО НА СИЛИМАРИН И НЕНАСИТЕНИ МАСТНИ КИСЕЛИНИ ПРИ КУЛТИВИРАНО ОТГЛЕЖДАНЕ НА МЕДИЦИНСКОТО РАСТЕНИЕ БЯЛ ТРЪН SILYBUM MARIANUM L.

(57) Изобретението се отнася до метод за регулиране на растежа, развитието и добива от семена и тяхното качество при отглеждане в полски условия на медицинското растение бял трън Silybum marianum L. Като лекарствено растение белият трън се използва заради семената си, в които се съдържат известни количества от биологично активни съединения от групата на флаванолигнаните, известни с общото название силимарин. Силимаринът е група от съединения с близък химичен строеж - силибин, изосилибин, силидианин и силикристин и техни изомери. Извлечен от семената, силимаринът се използва широко във фармацевтичната промишленост за получаване на лекарства със силни антиоксидантни свойства, използвани за лечение на увреждания на черния дроб. Предлага се метод за регулиране на добива от семена и тяхното качество при растението бял трън с помощта на оптимизирано листно минерално хранене и третиране с растежен регулатор. Комбинираното третиране на растенията с цитокининовия растежен регулатор тидиазурон през фаза розетка и листно подхранване с течен тор през вегетацията при различно съотношение на минералните елементи в него води до подобряване цъфтежната динамика, залагане на повече цветоносни стебла с по-големи цветни кошнички, които съдържат по-едри семена. Семената съдържат повече силимарин и ненаситени мастни киселини в липидната фракция след узряване.

2 претенции, 3 фигури

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Antioxidant activity of in vitro propagated *Stevia rebaudiana* Bertoni plants of different origins

Ely ZAYOVA, Ira STANCHEVA, Maria GENEVA*, Maria PETROVA, Lyudmila DIMITROVA
Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria

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Abstract: An efficient in vitro protocol for propagation of *Stevia rebaudiana* Bertoni is described. Multiple shoots were induced in vitro from shoot tip and nodal segments on Murashige and Skoog medium containing 6-benzylaminopurine, zeatin, or thidiazuron alone and in combination with naphthalene acetic acid or indole-3-acetic acid. A high frequency of shoot induction as well as maximum number of shoots per shoot tip explant was observed on Murashige and Skoog medium supplemented with 6-benzylaminopurine (1.0 mg L^{-1}) alone and combined with indole-3-acetic acid (0.1 mg L^{-1}). For root induction, in vitro shoots were transferred to rooting media containing naphthalene acetic acid, indole-3-acetic acid, or indole-3-butyric acid. The highest rooting frequency and the highest number of roots was observed in half-strength Murashige and Skoog medium supplemented with 0.1 mg L^{-1} indole-3-butyric acid. The rooted in vitro plants were successfully acclimatized in a growth chamber and transferred to the field. Leaf extracts of plants propagated in vitro and adapted to field conditions are characterized by high levels of water-soluble antioxidant capacity (expressed as equivalents of ascorbic acid), phenols, and flavonoids, and therefore by high total antioxidant potential, expressed as DPPH radical scavenging activity.

Key words: Acclimatization, micropropagation, nodal segments, shoot tips

1. Introduction

Stevia rebaudiana Bertoni, belonging to the family *Asteraceae*, is a perennial sweet herb. It is a native medicinal plant of Paraguay and is a new alternative source of calorie-free sweetener having no carbohydrates. The leaves of this plant produce diterpene glycosides (stevioside and rebaudiosides). Pure stevioside is 30 times sweeter than sugar (1–4). Recently, food-derived antioxidants, such as vitamins and phenolic phytochemicals, have received growing attention because they are known to function as chemopreventive agents against oxidative damage (5). The dry extract from the leaves also contains flavonoids, alkaloids, water-soluble chlorophylls and xanthophylls, hydroxycinnamic acids (caffeic, chlorogenic, etc.), neutral water-soluble oligosaccharides, free sugars, amino acids, lipids, essential oils, and trace elements (6). Plants constitute an important source of active natural products, which differ widely in terms of structure, biological properties, and ways of propagation. Therefore, it is of great interest to evaluate the nonenzymatic antioxidants and the water-soluble and lipid-soluble antioxidant capacities (expressed as equivalents of ascorbate and α-tocopherol), total phenolic compounds, flavonoids, and free radical scavenging activity of *Stevia rebaudiana* Bertoni propagated in different ways. Although phenolic compounds do not have any nutritional function, they may be important to human health because of their antioxidant potential (7). Therefore, the study of the importance and role of nonnutrient compounds, particularly phenolic acids, flavonoids, and high molecular tannins, as natural antioxidants has greatly increased (8). Natural antioxidants such as α-tocopherol and ascorbic acid are widely used because of their free radical scavenging activity (9). The leaf extract of the stevia plant has been used in the treatment of diabetes (10). It also enhances weight reduction, prevents dental caries, and has antimicrobial properties. It is reported that *S. rebaudiana* Bertoni also contains an antioxidant, steviol (11,12).

This species can be propagated by seed, by vegetative cutting, and by tissue culture. Seed germination is very poor, commonly due to infertile seed (13). Vegetative propagation by stem cutting is limited and requires enough stocks of stem cuttings (14,15). Thus, the development of an efficient alternative method for mass micropropagation of *S. rebaudiana* Bertoni is important for large-scale plant production. A number of protocols for in vitro propagation of this species have been described during recent years (16–23). According to Cenkci et al. (24), both

* Correspondence: boykova2@yahoo.com

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Morphological evaluation and antioxidant activity of *in vitro*- and *in vivo*-derived *E. purpurea* plants

Research Article

Ely Zayova, Ira Stancheva*, Maria Geneva, Maria Petrova, Rumiana Vasilevska-Ivanova

Institute of Plant Physiology and Genetics, BAS, Bulgaria

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Abstract: An effective *in vitro* protocol for rapid clonal propagation of Echinacea purpurea (L.) Moench through tissue culture was described. The in vitro propagation procedure consisted of four stages: 1) an initial stage - obtaining seedlings on Murashige and Skoog (MS) basal medium with 0.1 mg L^{-1} 6-benzylaminopurine, 0.1 mg L^{-1} α-naphthalene acetic acid and 0.2 mg L^{-1} gibberellic acid; 2) a propagation stage - shoot formation on MS medium supplemented with 1 mg L-1 6-benzylaminopurine alone resulted in 9.8 shoots per explant and in combination with 0.1 mg L^{-1} α-naphthalene acetic acid resulted in 16.2 shoots per explant; 3) rooting stage - shoot rooting on half strength MS medium with 0.1 mg L^{-1} indole-3-butyric acid resulted in 90% rooted microplants; 4) *ex vitro* acclimatization of plants. The mix of peat and perlite was the most suitable planting substrate for hardening and ensured high survival frequency of propagated plants. Significant higher levels were observed regarding water-soluble and lipid-soluble antioxidant capacities (expressed as equivalents of ascorbate and α-tocopherol) and total phenols content in extracts of *Echinaceae* flowers derived from *in vitro* propagated plants and adapted to field conditions in comparison with traditionally cultivated plants.

Keywords: *Purple coneflower • In vitro shoots • Seedlings • Morphological traits • Antioxidant activity*

Abbreviations

MS - Murashige and Skoog medium,
PGR - plant growth regulator,
BAP - 6-benzylaminopurine,
GA_3 - gibberellic acid,
AA - ascorbic acid,
IBA - indole-3-butyric acid,
NAA - α-naphthalene acetic acid.

1. Introduction

Echinacea purpurea L. Moench (Asteraceae) or purple coneflower is a widespread medicinal plant used in diverse range of herbal products. It was proved that Echinacea is one of the most promising immune strengtheners and modulators, with numerous scientific studies and rich clinical evidence in its favor [1-3]. The increasing demand in *E. purpurea* needed development of methods for rapid multiplication of plants and faster introduction of new cultivars with desired traits [4]. However, *E. purpurea* plants produced highly heterozygous progeny in the field [5]. In this regard, *in vitro* tissue cultures are proved to be valuable technique to produce genetically homogeneous plant material. There were identified 58 unique germplasm lines based on screening for antioxidant activity and concentrations of caftaric acid, chlorogenic acid, cichoric acid, cynarin, and echinacoside from clonal propagated seedling-derived plants [6].

Several *in vitro* techniques were developed in *E. purpurea* [3,4,7-9] as some of the genotypes showed high coefficient of *in vitro* propagation [8,10,11]. The application of biotechnological techniques might offer the possibility of producing large amount of uniform high-quality plants in a short period of time and limited space for obtaining a biomass as a source of biological active compounds [3]. Nevertheless, many questions about its *in vivo* and *in vitro* culture remain still unsolved. In Bulgaria, *E. purpurea* is grown on limited area and the information

* *E-mail: ira_stancheva@abv.bg*

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INFLUENCE OF SALT STRESS ON *EX VITRO* GROWTH AND ANTIOXIDATIVE RESPONSE OF TWO *PAULOWNIA* CLONES

K. Miladinova, T. Georgieva
Biotree
M. Geneva
Faculty of Biology, University of Sofia
Y. Markovska, K. Ivanova
Istitute of Plant Physiology and Genetics, Bulgarian Academy of Sciences

Abstract: *The morphological response of Paulownia tomentosa x fortunei* clone TF 01 *and Paulownia elongata x fortunei* clone EF 02, *grown in hydroponic at three levels of salinity, 50 mmol/l , 100 mmol/l, 200 mmol/l NaCl was compared. The content of malondialdehyde (MDA) was enhanced with increasing of salt stress. Activity of phenylalanine ammonia - lyase (PAL), which participate in the control of phenolic metabolism, was increased more in the leaves of Paulownia elongata x fortunei. Under salt stress phenolic and flavonoid contents were increased in the leaves of both clones selected. The results were discussed from the view of changes in synthesis of secondary metabolites and participation of separate classes of them in antioxidative response, characterizing different salt tolerance of Paulownia tomentosa x fortunei and Paulownia elongata x fortunei.*

Key words: *salinity; growth; phenylalanine ammonia - lyase; phenols, flavonoids*

High salinity is the most widespread abiotic stress and constitutes the most stringent factor in limiting plant distribution and productivity (Flowers et al., 1995:1). Salinity affects plant growth, metabolism and photosynthetic efficiency of crop plants (Maeda and Nakazawa, 2008:2; Misra et al., 1997:3). Much effort is being directed towards the identification of

physiological and biochemical processes that are affected by NaCl in order to increase salt tolerance (Apse et al., 2002:4). Molecular approaches to target highly tolerant plants are also difficult as these plants frequently survive the stress periods, being in a state of suspended metabolism. The study of plant stress tolerance is suggested for understanding and transfer of tolerance traits to sensitive crop plants in future (Demmig-Adams et al., 2002:5).

Paulownia is native from China. *Paulownia tomentosa* has been introduced into USA and Europe as an ornamental plant and is still widely used for this purpose. Trees introduced in Bulgaria reach 12 m average height and 13,4 cm average diameter during 7 years (Kalmukov, 1995:6). Over the last two decades *Paulownia* species has been extensively studied due to its ability to uptake nitrates and land contaminants, namely heavy metals (Wang et al., 2010:7). This high-yielding tree can be used for the production of energy, paper pulp and wooden building materials. The genetically tissue–cultured *Paulownia* seedlings produced by The World Paulownia Institute (WPI) allow production of biofuels after introducing of cultivars without detrimental impacts on food supply or the environment. Research on *in vitro* propagation of *P. elongata* and *P. fortunei* has been reported (Bergmann et al., 1997:8). Application of this technology for micropropagation of tree species offers a rapid means of producing clonal planting stock for afforestation, woody biomass production and it is effective way to maintain the genetic gain (Park and Bonga, 1992: 9).

Plants used in the current paper are propagated and rooted according technology registered of Biotree Ltd., Bulgaria. This laboratory is largest producer and supplier of genetically superior *Paulownia* tissue-cultures – *in vitro* seedlings. The farmers preferred *Paulownia tomentosa* x *fortunei* clone TF 01 due to fast development a uniform regular growth. *Paulownia elongata x fortunei* clone EF 02 is less branchy for the purpose of wood material formation. There is no information about its tolerance to salt stress and possibilities to improvement of saline soil utilization with these clones. The results derived from tissue cultures can be used to predict the responses of plants to environmental contaminants and to improve the design and thus reduce the cost of subsequent conventional whole plant experiments (Doran, 2009:10).

In this research, the effect of NaCl on growth, activity of phenylalanine ammonia lyase, phenolic and flavonoid contents in leaves of *Paulownia tomentosa x fortunei* clone TF 01 and *Paulownia elongata x fortunei* clone EF 02, grown in hydroponic after transplant the explants were compared so as to provide fundamental base for vegetation restoration in contaminated soils.

Materials and methods

Plant material. Seeds and *in vivo* explants from the species *P. tomentosa*, *P. elongata* and their hybrids with *P. fortunei* are used for developing of *in vitro* multiplication protocol. For induction of shoots, explants are cultured on Murashige and Skoog (MS) nutrient

Cadmium and Lead Effects on *ex vitro* Growth and Antioxidative Response of Two *Paulownia* Clones

KAMELIYA MILADINOVA*, TEODORA GEORGIEVA*, KATYA IVANOVA**, MARIYA GENEVA***, KALINA DANOVA****, YULIANA MARKOVSKA**

* *BioTree Ltd, 8 Iliensko shosse Str., 1220 Sofia, Bulgaria*

** *Faculty of Biology, University of Sofia, 8 Dragan Tsankov Bld., 1164 Sofia, Bulgaria*

*** *Istitute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. Georgi Bonchev Str., bl. 21, 1113 Sofia, Bulgaria*

**** *Institite of Organic Chemistry with Centre of Phytochemistry, Acad. Georgi Bonchev Str., bl. 9, 1113 Sofia, Bulgaria*

E-mail: pmmaster2001@yahoo.com

Abstract. In this study, the tolerance of *Paulownia tomentosa* x *fortunei* clone TF 01 and *Paulownia elongata x fortunei* clone EF 02, grown hydroponically at different levels of heavy metal exposition - 0.5, 2.5, 5.0 mg L^{-1} Cd and 5, 10, 20 mg L^{-1} Pb was compared in order to evaluate their phytoremediation potential. The highest accumulation of heavy metals occurred in the roots and the bioaccumulation coefficient increased in the order of Pb > Cd. These treatments caused significant reduction in total leaflet area of both clones, the leaf number, root and stem length changed in different manner. The elevated levels of lipid peroxidation at higher Cd and Pb concentrations indicated that these heavy metals caused oxidative stress in leaf tissues. The activity of phenylalanine ammonia - lyase (PAL), which participate in the control of phenolic metabolism, showed pronounced stimulation in the leaves of *Paulownia tomentosa* x *fortunei* after all treatments. Under heavy metal stress the content of some biologically active compounds, such as phenols and flavonoids increased in the leaves of *Paulownia elongata x fortunei* clone EF 02. The results are discussed from the view of the changes in synthesis and the participation of the separate classes of phenolics in antioxidative response, characterizing different heavy metal tolerance of *Paulownia tomentosa* x *fortunei* and *Paulownia elongata* x *fortunei*.

Key words: *Paulownia*, cadmium, lead, antioxidant defense, pollution.

INTRODUCTION

Heavy metal pollution of soils is one of the most important environmental problems because these metals are highly toxic for humans, animals, microorganisms and plants (A d r i a n o, 2001; G a s i c et al., 2006). Phytoextraction refers to the use of the harvestable parts of plants to remove pollutants, represents a green and environmental – friendly tool for cleaning metal – polluted soils compared with costly conventional chemical or physical remediation technologies (C l e m e n t e et al., 2005; F r e n c h et al., 2006). In order to achieve good phytoremediation efficiency, plants should accumulate high amounts of heavy metals, tolerate soil pollution and produce a great quantity of biomass in contaminated conditions (M c G r a t h et al., 2002). Widely used herbaceous hyperaccumulators are slow – growing plants and low – biomass producers, which accumulate only one specific element and possess low depth roots (R e e v e s et al., 2000). Deeper pollution and contamination caused by a number of metals require using as an alternative fast-growing woody species with deep root system and the ability to grow on nutrient-poor soil. *Populus nigra* has shown to be useful as a biomonitor in heavy metal contaminated regions (D j i n g o v a et al., 1995) and now *Populus* has been internationally accepted as a model system for physiological and molecular tree studies (T u s c a n et al., 2006). Over the last two decades *Paulownia* species has been extensively studied due to its ability to uptake nitrates and land contaminants, namely heavy metals (D o u m e t t et al., 2011; W a n g et al., 2010). This high yielding tree can be used for the production of energy, paper pulp and wooden building materials. The genetically tissue–cultured *Paulownia* seedlings produced by The World Paulownia Institute (WPI) allow production of biofuels without detrimental impacts on food supply or the environment. Research on *in vitro* propagation of *P. elongata* and *P. fortunei* has been reported by B e r g m a n n et al. (1997). Application of this technology for micropropagation of tree species offers a rapid means of producing clonal planting stock for afforestation, woody biomass production (P a r k et al., 1992). Plants in the current paper are propagated and rooted according technology registered by the Biotree Ltd., Bulgaria. The company's

Ex vitro Growth and Antioxidative Responses of Two *Paulownia* Clones to Zn Excess

KAMELIYA MILADINOVA*, TEODORA GEORGIEVA*, KATYA IVANOVA**,
MARIYA GENEVA***, KALINA DANOVA****, YULIANA MARKOVSKA**

* *Biotree, 8 Iliensko shose Str., 1220 Sofia, Bulgaria*

** *Faculty of Biology, University of Sofia, 8 Dragan Tsankov Bld., 1164 Sofia, Bulgaria*

*** *Istitute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. Georgi Bonchev Str., bl. 21, 1113 Sofia, Bulgaria*

**** *Institite of Organic Chemistry with Centre of Phytochemistry, Acad. Georgi BonchevStr., bl. 9, 1113 Sofia, Bulgaria*

E-mail: pmmaster2001@yahoo.com

Abstract. In previous experiments elevated applications of Zn (10, 20, 30 mg L^{-1}) caused impairment in growth parameters and antioxidative performance of *Paulownia tomentosa* x *fortunei* clone TF 01 and *Paulownia elongata x fortunei* clone EF 02, grown in hydroponic after transferring the explants from *in vitro* micropropagation. The plants were analyzed with reference to the distribution of Zn, the accumulation of biomass and the metal effects on lipid peroxidation and accumulation of biologically active compounds, such as phenols, flavonoids and anthocyanins. Zn induced a decrease in root, stem length, leaf number and total leaf area of both clones. Decrease in the ratio fresh mass/dry mass (FM/DM) in the roots, but not in the stems and leaves was established. The maximum accumulation of Zn occurred in roots, followed by stems and leaves of both clones. An enhanced levels of lipid peroxidation with increasing the concentrations of Zn indicated that this heavy metal caused oxidative stress in leaf tissues. The activity of phenylalanine ammonia - lyase (PAL), a key enzyme of phenylpropanoid metabolism, showed pronounced reduction in the leaves of *Paulownia tomentosa* x *fortunei* after treatments with 20 and 30 mg L^{-1} Zn. The total contents of phenols, flavonoids and anthocyanins changes in different manner after elevated concentrations of Zn. Stronger modifications, especially concerning leaf characteristics induced by 30 mg L^{-1} Zn, were consistent with physiological impairments while those induced by 10 mg L^{-1} Zn suggested a compensatory strategy for maintaining functional integrity of both clones.

Key words: *Paulownia*, zinc, growth parameters, antioxidant defense.

INTRODUCTION

Pollution with heavy metals, including micronutrients, such as zinc and cadmium, is one of the major environmental problems. Zinc inputs to the environment usually results from industrial activities, such as mining and smelting of metalliferous ores, electroplating, gas exhaust, energy and fuel production, fertilizer and pesticide application, and generation of municipal waste (K a b a t a – P e n d i a s, 2001). This heavy metal is essential in trace amounts for plants and organisms, but becomes toxic when present in bioavailable forms at excessive levels, adversely affecting leaf performance and plant growth similar to those induced by Cd (H e r m l e et al., 2007; W a n g et al., 2009). Although information about the quantitative Zn requirement of forest trees is sparse, the typical Zn concentration required for adequate growth of most crops is 15 – 20 mg/kg DM (M a r s c h n e r, 1995), and in contaminated soils it is easy to reach this threshold (B r a d l e y et al., 2007). Poplar and willow are promising as renewable energy fast-growing trees which tolerate and accumulate high levels of heavy metals, such as Zn and Cd, although significant clonal variations are established (L u n á č k o v á et al., 2003/4; G i a c h e t t i et al., 2006; H e r m l e et al., 2007). These tree species possess the best characteristics as ideal plants for phytoremediation (S h a h et al., 2007). Nevertheless, the studies on the physiological and molecular tolerance/defense mechanisms and heavy metal accumulation of woody species remain relatively unknown. Over the last two decades *Paulownia* species has been extensively studied due to its ability to uptake heavy metals (D o u m e t t et al., 2008; 2011). *Paulownia elongata* is used for the remediation of Pb, Zn, Cu and Cd polluted soils owing to its very high biomass productivity, rather than its metal accumulation potential (W a n g et al., 2010). Ultrastructural and physiological modifications induced by high zinc levels in *Paulownia tomentosa* are observed (A z z a r e l l o et al., 2012). Our preliminary investigations established some physiological aspects of response of two *Paulownia* clones to Cd and Pb in hydroponic experiments after transferring the explants obtained according to micropropagation technology registered of Biotree Ltd., Bulgaria (M i l a d i n o v a et al., 2012.

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RESEARCH PAPERS

EDTA Reduces Heavy Metal Impacts on *Tribulus terrestris* Photosynthesis and Antioxidants[1]

Y. Markovska[a], M. Geneva[b], P. Petrov[b], M. Boychinova[b], I. Lazarova[c], I. Todorov[b], and I. Stancheva[b]

[a] *Faculty of Biology, Sofia University "St. Kliment Ohridski", 8 D. Tzankov Blvd., 1164 Sofia, Bulgaria*

[b] *Department of Plant Mineral Nutrition and Water Relation, Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. G. Bonchev str., Bl. 21, 1113 Sofia, Bulgaria; fax- +359-2-873-9952, e-mail: ira_stancheva@abv.bg*

[c] *Faculty of Pharmacy, Department of Chemistry, Medical University of Sofia, Bulgaria*

Received August 21, 2012

Abstract—The effects of EDTA application to heavy metal-polluted soil on phytoextraction of heavy metals, leaf anatomy, gas exchange parameters, enzyme activities of C4 carbon cycle, antioxidant defense, and active compounds of *Tribulus terrestris* L. were evaluated. The addition of EDTA to the soil polluted with Cd and Pb markedly increased dry weight and Pb, Zn, and Cd contents in shoots. Plants responded to the action of EDTA by an increased stomatal conductance, photosynthetic and transpiration rates, water use efficiency, chlorophyll and carotenoid contents. The activities of C4 carbon cycle enzymes simultaneously increased, thus concentrating CO_2 for enhanced CO_2 assimilation and providing NADPH for the antioxidant system. Antioxidants, such as ascorbate, reduced glutathione, and flavonoids, increased more in the shoots of *T. terrestris* after the addition of EDTA. The activities of guaiacol peroxidase, catalase, and the enzymes of the ascorbate–glutathione cycle enhanced significantly in the presence of EDTA. Increased activities of antioxidant enzymes suggest that they have some additive functions in the mechanism of metal tolerance. EDTA application lowered the activity of phenylalanine ammonia-lyase and the content of total phenols, MDA, hydrogen peroxide, dehydroascorbate, and lipid-soluble antioxidant capacity expressed as α-tocopherol. Increased levels of total radical-scavenging activity are in correspondence with the activity of water-soluble antioxidant compounds in *T. terrestris* tissues. The content of furostanol saponins protodioscin, prototribestin, and rutin increased as a result of EDTA addition. The results obtained allowed us to assume that applied EDTA reduced a negative heavy metal impact on puncture vine photosynthesis and antioxidant potential.

Keywords: Tribulus terrestris, C4 carbon cycle enzymes, EDTA, heavy metals, leaf anatomy, antioxidant capacity, furostanol saponins

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INTRODUCTION

Tribulus terrestris L. is a flowering plant of the family Zygophyllaceae, native to warm temperate and tropical regions of the Old World in southern Europe, southern Asia, throughout Africa, and Australia. *T. terrestris* plant extracts contain saponins, flavonoids, amides, and alkaloids [1]. The biologically active furostanol saponin fraction of this plant finds application in the contemporary medicine as a component of drugs effective in treatment impotence and other sexual disorders [2].

T. terrestris grown on industrially polluted soil showed good ability for Cd, Pb, and Zn accumulation and could be used for phytoremediation of polluted soils [3]. The plant biomass production and the heavy metal (HM) concentration in the harvestable biomass are important factors for the practical efficiency of phytoextraction [4]. One strategy to achieve the higher HM removal efficiency is to enhance the concentrations of soluble HMs in the soil with the application of complexing agents [5]. EDTA is widely investigated due to its high complexing capability towards Pb, Cu, Cd, and Zn. That leads to an increase in the metal uptake by plants like *Brassica juncea, Helianthus annuus*, and *Zea mays* [6]. The medicinal plants could be grown as alternatives to edible crops in HM-polluted agricultural soils, because possess a significant

[1] This text was submitted by the authors in English.

Abbreviations: AGC—ascorbate–glutathione cycle; ASC—ascorbic acid; CAT—catalase; DHASC—dehydroascorbic acid; GPX—glutathione peroxidase; GR—glutathione reductase; GSH—reduced; GSSG—oxidized glutathione, GST—glutathione S-transferase; HM—heavy metal; NADP-ME—NADP-malic enzyme; PAL—phenylalanine ammonia-lyase; PEPC—phosphoenolpyruvate carboxylase; PPDK—pyruvate orthophosphate dikinase; SOD—superoxide dismutase.