Arch. Biol. Sci., Belgrade, 61 (2), 279-283, 2009 DOI:10.2298/ABS0901279T

MANGANESE EFFECTS ON IN VITRO DEVELOPMENT OF LESSER CENTAURY [*CENTAURIUM PULCHELLUM* (SW.) DRUCE]

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Abstract — To determine the manganese requirement necessary for optimal development of lesser centaury [*Centaurium pulchellum* (Sw.) Druce] in vitro, we investigated the effect of exogenously applied Mn on different developmental processes such as growth, flowering, fruiting, and seed germination. The application of Mn had no effect on stem length, except at the highest concentration of 10^{-2} M, which was inhibitory. In addition, *C. pulchellum* plants were capable of in vitro flowering and fruiting even on media without added Mn. However, Mn content in the media affected seed dimensions, since both length and width of the seeds increased with increasing Mn concentration. Moreover, both excess and absence of Mn in the media caused appearance of necrotic plants. Exogenously applied Mn had no effect on seed germination percentage, except at concentrations greater than 3×10^{-3} M.

Key words: *Centaurium pulchellum*, manganese, flowering, fruiting, germination, *in vitro*

UDC 581.145.1:581.14:546.711

INTRODUCTION

Manganese (Mn) is a micronutrient essential for all stages of plant development. It is involved in photosynthesis, respiration, and lignin and amino acid biosynthesis, in addition to performing a key function in the activation of several enzymes, including decarboxylating malate dehydrogenase, malic enzyme, isocitrate dehydrogenase, or nitrate reductase (Mukhopadhay and Sharma, 1991). Two Mn-containing enzymes have been identified in plants: Mn SOD and a 33-kD protein that is part of the oxygen-evolving water oxidizing complex in PSII (Burnell, 1988; Amesz, 1993). On the other hand, Mn can be detrimental when available in excess in the surroundings. Symptoms of Mn toxicity are quite diverse among plant species and include marginal chlorosis and necrosis of leaves in *Medicago sativa* L., *Brassica napus* L., *Lactuca sativa* L., and *Nicotiana tabacum* (Foy et al., 1978; Petolino and Collins, 1985), brown root discoloration in rapeseed (Moroni et al., 2003), wheat (Moroni et al., 1991), and soybean (Heenan and Cambell, 1981); and loss of apical dominance and enhanced formation of auxiliary shoots – "witches' broom" (Kang and Fox, 1980). Toxic Mn effects have also been described in different *in vitro* developmental processes, including callus induction and growth and shoot regeneration from callus (Petolino and Collins, 1985; Clairmont et al., 1986; Santandrea et al., 1997, 1998a). Moreover, high Mn levels have direct cytotoxic effects, causing extensive cytoplasmic injures, mitochondrial modification, and plasma membrane ruptures in the outer root cap and meristematic cells (Santandrea et al., 1998b). For all these reasons, it is very important to establish the optimal Mn dose for growth of certain plant species.

Lesser centaury [*Centaurium pulchellum* (Sw.) Druce] is an annual, herbaceous plant. This species is self-fertilized and forms dichasial inflorescences. It is widely spread in Europe, but is rather rare in Serbia, where its natural habitat is restricted to a few regions in Vojvodina (the northern part of Serbia). It is known that most species from the genus *Centaurium* are medicinal plants. This also applies to *C. pulchellum*, which is a rich source of secoiridoides and xanthones (Janković et al., 2002; Krstić et al., 2003). Fortunately, these plants are easily maintained *in vitro*, where they can be propagated through their natural life cycle, including fast vegetative growth, flowering, fruiting, and production of viable seeds. This interesting constitutive property is significant not only for preservation of the given species in Serbia, but for biotechnological investigations as well (Krstić et al., 2003; Todorović et al., 2006).

The aim of this study was to determine the super-, sub-, and optimal Mn concentrations required for different developmental stages during the *in vitro* growth of lesser centaury.

MATERIAL AND METHODS

Plant material and in vitro culture establishment

Seeds of *Centaurium pulchellum* (Sw.) Druce were collected in the area of Novi Sad (Vojvodina, Serbia). They were surface sterilized for 5 min with a 30% solution of commercial bleach and then rinsed three times with sterile distilled water. Sterilized seeds were germinated in distilled water under white light at 25 ± 2°C. Fourteen-day-old seedlings were planted on an agar medium containing 3% sucrose, 0.7% agar, and MS vitamins and salts (Murashige and Skoog, 1962) supplied with different concentrations of $MnSO_4$ (ranging from 0 to 10^{-2} M) and adjusted to pH 5.8. Three replicates of 30 seedlings each were cultured for each treatment. No phytohormones were added to the medium. All cultures were grown in a growth room under long-day conditions (16 h of light followed by 8 h of darkness) at constant temperature of 25 ± 2°C and relative humidity of 60-70%. White fluorescent tubes (Tesla, Pančevo, Serbia) provided a photon flux rate of 32.5 μmol $m^{-2}s^{-1}$ at the level of the samples.

Parameters such as plant stem length, presence of necrosis, and the number of flowers and capsules were recorded after 10 weeks.

Morphometric measurements of seeds

Seed length and width were measured under a Leica, LETZ DMRB microscope. Image analysis was performed using QWIN software. Statistical analyses were done with STATGRAPHICS software, Version 4.0 (STSC, Inc. and Statistical Graphics Corporation, 1985-1989, USA). Means were compared using the LSD multiple range test at a significance level of $p < 0.05$.

Seed germination

For seed germination experiments, only spherical and filled seeds were used. Replicates of approximately 30 seeds were placed in 6-cm Petri dishes (without filter paper) containing 2 ml of distilled water or test solution. Seeds were germinated under the same conditions as *in vitro* cultivated plants, i.e., under long days (16 h at 32.5 μmol $m^{-2}s^{-1}$ at the seed level) at 25 ± 2°C for 14 days. Germination was scored on the 14^{th} day after the onset of imbibition. Radicle protrusion was taken as the criterion for germination. All experiments were repeated twice, with three replicates. Results are presented as the percent of seed germination.

RESULTS AND DISCUSSION

As an essential micronutrient, Mn is required in low concentrations in media for *in vitro* plant cultures (Dodds and Roberts, 1985). However, exogenously applied Mn had no effect on stem length in *C. pulchellum*, except at the highest dose of 10^{-2} M, which was inhibitory (Fig. 1). Moreover, the plants were capable of *in vitro* flowering and fruiting on media without Mn added. Concentrations of Mn ranging from 10^{-6} to 10^{-3} M had no effect on these processes, while higher concentrations ($3x10^{-3}$ and 10^{-2} M) were inhibiting (Figs. 2 and 3).

Manganese is involved in photosynthesis, respiration, and activation of antioxidative enzymes (Mukhopadhay and Sharma, 1991; Santandrea et al., 2000). It has been shown that an excess of Mn inhibits chlorophyll synthesis and decreases the photosynthetic rate (Clairmont et al., 1986; Macfie and Taylor, 1992). As can be seen in Fig. 4, the optimal Mn concentrations in the growth media were from $3x10^{-5}$ to 10^{-3} M, while lower and higher concentrations caused appearance of necrotic spots on leaves. The observed necrosis is likely a consequence

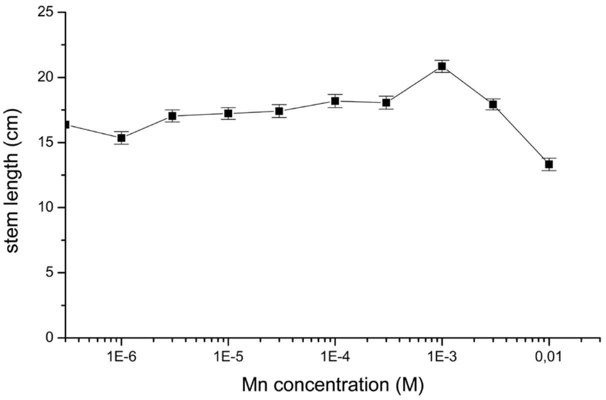

Fig. 1. Stem length in lesser centaury plants in vitro grown on different Mn concentrations.

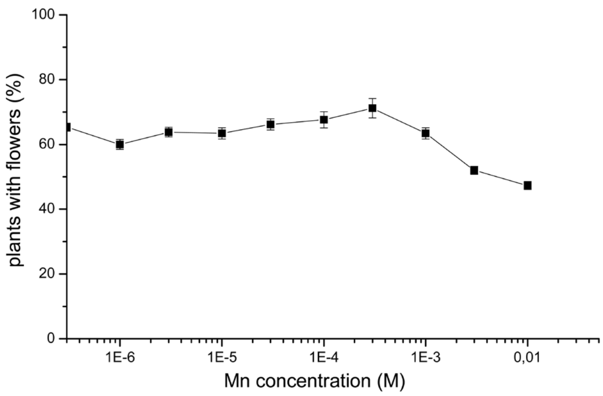

Fig. 2. Effect of different manganese concentrations on in vitro flowering of lesser centaury plants.

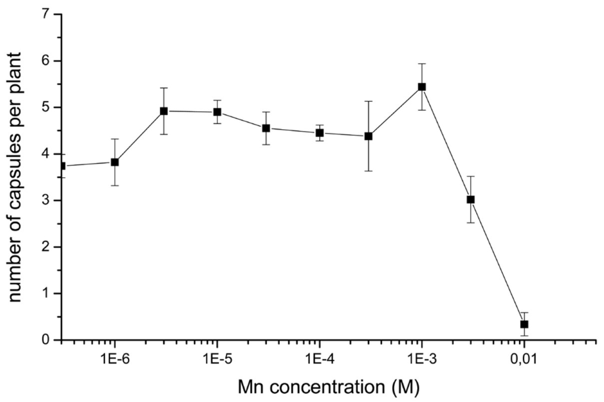

Fig. 3. Effect of different manganese concentrations on *in vitro* fruiting of lesser centaury plants.

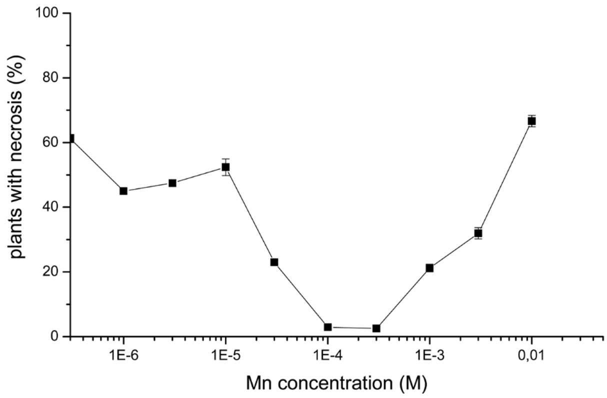

Fig. 4. Percent of necrotic plants grown *in vitro* on different manganese concentrations.

of oxidative stress imposed by either deficiency or excess of Mn. To be specific, when Mn is deficient, the activity of antioxidative enzymes is reduced, and so is the ROS-scavenging capacity of the plants. On the other hand, excessive uptake of Mn can cause direct generation of ROS through Fenton-like reactions (González et al., 1998).

Interestingly, both the length and width of seeds increased with Mn content in the media up to $3x10^{-4}$ M Mn, with the largest seeds produced from plants grown on Mn concentrations of from 3×10^{-4} to 3×10^{-3} M (Fig. 5).

For seed germination experiments, only spherical and filled seeds were used. The seeds of lesser centaury are positively photoblastic and require light for germination (Todorović et al., 2006). Under conditions of long days, the germination percentage did not differ significantly between seeds germinated in distilled water and those germinated in Mn solutions from 10^{-5} to $3x10^{-3}$ M. A strong inhibition of germination was observed only when exogenous Mn exceeded $3x10^{-3}$ M (Fig. 6). A similar effect was demonstrated for germination of Mn-tolerant cultivars of *Nicotiana tabacum* (Santandrea et al., 1997, 2000). In addition, it has been shown that lesser centaury can produce seeds with extremely high Mn contents. Interestingly, germination of these seeds was undisturbed. This is probably due to the fact that nearly all Mn in seeds is in bound form, which was proven by electron paramagnetic resonance spectroscopy (Todorović et al., 2008). The physi-

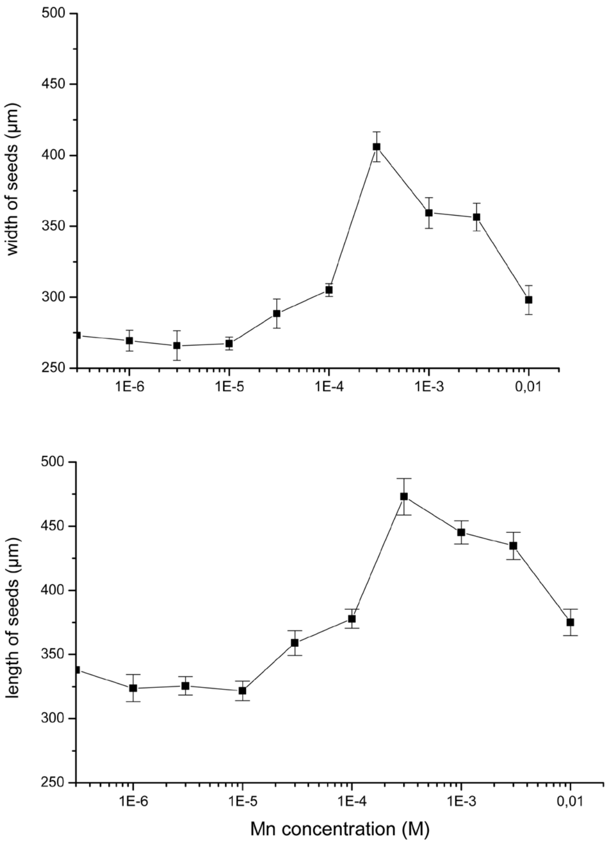

Fig. 5. Effect of different manganese concentrations on dimensions of *in vitro* produced seeds.

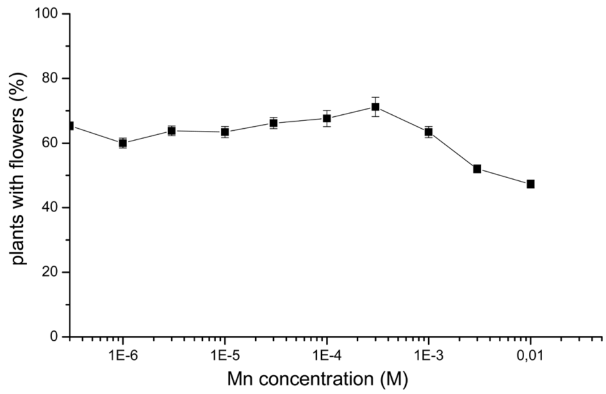

Fig. 6. Effect of manganese on light-induced germination of lesser centaury seeds.

ological, genetic, and molecular mechanisms of Mn tolerance in plants are not yet clear. The degree of tolerance or sensitivity varies widely in different species and environments and seems to be controlled in different ways (Mukhopadhay and Sharma, 1991; Santandrea et al., 2000).

It can be concluded that Mn is an essential micronutrient for the lesser centaury, and that even traces of Mn, probably present as impurities in the media, satisfy its requirements for Mn in all developmental stages. The rates of *in vitro* growth, flowering, fruiting, and seed germination of *C. pulchellum* were the same, regardless of the presence or absence of exogenous Mn. The majority of observed processes are insensitive to high doses of Mn, up to $3x10^{-3}$ M. The present results, along with the well-established model system for *in vitro* growth and development of lesser centaury, provide an opportunity to further study Mn-related defense mechanisms and the enzymes involved in these processes.

Acknowledgment — This work was supported by the Ministry of Science and Technological Development of the Republic of Serbia (Contract No. 143031).

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ЕФЕКАТ МАНГАНА ТОКОМ РАЗВИЋА СИТНЕ КИЧИЦЕ [*CENTAURIUM PULCHELLUM* (SW.) DRUCE] У КУЛТУРИ *IN VITRO*

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У овом раду проучавани су ефекти егзогено аплицираног мангана током процеса раста, цветања, плодоношења и клијања семена кичице ситне [*Centaurium pulchellum* (Sw.) Druce], гајене у култури *ин витро*. Резултати су показали да Mn нема ефекта на дужину стабла. Једино је највиша примењена концентрација Mn од 10^{-2} M имала инхибиторни ефект на посматрани процес. Биљке кичице ситне могу не само да расту, већ и да цветају и плодоносе на медијуму без аплицираног Mn. Међутим, димензије семена у многоме зависе од концентрације Mn у подлози. Са повећањем концентрације Mn у подлози, расте и ширина и дужина семена. Изостанак Mn у подлози, као и његове високе концентрације узроковале су појаву некротичних биљака. Концентрације Mn ниже од 3×10^{-3} M нису имале ефекта на проценат проклијалих семена.