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BRIEF COMMUNICATION

Nickel hyperaccumulation in shoot cultures of *Alyssum markgrafii*

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Abstract

Shoot cultures of *Alyssum markgrafii* O.E. Shulz, endemic nickel hyperaccumulating species of central Balkan, were established and maintained on Murashige and Skoog medium supplemented with 0.2 mg dm^{-3} benzyladenine (BA). Nickel in form of $NiCl_2 . 6 H_2O$ was supplemented at 22 different concentrations ranging from 0.0001 to 15 mM but none of them was lethal to cultures. High Ni^{2+} concentrations (10 mM or more) arrested shoot growth which, upon transfer to Ni-free medium, commenced *via* axillary bud proliferation. Shoots that developed from axillary buds through the subculture manifested increased tolerance to Ni^{2+} expressed as shoot elongation. Shoot multiplication and dry biomass production decreased with increase of Ni^{2+} in medium. Only the accumulation of Ni^{2+} in tissues increased with Ni^{2+} content of the medium. Apart from shoot cultures, high Ni^{2+} accumulation was registered in undifferentiated callus cultured on medium with 0.5 mg dm^{-3} BA and 0.5 mg dm^{-3} naphthylacetic acid. Highest content of accumulated Ni was 2.37 μg g^{-1}(d.m.) in shoots and 2.65 μg g^{-1}(d.m.) in callus, both measured on medium with 15 mM Ni^{2+}.

Additional key words: callus, *in vitro* culture, shoot cultures.

Nickel falls in the group of heavy metals and therefore is considered as toxic for plant growth and development. In low concentrations nickel can improve seed germination and growth of some species (Mishra and Kar 1974). Ni is a constitutive element of the enzyme urease involved in nitrogen metabolism (El-Shintinawy and El-Ansary 2000) and there have been well-documented suggestions that it should be listed among essential elements (Escew *et al.* 1984, Brown *et al.* 1987, Gerendas *et al.* 1999).

Some plant species are adapted to grow on soil containing high content of Ni^{2+}. Minguzzi and Vergnano (1948) showed that *Alyssum bertolonii* is not only tolerant but accumulates Ni^{2+} in its body. Such plants were later named metal hyperaccumulators. According to Krämer *et al*. (1996) there are more than 400 hyper-accumulating species among which 48 belong to genus *Alyssum*. Using hydroponic growth systems Krämer *et al*. (1996) showed that the basis for Ni^{2+} accumulation in *A. lesbiacum* is fast translocation of metal from roots into shoots using free histidine as chelating agent. In the other well-known Ni^{2+} hyperaccumulator, *Thlaspi goesingense* Hálácsy histidine overproduction was not correlated with Ni^{2+} hyperaccumulation (Persans *et al*. 1999). Thus it seems that the basis of Ni^{2+} tolerance in various species is founded on different mechanism. Metal tolerance in plants is also connected with the presence of metallo-thioneins (cysteine-rich proteins) and phytochelatins (low molecular mass polypeptides).

Alyssum markgrafii O.E. Schulz is a rare Balkan hyperaccumulating species that grows only on several serpentine “mountain-tops” in Serbia and Albania. It is an inconspicuous plant 20 - 50 cm tall with small hairy leaves. On the serpentine slopes of Mountain Goč (Serbia) where seeds were collected, plants contained 5.12 - 6.25 μg(Ni) g^{-1}(d.m.) and soil 1.52 - 3.12 μg(Ni) g^{-1} (Obratov *et al.* 1997). We established shoot cultures of

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Abbreviations: BA - benzyladenine; MS - Murahige and Skoog; NAA - naphthylacetic acid.
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A. markgrafii with the aim to investigate its tolerance to nickel and metal hyperaccumulating capacity under conditions of *in vitro* culture.

Seeds were surface sterilized for 20 min in commercial bleach containing 0.5 % NaOCl, rinsed thoroughly in autoclaved water and germinated in Petri plates on hormone-free basal medium containing Murashige and Skoog (1962; MS) inorganic salts and vitamins, 2 % sucrose and 0.64 % agar. Medium pH was adjusted to 5.8 prior to autoclaving performed for 20 min at 114 °C. Conditions of the growth room were: temperature 25 ± 2 °C, photoperiod 16 h, irradiance 45 μmol m^{-2} s^{-1}. Epicotyls excised from seedlings were subcultured on medium supplemented with 0.2 mg dm^{-3} benzyladenine (BA). Explants developed into shoot cultures that were further maintained by regular subculturing of axillary shoots. Experimental treatments, which were set with shoots 15 mm long, lasted 35 d. Culture vessels were 100-cm^3 wide neck Erlenmeyer flasks with 10 shoots growing on 40 cm^3 medium. Nickel was supplemented in form of $NiCl_2 \cdot 6\,H_2O$ same as in Heller`s medium (Heller 1953). Treatments containing 60 shoots were repeated 2 - 4 times in 22 different Ni^{2+} concentrations (0.0001 - 15.0 mM). Treatments with high Ni^{2+} concentrations were additionally repeated to collect material for analysis. All treatments were set with explants obtained from cultures that were not previously grown on nickel-supplemented medium. Parameters investigated in shoot cultures included: length of the main shoot, formation of axillary shoots (shoot multiplication), dry mass per plants, and accumulation of nickel in tissues. Callus induced in shoots cultured on MS medium supplemented with 1.0 mg dm^{-3} dichlorophenoxyacetic acid (2,4-D) was later maintained on medium with 0.5 mg dm^{-3} BA and 0.5 mg dm^{-3} naphthylacetic acid (NAA). Parameters investigated in callus tissue were: biomass increase per dry mass and accumulation of nickel in tissues. Effects of Ni^{2+} on callus growth and Ni^{2+} accumulation were studied in media supplemented with 0, 3, 5, 8, 10 and 15 mM Ni^{2+}. Treatments set with 18 - 24 callus explants were replicated 2 - 3 times. Initial mass of callus explants was ≈ 240 mg and subculture duration 35 d. Ni^{2+} content in shoots and callus was detected by atomic absorption spectroscopy (*Pye Unicam SP9*, Cambridge, UK) following material preparation by nitric acid - hydrogen peroxide method after Krishnamurty *et al.* 1976.

Epicotyls excised from germinated seedlings grew well on media supplemented with 0.2 mg dm^{-3} BA forming characteristic shoot cultures. This medium enabled also spontaneous rooting in 12.4 % cultures. Roots were thin and threadlike. Addition of nickel gradually decreased spontaneous rooting to 4.7 % in medium with 0.2 mM Ni^{2+}. At higher Ni^{2+} concentrations roots were absent.

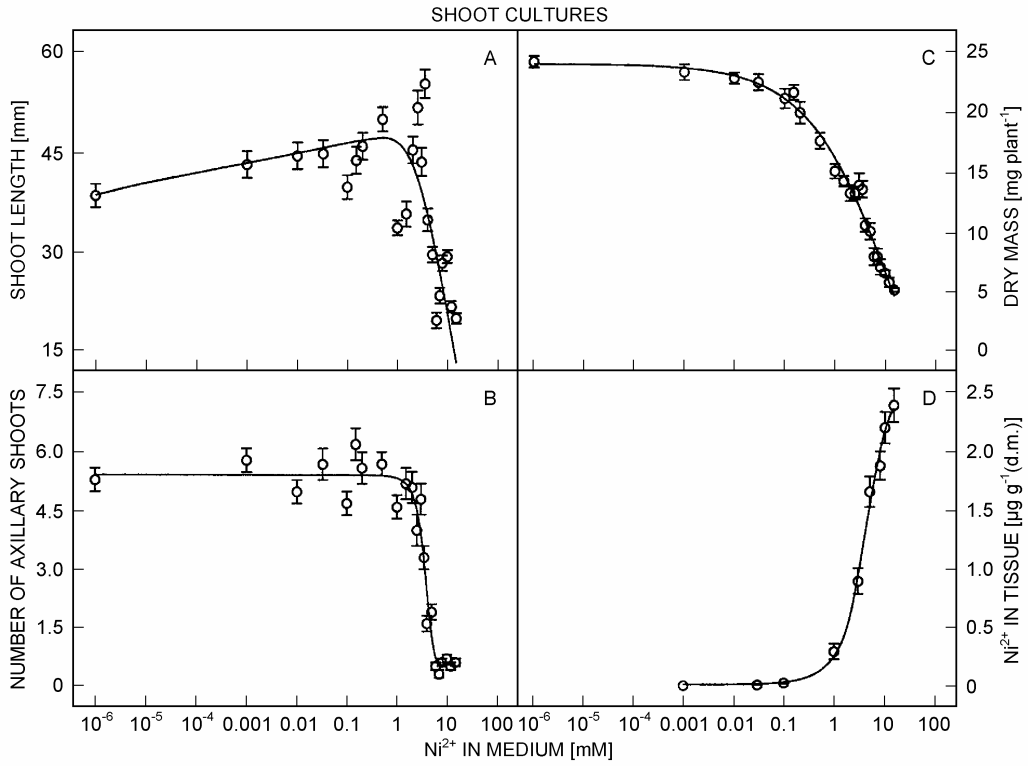

Fig. 1. Effect of Ni^{2+} on the growth of shoot cultures, means ± SE (n = 60 - 120): *A* - shoot elongation, *B* - number of axillary shoots, *C* - dry mass production. *D* - accumulation of nickel in tissue, means ± SE (n = 3).

The addition of 0.0001-1.0 mM Ni^{2+} (here considered as low concentration) did not affect the growth of shoots. In shoots the signs of deleterious Ni^{2+} effects were not observed until Ni^{2+} concentration in the medium reached 1.0 - 5.0 mM (here considered as moderate concentrations). These signs included: decrease of growth vigor and leaf chlorosis. Elongation of shoot cultures was apparently stimulated by Ni^{2+} since the highest values were registered at these Ni^{2+} concentrations (Fig. 1*A*). Further increase of Ni^{2+} concentration above 5.0 mM (considered here as high concentration) resulted in rapid inhibition of shoot elongation.

The terminal shoot of the explant and axillary shoots, which develop through the subculture, manifested different tolerance to Ni^{2+}. Axillary shoots were less sensitive to nickel and they reached highest lengths at 3.0 - 5.0 mM Ni^{2+}, concentrations that were already inhibitory for the terminal shoots (Fig. 2). Thus at moderate nickel concentrations growth of shoot cultures resulted mostly from the activation and elongation of axillary shoots.

Fig. 2. Effect of Ni^{2+} supplemented to the medium in different concentrations on the growth of shoot cultures (35 d): a - 15, b - 12, c - 10, d - 8, e - 5, f - 3, g - 0 (control) mM Ni^{2+}. *Bar* = 10 mm.

Shoot multiplication (Fig. 1*B*) was not affected in the presence Ni^{2+} in the medium up to the concentration 3.0 mM. Higher Ni^{2+} concentrations rapidly decreased shoot multiplication. Dry mass of plants (Fig. 1*C*) decreased gradually with the increase of Ni^{2+} in the medium. None of the investigated Ni^{2+} concentrations including the highest (10, 12 and 15 mM) were actually lethal for shoot cultures. At such high Ni^{2+} concentrations shoots just stopped growing, most leaves were chlorotic and some shoots perished from apical necrosis. However, when transferred from 15.0 mM Ni^{2+} to nickel-free medium, cultures recovered, forming axillary shoots that resumed normal growth. In this treatment 4.1 ± 0.4 axillary buds were activated reaching 21.1 ± 1.2 mm in length after 35 d (n = 60). Accumulation of Ni^{2+} in tissues (Fig. 1*D*) began when Ni^{2+} concentration in medium reached 0.1 to 1.0 mM. Then a significant increase of nickel accumulation occurred reaching a maximum of 2.37 μg(Ni) g^{-1} (d.m.) at 15.0 mM Ni^{2+}.

Finally, Ni^{2+} accumulation was investigated in callus derived from shoot cultures. This slow growing, undifferentiated callus was hard, compact and green. Callus was highly tolerant to nickel since the reduction of dry mass production did not reach 50 % even at 15.0 mM Ni^{2+} (Fig 3*A*). Callus hyperaccumulated Ni^{2+} in a manner similar to shoot cultures (Fig. 3*B*). The highest value 2.65 μg(Ni) g^{-1}(d.m.) was recorded on medium with 15.0 mM Ni^{2+}.

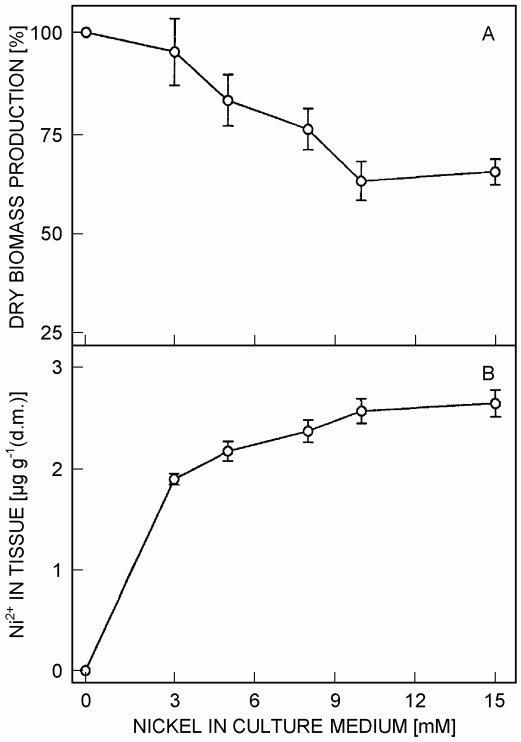

Fig. 3. Effect of Ni^{2+} on the growth of undifferentiated callus tissue: *A* - dry mass production, means ± SE (n = 18 - 24), *B* - accumulation of nickel in callus, means ± SE (n = 3).

Our results show that under conditions of *in vitro* culture *A. markgrafii* is as highly efficient nickel hyperaccumulator as in nature. The ability to hyperaccumulate nickel was apparently not restricted only to organized structures since it occurred in undifferentiated, non-organogenic callus. Hyperaccumulation in callus is an interesting finding since it indicates that the tolerance to nickel is a specific feature of cells and tissues of certain species and not the result of translocation. Further histological and histochemical investigation of this callus is required to pinpoint the intracellular localization of nickel.

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