



Bioinorganic chemistry

Influence of F₁ hybridization on the metal uptake behaviour of pine trees (*Pinus nigra* x *Pinus thunbergiana*; *Pinus thunbergiana* x *Pinus nigra*)

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ABSTRACT

Pine needles have been considered to be useful bio-indicators for air pollution. This phenomenon can be used for environmental studies for monitoring purposes. Additionally, this fact offers the possibility to study uptake and accumulation behaviour not only in different species, but also in hybrids obtained from common pine trees to inheritance processes. Therefore, needles of *Pinus nigra* Arnold and *Pinus thunbergiana* Franco as well as of their F₁ hybrids were investigated for essential and non-essential metals, such as Al, B, Ba, Ca, Cd, Co, Cu, Cr, Fe, K, Mg, Mn, Mo, Na, Ni, Pb, Sr, and Zn. The samples underwent acidic microwave-assisted digestion prior to analysis inductively coupled plasma atomic emission spectrometry (ICP-AES). Furthermore flavonoids were quantitatively determined to prove hybrid character.

Regarding all determined analytes, increase and decrease of uptake in the needles of the hybrids were evaluated in comparison to the needles of the parent pine species to see which parent is the dominant one. In the hybrids higher amounts of Al, Cd, Mo, Ni, Mg, Mn, and Zn were found. Different behaviour was registered for flavonoids than for metals, due to different metabolic pathways.

1. Introduction

During the last decades, heavy metal pollution has steadily become a serious environmental problem due to the toxicity and persistence of these species in the environment, not only in urban zones, but also in remote areas [1,2]. Through the food chain due to accumulation in crops or plants, heavy metals (e.g. cadmium and lead) may cause harmful effects on humans and animals [3].

Vegetation in general is a useful indicator of environmental (heavy) metal contamination due to root uptake and precipitation (rain and dry deposition) on the outer surface. Thus plants can be used to monitor pollution across both spatial and temporal scales. Pine needles represent adequate biomonitors of air pollutants since their waxy surfaces accumulate lipophilic gaseous pollutants and polluting particulates [4].

Regarding heavy metal pollution, needles from different pine species, such as *P. halepensis*, *P. sylvestris*, and *P. nigra* have been studied by various working groups in Europe and Asia [5–10]. Apart from the environmental impacts, the needle metal contents also vary during growth of the tree with significant differences found for one year old

shoots compared to older ones in *P. halepensis* [11]. Whereas the needle metal concentrations are more strongly affected by environmental conditions, accumulation in the stem increases with age of the tree [12].

Apart from the monitoring of airborne pollutions, the uptake from soil by roots also contributes to the metal content of needles. Pine needles are well known bio-indicators with differences found in dependency of species and growing area. Influence of the genetic background was found for nickel accumulation in *Alyssum* [13]. Thus it is supposed that F₁ hybridization can possibly influence also the uptake and accumulation behaviour of metals in pine species. Metals play an important role in plant physiology and problems related to the deficiency of elements are common in agricultural crops [14]. Plants use these elements as structural components in carbohydrates and proteins, as components of organic molecules indispensable for normal metabolism (such as magnesium in chlorophyll and phosphorus in ATP), enzyme activators (like potassium), and for maintaining osmotic balance [15–19].

P. nigra Arnold (European black pine) is widely used for monitoring

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studies, especially in Munich and surroundings [10,20] and its accumulation behaviour is well-understood [21]. The second pine tree species studied is *P. thunbergiana* Franco (Japanese or Korean black pine). For both of these species female and male parents were used resulting in two different F₁ hybrids, namely *P. thunbergiana* x *P. nigra* and *P. nigra* x *P. thunbergiana*. These hybrids produced by controlled hybridization have also been used for studying morphological and anatomical characteristics as well as basic needle volatile compounds such as terpenes [22,23]. Interspecific hybridization in forest trees has been on-going interest to forest geneticists and tree improvement scientists studying feasibility of transferring favourable traits among species (e.g. better resistance to pollution and damaging diseases, positive impact on the productivity and quality of plantations etc.).

Apart from metals the content of secondary metabolites such as flavonoids is also influenced by genetic background [23]. These phenolic compounds afford protection against UV radiation, pathogens and herbivores [24–27]. They are also important for normal plant growth and development [28]. It was shown that flavonoid transport in plants is improved by their complexation with metals [29]. Due to all above mentioned reasons the flavonoid content in pine needles is of importance for forestry. Furthermore, these compounds have been used in plant taxonomy, phylogenesis and for additional elucidation of hybridization events [30–33].

Thus the objective of the present project is the quantitative determination of Al, B, Ba, Ca, Cd, Co, Cu, Cr, Fe, K, Mg, Mn, Mo, Na, Ni, Pb, Sr and Zn as well as flavonoids in needles (one year old shoots) of pine hybrids and their female and male parents in order to investigate the influence of hybridization on metal uptake and flavonoid content.

2. Material and methods

2.1. Glassware and chemicals

Prior to use all glass- and plasticware were cleaned with diluted nitric acid (7 mol/L). Ultrapure water (> 18 M Λ /cm) was produced by an in-house-apparatus.

All reagents of p.a. quality, nitric acid (HNO₃) and hydrochloric acid (HCl), were supplied by Merck (Germany). For preparing the calibration standards the multi-element standard (ICP Multielement Standard IV; 1000 mg/L) from Merck (Germany) was used as stock solution. The certified reference material for method validation, namely strawberry leaves was purchased from LGC Standards (United Kingdom; LGC7162). The standards for organic compounds naringenin and quercetin were obtained from Sigma (Germany) and myricetin, isorhamnetin and kaempferol from Fluka (Switzerland). All organic solvents (methanol, diethylether, ethanol, formic acid, acetone) were obtained from Kemika (Croatia).

2.2. Samples

P. nigra Arnold (European black pine) is distributed along the Mediterranean Basin at low to moderate altitudes. It is characterized by 8–16 cm long, 1.5–2 mm wide, rigid needles, which persist for 4–7 years on the tree [34–35].

P. thunbergiana Franco (Japanese or Korean black pine) is native to Japan and South Korea. Its needles are acute, dark green, scabrous with minute marginal teeth, often twisted, with a size of 7–12 cm length and 0.7–1.2 mm width, which persist for 3–4 years [34,35].

P. thunbergiana x *P. nigra* and *P. nigra* x *P. thunbergiana* hybrids used in this study were produced by controlled hybridization during 1985 and 1989. The morphometric [22] and needle volatile composition analyses [23,36] confirmed their identity as F₁ hybrids. All trees sampled for this study were planted from seeds in the experimental plot in the Lisićine Arboretum (latitude: N 45° 40' longitude: E 17° 31', 150 m above sea level) in triangular-shaped plots with sides of 6 m length. The total planting area covers 0.55 ha and consists of the same

type of soil. The soil is luvisol, medium acid, poor in organic matter, at the lower scale for good nitrogen supply and deficient on available phosphorus [22]. The climatic conditions are annual precipitation of 918 mm and average temperature of 10.5 °C. The experimental plot is not placed in the central, but in a separated part of arboretum. There is no any significant anthropogenic activity near the sampling site except mowing meadows, pruning shrubs and hedges, removing dead plant material, maintaining paths and similar. The Arboretum is surrounded by forest and there is no agricultural land near the experimental plot.

Pine needles were collected in late June 2012 from three 23 year old *P. nigra*, three 30 year old *P. thunbergiana*, three 27 year old *P. thunbergiana* x *P. nigra*, and three 23 year old *P. nigra* x *P. thunbergiana* trees. One fully developed 1-year old shoot with needles from external and sunlit part of the crown was taken from each sampled tree. The fresh needles of all sampled trees from the same parental species or hybrids were bulked in equal mass proportion to form one pooled sample for each species/hybrid (*P. nigra*, *P. thunbergiana*, *P. thunbergiana* x *P. nigra* and *P. nigra* x *P. thunbergiana*).

2.3. Sample preparation for inorganic analysis

All samples were dried at 105 °C for 2 h to a constant weight, then homogenized and ground using a metal-free mortar. For the digestion, the MWS-2 Microwave System Speedwave BERGHOF (Germany) was used. Approximately 0.15 g weighed to the nearest 0.1 mg of the dried needles (triplicate for each sample) were mixed with 5 mL HNO₃ (7 mol/L) in the digestion vessels. The acidic digestion was carried out in three steps (1.–150 °C/10 min, 2. – 160 °C/10 min and 3. – 190 °C/20 min). The resulting clear solutions were then filled up to a total volume of 10.0 mL with ultrapure water. Blank solutions were prepared in the same manner.

2.4. ICP-AES

The elemental concentrations in the digests, blank and standard solutions were measured in triplicate with a Prodigy High Dispersive ICP-AES spectrometer working in a simultaneous mode (Teledyne Leeman, Hudson, NH, USA). The instrumental conditions applied are listed in Table 1. The emission lines selected for the determination of the elements were the following: Al (396.152 nm), B (208.956 nm), Ba (455.403 nm), Ca (396.847 nm), Cd (214.441 nm), Co (228.615 nm), Cr (267.716 nm), Cu (224.700 nm), Fe (238.204 nm), K (766.491 nm), Mg (280.271 nm), Mn (257.610 nm), Mo (202.030 nm), Na (589.592 nm), Ni (231.604 nm), Pb (220.353 nm), Sr (407.771 nm), and Zn (213.856 nm).

The matrix-matched standard solutions for external calibration were prepared by dilution from the above mentioned stock solution in the concentration range from 0.05 to 5.0 mg/L (modified when necessary according to the expected concentration ranges of the elements of

Table 1
ICP-AES operating conditions.

Instrument Spectrometer	Prodigy High Dispersive ICP High resolution Echelle polychromator Large format programmable array detector (L-PAD)
RF-Generator	40 MHz “free-running”
Output power	1.1 kW
Argon flow	Coolant: 18 L min ⁻¹ Auxiliary: 0.8 L min ⁻¹ Nebulizer: 1 L min ⁻¹
Peristaltic pump	1.0 mL min ⁻¹
Nebulizer	Pneumatic (glass concentric)
Spray chamber	Glass cyclonic
Plasma viewing	Axial
Replicates for each analysis run	3
Sample uptake delay	30 s

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