Environmental and Experimental Botany 107 (2014) 144–153

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Gas-exchange, photo- and antioxidant protection, and metal accumulation in I-214 and Eridano *Populus* sp. clones subjected to elevated zinc concentrations

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ARTICLE INFO

Article history:
Received 2 April 2014
Received in revised form 14 May 2014
Accepted 10 June 2014
Available online 18 June 2014

Keywords:
Antioxidant
Heavy metal
Phytoextraction
Phytoremediation
Hydroponics
Poplar

ABSTRACT

Rooted cuttings of Eridano and I-214 *Populus* clones were treated in hydroponics with high [Zn] to establish their phytoextraction capacity and physiological responses for phytoremediation. The Bio-concentration factor, Uptake ratio and Translocation factor revealed that the highest Zn accumulation occurred in roots. Lower accumulation at 1 mM [Zn] in Eridano aerial parts limited Zn toxicity on photosynthetic machinery. Increasing [Zn] negatively affected growth, net photosynthesis, stomatal conductance, maximum quantum yield, chlorophyll content and hydric parameters. The physiological impairment in I-214 at 1 mM [Zn] indicated a greater sensitivity to high [Zn] than Eridano. At 5 mM [Zn], high toxicity for both clones occurred. Upregulation of photoprotective and antioxidant responses was a consequence of Zn stress rather than a Zn tolerance mechanism. Increased de-epoxidation state of the xanthophyll-cycle, α-Tocopherol and reduced glutathione and decreased total phenolics in I-214 at 1 mM [Zn] suggested that it responded earlier to oxidative stress when compared to Eridano.

1. Introduction

Levels of heavy metals in soils have increased in recent decades as a consequence of contamination due to anthropogenic activities such as the application of pesticides, sewage sludge in soils, smelter and incineration emissions and mining activities, among many others (Chaney, 1993). Accumulation of heavy metals such as zinc (Zn) leads to severe damage of vegetation (Ernst and Joosse-van Damme, 1983). Furthermore, there are many sources of Zn contamination in soils and these are often associated with Pb, Cu, and Cd (Pedler et al., 2004).

Zinc is an essential micronutrient in plants and can act as a functional, structural and regulatory co-factor in a large number of enzymes (Barak and Helmke, 1993) such as RuBisCo (Brown et al., 1993). Zn plays an important role in plant metabolism (e.g. nitrogen metabolism, photosynthesis, transpiration and auxin synthesis) (Marschner, 1995) and has an essential function in the stability of plant cell membranes (Welch et al., 1982). Appropriate Zn concentrations in tissues increase plant productivity and growth however, becomes toxic at supraoptimal concentrations (Broadley et al., 2007). Symptoms of Zn toxicity in plants are similar to those found with Cd or Pb (Fodor et al., 2005). Excess Zn can directly affect plant growth, photosynthetic activity, water relations and metabolism (Apel and Hirt, 2004).

Zinc toxicity can affect water relations at multiple levels: impairment of water transport into root cells and through roots (Poschenrieder and Barceló, 1999), a decrease in leaf water content (Barceló and Poschenrieder, 1990; Bonnet et al., 2000) or impairment of stomatal conductance (g_s) through alterations of guard cell development and guard cell function which in turn decrease rates of transpiration and photosynthesis (Sagardoy et al., 2010).

Excess Zn inhibits photosynthesis at various stages and through different mechanisms. Zn has been shown to have a specific effect on the Calvin cycle (Chaney, 1993) and photosystem activities (Van Assche and Clijsters, 1986). Alterations in electron transport as a consequence of stress exposure result in the production of reactive oxygen species (ROS) (Asada, 1994). Also, excess Zn generates oxidative damage (Cakmak and Marshner, 1993) producing ROS (Kappus, 1985) and concomitantly induces the disruption of photosynthetic electron transport and the antioxidant defence system

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http://dx.doi.org/10.1016/j.envexpbot.2014.06.004
0098-8472/

(Cakmak, 2000). When ROS production exceeds the scavenging ability of a plant, oxidative damage can take place resulting in the degradation of cell membranes, nucleic acids and other cellular functions (Asada, 2006). In order to minimize ROS formation and prevent photoinhibition, plants increase thermal energy dissipation through the conversion of violaxanthin pigments to zeaxanthin in the xanthophyll-cycle (Demmig-Adams and Adams, 2006). On the other hand, ROS can be directly detoxified through several enzymatic pathways and antioxidant molecules such as ascorbate, α-Tocopherol and carotenoids (Foyer and Shigeoka, 2011). The antioxidant capacity of plants can be related to the tolerance to oxidative stress induced by high heavy metal exposure as reported (Zhu et al., 1999; Freeman et al., 2004; Liu et al., 2007). Information on the response of woody species to heavy metals is needed in order to optimize their use in the restoration of contaminated areas (Fernàndez et al., 2012; Romeo et al., 2014). Zn tolerant plants can be used to remove this metal by accumulating it in harvestable parts which can later be used to fertilize certain cereal crops, thereby increasing cereal grain yield (Zhang et al., 2013). Poplars are ideal candidates for the phytoremediation of contaminated substrates because of their fast growth, elevated water and nutrient usage, extensive root system (Mughini et al., 2013), high biomass production, and their capacity to absorb metals concentrate and tolerate them in their organs (Dos Santos Utmazian et al., 2007; Pietrini et al., 2010a). Plant capacity to accumulate Zn or Cd (unlike Cu, Pb or Ni) is dependent in part on their concentrations in soil and is probably due to their high solubility (Landberg and Greger, 1994). Zn uptake and translocation rates in some plant species is higher than those for Cd (Ali et al., 2000). *Populus* × *canadensis euramericana* Mönch.-I-214 clone and *P. deltoides* × *maximowiczii* – Eridano clone are largely used in poplar plantations in Italy for their adaptability to different environmental conditions. Their response and tolerance to heavy metals, especially Cd and Zn, have been reported (Sebastiani et al., 2004; Di Baccio et al., 2003; Zacchini et al., 2009; Romeo et al., 2014). In the present study, we have gone into greater depth in the study of the physiological effects of high [Zn] in order to expand our knowledge of this area, and especially in relation to the protection responses to stress at the chloroplast pigment and antioxidant level.

The general aims of this study were to assess (1) the Zn accumulation capacity of the *Populus* clones, I-214 and Eridano, and (2) their tolerance to Zn toxicity in order to provide relevant baseline information for future improvements in the field of phytoremediation techniques. A specific aim was to ascertain the contribution of photoprotective pigments and antioxidants to the tolerance of high Zn concentrations.

Populus cuttings were grown in hydroponic systems and exposed to 1 mM or 5 mM [$ZnCl_2$] threshold concentrations which have been described as inducing toxicity symptoms in poplar clones (Di Baccio et al., 2009). Measurements were performed on fully expanded leaves which had already developed before the application of Zn treatments and on fully expanded leaves developed after the application of treatments in order to evaluate the metal effect on plant development.

2. Materials and methods

2.1. Plant material and zinc treatments

Poplar woody cuttings (20-cm-long) obtained from Eridano (*Populus deltoides* × m*aximowiczii*) and I-214 (*Populus* × *canadensis euramericana* Mönch.) clones were collected from adult plants grown since 2001 in the IBAF experimental fields near Rome. Cuttings were rooted in spring (April) in pots filled with 6 L of water for 3 weeks. Thirty-six rooted cuttings of a similar size were selected and were placed in pots with 6 L of nutrient solution (Moore, 1974) at pH = 6.5. Cuttings were growing for 3 weeks in nutrient solution, which was replaced twice weekly. At the end of this period, groups of 6 cuttings were selected at random and placed in pots filled with nutrient solution containing 0 mM (control), 1 mM or 5 mM zinc chloride ($ZnCl_2$) for each clone. Hydroponic culture was performed in a greenhouse (Serveis de Camps Experimentals (UB)) under 65–75% relative humidity, 26 °C maximum air temperature and maximum photosynthetic photon flux density (PPFD) of approximately 1000 μmol m^{-2} s^{-1} at midday. Fully developed leaves before the Zn treatments were designated as old leaves, while fully developed leaves after the beginning of the treatments were designated as young leaves. After three weeks of treatment, sampling and measurements were carried out around midday (11:30 am–16:00 pm). Gas-exchange and chlorophyll fluorescence measurements were performed on uncut leaves of three plants per treatment. After these measurements were taken, plant parts were cut and separated into young leaves, old leaves, stems, woody cuttings and roots for biomass and Zn concentration determination of the dried material. Leaf samples from another three plants per treatment were selected and immediately frozen in liquid nitrogen, stored at −80 °C and lyophilized (Virtis Lyophiliser, Freezemobile 6EL, Gardiner, NY, USA). Lyophilized leaves were milled in a Cyclotec 1093/Foss Sample Mill (Tecator, Höganäs, Sweden) and thereafter all samples were stored until analyses of their chloroplast pigment, antioxidant content and carbon isotope composition were carried out.

2.2. Biomass determination and Relative Water Content

The samples of the different plant parts were weighed both for fresh weight (FW) and, after oven-drying at 60 °C until constant weight, for dry weight (DW). The relative water content (RWC) was calculated as [(FW − DW)/(FSW − DW) × 100], where FSW is fresh saturated weight (after rehydrating samples for 24 h in darkness at 4 °C). Leaf area (LA) was measured with a CI 2003 Laser Leaf Area Meter (CI-203) (CID, Inc., Camas, WA 98607, USA).

2.3. Zinc determination

Dried plants were carefully washed in distilled and MilliQ water. Leaves, stems and roots were ground to a fine powder in an agate mortar and woody cuttings were milled in a cutting mill Pulverisette 15 (Fritsch GmbH, Germany). Approximately 1 g of plant material was pre-digested in 5 mL of HNO_3 overnight at room temperature. Digestion was performed in a digestor block (JP Selecta) at 105 °C for three hours. A second digestion was done with 2.5 mL $HClO_4$ at 155 °C for two hours and afterwards at 180 °C for two hours. Zn content of different plant parts was determined by an Inductively Coupled Plasma Mass Spectrometry (ICP-MS PerkinElmer, model Elan-6000) at the Serveis Científico-Tècnics (UB). The Translocation factor (Tf), the Bio-concentration factor (BCF) and the Uptake ratio were calculated. Tf is defined as the ratio of metal concentration in aboveground parts to that in roots, and provides information about the capability of plants to translocate metals from the roots to the aerial parts. The BCF is defined as the ratio of metal concentration in the aerial parts and roots to that in the nutrient solution. The Uptake ratio provides information on the capability of the plant to extract the metal from the growing medium and to accumulate it in its structures. It was calculated as the total Zn content in each plant structure to the total Zn content in the different growth solutions at the end of the experiment. The Uptake ratio takes into account the metal content and the dry weight of each plant part, whereas the BCF only considers the metal concentration in each organ.