Environmental Pollution 159 (2011) 1058–1066

Contents lists available at ScienceDirect

Environmental Pollution

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

Changes in photosynthesis, mesophyll conductance to CO_2, and isoprenoid emissions in *Populus nigra* plants exposed to excess nickel

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The study reveals consequences of Ni stress on plant physiology, namely increasing diffusional limitation to photosynthesis and isoprenoid emissions.

ARTICLE INFO

Article history:
Received 23 September 2010
Accepted 18 October 2010

Keywords:
Isoprenoids
Photosynthesis
Mesophyll conductance
Nickel

ABSTRACT

Poplar (*Populus nigra*) plants were grown hydroponically with 30 and 200 μM Ni (Ni_{30} and Ni_{200}). Photosynthesis limitations and isoprenoid emissions were investigated in two leaf types (mature and developing). Ni stress significantly decreased photosynthesis, and this effect depended on the leaf Ni content, which was lower in mature than in developing leaves. The main limitations to photosynthesis were attributed to mesophyll conductance and metabolism impairment. In Ni-stressed developing leaves, isoprene emission was significantly stimulated. We attribute such stimulation to the lower chloroplastic [CO_2] than in control leaves. However chloroplastic [CO_2] did not control isoprene emission in mature leaves. Ni stress induced the emission of cis-β-ocimene in mature leaves, and of linalool in both leaf types. Induced biosynthesis and emission of isoprenoids reveal the onset of antioxidant processes that may also contribute to reduce Ni stress, especially in mature poplar leaves.

1. Introduction

Heavy metal pollution of soil and water is a problem of increasingly importance, because of the contamination of large areas worldwide due to the anthropogenic activity (e.g., industrialization, application of pesticides and fertilizers, mining, military applications, etc.) (Foy et al., 1978; Salt et al., 1998; Fargašová and Molnárová, 2010), and it has a strong impact on the environment and on the human health trough the food chain. Because of the different solubility most heavy metals may be available for living cells and are of some importance for organism and ecosystems under physiological conditions (Weast, 1984). Nickel (Ni), which is the 24th element in order of natural abundance in the Earth's crust (Garrett, 2000), is an essential mineral nutrient found in most natural soils at trace concentrations (Bai et al., 2006). However, high Ni concentrations may turn toxic to plants (Foy et al., 1978; Seregin and Kozhevnikova, 2006). Naturally elevated Ni concentrations can be observed in soils formed from serpentine (ultramafic) minerals (Kopittke et al., 2007). The sources of environmental Ni contamination are the production and processing of Ni, the recycling of Ni-containing products and waste disposal, land application of sludge, and the use of inorganic fertilizers and pesticides (Gimeno-García et al., 1996).

Several studies have shown that Ni excess inhibits photosynthesis (A) (Clijsters and Van Assche, 1985; Seregin and Kozhevnikova, 2006; Ahmed and Häder, 2010). Bazzaz et al. (1974) demonstrated that the diminished rate of photosynthesis in Ni-stressed sunflower was related to reduced stomatal conductance (g_s). This finding was confirmed by Ouzounidou et al. (2006) in a study on wheat. These authors also showed that Ni treatments reduced the maximum quantum yield of primary photochemistry, variable fluorescence (F_v) and chlorophyll concentration. Ni toxicity to photosynthetic apparatus was further showed by Drążkiewicz and Baszyński (2010), who found a marked decline of fluorescence induction kinetics as well as of chlorophyll and carotenoid concentrations in Ni-stressed plants of maize. However, the main mechanism primarily affecting photosynthesis in response to heavy metals, and to Ni stress in particular, is not clear. In fact, early literature reports the detrimental effects of heavy metals on g_s and the enzymatic capacity of the photosynthetic apparatus (Bertrand and Poirier, 2005; Seregin and Kozhevnikova, 2006). However, to the best of our knowledge, only the effect of zinc on mesophyll conductance to CO_2 (g_m) was described (Sagardoy et al., 2010). Being the second player in the diffusional limitations to A (Loreto

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doi:10.1016/j.envpol.2010.10.032

et al., 1992; Evans et al., 2009), g_m is a fundamental property of leaves. Mesophyll conductance influences photosynthetic capacity (Centritto et al., 2003; Flexas et al., 2008; Centritto et al., 2009), for it determines the chloroplastic CO_2 concentration (C_c), i.e., the [CO_2] available for photosynthesis in the chloroplasts. The first aim of this study was to investigate the effects of Ni stress on g_m and its relationship with photosynthetic capacity.

The second aim of this study was to investigate isoprenoid emissions in Ni-treated poplar saplings. Isoprenoid emissions, and isoprene in particular, have received attention not only because of their role in atmospheric chemistry but also for their putative defensive role in plant biology (Loreto and Velikova, 2001; Sharkey and Yeh, 2001). It has been demonstrated that isoprene emission modulates plant tolerance to heat (Sharkey and Singsaas, 1995; Velikova et al., 2006, 2009), pollutant and oxidative stress (Loreto and Velikova, 2001; Velikova et al., 2004). Isoprene biosynthesis is light dependent and is closely linked with carbon metabolism (Sharkey and Yeh, 2001), because about 80–90% of the carbon in the isoprene molecule originates from recently assimilated photosynthetic intermediates in non-stressed plants (Delwiche and Sharkey, 1993; Brilli et al., 2007). Despite this close dependence, there is an inverse relationship between isoprene emission and intercellular CO_2 concentration (C_i) in white poplar plants (Loreto et al., 2007). However, C_c and not C_i should drive the photosynthesis-dependent secondary metabolism. C_i has been used as a proxy of C_c with the implicit assumption that g_m is infinite, but this is not true in most of the cases (Loreto et al., 1992). Consequently, also isoprene dependence should be analyzed in terms of C_c instead of C_i.

Environmental stresses can affect emission of isoprenoids and the responses vary strongly depending on the physiological status of the plants, and severity and duration of the stress. For example, mild heat stress enhanced isoprene and monoterepene emissions (Sharkey and Loreto, 1993; Loreto et al., 1998; Staudt and Bertin, 1998; Velikova and Loreto, 2005). Moderate drought stress did not affect isoprene emission in *Populus alba* (Brilli et al., 2007) and *Quercus virginiana* (Pegoraro et al., 2004), but increased monoterepene emissions in *Quercus suber* (Staudt et al., 2008). However, both isoprene and monoterpene emissions decreased under severe drought (Pegoraro et al., 2004; Brilli et al., 2007; Fortunati et al., 2008) and then were induced at higher rates after recovering from drought stress (Brilli et al., 2007). In fact, apart from constitutive isoprenoid emissions, both abiotic and biotic stresses can induce the formation of an array of isoprenoids (Brilli et al., 2009; Loreto and Schnitzler, 2010). Understanding how stresses alter the constitutive emissions of isoprenoids while simultaneously inducing new emissions is crucial for quantitative prediction of isoprenoid emissions form stressed plants (Niinemets, 2010).

We hypothesized that exposure to heavy metal stress, as in the cases of other stresses, would affect the diffusional limitations to A as well as constitutive and induced isoprenoid emissions. To address the aforementioned questions, we designed an experiment in which *Populus nigra* plants were exposed to different concentrations of Ni. The reason for choosing poplar is that it is fast growing and, therefore, widely-used for bioremediation. Moreover, being poplar a strong isoprene emitter, the impact of heavy metal pollution on isoprenoid emissions needs to be assessed with high accuracy. We focused on the response of mature and developing leaves during Ni exposure, because these leaves are known to emit isoprene at different rates in poplar (Centritto et al., 2004).

2. Material and methods

2.1. Plant material and growing conditions

Woody cuttings of black poplar (*Populus nigra* L.) were rooted in perlite in a nursery. In April 2009, 20 cuttings were selected based on homogenous rutting pattern and uniformity of height (15–16 cm) and randomly assigned in 1.8 dm^3 pots. Plants were grown in hydroponic cultures in a climate chamber under controlled conditions: light intensity 350 μmol m^{-2} s^{-1}, day/night temperature 25/20 °C ± 2 °C, relative humidity 60–65%, and a photoperiod of 12 h. Plants were fertilized once a week with full-strength Hoagland solution.

2.2. Nickel treatment

Ni was added as nitrate ($Ni(NO_3)_2$) to a fresh nutrient solution at different concentrations: 0 μM (control), 30 μM (detected in preliminary experiments to provoke mild stress) and 200 μM (producing strong stress). There were five saplings per Ni treatments, which lasted two weeks. Two leaf types were analyzed: leaves that had already reached their fully expanded state (6th–7th from the plant apex, termed as "mature" leaves), and leaves that were still growing during Ni treatment (3rd–4th from the plant apex, termed as "developing" leaves).

2.3. Gas exchange and fluorescence measurements

Measurements of gas exchange, chlorophyll fluorescence and isoprenoid emissions (see below) were carried out 14 days after onset of Ni treatment. Steady-state A, g_s and transpiration were measured by a portable gas exchange system (LI-6400, Li-Cor, Lincoln, NE) equipped with a 6-cm^2 cuvette with a transparent window to allow illumination by natural light. The cuvette window was modified to accommodate a fluorescence probe (Fluorescence Monitoring System – FMS, Hansatech Instruments, UK). The tip of the optic fiber of the FMS was inserted in one corner of the cuvette window at an angle of 45° to the surface. The optic fiber could be placed about 1 cm from the leaf without shading it (Aganchich et al., 2009). Measurements were made in ambient [CO_2] (C_a, 380 μmol mol^{-1}) on individual leaves enclosed into a leaf cuvette under a rate of 0.44 L min^{-1} air flow, relative humidity within the cuvette at 50–55%, a leaf temperature of 25 °C and 500 μmol m^{-2} s^{-1} of light intensity. Then, gas exchange parameters and chlorophyll fluorescence yield were measured simultaneously. The maximum quantum yield of PSII in dark adapted leaves was estimated by the ratio between variable and maximal fluorescence, $F_v/F_m = (F_m - F_0)/F_m$. The efficiency of water-splitting apparatus was estimated by ratio between basal and variable fluorescence, F_0/F_v (Kriedemann et al., 1985). Mesophyll conductance to CO_2 was calculated by assuming that the electron transport rates calculated by gas exchange and by fluorescence match in the absence of internal resistances and photorespiration (Loreto et al., 1992). Oxygen concentration was lowered to 1.5% when testing leaf gas exchange under non-photorespiratory conditions as described in Loreto et al. (1994). The actual C_c was then calculated from the g_m value as shown elsewhere (Harley et al., 1992).

The A/C_i response curves were measured at 500 μmol m^{-2} s^{-1} light intensity and [CO_2] between 50 and 1400 μmol mol^{-1} after removing the stomatal limitation on A as described by Centritto et al. (2003) and Aganchich et al. (2009). The method of Sharkey et al. (2007) was used for fitting of A/C_c curves. The method fits the following parameters: maximum carboxylation rate allowed by Rubisco (V_{cmax}), rate of photosynthetic electron transport based on NADPH requirement (J_{max}), triose phosphate use (TPU) and day respiration (R_{day}). Stomatal limitation (L_s) of photosynthesis was estimated by using the following proportion $(A_0 - A)/A_0$ (Jones, 1985), where A is the mean of actual assimilation rate ($A_{C_a=380}$), and A_0 is the assimilation rate that would occur if resistances to CO_2 diffusion were zero (A at C_i equal to the growth C_a, $A_{C_i=380}$).

2.4. Isoprenoid emission measurements

To measure isoprenoid emissions, the leaf cuvette was disconnected from LI-6400 system, and the flow was diverted into a silcosteel cartridge packed with 200 mg of Tenax (Markes International Limited, UK). A volume of 6–12 L of air was pumped through the trap at a rate of 200 mL min^{-1}. The cartridge was analyzed by gas chromatograph coupled to a mass selective detector (Agilent Technologies, Wilmington, DE, USA). The GC was supplied with a thermal desorber UNITY (Markes International Limited). The GC was equipped with a splitless injector and an HP-5MS capillary column (30 m in length, 250 μm i.d. and 0.25 μm film thickness). The column oven temperature was kept at 40 °C for the first 5 min, then increased by 5 °C min^{-1} to 250 °C and maintained at 250 °C for 2 min. Helium was used as carrier gas. The concentration of each volatile compound was calculated by comparison with the peak area of a gaseous standard. The GC–MS was calibrated weekly using cylinders with standard of isoprene and α-pinene at concentration of 100 ppb (Rivoira, Milan, Italy). The compound identification was made using the NIST library provided with the GC/MS ChemStation software (Agilent). GC peak retention time was substantiated by analysis of parent ions and main fragments on the spectra.

2.5. Nickel determination

The same leaves used for physiological measurements were collected and dried at 80 °C until constant weight was reached. Mineralization was performed by treating 50 mg of powdered leaf samples with 4 ml of concentrated HNO_3 and 1 ml of H_2O_2 and by heating (TMD20 Heater System, Velp Scientifica, Milano, Italy) in two step procedure: 120 °C for 25 min followed by 250 °C for 15 min. The accumulation