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Physiological and growth responses to cadmium exposure in hydroponic culture of *Salicaceae* to select clones with phytoremediation ability

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1. GENERAL CONTEXT OF RESEARCH AND STATE OF THE ART

1.1 Heavy metals pollution

Heavy metals are ubiquitous environmental contaminants in industrialised societies. Soil pollution by metals differs from air or water pollution, because heavy metals persist in soil much longer than in other compartments of the biosphere (Lasat 2002). Over recent decades, the annual worldwide release of heavy metals reached 22,000 t (metric ton) for cadmium, 939,000 t for copper, 783,000 t for lead and 1,350,000 t for zinc (Singh et al. 2003). Sources of heavy metal contaminants in soils include metalliferous mining and smelting, metallurgical industries, sewage sludge treatment, warfare and military training, waste disposal sites, agricultural fertilizers and electronic industries (Alloway 1995). For example, mine tailings rich in sulphide minerals may form acid mine drainage (AMD) through reaction with atmospheric oxygen and water, and AMD contains elevated levels of metals that could be harmful to animals and plants (Stoltz 2004). Ground-transportation also causes metal contamination. Highway traffic, maintenance, and de-icing operations generate continuous surface and ground water contaminant sources. Tread ware, brake abrasion, and corrosion are well documented heavy metal sources associated with highway traffic (Ho and Tai 1988; Fatoki 1996; García and Millán 1998; Sánchez Martín et al. 2000). Heavy metal contaminants in roadside soils originate from engine and brake pad wear (e.g. Cd, Cu, and Ni) (Viklander 1998); lubricants (e.g. Cd, Cu and Zn) (Birch and Scollen 2003; Turer et al. 2001); exhaust emissions, (e.g. Pb) (Gulson et al. 1981; Al-Chalabi and Hawker 2000; Sutherland et al. 2003); and tire abrasion (e.g. Zn) (Smolders and Degryse 2002). Toxic heavy metals cause DNA damage, and their carcinogenic effects in animals and humans are probably caused by their mutagenic ability (Knasmuller et al. 1998; Baudouin et al. 2002). Exposure to high levels of these metals has been linked to adverse effects on human health and wildlife. Lead poisoning in children causes neurological damage leading to reduced intelligence, loss of short term memory, learning disabilities and coordination problems. The effects of arsenic include cardiovascular problems, skin cancer and other skin effects, peripheral neuropathy (WHO 1997) and kidney damage. Cadmium accumulates in the kidneys and is implicated in a range of kidney diseases (WHO 1997). The principal health risks associated with mercury are damage to the nervous system, with such symptoms as uncontrollable shaking, muscle wasting, partial blindness, and deformities in children exposed in the womb (WHO 1997). As

above-mentioned, heavy metals are present in soils and aqueous streams as both natural components or as a result of human activity (Raskin et al. 1994). Widespread low to medium pollution of agricultural land represents a specific problem and, in Europe, the polluted agricultural lands likely encompass several million of ha (Flathman and Lanza 1998). According to a report (ETCS 1998), there are 1,400,000 sites contaminated with heavy metals and/or organic pollutants in Western Europe alone. A European Union Council Directive (86/278/EEC, 1986) limited values for concentrations of heavy metals in arable soils to 3 mg kg^{-1} for Cd, 140 mg kg^{-1} for Cu, 75 mg kg^{-1} for Ni, 300 mg kg^{-1} for Pb, 300 mg kg^{-1} for Zn, and 1.5 mg kg^{-1} for Hg (Grčman et al. 2001). Besides, heavy metals are highly persistent in soils, with residence times in the order of thousands of years (McGrath 1987). Unless remediation action is undertaken, the availability of arable land for cultivation will decrease because of stricter environmental laws limiting food production on contaminated lands (Grčman et al. 2001), causing socioeconomic problems for the affected rural populations. Some metals are essential for life because they provide cofactors for metalloproteins and enzymes. On the other hand, at high concentrations, metals can act in a deleterious manner by blocking essential functional groups in biomolecules (this reaction has been reported mainly for non-redox-reactive heavy metals such as Cd and Hg) (Schutzendubel and Polle 2002), displacing essential metal ions, or modifying the active conformation of biological molecules (Collins and Stotzky 1989). Besides, they are toxic for both higher organisms and microorganisms (Garbisu and Alkorta 1997). Metal toxicity for living organisms is known to involve oxidative and/or genotoxic mechanisms and plants protect themselves by controlling root metal uptake and transport (Briat and Lebrun 1999). Inside plant cells, some proteins, such as ferritins and metallothioneins, and phytochelatins (glutathion-derived peptides) participate in excess metal storage and detoxification. Oxidative stress defense mechanisms also play an important role against metal toxicity in plants (Briat and Lebrun 1999). Sanità di Toppi and Gabbrielli (1999) reviewed several mechanisms of plant response to cadmium, such as phytochelatin-based sequestration and compartmentation processes, additional defense mechanisms based on cell wall immobilization, plasma membrane exclusion, stress proteins, stress ethylene, peroxidases, metallothioneins, etc.

1.2 Cadmium in the environment

Cadmium is a heavy metal naturally present in soil; it is non-essential and highly toxic to most organisms, having toxicity 2 to 20 times higher than many other heavy metals (Vassilev et al., 1998). Cadmium is the fourth most toxic metal to vascular plants (Jones, 1993; Oberlunder and Roth, 1978). It is placed in seventh position in the top ten priority hazardous substances list as provided by the American Agency for Toxic Substance and Disease Registry (Kamnev and Lelie, 2000), and therefore is considered a very serious pollutant. Total cadmium levels exceeding 8 mg kg^{-1}, or soluble (bioavailable) levels exceeding 0.001 mg kg^{-1}, are considered toxic to plants (Kabata-Pendius and Pendius, 1992; Bohn et al., 1985). The primary risk pathway associated with cadmium contaminated soils has been identified as the soil–plant–human pathway and the consumption of the crop or byproducts grown on these soils leads to its biomagnification in the food chain (Page et al., 1982). Anthropogenic pathways by which cadmium enters the environment are through industrial waste from processes such as electroplating, manufacturing of plastics, mining, paint pigments, alloy preparation, and batteries that contain cadmium (Adriano, 2001; Cordero et al., 2004). Household appliances, automobiles and trucks, agricultural implements, airplane parts, industrial tools, hand tools, and fasteners of all kinds (e.g., nuts, bolts, screws, nails) are commonly cadmium coated. Cadmium is also used for luminescent dials, in photography, rubber curing, and as fungicides (Adriano, 2001). Tobacco concentrates cadmium, leading to human exposure to this carcinogenic metal through smoking (Lugon-Moulin et al., 2004). Heavy metals enter soils through addition of sludge, composts, or fertilizers. Even with the strictest source control, domestic sewage sludge contains heavy metals because they are present in items washed down drains or toilets. Cadmium is given off from rubber when car tires run over streets, and after a rain, the cadmium is washed into sewage systems where it collects in the sludge. Composted sludge can contain high levels of cadmium. The composted sludge from Topeka, Kansas, which is applied to crop land, contains 4.2 mg/kg Cd (Liphadzi and Kirkham, 2006). Phosphate fertilizers are contaminated with cadmium. Zarcinas et al. (2004) attributed elevated levels of cadmium in soil and excessive concentrations of cadmium in cocoa (*Theobroma cacao*) in Peninsular Malaysia to input from phosphate fertilizers. People who smoke counterfeit cigarettes, which are packaged in the Far East or the Balkans and made to mimic legitimate brands, are exposed to increased concentrations of cadmium. The most likely origin of the excess cadmium is from heavy applications of cheap,

contaminated phosphate fertilizers (Booth, 2005; Stephens and Calder, 2005). Cadmium accumulates in animals, especially in the kidney, liver, and reproductive organs. Sheep in New Zealand are allowed to graze only a short period of time on pasturelands that have elevated cadmium concentrations due to repeated applications of cadmium-rich superphosphate fertilizer (Granel et al., 2002). The meat then has cadmium levels that are allowed in export. Elevated levels of cadmium in humans can cause kidney damage, and low levels of cadmium in the diet are linked renal dysfunction. Other diseases associated with cadmium exposure are pulmonary emphysema and the notorious Itai–Itai ("ouch–ouch") disease (Yeung and Hsu, 2005). It results in painful bone demineralization (osteoporosis), because cadmium replaces calcium in the bones. Cadmium poisoning has occurred worldwide. For example, it caused more than 100 deaths in Japan from 1922 to 1965 (Yeung and Hsu, 2005). Cadmium is one of the metals under scrutiny by the U.S. Environmental Protection Agency (EPA) (Hogue, 2004), and contamination from it occurs in more than 8% of hazardous waste sites in the United States (Yeung and Hsu, 2005).

1.3 Effects of cadmium toxicity in higher plants

Root tip damage is, together with decrease of root elongation rate, collapsing of root hairs or decrease of their number, decrease biomass, increase or decrease of lateral root formation, one of the main morphological and structural effects caused by cadmium (Hagemeyer and Breckle 1996). Inhibition of root extension growth can be the result of interference with cell division or with cell elongation. Trace elements have been shown to affect both processes. However, the result of stress depends not only on its cause, but also on its intensity (Hagemeyer and Breckle 1996). For example the dry mass production of roots was increased by low soil concentration of cadmium or zinc. But, at higher concentration of both metals, root growth was strongly inhibited (Hagemeyer et al. 1994). Cadmium also reduced the absorption of nitrate and its transport from roots to shoots, by inhibiting the nitrate reductase activity in the shoots (Hernandez et al., 1996). The inhibition of root Fe(III) reductase induced by cadmium led to Fe(II) deficiency, and it seriously affected photosynthesis (Alcantara et al., 1994). In a very general way, cadmium in plants causes leaf roll and chlorosis, and reduces growth, both in roots and in stems. This last effect is partly due to the suppression of the elongation growth-rate of cells, especially in the stem, because of an irreversible inhibition exerted by cadmium on the proton pump responsible for the process (Aidid and Okamoto, 1992).

Cadmium interacts with the water balance (Barceló and Poschenrieder, 1990; Costa and Morel, 1994) and damages the photosynthetic apparatus, in particular the light harvesting complex II (Krupa, 1988), and the photosystems II and I (Siedlecka and Baszynsky, 1993; Siedlecka and Krupa, 1996). In *Brassica napus* plants, cadmium lowered total chlorophyll content, carotenoid content, and increased the non-photochemical quenching (Larsson et al., 1998). Furthermore, cadmium inhibited the oxidative mitochondrial phosphorylation, probably increasing the passive permeability to H^+ of the mitochondrial inner membrane (Kessler and Brand, 1995). Cadmium also actively inhibits the stomatal opening, but how it does so has yet to be established. Probably the stomatal movements are not directly affected by cadmium, but rather are due to the strong interference of cadmium with movements of K^+, Ca^{2+} and abscisic acid in the guard cells (Barceló et al., 1986; Barceló and Poschenrieder, 1990). Reduction of chlorophyll content and of other pigments, such as carotenoids, is a common symptom of heavy metal toxicity (Baron et al., 1995; Krupa and Baszynski, 1995; Molas, 1997). In fact, the extent of loss of pigments (chlorphyll and carotenoids) is demonstrated to be a simple and reliable indicator of heavy metal toxicity in higher p1ants (Krupa et al., 1996). The effect seems to be common to higher plants, green algae and cyanobacteria. The decrease in chlorophyll content is contributed by both the inhibition of its biosynthesis and the induction of its degradation (Abdel-Basset et al., 1995; Molas, 1997). However, Baryla et al. (2001) showed that at least the cadmium dependent chlorosis in oil seed rape (*Brassica napus*) is not due to a direct intervention in chlorophyll biosynthesis. Instead, it is due to reduction in the chloroplast density per cell. Heavy metals can substitute for Mg in the chlorophyll, and since heavy metal substituted chlorophyll has been shown to lack photochemical properties of native chlorophyll, the process is implicated in the breakdown of photosynthesis (Kowalewska and Hoffmann, 1989; Kupper et al., 1998). The general physiological effects of heavy metal ions result from their ability to disrupt disulfide links in proteins and from the substitution of essential ions (Meharg, 1994). In general, heavy metals lower photosynthesis rates, affecting both light and dark reactions (Pietrini et. al., 2003, 2005). In addition to their direct action, determined in vitro, heavy metal ions induce specific indirect responses in plants which vary according to the concentration of metal ions used, length of exposure and the developmental stage of the plants at which the plants were subjected to stress. The metal ion treatment of plants induces lipid peroxidation in photosynthetic membranes (Sandmann and Boger, 1980), hydrolysis of membrane lipids and

release of free fatty acids (Skorzynska et al., 1991). Also, their action leads to inhibition of pigment (especially chlorophyll) biosynthesis (Vangronsveld and Clijsters, 1994), photosynthetic electron transport, Calvin-Benson cycle enzymes, protein synthesis and general disintegration of chloroplast membrane ultrastructure (Baszynski et al., 1988; Krupa and Baszynski, 1995; Maksymiec et al., 1995; Molas, 1997). The metal ion effects on the Rubisco (ribulose bisphosphate carboxylase oxygenase) may result from the substitution of metal ions at the Mg^{++} site in the ternary enzyme-CO_2-$metal^{+2}$ complex, or by reaction with the enzyme-SH groups (Clijsters and van Assche, 1985, Stiborova et al. 1986). The effects on in vivo primary photochemistry are indirect and arise from a lower utilization of ATP, NADPH, and from a higher thylakoid proton-gradient, resulting in a lower photochemical yield (Krupa et al., 1992, 1993). In general, the metal ion stress varies according to plant age, with plants at advanced growth stages showing more damage (Skorzynska and Baszynski, 1995, 1997; Skorzynska et al., 1995; Macsymiec and Baszynski, 1999; Tukendorf et al., 1997; Shaw and Rout, 1998). When relatively aged plants were treated with heavy metals, an increased damage was observed (Skorzynska and Baszynski, 1995). The threshold of metal ion toxicity seems to be specific to the metal, the plant and the age at which it is applied. A comparative study of the effect on the photosynthetic response of seagrass (*Halophila ovalis*) to Cu, Cd, Pb and Zn indicate that Cu and Zn are significantly more toxic than Pb or Cd (Ralph and Burchett, 1998). Even the specificity of heavy metal toxicity appears to be dependent on the growth stage of plant. The root growth study in metal treated plants revealed that Hg was more toxic than cadmium during germination, whereas cadmium was more toxic during the seedling stage (Shaw and Rout, 1998). Also, cadmium was found to produce oxidative stress (Hendry et al., 1992; Somashekaraiah et al., 1992), but, in contrast with other heavy metals such as Cu, it does not seem to act directly on the production of oxygen reactive species (via Fenton and:or Haber Weiss reactions) (Salin, 1988). On the other hand, cadmium ions can inhibit (and sometimes stimulate) the activity of several antioxidative enzymes. In *Helianthus annuus* leaves, cadmium enhanced lipid peroxidation, increased lipoxygenase activity and decreased the activity of the following antioxidative enzymes: superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase and dehydroascorbate reductase (Gallego et al., 1996). Cadmium treatment notably increased lipid peroxidation in pea plants (Lozano-Rodríguez et al., 1997), whereas no peroxidation was noticed in cadmium-exposed plants and hairy roots of *Daucus carota* (Sanità di Toppi et al., 1998).

Varying responses to cadmium-induced oxidative stress are probably related both to levels of cadmium supplied and to concentration of thiolic groups already present or induced by cadmium treatment. Thiols possess strong antioxidative properties, and they are consequently able to counteract oxidative stress (Pichorner et al., 1993).

1.4 Remediation technologies

Metal-contaminated soil can be remediated by chemical, physical or biological techniques (McEldowney et al. 1993). Chemical and physical treatments irreversibly affect soil properties, destroy biodiversity and may render the soil useless as a medium for plant growth. These remediation methods can be costly. Table 1 summarizes the cost of different remediation technologies.

Table 1 Cost of different remediation technologies (Glass, 1999)

Process	Cost (US$/ton)	Other factors
Vitrification	75–425	Long-term monitoring
Land filling	100–500	Transport/excavation/ monitoring
Chemical treatment	100–500	Recycling of contaminants
Electrokinetics	20–200	Monitoring
Phytoextraction	5–40	Disposal of phytomass

Among the listed remediation technologies, phytoextraction is one of the lowest cost techniques for contaminated soil remediation. There is a need to develop suitable cost-effective biological soil remediation techniques to remove contaminants without affecting soil fertility. Phytoremediation could provide sustainable techniques for metal remediation. Phytoremediation involves the use of plants to remove, transfer, stabilize and/or degrade contaminants in soil, sediment and water (Hughes et al. 1997). The idea that plants can be used for environmental remediation is very old and cannot be traced to any particular source. A series of fascinating scientific discoveries, combined with interdisciplinary research, has allowed phytoremediation to develop into a promising, cost-effective, and environmentally friendly technology. The term phytoremediation ("phyto" meaning plant, and the Latin suffix "remedium" meaning to clean or restore) refers to a diverse collection of plantbased technologies that use either naturally occurring, or genetically engineered, plants to clean contaminated environments (Cunningham et al. 1997; Flathman and Lanza 1998). Some

plants which grow on metalliferous soils have developed the ability to accumulate massive amounts of indigenous metals in their tissues without symptoms of toxicity (Reeves and Brooks 1983; Baker and Brooks 1989; Baker et al. 1991; Entry et al. 1999). The idea of using plants to extract metals from contaminated soil was reintroduced and developed by Utsunamyia (1980) and Chaney (1983). The first field trial on Zn and Cd phytoextraction was conducted by Baker et al. (1991).

1.5 Categories of Phytoremediation

Depending on the contaminants, the site conditions, then level of clean-up required, and the types of plants, phytoremediation technology can be used for containment or removal purposes (Thangavel and Subhuram 2004). Five main subgroups of phytoremediation have been identified: (1) phytostabilisation, plants reduce the mobility and bioavailability of pollutants in the environment either by immobilisation or by prevention of migration (Vangronsveld et al., 1995); (2) rhizofiltration, plant roots absorb metals from waste streams (Dushenkov et al., 1995); (3) phytovolatilization, plants extract certain metals from soil and then release them into the atmosphere by volatilization (Burken and Schnoor, 1999); (4) phytodegradation, plants and associated microbes degrade organic pollutants (Burken and Schnoor, 1997); and (5) phytoextraction, plants remove metals from the soil and concentrate them in the harvestable parts of plants (Kumar et al., 1995).

Initially, much interest focused on hyperaccumulator plants capable of accumulating potentially phytotoxic elements to concentrations more than 100 times than those found in nonaccumulators (Salt et al., 1998; Chaney et al., 1997; Raskin and Ensley, 2000). Metal concentrations in the shoots of hyperaccumulators normally exceed those in the roots, and it has been suggested that metal hyperaccumulation has the ecological role of providing protection against fungal and insect attack (Chaney et al., 1997). Exploitation of metal uptake into plant biomass as method of soil decontamination is limited by plant productivity and the concentrations of metals achieved (Baker et al., 1991). For instance, *Thlaspi caerulescens* is a known Zn hyperaccumulator, but its use in the field is limited because individual plants are very small and slow growing (Ebbs and Kochian, 1997). The ideal plant species to remediate a heavy metal-contaminated soil would be a high biomass producing crop that can both tolerate and accumulate the contaminants of interest (Ebbs and Kochian, 1997). Such a combination may not be possible, there may have to be a trade-off between

hyperaccumulation and lower biomass, and vice versa. Furthermore, the cropping of contaminated land with hyperaccumulating plants may result in a potentially hazardous biomass (Bañuelos and Ajwa, 1999).

1.6 Phytoremediation using trees

The potential use of trees as a suitable vegetation cover for heavy metal-contaminated land has received increasing attention over the last 10 years (Aronsson and Perttu, 1994; Glimerveen, 1996; EPA, 1999, 2000). Trees have been suggested as a low-cost, sustainable and ecologically sound solution to the remediation of heavy metal-contaminated land (Dickinson, 2000), especially when it is uneconomic to use other treatments or there is no time pressure on the reuse of the land (Riddell-Black, 1994). Benefits can arise mainly from stabilisation of the soil or waste, although in some cases phytoextraction may be sufficient to provide clean up of the soil. Before these benefits can be realised, the trees must become established on a site. On highly contaminated soils, or on mining wastes, tree establishment may be inhibited by high concentrations of heavy metals. Under such conditions root immobilisation, which would normally protect a plant, may not be able to prevent toxic amounts of metal being translocated to the aerial parts of the plant. In less-contaminated soils, other factors may limit plant growth; such as macronutrient deficiencies (Pulford, 1991) and physical conditions, especially those properties leading to poor waterholding, aeration and root penetration (Mullins, 1991). The physical and hydraulic conditions of a site are of primary importance to tree establishment. Phytostabilisation can result from either physical or chemical effects. Once the trees have become established, the vegetation cover can promote physical stabilisation of a substrate, especially on sloping ground. Long-term stability of the land surface can be achieved as the standing trees decrease erosion of the substrate by wind and water (Johnson et al., 1992). Trees have massive root systems, which help to bind the soil (Stomp et al., 1993), and the addition of litter to the surface quickly leads to an organic cover over the contaminated soil. In addition, transpiration of water by the trees reduces the overall flow of water down through the soil, thus, helping to reduce the amounts of heavy metals that are transferred to ground- and surface waters. Phytostabilisation of a heavy metal-contaminated substrate may also be achieved by causing chemical changes to specific metals, which result in their becoming less bioavailable. Chaney et al. (1997) identified two elements, Cr and Pb, which may be immobilised by a vegetation cover. They suggested that deep

rooting plants could reduce the highly toxic Cr(VI) to Cr(III), which is much less soluble and, therefore, less bioavailable (James, 2001). Although no mechanism for this was suggested, organic products of root metabolism, or resulting from the accumulation of organic matter, could act as reducing agents. It is known that Cr tends to be held in plant roots, whether supplied as Cr(VI) or Cr(III) (Pulford et al., 2001), which may also suggest reduction and immobilisation in the roots. Lead may be immobilised by the formation of the lead phosphate mineral chloropyromorphite in soils and within roots (Cotter-Howells et al., 1994), which has been shown to be formed in soils by *Agrostis capillaris* growing on lead/zinc mining wastes (Cotter-Howells and Caporn, 1996).

For the purposes of phytoextraction, Punshon et al. (1996) suggested that the following characteristics were beneficial:

a) ability to grow on nutrient-poor soil

b) deep root system

c) fast rate of growth

d) metal-resistance trait

In addition, an economically viable secondary use would be desirable. Trees have been shown to meet all of these requirements, the first three in particular. While a high metal content in agricultural crops is not desirable, and indeed is potentially dangerous, a higher metal content in trees is acceptable, as long as normal physiological activity is not affected (Labrecque et al., 1995).

1.7 The phytoremediation potential of poplars and willows

Several studies have shown the potential of willow for site reclamation and partial decontamination, as several species and clones of the genus *Salix* take up relatively high levels of heavy metals (Riddell-Black, 1994; Watson et al., 1999; Pulford et al., 2002). The same holds true for poplar. Poplars (or cottonwoods) are being used throughout North America to clean up sites that contain e.g. heavy metals, pesticides, and landfill leachates. Poplars are well suited for phytoremediation because they can remove contaminants in several ways, including degrading them, confining them, or by acting as filters or traps (Isebrands and Karnosky, 2001). Poplar and willow are often grown in short rotation coppice cultures (SRC), i.e. intensively managed plantations for rotations shorter than 15 years (Dickmann and Stuart, 1983; Macpherson, 1995). Plant material is selected for high biomass production, high growth

vigor, and disease resistance. Cultural management includes site preparation, high planting density, and coppicing (Dickmann and Stuart, 1983; Macpherson, 1995; Ledin and Willebrand, 1996). Coppicing refers to the cutting of a tree at the base of its trunk, resulting in the emergence of new shoots from the stump and/or roots (Blake, 1983). A coppice regime not only makes replanting of trees unnecessary for several rotations, but also results in a much higher biomass yield for several species (Sennerby-Forsse et al., 1992; Macpherson, 1995). Studies on phytoextraction have mainly focused on metal hyperaccumulating plants, as they accumulate 100–1000-fold the levels normally accumulated in plants, with no adverse effects on their growth (Reeves et al., 1999). However, hyperaccumulators are usually small with slow growth, and they have no economic value (Glass, 2000). In comparison with hyperaccumulators, trees tend to take up relatively small amounts of heavy metals, but they provide economic return of contaminated land through the production of biomass. Moreover, SRC has many additional ecological benefits, e.g. a positive impact on biodiversity, nutrient capture and carbon sequestration (Gordon, 1975; Perttu, 1995). Wood from SRC has traditionally been seen as a resource for the paper and pulp industry. But, in light of the greenhouse effect and the depletion of fossil fuels, SRC is now seen as a source of energy, because of the possibility of carbon sequestration and the substitution of fossil fuels. Furthermore, SRC on polluted land may reduce dust-blow, leaching and run-off of contaminated water (Watson et al., 1999; Isebrands and Karnosky, 2001).

Both biomass production and metal concentration should be taken into account when assessing the phytoextraction potential of a species or clone. Many studies have shown ‘‘toxic’’ metals to accumulate primarily in the root system; relatively high metal concentrations have also been found in leaves and bark (Rachwal et al., 1992; Landberg and Greger, 1996; Pulford et al., 2001; Thiry et al., 2002). As the amount of bark of a stem depends on its diameter, the shoot diameter distribution and population dynamics of the species or clone might also be considered. Large clonal variations in the number of shoots per stool and in the diameter distribution have already been demonstrated in poplar SRC (Laureysens et al., 2003) by repeated coppicing of the trees. Willow and poplar are considered best suited for this task because of their strong nature to coppice, their high capacity for metal uptake, and their high biomass production (Schnoor et al., 1996; Greger and Landberg, 1999; Robinson et al., 2000; Roselli et al., 2003). Salix spp grow well and fast in soils with large quantity of water and can tolerate moderate chilling and altitudes, while poplars can tolerate

moderately dry soils with discrete degree of salinity (Kuzminsky et al., 1999). At the same time the high transpiration capacity of salicaceae allows some poplar clones to extract from soils organic xenobiotics which are moderately hydrophobic. Plant density is also an interesting characteristic of salicaceae which can be as high as 8-10,000 per ha forming a dense and deep root system, useful to explore both superficial and deep ground contaminated soil. Moreover, salicaceae associate a very high biomass accumulation and transpiration rates with the ability of decontaminating soils from heavy metals (Robinson et al. 2000) and other contaminating substances such as hydrocarbon (Jordahl et al. 1997), herbicides (Gullner et al. 2001) and trichloroethylene (Newman et al. 1997). Furthermore, willows and poplars show a high intraspecific genetic polymorphism and a number of different genotypes could be available with a high degree of adaptability to a given climate and able to tolerate and uptake/degrade a given contaminant. For the genus *Populus,* for example, more than 30 species have been already classified, they all are diploids ($2n = 38$) and some species can be crossed. Hybrids are fertile and various pedigrees are also available. Further, the poplar genome is small (only 4 times that of *Arabidopsis*, and 400 times smaller than that of *Pinus*) and is available (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html). Bioinformatics is, thus, a powerful way to develop rapid analysis of genes involved in metabolic and physiological mechanisms useful for phytoremediation. The genus *Salix* is a member of the Salicaceae plant family. There are 400 species of willow, with more than 200 listed hybrids (Newsholme, 1992). The majority of the genus *Salix* grow in lowland wetland habitats and have evolved a number of varieties and hybrids (Sommerville, 1992). The large number of species and hybrids of *Salix* suggest a wide genetic variability within the genus. The genus features many species of high productivity and invasive growth strategies (Punshon et al., 1996). Many species, such as *S. caprea* and *S. cinerea*, and the hybrid *S. viminalis*, are known to colonise edaphically extreme soils (Dickinson et al., 1994).

1.8 Objectives

This thesis was realized in the frame of the PRIN project: this project aims to enhance the potentials to remediate the environmental contamination through biological systems. It is considered that poplars and willows can contribute to this objective for some reasons: a) poplars and willows have a fast rate of growth, high biomass productivity, high transpiration rate and grow easily from cuttings; b) there are experimental evidences of a wide genetic

variability between and within species concerning the ability to absorb and immobilize heavy metals inside the plants and their allocation to the various plant organs; c) exists a wide germplasm availability of both genera that include interspecific hybrids, partially of exotic provenance, and species of the national flora.

To evaluate phytoremediation capability and to explore genetic variability of some poplar and willow clones, a hydroponic screening for cadmium tolerance, accumulation and translocation was performed. Rooted cuttings were exposed for three weeks to 50 μM cadmium sulphate in a growth chamber and physiological parameters and cadmium content distribution among plant parts were evaluated.

In general, data obtained by hydroponic screening need to be confirmed by field performance trials, nevertheless Watson et al. (2003) have recently pointed out that results obtained in hydroponics and in field experiments are comparable.

In particular the work was structured in specific topics, according to the following different objectives:

1) Selection of poplar clones for heavy metal phytoextraction potential by analysis of growth and eco-physiological parameters and cadmium uptake. The aim of this study was to investigate the cadmium extraction, shoot distribution and tolerance of 10 poplar clones, preliminary selected for higher abilities of cadmium uptake among a larger number of clones from a germplasm collection established in central Italy. The evaluation of phytoremediation potential of these clones has been performed combining all chemical, biochemical, physiological and growth parameters.

2) Metal tolerance, accumulation and translocation in poplar and willow clones treated with cadmium in hydroponics. This study was aimed at evaluating the response of different poplar and willow clones for cadmium tolerance, accumulation and translocation in a hydroponic culture. The characterisation of several Salicaceae clones for the effectiveness to tolerate and bio-concentrate cadmium could be very interesting in specifying the potentiality of these plants to phytoremediate cadmium-polluted soils.

3) Imaging and characterisation of the damage exerted by cadmium on leaves of poplar and willow clones with different translocation capacity by chlorophyll fluorescence analysis. The aim of this study was to investigate the effects caused by cadmium exposure, at physiological level, on poplar and willow clones with different ability to translocate metal in leaves. These effects were analysed by gas exchanges and imaging chlorophyll fluorescence

analysis to evaluate the extent and the pattern of the damage produced by cadmium on leaves. Data obtained were discussed in order to depict different strategies of poplar and willow clones in accumulating and distributing cadmium over leaf blade and also to evaluate metal tolerance by an early screening through imaging chlorophyll fluorescence.

2. SELECTION OF POPLAR CLONES FOR HEAVY METAL PHYTOEXTRACTION POTENTIAL BY ANALYSIS OF GROWTH AND ECO-PHYSIOLOGICAL PARAMETERS AND CADMIUM UPTAKE.

2.1 Introduction

Many plant species can uptake quantities of cadmium from the soil or water contaminated around their root ambient (Perfus-Barbeoch et al., 2002). However, once that ions of this toxic heavy metal are inside roots they rarely move freely for long distance due to their high binding ability to the soft bases of nitrogen, sulphur or oxygen atoms widely present in many functional and structural groups of most biomolecules (Rauser, 1999). Thus, in general, between 70-85% (Wu, 1990) of the absorbed cadmium in many plants remains in roots where it can accumulates to concentration around some micromolars before it begins to significantly alter some functions and (ultra)-structural characteristics of this organ (Ederli et al., 2004). Chlorosis, lower carbon and nitrogen metabolic activities, and growth reduction are typical effects reported for most plants exposed to toxic amount of cadmium (Sanità di Toppi and Gabbrielli, 1999; Pietrini et. al., 2003). Alterations include also the reduced root absorption of some essential mineral elements (e.g. Mg and Fe) and thus, all consequent dysfunctions and structural changes in the above ground organs which can be attributed to the lack of these and other as well essential elements (Das et al., 1997). On another hand, in some species, those which have evolved for surviving in metalliferous soils, most of the high quantity absorbed heavy metals (between 0.1 and 1% of plant dry weight) results safely hyperaccumulated in shoots (Zhao et al., 2002). According to a systematic study, this hyperaccumulation, localisation and high tolerance of metals in shoots can also be observed in at least 45 families of higher plants (Schnoor, 2002). If such characteristics would be also observed in high biomass plants, trees for example, the extraction of metals like cadmium from mildly contaminated soils could be proposed with higher probability of success in environmental applications. On biomass basis, in fact, it is not excluded that the absolute amount of cadmium moving from the soil to the plant is higher in some trees than in some hyperaccumulators. Further, many trees, in addition to be fast growing characteristic, are also deep-rooted (useful in the case of immobile contaminants at depth), and easily propagating. Poplars and willows exhibit all of the above mentioned properties making them good candidates for cadmium phytoextraction. Moreover, cultural management of these Salicaceae

by means of short rotation coppice cultures is another interesting aspect to be considered for phytoremediation strategies (Ceulemans et al., 1992; Scarascia-Mugnozza et al., 1997). A large body of literature exists on the uptake capacity of cadmium by willow species and genotypes in field trials, greenhouse and hydroponic pot experiments (Landberg and Greger, 1996; Punshon and Dickinson, 1999; Pulford et al., 2002; Dickinson and Pulford, 2005; Cosio et al., 2006; Maxted et al., 2007). Many authors have reported the difference between willow and poplar clones in the partitioning of heavy metals within tree organs (Robinson et al., 2000; Lunáčková et al., 2003; Fischerova et al., 2006; Unterbrunner et. al., 2007). Nevertheless, only few works investigate responses of different poplar clones to the presence of cadmium in the growing medium (Lunáčková et al., 2003/4; Pilipovic et al., 2005; Dos Santos Utmazian et al., 2007). However, given the high variability of salicaceae in the environmental adaptation, there is a big scope to search for superior cadmium phytoextractor species or genotypes, particularly within poplars. The genus *Populus* is geographically widespread in various climatic areas and its presence can be observed in the severe soil conditions (pioneer species) that characterise heavily contaminated areas (Pulford and Watson, 2003). In screening (Dos Santos Utmazian et al., 2007) among some *Populus nigra* genotypes for cadmium removal from hydroponic medium only around 1% of the total absorbed cadmium is reported to accumulate in leaves. This low leaf cadmium content, with respect to willow where it is around 30% of the total absorbed (Robinson et al., 2000), is considered somehow as a positive characteristic which allows that only one third of cadmium return to the soil at the end of the growth cycle. Of course, it is fundamental that the amount missing in poplar leaves compared to willow is found all in stems. With this study we aimed to investigate the cadmium extraction, shoot distribution and tolerance of 10 poplar clones, preliminary selected for higher abilities of cadmium uptake among a larger number of clones from a germplasm collection established in central Italy. The evaluation of phytoremediation potential of these clones has been performed combining all chemical, biochemical, physiological and growth parameters.

2.2 Materials and methods

Plant material and growth conditions

Stem cuttings (20-cm-long) of *Populus x euramericana* - clones A4A, Luisa Avanzo and I-214*, Populus deltoides* - clone Lux*, Populus x interamericana* - clone 11-5*, Populus nigra* -

clones Poli and 58-861, *Populus alba* - clone 6K3 and 14P11, *Populus trichocarpa* - clone Nisqually were rooted and grown in pots filled with third-strength Hoagland's nutrient solution, pH 6.5 (Arnon and Hoagland, 1940). Cuttings were grown in a controlled climate chamber equipped with metal halide lamps (Powerstar HQI-TS; Osram, Munich, Germany) providing a photon flux density of 300 μmol m^{-2} s^{-1} for 14 h at 25°C. During the 10 h dark period the temperature was 20°C. The relative humidity was 70-80%. Plants were allowed to develop roots and grow three weeks in hydroponics before the cadmium treatment was started. At the end of this period cuttings of each clone were selected, weighed and randomly assigned to two groups of treatment with Hoagland's solution containing 0 μM (control) and 50 μM $CdSO_4$ (Sigma, St. Louis, USA) for three weeks. The nutrient solutions were completely replaced twice a week to prevent depletion of metals, nutrients and oxygen. Each treatment group consisted of five cuttings of each clone.

Biomass partitioning

At the end of the experiment all plants were harvested and washed without damaging the roots. Plant material was separated into roots, original cutting and shoots (leaves and secondary stems) and placed in a drying cabinet at 80°C until a constant weight was reached. Root, original cutting and shoot dry biomass was measured.

Cadmium determination

Dried samples of shoots, original cuttings and roots were weighed and ground. Approximately 0.2 g of material from each sample was weighed. Concentrated nitric acid (10 ml) was added to each tube and the mixtures heated on a heating block until a final volume of ca. 3 ml was reached. The samples were then diluted to 10 ml using deionised water and stored in plastic containers (Robinson et al., 2000). Metal determination was performed using an atomic absorption spectrophotometer (Perkin Elmer, Norwalk, CT, USA).

Gas exchange and chlorophyll fluorescence measurements

Net photosynthesis (A, A_{max}), stomatal conductance (g_s), transpiration (E), instantaneous water use efficiency (*i*WUE), efficiency of (PSII) photosystem II (F_v/F_m), quantum yield of electron transport through PSII ($\Delta F/F_m$) and quenching coefficient (qP and NPQ) were measured in the cuvette on the third fully expanded leaf with gas exchange system (HCM

1000, Walz, Germany), configured for simultaneous measurement of chlorophyll fluorescence (MINI-PAM, Walz, Germany). The relative humidity of air entering the cuvette was set at 50% and air and cuvette temperature was 25°C. CO_2 partial pressure was set at 370 µbar bar^{-1}. A white light source (KL 1500; Schott, Mainz, Germany) was used to vary the incident PPFD on the leaf surface between 300 and 700 µmol m^{-2} s^{-1} PPFD. Values for net CO_2 assimilation rate (A, A_{max}) and stomatal conductance (g_s) were calculated using the equations of von Caemmerer and Farquhar (1981). Instantaneous water use efficiency (*i*WUE) was calculated as the ratio between net photosynthesis (A) and transpiration (E) (Condon et al., 2002). The fluorescence quantum yield of electron transport through PSII ($\Delta F/F_m$) was estimated by dividing the difference between the maximum fluorescence (F_m) and the steady-state fluorescence (F_s) in the illuminated leaf ($\Delta F = F_m - F_s$) by F_m, as reported in Genty et al. (1989). Chlorophyll fluorescence quenching parameters were calculated as according to Schreiber et al. (1986). The efficiency of PSII (F_v/F_m) was estimated with Plant Efficiency Analyser (PEA, Hansatech, King's Lynn, Norfolk, UK) from the ratio of variable ($F_v = F_m - F_o$) to maximum fluorescence (F_m) measured on 30 min dark-adapted leaves. Plants were harvested the day after these measurements were completed to assess dry matter.

Pigment analysis

Two square centimeters of the same leaf used for photosynthesis and fluorescence measurements were ground under dim light in a mortar containing liquid N_2. When the leaf was reduced to a fine powder, 2 ml of methanol were added to extract the pigments. The samples were centrifuged at 12000 x *g* at 5°C for 10 min, and the supernatant was removed and used for pigment determinations. Absorbance was measured at 470, 652.4 and 665.2 nm with a spectrophotometer (Perkin Elmer, Norwalk, CT, USA). The extinction coefficients and the equations reported by Wellburn (1994) were used to calculate chlorophyll a and b and total carotenoid contents. Five replicates were done for each measurement.

Statistical analysis

Data for all eco-physiological and growth parameters were subjected to analysis of variance (ANOVA) using the SPSS software supplemented with multiple-comparison test of the means using the Tukey-Kramer method with a significance level of $P < 0.05$. We also used hierarchical cluster analysis to classify the clones basing on the results of our experiment.

2.3 Results

ANOVA results for the effects of cadmium treatment, poplar clones and their interaction were reported in Table 1. Effects due to cadmium were detected for all parameters except original cutting dry weight, root to shoot ratio and dark respiration. Differences among poplar clones were found for all parameters. Significant interactions between poplar clones and cadmium treatment were found in all parameters except original cutting dry weight and dark respiration.

Biomass production

Cadmium treatment reduced root and shoot dry masses in comparison to control in all clones except Lux (Fig. 1a), while there was no effect on original cutting dry mass (Fig. 1c). Moreover cadmium treatment caused an increase in the root to shoot ratio in Lux, I-214, and 6K3 with respect to control, while most of clones reduced this ratio (Fig. 1d). In particular, 11-5 and 14P11 decreased their root dry mass by around 80% of the control values (Fig. 1a). Besides, 11-5, 14P11, 6K3 and I-214 reduced their shoot dry mass with respect to control by around 65% (Fig. 1b). Under cadmium treatment, root and shoot dry masses differed significantly among poplar clones. In particular, Lux and A4A showed the highest values for root dry mass while 58-861 and Poli had the lowest ones (Table 2). On the other hand, A4A and Luisa Avanzo showed the highest values for shoot dry mass while I-214 and 58-861 showed the lowest ones (Table 2). Original cutting dry mass and root to shoot ratio differed significantly among poplar clones as well.

Table 1. ANOVA results for the effects of cadmium treatment, poplar clones and their interaction, on root, shoot, original cutting dry weight, root to shoot ratio, net photosynthesis (A and A_{max}), stomatal conductance (g_s), dark respiration (R_d), transpiration (E), instantaneous water use efficiency (*i*WUE), five chlorophyll fluorescence parameters (F_v/F_m, F_o, $\Delta F/F_m$, qP, NPQ), chlorophylls (Chl a, Chl b and Total Chl) and carotenoids (Total Car) content. Significance of the main effects and their interaction are indicated as * = $P < 0.05$, ** = $P < 0.01$, ns = not significant.

Parameter	Treatment	Poplar clones	Interaction
Root	**	**	**
Shoot	**	**	**
Original cutting	ns	**	ns
Root/Shoot	ns	**	**
A	**	**	**
A_{max}	**	**	**
g_s	**	**	**
R_d	ns	**	ns
E	**	**	**
*i*WUE	*	**	*
F_v/F_m	**	**	**
F_o	**	**	**
$\Delta F/F_m$	**	**	**
qP	**	**	**
NPQ	**	**	**
Chl a	**	**	**
Chl b	**	**	**
Total Chl	**	**	**
Total Car	**	**	**

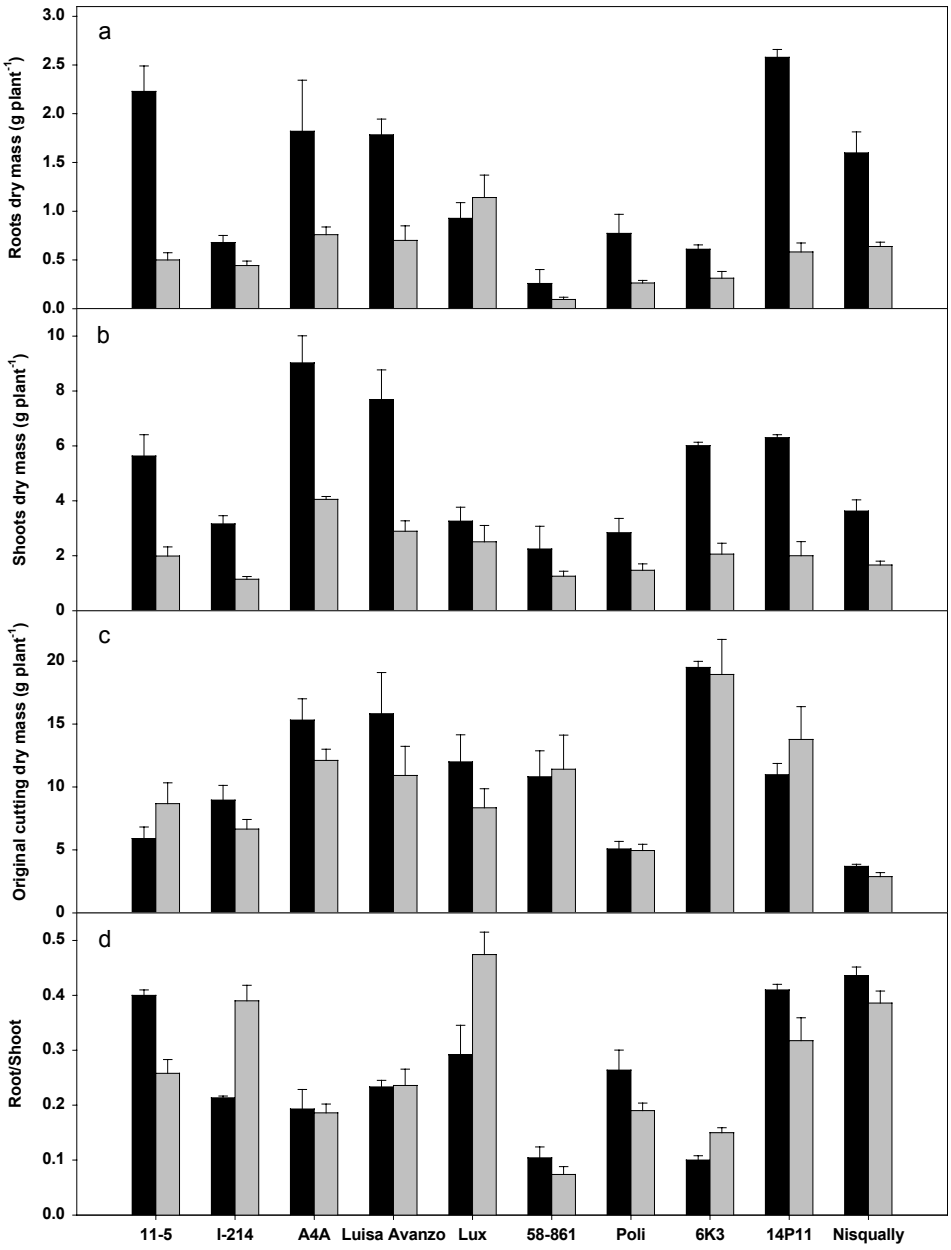

Figure 1. Effect of cadmium on biomass and root to shoot ratio: dry mass of roots (a), shoots (b), original cutting (c) and root to shoot ratio (d) measured at the end of the experiment in plants of 10 poplar clones grown in the presence of 0 μM (control, black bars) and 50 μM (grey bars) $CdSO_4$. Values are the mean of five replicates. Error bars indicate standard error.

Table 2. Dry biomass (g plant^{-1}) of roots, shoots and original cutting and root to shoot ratio measured in plants of 10 poplar clones grown in the presence of 50 μM $CdSO_4$ at the end of the experiment. Within a column, means values with a same letter were not significantly different ($P < 0.05$, ANOVA; Tukey-Kramer mean comparisons test). Values are the mean of five replicates.

Poplar clones	Roots	Shoots	Original cutting	Root/Shoot
11-5	0.50 bc	1.99 bc	8.67 bcd	0.26 cd
I-214	0.44 bc	1.14 d	6.65 bcd	0.39 ab
A4A	0.76 ab	4.05 a	12.11 abc	0.19 de
Luisa Avanzo	0.70 ab	2.90 b	10.90 abc	0.23 cd
Lux	1.14 a	2.51 b	8.35 bcd	0.47 a
58-861	0.09 d	1.26 cd	11.41 abc	0.07 e
Poli	0.26 c	1.47 cd	4.95 cd	0.19 de
6K3	0.31 c	2.06 bc	18.95 a	0.15 de
14P11	0.58 b	2.00 bc	13.78 ab	0.31 bc
Nisqually	0.64 ab	1.66 c	2.88 d	0.39 ab

Cadmium content

The metal content in plant parts was calculated by multiplying dry weight of part by metal concentration (data not shown) (Table 3). The highest cadmium content in roots was measured in Lux, Nisqually and Luisa Avanzo while the lowest ones were measured in 58-861 and 6K3. Cadmium content in shoots was significantly higher in A4A and Lux than other clones. The lowest values were found in Poli and 58-861. On the other hand, 14P11 and Poli exhibited the highest values of cadmium content in original cuttings while Nisqually had the lowest one. The metal content in whole plant was different among poplar clones. In particular, Lux, A4A and Luisa Avanzo showed the highest values while 58-861 and 6K3 had the lowest ones. The extent of metal translocation to the above ground organs was estimated on percentage of distribution between plant parts (Table 4). For most poplar clones the highest cadmium content was found in roots; it was intermediate in original cuttings and lowest in shoots. Nevertheless three clones accumulated a large amount of cadmium in the original cutting. In fact 58-861, Poli and 6K3 accumulated about 45% of cadmium in the original cutting and only about 1% in shoots.

Table 3. Cadmium content (mg plant part^{-1}) in roots, shoots, original cutting and whole plant measured in plants of 10 poplar clones grown in the presence of 50 μM $CdSO_4$ at the end of the experiment. Within a column, means values with a same letter were not significantly different ($P < 0.05$, ANOVA; Tukey-Kramer mean comparisons test). Values are the mean of five replicates.

Poplar clones	Roots	Shoots	Original cutting	Whole plant
11-5	4.83 bc	0.33 bc	1.24 bc	6.39 bc
I-214	4.37 bc	0.23 bc	1.19 bc	5.79 bc
A4A	5.85 ab	0.71 a	1.99 ab	8.55 ab
Luisa Avanzo	6.07 ab	0.20 c	0.98 bc	7.25 ab
Lux	7.42 a	0.55 ab	1.84 ab	9.81 a
58-861	1.75 d	0.04 c	1.51 ab	3.30 d
Poli	2.91 cd	0.03 c	2.05 a	4.99 bc
6K3	1.91 d	0.08 c	1.83 ab	3.82 cd
14P11	4.71 bc	0.11 c	2.22 a	7.04 b
Nisqually	6.18 ab	0.08 c	0.75 c	7.01 b

Table 4. Distribution of cadmium content (%) among roots, shoots and original cutting measured in plants of 10 poplar clones grown in the presence of 50 μM $CdSO_4$ at the end of the experiment. Within a column, means values with a same letter were not significantly different ($P < 0.05$, ANOVA after angle transformation (arc sin $\sqrt{\%}$); Tukey-Kramer mean comparisons test). Values are the mean of five replicates.

Poplar clones	Roots	Shoots	Original cutting
11-5	75.54 abc	5.09 ab	19.37 cd
I-214	75.52 abc	3.96 b	20.52 cd
A4A	68.43 bcd	8.29 a	23.28 cd
Luisa Avanzo	83.75 ab	2.75 c	13.50 de
Lux	75.63 abc	5.65 ab	18.72 cd
58-861	53.07 de	1.20 de	45.73 ab
Poli	58.22 de	0.66 e	41.12 ab
6K3	50.11 e	2.01 c	47.88 a
14P11	66.95 bcd	1.52 cd	31.53 bc
Nisqually	88.22 a	1.10 de	10.68 e

Gas exchange parameters

Significant treatment effects were found for leaf gas exchange parameters, measured during the experiment. Cadmium treatment reduced net photosynthesis both in growth (A) and in saturating light conditions (A_{max}) in comparison to control in all clones (Fig. 2 b,c). In particular, A4A and Nisqually decreased their assimilation rate (A and A_{max}) by around 85-90% of the control values. Under cadmium treatment, 11-5, I-214, Luisa Avanzo, Lux and Poli exhibited the highest rates for A and A_{max} while, A4A and Nisqually had the lowest ones (Table 5). The treatment negatively affected stomatal conductance (g_s) and transpiration (E) rates of all poplar clones. In fact, most of clones reduced their stomatal conductance and transpiration rates with respect to control by around 80-90% (Fig. 2d,e). Under cadmium treatment, Lux and 11-5 had the highest values for g_s and E while Nisqually had the lowest one (Table 5). Under cadmium treatment most of poplar clones showed an increase of instantaneous water-use efficiency (*i*WUE) values with respect to control (Fig. 2f). In particular, 6K3 exhibited the highest value while A4A and Lux had the lowest ones (Table 5). The treatment with cadmium did not affect dark respiration (R_d) (Fig. 2a). However, R_d was different among poplar clones. Specifically, Lux showed the highest value for R_d while Nisqually had the lowest one (Table 5).

Chlorophyll fluorescence parameters

The efficiency of PSII (F_v/F_m) was generally affected by poplar clones and cadmium treatment (Fig. 3a). Several clones maintained the same level of F_v/F_m in treatment and control conditions. Besides A4A, Lux, I-214, showed a reduction of this value with respect to control. Under cadmium treatment 58-861 exhibited the highest F_v/F_m absolute value differing significantly with those of A4A, I214, Lux, 6K3 and Poli (Table 6). Cadmium treatment increased the basal fluorescence emission (F_o) in comparison to control in all clones except Luisa Avanzo, 58-861 and Nisqually (Fig. 3b). In fact, at the end of the treatment A4A, Lux and 6K3 exhibited the highest values for F_o while 58-861 had the lowest one (Table 6). The fluorescence quantum yield of electron transport through PSII ($\Delta F/F_m$) was generally affected by cadmium treatment and showed a trend similar to that of F_v/F_m (Fig. 3c). In particular, A4A showed a value of $\Delta F/F_m$ significantly lower than other clones (Table 6). Photochemical (qP) and non-photochemical quenching of fluorescence (NPQ) were generally affected by clones and cadmium treatment (Fig. 3d,e). These parameters decreased and increased with

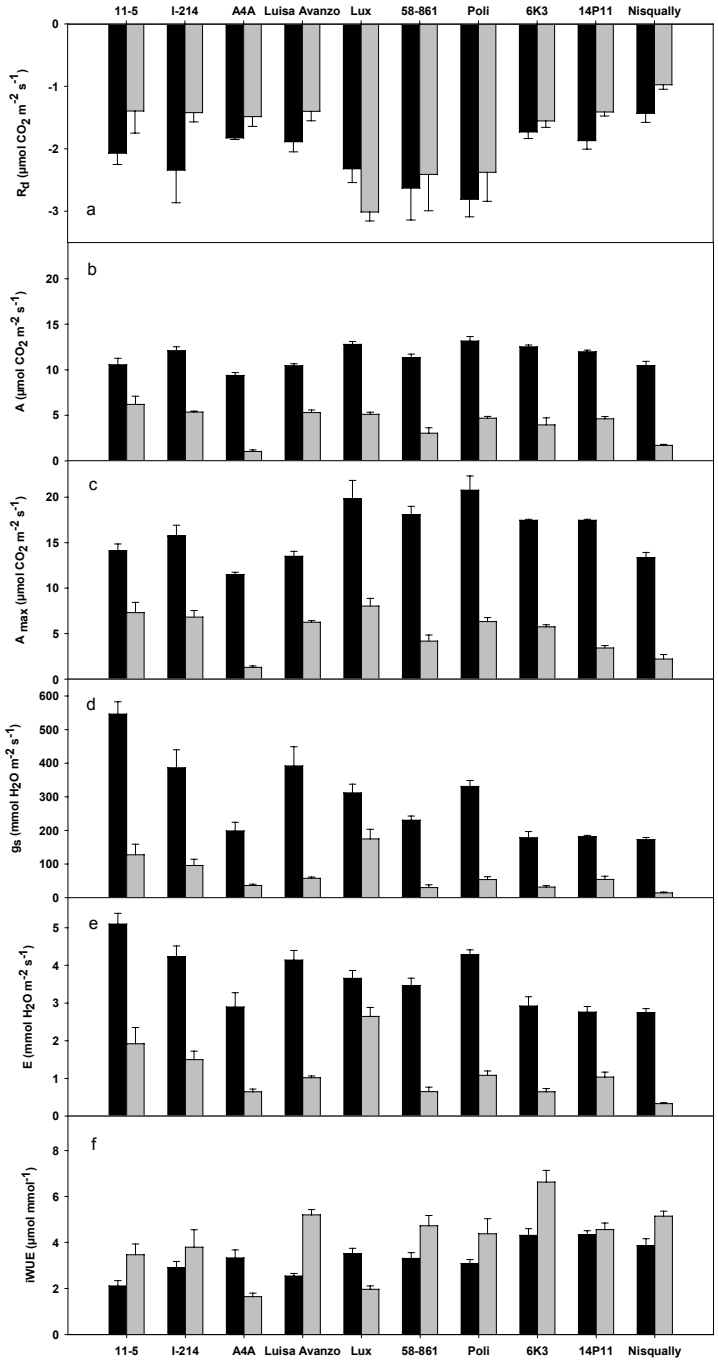

Figure 2. Effect of cadmium on gas exchange parameters: dark respiration, R_d (a), net photosynthesis in growth A (b) and saturating light conditions A_{max} (c), stomatal conductance, g_s (d), transpiration, E (e), instantaneous water use efficiency, *i*WUE (f), measured at the end of the experiment on the third fully expanded leaf in plants of 10 poplar clones grown in the presence of 0 µM (control, black bars) and 50 µM (grey bars) $CdSO_4$. Values are the mean of five replicates. Error bars indicate standard error.

Table 5. Net photosynthesis A and A_{max} (μmol CO_2 m^{-2} s^{-1}), dark respiration R_d (μmol CO_2 m^{-2} s^{-1}), stomatal conductance g_s (mmol H_2O m^{-2} s^{-1}), transpiration E (mmol H_2O m^{-2} s^{-1}), and instantaneous water use efficiency *i*WUE (μmol $mmol^{-1}$) measured on the third fully expanded leaf in plants of 10 poplar clones grown in the presence of 50 μM $CdSO_4$ at the end of the experiment. Within a column, means values with a same letter were not significantly different ($P < 0.05$, ANOVA; Tukey-Kramer mean comparisons test). Values are the mean of five replicates.

Poplar clones	A	A_{max}	R_d	g_s	E	*i*WUE
11-5	6.20 a	7.31 ab	-1.39 ab	127.90 ab	1.92 ab	3.46 b
I-214	5.36 ab	6.82 ab	-1.42 ab	95.86 b	1.50 b	3.79 b
A4A	1.03 d	1.31 e	-1.49 ab	36.17 c	0.65 cd	1.64 c
Luisa Avanzo	5.30 ab	6.31 ab	-1.40 ab	57.23 bc	1.02 c	5.21 ab
Lux	5.11 ab	8.01 a	-3.01 c	174.75 a	2.65 a	1.96 c
58-861	3.04 bcd	4.22 cd	-2.41 bc	29.90 c	0.65 cd	4.72 b
Poli	4.68 ab	6.31 ab	-2.38 bc	53.70 bc	1.09 c	4.38 b
6K3	3.95 bc	5.81 bc	-1.55 ab	31.32 c	0.65 cd	6.62 a
14P11	4.63 ab	3.41 d	-1.41 ab	53.95 bc	1.04 c	4.55 b
Nisqually	1.70 cd	2.22 de	-0.97 a	14.56 d	0.33 d	5.14 ab

respect to the presence of cadmium, in accordance with the lower photosynthesis and higher dissipation as heat of the absorbed energy. Under cadmium treatment, A4A exhibited the lowest qP absolute value differing significantly with those of other clones (Table 6). On the other hand, NPQ showed a different trend. A4A was the clone with the highest NPQ absolute values differing significantly with those of Nisqually, 6K3, 58-861 and I-214.

Photosynthetic pigments

Significant treatment and clone effects were found for all photosynthetic pigments, measured during the experiment. Most of clones were affected by cadmium treatment that generally reduced a, b and total chlorophyll content in comparison to control (Fig. 4a,b,c). In particular, A4A and Lux decreased their chlorophyll a, b and total chlorophyll content by around 75% of the control values, while Poli and Luisa Avanzo maintained similar values both in treatment and in control conditions. Under cadmium treatment, chlorophyll a, b and total chlorophyll content differed significantly among poplar clones. Specifically, Poli had the highest value

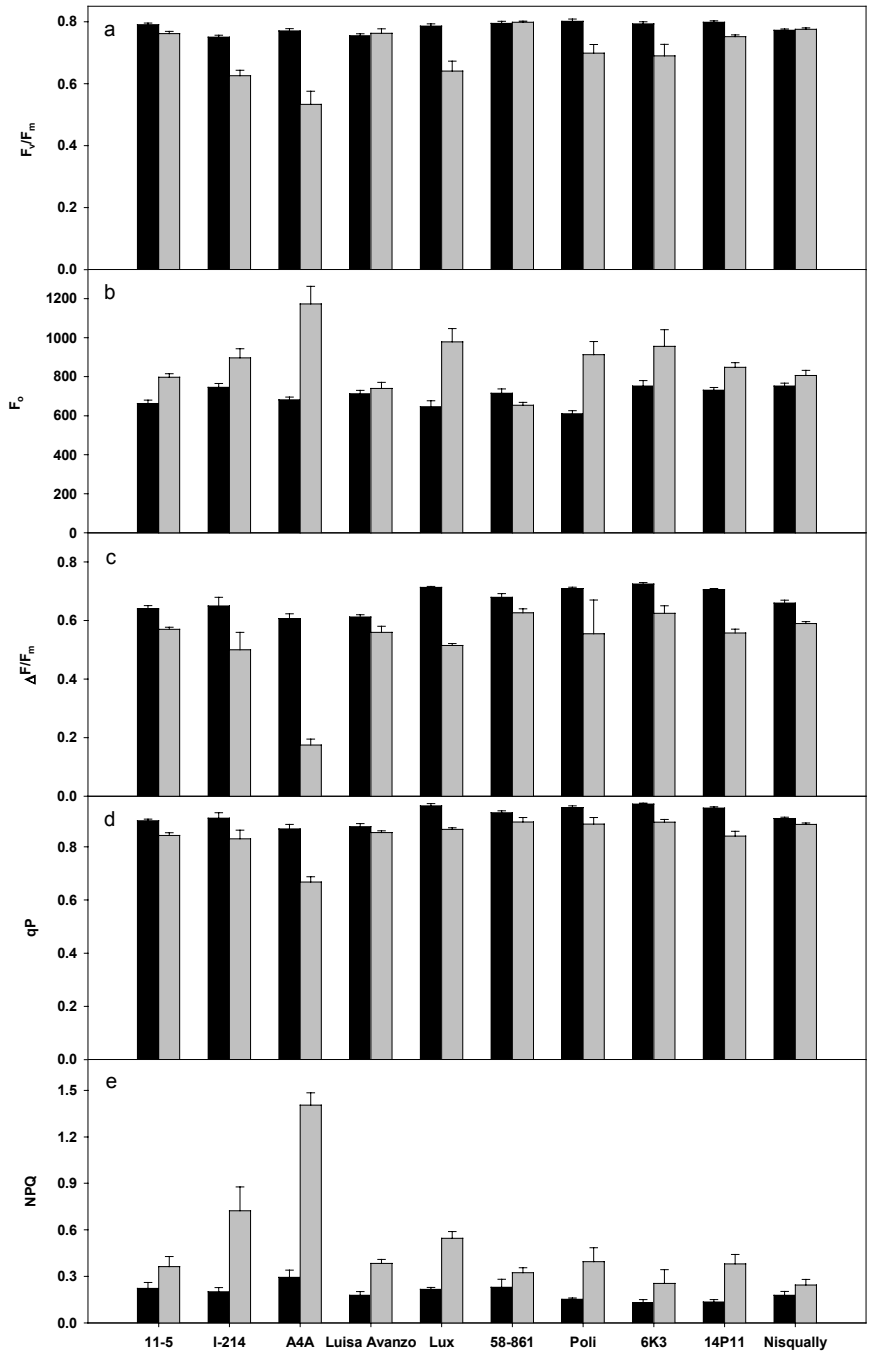

Figure 3. Effect of cadmium on chlorophyll fluorescence parameters: fluorescence ratio F_v/F_m (a) and basal fluorescence F_o (b) of dark adapted leaves, fluorescence estimation of PSII quantum yield, ($\Delta F/F_m$) (c) photochemical qP (d) and nonphotochemical quenching NPQ (e) measured at the end of the experiment on the third fully expanded leaf in plants of 10 poplar clones grown in the presence of 0 μM (control, black bars) and 50 μM (grey bars) $CdSO_4$. Values are the mean of five replicates. Error bars indicate standard error.

while A4A and Lux showed the lowest ones (Table 7). Total carotenoid content increased significantly in comparison to control in Luisa Avanzo and 11-5, while showed a reduction in the other clones (Fig. 4d). At the end of the treatment, 6K3 and Poli had the highest carotenoid content values while A4A and I-214 exhibited the lowest ones (Table 7).

Table 6. Fluorescence ratio F_v/F_m and basal fluorescence F_o of dark adapted leaves, fluorescence estimation of PSII quantum yield $\Delta F/F_m$, photochemical qP and non-photochemical quenching NPQ measured on the third fully expanded leaf in plants of 10 poplar clones grown in the presence of 50 μM $CdSO_4$ at the end of the experiment. All data are expressed in relative units. Within a column, means values with a same letter were not significantly different ($P < 0.05$, ANOVA; Tukey-Kramer mean comparisons test). Values are the mean of five replicates.

Poplar clones	F_v/F_m	F_o	$\Delta F/F_m$	qP	NPQ
11-5	0.76 ab	797.0 bc	0.57 ab	0.84 a	0.36 bc
I-214	0.63 cd	896.6 bc	0.50 bc	0.83 a	0.72 b
A4A	0.53 d	1172.5 a	0.18 d	0.67 b	1.41 a
Luisa Avanzo	0.76 ab	740.0 bc	0.56 ab	0.85 a	0.39 bc
Lux	0.64 cd	978.0 ab	0.51 bc	0.86 a	0.55 bc
58-861	0.80 a	654.2 c	0.63 a	0.89 a	0.32 c
Poli	0.70 bc	912.7 b	0.55 ab	0.89 a	0.40 bc
6K3	0.69 bc	956.0 ab	0.63 a	0.89 a	0.25 c
14P11	0.75 ab	848.5 bc	0.56 ab	0.84 a	0.38 bc
Nisqually	0.78 ab	806.5 bc	0.59 ab	0.88 a	0.24 c

Cadmium tolerance

We classified the 10 poplar clones into four groups by means of hierarchical cluster analysis based on a comparison of all the data obtained (for all parameters) during the experiment (Fig. 5). Based on this analysis, clones 11-5, Luisa Avanzo and Poli appear to be cadmium-tolerant; clones I-214, Lux, 6K3 and 14P11 moderately cadmium-tolerant, clones 58-861 and Nisqually barely metal-tolerant; and A4A highly cadmium- susceptible.

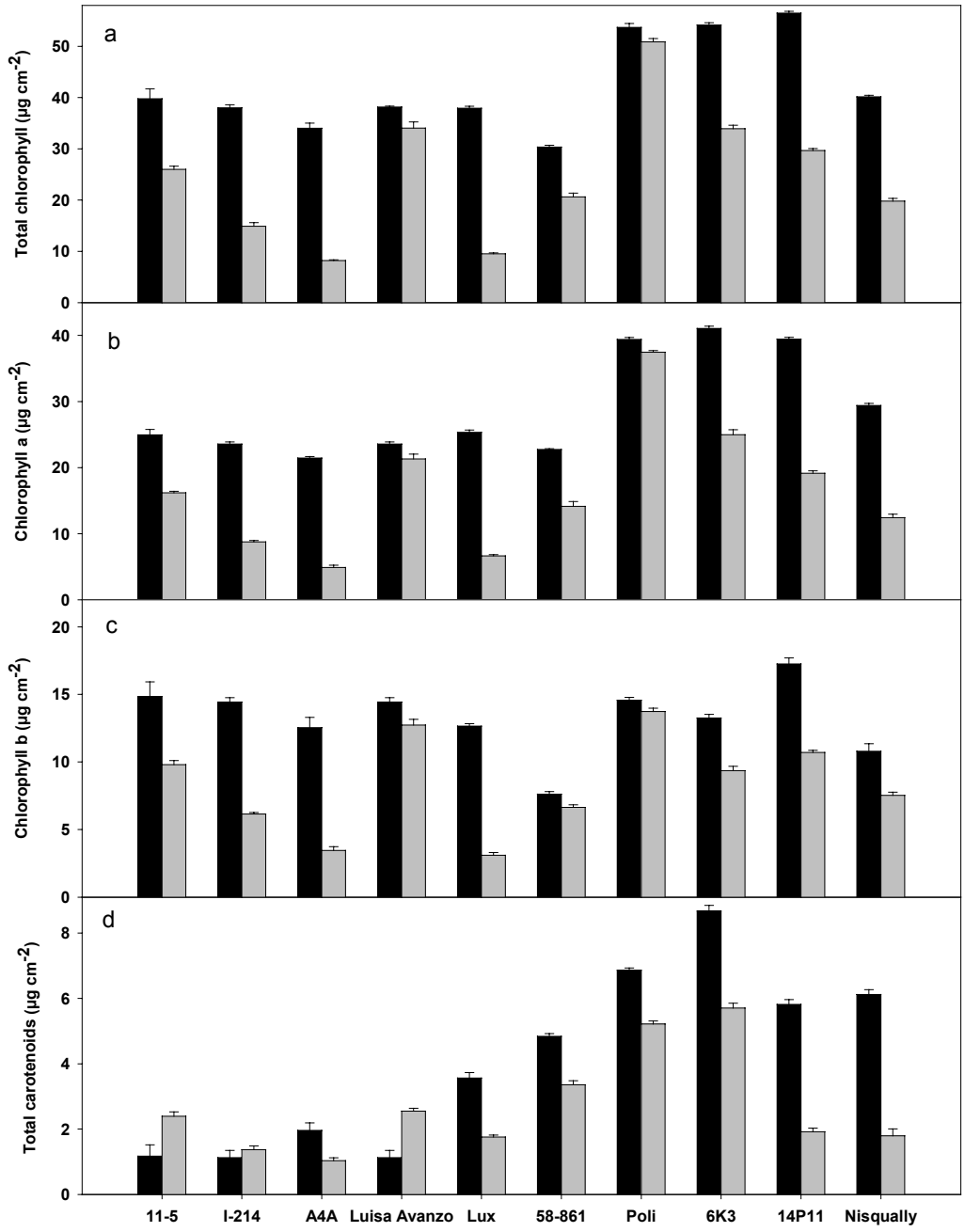

Figure 4. Effect of cadmium on pigment content: total chlorophyll (a), chlorophyll a (b), chlorophyll b (c) and total carotenoid (d) contents measured at the end of the experiment on the third fully expanded leaf in plants of 10 poplar clones grown in the presence of 0 µM (control, black bars) and 50 µM (grey bars) $CdSO_4$. Values are the mean of five replicates. Error bars indicate standard error.

Table 7. Total chlorophyll (tot chl), chlorophyll a (chl a), chlorophyll b (chl b) and total carotenoid (tot carot) contents (μg cm^{-2}) measured in plants of 10 poplar clones grown in the presence of 50 μM $CdSO_4$ at the end of the experiment. Within a column, means values with a same letter were not significantly different ($P < 0.05$, ANOVA; Tukey-Kramer mean comparisons test). Values are the mean of five replicates.

Poplar clones	tot chl	chl a	chl b	tot carot
11-5	25.99 d	16.19 e	9.80 cd	2.39 cd
I-214	14.91 f	8.76 g	6.15 f	1.37 fg
A4A	7.96 g	4.78 i	3.18 g	1.03 g
Luisa Avanzo	34.03 b	21.30 c	12.73 b	2.55 c
Lux	9.32 g	6.47 h	2.85 g	1.72 ef
58-861	20.42 e	13.94 f	6.48 f	3.23 b
Poli	50.78 a	37.17 a	13.61 a	5.11 a
6K3	33.77 b	24.74 b	9.03 d	5.55 a
14P11	29.67 c	19.15 d	10.52 c	1.91 de
Nisqually	19.81 e	12.44 f	7.37 e	1.79 ef

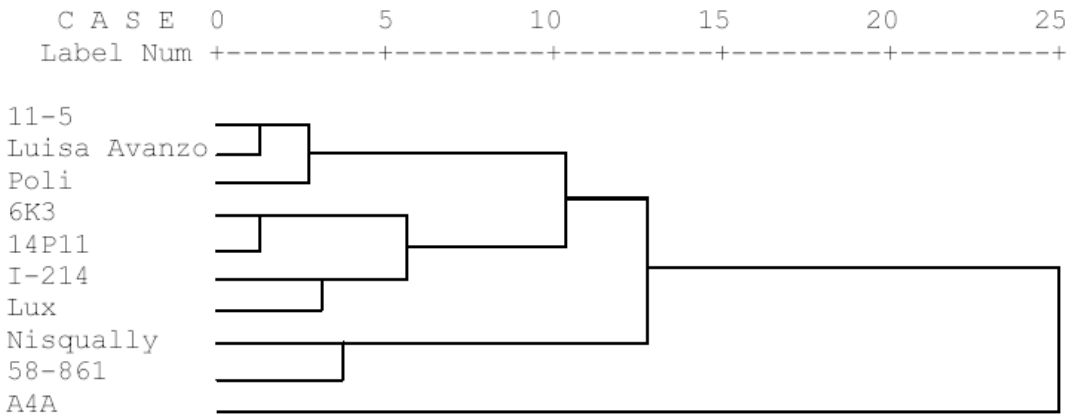

Figure 5. Cluster analysis dendrogram of the performance of the 10 poplar clones grown in the presence of 0 μM (control) and 50 μM $CdSO_4$.

2.4 Discussion

We focused our research work on 10 poplar clones of different species exposed to 50 μM $CdSO_4$ for 3 weeks and we analysed their growth and eco-physiological responses to individuate clones with best metal uptake capacity and tolerance.

As reported by many authors, growth inhibition is one of the main symptoms of cadmium phytotoxicity. In the present experiment we found different responses to metal exposure among clones, ranging from severe to slight reduction of root and shoot biomass production compared to the control. Shoot and root biomass reduction was found at the end of the treatment in all clones except Lux (Fig. 1a,b). Under cadmium treatment A4A, Lux and Luisa Avanzo showed the highest values of shoot and root dry mass (Table 2). In agreement with other authors (Shulka et al., 2003; Šottníková et al., 2003) we found that roots responded to cadmium presence more sensitively than shoots, but this effect is mainly due to the higher cadmium concentration in roots. Nevertheless, we found an increase in the root to shoot ratio in Lux, I-214 and 6K3 with respect to control, while most of clones showed a decrease of the ratio (Fig 1d). This is probably due to a different strategy of the biomass allocation (Lunáčková et al. 2003/4; Hagemeyer and Breckle, 1996). Another root toxicity symptom observed was root browning (data not shown). It has been reported that root browning was due to an enhanced suberization or lignification of roots tips that consequently lost their capacity for nutrient uptake (Kahle, 1993; Hagemeyer and Breckle, 1996; Schützendübel and Polle, 2002). Although cadmium probably has a significant effect on root hairs (Gussarson, 1994), this was not observed in the tested clones due to the lack of root hairs in plants grown in hydroponics (Hagemeyer and Breckle, 1996). However, the degree of growth reduction and metal accumulation in response to cadmium are strictly dependent on species and clones (Landberg and Greger, 1996; Punshon and Dickinson, 1999). With regard to the metal accumulation, our results are in line with previous investigations (Cosio et. al., 2006) carried out in hydroponics and showed that for all tested clones the highest cadmium content was found in roots (Table 3). Among clones, Lux and A4A showed the highest values of cadmium content in shoot tissue, while Poli exhibited the highest one in the original cutting. As described in results section, the investigated clones varied substantially in their distribution pattern of cadmium among root, shoot and original cutting. If we consider the data reported in Table 4 we can distinguish three principal patterns of metal distribution among root, shoot and original cutting:

a) Low percentage of cadmium in roots (< 67%) associated with high percentage of cadmium in original cutting (> 31%) and low percentage in shoot (< 2%), indicating limited transfer to shoot (low shoot accumulators): 58-861; Poli; 6 K3; 14P11.
b) Medium percentage of cadmium in roots (67-76%) associated with medium percentage of cadmium in original cutting (18-23%) and high percentage in shoot (> 4%), indicating high metal uptake and efficient transport to shoot (shoot accumulators): 11-5; I-214; A4A; Lux.
c) High percentage of cadmium in roots (> 83%) associated with low percentage of cadmium in original cutting (<13%) and medium percentage in shoot (1-3%), indicating high metal uptake but inefficient transfer to shoot (root accumulators): Luisa Avanzo; Nisqually.
Accumulation of metal primarily in harvesting organs, (i.e. woody structures of stem and branches) instead of leaves, which return metals to ground annually due to shedding, is an important trait for a phytoremediation species. In fact, metal accumulation in leaves may have some adverse effects on the environment, e.g. metal accumulation in the topsoil via leaf decomposition (Vandecasteele et al., 2003) or metal contamination for the food chain (Vandecasteele et al., 2002). On the contrary, decomposition of wood and bark tissues is slow. Accumulated metals can be immobilised in a metabolically inactive compartment for a considerable period of time (Lepp, 1996), if the contaminated trees are not reused for other purposes which accelerate the return of the heavy metals to the environment. According to Dickinson and Lepp (1997) our results showed that metal content in wood are commonly lower than in roots, but wood represents a much more significant proportion of total biomass, so metal content of wood can contribute for a major part of the total amount of metal in a tree.
Other important indicators to evaluate clones response to cadmium exposure are the eco-physiological parameters. In fact, the photosynthetic apparatus appears to be especially sensitive to cadmium (for reviews see Krupa, 1999; Sanità di Toppi and Gabbrielli, 1999; Joshi and Mohanty, 2004; Pietrini et al., 2005).
In plants cadmium affects a number of physiological processes, directly or indirectly. In the former case, cadmium interferes with chlorophyll biosynthesis and degradation, assembly of pigment protein complexes and thylakoids, the electron transport chain, Calvin cycle enzymes, sugar transport and consumption and oxidative stress (Stiborova et al., 1986; Krupa, 1988; Becerril et al., 1988; Boddi et al., 1995; Siedlecka et al., 1997; Seregin and Ivanov, 2001). In the latter case, the heavy metal disturbs water and ion uptake which consequently negatively affects the plant water status (Seregin and Ivanov, 2001). In our study

photosynthesis and stomatal conductance of all clones decreased in response to cadmium treatment compared with the control; values for some clones, such as A4A and Nisqually were considerably reduced (Fig. 2b,c,d). Clones that are sensitive to cadmium often exhibit severe reduction in CO_2 assimilation and stomatal conductance (Barcelo and Poschenrieder, 1990; Baryla et al., 2001). As reported by Pietrini et al. (2003) considering the sensitivity of photosynthesis to cadmium, it can be expected that an effective cadmium tolerance must include the ability to widely protect and maintain photosynthetic activity. The high accumulation in the roots and low transport of heavy metals to the shoot is probably a mechanism evolved to protect plant organs involved in photosynthesis (Landberg and Greger, 1996). Our data showed that clone A4A, which had the highest content of cadmium in the shoot, exhibited a very low level of photosynthetic activity (Table 5). Nevertheless, Nisqually showed a very low photosynthetic rate despite its low cadmium content in shoot tissue. This decrease in the activity of the photosynthetic apparatus may be related to general indirect effect of cadmium ions by changes in the root system and, in particular, due to water stress induced by cadmium. In fact, cadmium treatment could have negatively affected water absorption and transport and suppressed transpiration causing a strong reduction in photosynthesis in Nisqually (Sheoran and Singh, 1993; Prasad, 2003). Dark respiration data showed a different behaviour among clones; nevertheless these results are in line with many authors which found both a reduction (Losch and Kohl, 1999; Seregin and Ivanov, 2001) and an increase of dark respiration (Arisi et al., 2000; Van Assche and Clijsters, 1990) in plants exposed to cadmium. As reported by Stomp et al. (1993), high transpiration rate is one of woody plant characteristics for effective phytoextraction. In this work, most clones reduced their transpiration rate with respect to control; nevertheless, clones Lux, 11-5 and I-214 were less affected by the treatment (Fig. 2e). The decreased transpiration observed in treated plants implies that cadmium also affects water relations (Hagemeyer and Waisel, 1989). The consequence was that most clones showed an increase of *i*WUE with respect to control. However, clones Lux, I-214 and 14P11 maintained *i*WUE values near or slightly lower than control plants showing an efficient regulation capacity of water balance (Fig. 2f). Another effective way to analyse the influence of stressors (also heavy metals) on photosynthesis in vivo is to detect chlorophyll fluorescence and to evaluate the quenching components as these methods have the advantage of being both non-invasive and non-destructive (Schreiber and Bilger, 1987). As reported by many authors (Greger and Ogren, 1991; Krupa et al., 1992;

Pietrini et al., 2003; Linger et al., 2005), concentrations of cadmium that significantly suppressed the growth and pigment biosynthesis only marginally decreased the efficiency of PSII (F_v/F_m) in dark adapted leaves. Our data showed a similar response for most of studied clones. In fact, cadmium treatment strongly reduced F_v/F_m in comparison to control in A4A, while it slightly decreased in I-214 and Lux (Fig. 3a). The F_v/F_m ratio depends on the variations of F_o and F_m. In particular, as reported by Gilmore et al. (1996), F_o increases when the photochemical apparatus is damaged or, more specifically, when the number of functional chlorophylls not connected to the reaction centres of PSII increases. The decrease of F_o is, on the contrary, an indication of a high-energy dissipation in the minor antenna. Our data indicated that cadmium treatment significantly increased F_o in comparison to control in all clones except Luisa Avanzo, Nisqually and 58-861 (Fig. 3a). These last clones showed a reduction of F_m (data not shown) proportional to F_o, thus explaining the maintenance of high F_v/F_m ratio and possibly indicating balanced damage, or close regulation of, energy harvesting and energy conversion capacities. Although cadmium did not cause a reduction in F_v/F_m in all clones, it did induce a reduction in photosynthetic electron transport. Differences in the fluorescence parameters, specifically of $\Delta F/F_m$, qP and NPQ, are indications of reduced electron transport between cadmium treated and control plants (Fig. 3c,d,e). The quantum yield of electron transport through PSII ($\Delta F/F_m$) (Genty et.al., 1989) and photochemical quenching, qP, reflecting the number of open reaction centres, are indicators for the capacity of photochemical processes. Non-photochemical quenching component, NPQ (or qN), unites processes that are associated with heat dissipation and most of the time-reversible inactivation of PSII reaction centres to prevent destruction of the photosynthesis apparatus (Krause and Weis, 1991; Horton et al., 1996). Some authors have found that plants exposed to cadmium reduced $\Delta F/F_m$ and qP and increased NPQ with respect to control (Krupa et al., 1993; Di Cagno et al., 1999; Linger et al., 2005). Our results are in agreement with previous investigations and exhibited similar responses. Nevertheless, this effect was particularly evident in clone A4A which showed a decrease in $\Delta F/F_m$ and an increase in NPQ near three times lower and higher than control, respectively (Fig. 3c,e). NPQ increased in all clones with respect to control, indicating that mechanisms able to dissipate excess excitation energy were involved, and implying that energy consumption is inhibited by cadmium, probably through an inhibition of enzymes of the Calvin cycle (Stiborova, 1988; Sheoran et al., 1990; Chung and Sawhney, 1999). As reported by Krupa et al. (1993) and Skorzynska and Baszynsky

(1997) a slight increase in NPQ indicates a higher dissipation of absorbed energy as radiationless decay and protects the leaf from a damage; nevertheless an excessive enhancement of NPQ could be the symptom of incapacity in down-regulation of PSII efficiency to reduce the electron pressure in electron transport chain and switch over energy consumption to heat dissipation (Linger et al., 2005).

The reduction of chlorophyll and carotenoid content is another common symptom of cadmium toxicity (De Filippis et al., 1981; Sheoran et al., 1990; Krupa et al., 1993). Such a decrease in chlorophyll content may be caused by both the inhibition of its biosynthesis and the induction of its degradation (Stobart et al., 1985; Abdel-Basset et al., 1995; Boddi et al., 1995). Our data indicated that cadmium treatment decreased chlorophyll content (both a and b) in comparison to control in all clones except Luisa Avanzo and Poli (Fig. 4a,b,c). In particular, the values of clones A4A and Lux were considerably reduced. These results confirm that clones with higher cadmium content in shoot tissue exhibited an evident leaf chlorosis. Larsson et al. (1998) showed that in *Brassica napus* plants, cadmium lowered total chlorophyll content, carotenoid content, and increased the non-photochemical quenching. Our results are in agreement with this last indication but showed a sensible increase of carotenoids in clones 11-5 and Luisa Avanzo (Fig. 4d). It is known that carotenoids are involved in the safe heat dissipation of excess absorbed energy and singlet oxygen (Asada and Takahashi, 1987), this last formed when an excess of photons are loaded to the light harvesting antenna. Thus, it is reasonable to assume that the increase or the maintenance of high level of carotenoids (probably xantophylls) in some clones is a protective process induced by cadmium to enhance the heat dissipation capacity.

2.5 Conclusions

We observed the responses to cadmium treatment of 10 poplar clones. The results indicated that some clones were better able to survive, grow and accumulate cadmium than others. In fact these clones adapted to cadmium presence, by means of growth and eco-physiological changes, such as maintenance of photosynthesis and transpiration, cadmium allocation in roots or original cuttings, developing of protective mechanisms and changing the allocation pattern of biomass. Among the clones that we studied, 11-5, Luisa Avanzo and Poli appear to be metal-tolerant; I-214, Lux, 6K3 and 14P11 seem to be moderately metal-tolerant; 58-861 and Nisqually appear to be barely metal-tolerant; and A4A may be considered highly

cadmium-susceptible. Among the physiological parameters analysed in this work, transpiration rate, net photosynthesis and chlorophyll content provided useful information to asses differences in tolerance response. On the basis of this classification, if we consider metal accumulation, distribution and tolerance capacity, we could individuate the best application for each clone in phytoremediation strategy. In particular, metal tolerant clone 11-5, able to accumulate cadmium on shoot tissue, could be of interest for phytoextraction. The other metal tolerant clone Luisa Avanzo, able to accumulate cadmium mainly on roots, could be used for rhizofiltration or phytostabilization. Finally the clone Poli, able to allocate a high percent of cadmium on stem tissue, could be efficiently used for phytoremediation in short rotation coppice cultures. This classification confirms the high genetic variability of the *Populus* genus. A large part of this variability is under a moderate to strong genetic control (Farmer, 1996). The molecular analysis of this genetic diversity could be a useful tool for further screening and selection of poplar clones with best physiological and morphological response to heavy metals stress.

3. METAL TOLERANCE, ACCUMULATION AND TRANSLOCATION IN POPLAR AND WILLOW CLONES TREATED WITH CADMIUM IN HYDROPONICS

3.1 Introduction

The enhanced level of pollutants in soil and water due to industrialisation is one of the major environmental problems at global scale. In particular, cadmium is considered one of the most widespread pollutants, having toxic effects on plants and animals. Cadmium enters the environment from industrial processes, heating systems, urban traffic, phosphate fertilizers and mineralisation of rocks (Rauser and Muwly, 1995). Plants exposed to toxic cadmium concentration undergo a stress condition, revealed by damage symptoms such as chlorosis, growth inhibition, reductions in water and nutrient uptake, alteration of enzyme activity and photosynthesis impairment (Sanità di Toppi and Gabbrielli, 1999; Pietrini et al., 2003). To remove cadmium and other pollutants from contaminated areas, unconventional techniques that utilise biological processes have been successfully applied. In particular, plants can be used for removing heavy metals from soil and accumulate them in the harvestable parts. This technology, called phytoextraction (Kumar et al., 1995; Raskin et al., 1997, Padmavathiamma and Li, 2007), is less expensive and environmental disruptive than conventional remediation systems that consist mainly in the excavation and incineration of soil (Cunningham and Ow, 1996). Other advantages of utilising plants to clean up contaminated areas are the production of biomass, which can be eventually used for producing energy and other commodities. The efficiency of the phytoextraction depends largely on the metal bioavailability in the contaminated matrix as well as on several plant characteristics such as the capability to hyperaccumulate metals, also the not essential ones, a fast growing, a depth and large root system and the ability to translocate metals in the aerial parts.

In the last years, forest trees have been studied for assessing the potentiality to remediate heavy-metal contaminated sites (Rosselli et al., 2003; Pulford and Watson, 2003; Unterbrunner et al., 2007). Some aspects of forest tree biology and cultivation sound very interesting for phytoremediation strategy. Among them, the large biomass yield that can be used for energy production, an extended and depth root apparatus, a low impact on trophic chains and the adaptability of some tree species to grow in marginal soils. Respect to hyperaccumulating plants, metal uptake by trees is reported to be smaller but, on an hectare basis, the removal of heavy metals from soil could be more effective due to the higher

biomass production, given the general good association for non hyperaccumulating plants between the driving force of water transpiration and metal extraction.

Several studies have focused the potentiality of willows and poplars for phytoextraction (Riddell-Black, 1994; Punshon and Dickinson, 1999; Robinson et al., 2000; Pulford et al., 2002; Laureysens et al., 2004a; Kuzovkina and Quigley, 2005). In fact, these Salicaceae are reported to be adapted to grow in severe soil conditions that characterise contaminated areas, besides their capability to accumulate heavy metals (Pulford and Watson, 2003). Cultural management of willows and poplars by means of short rotation coppice cultures (SRC) is another interesting aspect to be considered for phytoremediation strategies (Ceulemans et al., 1992; Scarascia-Mugnozza et al., 1997; Perttu, 1999; Rockwood et al., 2004). In this context, Dickinson and Pulford (2005) have reported that willow SRC can be utilised as an efficient and cost-effective method to remove cadmium contamination from agricultural soils. Moreover, a significant clonal variability for heavy metal accumulation in poplar and willow was found (Landberg and Greger, 1996; Watson et al., 1999; Laureysens et al., 2004b).

Most of the studied conducted on trees evidenced that heavy metals accumulation pattern show a predominant compartmentalisation in the roots and a low translocation to the shoots. This is probably the major constraint to overcome for a more efficient utilization of these species to clean up soils from metal contamination. Then, an appropriate trait to screen out forest plant material is the ability to translocate the absorbed metal into the aerial parts, especially in stem tissues that are not renewable as foliage and that can be harvested and utilised for energy production.

Many authors have reported differences among willow and poplar clones in the partitioning of heavy metals within tree organs (Mills et al., 2000; Robinson et al., 2000; Lunáčková et al., 2003; Robinson et al., 2005; Fischerova et al., 2006; Unterbrunner et al., 2007). Nevertheless, only few works have compared the responses of willow and poplar clones to the presence of cadmium in an hydroponic system (Šottníková et al., 2003; Lunáčková et al., 2003/4; Dos Santos Utmazian et al., 2007). Hydroponic culture is a very useful tool to carry out a selection inside a considerable number of individuals. In fact, it allows to reduce growing and treating time of the plants, the space requested for the experiment, the variability due to the environmental factors. In general, data obtained by hydroponic screening need to be confirmed by field performance trials, even if Watson et al. (2003) have recently pointed out that results obtained in hydroponics and in field experiments are comparable.

This study was aimed at evaluating the response of different poplar and willow clones for cadmium tolerance, accumulation and translocation in an hydroponic culture. The characterisation of several Salicaceae clones for the effectiveness to tolerate and bio-concentrate cadmium could be very interesting in specifying the potentiality of these plants to phytoremediate cadmium-polluted soils.

3.2 Materials and methods

Plant material and growth conditions

Previously rooted stem cuttings (20-cm-long) taken from poplar and willow clones (listed in Table 1) were divided in two stocks to be treated in hydroponics with 0 (control) or 50 µM $CdSO_4$ (Sigma, St. Louis, USA) for three weeks. Particular attention was paid to choose homogenous rooted cuttings to introduce randomly in the experimental treatment that consisted in pots filled with third-strength Hoagland's nutrient solution, pH 6.5 (Arnon and Hoagland, 1940). Cuttings were grown in a controlled climate chamber equipped with metal halide lamps (Powerstar HQI-TS; Osram, Munich, Germany) providing a photon flux density of 300 µmol m^{-2} s^{-1} for 14 h at 25°C. During the 10 h dark period the temperature was 20°C. The relative humidity was 60-70%. The nutrient solutions were completely replaced twice a week to prevent depletion of metals and nutrients and expose plants to a constant metal concentration. An aeration system based on pumps was used to avoid oxygen deprival. Each treatment group consisted of five cuttings of each clone. At the end of the experimental period, control and treated plants were harvested and washed without damaging the roots. After leaf and root measurements, plants were separated in aerial part (leaves, secondary stems and original cutting) and roots. The leaf area was measured using a leaf area meter Li 3000 (Licor, Nebraska, USA) and then each plant parts was dried in an oven at 80°C until a constant weight was reached.

Cadmium determination

Metal concentration was measured by an atomic absorption spectrophotometer (Perkin Elmer, Norwalk, CT, USA) on digested samples of aerial parts and roots. Dried materials were accurately weighed and mineralised with concentrated nitric acid under heating according to Robinson et al. (2000).

Bio-concentration factor (BCF), translocation factor (Tf) and tolerance index (Ti) calculation

According to Zayed et al. (1998), the bio-concentration factor (BCF) of total plant, root system and aerial part (stem + leaves and secondary stems) for cadmium was calculated as follows:

$$BCF = \frac{\text{cadmium concentration in the harvested plant material (mg Kg}^{-1})}{\text{cadmium concentration in the solution (mg Kg}^{-1})}$$

The translocation factor (T*f*) was calculated to evaluate the capability of plant to accumulate the metal, absorbed by roots, in the aerial part:

$$Tf = \frac{\text{cadmium concentration in the aerial part (mg Kg}^{-1})}{\text{cadmium concentration in the roots (mg Kg}^{-1})} \times 100$$

Tolerance index (T*i*) was calculated to measure the ability of plant to grow in presence of a given concentration of metal, according to Wilkins (1978):

$$Ti = \frac{\text{dry weight of plants grown in cadmium solution}}{\text{dry weight of plants grown in control solution}} \times 100$$

Dry weight of plant was referred to roots, secondary stems and leaves.

Statistical analysis

Data reported refer to single typical experiment with five replicates. Data were processed with analysis of variance (ANOVA) by using the SPSS software tool and the means were compared using LSD test with a significance level of $P \leq 0.05$, unless otherwise stated.

3.3 Results

Effects due to cadmium were detected for all parameters except for mean root number. Differences among poplar or willow clones were found for all parameters. A significant interaction between poplar or willow clones and cadmium treatment was found for all morphophysiological parameters analysed except for mean root number in willow clones (Tables 2A and 2B). In Figure 1 a comparison between representative willow and poplar plants treated or not with cadmium is reported. No chlorosis symptoms were revealed in both plant species. Shoot and root growth was not particularly affected by cadmium exposition in willow while a significant reduction occurred in poplar. Damage exerted by cadmium at leaf level is an important aspect to evaluate in plants screened for phytoremediation since an efficient photosynthetic apparatus allows plants to maintain an effective transpiration flux that drives metals from roots to aerial parts. Total leaf area is a sensitive parameter to cadmium presence in the growth medium. Figure 2A shows the total leaf area of poplar clones subjected or not to 21 days of cadmium treatment in an hydroponic experiment. All clones revealed a dramatic reduction in total leaf area caused by cadmium exposition. The heavy metal treatment affected especially the clones 11-5, I-214, L.Avanzo and 14P11 that showed higher leaf area reduction. A4A, that exhibited the highest total leaf area in control condition and Nisqually, that on the contrary expressed one of the lowest one, revealed less inhibition, respect to the other clones, as a consequence of metal exposition. In willow (Figure 2B), 6-02 and 2-03 clones resulted particularly affected by cadmium treatment, while the other clones showed no total leaf area reduction. Rooting system plays a key role in the interaction between contaminants and plant. In poplar and willow clones the effect of cadmium on rooting system was analysed measuring some morphological parameters such as mean number of roots per plant, mean root length per plant and total root length (extension of the primary root system). Cadmium treatment affected the mean number of roots per plant only in 5 poplar clones (Figure 3). In particular, clones 6K3, 14P11 and 58-861 evidenced a dramatic

Table 1. Clones of poplar and willow tested in the experiment

Populus x euramericana - clones A4A, Luisa Avanzo and I-214
Populus deltoides - clone Lux
Populus x interamericana - clone 11-5
Populus nigra - clones Poli and 58-861
Populus alba - clones 6K3 and 14P11
Populus trichocarpa - clone Nisqually
Salix alba - clone SS5
Salix alba - clone SP3
Salix alba - clone 6-03
Salix alba - clone 2-03
Salix sp. - autochthonous clone Quirani (collected near sulphur springs 30 Km N-E of Rome)
Salix sp. - autochthonous clone Cretone (collected near sulphur springs 30 Km N-E of Rome)

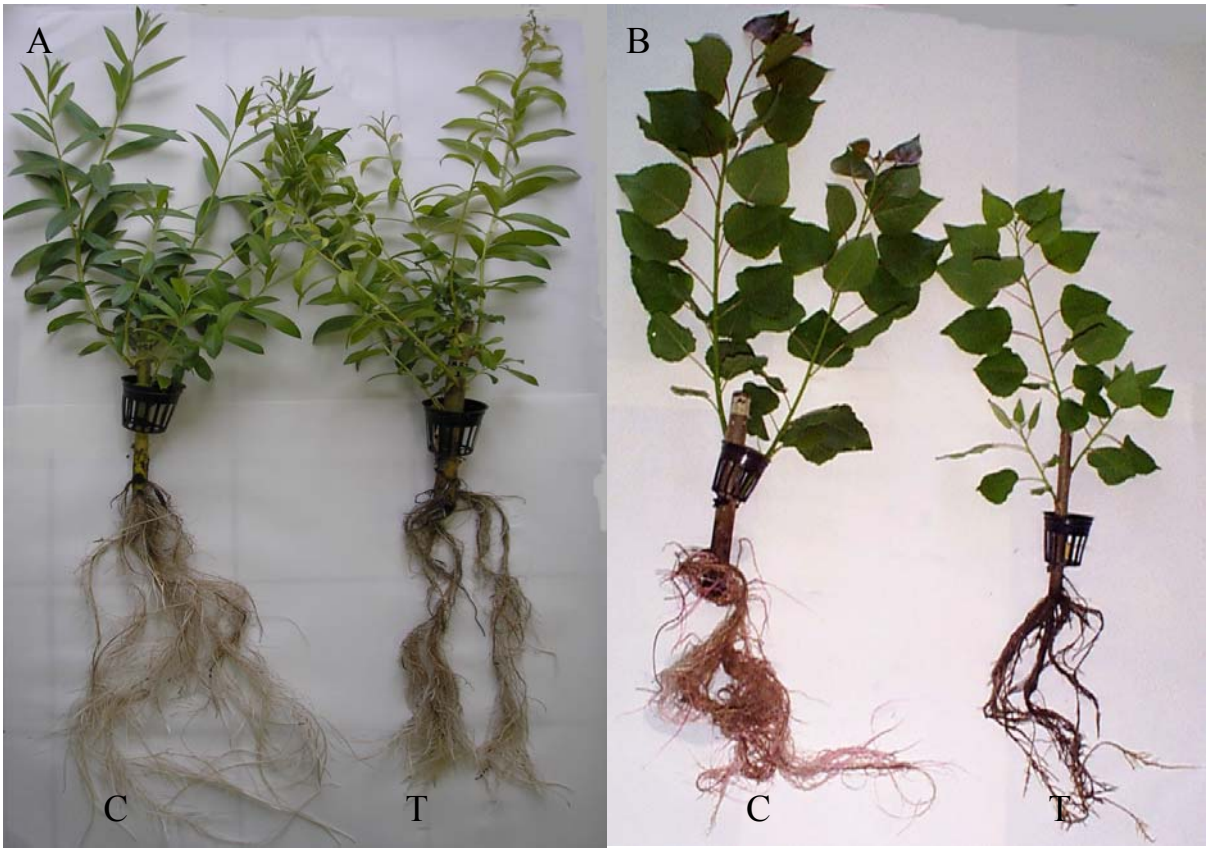

Figure 1. Morphological aspect of willow (A) and poplar (B) plants exposed to 50 μM $CdSO_4$ (T) compared to control (C).

Table 2A. ANOVA results for clone and Cd treatment effects on some morpho-physiological parameters in poplar cuttings grown in hydroponic solution.

Effect	Total leaf area	Mean root length	Mean root number	Total root length
Clone	***	***	***	***
Cd treatment	***	***	ns	***
Clone X Cd treatment	***	**	*	**

Significance of the main effects and the interaction between them are indicated as * = $P< 0,05$, **= $P< 0,01$, ***= $P< 0,001$, ns = not significant

Table 2B. ANOVA results for clone and Cd treatment effects on some morpho-physiological parameters in willow cuttings grown in hydroponic solution.

Effect	Total leaf area	Mean root length	Mean root number	Total root length
Clone	**	***	***	***
Cd treatment	ns	***	ns	*
Clone X Cd treatment	***	*	ns	*

Significance of the main effects and the interaction between them are indicated as * = $P< 0,05$, **= $P< 0,01$, ***= $P< 0,001$, ns = not significant

reduction of the number of roots as a consequence of heavy metal exposure while Luisa Avanzo showed a less remarkable decrease of this root parameter. It is worth to note that in

clone Lux cadmium treatment provoked a slight stimulation of root emission respect to

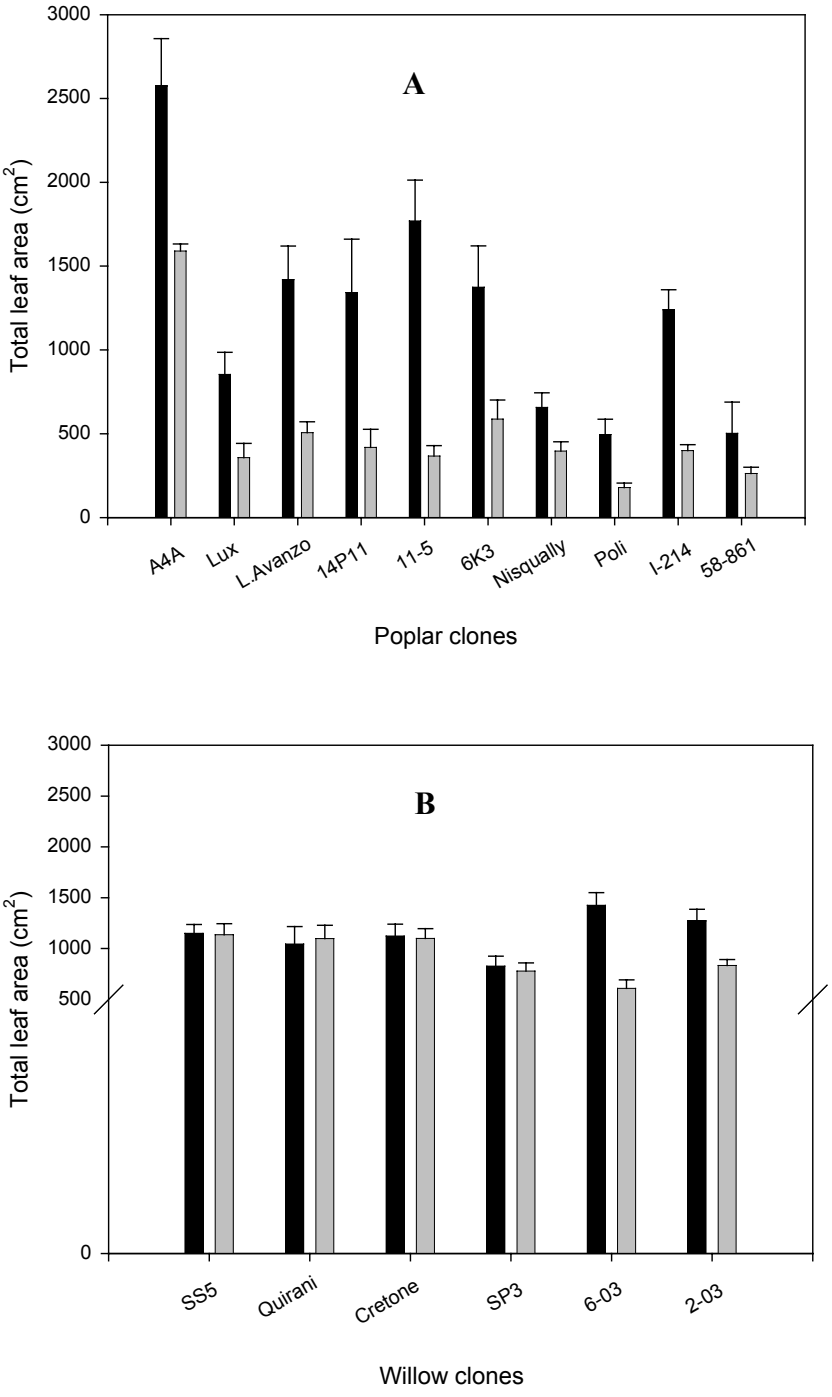

Figure 2. Total leaf area (cm^2) measured at the end of the experiment on plants of poplar (A) and willow (B) grown in the presence of 0 μM (control, black bars) and 50 μM (grey bars) $CdSO_4$. Values are the mean of five replicates. Error bars indicate standard error.

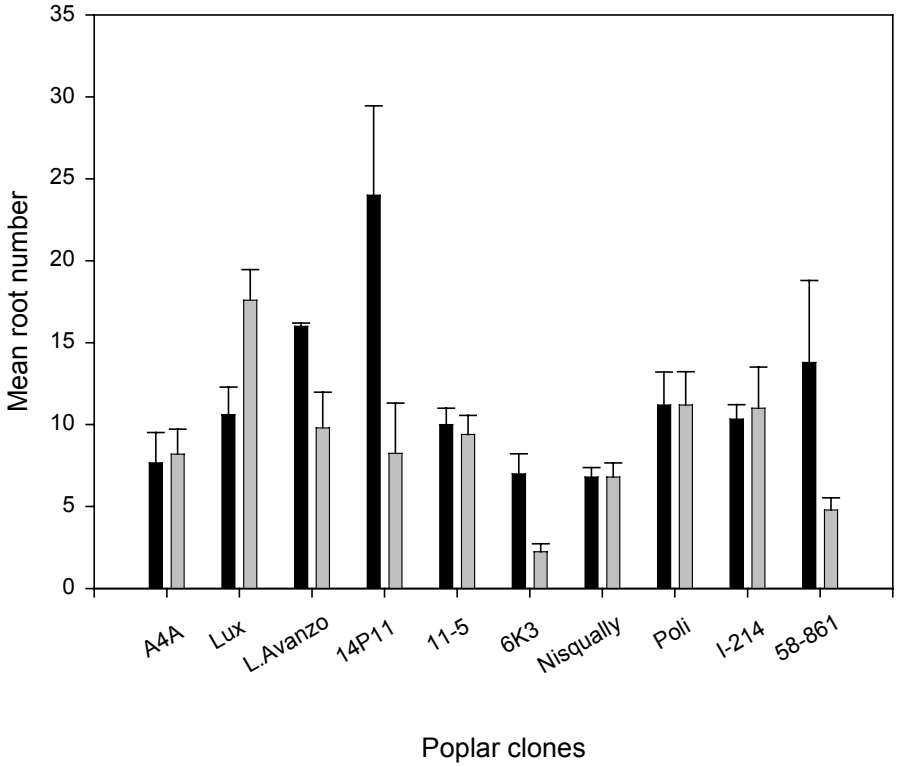

Figure 3. Mean root number measured at the end of the experiment on plants of poplar grown in the presence of 0 μM (control, black bars) and 50 μM (grey bars) $CdSO_4$. Values are the mean of five replicates. Error bars indicate standard error.

control. Results on willow clones did not evidence any effect of metal treatment for this root characteristic (data not shown). Mean root length per plant in poplar (Figure 4A) was notably affected by cadmium treatment. In fact, 8 clones showed a significant reduction of this root parameter with clone 11-5 that revealed the highest decrease. Clones Lux and I-214 were unaffected by metal treatment. In willow clones (Figure 4B), SS5 and Quirani evidenced no mean root length inhibition by cadmium treatment that on the contrary affected more or less dramatically this root parameter in the other clones. The extension of the primary root system, expressed as total root length, in poplar (Figure 5A) was negatively influenced by metal treatment in six clones by ten with a dramatic reduction in clones 14P11, 6K3 and 58-861, and a consistent lowering in clones Luisa Avanzo, 11-5 and Poli. Clones Nisqually, A4A, I-214 and Lux were statistically not affected by cadmium treatment. Clone Lux showed the most extended root system under metal treatment among the tested poplar clones. In willow clones (Figure 5B), total root length was negatively affected by metal treatment in Cretone,

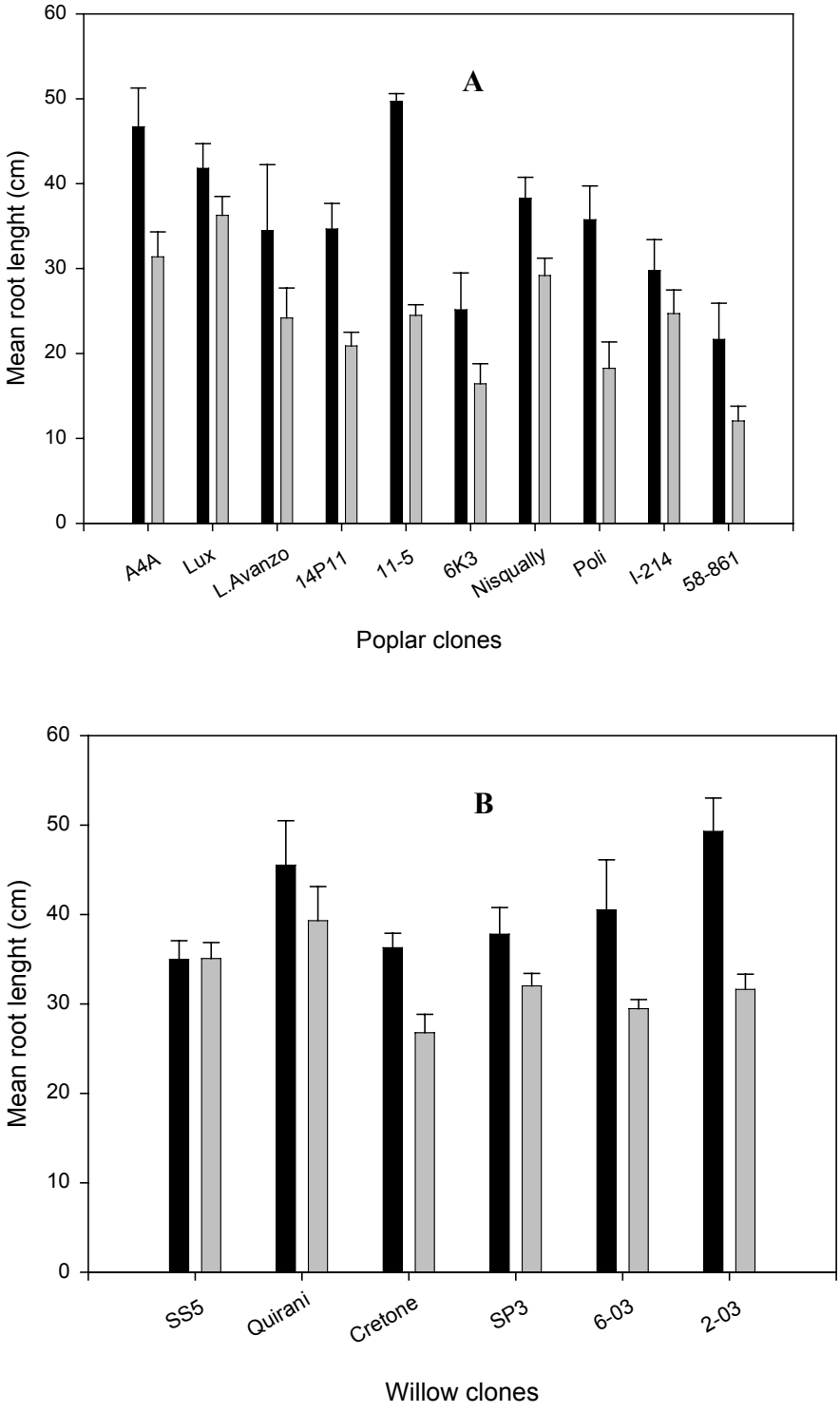

Figure 4. Mean root length (cm) measured at the end of the experiment on plants of poplar (A) and willow (B) grown in the presence of 0 μM (control, black bars) and 50 μM (grey bars) $CdSO_4$. Values are the mean of five replicates. Error bars indicate standard error.

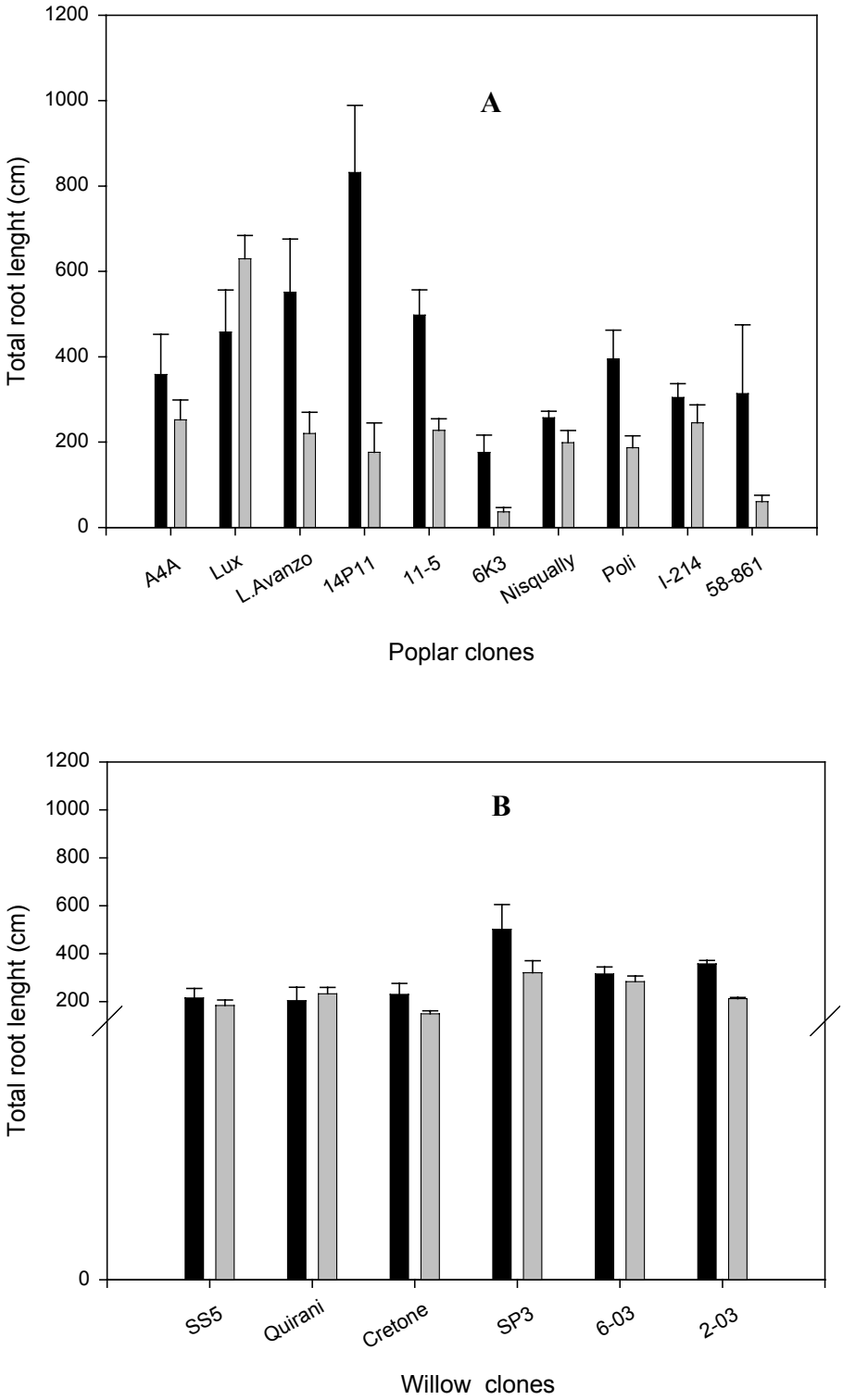

Figure 5. Total root length (cm) measured at the end of the experiment on plants of poplar (A) and willow (B) grown in the presence of 0 μM (control, black bars) and 50 μM (grey bars) $CdSO_4$. Values are the mean of five replicates. Error bars indicate standard error.

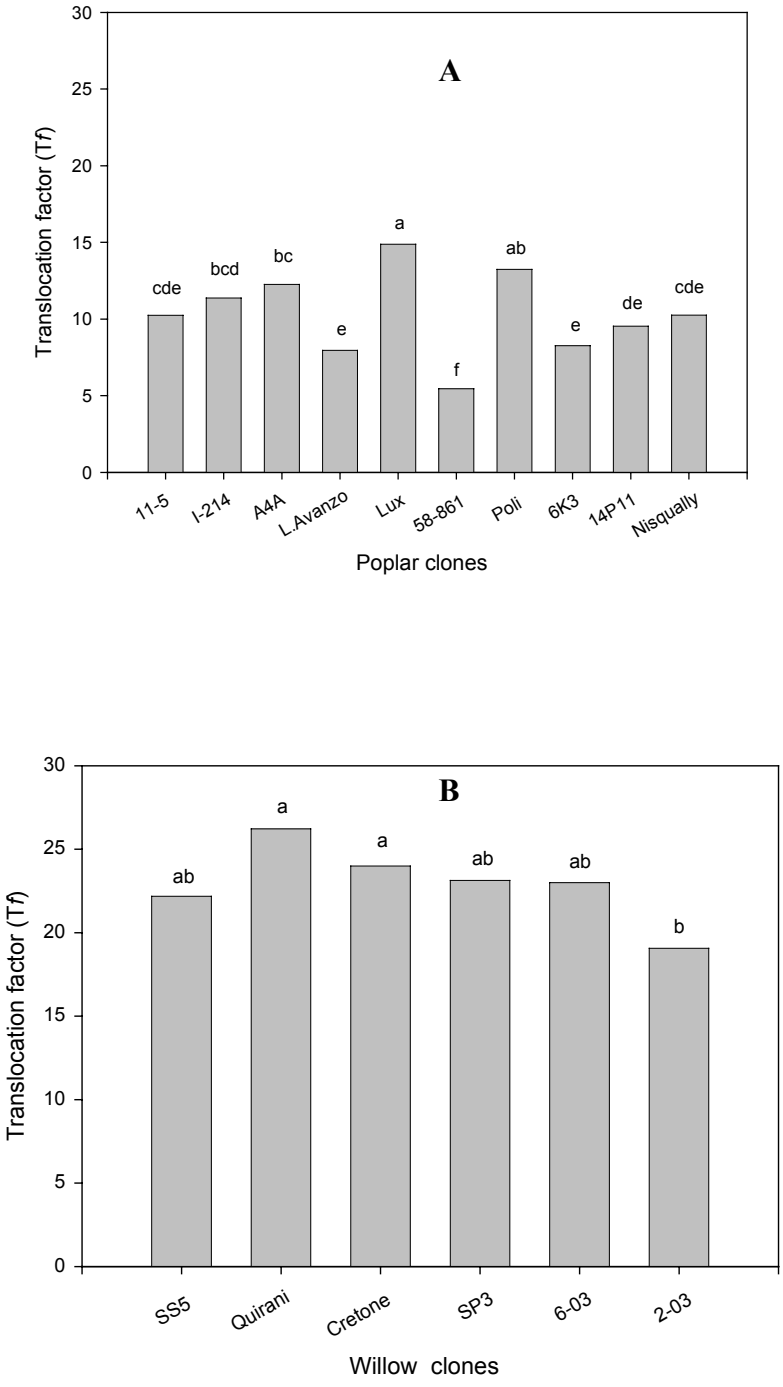

Figure 6. Translocation factor (T*f*) calculated at the end of the experiment on plants of poplar (A) and willow (B) grown in the presence of 50 μM $CdSO_4$. For comparison of means, ANOVA after angle transformation (arc sin $\sqrt{\%}$), followed by LSD test ($P \leq 0.05$), were performed. Different letters above bars indicate significant differences. Values are the mean of five replicates.

Table 3A. Cadmium concentration ($mg \cdot Kg^{-1}$) in roots, aerial part and total plant of poplar cuttings grown in hydroponics for three weeks in 50 μM $CdSO_4$. In columns, different letters represent significantly different values (LSD test, $P \leq 0.05$). Values are the mean of five replicates.

Poplar clones	Roots	Aerial part	Total plant
11-5	9610 bcd	325 cd	9935 bc
I-214	9881 bc	383 abc	10264 bc
A4A	7737 cde	340 bc	8077 cd
L.Avanzo	8575 cde	172 e	8747 bcd
Lux	6802 d	445 ab	7247 d
58-861	18389 a	165 e	18554 a
Poli	10820 b	466 a	11286 b
6K3	6089 e	126 e	6216 d
14P11	7964 cde	216 de	8181 bcd
Nisqually	9701 bcd	311 cd	10012 bc

Table 3B. Cadmium concentration ($mg \cdot Kg^{-1}$) in roots, aerial part and total plant of willow cuttings grown in hydroponics for three weeks in 50 μM $CdSO_4$. In columns, different letters represent significantly different values (LSD test, $P \leq 0.05$). Values are the mean of five replicates.

Salix clones	Roots	Aerial part	Total plant
SS5	4235 b	602 b	4837 b
Quirani	4199 b	814 a	5014 b
Cretone	3708 b	591 b	4300 b
SP3	4022 b	621 b	4644 b
6-03	5776 a	869 a	6646 a
2-03	3835 b	410 c	4245 b

SP-3 and 2-03 while no effect was observed in SS5, Quirani and 6-03. SP-3 and 6-03 evidenced the longest root extension among the willow clones grown in cadmium added solution. In Table 3A the concentration of cadmium detected in cuttings of poplar clones exposed for three weeks to 50 μM CdSO4 is reported. Metal concentration in control cuttings was below the threshold of detection.

Table 4A. Bio-concentration factor (BCF) of in roots, aerial part and total plant of poplar cuttings grown in hydroponics for three weeks in 50 μM $CdSO_4$. In columns, different letters represent significantly different values (LSD test, $P \leq 0.05$). Values are the mean of five replicates.

Poplar clones	Roots	Aerial part	Total plant
11-5	155 bc	5,1 cde	160 bc
I-214	159 bc	6,2 ab	166 bc
A4A	125 bcd	5,5 bcd	130 bcd
L. Avanzo	138 bcd	2,7 fg	141 bcd
Lux	110 cd	7,2 ab	117 bcd
58-861	297 a	2,6 fg	300 a
Poli	175 b	7,5 a	181 b
6K3	98 c	2,1 g	100 d
14P11	128 bcd	3,5 efg	132 bcd
Nisqually	160 b	4,3 def	164 bc

Table 4B. Bio-concentration factor (BCF) of in roots, aerial part and total plant of willow cuttings grown in hydroponics for three weeks in 50 μM $CdSO_4$. In columns, different letters represent significantly different values (LSD test, $P \leq 0.05$). Values are the mean of five replicates.

Salix clones	Roots	Aerial part	Total plant
SS5	68 b	9,7 b	78 b
Quirani	68 b	13,2 a	81 b
Cretone	60 b	9,5 b	69 b
SP3	65 b	10,1 b	75 b
6-03	93 a	14,1 a	107 a
2-03	62 b	6,6 c	68 b

Cadmium concentration of the whole plant differed remarkably among the clones, ranged from 18554 ppm for 58-861 to 6216 ppm for 6K3. Poli also evidenced high cadmium concentration at whole plant level while A4A, Lux and 6K3 showed a lower metal

accumulation respect to the other clones. Cadmium concentration detected in the analysed plants was mainly due to root accumulation and reflected, among clones, the distribution observed at whole plant level. On the contrary, in the aerial part of plant, accumulation of cadmium among clones varied from 466 ppm for Poli to 126 ppm for 6K3. Remarkable metal accumulation was also evidenced in clones Lux, and I-214, while the lowest concentration was detected in 14P11, L.Avanzo, 58-861 and 6K3. In willow (Table 3B), cadmium accumulation among clones was very homogeneous with the highest value detected, at whole plant level, in clone 6-03. The same behaviour was observed for root cadmium content that on average represented near the 87 % of the total concentration measured in plants. On the contrary, in the aerial part of plant more variability occurred since the highest metal accumulation was found in clones 6-03 and Quirani while the lowest in 2-03. To evaluate the capability of poplar and willow clones to extract and accumulate cadmium into the plant, the bio-concentration factor (BCF) was calculated. In Table 4A, BCF of poplar clones, referred to total plant, root system and aerial part of plant, is reported. Clones 58-861 and Poli showed the highest total plant BCF while 6K3 the lowest one. Root BCF represents near the 97% of the total BCF. Aerial part BCF revealed Poli as the poplar clone with the more pronounced capability to accumulate cadmium in leaf and stem tissues. Data regarding BCF for willow clones are shown in table 4B. Both total and root BCF evidenced 6-03 clone as the more efficient cadmium bio-accumulator among willow clones screened. BCF of the aerial part evidenced that clones Quirani and 6-03 presented a more pronounced ability in bio-accumulating cadmium in the above ground tissues while clone 2-03 evidenced a less efficiency for this purpose. The capability of poplar and willow clones to accumulate cadmium in the above ground tissue was better highlighted calculating the translocation factor (T*f*), that indicates the amount of the absorbed metal by roots that reaches the aerial part of the plant, as percentage (Figures 6A and 6B). In poplar (Figure 6A), highest T*f* values were observed for clones Lux and Poli while the lowest ones were evidenced by 58-861. Calculation of T*f* for willow plants highlighted a similar translocation capability among clones except for 2-03 that showed the lowest value (Figure 6B).

3.4 Discussion

The possibility for phytoremediation to have a wide and successful application is mainly based on the availability of selected plant materials to be efficiently utilised for the different type of contaminated substrate (soil, water, sludge etc.). Due to their growth, genetic and cultural characteristics, poplar and willow clones could fit in well with this purpose. In this work we aimed to compare the behaviour of a significant number of clones, included within a large collection of species of *Populus* and *Salix* collected within Italian population, with regard to cadmium tolerance, accumulation and translocation to aerial parts. These aspects are fundamental to specify in plants that are candidates for phytoremediation and must be the driving criteria to perform an efficient screening for this scope. Metal tolerance, and thus the protection of integrity and functionality of the primary physiological and metabolic processes (Pietrini et al. 2003), is a necessary pre-requisite for a plant that can be proposed for a phytoremediation use. Nevertheless, this characteristic must be the consequence of a combination of metal absorption and limitation of the damaging effect exerted by the metal itself, and not be merely due to metal exclusion. Tolerance at root level, that means the preservation of the selective property of cell membrane and the regulation of the influx/efflux processes, represents the first step to allow metal absorption and loading into the xylem vessels minimising nutrient uptake disturbance. In the present work, poplar and willow clones showed remarkable differences in tolerance to cadmium by rooting system, analysed by following morphological parameters such as mean number of roots per plant, mean root length per plant and total root length (Figs 2, 3 and 4). In general, metal treatment negatively affected the length of the roots more than their number, a root parameter that in willow was completely unaffected. This is consistent with data reported by Šottníková et al. (2003) and Lunáčková et al. (2003/2004). A more pronounced effect of cadmium in reducing root length rather than root number was also reported by Punshon and Dickinson (1999) in willow. In the present work, total root extension was not significantly reduced in the 40% of poplar clones and in the 50% of willow clones, evidenced this last species as slightly more tolerant to cadmium respect to poplar at root level.

Absorbed metal can be loaded on xylem vessels by binding to organic acids, thiol and amine compounds (Kramer et al., 1996; Keltjens and van Beusichem, 1998; Rauser, 1999), to be transported into shoots. In leaf, cadmium can represent a very toxic agent, since it can destroy thylacoidal membranes and alter enzyme activities, resulting in a reduction of photosynthesis

(Becerril et al., 1988; Pietrini et al., 2005). For this reasons, mechanisms that restrict the movement, the action and the damaging effect of metal must be promptly activated in leaves of a metal-accumulating plant, in order to maintain an efficient transpiration flux that

Table 5. Comparison between poplar and willow for tolerance index (T*i*), bio-concentration factor (BCF) and translocation factor (T*f*). Data refer to mean value of cuttings from all clones grown in hydroponics for three weeks in 50 μM $CdSO_4$. In columns, different letters represent significantly different values (t-test, $P \leq 0.01$). For T*i* and T*f*, data angle transformation (arc sin $\sqrt{\%}$) was performed.

Plant species	T*i*	BCF	T*f*
Poplar	45 b	159 a	10 b
Willow	73 a	80 b	23 a

Table 6. Comparison between poplar and willow for cadmium concentration ($mg \cdot Kg^{-1}$) in roots, aerial part and total plant. Data refer to mean value of cuttings from all clones grown in hydroponics for three weeks in 50 μM $CdSO_4$. In columns, different letters represent significantly different values (t-test, $P \leq 0.01$).

Plant species	Roots	Aerial part	Total plant
Poplar	10001 a	284 b	10348 a
Willow	4295 b	651 a	4947 b

represents the driving force to move metal from roots to the aerial parts. Among these mechanism, metal storage at cell (vacuole) or tissue (trichomes, veins) level (Clemens, 2001; Vollenweider et al., 2006), chelation by organic ligands (Hall, 2002) and antioxidative defence (Schützendübel and Polle, 2002) have been reviewed as tolerance response that can avoid or limit damaging effects on leaf growth and physiology. In the present work, effect of cadmium on leaves of poplar and willow clones was evaluated by measuring the total leaf area (Figure 2). Poplar clones showed, on average, a more pronounced leaf area reduction than willow ones. In fact, in this last species the leaf area of only two clones by six was

negatively affected by metal presence while, in poplar, differently to Pilipovic et al. (2005), all clones presented a remarkable reduction of this parameter. A reduction in leaf area in poplar and willows species following cadmium treatment was also found by in Lunáčková et al. (2003). Comparison between the two species for that regards cadmium tolerance was better highlighted by the analysis of the tolerance index, T*i* (Table 5). On the basis of the dry biomass of total plant, on average of all clones analysed, Ti revealed that willow tolerate cadmium much more than poplar. Different cadmium tolerance found in this trial for poplar and willow can be appreciated in Figure 6. According to the scheme proposed by Lux et al. (2004), willow clones tested in this work can be defined as highly tolerant (T*i* >60) while poplar clones as medium tolerant (T*i* between 35 and 60). In this context, respect to data presented in this work, a relatively higher T*i* of root and leaves in some willows and poplar clones was reported by Dos Santos Utmazian et al. (2007) but cadmium concentration was several fold less than that used in our experiment. A remarkable difference in cadmium tolerance among willow clones was found by Punshon and Dickinson (1999) and Kuzovkina et al. (2004). Cadmium accumulation in poplar was very different among clones while in willow was more homogeneous (Tables 3A and 3B). On average, as showed in Table 6, poplar evidenced a capability to accumulate cadmium in plant twice respect to willow, even if this accumulation is quite completely confined in roots. On the contrary, willow showed a higher ability respect to poplar to accumulate the metal in the aerial part. These data are in line with those previously reported by Robinson et al. (2000) and Lunáčková et al. (2003/2004). On the contrary, Unterbrunner et al. (2007) evidenced that *Populus tremula* accumulated cadmium more in the above ground tissues than in roots while *Salix caprea* showed an opposite behaviour, even if these performances were obtained directly on contaminated sites. In this context, the calculation of the bioconcentration factor (BCF) can give further information to compare the capability for these Salicaceae to extract metal from a contaminated matrix respect to other species, for example hyperaccumulating plants. Even if poplar and willow clones tested in this work exhibited very different values in their root and aerial part BCF, these values are higher than that reported for other plant species, also those classified as hyperaccumulators (Ghosh and Singh, 2005). It must be noticed that BCF is certainly affected by the nature of the contaminated medium being the bio-availability of Cd in hydroponics higher respect to soil, that was the substrate used by Ghosh and Singh (2005). BCF for cadmium in the aerial part of willow, evaluated in field and pot experiments (see

Dickinson and Pulford, 2005), was generally lower than that found in our experiment, where it ranged from 6,6 to 14,1. These values are also higher than previously reported (Mattina et al., 2003; Marchiol et al., 2004; Zhuang et al., 2007) for many herbaceous species in which, also at root level, BCF was dramatically lower than that calculated for poplar and willow clones investigated in the present paper. On average, poplar clones showed a two times higher capability to remove metal from the solution respect to willow (Table 5), expressed as total BCF. The ability to accumulate metal in the aerial parts respect to roots can be better highlighted calculating the translocation factor (T*f*). In this work willow exhibited a more than double T*f* than poplar (Table 5). Interestingly, T*f* value of willow was very similar to that obtained for Brassica Juncea, an hyperaccumulating plant, by Ghosh and Singh (2005). A higher cadmium translocation capability (expressed as leaf:root ratio) of willow respect to poplar was also reported by Dos Santos Utmazian et al. (2007) in an hydroponic experiment. It is worth to mention that T*f* of Salicaceae clones measured in this work is lower than that found in herbaceous plants cultivated in pots by Mattina et al. (2003) and Marchiol et al. (2004) or in wetlands (Deng et al., 2004), while BCF is generally higher. This consideration confirms that Salicaceae plants have a considerable potentiality to remove cadmium from contaminated medium that could be increased ameliorating metal transport to shoots. The comparison between poplar and willow for cadmium accumulation and distribution, evidenced by BCF and T*f* mean values, revealed that these two Salicaceae species could be differently useful for a specific phytoremediation purpose. In fact, poplar, that showed in this work a remarkable ability to bio-concentrate cadmium in the root system, could be efficiently used in remediation of polluted water (rhizofiltration) or in contaminated site where the main goal is to limit or avoid metal movement to percolating water to the water layer along the soil profile (phytostabilisation). On the contrary, willow clones tested in this trial highlighted an interesting potentiality to translocate and concentrate cadmium in the above ground organs associated to a notable tolerance. Then, these results indicate this species as very suitable to move pollutant from soil to the harvestable parts of plants (phytoextraction) that, if cultured in SRC management, could yield also biomass for energy production, realising a double ecological service. The effective capability of these Salicaceae clones to accumulate cadmium in the above ground organs is currently under evaluation for the whole growing season in an outdoor mesocosm system, a culture technique characterised by more similar environmental conditions to open-field respect to hydroponics. Preliminary results regarding BCF and T*f*

showed only a slight decrease in plants cultivated in mesocosm respect to hydroponics (data nor shown), confirming the potentiality of poplar and willow to extract cadmium to a contaminated matrix and accumulate it in the aerial part. Metal translocation in the above ground organs is a biochemical process of main interest for an effective utilisation of plants to remediate polluted sites. In fact, a more efficient mobilisation of metals from root towards the above ground organs could reduce the damaging effects exerted by these pollutants on root physiology and biochemistry, maintaining uptake effectiveness and allowing metal removal from the contaminated substrate along time. Then, metal tolerance, bioaccumulation and translocation capability must be considered together to evaluate species, clones or individuals with interesting perspectives for phytoremediation in order to characterise the biochemical and molecular traits involved in these processes. Results obtained in this trial evidenced that poplar clones showed divergent behaviour for cadmium bio-concentration and allocation in plant parts. Two clones of *Populus nigra*, Poli and 58-861, exhibited peculiar and interesting performances to be better elucidated. In fact, both clones showed the highest metal accumulation among poplar clones but Poli evidenced a remarkable ability to bio-concentrate cadmium in the aerial part while 58-861 exhibited the lowest ability for this trait. Willow clones were more homogeneous for cadmium tolerance and accumulation and no clear indications were obtained since the best cadmium accumulator clone, 6-03, was however the most affected by cadmium exposure at leaf level. Interesting responses were then obtained in the clone SS5 and in the autochthonous clone Quirani. It is worthwhile to mention that clone Quirani was collected in a sulphur area and Cd is a well known sulphur-philic metal. Studies are in progress to characterise poplar and willow clones for that concerns the biochemical and molecular processes involved in the accumulation and translocation of cadmium in the above ground organs.

4. IMAGING AND CHARACTERISATION OF THE DAMAGE EXERTED BY CADMIUM ON LEAVES OF POPLAR AND WILLOW CLONES WITH DIFFERENT TRANSLOCATION CAPACITY BY CHLOROPHYLL FLUORESCENCE ANALYSIS

4.1 Introduction

Cadmium, which is a highly toxic heavy metal, is dispersed in natural and agricultural environments principally through human activities such as mining, refining, municipal waste incinerators, and fossil fuel combustion sources (Wagner, 1993), as well as natural rock mineralization processes (Baker et al., 1990). Major inputs of cadmium into agricultural soils are due to the application of phosphatic fertilizers (McLaughlin et al., 2000), soil amendments with municipal sewage sludges and atmospheric deposition (Weissenhorn and Leyval, 1995). Cadmium is relatively mobile in plants and elevated concentrations of metal induce inhibition of various processes in plant metabolism (reviewed by Sanità & Gabbrielli, 1999; Joshi & Mohanty, 2004; Küpper & Kroneck, 2005). To remove cadmium and other pollutants from contaminated areas, unconventional techniques that utilise biological processes have been successfully applied. In particular, plants can be used for removing heavy metals (phytoremediation) from soil and accumulate them in the harvestable parts. This technology (Kumar et al., 1995; Raskin et al., 1997) is less expensive and environmental disruptive than conventional remediation systems that consist mainly in the excavation and incineration of soil (Cunningham and Ow, 1996). Among plant species, several studies have described the potentiality of willows and poplars for phytoextraction (Riddell-Black, 1994; Punshon and Dickinson, 1997; Robinson et al., 2000; Pulford et al, 2002, Laureysens et al, 2004a). In phytoremediation strategy, leaves play a crucial role in the extraction of metals from contaminated soil and water as the absorption and translocation of mineral elements in plants is largely determined by leaf transpiration (Stomp et al., 1993; Marschner, 1995). Moreover, leaf is also a target organ for metal accumulation and induction of oxidative stress (Laureysens et al, 2004a; Smeets et al., 2008). For instance, cadmium may affect photosynthesis at different levels, including stomatal conductance, Calvin cycle enzyme activity, photosynthetic pigments, thylakoid ultrastructure, and electron transport activity (Krupa and Baszynski, 1995; Vassilev and Yordanov, 1997). As reported by Pietrini et. al. (2003) considering the sensitivity of photosynthesis to cadmium, it can be expected that an

effective cadmium tolerance must include the ability to widely protect and maintain photosynthetic activity. In this context, the high accumulation in the roots and low transport of heavy metals to the shoot is probably a mechanism evolved to protect plant organs involved in photosynthesis (Landberg and Greger, 1996). Generally, plants can withstand heavy-metal accumulation until the metal reaches the toxicity threshold in the tissue. Nevertheless, poplar and willow clones showed a very different ability to accumulate metal in leaves, varying between 1% and 30% of the total absorbed cadmium (Dos Santos Utmazian et al., 2007; Robinson et al., 2000). This large range of metal accumulation in shoot tissue results in a remarkable variability in tolerating cadmium by poplar and willow clones. Then, a better evaluation of the damage induced by metals and a characterisation of physiological and biochemical processes involved in metal tolerance appears to be fundamental to select plants with enhanced capability to efficiently remove these pollutants from contaminated matrix. In this context, as a powerful method to analyse the effects produced by metal accumulation on leaves and to evaluate the tolerance to cadmium we use the chlorophyll fluorescence imaging that allows the monitoring of the spatial distribution of photosynthetic activity without destruction of leaf tissue (Daley 1995, Lichtenthaler and Miehé 1997, Govindjee and Nedbal 2000, Omasa et al. 2002). Many useful fluorescence parameters, such as F_v/F_m, ΦPSII, q_P NPQ, have been developed from saturation pulse induced fluorescence analysis (Genty et al., 1989, Bilger and Björkman, 1991; Krause and Weis, 1991, Govindjee, 1995, Maxwell and Johnson, 2000, Müller et al. 2001) and used to estimate photosynthetic activity under actinic light. The importance of fluorescence imaging as a powerful method to put in evidence heterogeneity in the photosynthetic function across a leaf has been demonstrated by image analysis of the quantum efficiency of PSII in leaves subjected to several stresses, in particular: herbicides (Barbagallo et al., 2003), pathogens (Berger et al., 2004), low temperature (Oxborough and Baker, 1997), CO_2 concentration (Bro et al., 1996). Nevertheless, only few works describe toxic effects exerted in plants by soil contaminants such as cadmium by chlorophyll fluorescence imaging (Ciscato and Valcke, 1998; Chaerle et al., 2007).

The aim of this study was to investigate the effects caused by cadmium exposure, at physiological level, on poplar and willow clones with different ability to translocate metal in leaves. These effects were analysed by gas exchanges and imaging chlorophyll fluorescence analysis to evaluate the extent and the pattern of the damage produced by cadmium on leaves. Data obtained were discussed in order to depict different strategies of poplar and willow

clones in accumulating and distributing cadmium over leaf blade and also to evaluate metal tolerance by an early screening through methods such gas exchanges and imaging chlorophyll fluorescence.

4.2 Materials and methods

Plant material and growth conditions

Stem cuttings (20-cm-long) of *Populus x euramericana* - clones A4A and I-214*, Populus nigra* - clone Poli*, Salix alba* - clone SS5 were rooted and grown in pots filled with third-strength Hoagland's nutrient solution, pH 6.5 (Arnon and Hoagland, 1940). Cuttings were grown in a controlled climate chamber equipped with metal halide lamps (Powerstar HQI-TS; Osram, Munich, Germany) providing a photon flux density of 300 µmol m^{-2} s^{-1} for 14 h at 25°C. During the 10 h dark period the temperature was 20°C. The relative humidity was 60-70%. Plants were allowed to develop roots and grow three weeks in hydroponics before the cadmium treatment was started. At the end of this period cuttings of each clone were selected, weighed and randomly assigned to two groups of treatment with Hoagland's solution containing 0 µM (control) and 50 µM $CdSO_4$ (Sigma, St. Louis, USA) for three weeks. The nutrient solutions were completely replaced twice a week to prevent depletion of metals, nutrients and oxygen. Each treatment group consisted of five cuttings of each clone.

Gas exchange measurements

At the end of the treatment, net photosynthesis (A), stomatal conductance (g_s) and transpiration (E) were measured in the cuvette on the third fully expanded leaf with a gas exchange system (HCM 1000, Walz, Germany). The relative humidity of air entering the cuvette was set at 60% and air and cuvette temperature was 25°C. CO_2 partial pressure was set at 370 µbar bar^{-1}. A white light source (KL 1500; Schott, Mainz, Germany) was used to vary the incident PPFD on the leaf surface. Measurements were carried out at PPFD of 300 µmol m^{-2} s^{-1}. Values for net CO_2 assimilation rate (A) and stomatal conductance (g_s) were calculated using the equations of von Caemmerer and Farquhar (1981). Gas exchange determinations were obtained from n = 5 fully developed leaves of poplar and willow.

Imaging of chlorophyll fluorescence

Chlorophyll fluorescence imaging analysis was performed on the same leaf used for gas exchange measurements by a MINI-Version of the Imaging-PAM fluorometer (Walz, Effeltrich, Germany). The 24x32 mm area imaged by the MINI-Version is illuminated by a very powerful Luxeon LED array consisting of 4 groups of 3 LEDs equipped with 4 individual short-pass filters. This instrument employs the same blue LEDs, with a peak wavelength at 450 nm, for pulse modulated measuring light, continuous actinic illumination and saturation pulses. Another set of LEDs (total of 32 LEDs) provided the pulse-modulated light for assessment of PAR-absorptivity at 650 nm and 780 nm. The charge-coupled device (CCD) camera has a resolution of 640 × 480 pixel. Pixel value images of the fluorescence parameters were displayed with the help of a false colour code ranging from black (0.000) through red, yellow, green, blue to pink (ending at 1.000) (Berger et al., 2004). All measurements were carried out with maximal distance between camera and leaf. Leaves were dark adapted for at least 30 min prior to determination of F_o and F_m. The maximum quantum yield of PSII photochemistry (F_v/F_m), was determinate as $F_m - F_o/F_m$. Then, plants were adapted for 15 minutes at a light intensity of 300 µmol m^{-2} s^{-1} and saturating pulse were applied in order to determine the maximum fluorescence (F'_m) and the steady-state fluorescence (F_s) during the actinic illumination. Saturation pulse images and values of various chlorophyll fluorescence parameters were captured. The quantum efficiency of PSII photochemistry, ΦPSII, was calculated according to Genty et al. (1989) by the formula: ($F'_m - F_s$)/F'_m). The coefficient of photochemical quenching, q_P, is a measurement of the fraction of open centres calculated as ($F'_m - F_s$)/ ($F'_m - F'_o$) (Schreiber et al., 1986). The value of F'_o was estimated using the approximation of Oxborough and Baker (1997), $F'_o = F_o/(F_v/F_m + F_o/F'_m)$. Calculation of quenching due to non-photochemical dissipation of absorbed light energy (NPQ) was determined at each saturating pulse, according to the equation NPQ = ($F_m - F'_m$)/ F'_m (Bilger et al., 1991). The measured values of NPQ were divided by four for the display of values < 1.000. Chlorophyll fluorescence determinations were obtained from n = 5 fully developed leaves of poplar and willow.

Cadmium determination

At the end of the experiment, leaves, previously analysed for gas exchange and chlorophyll fluorescence, were harvested and placed in a drying cabinet at 80°C until a constant weight

was reached. Dried samples of leaves were weighed and ground. Concentrated nitric acid (10 ml) was added to each tube containing 0.2 g of dry material and the mixtures heated on a heating block until a final volume of ca. 3 ml was reached. The samples were then diluted to 10 ml using deionised water and stored in plastic containers (Robinson et al., 2000). Metal determination was performed using an atomic absorption spectrophotometer (Perkin Elmer, Norwalk, CT, USA).

Statistical analysis

Experimental data were subjected to analysis of variance (ANOVA) using the SPSS software supplemented with multiple-comparison test of the means using the LSD with a significance level of $P < 0.05$.

4.3 Results

Cadmium content

Metal content in leaf tissue was calculated by multiplying dry weight of leaf by metal concentration. In Table 1 cadmium content in leaves of poplar and willow clones at the end of the treatment was reported. Clone SS5 showed the maximum value of metal content, followed by A4A, I-214 and finally Poli.

Table 1. Cadmium content (mg plant part^{-1}) in leaves measured in plants of poplar and willow clones grown in the presence of 50 μM $CdSO_4$ at the end of the experiment. Within a column, means values with a same letter were not significantly different ($P < 0.05$, ANOVA; LSD mean comparisons test). Values are the mean of five replicates.

Clones	Leaves
Poli	0.02 d
I-214	0.18 c
A4A	0.54 b
SS5	1.35 a

Gas exchange parameters

Control plants of all clones showed similar values of net photosynthesis, stomatal conductance and transpiration. Cadmium treatment significantly reduced net photosynthesis (A) in comparison to control in all clones (Fig. 1 A). In particular, A4A decreased assimilation rate by around 80% of the control values, while SS5 reduced net photosynthesis with respect of control by around 20%. Among treated clones assimilation rate differed significantly. Specifically, SS5 showed the highest rate for A, followed by Poli, then I-214 and finally A4A. The treatment negatively affected stomatal conductance (g_s) and transpiration (E) of all clones (Fig. 1 B,C). Nevertheless, while poplar clones Poli, I-214 and A4A significantly reduced their stomatal conductance and transpiration with respect to control, willow clone SS5 showed values slightly lower than control. Under cadmium treatment, SS5 had the highest values for g_s and E while A4A had the lowest ones.

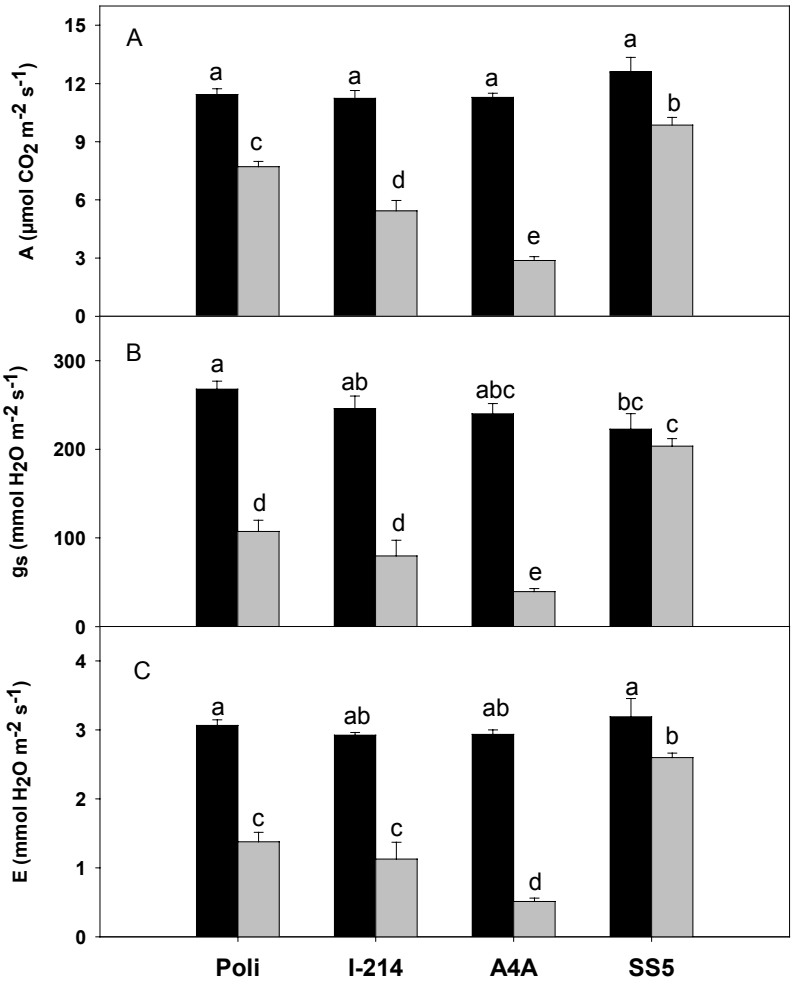

Figure 1. Effect of cadmium on gas exchange parameters: net photosynthesis in growth light conditions A (A), stomatal conductance, g_s (B) and transpiration, E (C), measured at the end of the experiment on the third fully expanded leaf in plants of poplar and willow clones grown in the presence of 0 µM (control, black bars) and 50 µM (grey bars) $CdSO_4$. For comparison of means, ANOVA followed by LSD test ($P <= 0.05$), were performed. Different letters above bars indicate significant differences. Values are the mean of five replicates. Error bars indicate standard error.

Chlorophyll fluorescence parameters

Chlorophyll fluorescence parameters in control leaves of poplar and willow clones are shown in Table 2. Most of chlorophyll fluorescence parameters showed similar or slightly different values among clones. F_v/F_m ratio was similar in all tested clones. The highest values of F_o and F_m were found in I-214, while the lowest values were obtained in Poli. A4A and I-214 had lower values of ΦPSII compared to Poli and SS5. Q_P was lower in A4A differing significantly

Table 2. Chlorophyll fluorescence parameters in control leaves of poplar and willow clones. F_o, minimum chlorophyll fluorescence yield obtained with dark-adapted leaf; F_m, maximum chlorophyll fluorescence yield obtained with dark-adapted leaf; F_v/F_m,, maximal quantum efficiency; ΦPSII, effective quantum yield of photochemical conversion in PSII; q_P, photochemical quenching; NPQ, non-photochemical quenching. Values are means of 5 samples. For comparison of means, ANOVA followed by LSD test, calculated at 95% confidence level, were performed. Values followed by the same letter indicate no significant differences.

Clones	F_o	F_m	F_v/F_m	ΦPSII	q_P	NPQ
Poli	0.073 c	0.328 c	0.777 a	0.506 a	0.765 a	0.826 c
I-214	0.110 a	0.487 a	0.774 a	0.457 b	0.777 a	1.072 a
A4A	0.086 b	0.378 b	0.772 a	0.454 b	0.723 b	1.107 a
SS5	0.088 b	0.384 b	0.770 a	0.492 a	0.786 a	0.896 b

Table 3. Chlorophyll fluorescence parameters in cadmium treated leaves of poplar and willow clones. F_o, minimum chlorophyll fluorescence yield obtained with dark-adapted leaf; F_m, maximum chlorophyll fluorescence yield obtained with dark-adapted leaf; F_v/F_m, maximal quantum efficiency; ΦPSII, effective quantum yield of photochemical conversion in PSII; q_P, photochemical quenching; NPQ, non-photochemical quenching. Values are means of 5 samples. For comparison of means, ANOVA followed by LSD test, calculated at 95% confidence level, were performed. Values followed by the same letter indicate no significant differences.

Clones	F_o	F_m	F_v/F_m	ΦPSII	q_P	NPQ
Poli	0.096 c	0.424 a	0.773 a	0.377 a	0.653 a	1.549 c
I-214	0.117 b	0.427 a	0.726 b	0.305 b	0.591 b	1.789 b
A4A	0.132 a	0.447 a	0.704 c	0.199 c	0.467 c	2.281 a
SS5	0.082 d	0.349 b	0.765 a	0.352 a	0.648 a	1.737 b

from the other clones. Non photochemical quenching, NPQ, showed higher values in A4A and I-214 compared to Poli and SS5. Chlorophyll fluorescence parameters in leaves of poplar and willow clones measured at the end of cadmium treatment are reported in Table 3, while the corresponding images are shown in Figure 2. In Figure 2 a representative image of a single leaf for each clone is presented. Chlorophyll fluorescence parameters were differently affected by cadmium treatment in leaves of poplar and willow clones. The highest values of

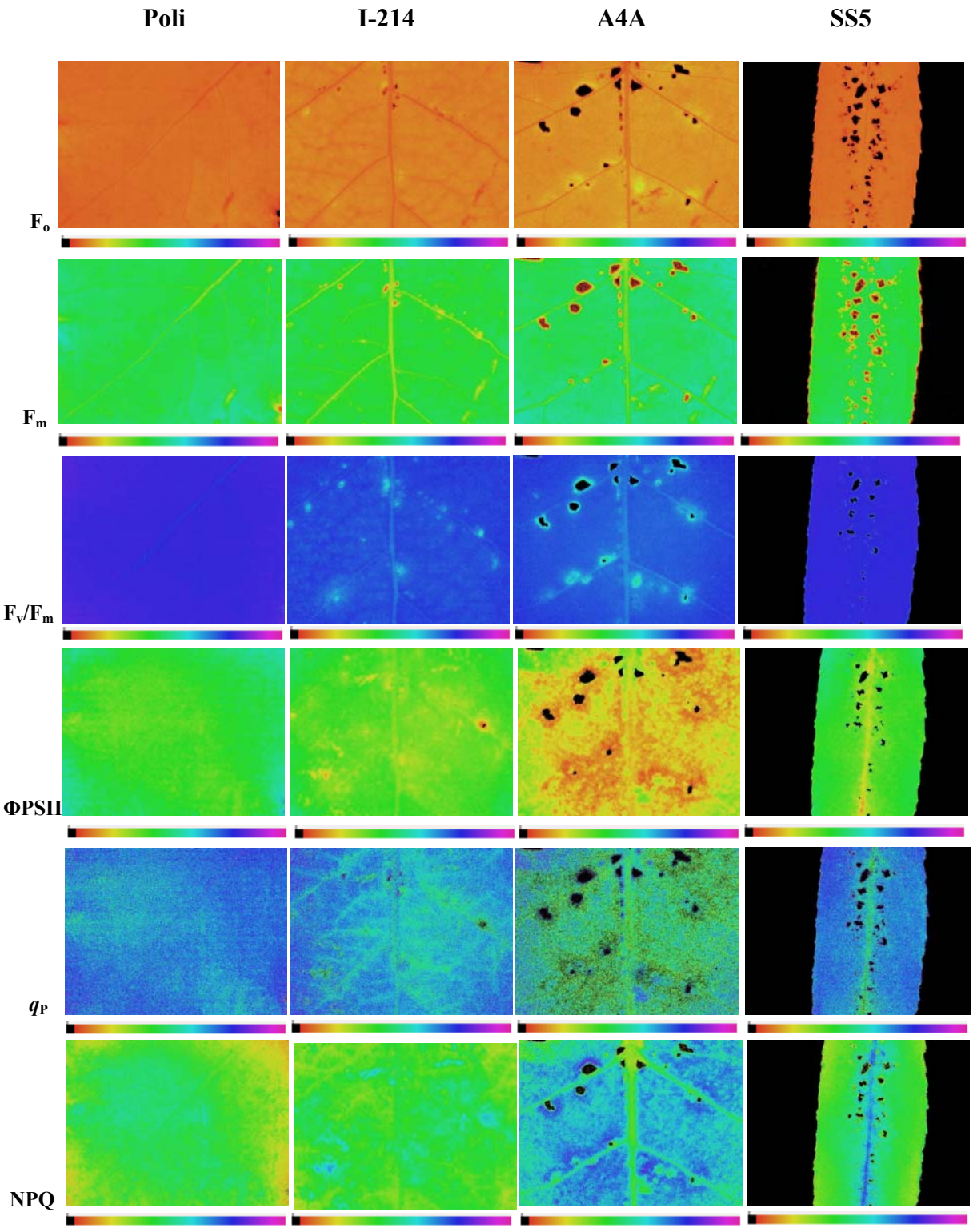

Figure 2. Chlorophyll fluorescence images of F_o, F_m and F_v/F_m after dark-adapted leaf and ΦPSII, q_P and NPQ at steady-state with actinic illumination of 300 μmol photons m^{-2} s^{-1} measured at the end of the experiment in leaves of poplar and willow clones grown in the presence of 50 μM $CdSO_4$. The false colour code depicted at the bottom of each image ranges from 0.000 (black) to 1.000 (pink).

F_o and F_m were measured in A4A, while the lowest values were obtained in SS5 (Table 3 and Fig. 2, F_o and F_m). At the end of the treatment clones Poli and SS5 exhibited the highest values of the maximum quantum yield of PSII photochemistry (F_v/F_m) differing significantly with those of I-214 and A4A (Table 3 and Fig. 2, F_v/F_m). The quantum efficiency of PSII photochemistry (ΦPSII) was generally affected by cadmium treatment; in particular A4A had the lowest value of ΦPSII while Poli and SS5 showed the highest ones (Table 3 and Fig. 2, ΦPSII). Photochemical quenching q_P was lower in A4A differing significantly from the other clones (Table 3 and Fig. 2, q_P). Non-photochemical quenching (NPQ) showed the highest value in A4A while Poli had the lowest one (Table 3 and Fig. 2, NPQ).

4.4 Discussion

In leaf, cadmium can represent a very toxic agent, since it can destroy thylacoidal membranes and alter enzyme activities, resulting in a reduction of photosynthesis (Becerril et al., 1988; Pietrini et al., 2003). For this reasons, mechanisms that restrict the movement, the action and the damaging effect of metal must be promptly activated in leaves of a metal-accumulating plant, in order to maintain both an efficient photosynthetic activity and a transpiration flux that represent the driving force to move metal from roots to the aerial parts. Among these mechanisms, metal storage at cell (vacuole) or tissue (trichomes, veins) level (Clemens, 2001; Vollenweider et al., 2006), chelation by organic ligands (Hall, 2002) and antioxidative defence (Schützendübel and Polle, 2002) have been reviewed as tolerance response that can avoid or limit damaging effects on leaf growth and physiology.

In the present experiment effects of cadmium on poplar and willow clones with different translocation capacity to leaves were examined by gas exchanges and imaging chlorophyll fluorescence analysis to evaluate the extent and the pattern of the damage produced by metal on leaf. Evaluation of photosynthetic apparatus to ascertain toxicity of cadmium at leaf level was chosen as it is reported by many authors that enzymes and reactions involved in photosynthesis are especially sensitive to this heavy metal (for reviews see Krupa, 1999; Sanità di Toppi and Gabbrielli, 1999; Joshi and Mohanty, 2004; Pietrini et al., 2005).

In our study photosynthesis, stomatal conductance and transpiration of all clones decreased in response to cadmium treatment compared with the control; nevertheless, while values for clone A4A were considerably reduced, clone SS5 was slightly affected by the treatment (Fig. 1). Our data indicated that leaves of clones with different metal content showed dissimilar

physiological responses. In particular, as regards poplar clones Poli, I-214 and A4A, we found a direct relation between metal content in leaves (Table 1) and gas exchange parameters (Fig. 1). In fact, in these clones, an increasing content of cadmium in leaves caused a reduction of assimilation rate, stomatal conductance and transpiration. On the contrary willow clone SS5, having the highest cadmium content in leaves, showed only a slight reduction of gas exchange parameters with respect to the other clones. In order to better elucidate this different behaviour and to investigate the heterogeneities of damage symptoms in the leaf caused by cadmium presence, chlorophyll fluorescence images were analysed. Chlorophyll fluorescence imaging system is well established as non-destructive method for detecting and diagnosing plant stresses. This technique has been shown to be capable of revealing spatial and temporal changes during plants stress development (Chaerle and Van Der Straeten, 2000, 2001; Soukupova et al., 2003; Berger et al., 2007) as well as environmental effects on several aspects of whole plant physiology (Gielen et al., 2005). Our results indicated that in control conditions all tested clones showed similar values in gas exchange parameters (Fig. 1) and only a slight difference in chlorophyll fluorescence parameters (Table 2). It is worth to mention that chlorophyll fluorescence images of clones grown in control conditions did not show a heterogeneous distribution of light utilisation and photosynthetic activity over the leaf surface (data not shown). Respect to control plants, leaves of treated cuttings showed patchy chlorosis and necroses, as symptoms of cadmium toxicity, differently among clones. As reported by some authors (Dannehl et al., 1995; Araus et al., 1998) a reduction of F_v/F_m is related to a decrease of chlorophyll content. Chlorotic areas were observed in clone A4A and I-214, while no evident chlorosis was found in SS5 and Poli (Fig. 2, F_v/F_m). Necroses were detected as a small dots near lateral and central veins especially in SS5 and A4A and partially in I-214. No necroses were observed in Poli (Fig. 2). Heterogeneity of images (Fig. 2) suggests that pigment composition and concentration and stomatal function differ over leaf blade, contributing to spatial differences in photochemical activity (Chaerle et al., 2003; Terashima, 1992). Chlorophyll fluorescence parameters in cadmium treated leaves of poplar and willow clones are reported in Table 3. Our data exhibited that all chlorophyll fluorescence parameters showed significant different values among tested clones. The highest observed level of F_o, measured in A4A and I-214, could be attributed to an increase in the fraction of PSII reaction centres, in a photoinactivated status (Barber, 1998), which would lead to a decrease in the PSII photochemical capacity (Table 3). These effects caused lower F_v/F_m and displayed a lower

maximum quantum yield of PSII photochemistry (Butler et al., 1978; Rohàcek, 2002). Higher values of F_m and F_o in clones I-214 and A4A also resulted in lower values of ΦPSII, q_P and higher values of NPQ (Table 3). ΦPSII images indicate the distribution of the yield of linear electron transport through PSII (Genty and Meyer 1995, Bro et al. 1996, Meyer and Genty 1998, Meyer and Genty 1999). Higher values of ΦPSII in SS5 and Poli, indicate that a greater percentage of the absorbed quanta were converted into chemically fixed energy by photochemical charge separation at PSII reaction centres. The remaining quanta were dissipated into heat and fluorescence. NPQ reflects heat-dissipation of excitation energy in the antenna systems and is a good indicator for excess light energy (Cheng et al., 1999; Demmig-Adams et al., 1996; Rohàcek, 2002). An image of NPQ indicates the distribution and the strength of the intrathylakoid pH gradient and the ability of chloroplasts to dissipate excess excitation energy as heat on the leaf (Daley et al. 1989, Osmond et al. 1998, Müller et al. 2001). Therefore, NPQ images have been used as indicators of stomatal patchiness, because heat dissipation depends on stomatal closure (Daley et al. 1989, Mott 1995, Eckstein et al. 1996, Osmond et al. 1998). As reported by Krupa et al. (1993) and Skorzynska and Baszynsky (1997) a slight increase in NPQ indicates a higher dissipation of absorbed energy as radiationless decay and protects the leaf from a damage; nevertheless an excessive enhancement of NPQ, as reported in clone A4A, could be the symptom of incapacity in down-regulation of PSII efficiency to reduce the electron pressure in electron transport chain and switch over energy consumption to heat dissipation (Linger et al., 2005). The aforementioned relation between cadmium content in leaves of poplar clones and the effect caused on photosynthetic activity can be also revealed by chlorophyll fluorescence parameters (Table 3) and their corresponding images (Fig. 2). In particular, as regards poplar clones Poli, I-214 and A4A, we found that changes of these parameters are related to cadmium content. These variations are also in accordance with the lower photosynthesis and higher dissipation as heat of the absorbed energy measured on leaves of different clones. Willow clone SS5, in spite of the highest cadmium content in leaves, showed most of values of chlorophyll fluorescence parameters similar to Poli, clone with the lowest metal content in leaves (Table 3). This different behaviour of willow with respect to poplar clones could be explained by means of the analysis of images of chlorophyll fluorescence parameters F_o, F_m, F_v/F_m, ΦPSII, q_P and NPQ measured at the end of the experiment (Fig. 2). These images show, in all tested clones except for Poli, a heterogeneous distribution of light utilisation and photosynthetic activity over the

leaf surface. A remarkable increase of necrotic areas (black dots) in relation to cadmium content in leaves was observed (see clones I-214, A4A and SS5). This is consistent with recent findings of some authors (Cosio et al. 2005; 2006), that showed a correspondence between the cadmium spots observed on autoradiographs and the necrotic dots observed on the margin of the willow leaves. Moreover, the structural connection between cadmium accumulation and necrosis was also found at the microscopic level in willow leaves (Vollenweider et al. 2006). As reported in Figure 2, the reduction or the enhancement of F_o, F_v/F_m, ΦPSII, q_P and NPQ values around the necrotic areas was different depending on tested clones. In particular, clone Poli maintained a high photosynthetic activity and a homogenous distribution of the chlorophyll fluorescence parameters owing to low cadmium content in leaves (see Fig. 2 and Table 3); clone I-214 showed some small necrotic areas near the central and lateral veins that caused, around these dots, a slight reduction in the photosynthetic activity (see Fig 2 and Table 3, ΦPSII, q_P) and an increase of dissipation of absorbed energy as heat (see Fig 2 and Table 3, NPQ); clone A4A exhibited a large number of necrotic areas both near the central and lateral veins that drastically and diffusely reduced the efficiency of light energy utilisation (see Fig 2 and Table 3, ΦPSII, q_P) and increased the need to dissipate the energy not used for photosynthesis (see Fig 2 and Table 3, NPQ); finally, clone SS5 expressed the higher number of necrotic areas with respect to other clones, prevalently located along the central vein, but areas around necrotic dots maintained a high photochemical efficiency without to be damaged (see Fig 2 and Table 3, ΦPSII, q_P) in spite of the high cadmium content found in leaves. Our results showed that chlorophyll fluorescence images of ΦPSII and NPQ were better indicators of early stress caused by cadmium presence with respect to F_v/F_m. In fact, while images of F_v/F_m only showed a partial distribution of the damage, images of ΦPSII and NPQ exhibited the real detrimental effect of cadmium presence over the leaf surface. A probable explanation of the high capacity to tolerate the metal presence in leaves of willow could be, according to Vollenweider et al. (2006), that the main cadmium sinks in the leaves were not in the mesophyll but in the veins, at least as long as the cadmium storage remained under control. The likely advantages of such a sink strategy for the willow leaves included: the storage in a tissue with a low sensitivity to cadmium toxicity, the proximity to metal cycling routes and the metal confinement away from the leaf blade. Besides, the different behaviour between willow and poplar clones, as regards the damage produced by cadmium at leaf level, could be caused by lacked synthesis of phytochelatins in willow. Phytochelatins (PCs) are induced by, and bind

to, heavy metals. A recent study (Chen et al., 2006) showed that PCs have the ability to undergo long-distance transport in a root-to-shoot and shoot-to-root direction in transgenic *Arabidopsis thaliana*. In heavy metal tolerance, PCs have shown various effects (De Knecht et al. 1992, De Vos et al. 1992; Chen and Goldsbrough 1994, Howden et al. 1995). Landberg and Greger (2004) investigated the response to heavy metals in *Salix viminalis* clones with different tolerance, analysing the presence of phytochelatins in response to a challenge with heavy metals Cd, Cu, Ni, Pb and Zn. It was shown that none of the clones exhibited induction of phytochelatins in any condition of treatment. Moreover, as reported by Riesen and Feller (2005), the mobility of heavy metals in phloem plays a critical role in the redistribution of micronutrients and pollutants. Probably, in willow, a reduced mobility of the metal inside and between cells, due to the absence of PCs, could limit its diffusion and decrease the damage at leaf level. On the contrary the presence of PCs in poplar (Arisi et al., 2000; Gawel et al., 2001) could widely diffuse cadmium in the leaf and cause detrimental effects. According to these findings, our study evidenced that only poplar leaves with a very low metal content were able to maintain a high photosynthetic activity.

4.5 Conclusions

We studied the response to cadmium treatment in leaves of poplar and willow clones. The results indicated that willow clone SS5 was more tolerant at leaf level than other poplar clones, except Poli, since maintained a high photosynthetic activity. Nevertheless, while poplar clone Poli showed a notably low cadmium content willow clone SS5 exhibited the highest one. Such a discrepancy could be ascribed to a different mobility of heavy metals inside and between cells in the tested clones. In particular, SS5 probably bound cadmium in the veins and in necrotic areas on leaf surface without to damage the rest of the leaf blade while poplar clones showed a wide diffusion of the damage. In this investigation chlorophyll fluorescence imaging has been used as a useful and intuitive technique in the context of plant photosynthetic performance under cadmium stress. Moreover, among chlorophyll fluorescence measured parameters, images of ΦPSII and NPQ make possible the early detection of cadmium stress better than F_v/F_m in plants grown in controlled environment and could be used as a fast screening system to evaluate the tolerance capability of plants to heavy metal also in field experiment.

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