Arbuscular mycorrhizal fungi as a tool to ameliorate the phytoremediation potential of poplar: biochemical and molecular aspects

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Poplar is a suitable species for phytoremediation, able to tolerate high concentrations of heavy metals (HMs). Arbuscular mycorrhizal fungi (AMF) form symbiotic associations with the roots of most land plants; they improve nutrient uptake and enhance phytoextraction of HMs while alleviating stress in the host plant. This review summarizes previous results from field and greenhouse studies conducted by us and dealing with this topic. In a field trial on a highly Zn- and Cu-contaminated site, differences in plant survival and growth were observed among 168 clones originating from natural populations of *Populus alba* L. and *Populus nigra* L. from northern Italy. After two and a half years from planting, the density, activity and metabolic versatility of the culturable fraction of the soil bacteria in the HM-polluted field was higher in the soil close to where larger poplar plants were growing, in spite of comparable HM concentrations recorded in these soils. One well-performing clone of *P. alba* (AL35), which accumulated a higher concentration of both metals and had high foliar polyamine (PA) levels, was used for further investigation. In a greenhouse study, AL35 cuttings pre-inoculated with AMF (*Glomus mosseae* or *Glomus intraradices*) and then transferred to pots containing soil, collected from the HM-polluted site, displayed growth comparable to that of controls grown on unpolluted soil, in spite of higher Cu and Zn accumulation. Such plants also showed an overall up-regulation of metallothionein (MT) and PA biosynthetic genes, together with increased PA levels. A genome-wide transcriptomic (cDNA-AFLP) analysis allowed the identification of a number of genes, mostly belonging to stress-related functional categories of defense and secondary metabolism, that were differentially regulated in mycorrhizal *vs*. non mycorrhizal plants. A proteomic analysis revealed that, depending on sampling time, changes in protein profiles were differentially affected by AMF and/or HMs. It is concluded that soil-borne microorganisms affect plant performance on HM-polluted soil. In particular, mycorrhizal plants exhibited increased capacity for phytostabilization of HMs, together with improved growth. Their greater stress tolerance may derive from the protective role of PAs, and from the strong modulation in the expression profiles of stress-related genes and proteins.

Keywords: Arbuscular Mycorrhizae, Copper, Phytoremediation, Poplar, Proteome, Soil Bacteria, Transcriptome, Zinc

Introduction

Many large areas around the world are contaminated with heavy metals (HMs) and/or organic compounds; most of these have not been remediated due to the high cost and technical drawbacks of currently available technologies. HMs tend to accumulate in soils and aquatic sediments and can enter the food chain leading to the biomagnification phenomenon thereby representing a risk to the environment and to human health (Clijsters et al. 1999). Some essential elements, such as copper (Cu) and zinc (Zn), may be present in soils and waters at potentially toxic levels mainly as a result of agricultural and industrial practices (Ali et al. 2004).

Alternative techniques for the clean-up of polluted soil and water, such as the cost-effective and less disruptive phytoremediation, have gained acceptance in recent years (Pilon-Smits 2005, Thewys et al. 2010). Trees have been suggested as suitable for phytoremediation due to their high biomass production (Dickinson & Pulford 2005) and because tree plantations can be multi-purpose (Tognetti et al. 2013). Poplar has many characteristics suitable for phytoremediation: a fast rate of growth, a deep and wide-spreading root system and a metal-resistance trait (Aronsson & Perttu 2001, Di Baccio et al. 2011, Punshon & Dickinson 1997, Sebastiani et al. 2004). In Italy, *P. alba* (white poplar) and *P. nigra* (black poplar) populations of the Ticino river valley constitute a hotspot of biodiversity (Castiglione et al. 2009, 2010, Fossati et al. 2004). Their genetic variability is being exploited for the selection of genotypes having interesting traits, such as tolerance to pollutants. The remarkable clonal variability of poplar allows to identify genotypes with a greater ability to accumulate/tolerate pollutants including heavy metals (Dos Santos Utmazian et al. 2007, Kopponen et al. 2001, Laureysens et al. 2004, Punshon & Dickinson 1997, Zalesny et al. 2005). Finally, poplar offers the advantage that, being the first "model tree species" whose genome has been sequenced (Tuskan et al. 2004), physiological and molecular mechanisms at the basis of metal tolerance can be more easily investigated at the transcriptomic level (Di Baccio et al. 2011).

Plant symbiotic fungi, such as mycorrhizae, and soil bacteria can confer increased tolerance to stress (Gamalero et al. 2009). Arbuscular mycorrhizal fungi (AMF) form associations with the roots of the vast majority of land plants; the fungus colonizes the roots and forms arbuscules within root cortical cells thus improving plant nutrient uptake, especially phosphorus (Smith & Read 1997). Moreover, increasing evidence shows that symbiotic fungi contribute to plant adaptation to multiple biotic and abiotic stresses (Gohre & Paszkowski 2006, Lebeau et al. 2008, Lingua et al. 2002, Liu et al. 2007, Rodriguez & Redman 2008, Smith et al. 2010). In the case of HMs, the beneficial ef-

fect varies according to plant and fungal species, metal and concentration (Bois et al. 2005, Lebeau et al. 2008, Takacs et al. 2005, Todeschini et al. 2007). The mechanisms by which AMF offer protection from stress have not been clarified, although decreased metal uptake has been reported in some cases (Christophersen et al. 2012, Mrnka et al. 2012). The potential of plant-microbe interactions in enhancing phytoremediation potential has been reviewed extensively elsewhere (Doty 2008, Lebeau et al. 2008, Rajkumar et al. 2012). Also in poplar, the effects of bacterial endophytes (van der Lelie et al. 2009), and of endo- and ectomycorrhiza (Mrnka et al. 2012) on phytoremediation capacity have been described.

Information regarding basic molecular processes underlying metal detoxification/tolerance is scarce especially in tree species. Metallothioneins (MTs) are among the plant components that respond to metal stress. MTs are small proteins encoded by a multigene family whose members appear to be differentially regulated in relation to organ and developmental stage, and in response to a number of stimuli including HMs (Cobbett & Goldsbrough 2002). A role for MTs in HM detoxification and homeostasis has been proposed either because they bind to HMs or because they function as antioxidants (Akashi et al. 2004). The evidence is largely based on MT gene expression studies and yeast complementation experiments with plant MT genes, and some of it comes from studies on poplar species or hybrids (Balestrazzi et al. 2009, Castiglione et al. 2007, Hassinen et al. 2009, Kohler et al. 2004).

Polyamines (PAs) are organic polycations regarded as plant growth regulators (Bagni & Torrigiani 1992); putrescine (*Put*), spermidine (*Spd*) and spermine (*Spm*), the most abundant PAs in plants, occur both in free and conjugated forms, the latter mainly represented by phenylamides (products of the covalent binding of PAs with hydroxycinnamic acids - Martin Tanguy 1997). Due to their transcriptional and translational effects free PAs are essential for normal growth and development of eukaryotic organisms (Kusano et al. 2008). In addition, there is abundant evidence for a stress protective role of PAs. Such evidence clearly arises from increased tolerance to multiple types of abiotic stress in plants that over-express PA biosynthetic genes (Abou-Shanab et al. 2008, Alcazar et al. 2010, Groppa & Benavides 2008, Prabhavathi & Rajam 2007), and from experiments in which plants were treated with exogenous PAs (Groppa et al. 2007, Prabhavathi & Rajam 2007, Velikova et al. 2000). Although the exact mechanism is still unclear, PAs may act as free radical scavengers, stabilize membranes and retard senescence (Sharma & Dietz 2006). Up-regulation of PA metabolism has been reported in poplars exposed to high Zn or Cu concentrations under *in vitro* (Franchin et al. 2007) or greenhouse/pot (Lingua et al. 2008) conditions and has been shown to correlate with the extent of metal tolerance.

The present review summarizes the results of our studies carried out in the last ten years, aimed at: (i) field screening for metal tolerance and accumulation of poplar clone collections established on a Cu- and Zn-polluted site; (ii) analyzing the microbial populations present in the poplar plantation on the polluted site; (iii) investigating the effect of AMF on a tolerant poplar clone selected on the polluted site; (iv) monitoring the changes in some molecular/biochemical parameters involved in stress responses; and (v) analyzing the transcriptome and proteome changes elicited in leaves by HM and/or AMF.

Field screening of tolerant poplar clones

After assessing their genetic dissimilarity through Amplified Fragment Length Polymorphism (AFLP) analysis, more than 2000 cuttings of 168 different poplar clones (40 clones of *P. alba* and 128 clones of *P. nigra*) collected along the banks of the Ticino river (near Pavia, Italy) were planted in March 2003 on a highly Cu- and Zn-polluted soil (ca. 900 and 1200 mg kg^{-1} dry weight soil, respectively in the top 60-cm layer) close to an industrial plant. The clones were screened for survival and growth at the end of two consecutive growing seasons; metal accumulation in plant organs and leaf PA concentrations were determined in the best performing ones (Castiglione et al. 2009). After the first growth season, survival ranged from 0 to 80% (Tab. 1); clones with more than 45% survival varied from 20 to 28%, the latter in the *P. alba* collection named AL. This confirmed the large clonal variability of poplar, according to previous reports on Salicaceae (Aravanopoulos et al. 1999) and their mainly sexual reproduction (Smulders et al. 2008). Survival after the second growth season positively correlated (R^2=0.75-0.95) with plant size at the end of the first season, thus confirming the evidence that individuals with a larger biomass had a higher probability of survival (Zalesny et al. 2005).

Six of the best-performing poplar clones were selected for further analyses. After the first growth season the highest amount of Zn was found in the leaves, as compared with stem and roots, in agreement with previous results in *Salix* and *Populus* (Dos Santos Utmazian & Wenzel 2007, Laureysens et al. 2004) indicating that Zn is efficiently translocated from the roots and accumulated in the leaves (Tab. 1). The clone named AL35 exhibited the highest concentration of Zn in all three organs as compared with the other clones. AL35 also displayed a high capacity for accumulating Cu, whose concentration exceeded several fold that found in other clones, particularly in the roots; the latter is in accordance with the low translocability of this metal (Borghi et al. 2007, Kopponen et al. 2001, Todeschini et al. 2007).

The measure of foliar free and conjugated PA levels in the same plants confirmed that AL35 has outstanding features (Tab. 2). Although clonal variability was less accentuated than variability in metal accumulation capacity, AL35 stood out for its very high PA levels, with a prevalence of conjugated forms, which reached, in the case of *Put*, a concentration 18-fold higher than that of other clones. The prevalence of conjugated PAs relative to the free forms is consistent with the abundance of phenolic compounds in poplar tissues (Tsai et al. 2006). The strong positive correlation across clones between foliar levels of PAs and root Cu concentration (R^2 = 0.79 for total PAs) suggested that Cu rather than Zn drove the long-term PA response. In fact, Cu is considered more toxic than Zn, and has been demonstrated to induce differential responses in terms of growth and PA accumulation also

Tab. 1 - Survival percentage and metal concentrations in selected poplar clones (AL22, AL35, NG12, NG19, SN26 and SN56) growing on a contaminated site at the end of the first growth season. Data are the mean ± SE.

Poplar clone	Survival percentage (%)	Metals (mg kg^{-1} dry weight)					
		Cu			Zn		
		Leaves	Stems	Roots	Leaves	Stems	Roots
AL22	66.7	404 ± 22	37 ± 8	78 ± 53	845 ± 77	125 ± 27	97 ± 30
AL35	75	236 ± 50	109 ± 13	568 ± 174	2533 ± 234	710 ± 23	1159 ± 219
NG12	75	162 ± 7	34 ± 1	71 ± 6	2012 ± 246	672 ± 26	263 ± 9
NG19	50	190 ± 10	25 ± 10	50 ± 10	1076 ± 156	238 ± 10	137 ± 10
SN26	66.7	171 ± 10	57 ± 18	178 ± 18	1889 ± 223	552 ± 22	182 ± 4
SN56	75	132 ± 13	20 ± 23	81 ± 23	879 ± 189	151 ± 6	202 ± 62

Tab. 2 - Free and conjugated polyamine levels in selected poplar clones (AL22, AL35, NG12, NG19, SN26 and SN56) growing on a contaminated site at the end of the second growth season. Data are the mean ± SE. (*Put*): putrescine; (*Spd*): spermidine; (*Spm*): spermine.

Poplar clone	Polyamines (nmol g^{-1} fresh weight)					
	Free			Conjugated		
	Put	*Spd*	*Spm*	*Put*	*Spd*	*Spm*
AL22	20.4 ± 4.6	32.4 ± 3.4	20.2 ± 0.6	284.0 ± 41.0	102.0 ± 20.0	8.0 ± 1.0
AL35	360.8 ± 17.8	54.1 ± 3.4	0.0	1853.8 ± 151.5	287.7 ± 11.8	0.0
NG12	35.9 ± 5.3	22.7 ± 2.4	3.8 ± 0.1	205.4 ± 54.1	74.1 ± 6.0	0.0
NG19	33.3 ± 2.2	25.3 ± 5.9	6.5 ± 1.8	162.3 ± 0.8	221.4 ± 13.0	0.0
SN26	54.3 ± 0.9	67.0 ± 2.6	25.6 ± 2.8	115.0 ± 45.8	129.5 ± 55.0	31.0 ± 3.0
SN56	22.4 ± 1.2	57.1 ± 2.9	10.4 ± 0.3	109.0 ± 15.0	105.0 ± 15.0	0.0

in micropropagated white poplar (cv. "Villafranca") shoots cultured *in vitro* (Franchin et al. 2007). Thus, in clone AL35 grown on the polluted site, the higher free and conjugated *Put* levels were associated to the very high concentration of both HMs accumulated in all three organs. Given that AL35 also exhibited a very high survival rate (75%), its capacity to synthesize and accumulate large amounts of *Put* likely contributed to its tolerance to HMs. It has been suggested that PAs may protect the plant through reduction of oxidative damage/lipid peroxidation by free radical scavenging and even metal chelation (Groppa & Benavides 2008, Lovaas 1997, Sharma & Dietz 2006).

Survey of soil microbial populations associated to poplar in the polluted site

The importance of rhizosphere bacteria in growth and development of their host plants, and the use of microorganisms or their genes for engineering plants in enhancing phytoremediation is still underestimated (Doty 2008, Frey et al. 2010), even though there is clear evidence that metal phytoextraction and accumulation in plants can be affected by soil microorganisms (Abou-Shanab et al. 2008, Shilev et al. 2001, Solis-Dominguez et al. 2011).

A second experimental plot was established next to the previous one on the same Cu- and Zn-polluted site (where the first poplar clone screening was performed) using the best performing white and black poplar clones from the previous trial and the commercial hybrid poplar clone I-214 (*Populus* × *canadensis* Moench) as a spacer between different clones (Gamalero et al. 2012). Although the overall survival of the clones confirmed previous results, cuttings from the same clone showed a marked variability in terms of survival and growth in different zones of the field ("position effect"). After checking that there were no significantly different Cu, Zn and phosphorus concentrations in the soil in the different zones, we turned our attention to the soil microbial populations. After two and a half years from planting, microbiological and molecular analyses were focused on microbial populations collected in proximity of the roots of large and small I-214 trees, as well as in the soil with no plants (bulk soil - Gamalero et al. 2012). I-214 trees were chosen because they were more abundant than any other tested clone (being used as spacer, their number equaled the sum of all the individuals of the other clones under study); furthermore, they were very evenly distributed on the area chosen for the study. In agreement with the "rhizosphere effect" (Hartmann et al. 2008), results showed that the density, activity and metabolic versatility of the culturable fraction of the bacteria tended to increase from the bulk soil to the soil collected beneath the large I-214 trees. Overall, about 100 culturable bacterial strains were isolated and identified from the three different soil samples (bulk soil, small I-214, and large I-214). The Denaturing Gradient Gel Electrophoresis (DGGE) profiles of the culturable fraction revealed differences in bacterial populations depending upon the soil sample. In bulk soil, all the isolated strains were Gram positive, including sporulating (*Bacillus* sp.) and non sporulating (*Arthrobacter* and *Streptomyces* spp.) species. Two Gram negative species (*Chryseobacterium soldanellicola* and *Variovorax paradoxus*) were preferentially associated with the poplar-planted soils. *C. soldanellicola* has never been reported to be tolerant to HMs, and it was previously isolated only from roots of sand-dune plants (Park et al. 2006). On the other hand, *V. paradoxus* has been found to promote plant growth under stress conditions probaly due to its ability to synthesize 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme which modulates the level of the stress hormone ethylene in plants (Belimov et al. 2001). In the soil surrounding the large I-214 poplar plants, *Flavobacterium* was the prevalent genus. Flavobacteria are found in the soil and rhizosphere where they tolerate high levels of major pollutants, including HMs (Kuffner et al. 2008, Piotrowska-Seget et al. 2005), and promote plant growth under natural and stressful conditions (He et al. 2010, Kuffner et al. 2008). Both culture-independent methods for the whole bacterial community and DGGE analysis of the culturable fraction revealed a larger biodiversity in the poplar-associated soil samples as compared to the bulk soil, strongly suggesting that poplar trees select and increment the rhizosphere-associated microflora, possibly through the release of root exudates.

Arbuscular mycorrhizal fungi improve biomass production on polluted soil

Given the interesting features of AL35, the next step was to investigate the role of AMF in poplar tolerance to Cu and Zn using this clone. To this aim, poplar cuttings were pre-inoculated as described in Lingua et al. (2008), or not inoculated (controls), with either *Glomus mosseae* (Gm) or *G. intraradices* (Gi), now *Funneliformis mosseae* and *Rhizophagus intraradices*, respectively, according to the reviewed Glomeromycota classification by Schüßler & Walker (2010). After one month cuttings were transferred to pots containing the same polluted (P) soil collected from the experimental site, or non polluted (NP) agricultural soil, and grown

Tab. 3 - Leaf, stem and root biomass (mean g dry weight $plant^{-1}$) of *P. alba* clone AL35 after two seasons of growth (S3) in the greenhouse on unpolluted (NP) or polluted (P) soil in the absence or in the presence of either *G. mosseae* (Gm) or *G. intraradices* (Gi). Data are the mean ± SE (n=3).

Treatment	Biomass		
	Leaves	Stems	Roots
NP	3.88 ± 0.82	10.31 ± 4.71	5.32 ± 2.94
NP-Gm	0.76 ± 0.01	12.69 ± 3.14	3.13 ± 1.15
NP-Gi	2.17 ± 0.23	7.20 ± 0.91	3.29 ± 0.16
P	0.51 ± 0.07	1.31 ± 0.08	0.79 ± 0.07
P-Gm	2.82 ± 0.16	8.24 ± 2.93	4.88 ± 1.80
P-Gi	0.44 ± 0.04	8.31 ± 0.56	3.40 ± 0.84

Tab. 4 - Cu and Zn concentrations in leaves, stems and roots of *P. alba* clone AL35 after two seasons of growth (S3) in the greenhouse on unpolluted (NP) or polluted (P) soil in the absence or in the presence of either *G. mosseae* (Gm) or *G. intraradices* (Gi). Data are the mean ± SE.

Treatment	Metals (mg kg^{-1} dry weight)					
	Cu			Zn		
	Leaves	Stems	Roots	Leaves	Stems	Roots
NP	13.76 ± 1.3	8.45 ± 0.6	37.13 ± 3.2	385.22 ± 34.6	82.09 ± 7.2	92.24 ± 8.2
NP-Gm	12.08 ± 0.9	5.71 ± 0.5	14.21 ± 1.2	269.22 ± 24.2	82.99 ± 7.5	43.79 ± 3.8
NP-Gi	13.01 ± 1.2	5.73 ± 0.5	15.72 ± 1.4	284.97 ± 26.0	76.19 ± 6.9	37.87 ± 3.3
P	20.16 ± 1.7	19.07 ± 1.7	97.56 ± 8.6	387.12 ± 34.9	126.96 ± 11.2	98.50 ± 8.8
P-Gm	31.86 ± 2.7	7.72 ± 0.6	605.47 ± 54.3	532.63 ± 47.8	63.76 ± 5.7	212.11 ± 19.1
P-Gi	26.90 ± 2.3	5.66 ± 0.5	244.69 ± 21.8	461.18 ± 41.7	116.40 ± 10.5	115.76 ± 10.3

for two vegetative seasons (2006-2007) in a greenhouse. Leaf sampling was performed four (S1, July 2006), six (S2, September 2006) and 16 months (S3, July 2007) after cutting transplanting. At the end of the experiment (S3), the extent of mycorrhization (5-23%), though fairly low, was in line with previous reports (Quoreshi & Khasa 2008). At this time, plant biomass was severely affected by HMs in the absence of AMF (up to 85% growth inhibition - Tab. 3). Interestingly, both fungal species restored plant biomass to control levels (Tab. 3), despite the generally higher HM accumulation in plant organs of mycorrhizal plants compared with those of non mycorrhizal ones (Tab. 4). In particular, Cu concentration was enhanced by both AMF in leaves and roots, while that of Zn was only enhanced by Gm in the roots. The total amount of metal accumulated by poplar organs, especially the roots, also increased dramatically in mycorrhizal plants (*i.e.*, 10 and 37 fold in the case of Cu, and 12.5 and 5 fold in the case of Zn for Gm and Gi, respectively - Cicatelli et al. 2010). In some non-woody plants, both monocot and dicot, increased uptake of metals (As, Cu, Zn) was reported in mycorrhizal plants as compared with non-mycorrhizal ones (Hua et al. 2010, Tseng et al. 2009).

Growth inhibition is a frequent symptom of HM phytotoxicity. Although the two fungal species can exert differential effects (Lingua et al. 2008) in AL35 plants grown on P soil the restoration of biomass production by pre-inoculation with AMF indicates that mycorrhization exerted a strong protective effect against HM toxicity. The higher phosphorus concentration in mycorrhizal roots suggests that growth recovery was due at least in part to the improved nutritional status, and certainly not to the reduced HM uptake. The mechanism by which the fungal symbionts exerted their protective role has been the focus of our further investigations.

Metallothioneins are up-regulated in mycorrhizal plants

Mycorrhizal plants grew better than their non-mycorrhizal counterparts though accumulating more Cu and Zn, suggesting the presence of detoxifying mechanisms arising from cellular molecular and biochemical processes. Quantitative RT-PCR was performed to investigate the steady-state transcript levels of the poplar MT multi-gene family in mycorrhizal plants compared with non-mycorrhizal ones grown on either P or NP soil (Cicatelli et al. 2010). In P soil, both AMF strongly up-regulated the expression of both *a* and *b* isogenes of *PaMT1, PaMT2* and *PaMT3* both at the first (S1) and third (S3) leaf sampling (Tab. 5). The largest increase was observed for *PaMT1a* in leaves of Gm plants. Overall, these results suggest that MTs may indeed afford protection against HM-induced stress, as previously reported in *Pisum* (Rivera-Becerril et al. 2005). MTs probably exert an antioxidant function as reported in transgenic *P. alba* cv. Villafranca plantlets over-expressing a pea MT2 gene (Balestrazzi et al. 2009). Although no correlation was found between MT expression and leaf metal concentration at S3 (but not S1), roots of mycorrhizal plants had accumulated one or both metals at higher concentration than controls. This would suggest that a signal coming from roots induced an up-regulation of all leaf MT mRNAs even before the plants had translocated and accumulated the metals at the leaf level. Moreover, since the fungus alone did not induce up-regulation of any of the MT isogenes on NP soil (Cicatelli et al. 2010), it appears that both high metal concentration and AMF are needed to trigger such induction. This has been confirmed by more recent results from a genome-wide transcriptomic analysis (Cicatelli et al. 2012). By contrast, in *Brassica* and in tomato, a decrease in MT expression was reported in mycorrhizal as compared with non-mycorrhizal plants. However, in tomato this was associated with lowered metal concentration in mycorrhizal plants (Dabrowska et al. 2012, Ouziad et al. 2005), in contrast to the results obtained from the clone AL35.

Tab. 5 - Modulation of poplar MT (MT1 to 3, isoforms *a* and *b*), ADC and SPDS (1 and 2) gene transcript levels in leaves of *P. alba* clone AL35 after 4 months (S1) and 16 months (S3) of growth in the greenhouse on polluted (P) soil in the absence or in the presence of either *G. mosseae* (Gm) or *G. intraradices*. Leaf mRNA levels were quantified by real-time RT-PCR and normalized with respect to actin. Arrows indicate alterations in gene expression relative to non mycorrhizal plants (on P soil).

Genes	S1		S3	
	P-Gm	P-Gi	P-Gm	P-Gi
PAMT1a	↑	↑	↑	↑
PAMT1b	↑	↑	↑	↑
PAMT2a	↑	↑	↑	↑
PAMT2b	↑	↑	↑	↑
PAMT3a	↓	↑	↑	↑
PAMT3b	↑	↑	↑	↑
PaADC	↑	↑	↔	↔
PaSPDS1	↓	↑	↑	↑
PaSPDS2	↓	↑	↓	↑

Plant PA metabolism positively responds to mycorrhization

In the field trial on the polluted site, the high-performing AL35 clone exhibited an endogenous concentration of *Put* that was 18-fold higher than in the other clones. This corroborates the idea that PAs have a major role in conferring metal tolerance to plants. In subsequent greenhouse experiments performed with this clone, a different response to AMF inoculation was observed for genes encoding for the PA biosynthetic enzymes arginine decarboxylase (ADC), responsible for *Put* biosynthesis, and spermidine synthase (SPDS), which forms *Spd* from *Put*. Transcriptional changes associated with colonization by *G. mosseae* or *G. intraradices* in roots of *Medicago truncatula* Gaertn. also

revealed several hundred genes that were either up- or down-regulated by only one of the two fungal species (Hohnjec et al. 2005). At first sampling (S1), an up-regulation of *PaADC* occurred in leaves of mycorrhizal AL35 plants grown on P soil relative to non-mycorrhizal controls, while at third sampling (S3) *PaADC* was down-regulated in the same plants (Tab. 5). *PaADC* up-regulation is in line with the purported role of this enzyme in plant responses to different stresses including metal stress (Alcazar et al. 2010, Groppa & Benavides 2008, Prabhavathi & Rajam 2007). No *Put* was accumulated possibly because it was transformed into *Spd* and/or *Spm*. At third sampling, *PaSPDS1* and *PaSPDS2* transcripts were both induced by AMF (Tab. 5). As a result of *PaSPDS* up-regulation, free *Spd* titres were higher in the presence than in the absence of AMF and correlated with improved plant growth; conjugated *Spd* and *Spm* levels were also dramatically enhanced in plants inoculated with *G. intraradices* relative to uninoculated controls. Although enhanced *Put* titres seem to be the common physiological response to HM stress (Castiglione et al. 2009, Franchin et al. 2007, Groppa & Benavides 2008, Lei et al. 2007), the accumulation of the higher PAs *Spd* and *Spm* seems to be typical of mycorrhizal plants grown under abiotic stress (Sannazzaro et al. 2007). The positive role of PAs, especially *Spd* and *Spm*, possibly relies on their polycationic nature allowing a high biological activity (Hanfrey et al. 2002), such as binding with charged macromolecules. PAs may also afford protection from HM-induced stress by exerting an antioxidant activity, and/or by chelating metals (Groppa et al. 2007, Kuthanova et al. 2004). Moreover, the singlet oxygen quenching capacity of phenylamides and their free radical scavenging properties have been established (Edreva et al. 2007). These molecules are regarded as end-products of cellular metabolism (secondary metabolites), and long-distance signalling from mycorrhizal roots has been previously reported (Copetta et al. 2006, Guerrieri et al. 2004), resulting in the enhancement of secondary metabolite production in leaves.

The leaf transcriptome and proteome are modulated in mycorrhizal plants

Transcriptome analysis

Given the beneficial effects of AMF inoculation on poplar trees grown on HM-contaminated soil and the modified expression of major stress-related genes by fungal symbiosis, the hypothesis that a broader range of genes is involved in the improved growth performance of mycorrhizal AL35 plants on P soil relative to non-mycorrhizal ones was pursued. A genome-wide transcriptomic analysis was conducted by cDNA-AFLP on leaves of AL35 plants grown on NP or P soil, the latter in the presence or in the absence of *G. mosseae* or *G. intraradices* (Cicatelli et al. 2012). A comparison of the cDNA-AFLP patterns of all four experimental conditions at S1 revealed that a large number of transcription-derived fragments (TDFs) were differentially modulated, and that AMF inoculation strongly modified the leaf transcriptome, partially restoring it to the control profile (Cicatelli et al. 2012). Most of the sequenced TDFs had similarities with database entries with known function and thus the identified TDFs were assigned to functional categories. The largest group of cDNA sequences (18% of the total) corresponded to secondary plant metabolism, while defense, photosynthesis/energy, and intracellular traffic categories were also well represented (11%). Thirteen genes whose expression was altered in mycorrhizal plants plus MT, ADC, and SPDS genes were selected as representing important functional categories, including secondary metabolism and defense, and analyzed by qRT-PCR.

Tab. 6 - Modulation of transcript levels of genes selected, following a cDNA-AFLP analysis, in leaves of *P. alba* clone AL35 after 4 months (S1) of growth in the greenhouse on polluted (P) soil in the absence or in the presence of either *G. mosseae* (Gm) or *G. intraradices* (Gi). Leaf mRNA levels were quantified by real-time RT-PCR and normalized with respect to actin. Arrows indicate alterations in gene expression relative to non mycorrhizal plants grown on NP soil.

Genes	S1		
	P	P-Gm	P-Gi
Clathrin protein	↑	↓	↓
Phytochelatin synthase	↑	↔	↑
Chlorophyll binding protein	↔	↑	↑
Pyridoxine-5'-phosphate oxidase	↔	↑	↑
Nuclear transport factor 2	↔	↑	↑
Teosinte-branched-like protein	↔	↑	↔
S-adenosylmethionine-dependent methyltransferase	↔	↑	↔
Glutathione synthase	↔	↑	↑
Thaumatin like protein	↔	↔	↑
Remorin protein	↓	↔	↑
Arginine decarboxylase	↓	↔	↔
Prephenate dehydratase	↓	↑	↔
Polygalacturonase	↓	↔	↔
Peroxidase	↓	↑	↑
Glutamine synthase	↓	↑	↔
Auxin responsive protein	↓	↑	↔

Results showed that the vast majority of the selected genes was not affected or down-regulated on P soil, but up-regulated by one or both AMF, especially *G. mosseae* (Tab. 6). This group includes defense genes such as thaumatin-like protein (TLP), glutathione synthase (GSH) and several MTs, but also genes involved in primary metabolism and transcription. Another group was down-regulated by HMs and restored to control levels or up-regulated in mycorrhizal plants (Tab. 6). They mainly belong to defense (MTs, remorin, peroxidase) and secondary metabolism (prephenate dehydratase) categories; the latter category included also ADC gene. Thus, most genes whose expression was strongly altered belong to stress-related functional categories. Most of these genes are reported for the first time in response to HM stress, and in particular to AMF colonization. It was also shown based on their expression pattern that the fungal species behave differently. In particular, *G. mosseae* appears to have a better capacity than *G. intraradices* to activate the plant's defense system as previously hypothesized in the case of cv. Villafranca, which was able to recover growth when cultivated on artificially Cu or Zn polluted soil (Lingua et al. 2008). These results were further corroborated by means of a MSAP (Methylation Sensitive Amplified Polymorphism) analysis aimed at highlighting epigenetic modifications of the DNA in the presence of HMs and/or AMF (Cicatelli et al. 2014).

A cDNA microarray approach was pursued in order to investigate the expression of some genes involved in antioxidant metabolism and metal homeostasis in leaves and roots of AL35 poplar plants inoculated with *G. mosseae* (Pallara et al. 2013). A total of twenty-six genes were considered, including eight superoxide dismutases (4 *CuZnSod*, 2 *FeSod*, 2 *MnSod*), three catalases (*Cat*), three ascorbate peroxidases (*Apx*), one dehydroascorbate reductase (*Dhar*), one γ-glutamylcysteine synthase (*Ecs*), two glutathione reductases (*Grc*), six metallothioneins (*MT*, only in roots), one metal transporter (*Mtp*), and one phytochelatin synthase (*Pcs*). Microarray data were validated by RT-qPCR on five of the above-mentioned genes. Plants

grown on P soil generally exhibited higher transcript levels. However, some of the responses were clearly tissue specific; for instance, *Mtp* and *Pcs* were down-regulated in roots, but up-regulated in leaves. Similarly, *MT1a* and *MT1b* were down-regulated in roots. In general, ANOVA indicated that the metal treatment affected the transcription of a larger number of genes involved in antioxidant metabolism in leaves than in roots, suggesting that metals in roots might be more abundant in the apoplast than in the symplast. Mycorrhizal plants grown on P soil grew better and showed a down-regulation of most antioxidant genes, in spite of a higher concentration of metals in their tissues, suggesting a higher degree of protection in this plant and a lower need of activating antioxidant pathways implicated in ROS scavenging. Similar results were previously reported in the case of mycorrhizal *Pisum sativum* (Rivera-Becerril et al. 2005) and *Medicago truncatula* (Aloui et al. 2011) under Cd stress.

Proteomic analysis

A proteomic analysis was performed on the leaves of clone AL35 inoculated or not with *G. intraradices* and grown in the greenhouse on P or NP soil (Lingua et al. 2012). Protein expression was evaluated four (S1), six (S2) and 16 (S3) months after transplanting of the cuttings. Though the description of all proteins modulated in the four different treatments and at three time points is beyond the scope of this paper, some general observations can be summarized here. Most of the identified proteins showing variations concerned the functional groups of "photosynthesis and carbon fixation" and "sugar metabolism". This is not surprising considering the analyzed organ. Secondly, the expression profiles varied significantly with time: at S1, 22 spots showed a differential expression between the four treatments, 52 at S2, and 66 at S3. At the first sampling time, a relevant number of proteins were involved in the functional group "protein folding", while at the second and third sampling times this group was less represented and the previously missing groups of "oxidative damage" and "glutathione metabolism" were represented. Furthermore, at S1 all the modulated spots were affected by fungal inoculation (as shown by two-way ANOVA), while only 25% of them were affected by the metal treatment. At S2 the situation was reversed with most of the spots (94%) affected by the metal treatment and only 42% of them influenced by the fungal inoculation. At the last sampling, both factors were equally important.

Conclusions

Our results demontrate the feasibility of establishing a multi-clonal poplar stool bed on a highly Cu- and Zn-contaminated soil for phytoremediation purposes. The broad genetic variability of natural poplar collections allow high performing clones to be selected and used for soil recovery. One of the selected clones (AL35) showed outstanding features for both Cu and Zn phytoextraction and/or phytostabilization purposes. In addition, results show that plant association with bacterial and fungal microorganisms is able to greatly improve plant growth performance on a metal-polluted soil. Evidence is provided that stress recovery may arise from the protective role of MTs and PAs, whose genes are specifically up-regulated by the plant-AMF interaction. Large-scale molecular analyses confirm that the symbiosis determines several changes at the transcriptional/translational levels, which presumably underlie the improved performance of plants under stressful conditions. Data resulting from the proteomic analysis shall be integrated and compared with those from transcriptomic and biochemical analyses, according to a system biology approach, in order to exploit the different sensitivity of the various techniques, and provide a general framework for the plant's response to HMs. In the future, the acquired knowledge on candidate genes/proteins involved in AMF-enhanced poplar tolerance could represent the basis for molecular engineering efforts to obtain tolerant plants to be used in phytoremediation activities.

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