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ORIGINAL PAPER

Genotypic differences explain most of the response of willow cultivars to petroleum-contaminated soil

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Abstract

Key message **Highly tolerant and productive willow cultivars have been identified as potential candidates for phytoremediation of contaminated soil with petroleum hydrocarbons (PHs) in southern Quebec, Canada.**

Abstract Tolerance of *Salix* to various organic and inorganic contaminants has been well documented and can vary widely among genotypes. In this study, we compared the responses and tolerances of 11 willow cultivars grown in soil contaminated with petroleum hydrocarbons. The study aimed to identify cultivars with the best performance and capacity to establish under these conditions. Using cuttings of the selected willow genotypes, a high-density field experiment was set up on a former industrial site encompassing two distinct sectors: one contaminated (900 m^2), and the other uncontaminated (2,400 m^2), which was used as control following a randomized block design. Trees were monitored over two growing seasons by recording a series of growth parameters and physiological measurements (leaf area, chlorophyll concentration, stomatal conductance and nutrient concentration). Genotypic differences explained most of the responses of cultivars observed to soil contamination. *S. miyabeana* (SX67 and SX61) achieved the highest biomass production, while *S. nigra* (S05) *and S. acutifolia* (S54) had the highest photosynthetic capacity. While the cultivars *S.* × *dasyclados* (SV1), *S. purpurea* ('Fish Creek') and *S. caprea* (S365) appeared to be negatively affected by the presence of contaminants, the establishment and development of S05, *S. eriocephala* (S25) and *S. purpurea* × *S. miyabeana* ('Millbrook') was much less influenced by soil pollution. Our results will help to guide cultivar selection in future phytoremediation projects.

Keywords Salix physiology · Organic compound · Phytoremediation · Biomass

Introduction

Soil contamination from a range of sources is increasingly problematic around the world. The high financial and environmental costs associated with conventional decontamination techniques have motivated the search for more affordable and sustainable approaches (Cunningham et al. 1995; Cunningham and Ow 1996; Newman and Reynolds 2004). Researchers have turned to phytoremediation as a low-cost alternative with minimal negative environmental impacts (Di Baccio et al. 2011). Phytoremediation uses plants to stabilize, reduce or degrade organic and inorganic pollutants present in air, water or soil as a result of human activities (Trapp and Karlson 2001; Susarla et al. 2002; Zalesny et al. 2005; Komives and Gullner 2006). Several distinct mechanisms are included in phytoremediation, i.e., phytodegradation, phytoextraction and phytostabilisation (Schnoor et al. 1995; Gerhardt et al. 2009; Vangronsveld

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et al. 2009). Given the low water solubility of hydrophobic organic compounds such as PAHs and PCBs, these contaminants are only very minimally bioavailable to plants and can only rarely be extracted from the ground. However, there is evidence that some of these compounds can be absorbed by plants (Conger 2003; Newman and Reynolds 2004; Vangronsveld et al. 2009). Most frequently, plants and their rhizosphere microflora are used to degrade or transform contaminants into less toxic compounds (Macek et al. 2000).

Willows possess many features that make them an excellent choice as a model species in phytoremediation field experiments. These include rapid growth, high biomass production, high genetic variability, important capacity to transpire water efficiently to maintain a high transpiration rate, well-developed root system and the ability to absorb and accumulate a wide range of pollutants (Corseuil and Moreno 2001; Vervaeke et al. 2003; Kuzovkina and Quigley 2005; Yu et al. 2008; Mleczek et al. 2010; Drzewiecka et al. 2012; Gąsecka et al. 2012). The presence of contaminants in soil at levels exceeding thresholds for phytotoxicity is known to have a negative impact on metabolism of plants, affecting absorption of nutrients, photosynthetic performance and gas exchange, growth and development (Kukkola et al. 2000; Wu et al. 2010).

Several studies have demonstrated that the presence of toxic agents in the environment leads to a decrease in size and biomass of roots, shoots, and leaves, length, and premature senescence of leaves (Morris et al. 2000). In this study, we compared various physiological responses as well as productivity and tolerance of 11 willow cultivars grown on a site contaminated by petroleum. The aim of the study was to assess their ability to establish in conditions of severe stress related to the presence of organic compounds such as PAHs, PCBs and C10–C50 petroleum hydrocarbons. Our objective was to identify cultivars with the best performance and capacity to establish under these conditions which would, therefore, be ideal candidates for future phytoremediation projects in southern Québec, Canada.

Materials and methods

Experimental design, site description and willow material

A high-density field experiment was established in May 2011 near a former petrochemical plant in Varennes, southern Québec, Canada (45°46′N, 73°22′W). The region has a continental climate characterized by an average annual temperature of 6.6 °C and 984 mm of precipitation (including 186 cm of snow). Mean temperature and total yearly precipitation at the site are presented in Supplementary Table 1. Eleven willow cultivars were selected for the study (Table 1): *S. purpurea* 'Fish Creek'; *S. miyabeana* SX67; *S. miyabeana* SX61*; S. nigra* S05*; S. eriocephala* S25*; S. caprea* S365*; S.* × *dasyclados* SV1*; S. acutifolia* S54*; S. alba* S44*; S. viminalis* S33 *and S. purpurea* × *S. miyabeana* 'Millbrook' (Dickmann and Kuzovkina 2008; Newsholme 1992). Cuttings were planted in June 2011 and coppiced at the end of October, in a randomized block design on an area encompassing two distinct sectors: one moderately contaminated (900 m^2) with petroleum hydrocarbons (PHC) and another (2,400 m^2), uncontaminated, which was used as control. Soil characteristics of each sector are presented in Table 2. The experimental design comprised three blocks on the contaminated sector and four on the control. Each block covered 300 m^2 and was split into 12 plots, one cultivar plus a control per plot. Cuttings were planted at a density of 30,000 ha^{-1}. Five rows spaced by 1 m were planted in each plot. Each row comprised 75 cuttings distanced by 30 cm from one another. The rows on the border of each plot served as a buffer and were not used in the experiment. Willows were monitored over two growing seasons (2012 and 2013) by recording a series of growth and physiological measurements (leaf area, chlorophyll concentration, stomatal conductance and nutrient leaf status).

For the height, diameter, number of shoots, chlorophyll concentration and leaf area, four blocks were monitored on the control site (32 trees) and three were monitored on the polluted site (24 trees). The same sampling strategy was used for the biomass yield (16–12 trees). For the stomatal conductance, three blocks were monitored on each sector (15–15 trees). Three blocks per site were selected for the nutrient status of leaves analysis as well (3–3 trees).

Soil analysis

Soil samples were taken for organic compound analysis prior to planting. Twelve core samples were gathered from the top 30 cm of the bulk soil in each of the seven blocks and pooled for analysis. Pooled samples were sent to AGAT Laboratories [specializing in laboratory analysis and accredited by the Standards Council of Canada (SCC)] for soil analysis and to determine the concentration of all polychlorinated biphenyls congeners (PCBs), total polycyclic aromatic hydrocarbons (PAHs), monocyclic aromatic hydrocarbons (MAHs), halogenated hydrocarbons (THHs) and petroleum hydrocarbons C10–C50 by the GC–MS method. Soil samples were also taken for physicochemical characterisation following the procedure described in Cloutier-Hurteau et al. (2013). The contamination level at the site was assessed following the generic criteria for soils from the Soil Protection and

Table 1 Willow (*Salix* spp.) cultivars used in the project

Name	Authority	Genotype	Characteristics	Origin/distribution[a]
Fish Creek	SUNY-ESF, NY, USA	*Salix purpurea*	High biomass production and JGI-DOE sequencing project underway Introduced species	Northern Africa, Europe and Russia
SX67	University of Toronto, ON, Canada	*Salix miyabeana*	High biomass production Cultivated species	Native of Honshu and Hokkaido, Japan, Russian Far East, Korea, northeast and north China and Mongolia
SX61	University of Toronto, ON, Canada	*Salix miyabeana*	High biomass production Cultivated species	
S05	Unknown	*Salix nigra*	Indigenous species	Eastern North America (southern Canada to northern Florida, west from Minnesota and Texas)
S25	University of Toronto, ON, Canada	*Salix eriocephala*	Indigenous species	Northern and eastern North America, from New Brunswick to British Columbia and south to Virginia, Colorado and California
S365	University of Toronto, ON, Canada	*Salix caprea*	Introduced species, hybrid	British Isles, lowlands of Europe and central Asia
SV1	University of Toronto, ON, Canada	*Salix* × *dasyclados*	High biomass hybrid	Northern Europe, Russia to west Asia
S54	Quebec Natural Resources Ministry (QMNR)	*Salix acutifolia*	High biomass production Cultivated species	Native of Poland and Russia, extending to east Asia
S44	QMNR	*Salix alba*	High biomass production Introduced species	Europe, British Islands, Asia Minor, western Siberia, northern Africa
S33	QMNR	*Salix viminalis*	High biomass production and genetic maps available	Eurasia except Far East
Millbrook	SUNY-ESF, NY, USA	*S. purpurea* × *S. miyabeana*	High biomass production	Hybrid

Modified from Bell et al. (2014)

[a] Dickmann and Kuzovkina (2008), Newsholme (1992)

Contaminated Sites Rehabilitation Policy of the Quebec Ministry of Sustainable Development, Environment, Wildlife and Parks (http://www.mddefp.gouv.qc.ca/sol/terrains/politique-en/appendix2-table1.htm). Results of both analyses are presented in Table 2.

Measurements and harvesting

Biometric analysis and chlorophyll measurements

Chlorophyll concentration and biometric parameters, including height and diameter of principal shoot and number of shoots, were monitored monthly over two growing seasons (from June to September on year 2 and 3). The chlorophyll level was measured twice for each individual with the Chlorophyll Meter from atLEAF+, Wilmington, USA. Relative chlorophyll concentration was converted to a SPAD value before calculation into total chlorophyll concentration (mg/cm^2) using the converter on the atLEAF+ website (http://www.atleaf.com/) (Schaper and Chacko 1991; Roitto et al. 2005; Uddling et al. 2007; Zhu et al. 2012). Leaf area data were collected at the end of the first growing season. The area of the first five fully expanded leaves from the apex of eight trees per plot was calculated using the Area Meter MK2 from Delta-T Devices, for a total of 32 stools per cultivar on the control sector and 24 on the polluted one. Shoot biomass was harvested at the end of November 2013, when the shoots were 2 years old. The fresh aboveground woody material of the selected trees was weighed in the field using an industrial scale (SAFIR S100 from Gram Precision, Barcelona, Spain). To estimate dry weight from measured fresh weight across the trial, a moisture content of 50 % was assumed. Although this value could vary, data from

Table 2 Soil analysis for mean hydrocarbon content and physicochemical properties of uncontaminated and contaminated sites made in 2011, before planting

Hydrocarbon content (mg/kg)	Soils		Physicochemical properties	Soils	
	Control	Polluted		Control	Polluted
BPCs (total congeners)	<0.05	0.499*	Electrical conductivity (µS/cm)	143	261*
MAHs—xylene	<0.2	0.3	pH	6.65	7.12
MAHs—ethylbenzene	<0.2	0.4	Organic matter (g/kg)	26.3	33.2
C10–C50	<100	957	CEC (cmol (+)/kg)	27.9	34.1*
Acenaphthene	<0.1	5.63*	Density (g cm^{-3})	1.51	1.00
Acenaphthylene	<0.1	0.43*	P-Mehlich III (mg kg^{-1})	0.47	0.81*
Anthracene	<0.1	3.43	N total (g/kg)	2.8	3.0
Benzo(a)anthracene	<0.1	0.27	Ca (cmol (+)/kg)	20.3	27.4*
Chrysene	<0.1	0.33	Al (cmol (+)/kg)	0.136	0.067
Fluoranthene	<0.1	1.17	K (small (+)/kg)	1.8	1.6
Fluorene	<0.1	3.77	Mg (cmol (+)/kg)	5.5*	4.7
Naphthalene	<0.1	2.10			
Phenanthrene	<0.1	20.3*			
Pyrene	<0.1	1.77			
Methyl-1naphthalene	<0.1	4.83			
Methyl-2naphthalene	<0.1	4.87			
Dimethyl-1.3naphthalene	<0.1	7.80			
Trimethyl-2.3.5naphthalene	<0.1	2.20			

Values observed in the "Control" column of the hydrocarbon concentration analyses represent the quantification limit. Asterisks indicate a significant difference between the two types of soil. The position of the asterisk indicate which soils has the highest mean value

four previous studies (Labrecque and Teodorescu 2005; Guidi et al. 2013; Mosseler et al. 2014a, b) all showed a value of 50 % with less than 2 % of variation, suggesting it would be unlikely to impact biomass yield assessment in these system.

Stomatal conductance

Stomatal conductance was measured from June to September during each of the two growing seasons, and the average per year of the measurements was used for statistical analysis. The top ten mature leaves on each tree were measured twice using two separate Leaf Porometers (Model SC-1, Decagon Devices, Pullman, WA, USA), to measure the stomatal conductance of each genotype on both contaminated and uncontaminated soil simultaneously.

Nutrient concentration

About 15–20 fully developed leaves from the upper portion of the main shoot of one chosen tree for each cultivar on each block were harvested at the end of September of the second growing season for nutrient concentration analysis. 25 g of the leaf material was placed in a cooler and sent to Agridirect Laboratories for analysis. Samples were treated for quantification of nitrogen, phosphorus, potassium, calcium, magnesium and trace mineral elements (Al, Na, B, Cu, Fe, Mn, Mo, Zn) following an adapted version of AOAC Official Method, 16th edition (http://www.agridirect.ca/systeme/nosmethodes.asp#tissus).

Statistical analysis

Physiological responses and biomass productivity of the 11 willow genotypes on contaminated and uncontaminated soils were compared using JMP® 8.0 (SAS Institute, Cary, NC, USA). The effects of the two experimental factors, i.e., "willow genotype" and "the presence or absence of contamination" were assessed with a two-way analysis of variance (ANOVA). Homogeneity of the variance and normality of the data were checked visually and a logarithmic transformation was performed when necessary. Significance of differences between means was tested by Tukey's HSD test or the Student's *t* test, depending on the data, and was considered significant at $\alpha = 0.05$. Further analyses were carried out when the *P* value of the ANOVA test was under but very close to significance. A non-parametric Wilcoxon test was performed on the raw data to quantify the difference between the two sectors (uncontaminated and contaminated).

Results and discussion

All monitored growth and physiological parameters varied by willow genotype during both growing seasons, while contamination had a lower impact, or, in some cases, no significant impact. The overall effects of genotype and contamination, and the combination of genotype and soil contamination on nutrient concentration, growth parameters and physiological variables are shown in Supplementary Tables 2 and 3.

Growth and yield

Growth parameters, i.e., height, diameter and number of shoots per stool, varied strongly according to genotype. The "treatments" factor (contaminated versus uncontaminated) had a limited impact on these parameters. The height and the diameter were significantly affected by contamination but less than the genotype on the total variance. The treatment explained 24.4 % of the variability of height and 12.8 % of the diameter compared to 29.2 and 22.5 %, respectively, of the variability explained by the genotype (Fig. 1). Eight of the eleven cultivars tested had a significantly shorter shoot on the contaminated site. Only S05, S33 and S54 were not affected with a diminution of height of only 9, 6 and 24 %, respectively. 'Fish Creek' was the only cultivar with a significantly lower number of shoots on contaminated versus uncontaminated soil (25 % less shoot), a pattern displayed by S365 and 'Fish Creek' for diameter size (loss of 36 % in diameter size for S365 and 16 % for 'Fish Creek') (Fig. 1). *S. alba* (S44) had the longest main shoot and the largest shoot diameter among cultivars on both uncontaminated and contaminated sectors. S33 had the lowest value of height and diameter on the uncontaminated sector, while S365 had the lowest value on the contaminated one. 'Fish Creek' had the highest number of shoots per stool on both uncontaminated and contaminated soils, while S44 had the lowest number of shoots on both sector.

A study have reported that willow growth habit tends to be related to number of shoots and diameter (Tharakan et al. (2005). Genotypes producing several shoots generally have lower diameters ('Fish Creek', S365) and those that produce less shoots tend to have larger diameters (SV1, S44, S25, 'Millbrook', SX67 and SX61). The same pattern

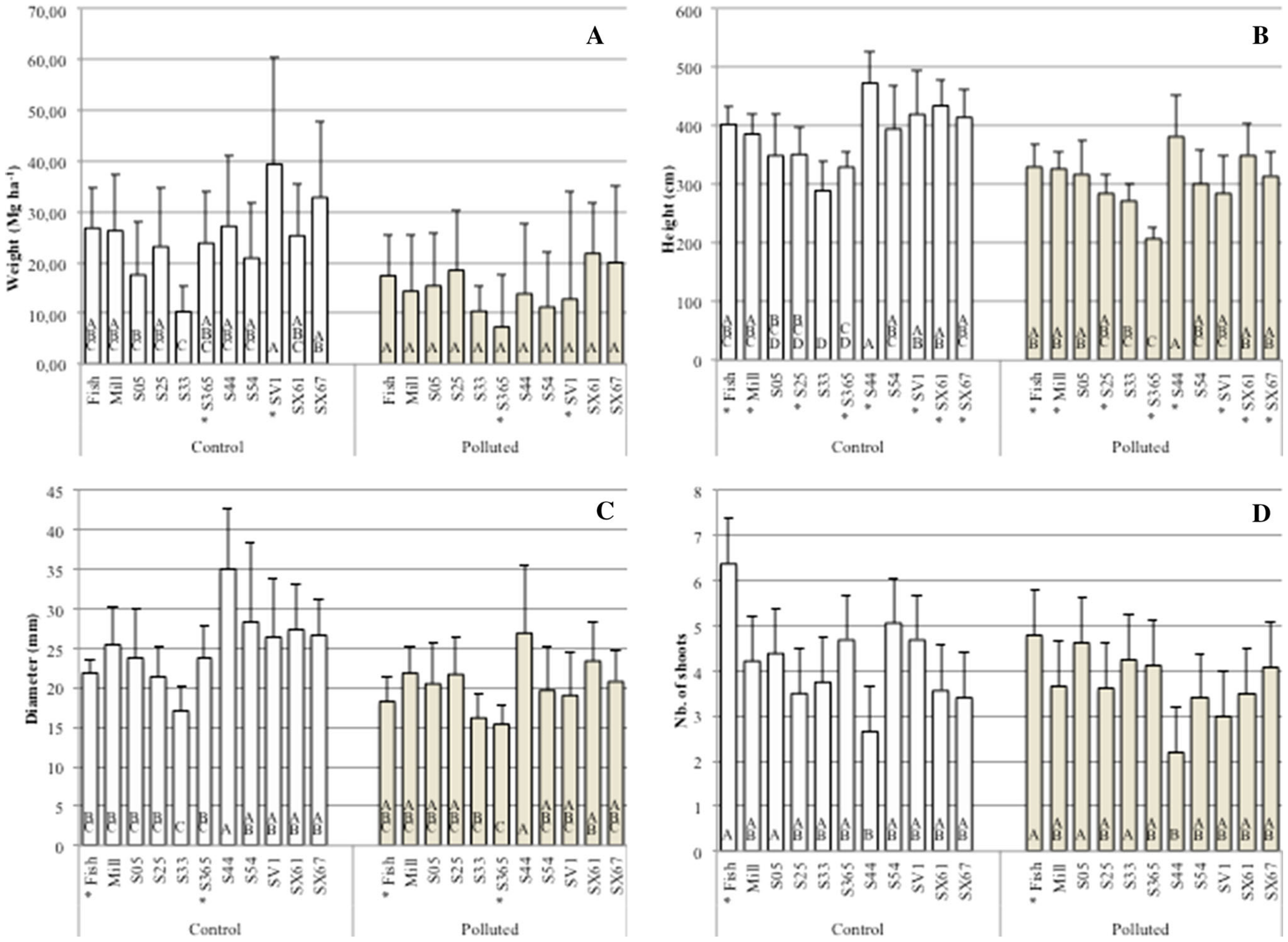

Fig. 1 Shoot growth data for all harvested willows in 2013 (mean and standard deviation) on control and polluted soils. Analyzed using Tukey's HSD test ($\alpha = 0.05$). *Asterisk* indicates significant difference between soil contaminations, for a particular cultivar

holds for groups with intermediate shoot production and dimensions (S54, S05). Based on our results, to achieve maximum biomass production, cultivars with a potential for low shoot production, but with higher diameter and height should be prioritized during the selection of cultivars for phytoremediation. Indeed, cultivars with high biomass production were also taller and had the larger shoot diameters (for example SX61, SX67). By contrast, the cultivars that produced many shoots (S33, S365) had a low biomass production.

Mean biomass production of willow cultivars was different among genotype and soil contamination. An interaction was also detected between the two factors which indicates a difference in the effect of treatment between cultivars (Supplementary Data Table 2). Soil contamination had a significant effect on yield of SV1 and S365 (loss of 68 and 69 % in biomass, respectively (Fig. 1). On the uncontaminated site, SV1 produced the largest amount of biomass, while SX61 was the most productive on the contaminated area. S33 and S365 had the lowest aboveground biomass on uncontaminated and contaminated sectors, respectively.

Several factors, including physicochemical composition of soil, temperature, amount of precipitation and nutrient characteristics can influence the yield of willow planted in short rotation intensive culture (Weih and Nordh 2002; Labrecque and Teodorescu 2003). Studies in Denmark and Québec on willow performance on various sites have shown that it tends to achieve optimum growth on poorly drained sites and in soil with a high organic matter levels (Labrecque and Teodorescu 2001; Sevel et al. 2012). The transpiration rate of willow being very high, the presence of a large amount of clay facilitates water retention and thus reduces the risk of drought-related stress. In general, willow performances on the contaminated soil were not as bad as expected. The characteristics of the contaminated soil were generally more favorable to growth and the Wilcoxon test showed that there is some significant differences between the two sectors. We monitored a significantly higher electrical conductivity (261 versus 143 μS/cm), a higher organic matter content (33.2 versus 26.3 g/kg), significantly greater cation exchange capacity (34.1 versus 27.9 cmol(+) kg^{-1}) and significantly higher phosphorus concentration (0.81 versus 0.47 ppm). These characteristics may explain the good performance of the cultivars on the contaminated site and why contamination had no significant effect on most physiological parameters. The summer of 2011 was characterized by high levels of precipitation, over the average for this location (477.6 mm between June and September compared to 385.1 mm). Although mean temperature was slightly above average, these climatic conditions are extremely beneficial for willow establishment (Labrecque and Teodorescu 2003) and could explain the very low mortality rate observed during the first summer.

Previous studies conducted in Québec (Labrecque and Teodorescu 2003, 2005) using S33 and SV1 found these cultivars to have high biomass production. Our results suggest this success may not extend to all growing conditions. While SV1 produced the largest amount of biomass on the control sector, it performed far less well in polluted soil (loss of biomass of 67.5 %). This finding is of particular interest because it shows that contamination by PHC has a significant negative effect on this cultivar's biomass production, making it an unsuitable candidate for future phytoremediation projects. The biomass production of S33 was also far below the values published by previous studies (Labrecque et al. 1997; Labrecque and Teodorescu 2003, 2005) but in this case, the poor field performances of this cultivar was mostly due to damages caused by the potato leafhopper (*Empoasca fabae* Harris) which is common on *S. viminalis* cultivars (Keoleian and Volk 2005; Guidi et al. 2013). The vulnerability of these cultivars to this insect, common to southern Québec, could also justify excluding this cultivar from potential phytoremediation candidates. While other insects were found on the plantation site, including *Tuberolachnus salignus,* particularly numerous on 'Fish Creek' shoots on the control sector, they did not appear to affect tree growth.

If no significant differences in biomass production were detected between the cultivars on the polluted sector, it is interesting to compare the yield of some cultivars on each sector. For instance, the performance of SX61, on the polluted sector was very good considering that the biomass produced was only 13.8 % less compared to the biomass produced on the control sector. On the contrary, the yield produced by SX67 after two growing seasons dropped drastically with a diminution of 39.2 % on the polluted site compared to the control site. S05 experienced the less significant diminution in production with a difference of only 12.3 % between the polluted sector and the control sectors, making this cultivar the least influenced by pollution.

Chlorophyll level and standard leaf area

Willow genotype had the greatest impact on the variance of chlorophyll concentration and leaf area in the selected cultivars. The treatment explained 5.8 % of the variability of chlorophyll concentration while 30.0 % of the variability is explained by the genotype. The effect of soil contamination was far more subtle, significantly affecting the chlorophyll concentration of S365 and S54 (diminution of 23 and 17 % of chlorophyll, respectively) and the leaf area of S365 and SV1 (diminution of 50 % of mean leaf area for both cultivars) (Fig. 2). 'Fish Creek' had the

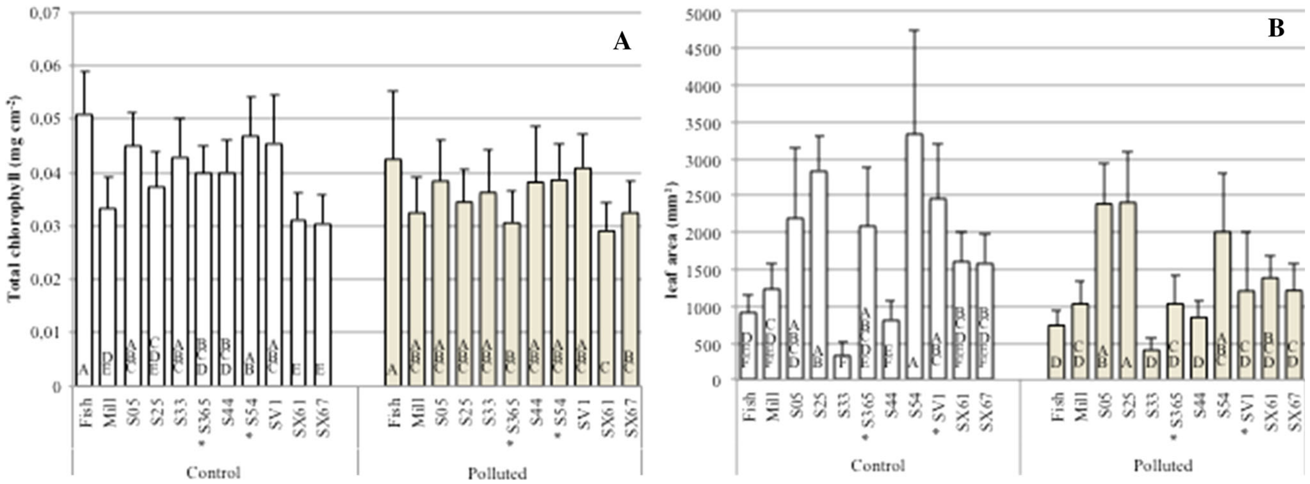

Fig. 2 Leaf chlorophyll concentration (**a**) and leaf area (**b**). Analyzed using Tukey's HSD test ($\alpha = 0.05$). The *asterisk* indicates significant difference between conditions, for a particular cultivar. The chlorophyll concentration assessment was undertaken in 2013, while the leaf area was measured at the end of the first growing season (2012)

highest concentration of chlorophyll in leaves under both conditions, while SX61 leaves had the lowest concentration on the uncontaminated sector and SX67 on the contaminated one. The cultivar with the largest leaves on the uncontaminated sector was S54, and on the contaminated one, S25. S33 developed the smallest leaves on both uncontaminated and contaminated soil.

Several willow cultivars have been found to be very effective at absorbing available nitrogen in their rhizosphere (Ericsson 1981; Labrecque and Teodorescu 2001). Since the chlorophyll amount in leaves is strongly influenced by the amount of nitrogen present in plant and therefore nitrogen available in the soil, small variations in chlorophyll level between the two tested sectors could be explained by the edaphic properties of the contaminated soil. In fact, Table 2 shows that the contaminated site contains a higher proportion of nitrogen, which could explain why chlorophyll production was not affected by the presence of contaminants. Although chlorophyll is a stress-sensitive pigment, the majority of the selected genotypes responded well to this stress, and their photosynthetic capacity did not decrease. The highest chlorophyll concentration was measured in cultivar SX67. On the contaminated sector, SV1 and S365 showed the greatest drop in leaf area, with a decrease in excess of 50 %, while S05 leaf size increased slightly, more than 9 %. The generally lower leaf area values measured on the contaminated sector probably reflect plant response to environmental stress, which has been observed frequently (Drzewiecka et al. 2012; Gąsecka et al. 2012; Zhou et al. 2013). The huge difference in leaf size between the different cultivars on the contaminated site and mostly, the very unequal effect of contamination on leaf development could be a consequence of a different partitioning of nutritive resources within the plant. Genotype can play an important role in carbon allocation during a stressful situation such as soil contamination. Some plants might favor root development to the detriment of aerial growth (SV1 and S365) to compensate, while others might favor leaves development to assure a good photosynthetic and transpiration rate in stressful situation (S05). It would be interesting to conduct further analysis on roots growth and starch accumulation to validate this hypothesis.

Stomatal conductance

All variations observed in stomatal conductance values were attributable to genotype, while contamination had no significant impact. The treatment explained 0.6 % of the variability of stomatal conductance, while 39.7 % of the variability is explained by the genotype. S365 and SV1 both had the highest values of stomatal conductance on the uncontaminated soil, while SV1 and S33 had the highest values on the contaminated soil (Fig. 3). SX67 and SX61 showed the lowest values on the uncontaminated and contaminated sectors, respectively.

Stomatal conductance is known to be influenced by environmental stress and the presence of contaminants in soil (Farquhar and Sharkey 1982; Hermle et al. 2007). Nonetheless, the measurements taken during the summer of 2013 show that such variations were entirely associated with genotypic factors. For example, we observed that S33 and SV1 had a high density of trichomes on the abaxial leaf surface (data not shown). These appendages play a very important role in regulating leaf temperature, evapotranspiration, and in the degree of stomatal opening (ESAU

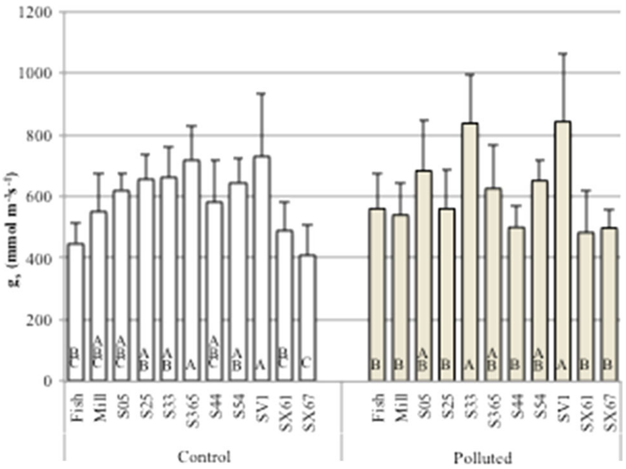

Fig. 3 Mean stomatal conductance of collected data between June and September 2013. Analyzed using Tukey's HSD test (α = 0.05)

1965). Stomatal conductance levels in these two cultivars are, therefore, probably the result of this characteristic, which reduces the adverse effects of excessive water loss in times of stress. Some genotypes had higher conductance values on the contaminated sector ('Fish Creek', S05, S33, SV1 and SX67), some lower values (S25, S365 and S44) and others very similar values ('Millbrook, S54, and SX61) regardless of soil quality. No general trend was observed in regard to other physiological traits of leaves.

Nutrient concentration and proportion

All foliar nutrient concentration variables (N, P and K) were significantly different according to the genotype but only P was affected by soil contamination (Supplementary Table 3).

Nitrogen use efficiency (NUE) is known to be a limiting factor for willow growth under temperate climatic conditions, such as those of the study site (Weih and Nordh 2002). Following the concept elaborated in this study, the phenology of bud burst and how quickly the willow start to grow is decisive for a high biomass production. It has been shown that willows which start their growing season earlier in spring have better chances of producing a large amount of biomass. The "early starting" clones tend to use their available nitrogen earlier in the season which lead to a lower growth rate at the end of the summer and lower leaf N concentration. Conversely, "late starting" clones start by accumulating N before using it for growth so they end the season with higher concentration of N in their leaves. We observed this tendency in our study as well. In spring 2012, after coppicing, the cultivars SX67 and SX61 started their growing season extremely rapidly and were the bigger trees on the field (data not shown). They also produced more biomass in contaminated soil and had the lowest level of nitrogen in their leaves at the end of the experiment. S365 and S33 were the smallest trees on the contaminated site in June 2012, produced the lowest amount of biomass, and had a rather high foliar nitrogen concentration. This correlation between bud burst, biomass production and nitrogen use efficiency is quite interesting and could be used for better understanding of the ecophysiological responses of willows.

According to Kopinga and Burg (1995), the proportion of K, P and Mg in relation to N concentration (for $N = 100$) and the cation quotient for the relative deficiency of K in the leaves of deciduous trees can be used to evaluate nutrient status and classify it as "sufficient", "nearly sufficient" or "insufficient". These authors tested three willow species in their study (*S. viminalis, S. alba and S. triandra*), and determined so-called "normal values" for N (2.3–3 %), P (0.17–0.21 %), K (0.85–1.2 %) and Mg (0.17–0.30 %). Considering this (Table 3) we can conclude that all cultivars had a normal nutrient intake on the polluted sector and that several even had an optimal concentration of nutrients in their leaves. For the proportions of P, K and Mg with respect to N, the nutritional status of the plants in our study was generally optimal, on both the control and contaminated sectors (Table 4 in Supplementary data). Those cultivars which did not have "optimal" status could still be considered "sufficient", and no clones showed a nutritional deficit.

Conclusions

The willow cultivars tested in this study were robust and tolerated well the soil contaminated with PHC. Our results show that most variability in physiological responses was due to genotype and not to the presence of contaminants in the soil. The more favorable physicochemical conditions of the polluted site probably helped minimize the effect of contaminants on clone growth. SX67 and SX61, produced the greatest biomass, while S05 and S54 showed the highest photosynthetic capacity. The growth and productivity of some cultivars, notably S365 and SV1, were particularly influenced by the presence of contaminants. Only S05 performed similarly on both polluted and unpolluted soil. Not only did none of its physiological parameters appear to be influenced significantly by pollution, but it also exhibited superior performance on the polluted site in regard to leaf area and number of shoots. In terms of biomass production, the performances of both SX61 and SX67 cultivars were not that surprising. Those two are known to achieve large heights and produce a lot of woody material (Labrecque and Teodorescu 2005). Regarding the cultivar S33, the measurements presented in the figures should be taken with caution since the trees monitored on

Table 3 Measured concentration of nitrogen, phosphorus and potassium in *Salix* spp. leaves

Cultivars	Nitrogen (%)		Phosphorus (%)		Potassium (%)		Calcium (%)		Magnesium (%)	
	Control	Polluted	Control	Polluted	Control	Polluted	Control	Polluted	Control	Polluted
FishCreek	2.80	2.50	0.448	0.295	1.15	0.96	2.6	2.58	0.387	0.353
Millbrook	2.27	2.37	0.288	0.261	1.37	1.27	2.56	2.56	0.343	0.297
S05	2.63	2.57	0.329	0.293	1.29	1.52	1.95	1.72	0.420*	0.287*
S25	2.67*	2.37*	0.279	0.263	1.56*	1.29*	2.05	2.38	0.347	0.347
S33	3.07	2.67	0.409	0.362	1.54	1.32	1.53	1.53	0.32	0.343
S365	2.97	2.70	0.323	0.332	1.44	1.37	1.78	2.02	0.377	0.39
S44	2.70	2.93	0.329	0.332	1.87*	1.26*	1.61	2.45	0.367	0.413
S54	2.73	2.47	0.259	0.245	1.56	1.39	2.22	1.97	0.337	0.333
SV1	2.70	2.73	0.453	0.406	1.30	0.95	1.93	2.02	0.417	0.397
SX61	2.13	2.30	0.225	0.185	1.31	1.53	2.52	2.79	0.303	0.273
SX67	2.20	2.30	0.267	0.246	1.89*	1.21*	2.94	2.51	0.237	0.25

Asterisk indicates significant difference between treatments

the control site were affected by a pest (potato leafhopper), while no sign of infestation was present on the polluted site.

Previous studies have shown that low concentrations of PAH and other organic compounds can have no notable effect on biomass yields and, in some cases, can even enhance the growth and transpiration of willow trees (Thygesen and Trapp 2002; Vervaeke et al. 2003; Ucisik and Trapp 2006, 2008). Growth stimulation of plants, when exposed to small amounts of phytotoxic compounds, is known as hormesis and can occur when the concentration of a pollutant does not reach a high enough level to create permanent damage to the plant. In this case, the presence of a stress will rather cause the activation of the metabolism of the plant and promote its growth. This could give an insight into the high tolerance of our cultivars and the low impact of contamination on the physiological processes in the plants (Laughlin et al. 1981; Calabrese 2004; Calabrese and Blain 2009).

The high tolerance of S05 on the contaminated site is very interesting. Although this cultivar has been used in a few phytoremediation studies (Susarla et al. 2000; Punshon et al. 2003; Kuzovkina et al. 2004), its high tolerance to PHC contamination on the Varenne's site was unexpected. *S. nigra* S05 is native to eastern Canada, which would be preferable to the use of exotic germplasm for many projects. We are confident that these promising findings can help inform cultivar selection and improve phytoremediation techniques in Québec and around the world.

Author contribution statement Labrecque M., Pitre F., and Guidi W. made the conception and the design set up. Grenier V. and Guidi W. worked on data acquisition. Grenier V., Guidi W., Labrecque M., and Pitre F. worked on the analysis and interpretation of data. Grenier V. made the statistical analysis and the drafting of the manuscript. Labrecque M., Pitre F., and Guidi W. brought a critical revision and Labrecque M. and Pitre F. made the final approval.

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Conflict of interest The authors declare that they have no conflict of interest.

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