



Tolerance of Japanese knotweed s.l. to soil artificial polymetallic pollution: early metabolic responses and performance during vegetative multiplication

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Tolerance of Japanese Knotweed s.l. to soil artificial polymetallic pollution: early metabolic responses and performance during vegetative multiplication --Manuscript Draft--

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Abstract:	<p>The expansion of invasive Japanese Knotweed s.l. is of particular concern because of its aptitudes to rapidly colonize diverse environments, especially anthropized habitats generally characterized by their pollution with heavy metals. Whether the presence of heavy metals impacts the performance traits of this plant is a central question to better understand its invasive properties, though no controlled approach to assess these effects were yet reported.</p> <p>In this aim, we undertook greenhouse experiments where rhizome fragments of Japanese Knotweed s.l. (<i>Fallopia japonica</i> and <i>Fallopia x bohemica</i>) were grown during 1 and 3 months, in soil pot artificially polluted or not with heavy metals added in mixture (Cd, Cr, Pb, Zn). Our results showed that (i) the presence of heavy metals delayed rhizome regeneration and induced lowered plant part weights but did not affect plant height after three months; (ii) the effect of metals on the metabolic profiles of belowground part extracts was only detectable after 1 month and not after 3 months of growth, though, it was possible to highlight the effect of metals independently of time and genotype for root extracts, and torosachrysone seemed to be the most induced compound; (iii) the hybrid genotype tested was able to accumulate relatively high concentrations of metals, over or close to the highest reported ones for this plant for Cr, Cd and Zn, whereas Pb was not accumulated.</p> <p>These findings evidence that the presence of heavy metals in soil has a low impact on <i>Fallopia</i> spp. overall performance traits during rhizome regeneration, and has a rather stimulating effect on plant growth depending on pollution level.</p>
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	<p>the Authors took into account the most of suggestion, however, in my opinion this report cannot be acceptable at this moment, because of the lack of some important data. The manuscript is dedicated to two invasive species of the Fallopia genus (Fallopia japonica and the hybrid - Fallopia x bohemica). In this study the results connected with heavy metals content in investigated plants are incomplete. The author did not shown the concentration of heavy metals in both species. In my opinion the heavy metals in Fallopia japonica should be done. It is important, because both taxa belongs to the most invasive plants in Europe. Therefore, I recommended to fill in the missing results.</p> <p>Analysis were undertaken (in the same laboratory as for F. x bohemica) and showed that overall, F. japonica accumulate metals in the same ranges of what was found with F. x bohemica, though differences exist:</p> <p>In highly polluted conditions (M3 treatment) F. japonica accumulated more metals than the hybrid for all the metal tested tested in the study and in all plant parts except for Cd concentration in roots which was greater for F. x bohemica.</p> <p>It is also interesting to notice that, except for Zn, F. japonica accumulated very limited amounts of metals compared to F. x bohemica in the control M0 condition. Though, its translocation factor varies not much in the presence of high metal concentrations (from 3 to 5 for Cd and from 2 to 3 for Zn in M0 and M3 conditions respectively) compared to F. x bohemica for which the translocation factors of these two metals were even inverted with metal contamination (from 0,5 to 3 for Zn and from 0,1 to 1 for Cd).</p> <p>According to this, modifications were made in the manuscript in the Material and Methods (lines 176-180), Results (lines 275-283) and Discussion (lines 311-314) of the revised manuscript.</p> <p>Figure 7 was also modified.</p>
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Tolerance of Japanese Knotweed *s.l.* to soil artificial polymetallic pollution: early metabolic responses and performance during vegetative multiplication

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Key-words: Metallic trace elements, *Fallopia* spp. (syn. *Reynoutria* spp.), Plant performance traits, Metabolite profiling, Plant secondary metabolites.

Abstract

The expansion of invasive Japanese Knotweed *s.l.* is of particular concern because of its aptitudes to rapidly colonize diverse environments, especially anthropized habitats generally characterized by their pollution with heavy metals. Whether the presence of heavy metals impacts the performance traits of this plant is a central question to better understand its invasive properties, though no controlled approach to assess these effects were yet reported.

In this aim, we undertook greenhouse experiments where rhizome fragments of Japanese Knotweed *s.l.* (*Fallopia japonica* and *Fallopia x bohemica*) were grown during 1 and 3 months,

in soil pot artificially polluted or not with heavy metals added in mixture (Cd, Cr, Pb, Zn). Our results showed that (i) the presence of heavy metals delayed rhizome regeneration and induced lowered plant part weights but did not affect plant height after three months; (ii) the effect of metals on the metabolic profiles of belowground part extracts was only detectable after 1 month and not after 3 months of growth, though, it was possible to highlight the effect of metals independently of time and genotype for root extracts, and torosachrysone seemed to be the most induced compound; (iii) the hybrid genotype tested was able to accumulate relatively high concentrations of metals, over or close to the highest reported ones for this plant for Cr, Cd and Zn, whereas Pb was not accumulated. These findings evidence that the presence of heavy metals in soil has a low impact on *Fallopia* spp. overall performance traits during rhizome regeneration, and has a rather stimulating effect on plant growth depending on pollution level.

Introduction

The increased prevalence of heavy metals in the environment, which is largely due to the intensification of anthropogenic activities, is of particular concern because it affects living organisms at the whole ecosystem level. Concerning plants, two main adaptive strategy may be used to cope with an excess of heavy metals (Baker, 1981) : (i) *exclusion* which is the most common strategy used, where metal concentration in shoots remains low and constant while external concentration is varying, or (ii) *accumulation* where metal is more translocated in shoots than in roots, with the distinction between *indicators* that concentrate metals at levels close to that of soil and *hyperaccumulators* which are able to accumulate metals at very high levels (van der Ent et al. 2013; 2015). In order to assess the effects of heavy metals on plants and better understand the molecular mechanisms involved in plant tolerance to metal stress, omics tools have been used to

characterize stress-induced metabolites, phytohormones, transcription factors or proteins (Singh et al. 2016), as well as key genes acquired during plant evolution conferring hypertolerance (Hanikenne and Nouet, 2011). Among the metabolic changes occurring during metal exposure, plant secondary metabolites like polyphenols have been quite less studied compared to primary ones or plant hormones for example, at least qualitatively, and only an induction of polyphenol biosynthesis of metal exposed plants has been reported in a few studies, as recently reviewed by Singh et al. (2016). Though, these compounds are well-known free-radical scavengers that protect cells from oxidative stress, they also possess strong metal chelating properties, especially polymers like tannins, and they also act as molecular signals involved in many plant biotic interactions from symbiosis establishment and insect visual attraction to disease resistance (Cheynier et al. 2013). This implies that the metal-induced changes in plant polyphenol biosynthesis also probably affects plant biotic interactions, especially in the root system which is directly exposed to metal toxicity and play a major role in shaping rhizosphere microbial communities through root exudation (*e.g.* Michalet et al. 2013). Some authors hypothesised that this modulation of root exudates composition under contaminant exposure might select for microbes that confer plant tolerance to the stress and is referred to as the “plant call for support” theory (Thijs et al. 2016). Indeed plant-associated microbes are believed to play a great role in plant adaptation to metallic stress and this area of research has recently taken much attention, since it could be a way to drastically improve phytoremediation (Rajkumar et al. 2012; Thijs et al. 2016). Plant-microbiome interactions are also described as important drivers for the establishment of weed and invasive species (Trognitz et al. 2016). Whether invasive plant species are positively influenced by soil metal pollution, at least for those that are able to grow on metalliferous area, remains an open question that has not yet been clearly examined in the literature. Indeed, metal-polluted environments like urban or industrial settings are disturbed habitats where contaminants exert a strong selective pressure

76 which render them more prompt to biological invasions (*i.e.* the so-called “empty niche” theory,
 77 as early stated by Elton in 1958).
 78 A well-known invasive species able to grow on metal polluted soil is the plants belonging to
 79 the genus *Fallopia* (syn. *Reynoutria*, Polygonaceae) which includes two Asian herbaceous
 80 perennial polyploid species (*F. japonica* and *F. sachalinensis*) and their interspecific hybrid *F.*
 81 *x bohemica*, forming a polyploid complex of species, generally referred to as Japanese
 82 Knotweed *s.l.* (Bailey et al. 2009). Because of their high performance traits (Bailey et al. 2009)
 83 leading to dominance in the community, these species impact ecosystem functioning
 84 (Dassonville et al. 2011) and pose severe socio-economical issue in Europe and North America
 85 as no conventional method to efficiently control their expansion is currently known. Actually,
 86 the annual cost to control Japanese Knotweed is estimated to be of more than 1.5 billion £/year
 87 only for Great Britain, representing one third of the total annual cost for alien weed management
 88 (Williams et al. 2010). Their capacity to tolerate abiotic stress (Rouifed et al. 2012) and their
 89 strong competitive aptitudes allow them to rapidly colonize various environments. They mainly
 90 spread by rhizome (or stem) regeneration through vegetative reproduction, though the hybrid
 91 is also able to produce seeds and back-crosses with parental species (Bailey et al. 2009,
 92 Soltysiak and Brej, 2012; Groeneveld et al. 2014; Gaskin et al. 2014). The later (*F. japonica*
 93 and *F. sachalinensis*) are native to Eastern Asia including volcanic areas presenting very high
 94 metallic trace elements concentrations in soil: actually more than 90 and 9000 mg/kg of Cd and
 95 Zn were respectively found in metalliferous site of Japan where these species naturally grow
 96 (Nishizono et al. 1989). This may in part explain why they are mainly found in anthropized
 97 environments like railroads or industrial and urban areas, and that these disturbed habitats seem
 98 to promote specifically their expansion (Soltysiak and Brej, 2012; Soltysiak and Brej, 2014).
 99 Japanese knotweed *s.l.* are able to accumulate metallic trace elements and the ability to
 100 concentrate toxic metals in plant parts of these species has been extensively studied, but

exclusively from field measurements (e.g. Soltysiak et al. 2011; Rahmonov et al. 2014; Širka et al. 2016). Most of these studies reported higher amounts of metals accumulated in underground parts of the plant but no clear correlations between a rather accumulated type of metal or relationships between soil concentrations and plant metal accumulation could be made from these studies due to the variability of the published values.

Facing this, controlled studies need to be developed in order to better understand the impact of the presence of metals in soils on Japanese Knotweed *s.l.* performance traits, especially during vegetative propagation. In this aim we undertook greenhouse experiments, where rhizome fragments of genotypes of Japanese Knotweed *s.l.* were grown during one and three months respectively, in soil pot artificially polluted or not with heavy metals in mixture (Cd, Cr, Pb, Zn) at three different concentrations from faintly polluted to highly polluted soils respectively. These metals were chosen because they are among the main pollutants generated by anthropic activities and are often found in urban soils, and also because two of them are essential trace elements (Cr and Zn), whereas the two others are toxic to most organisms (Cd, Pb). To assess if performance traits were impacted, the growth kinetic as well as final plant part dry weights were recorded. A metabolic profiling approach on belowground plant parts (roots and rhizomes separately) and based on UV-absorbing compounds was also achieved in order to target more specifically phenolic and other aromatic compounds that might be induced during metal stress.

Materials and Methods

Greenhouse experiments settings

Two independent microcosm experiments of one month and three months respectively were conducted in greenhouse. For the one month experiment which occurred between February and March 2014, a loamy Luvisol (WRB, 2006) continuously cropped with maize and collected at La Côte Saint-André (Isère, France) possessing the following characteristics: sand 37.5%; loam

42.7%; clay 19.8%; OM 2.23%; pH 6.4; CEC: 8.79 cmol.kg⁻¹ and exempt of heavy metal pollution, was sieved (2 mm mesh size) and rehydrated to its field capacity (28% m/v) by adding either Ultra-Pure water (UP) (control, M0) or a solution containing Cd, Cr, Pb and Zn (added as chloride salts) at concentrations close to common recognized soil pollution threshold (M2 treatment; Baize et al. 2007), ten times lower (M1 treatment) or ten times higher (M3 treatment) and summarized in **Table 1**. Then 200 g of moistened soil were placed into 0.3 L plastic pots and rhizome fragments of 2 genotypes of Japanese Knotweed *s.l.* (*F. x bohemica* or *F. japonica*) were planted. These rhizome fragments were obtained from individuals morphologically identified (F. Piola, personal communication) using leaf shape and size (Bailey et al. 2009) and collected from the field on the riverbanks of the Suran river (Ain, France) for *F. x bohemica* and on the riverbanks of the Valencizes river (Rhône, France) for *F. japonica* and that were acclimated for more than a year to greenhouse conditions in order to avoid any “mother effect”. For each treatment, five budding rhizome fragments from each genotype were cut into pieces (average weight of 0.7 ± 0.1 g), planted in soil and grown in greenhouse (13h day 22°C/11h night 18°C) with light intensity of about 8-10 klux. Soil moisture was manually controlled by adding water every three days. For the three months experiment which occurred between January and March 2015, the same procedure was repeated but with modifications concerning the amount of soil added in pots (600 g in order to allow the plant to grow for three months; 0.75 L pots). Metal concentrations were also modified because of the absence of regeneration of rhizome for the initial M3 dose chosen in the one month experiment, and in order to keep roughly an order of magnitude of difference between each treatment. Concentrations were approximatively divided by two for M1 and M2 treatments and by three for M3 treatment (**Table 1**).

Growth traits

For the three months experiment, during growth, the height of each plant was notified three times a week by measuring the distance between topsoil and the highest photosynthetic part of the plant. At the time of collection, plants were carefully excavated from soil, and roots were rinsed with tap water. Aboveground parts (stems + leaves) were separated from belowground parts (rhizome + roots) and both were immediately frozen in liquid nitrogen and freeze-dried before being weighed.

Metabolite profiling of belowground parts

For both experiments, after freeze-drying fine roots were separated from rhizomes, and about 100 mg of each were grounded (TissueLyserII® - Qiagen; F= 300/sec., 3 min.) and sonicated with a 1 mL mixture of MeOH-H₂O (1:1) for 15 minutes. After the supernatant was removed, the remaining powder was sonicated with 1 mL MeOH and both supernatants were pooled and concentrated under vacuum (Speedvac® – Labenco) before being dissolved at 10 mg/mL in MeOH 80% and stored at -20°C before analysis.

Samples were analysed by UHPLC-DAD ESI-QTOF (Agilent 1290 infinity linked with Agilent QTOF 6530), using poroshell 120 EC-18 column (2.7 µm, 3.0 x 100 mm, Agilent) with a gradient of 0.1% formic acid in water (A) and acetonitrile (B) as follows: 1% of B from 0 to 1.5 min, and growing with a linear gradient to 15% of B at 8 min ; 60% of B at 14 minutes and 100% of B at 16 min for 1 min. All solvents were LC-MS grade (Optima). The flow rate was adjusted at 1.2 mL/min and the injection volume was 2 µL. UV spectra were recorded between 190 and 600 nm. The ESI source was optimized as follows: positive ionization mode, scan spectra from m/z 100 to 2000, capillary voltage 3.5 kV, fragmentor 120 V, fixed collision-induced dissociation (CID) energy at 20 eV. Nitrogen was used as the nebulizing gas with a flow rate of 12 L/min and a temperature of 310°C at 40 psi.

Compounds were identified by analysis of their UV, HRMS and MS/MS spectra using MassHunter Qualitative Analysis (Agilent Technologies, USA)

Heavy metal contents in plants and soil

Metal content in soil (three biological replicates of *F. x bohemica* rhizosphere soil) was analysed by ICP-MS at the CRPG of Vandoeuvre-les-Nancy and metal content in plant parts was analysed by ICP-MS (and ICP-radial for Zn) by USRAVE (INRA-Centre de Bordeaux ; CoFrac-certification n°1-1838). Three replicates of both *F. x bohemica* and *F. japonica* growing in M0- and M3-treatments were analysed

Statistical analysis

Growth traits

Individual plant heights measured during the three months experiment were analysed with a dose/response model (package drc, Knezevic et al. 2007). Data obtained from both genotypes tested were pooled because they did not differ significantly.

Concerning plant part dry weights, differences between treatments, genotypes and the interaction between the two factors were analysed using variance analysis (ANOVA) on RStudio software (version 3.2.2).

Metabolite profiling

Peak areas were normalised by using relative values, *i.e.* percentages of each compound in the chromatogram. PCA (Principal Component Analyses) were performed using R studio software with “ade4” package. The compounds showing variations in their proportion between treatments were analysed using ANOVA, followed by a Tukey-HSD test. All tests were done in respect with the rules of normality and homoscedasticity assumptions.

For the one month experiment, 26 samples were analysed giving 80 peaks for rhizome extracts and 114 peaks for root extracts, whereas for the three months experiment, 42 samples were analysed with 107 peaks for rhizome extracts and 101 peaks for root extracts.

In order to compare the one month and three months experiments together, all extracts obtained during both experiments from the different biological replicates were pooled according to time

of collection, treatment and genotype (giving 14 pooled extracts for roots and rhizome respectively) and analysed with the same procedure of chromatography and data treatment (62 peaks analysed). As metal levels of both experiments were of the same order of magnitude data were pooled in one single matrix.

Results

Growth traits

Plant height in function of time and treatment is presented in **Figure 1**. Data obtained for both genotypes were analysed together because their response did not differ significantly. Pairwise comparison of parameters of the Log logistic model between treatments showed that concentration of metals affected growth of plants during time: the higher the concentration of metals, the later the plants reached 50% of their maximal height. Plants in M0 and in M1 treatments reached half of their height in 19 days. Plants in M2 treatment reached half of their height 6 days later (25 days, $p < 0.001$), and plants in M3 treatment needed further 5 days (30 days, $p < 0.001$). However, plants treated with M2 concentration reached the highest height (11.6 cm), not significantly different from plants with M3 concentration (10.9 cm, $p = 0.155$), but significantly higher than plants in M0 and M1 concentrations (10.3 cm, $p < 0.001$ and 10.4 cm, $p < 0.001$, respectively).

F. x bohemica had significantly higher aboveground ($F = 4.76$, $p = 0.035$) and belowground ($F = 5.32$, $p = 0.026$) weights than *F. japonica* after 3 months of growth. However, the genotype did not influence the response of plants to the metal treatment (interactions genotype x treatment non significant). Control plants (M0) showed significantly higher aboveground ($F = 2.95$, $p = 0.044$) and belowground ($F = 5.86$, $p = 0.002$) weights, but treatment affected differently aerial and underground parts: plants treated with M1 concentration showed the

lowest aboveground weight whereas plants treated with M2 and M3 concentrations showed the lowest belowground weight.

Metabolite profiling of belowground parts

PCA based on metabolic profiles obtained from rhizome and root extracts after one and three months of growth (**Fig. 3** & **Fig. 4** respectively) allowed in each case to distinguish both genotypes tested along axis 1, except for root extracts at 3 months for which this distinction was made along axis 2 (**Fig. 3A, 3C** & **Fig. 4A, 4C**). The effect of metal treatments was detectable for both plant parts after one month of growth since M2-treated plants were relatively well separated from M0-treated plants along axis 2, and this was especially true for roots (**Fig. 3B, 3D**). Since the M3 treatment chosen in the one month experiment was too toxic to allow rhizome regeneration, no data could have been obtained from this group which is then lacking in the PCA. After three months of growth, it was not possible to distinguish any of the metal treated groups from the control (**Fig. 4B, 4D**).

In order to better characterize the effect of metal pollution on plant belowground metabolites, biological replicate extracts of both plant parts were pooled according to time, genotype and metal treatment, leading to 14 new pooled samples which underwent the same chromatographic analysis and data treatment procedure (**Fig. 5**). This allowed for both plant parts to distinguish the effect of time which was mainly explained by axis 1 (**Fig. 5A, 5D**), from that of genotype which was mainly explained by axis 2 (**Fig. 5B, 5E**). It was also possible to discriminate metal treated groups along axis 3 but this was only the case for root extracts (**Fig. 5F**) and not for rhizome extracts (**Fig. 5C**), even when considering lower eigen values.

One of the compound for which the proportions were the most increased with metal concentrations and strictly explaining axis 3, was identified as torosachrysone, a dihydroanthracenone derivative, by comparing its UV, HRMS and MSMS spectra with literature data (**Fig. 6**). Other anthracene derivatives including anthraquinone and dianthrone

were also found to be increased in their proportions in root extracts at one and three months (data not shown).

Heavy metal contents in soil and plant parts of *F. x bohemica*

The content of metallic trace elements in M3-treated soil after the three months experiment was relatively close to the theoretical concentrations expected (i.e. resulting from the artificial contamination of soil experimented in this study) for Cd (7.2 ± 0.5 mg/kg vs 5.5 mg/kg in theory), Cr (332.0 ± 39.2 mg/kg vs 440.0 mg/kg in theory) and Zn (923.7 ± 70.5 mg/kg vs 880.0 mg/kg in theory), whereas for Pb, the measured concentration was approximatively divided by three compared to its theoretical concentration (95.9 ± 8.3 mg/kg vs 295.0 mg/kg in theory) (**Fig. 7** and **Table 1**).

The control soil M0 that received no metallic treatment showed low heavy metal concentrations as expected, in the range of what is naturally found for this type of soil, and could thus be considered as non-polluted.

F. x bohemica accumulated metals even if no artificial pollution was added (i.e. M0-treatment; **Fig. 7A-D-left**), and especially in underground parts: similar contents than those found in soil were actually measured for Cr and Zn (**Fig. 7B** and **7D** respectively). In contrast Cd was accumulated approximatively five times more (**Fig. 7A**) and Pb six time less (**Fig. 7C**). These metals did not seem to be translocated to aerial parts in these conditions since metal concentrations were very low and close to the limit of quantification for Cd, Cr and Pb.

When artificial metallic pollution was added at the highest concentration tested (i.e. M3-treatment; **Fig. 7A-D-right**), each metal except Pb was more accumulated in *F. x bohemica* plant parts and the translocation factor ($[\text{metal}]_{\text{shoots}}/[\text{metal}]_{\text{roots}}$) of Cd was increased ten times compared to control (1.0 vs 0.1; **Fig. 7A**) and six times for Zn (3.3 vs 0.5; **Fig. 7D**). The highest concentrations of metal in *F. x bohemica* measured for this treatment were of 507.0 mg/kg for

Zn in aerial parts, and of 127.0 and 14.0 mg/kg for Cr and Cd respectively in belowground parts.

By comparison, in the M3 condition *F. japonica* accumulated more of each metal than the hybrid, especially Zn and Cd, for which, concentrations were as high as 833.0 and 19.5 mg/kg respectively in aerial parts of some individuals, and of up to 244.0 mg/kg for Cr in belowground parts whereas Pb was also poorly accumulated (Fig. 7A-D right).

It is also interesting to notice that, except for Zn, *F. japonica* accumulated very limited amounts of metals compared to *F. x bohemica* in the control M0 condition (Fig. 7A-D left). Though, its translocation factor varies not much with the presence of high concentrations of metal (from 3 to 5 for Cd and from 2 to 3 for Zn in M0 and M3 conditions respectively) compared to *F. x bohemica*.

Discussion

In this study we assessed the effect of an artificial polymetallic pollution of soil on *Fallopia* spp. performance, and more precisely its impact on rhizome regeneration and growth occurring in the early stages of vegetative propagation, as well as its impact on belowground secondary metabolism.

Our results showed that the presence of heavy metals in soil induced a delay for plants to reach half of their maximal height, delay that was more pronounced with high metal concentrations. Though, after three months of growth, plant final height of both genotypes was not impacted by metal pollution and it was even significantly greater for M2-treated group compared to control. This implies that plant growth speed was higher when metals were present. The observed delay in rhizome regeneration induced by metallic pollution could be correlated with the fact that metabolic changes in belowground parts of the plant were only observed after one month of growth and not after three. This suggests that the delay in rhizome regeneration induced by the presence of metals is due to plant investment in the production of secondary

metabolites rather than in primary metabolites needed for cell and tissue multiplications (as demonstrated in the “grow or defend” theory; Herms and Mattson, 1992). It also correlates with the fact that lowered plant part weights were measured in metal-treated groups. One of the plant growth traits affected by metal treatment was belowground part weight which was reduced when metals were added at high concentrations suggesting a toxic effect. On the other hand, aerial parts were not affected by these treatments indicating that this plant shows an overall good tolerance to metal stress regarding the concentrations added. The fact that aerial part weight was affected with low doses of metals (i.e. M1-treatment) reveals that this plant might be very sensitive to soil metal concentrations.

Concerning plant metal accumulation, the reported values of this study are in the range of the highest published, except for Pb which was not accumulated and Cr which was found at concentrations up to three times more than those reported in underground parts and up to ten times in aerial parts (**Fig. 6** and **Table 2**). The fact that Cr was present at concentrations up to 8 times higher in soil may explain in part these results. Of particular interest is the fact that Zn and Cd translocation factors were increased in the case of *F. japonica* and even inverted in the case of *F. x bohemica* with Cd and Zn, when plants were exposed to high metal concentrations, suggesting a change in plant nutritional strategy. Although these observations are interesting for the potential valorisation of this plant in phytoremediation of contaminated sites, caution must be taken for the extrapolation of these results to what occurs *in situ*, since we only worked on young shoots which may accumulate more than old ones, and as stressed by Van der Ent et al. (2012), these types of controlled-approaches have very limited relevance in explaining what occurs in natural environment although the polymetallic contamination applied in our study is probably closer to what happens in urban ecosystems for example. In all cases further mesocosm and field studies are required to confirm the results obtained.

The consequences of metal accumulation in the plant and its relative high tolerance and sensitivity to polymetallic stress let us hypothesize that these traits may serve the plant to rapidly colonize diversely polluted environments, where other plant species might be excluded because of the presence of metals in soils. Herbivory may also be limited because of metal accumulation in plant aerial parts which may have a repellent effect on predators (especially young shoots that may be more prompt to herbivory attacks; Price 1991). The molecular mechanisms involved in metal tolerance remain to be elucidated, though the metabolic profiling approach undertook in this study might bring some clue about the secondary metabolites directly or indirectly involved in metal tolerance. Torosachryson was identified as one of the compound for which the relative proportions were the most increased in roots with metal concentrations, this independently of plant age or genotype. To our knowledge this is the first report of this compound in this genus. It has been described to be involved in anthraquinone biosynthesis (Abdel-Rahman et al. 2013), compounds that are well known in *Fallopia* spp. (Piola et al. 2013), and that have also been characterized with higher relative proportions in metal-treated plant roots in this study (data not shown). The exact role of torosachryson in metal tolerance and/or accumulation is still to be elucidated but from the results of our study, it seems to be well involved directly or indirectly in plant adaptation to metallic stress. Following the “plant call for support theory” emitted by Thijs et al. (2016), this compound could have an impact on the selection of rhizosphere microbes possessing the ability to improve plant tolerance to metallic stress. Further experiments including field studies should be conducted in order to confirm these findings, for example the correlation between torosachryson content in roots and heavy metal content in plant or soil collected *in situ* could be interesting to analyse. The production of torosachryson by fungal strain has also been reported (Gill, 2003; Müller et al. 2004) leading to the hypothesis that this compound might be produced by fungal endophytes. The presence of such associated microbes has recently been shown in this plant through the

identification of Dark Septates Endophytes (DSE) microsclerotia colonizing roots (Gucwa-Przepióra et al. 2016). These fungal endophytes, which are characterized by their melanised and septate hyphae, are frequent associated root endophytes of herbaceous plants. They have been described as important determinants influencing plant tolerance to heavy metal stress because they are able to tolerate high concentration of metallic trace elements, notably through the increase of melanin which enables to decrease metal toxicity (Ban et al. 2012). Whether these micro-organisms play a role in *Fallopia* adaptation to heavy metal stress remains to be confirmed, but the findings of our study in the light of recent literature on the subject let us suggest that it is the case.

Conclusion

To conclude, our findings evidenced that the presence of toxic metals in soil had a low impact on Japanese Knotweed *s.l.* overall performance traits during vegetative propagation, and had a rather stimulating effect on plant growth depending on pollution level:

- the presence of metal in soil induced a change in plant belowground secondary metabolism which only occurs at early stages of plant vegetative propagation and this might be correlated with the observed metal-induced delay in rhizome regeneration and with lowered plant part weights under metal stress.

- on the other hand the fact that plant final height was not affected (or even increased at some concentrations), and that plant metal accumulation and nutritional strategies were modified depending on metal concentrations in soil, might suggest that this plant is very sensitive to the presence of metal in soil and that it may explain, at least in part, its capacity to rapidly colonize anthropized metal-polluted environments. All these results need to be confirmed with further *in situ* studies and/or controlled mesocosm studies using naturally polluted soils.

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Table legends:

Table 1. Theoretical metal concentration achieved in soil (in mg/kg) after artificial pollution in function of treatment

Table 2. Highest reported values of heavy metal concentrations (in mg/kg) in soil and plant parts of *Fallopia* spp. Reference literature in which values were published is indicated:

^aRahmonov et al. 2014; ^bSoltysiak et al. 2011; ^cŠirka et al. 2016.

Figure legends:

Figure 1. Evolution of mean height (cm) of plant individuals during time (days after plantation), displayed in function of metal treatment for the three months experiment. Small figures represent raw data (\circ M0, Δ M1, $+$ M2, \times M3) and curves were calculated with the model.

Figure 2. Plant aboveground (**A**) and belowground (**B**) dry weights in function of metal treatment (mean \pm S.E.). Statistically significant changes compared to control M0 are highlighted (* $p \leq 0.05$, ** $p \leq 0.01$).

Figure 3. Principal Component Analyses (PCA) of rhizome (**A**, **B**) and root (**C**, **D**) extracts obtained after 1 month of growth and based on UV-absorbing compound chromatographic profiles integrated at 280 nm and expressed as relative peak areas. Genotypes (**A**, **C**) and metal treatments (**B**, **D**) are displayed following PC1 and PC2: for rhizome extracts PC1=24.4% and PC2=20.3% (N=26 samples; 80 peaks) and for root extracts PC1=33.4% and PC2=15.4% (N=26 samples; 114 peaks).

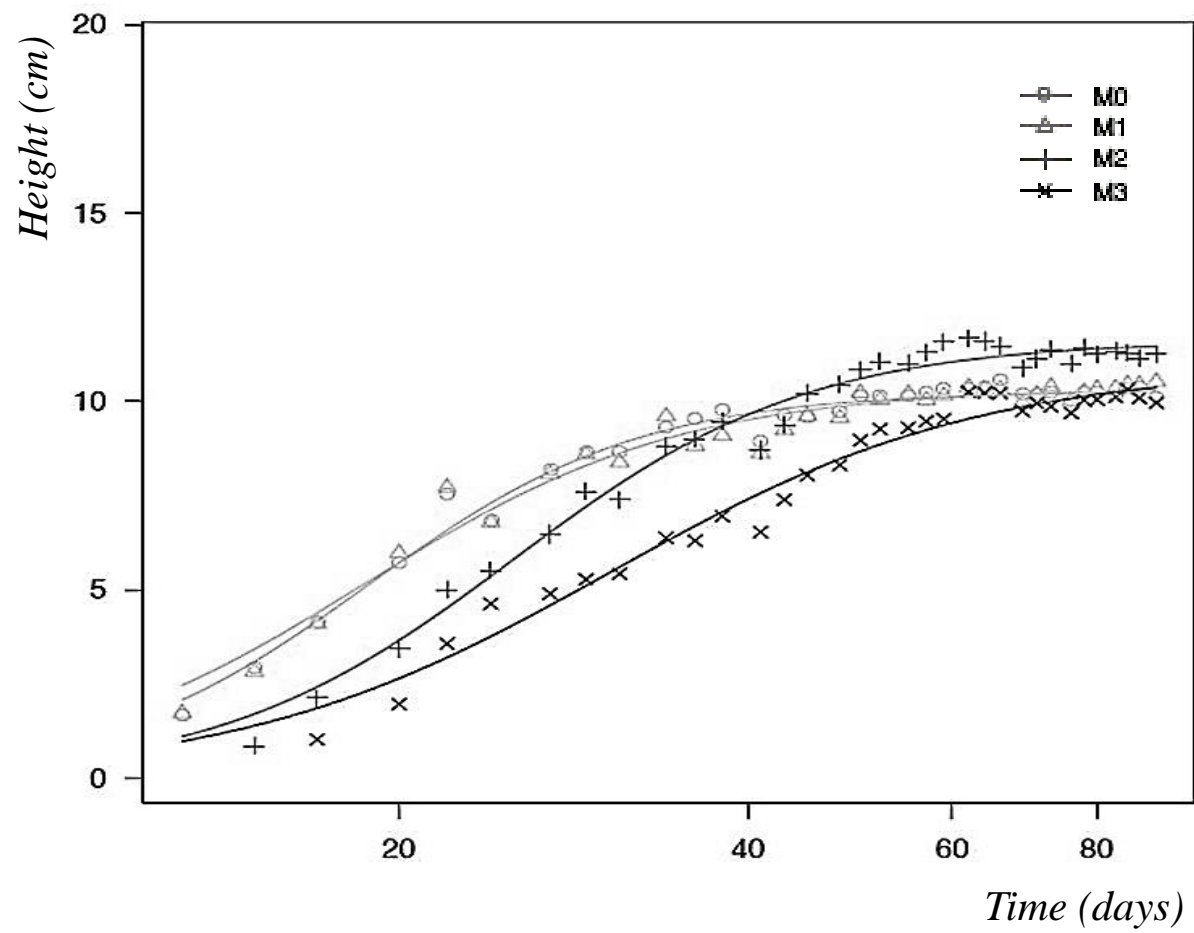
Figure 4. Principal Component Analyses (PCA) of rhizome (**A**, **B**) and root (**C**, **D**) extracts obtained after 3 months of growth and based on UV-absorbing compound chromatographic profiles integrated at 280 nm and expressed as relative peak areas. Genotypes (**B**, **C**) and metal treatments (**B**, **D**) are displayed following PC1 and PC2: for rhizome extracts PC1=27.9% and PC2=17.6% (N=45 samples; 107 peaks) and for root extracts PC1=23.9% and PC2=15.2% (N=45 samples; 101 peaks).

Figure 5. Principal Component Analyses (PCA) of rhizome (**A**, **B**) and root (**C**, **D**) pooled extracts and based on UV-absorbing compound chromatographic profiles integrated at 280 nm and expressed as relative peak areas. Duration of experiment (**A**, **D**) and genotypes (**B**, **E**) are displayed following PC1 and PC2, whereas metal treatments (**C**, **F**) are displayed following

PC1 and PC3: for rhizome extracts PC1=26.0%, PC2=17.3% and PC3=6.9% (N=14 samples; 64 peaks) and for root extracts PC1=20.6%, PC2=12.6% and PC3=9.1%. (N=14 samples; 62 peaks).

Figure 6: Molecular structure of torosachrysone (**A**), its experimental UV spectra (**B**) which is similar to what was described by Müller et al. (2000) for the compound (R)-[methoxy-¹³C]torosachrysone (R)-2, and its experimental HRMS (m/z : $[M+H]^+$ calc. for C₁₆H₁₇O₅: 289.1076, found 289,1074; match score 98%) and MSMS spectra in positive mode (**C**). The later was identical to the data presented by Abdel-Rahman et al. (2013) in which a fragmentation pattern is also proposed for this molecule, pattern confirmed by HRMS data of this study.

Figure 7: Heavy metal content (Cd: **A**; Cr: **B**; Pb: **C**; Zn: **D**; in mg/kg) in soil and plant parts of *F. japonica* (Fj) and *F. x bohemica* (Fxb) grown in M0- and M3-treated groups (mean \pm S.D. of 3 biological replicates in each case). BP: belowground parts; AP: aboveground parts.

**Figure 1**

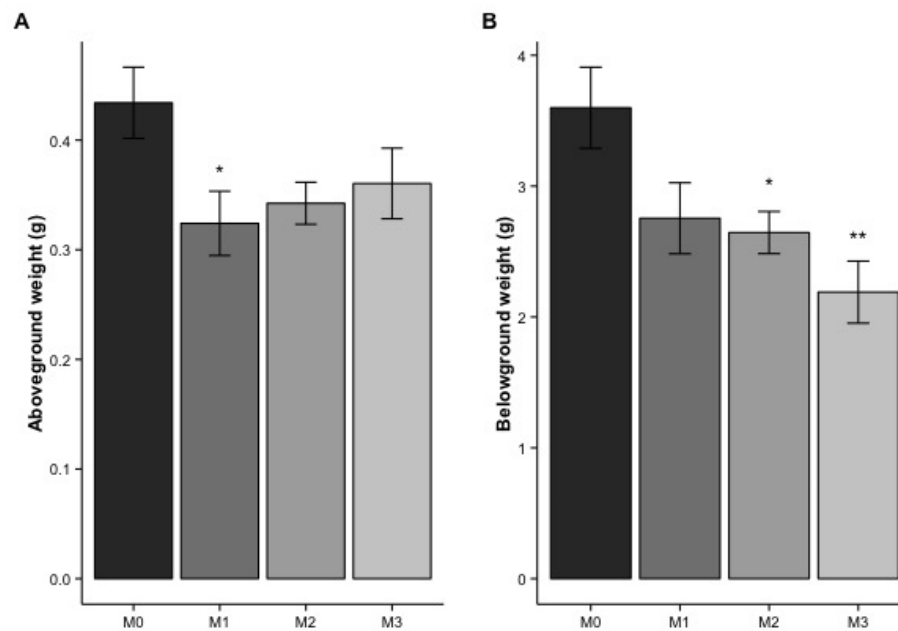


Figure 2

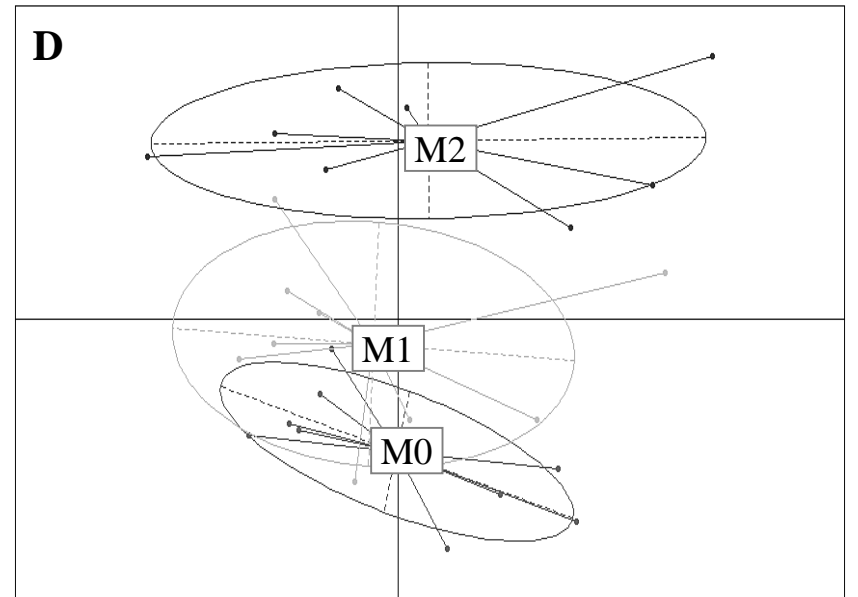
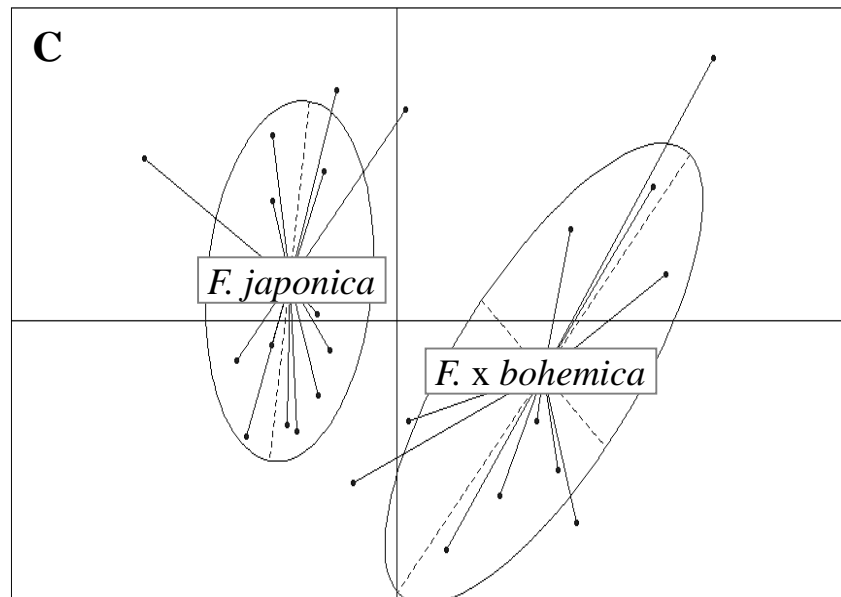
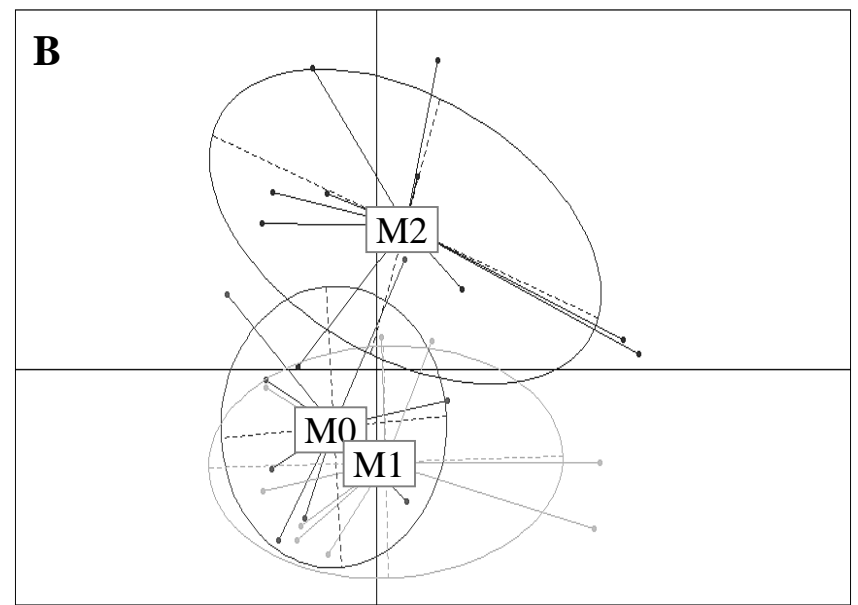
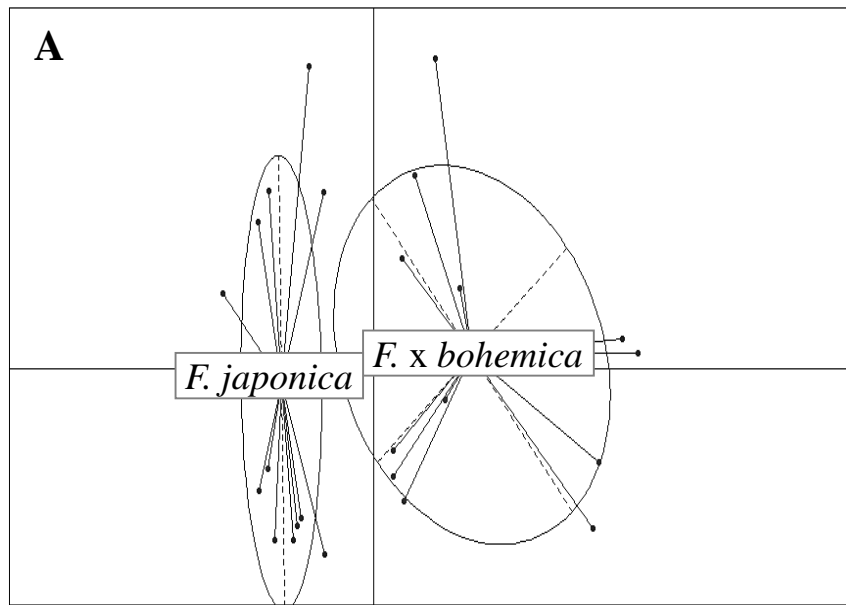


Figure 3

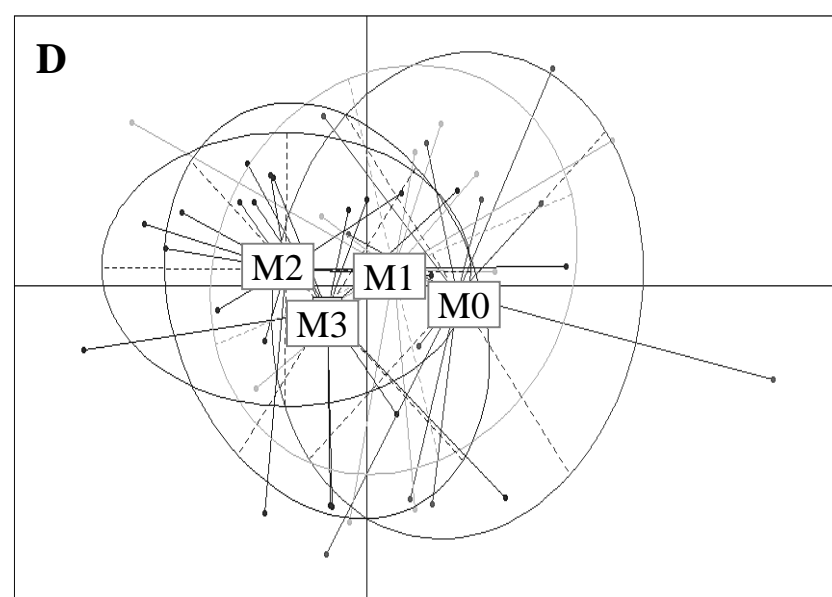
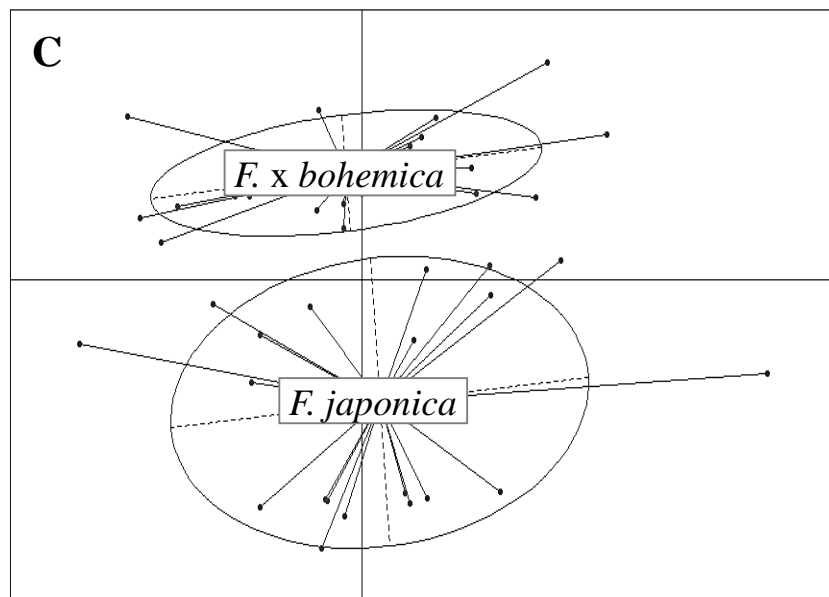
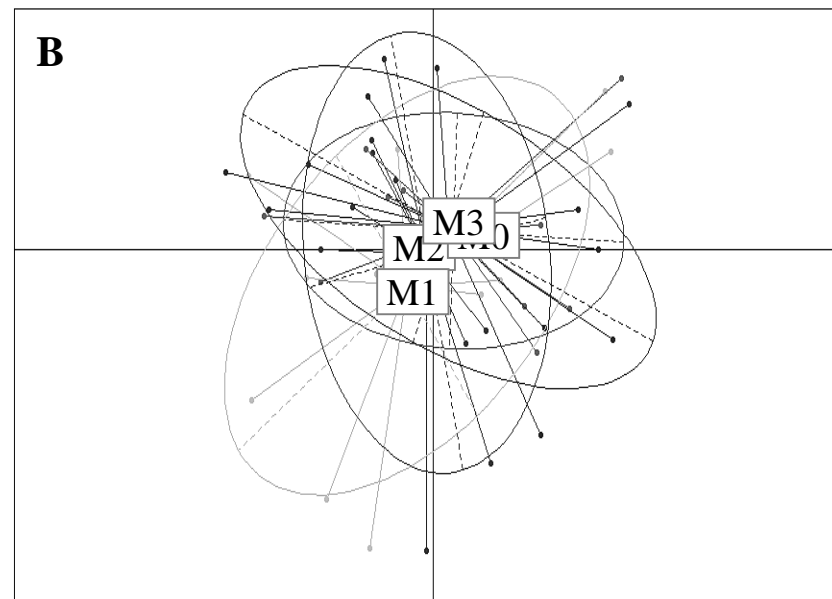
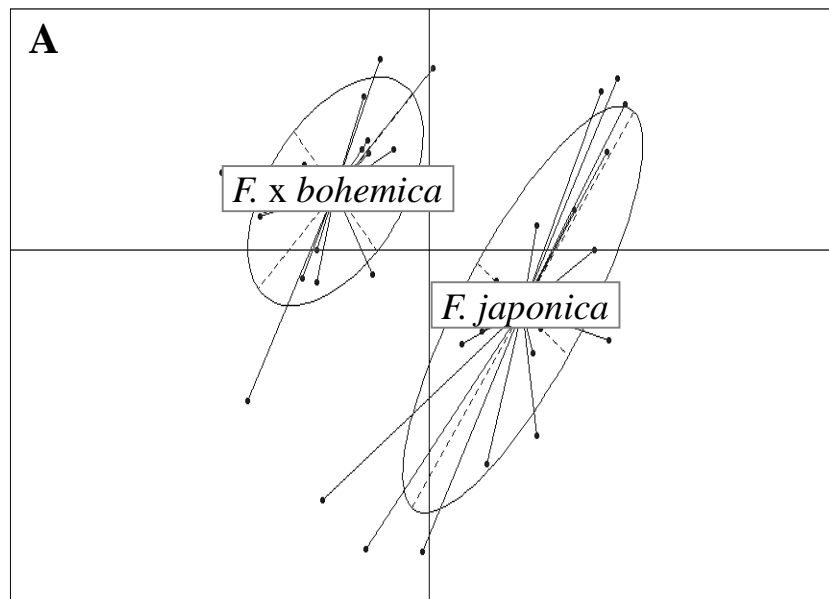


Figure 4

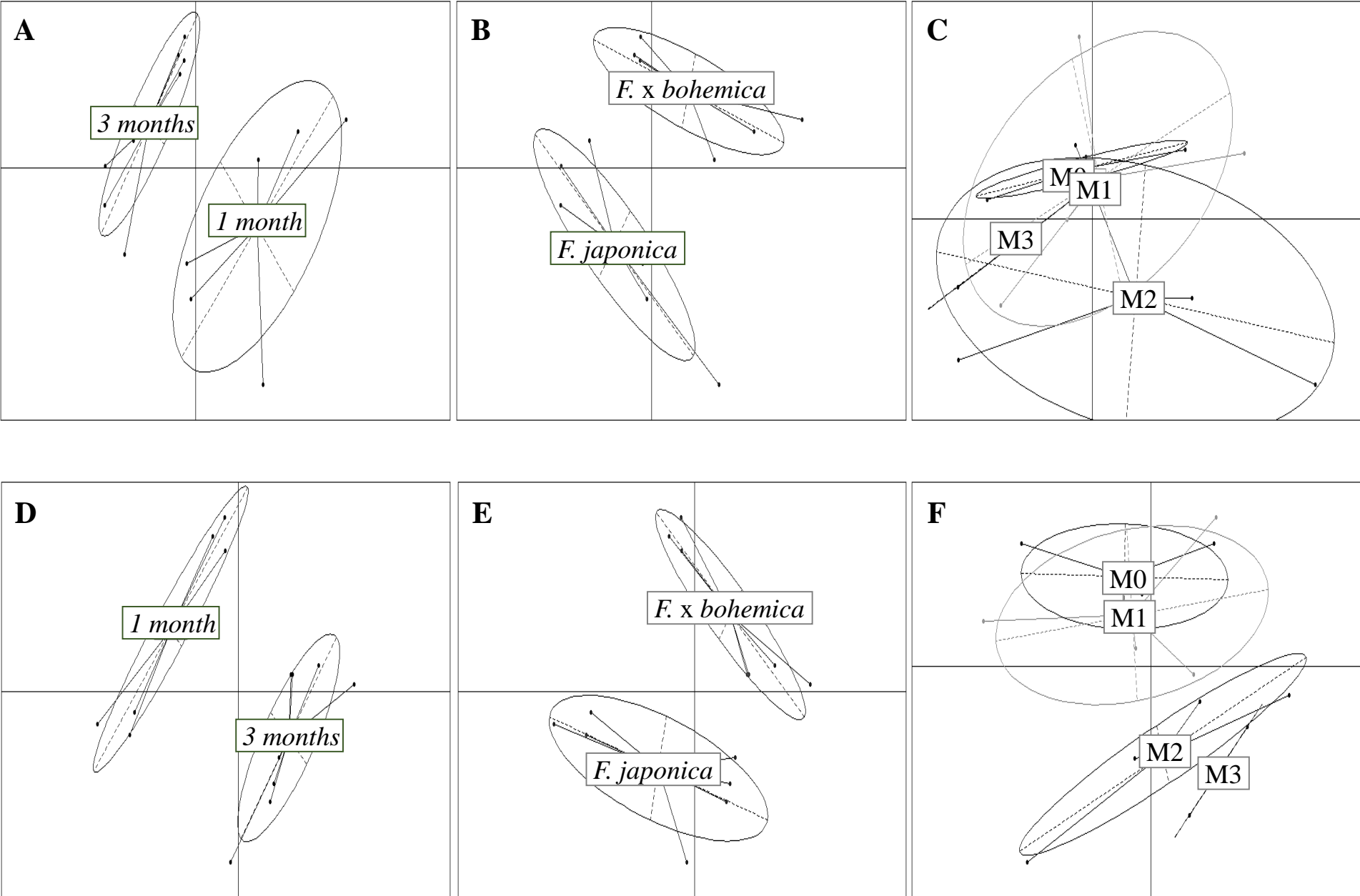


Figure 5

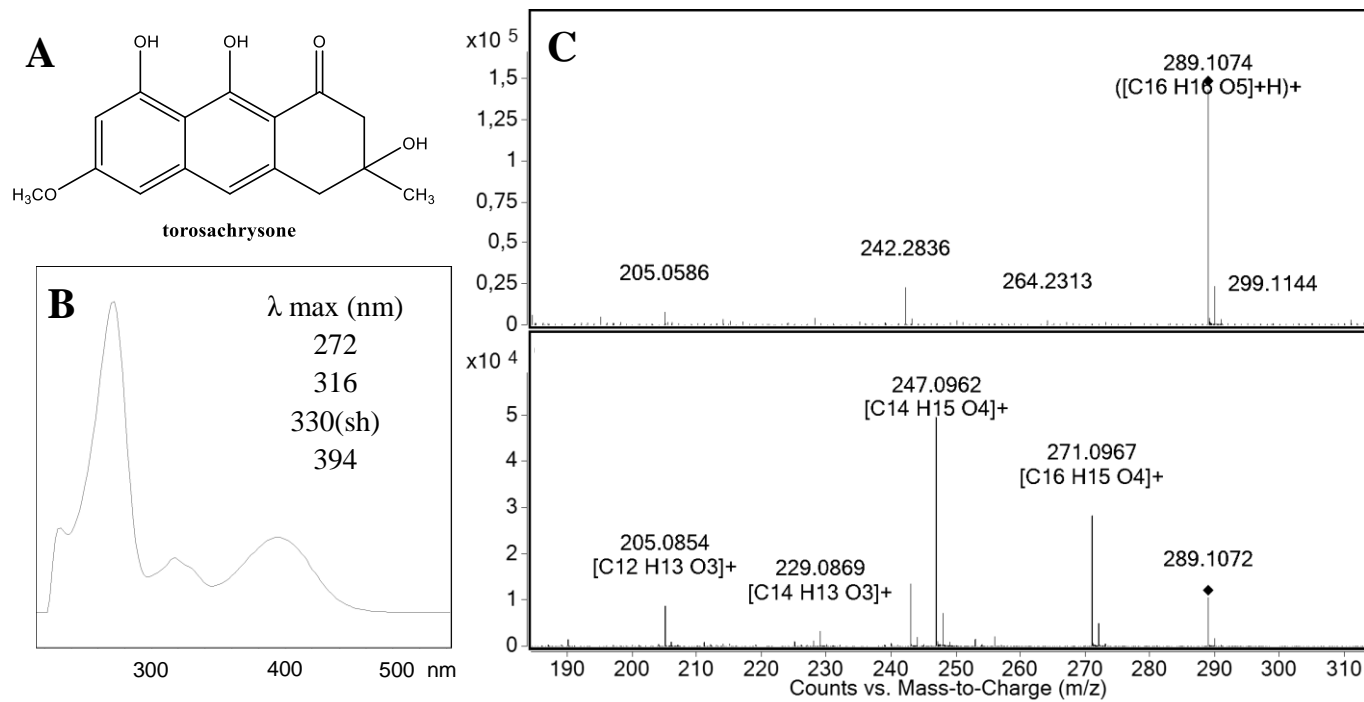


Figure 6

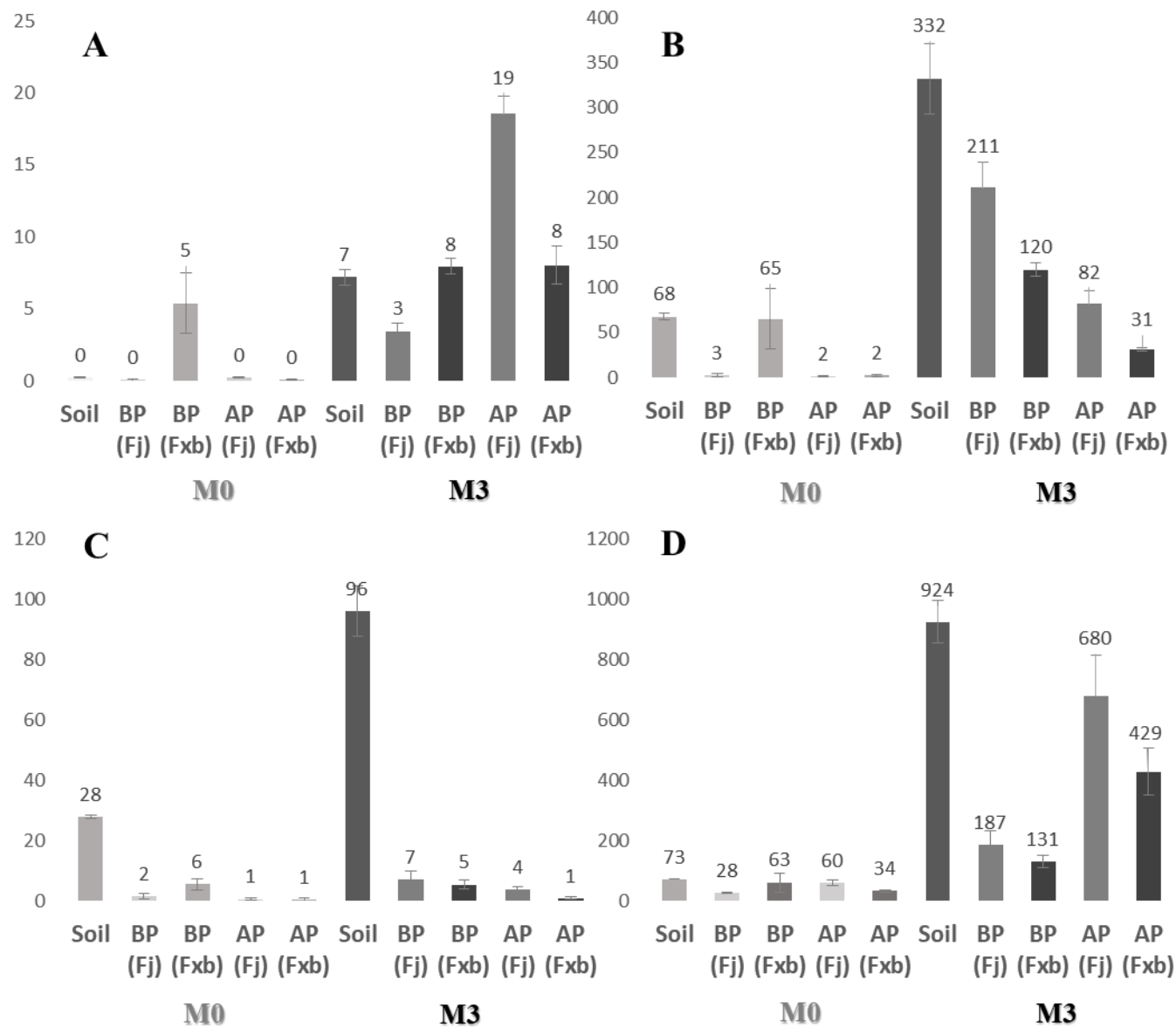


Figure 7

Metals	Non polluted (M0)	Polluted +/- (M1)		Polluted + (M2)		Polluted ++ (M3)	
		1 month	3 months	1 month	3 months	1 month	3 months
Cd	0	0.2	0.1	2	1	20	6
Cr	0	15	9	150	90	1500	440
Pb	0	10	6	100	60	1000	295
Zn	0	30	18	300	180	3000	880

Table 1

Metals	Soil	Underground parts	Aerial parts
Cd	6.7 ^a	19.7 ^b	6.7 ^b
Cr	59.8 ^b	40.9 ^b	2.7 ^c
Pb	203.0 ^b	27.3 ^b	10.7 ^c
Zn	2623.0 ^b	357.0 ^b	541.7 ^a

Table 2



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Supplementary Material

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