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SHORT NOTES

Phlorizin released by apple root debris is related to apple replant disease

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Summary. Autotoxic compounds are likely to be among the causes of apple replant disease, but their secretion is low during plant life. Using targeted metabolomics, the changes in soil phenolic profile were analyzed after the addition of apple roots, and their potential autotoxicity was assessed on apple seedlings. The addition of apple roots severely damaged the plants, attributed to autotoxic action of the phenolic compound phlorizin. Prolonged residence time of the roots in the soil before planting reduced their negative action, probably due to the degradation of phlorizin.

Key words: allelochemicals, phenolic compounds, soil, autotoxicity, continuous cropping obstacle.

Introduction

Apple replant disease (ARD) is a complex syndrome arising from the repeated replanting of apple trees in the same soil; the main symptom is reduced plant growth, particularly root biomass. This syndrome is related to biotic factors (i.e. increased concentrations of pathogenic fungi, decrease in plant growth promoting bacteria) and, possibly, abiotic factors in soil, although the precise etiology is still unclear (Mazzola and Manici, 2012). One of the possible biotic causes of ARD is autotoxicity, in which the phenolic compounds released by roots may play an important role (Huang *et al.*, 2013). The roots of apple trees can release several different phenolic compounds and some of them (phlorizin, *p*-hydroxybenzoic acid, *p*-hydroxy hydrocinnamic acid, phloroglucinol) were found in liquid cultures (Börner, 1959). However, root exudation of these substances is quite low during the lifespan of apple plants (Hofmann *et al.*, 2009). On the other hand, phenolic compounds released from decomposing apple leaves and roots (1% in soil) may reach high concentrations, as demonstrated by Politycka and Adamska (2003). In the present study, we increased the quantity of root material added to soil by up to 20% of its volume.

In-field studies investigating the causes of ARD are of extremely difficult interpretation, because of the high number of factors that could be involved. We therefore studied the phenomenon with an artificial setup under controlled conditions, where only the factor 'effect of roots on new plants' varied. Sampling was performed at 0, 3 and 7 months at the most active temperature (20°C), to specifically identify and quantify the phenolic compounds released during the decay of apple roots, using Ultra High Performance Liquid Chromatography (UHPLC) coupled to a mass spectrometer. Furthermore, we tested root autotoxic potential on apple seedlings in soil.

Materials and methods

Experimental design and plant growth measures

Healthy roots (<3 mm diam) were collected from explanted apple trees (rootstock M26) in the Tren-

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tino-South Tyrol region (Italy) on 26 January 2015. They were ground and mixed (1:5, v:v) with sieved soil (loam; pH 7.7; 52 g kg^{-1} of organic matter) taken from an uncultivated area (treatment R3). The soil was divided into two portions that were used to repeat the experiment twice in the same conditions. Sieved soil without any addition of ground roots served as an untreated control (treatment C3). After gentle watering (20 mL kg^{-1} of soil), both soils (R3 and C3) were kept under controlled conditions (20°C) in the greenhouse for 90 d. The same protocol was repeated three months later (4 May 2015) using the soil collected in January, which was kept in natural conditions in the meantime, and a soil mixed with root debris (treatment R0) and an untreated control soil (C0) were obtained. Apple seedlings, grown in peat from seeds of the cv. Fuji in peat, were transplanted at the age of 90 d into the four treated soils (R0, C0, R3, C3), with three soil samples being collected from each soil treatment for analysis of phenolic compounds before transplanting (time T1). The soil samples were also checked for absence of the three main apple tree pathogens, *Armillaria* spp., *Phytophthora cactorum* and *Rosellinia necatrix*, using diagnostic PCRs, according, respectively, Lochman *et al.* (2004), Bhat and Browne (2010) and Pasini *et al.* (2016). Fifteen replicates (pots) per soil treatment, having one seedling each, were held at 20 ± 0.5°C in a greenhouse. After 120 d, the chlorophyll content of the apple seedling leaves was measured (SPAD502, Spectrum Technologies) and the fresh weights of whole plants and roots were assessed. At the same time, three soil samples per treatment were taken from the pots and subjected to phenolic compound analysis (time T2). During the experiment the plantlets did not show any symptoms ascribable to root infections of microbial pathogens.

Analysis of phenolic compounds

Samples were extracted as described in Vrhovsek *et al.* (2012). After evaporation of methanolic fractions, samples were applied to a preconditioned ENV+ Isolute C18 SPE column. Preconditioning was performed by purging the column with 10 mL of methanol and 20 mL of water. After loading a sample onto the column, it was washed with 10 mL of water. Polyphenols, retained in the column, were eluted with 20 mL of methanol. Solvent was evaporated using a rotavapor and the residues were dissolved in 500 μL of a methanol/water mixture (2:1). Samples were injected before and after concentration using SPE. Phenolic compounds were analyzed according to Vrhovsek *et al.* (2012), with a method that allows the detection of a total of 135 different phenolic compounds. Briefly, UHPLC (Waters Acquity UPLC - Milford) coupled to a mass spectrometer (Waters Xevo TQMS - Milford) was used. Separation of the compounds was achieved on a Waters Acquity HSS T3 column 1.8 μm, 100 mm × 2.1 mm (Milford), kept at 40°C. Mobile phase A was water containing 0.1% formic acid; mobile phase B was acetonitrile containing 0.1% formic acid. The chemicals used for the analysis were purchased from Sigma Aldrich.

Statistical analyses

Statistical analyses was performed with PAST, version 2.17 (Hammer *et al.*, 2001) and Statistica 9 software (StatSoft). An F-test was used to demonstrate non-significant differences between the two repetitions of the experiment ($P>0.05$) and data on plant growth were pooled. Since the distribution of data was not normal, statistically significant differences between treatments ($P<0.05$) were assessed with the Kruskal-Wallis test with Mann Whitney pairwise comparisons (Bonferroni corrected). During analysis of the phenolic compounds, values below the Limit Of Detection (LOD) were substituted with LOD/√2 (Verbovšek, 2011). Once homogeneity of variance assessed with Levene's test ($P>0.05$) was satisfied, non-metric multidimensional scaling (NMDS), one-way analysis of similarities (ANOSIM), similarity percentage analysis (SIMPER) and the Wilcoxon test were employed to assess the difference in composition in the phenolic profile of soils. Pearson's correlation was calculated to determine the relationship between the concentrations of phenolic compounds and plant weights.

Results and discussion

Diagnostic PCRs (*Armillaria* spp., *Phytophthora cactorum*, *Rosellinia necatrix*) did not amplified any products, therefore we excluded the presence of apple root pathogens in the soil treated with roots. The soil treatments affected seedling growth. In particular, seedlings planted in soil immediately after mixing with root debris (treatment R0) showed lower chlorophyll content and total seedling weight com-

pared with all other treatments (Table 1, Kruskal-Wallis and Mann Whitney pairwise test, $P<0.05$). The mean root weight in the R0 treatment was only significantly less than R3 and C3 treatments. The addition of apple roots to soil just before planting therefore significantly impaired the health of the seedlings, showing marked autotoxic effects on the plants and not just on their root systems.

Our results indicate that this autotoxic effect of roots on new plants was visible in the soil, and not only in water cultures (Börner, 1959). In contrast, Politycka and Adamska (2003) found a stimulating or slightly inhibiting effect of apple roots on radical growth of cucumber, results that could be due to the use of a different plant species and/or lower concentrations of apple roots in the soil. The artificial experimental set up allowed us to separate the effect of roots on new plants, without confounding effects from other factors.

Fourteen phenolic compounds were detected in soil samples at time T1 (preplanting). The concentrations of these compounds were generally low, with the exception of phlorizin, phloretin and narigenin (Table 2). An NMDS (stress = 0.078, R^2 axis 1 = 0.992, axis 2 = 0.085) on Euclidean distances of the dataset indicated that data points representing the samples from R0 soil clustered together, separated from the other cluster, which comprised samples from the R3, C0 and C3 treatments (Figure 1A). A one-way ANOSIM with Bonferroni-corrected pairwise comparisons, confirmed the difference between the phenolic profile of R0 samples and all the other samples ($P<0.05$).

The concentration of four phenolic compounds, *p*-coumaric acid, quercetin-3-rhamnoside, phloretin and phlorizin, significantly increased in R0 treatment soils, compared to C0 (Wilcoxon test, $P<0.05$). These compounds are all considered to be allelochemicals in apple and in other plants (Huang *et al.* 2013; Inderjit and Dakshini, 1995). In the R0 treatment, the concentrations of all these compounds, but not *p*-coumaric acid, were also significantly greater than those in R3, meaning that after 3 months of roots in the soil, these substances had degraded. A significant negative correlation was found between the sum of the concentrations of the single phenolic compounds measured at T1 and total plant weight (Pearson correlation r = -0.89, $P<0.05$), so a high concentration of polyphenols at planting corresponded to diminished plant growth. In order to detect which phenolic compounds were most responsible for the difference in R0 soils, SIMPER was used. This indicated phlorizin as the phenolic compound contributing to more than 90% of intergroup dissimilarity between R0 and the other treatments, and phloretin as the second most important compound (approximately 5%). In the R0 samples, phlorizin and phloretin reached average concentrations, respectively, of 77.4 (± 8.0) and 3.7 (± 0.9) $\mu g\ g^{-1}$, while in the other samples phorizin concentrations were < 0.1 $\mu g\ g^{-1}$ and phloretin < 0.06 $\mu g\ g^{-1}$.

We therefore confirm the trend for polyphenol concentrations observed by Politycka and Adamska (2003), although they measured total phenolic content, which also comprises other high molecular weight polyphenols, such as proanthocyanidins. Phlorizin and phloretin are the main flavonoids produced by apple plants and are usually stored in bark and roots (Gosch *et al.*, 2010). These polyphenols inhibit root and shoot growth in water culture (Börner, 1959), and phlorizin can specifically inhibit the respiratory rate and enzyme activities of the tricarboxylic acid cycle in apple roots (Wang *et al.*, 2012; Yin *et*

Table 1. Means (± standard errors) of measurements for apple seedlings after 4 months growth in soils amended with old apple roots at different times and in control soils. Letters in each column indicate statisically significant differences ($P<0.05$). R3 = soil with roots amended 3 months before planting; C3 = control soil of the R3 treatment; R0 = soil with roots amended just before planting; C0 = control soil of the R0 treatment.

Treatment	Whole plant fresh weight (g)	Root fresh weight (g)	Chlorophyll content (SPAD)
R3	5.53 ± 0.34 *a*	3.29 ± 0.26 *a*	33.9 ± 0.8 *a*
C3	6.70 ± 0.52 *a*	3.69 ± 0.31 *a*	38.0 ± 0.9 *b*
R0	3.19 ± 0.16 *b*	2.21 ± 0.14 *b*	24.5 ± 1.2 *c*
C0	5.90 ± 0.54 *a*	2.93 ± 0.24 *ab*	38.6 ± 1.1 *b*

Table 2. Mean concentrations (μg g^{-1}, ± standard errors) of the phenolic compounds in soil at planting time (T1), measured with UHPLC and mass spectrometery. R3 = soil with roots amended 3 months before planting; C3 = control soil of the R3 treatment; R0 = soil with roots amended just before planting; C0 = control soil of the R0 treatment.

Phenolic compound	R3	C3	R0	C0
Anthranilic acid	0.0015 ± 0.010	0.0009 ± 0.0004	0.0040 ± 0.0024	0.0023 ± 0.0014
4-Aminobenzoic acid	0.0004 ± 0.002	0.0002 ± 0.0000	0.0003 ± 0.0001	0.0002 ± 0.0000
P-hydroxybenzoic acid	0.0086 ± 0.0037	0.0160 ± 0.0074	0.0235 ± 0.0096	0.0053 ± 0.0027
Cinnamic acid	0.0736 ± 0.0616	0.0734 ± 0.0726	0.0957 ± 0.0796	0.0173 ± 0.0170
Vanillin	0.0048 ± 0.0005	0.0040 ± 0.0004	0.0056 ± 0.0005	0.0050 ± 0.0002
Vanillic acid	0.0008 ± 0.0002	0.0009 ± 0.0001	0.0010 ± 0.0002	0.0009 ± 0.0002
2,6-Dioh-benzoic acid	0.0217 ± 0.0114	0.0109 ± 0.0021	0.0551 ± 0.0416	0.0436 ± 0.0348
P-coumaric acid	0.0479 ± 0.0334	0.0496 ± 0.0465	0.0916 ± 0.0553	0.0211 ± 0.0185
Caffeic acid	0.0010 ± 0.001	0.0058 ±0.0023	0.0036 ± 0.0011	0.0034 ± 0.0016
Ferulic acid	0.0707 ± 0.0433	0.0392 ± 0.0381	0.1339 ± 0.0852	0.1092 ± 0.1081
Phloretin	0.0107 ± 0.080	0.0024 ± 0.0016	3.6734 ± 0.8509	0.0104 ± 0.0091
Phlorizin	0.0707 ± 0.0000	0.0707 ± 0.0000	77.4076 ± 8.0480	0.0707 ± 0.0000
Naringenin	0.1536 ± 0.1275	0.0230 ± 0.0195	0.1752 ± 0.1153	0.1683 ± 0.1648
Quercetin-3-rhamnoside	0.0124 ± 0.0059	0.0243 ± 0.0097	0.1562 ± 0.0786	0.0119 ± 0.0084

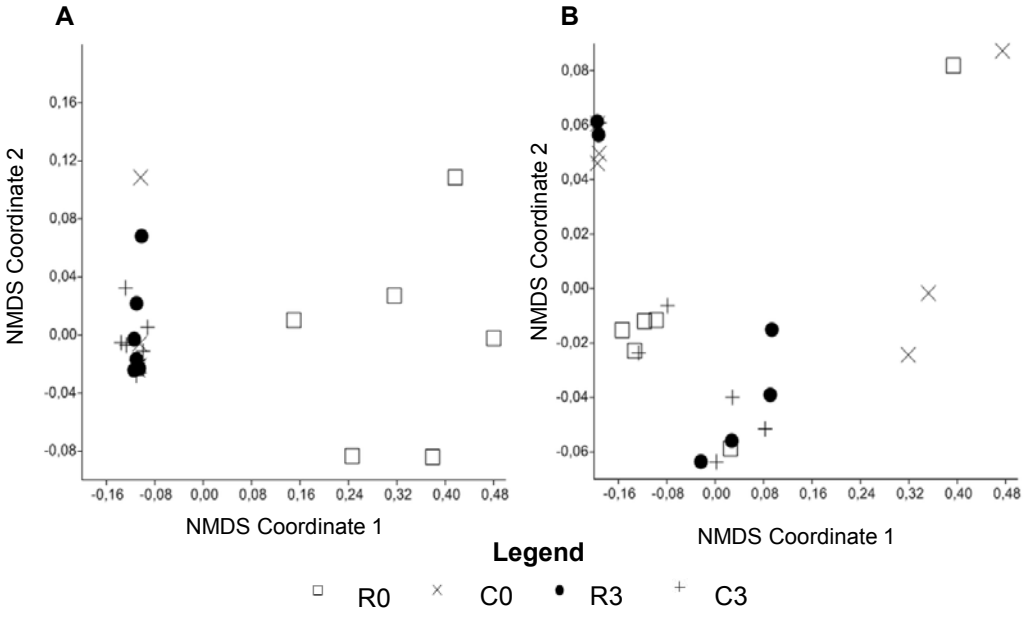

Figure 1. Non Metric Multidimensional Scaling (NMDS) based on Euclidean distances of soil samples amended with old apple roots at different times and control soils. R3 = soil with roots amended 3 months before planting; C3 = control soil of the R3 treatment; R0 = soil with roots amended just before planting; C0 = control soil of the R0 treatment. Each point represents the phenolic profile of one sample. a) at planting time (T1); b) after 4 months of seedlings growth (T2).

al., 2016*)*. The concentration of phlorizin and phloretin in R3 treatment soils was comparable with that in control soils, indicating that the 3 months when the ground roots remained in the soil were sufficient to allow degradation of these compounds.

These results suggest that in orchards the concentration of phlorizin in soil should be measured before replanting to assess the level of autotoxicity, using this compound as an indicator of soil health. We ascertained that concentrations of 77 μg g^{-1} in soil were detrimental for apple seedlings. Leaving several months between explanting and replanting is also recommended, especially because the degradation of phenolic compounds is much slower in winter, when the soil temperatures are low (Politycka and Adamska, 2003), and the release of phenolic compounds from intact roots could be gradual.

Eleven phenolic compounds were detected in soils from sampling at time T2. Again in this case, the concentrations were low (Table 3). As compared to T1, a lower number of benzoic acid derivatives was found. At this time, the NMDS on Euclidean distances (stress = 0.01, R^2 axis 1 = 0.99, axis 2 = 0.1) did not show any clustering of the samples (Figure 1B), a fact that was confirmed by one-way ANOSIM, which found no significant differences in the phenolic profile in the different treatments (P>0.05). The only phenolic compound that significantly increased in all soil treatments at T2 as compared to T1 was vanillic acid (Wilcoxon test, P<0.05), suggesting possible exudation from seedling roots, as happens in other plant species (Kong *et al.*, 2006). Four months after planting the seedlings, the concentrations of phlorizin and phloretin in R0 soils, which were very high in T1, dropped significantly (Wilcoxon test, P<0.05), although weights of seedlings planted in this soil were reduced. This suggests that the initial stress caused by high concentration of phlorizin can impair plant health for long periods, as the plants remained stunted even when the concentration of the compound decreased significantly.

In conclusion, this study confirmed that the presence of apple root debris in soil can significantly impair the growth of apple seedlings, and that this negative effect disappears when phenolic compounds (mainly phlorizin and phloretin) have degraded. If the seedlings are planted just after the addition of roots, the initial negative impact on subsequent growth persists over time, despite the reduction in concentrations of phenolic compounds. Assessment of phlorizin could therefore be the basis for developing an indicator of ARD risk in orchard soils, or to determine the appropriate time for replanting to avoid ARD.

Table 3. Mean concentrations (μg g^{-1}; ± standard errors) of phenolic compounds in soil after 4 months of seedlings growth (T2), measured with UHPLC and mass spectrometery. R3 = soil with roots amended three months before planting; C3 = control soil of the R3 treatment; R0 = soil with roots amended just before planting; C0 = control soil of the R0 treatment.

Phenolic compound	R3	C3	R0	C0
P-hydroxybenzoic acid	0.0206 ± 0.0018	0.0265 ± 0.0063	0.0271 ± 0.0054	0.0275 ± 00.67
Vanillin	0.0032 ± 0.0005	0.0027 ± 0.0004	0.0040 ± 0.0007	0.0026 ± 0.0004
Vanillic acid	0.0222 ±0.0025	0.0266 ± 0.0024	0.0296 ± 0.0043	0.0316 ± 0.0058
Syringaldehyde	0.0012 ±0.0003	0.0009 ± 0.0002	0.0008 ± 0.0002	0.0009 ± 0.0002
Esculin	0.0004 ±0.0000	0.0007 ± 0.0003	0.0018 ± 0.0010	0.0004 ± 0.0000
P-coumaric acid	0.0028 ±0.0008	0.0020 ± 0.0003	0.0041 ± 0.0005	0.0033 ± 0.0005
Ferulic acid	0.0020 ±0.0005	0.0012 ± 0.0001	0.0023 ± 0.0005	0.0012 ± 0.0002
Phloretin	0.0054 ±0.0020	0.0042 ± 0.0029	0.0105 ± 0.0023	0.0064 ± 0.0029
Phlorizin	1.2200 ± 0.3982	1.1454 ± 0.3057	1.4584 ±0.6136	2.0692 ± 0.9222
Taxifolin	0.0073 ± 0.0029	0.0069 ± 0.0025	0.0081 ±0.0030	0.0073 ± 0.0030
Dihydrokaempferol	0.0049 ± 0.0008	0.0040 ± 0.0003	0.0057 ± 0.0021	0.0141 ± 0.0062

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