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Root exudation of phloridzin by apple seedlings (*Malus x domestica* Borkh.) with symptoms of apple replant disease

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Summary

This study investigates the occurrence of the flavonoid phloridzin (phloretin-2'-*O*- β -D-glucoside) in root exudates of apple seedlings showing growth reduction related to apple replant disease (ARD). The disease is most likely caused by a complex of soil-borne fungi and bacteria, but the etiology remains to be elucidated. Information on specific exudation processes in the rhizosphere of apple seedlings could contribute to our understanding of the conditions triggering ARD development.

To procure ARD symptoms, apple seedlings (*Malus x domestica* Borkh.) were grown in ARD-conductive soil. Root exudates were collected by submerging the roots in a solution of 0.05 mM CaCl₂ for a period of 4 h. The fraction of phenolic root exudates was analyzed using HPLC/DAD (high performance liquid chromatography/diode array detector).

Results suggest that (i) phloridzin is a constant root exudate of apple seedlings. It was the most abundant phenol in the collected exudates from replant-diseased as well as healthy seedlings. (ii) Phloridzin exudation, related to root dry matter, was the most intensive at the onset of ARD symptom development and lower during the period when symptoms were most severe or outgrown. (iii) In comparison to healthy seedlings, the phloridzin exudation of apple replant-diseased seedlings was significantly higher only at the onset of ARD symptom development, suggesting a response of the plants to infection.

The finding of phloridzin in the root exudates of *Malus x domestica* Borkh. might have consequences for research on the etiology of ARD. Specialized pathogenic microorganisms could be attracted by this distinct compound. Since it is very characteristic of apple plants, phloridzin might be the compound that ARD-causing microorganisms utilize to recognize their host. For practical applications, phloridzin root exudation could therefore be a parameter in evaluating ARD-susceptibility of different rootstocks.

Introduction

Apple replant disease is a soil-borne disease, which has been reported to occur in apple-growing areas throughout the world, in Europe (SAVORY, 1966; HOESTRA, 1968; MANICI et al., 2003), North America (WILLET et al., 1994), and Australia (DULLAHIDE et al., 1994). Symptoms include general growth reduction, browning of roots, and stunting of the shoot (SAVORY, 1966; HOESTRA, 1968; CARUSO et al., 1988). These symptoms have been described for *Malus* as well as other species of the *Rosaceae* family, subfamily *Maloideae* (OTTO and WINKLER, 1995).

The fact that sterilization of soil leads to healthy plants supports the hypothesis that ARD is caused by soil-borne microorganisms. Pathogens suggested to be involved are *actinomycetes* (OTTO and WINKLER, 1977; WESTCOTT et al., 1986), bacteria such as *Bacillus subtilis* (UTKEDE and LI, 1988), and fungi, e.g., *Pythium intermedium* (SEWELL, 1980; MANICI et al., 2003). MAZZOLA (1998) suggests that rather than by a single organism, ARD is caused by a complex of several microbial species.

To reduce effects of apple replant disease, orchard management relies on land shifting or sterilization of affected soil by fumigation, e.g. with methyl bromide. Because of its general toxicity to soil organisms, several studies of the past years have focused on finding alternative techniques to fumigation, like organic soil amendments or alternative rootstock genotypes to combat apple replant disease (MAZZOLA et al., 2001; RUMBERGER et al., 2004; WILSON, 2004; YAO et al., 2005). In order to find effective techniques, research on the underlying mechanisms of the disease is still necessary. Information on rhizosphere processes during the disease might be pivotal in explaining the involvement of microorganisms in ARD and might provide new strategies against ARD.

We hypothesize that specific biochemical conditions in the rhizosphere of apple roots, like the root exudation of certain compounds, trigger plant-microbe interactions that lead to the disease. It has been suggested that particular or rare compounds from root exudates of a given plant species might have specific functions in the rhizosphere of that species (SCHROTH and HILDEBRAND, 1964; ROVIRA, 1969; WERNER, 2001). For flavonoids, BELAÑOS-VASQUEZ and WERNER (1997) and JAIN and NAINAWATEE (2002) suggested a role of signal molecules for microorganisms in the rhizosphere. Signal molecules in root exudates might attract specialized microorganisms to their host plants. This could help explain the specificity of certain plant-microbe interactions.

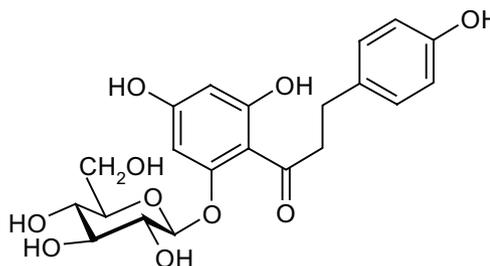


Fig. 1: Phloridzin (phloretin-2'-*O*- β -D-glucoside)

The flavonoid phloridzin (phloretin-2'-*O*- β -D-glucoside, Fig. 1) could be such a root exudate with specific functions in the rhizosphere of apple seedlings. It is a compound characteristic to the genus *Malus*, yet not exclusive to that genus, since it has been detected also in *Fragaria* (HILT et al., 2003). In apple plants, phloridzin has been detected in roots, leaves, buds and fruit (BÖRNER, 1959; RAA, 1968; ANTOLOVICH et al., 2000; HRAZDINA, 2003), but not yet reliably identified as a part of the root exudates (SZABÓ and WITTENMAYER, 2000).

BÖRNER (1959) was the first to examine the role of phloridzin in relation to the symptoms of apple replant disease (ARD). He determined phloridzin concentrations in apple plant residues from soil of former apple orchards to assess possible toxic effects on newly planted apple trees. The concentrations and persistency of phloridzin were too low to explain the occurring symptoms. BÖRNER later suggested that, instead of leaching from apple plant residues,

phloridzin in soil could also originate from active root exudation of apple plants and could thus be directly released into the rhizosphere (BÖRNER, 1960).

The objectives of our study were to (i) prove the presence of phloridzin in the fraction of phenolic root exudates of *Malus × domestica* Borkh., (ii) quantify changes in phloridzin exudation in relation to development of replant disease symptoms, and (iii) investigate differences in phloridzin exudation between healthy and ARD-infected plants.

Materials and methods

Preparation of cultivation substrates

The original soil was collected from the topsoil (approx. 0-30 cm) of a previously cleared apple orchard site suspected to be affected by ARD. This soil was sieved (5 mm mesh size) for homogenization and in order to remove large plant residues. Part of the soil was sterilized by gamma radiation (^{60}Co , radiation dose 35 kGy, Gamma Service Produktbestrahlung, Radeberg, Germany). Chemical soil parameters of the original and sterilized soil were compared using standard methods of soil analysis. No differences in pH or plant-available macro- and micronutrients were observed.

The cultivation substrates, hereafter referred to as "ARD-conductive soil" and "sterilized ARD soil", were prepared by mixing the original or gamma-radiated soil with an equal amount of coarse quartz sand (1-2 mm). The sand was added to improve aeration in the pots and facilitate root growth.

Apple seedling cultivation

In order to investigate the possibility of phloridzin root exudates being involved in the development of ARD, we carried out a three-month in-vitro experiment with apple seedlings grown in ARD-conductive soil and sterilized soil for control. Apple seedlings were grown from seeds (G. J. Steingaesser, Miltenberg, Germany) of *Malus × domestica* Borkh., a variety used for tall tree rootstocks. When three pairs of primary leaves had developed, the seedlings were transplanted into the prepared substrates. The pots had volumes of 0.2 to 0.9 l, in accordance with expected plant size at future dates of the root exudates collection. They were lined with thin plastic bags (perforated at the bottom) to prevent root system damage at the time of removing the plants from containers for measurements. Seedlings were watered daily to maintain the water-holding capacity of the substrate at 80%. Plants were sprayed with dimethoate (against aphids) and also with sulfur and fenarimol (as alternating applications, against mildew). Fertilization was not included in the experiment. Seedlings did not show any symptoms of nutrient deficiency.

Collection of root exudates

We collected root exudates of the grown seedlings by submersion of the roots in a solution of 0.05 mM CaCl_2 (EGLE et al., 2003). This approach is similar to the dipping method described and evaluated by GRANSEE and WITTENMAYER (2000), who concluded that the method is suitable for nearly complete sampling of root exudates. Collection of root exudates was started 30 days after the transfer of the seedlings into the substrates and repeated on every ninth day for a total of ten collection dates, corresponding to 30, 39, 48, 57, 66, 75, 84, 93, 102 and 111 days after the transfer into the substrates.

The seedlings were carefully removed from the pots and substrate. The roots were gently rinsed with water to prevent damage of the root system. Subsequently, the entire root systems of three intact seedlings were submerged in a solution of 0.05 mM CaCl_2 in a glass

beaker wrapped with aluminum foil to prevent the roots from light exposure. The plants were set up in a climate chamber (22°C, 70 % relative humidity, light intensity 500 $\mu\text{m}^2 \text{s}^{-1}$). After one hour of equilibration, the solution was discarded and replaced with fresh solution of 0.05 mM CaCl_2 . The plants were then allowed to continue exudation into the solution for 4 h. The total amount of root exudates is attributed to this time period. The solution containing the root exudates was shock-frozen in liquid nitrogen, lyophilized at -25°C, and stored in the dry state at -70°C.

Separation and quantification of the phenolic component

For analysis of the phloridzin contents in the acquired root exudates, we chose an established HPLC-method originally applied in food technology for the detection of phenolic compounds in fruit juices (SCHIEBER et al., 2001). The phenolic compounds of the root exudates were separated by solid phase extraction using reverse-phase cartridges (DSC18, 3 ml, 500 mg, particle size 56 μm , Supelco, Bellefonte, PA, USA). About 25 mg of the lyophilized sample was dissolved in 2 ml of 0.5% acetic acid. Prior to extraction, the columns were activated with 2 ml of methanol and conditioned with 2 ml of 0.5% acetic acid. The phenolics were eluted from the column with 2 ml of methanol. The solvent was evaporated at 25 °C *in vacuo* and the phenolics were redissolved in 0.2 ml acetonitrile and 0.8 ml 2 % acetic acid for HPLC analysis. The C18 columns were weighed before and after extraction to determine the amount of insoluble residues. HPLC analysis of the phenols was performed as described by SCHIEBER et al. (2001) using a reversed-phase stationary phase (125 Aqua C18 column, 250 x 4.6 mm I.D., particle size 5 μm , Phenomenex, Torrance, CA, USA). The HPLC system (Merck/VWR International, Darmstadt, Germany) consisted of a degasser L-7614, HPLC-pump L-7100, column thermostat L-7350 and a diode-array-detector L-7455. Phloridzin was identified by comparison of the retention time with that of the authentic reference substance (Fluka, Seelze, Germany) and by LC-MS/MS (liquid chromatography/tandem mass spectrometry).

Determination of plant parameters

Upon collection of root exudates, plant parameters were determined in order to relate them to phloridzin exudation and quantitatively describe the growth reduction of replant-diseased apple seedlings. The following root and shoot parameters were measured: root surface (SATTELMACHER et al., 1983), root and shoot dry weight (gravimetrically), and leaf surface by digital image analysis (Software Analysis 3.0, Soft Imaging System GmbH, Münster, Germany).

Statistics

For measurements, nine apple seedlings per cultivation substrate were harvested. The nine seedlings were pooled in groups of three for collection of exudates, allowing three replicates in HPLC analysis ($n=3$). Plant parameters were determined individually for the selected nine plants ($n=9$).

For data analyses, SPSS 11 software was used (Chicago, IL, USA). Normal distribution was confirmed with the Kolmogorov-Smirnov-test. Significant differences were detected using one factorial analysis of variance and Duncan-test.

Results

Apple replant disease symptoms

Apple seedlings grown in soil conductive to ARD displayed significant growth reductions of shoots and roots and were considered

"apple replant-diseased plants", whereas seedlings grown in the same but sterilized soil did not show any symptoms of ARD and were thus considered „healthy plants“. After the cultivation period of 111 days, seedlings grown in ARD soil had built up only 34% of the shoot dry matter of healthy plants. Leaf area was 40% and root dry matter was 30% compared to healthy plants (data not shown). Growth reduction is visualized in Fig. 2 a - c. The prerequisite for the comparison of the phloridzin root exudation of diseased and healthy plants was thus achieved.

Phloridzin in root exudates

Phloridzin was found to be the most abundant compound in the phenolic component of the root exudates of both replant-diseased and healthy apple seedlings throughout the measurement period (81 d). Beside phloridzin (phloretin-2'-O- β -D-glucoside), one more compound was identified in the phenolic root exudates, namely phloretin (4,2',4',6' tetrahydroxydihydrochalcon), the aglycone of phloridzin. Authentication was achieved by LC-MS/MS (quasi molecular ion peak [M-H]⁻ at m/z 435 for phloridzin, and a fragment of m/z 273 for the phloretin aglycon, HILT et al., 2003). Other compounds were present unregularly or in small quantities and could not be identified. Fig. 3 shows a typical chromatogram indicating the spectrum of various substances of the phenolic fraction of the root exudates with phloridzin being the dominant compound.

Total root exudates, defined as the soluble component of the lyophilized exudation solution, increased with plant age (Tab. 1). Healthy plants did not consistently exude significantly higher amounts of total root exudates, yet there was a tendency toward this for the last four measurements (Tab. 1).

Concentrations of phloridzin in the root exudates were significantly higher for diseased seedlings 30 days after transfer into ARD-conductive soil (Tab. 1), indicating quantitative and qualitative changes in root exudation as a first response to infection. Subsequent measurements of phloridzin concentrations did not show differences between the treatments.

When phloridzin exudation was considered in relation to synthesizing capacity of seedlings, such as root dry weight and leaf area, the same pattern was noticed: phloridzin exudation was significantly higher for the replant-diseased seedlings only at the first measurement (30 days after transplanting into cultivation substrates, Tab. 2). For subsequent analyses of exudates, a general trend of higher phloridzin exudation by replant-diseased seedlings could be observed (Tab. 2).

Discussion

Amounts of exuded phloridzin

In comparison to carbohydrates or organic acids (SZABÓ and WITTENMAYER, 2000), phenolic compounds occur at a lower concentration in apple seedlings and in their root exudates. The phenolic compound phloridzin was exuded by apple seedlings in the range of only a few micrograms per plant during a four-hour time period. This is two magnitudes lower than the amount of, for example, citric acid, as found by EGGLE et al. (2003) using the same method for quantification of root exudates in lupin. In the study by HRAZDINA (2003), values for phloridzin contents in roots of (micropropagated) apple seedlings are in a range of 400 to 1200 $\mu\text{g g}^{-1}$ root fresh weight. Our results for exudation of phloridzin per g root dry weight were in the range of 2 to 30 μg . Assuming a root dry matter content of 20%, we can state that less than one-hundredth of the phloridzin amount accumulated in the root was released into the rhizosphere. Phloridzin should be apparently considered a trace substance and not a significant carbon source for potential root-pathogenic organisms.

For replant-infected seedlings, leakage from cells might occur, since

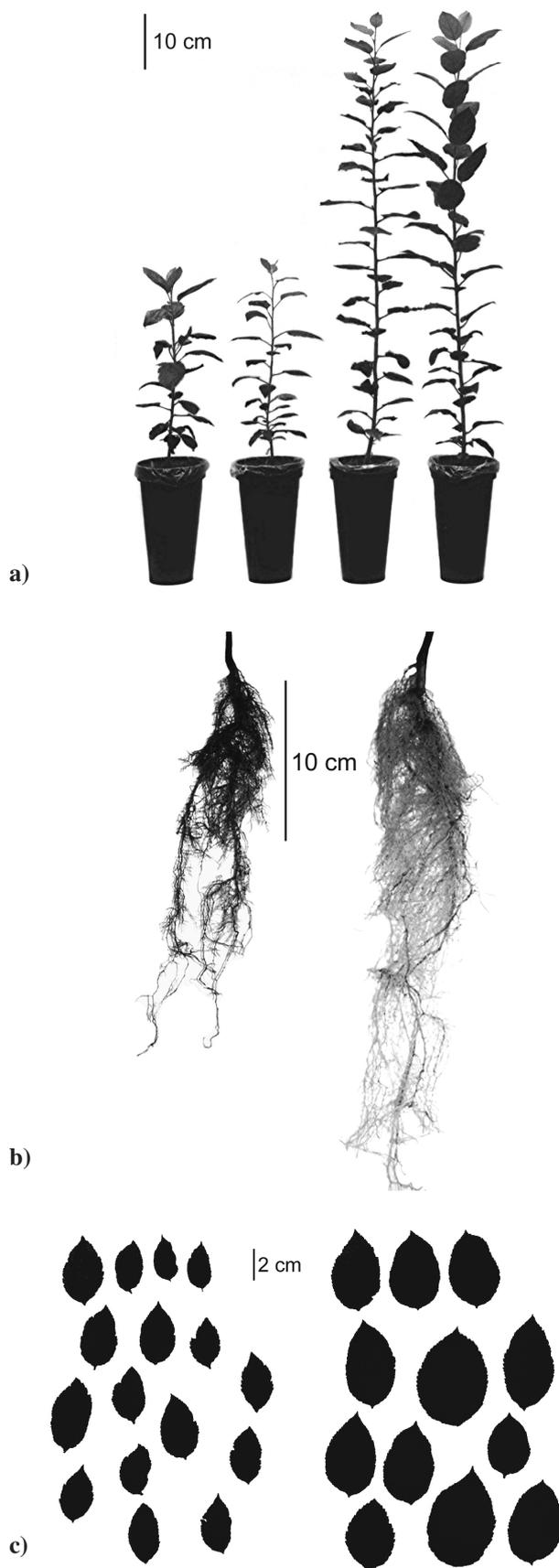


Fig. 2: a - c Significant growth reduction of apple replant-diseased (left) compared to healthy (right) apple seedlings, in parentheses time after transfer of seedlings to cultivation substrate. a) Shoots (111 d); b) Root system (93 d); c) Leaves (111 d).

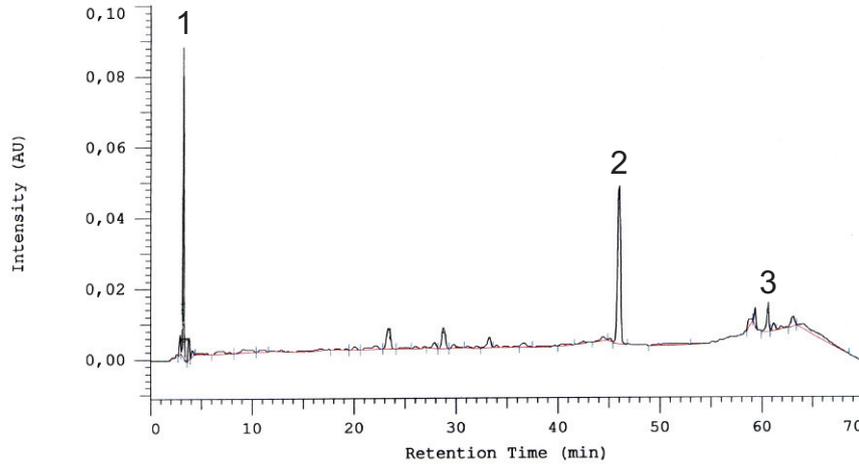


Fig. 3: HPLC-chromatogram of phenolic root exudates of replant-diseased apple seedlings (30 days after transplanting in ARD-conductive soil). 1 – injection peak, 2 – phloridzin, 3 – phloretin.

Tab. 1: Root exudates (mg) released per seedling and concentration of phloridzin (%) in exudates during 4 h. Comparison between apple seedlings cultivated in apple replant disease conductive soil or in the same but sterilized soil.

Time after transfer to soil (d)	Root exudates per plant (mg) ^a				Phloridzin concentration in root exudates (%) ^a					
	ARD-conductive soil		sterilized ARD soil		ARD-conductive soil		sterilized ARD soil		<i>P</i>	
30	0.77 ^b	(0.18) ^c	1.17	(0.09)	*	0.14	(0.04)	0.03	(0.00)	**
39	1.08	(0.13)	0.78	(0.08)	*	0.11	(0.08)	0.14	(0.01)	n.s.
48	0.66	(0.14)	0.70	(0.14)	n.s.	0.28	(0.16)	0.25	(0.19)	n.s.
57	0.68	(0.47)	1.00	(0.71)	n.s.	0.31	(0.20)	0.10	(0.05)	n.s.
66	0.63	(0.27)	1.11	(0.29)	*	0.34	(0.22)	0.24	(0.14)	n.s.
75	1.78	(0.33)	0.68	(0.57)	*	0.20	(0.16)	0.42	(0.19)	n.s.
84	2.03	(0.11)	2.79	(2.50)	n.s.	0.19	(0.06)	0.29	(0.14)	n.s.
93	2.70	(0.33)	4.94	(0.69)	n.s.	0.13	(0.05)	0.19	(0.06)	n.s.
102	3.34	(1.27)	5.30	(1.69)	n.s.	0.32	(0.22)	0.14	(0.03)	n.s.
111	3.22	(0.95)	12.41	(7.81)	n.s.	0.16	(0.05)	0.30	(0.13)	n.s.

^a n=3

^b mean

^c standard deviation of the mean

^d degree of significance of *P*: *P* < 0.05*, *P* < 0.01**, *P* < 0.001***, n.s. not significant

Tab. 2: Phloridzin exudation (µg) per g root dry weight and per dm² leaf area during 4 h. Comparison between apple seedlings cultivated in apple replant disease conductive soil or in the same but sterilized soil.

Time after transfer to soil (d)	Exuded phloridzin per root dry weight (µg g ⁻¹) ^a				Exuded phloridzin per leaf area (µg dm ⁻²) ^a					
	ARD-conductive soil		sterilized ARD soil		ARD-conductive soil		sterilized ARD soil		<i>P</i>	
30	30.2 ^b	(2.4) ^c	6.8	(2.5)	***	5.2	(0.5)	1.5	(0.3)	***
39	15.6	(9.4)	8.1	(0.3)	n.s.	4.1	(2.4)	2.6	(0.2)	n.s.
48	9.4	(2.7)	5.7	(3.2)	n.s.	4.0	(1.1)	2.2	(1.2)	n.s.
57	5.8	(3.7)	1.7	(0.1)	n.s.	3.8	(2.5)	0.7	(0.1)	n.s.
66	6.4	(5.1)	5.0	(2.2)	n.s.	4.2	(3.7)	2.2	(0.6)	n.s.
75	7.5	(5.5)	2.7	(0.9)	n.s.	2.6	(1.8)	0.6	(0.3)	n.s.
84	6.6	(2.2)	6.8	(1.5)	n.s.	2.1	(0.5)	1.3	(0.2)	n.s.
93	5.8	(2.5)	5.4	(1.5)	n.s.	2.3	(0.8)	1.8	(0.6)	n.s.
102	11.8	(8.5)	2.8	(1.1)	n.s.	5.2	(3.0)	1.2	(0.6)	n.s.
111	4.5	(2.3)	10.6	(11.7)	n.s.	1.9	(0.6)	5.3	(4.7)	n.s.

^a n=3

^b mean

^c standard deviation of the mean

^d degree of significance of *P*: *P* < 0.05*, *P* < 0.01**, *P* < 0.001***, n.s. not significant

the root cortex of infected plants becomes heavily damaged, as described by CARUSO et al. (1989) in a histological study. To prevent contamination of the exudates with leaked compounds during the collection, the equilibration step was applied. The raised phloridzin values for the first measurement (30 d upon the transfer into cultivation substrate) in our study might point to leakage as a result of cell damage inflicted by root pathogens. However, the results indicate that the total root exudation was not consistently higher for the diseased plants. The trend was even the opposite towards the end of the observation period, when the healthy plants had developed root systems three times larger than the diseased plants, with an adequately higher capacity to exude compounds into the rhizosphere. Thus the raised phloridzin content at the early-measurement point was genuine. Furthermore, at this early measurement point, the phloridzin concentration in the exudates was increased, suggesting a qualitative plant response to the contact with the ARD-conductive soil.

Phloridzin – a constant component in root exudate of apple seedlings

In previous approaches (SZABÓ and WITTENMAYER, 2000; WITTENMAYER und SZABÓ, 2000), a specific substance was searched for within the root exudates of *Malus* species that could attribute the specificity of ARD to a rhizosphere interaction, involving a distinct root exudate and an attracted specific pathogen. The results of this study show that the suspected phloridzin indeed occurs in root exudates of the examined apple seedlings. It was exuded in detectable amounts throughout the entire observation period.

OTTO et al. (1993), who investigated the infestation of apple seedling roots with actinomycetes in relation to ARD, observed that apple seedlings were susceptible to infection while the shoot was growing, whereas seedlings with a dormant terminal bud did not show newly infected root tissue. This observation gave rise to the hypothesis that a *Malus*-specific compound may be exuded during a certain time period, and during that period the compound serves as an attractant to pathogens. This hypothesis was disproved by our finding that phloridzin was exuded throughout the measurement period, and not at specific periods only. However, the fact that a higher exudation of phloridzin was found at the very beginning of the measurements, points to a quantitative and qualitative response of the plants just shortly upon transfer to the ARD-conductive soil, i.e., during the period when most likely the infection occurs.

In a study on the influence of polyphenols on the resistance of apple leaves to the fungal pathogen *Venturia inaequalis*, RAA (1968) found an antimicrobial effect produced by phloridzin. Taking this finding into account, the higher root exudation of phloridzin could be interpreted as a defense reaction of apple seedlings, though not an effective one, against microorganisms involved in ARD. In another study of the role of phloridzin in plant disease (DREYER and JONES, 1981), two opposite effects were suggested: repellence as well as attraction. These reversal effects have been explained by HARBORNE (1994). Secondary plant substances, whose primary function is in defense, might become attractant substances if a microorganism would have overcome the repellency in order to avoid competitors. Since phloridzin occurs only in small amounts, the original function in defense might be ineffective, as we found. Meanwhile its second possible function as attractant or signal molecule should be considered.

Conclusions

With this study we were able to (i) confirm the occurrence of phloridzin in root exudates of *Malus x domestica* Borkh. seedlings. Phloridzin was the dominant compound in the fraction of detected

phenolic root exudates. This substance was present in all samples throughout the observation period in replant-diseased as well as healthy plants. Therefore, we conclude that phloridzin is a constant component of root exudates in *Malus*.

(ii) In apple replant-diseased plants, phloridzin exudation was the most intensive at the onset of ARD symptom development and lower during the period when symptoms were most severe or outgrown. We therefore deduce that the symptom expression itself did not stimulate phloridzin exudation. We suggest that the higher phloridzin exudation at the onset of the disease can be considered as a response of the plants to infection.

This is also supported by (iii) a significant difference in the amount and concentration of exuded phloridzin between the diseased and healthy apple seedlings 30 days after the transplanting. Seedlings cultivated in ARD-conductive soil exuded significantly more phloridzin compared to healthy seedlings.

Considering our results, we assume two functions for phloridzin exudation: the primary one, as a part of the defense mechanism, but failing to suppress disease development, supported by results (ii) and (iii); and the secondary, as a host-signaling compound utilized by microorganisms, which is supported by result (i). As a constant component of the root exudates of apple plants, phloridzin might have become an attractant for microorganisms that have developed resistance to this plant defense mechanism. This possible secondary function of phloridzin should be taken into account in future investigations on possible ARD pathogens. For future testing of apple rootstocks we propose to screen seedlings with different phloridzin exudation patterns for their susceptibility to ARD.

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