Polyphenols from *Rheum* Roots Inhibit Growth of Fungal and Oomycete Phytopathogens and Induce Plant Disease Resistance

Marit Gillmeister,^{1,†} Silvia Ballert,¹ Anja Raschke,² Joerg Geistlinger,¹ Kathrin Kabrodt,¹ Helmut Baltruschat,¹ Holger B. Deising,² and Ingo Schellenberg¹

¹ Institute of Bioanalytical Sciences (IBAS), Anhalt University of Applied Sciences, 06406 Bernburg, Germany

² Institute for Agricultural and Nutritional Sciences – Phytopathology and Plant Protection, Martin Luther University Halle-Wittenberg, 06120 Halle (Saale), Germany

Abstract

A growing world population requires an increase in the quality and quantity of food production. However, field losses due to biotic stresses are currently estimated to be between 10 and 20% worldwide. The risk of resistance and strict pesticide legislation necessitate innovative agronomical practices to adequately protect crops in the future, such as the identification of new substances with novel modes of action. In the present study, liquid chromatography mass spectrometry was used to characterize *Rheum rhabarbarum* root extracts that were primarily composed of the stilbenes rhaponticin, desoxyrhaponticin, and resveratrol. Minor components were the flavonoids catechin, epicatechin gallate, and procyanidin B1. Specific polyphenolic mixtures inhibited mycelial growth of several phytopathogenic fungi and oomycetes. Foliar spray applications with fractions

containing stilbenes and flavonoids inhibited spore germination of powdery mildew in *Hordeum vulgare* with indications of synergistic interactions. Formulated extracts led to a significant reduction in the incidence of brown rust in *Triticum aestivum* under field conditions. *Arabidopsis thaliana* mutant and quantitative reverse-transcription polymerase chain reaction studies suggested that the stilbenes induce salicylic acid-mediated resistance. Thus, the identified substances of *Rheum* roots represent an excellent source of antifungal agents that can be used in horticulture and agriculture.

Keywords: cereals and grains, cultural and biological practices, disease management, antifungal compound, flavonoid, phytopathogenic fungi, plant defense, powdery mildew, *Rheum*, salicylic acid, stilbene.

An increasing demand for environmentally and socioeconomically acceptable alternatives to chemical fungicides has intensified research into new bio-based strategies in crop protection (Cáceres et al. 1991; Yoon et al. 2013). The need for new and innovative plant protection products is mainly driven by resistance management and legal registration. The fungicide market is dominated by compound classes with only a few distinct modes of action. Accordingly, about 70% of the global fungicide market is dominated by fungicides with a high to medium resistance risk (Leadbeater 2015). Phytopathogenic fungi are able to adapt to fungicidal modes of action through mutation, leading to a loss of fungicide efficacy (Aznar et al. 2015; Hahn 2014; Hollomon 2015). In addition to target site resistance (Kim et al. 2003; Lesemann et al. 2006; Sierotzki et al. 2000), pathogens have also acquired resistance mechanisms based on drug efflux transport and other detoxification methods (Ammar et al. 2013; Pedras and Ahiachonu 2005; Rupp et al. 2017; Yang et al. 2018). Furthermore, the number of synthetic fungicides available on the market is decreasing, because many cannot be reregistered due to changing crop protection legislation (Chapman 2014; Deising et al. 2017; Thompson et al. 2008).

In order to control economically important plant pathogens, alternatives have been developed based on various plant species that harbor diverging plant protective ingredients (Chen and Dai 2014;

Copping and Menn 2000). Some of these act via the natural plant defense system (i.e., systemic acquired resistance [SAR]). Mechanisms that activate SAR were examined in detail at the biochemical and molecular levels in *Cucumis sativus* (cucumber), *Nicotiana tabacum* (tobacco), and *Arabidopsis thaliana* (Lawton et al. 1993; Shine et al. 2019; Ward et al. 1991). SAR-related host defense responses included transcriptional induction of genes that encode pathogenesis-related (PR) proteins, formation of reactive oxygen species, and callose deposition to strengthen the cell wall (Conrath 2006; Schneider et al. 1996). The importance of novel mode-of-action chemicals capable of activating SAR for unique crop protection strategies has been fully acknowledged (Dong 1998; Gozzo and Faoro 2013; Ryals et al. 1996), and a considerable number of natural and synthetic substances have been evaluated with regard to their ability to induce resistance in plants (Kessmann et al. 1994). Surprisingly, even fungicides act as priming agents and, thus, exhibit direct and indirect modes of protecting plants against diseases. For instance, a strobilurin fungicide promotes resistance of tobacco against *Tobacco mosaic virus* and *Pseudomonas syringae* pv. *tabaci* by priming the plants for faster accumulation of PR-1 proteins (Herms et al. 2002). Furthermore, synthetic benzothiadiazole (BTH) shows SAR-inducing effects in powdery mildew-infected *Triticum aestivum* (wheat) (Görlach et al. 1996). Likewise, chemical derivatives mimicking the resistance-inducing activity of salicylic acid (SA), such as 2,6-dichloroisonicotinic acid, have also been shown to induce SAR (Kauss et al. 1992; Malamy and Kleissig 1992; Morris et al. 1998). Protective plant activator Bion has been introduced to the market as an efficient alternative to conventional agents and is well tolerated by plants. However, farmers prefer reliable curative commercial fungicides and the Bion agent BTH has not been widely used (Conrath et al. 2015). Therefore, the main task for successful antifungal strategies is to develop substances with a low risk of resistance (i.e., direct antifungal activity and priming capacity), which combine sustainability, broad-spectrum efficacy, and low toxicity in humans, animals, host plants, and ecosystems (Beckers and Conrath 2007; Chollet et al. 2014).

For example, root extracts of *Rumex crispus* reduce disease symptoms of powdery mildews in *Hordeum vulgare* (barley) and

[†]Corresponding author: M. Gillmeister; E-mail: marit.gillmeister@hs-anhalt.de

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cucumber as effectively as the synthetic fungicide fenarimol (Kim et al. 2004). Substances isolated from *R. crispus* also show fungicidal activity against *Blumeria graminis* f. sp. *hordei* and have been identified as chrysophanol, paretin, and nepodin (Choi et al. 2004). A leaf extract from *Reynoutria sachalinensis* has been commercialized (Milsana) due to its effectiveness in combating powdery mildew in cucumber. Intriguingly, the latter studies have shown that the active ingredients in *R. sachalinensis* (mainly anthraquinone physcion) do not primarily exhibit antifungal properties but, rather, induce resistance pathways in plants (Herger and Klingauf 1990; Konstantinidou-Doltsinis and Schmitt 1998).

Due to their large quantities of complex polyphenolic substances, the roots of approximately 60 species of the genus *Rheum* (rhubarb) may prove to be a promising source of novel plant protection compounds (Kuhl and DeBoer 2008). Bioactive phenolic compounds have been identified in *Rheum* roots, including stilbenes, flavonoid-type catechins, and anthraquinones (Ye et al. 2007). Flavonoids and related polyphenols are known to inhibit spore germination of fungal phytopathogens, as exhibited in the isoflavonoid phytoalexin maackiain in *Cicer bijugum* (wild chickpea), by interfering with conidial germination of *Botrytis cinerea* (Harborne and Williams 2000; Stevenson and Haware 1999). The stilbene phytoalexin resveratrol (RES) also stimulates plant defense responses (Adrian et al. 1997; Gonzalez Ureña et al. 2003; Langcake and Pryce 1976; Pezet et al. 2004a,b; Pocięcha et al. 2014). Increased resistance to *B. cinerea* in vivo, mediated by fungitoxic RES, was due to stilbene synthase genes transferred to tobacco for phytoalexin biosynthesis (Hain et al. 1993). Stilbenes have also been generally shown to inhibit spore germination and hyphal growth (Kumar and Nambisan 2014). Extracts of anthraquinones from plant species such as *Rheum emodi* or *R. officinale*, including physcion or rhein, have been described as potent antifungal agents (Agarwal et al. 2000; Yang et al. 2009); however they may no longer be used as commercial pesticides in the European Union (EU; 2008/986/EC).

The aim of the current study was to investigate the impact of anthraquinone-depleted root extracts from *R. rhubarbarum* against fungal and oomycete plant diseases, with a strong focus on biotrophic *B. graminis* f. sp. *hordei*. The assays ranged from mycelial growth tests to large-scale field trials, combined with greenhouse studies on the antifungal and resistance-inducing properties of the obtained root extracts. The chemical characterization of *Rheum* bioactive secondary metabolites (i.e., flavan-3-ols and stilbenes) found epicatechin gallate (ECG), RES, and other related compounds to be highly efficient in controlling plant diseases. They featured direct antifungal properties and were found to induce immune responses in host plants.

Materials and Methods

Extraction and fractionation of *Rheum* root compounds. *R. rhubarbarum* L. 'The Sutton' (Rrha_E) was cultivated at Anhalt University in Bernburg-Strenzfeld, Germany (51°82' N, 11°70' E, 80 m above sea level) on a loess chernozem (pH 7.0 to 7.4), and harvested after about 7 years of cultivation. After undergoing cleaning and drying (60°C), its roots were extracted using a multistep laboratory method consisting of solid/liquid and liquid/liquid extraction with different polar solvents (methanol and ethyl acetate) followed by lyophilization (Baltrusch et al. 2013). Unformulated polyphenol-enriched ethyl acetate phases and, for field trials, a 30% (wt/vol) formulated ethyl acetate phase of Rrha_E (HELM AG, Hamburg, Germany) were used in further investigations. Crude extracts from *R. officinale* BAILL. (Roff_{E39}/Roff_{E18}; accession number RHEUM 39/18; IPK, Gatersleben, Germany) and one from *Rumex obtusifolius* L. (RUo_E; accession number RUM 48; IPK) were obtained accordingly (Supplementary Table S1). Roff_{E39}, Roff_{E18}, and RUo_E were applied in mycelial growth tests and barley leaf segment assays (LSAs). Aliquots of the four mixed phases (Rrha_E, Roff_{E39}, Roff_{E18}, and RUo_E) were used for liquid chromatography mass spectrometry (LC-MS) analysis and for testing.

Fractionation of the Rrha_E mixed phase (crude extract) was performed using a preparative-scale high-performance liquid chromatography (HPLC) system (Varian Pro/PrepStar; Agilent

Technologies, Waldbronn, Germany), equipped with ZORBAX Eclipse XDB-C18 (30.0 by 250 mm, 5 µm) and Prep Guard-C18 (21.2 mm, 10 µm) columns. The mobile phase consisted of 0.5% (vol/vol) acetic acid and methanol (gradient elution of 23 to 60% methanol for the first 40 min, then rising to 100% for 7 min, and held at 100% methanol for another 8 min). Run time was 65 min at a flow rate of 34 ml min⁻¹, 30°C, and 280 nm detection wavelength (injection volume ≥ 7.5 ml). Per fraction, volumes of 54 identical HPLC runs were collected, mixed, evaporated, and freeze dried. Aliquots of the Rrha_E fractions were used for LC-MS analysis and for testing.

All freeze-dried extracts and fractions that were used in subsequent experiments were dissolved in distilled water prior to use. Extracts are, in principle, available upon request but, due to the laboratory production methods, a case-by-case decision has to be made for specific requests.

Plant material, phytopathogens, and growth conditions. Rrha_E was tested with two in vivo model systems including biotrophic plant–fungus pathosystems: (i) *H. vulgare* L. 'Lawina'–*Blumeria graminis* (DC.) E. O. Speer f. sp. *hordei* and (ii) *A. thaliana* (L.) Heynh. acc. Col-8 or deficiency mutants–*Erysiphe cruciferarum* Opiz ex L. Junell. *Arabidopsis* mutants *eds5-1*, *eds8*, *ein2-1*, *jar1-1*, *MYB51*, *npr1-1*, *npr1-3*, *pad2-1*, and *pad4-1* were used for mutant screening. The barley plants used in the LSAs and histochemical studies were cultivated in a greenhouse in type T Fruhstorfer potting soil (Hawita-Group, Vechta, Germany) for 2 to 3 weeks at 19.5°C and 51.8% relative humidity. *Arabidopsis* plants, used in the mutant screening and transcript abundance analyses, were grown in a climate chamber for 8 weeks under a light regime of 12 h of light and 12 h of darkness with 2,080 ± 6% lux light intensity at 20°C and 81% relative humidity. *B. graminis* f. sp. *hordei* and *E. cruciferarum* isolates were maintained in climate chambers (light regime of 12 h of light and 12 h of darkness with 7,000 ± 6 and 34,450 ± 6% lux light intensity, 20 and 18°C, and 75 to 82% relative humidity) by infecting plants every 1 to 2 weeks.

In vitro mycelial growth tests were conducted with necrotrophic and hemibiotrophic fungi and oomycetes. *Alternaria brassicicola* (Schwein.) Wiltshire, *Fusarium culmorum* (Wm. G. Sm.) Sacc., *F. oxysporum* f. sp. *lycopersici* W. C. Snyder & H. N. Hansen, and *Magnaporthe oryzae* B.C. Couch were cultivated on potato dextrose medium; *Phytophthora capsici* Leonian isolates (LT15341, LT1232, and 2131103) on V8 juice medium; and *Venturia inaequalis* (Cooke) G. Winter on a malt extract glucose medium at 22°C for 1 to 3 weeks. Field trials were performed with the winter wheat (*T. aestivum* L.) cultivars Pamier and Julie E, grown on an experimental field site in Bernburg-Strenzfeld, Germany (annual average temperature of 9.4 to 11.0°C, average relative humidity of 76 to 80%). The trial was repeated in two seasons (2015 and 2017). Plant material and phytopathogens used for in vitro tests, in vivo assays, and field trials are listed in Supplementary Table S2.

Plant treatments. Leaf treatments for in vivo assays were conducted by spraying plants with a membrane-vacuum pump with glass sprayer (model N022 AN.18; KNF Neuberger GmbH, Freiburg, Germany and Hofmann Glastechnik GmbH, Staudt, Germany) using 1 bar of pressure 48 h before pathogen inoculation. Each treatment group was sprayed with the same spray volume. All control plants used in the in vivo assays were treated with distilled water. The barley plants used in the LSAs and histochemical studies were treated with concentrations ranging from 8 to 500 parts per million (ppm; distilled water-based solutions). *Arabidopsis* plants used in the mutant screening and transcript abundance analyses were treated with extract concentrations of 0.5% (wt/vol) and 0.1% (wt/vol), respectively. Wheat plants intended for the field trials were treated with extract or fungicide dosages of up to 800 g ha⁻¹. Fungicides used in the field trials were purchased from BASF SE, Ludwigshafen, Germany. For controls, an equal volume of the plant extract solvent (distilled water) was sprayed. BREAK-THRU S 240 (B-T S 240, polyether trisiloxane; Evonik Industries AG, Essen, Germany) was deployed as a 0.1% (vol/vol) adjuvant for leaf treatments with extracts, fractions, or standard substances and treatments with water (controls). Plants for transcript abundance analyses were not treated with adjuvants. In field trials, B-T S 240 was used in dosages of 300 ml ha⁻¹. Two

treatments were applied with flat spray nozzles at developmental stages DS 33-37 (flag leaf) and DS 51-61 (ear emergence) in randomized field plots. Dip inoculation of leaves with fungal conidia for barley and *Arabidopsis* LSAs (Hückelhoven et al. 1999; Johansson et al. 2014) led to an average of 1.1 and 1.2 conidia per mm² leaf area respectively, while a higher conidial density (3.4 conidia per mm²) was applied on leaves used in the quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analyses.

LSAs, histochemical studies, mycelial growth tests, and field trials. The barley LSAs were evaluated by visually estimating the number of *B. graminis* f. sp. *hordei* pustules on 7-cm-long leaf segments ($\geq$ two biological and $\geq$ nine technical replicates). Synergistic effects in barley LSAs were calculated according to Colby (1967). LSAs for *Arabidopsis* (mutant screening) were evaluated by visually estimating the infestation intensity ($\geq$ 3 biological and 16 technical replicates). Disease severity was recorded in percentage of mycelium-covered leaf surface for both assays.

For the histochemical observations, infected leaves were stained with 3,3'-diaminobenzidine or acetic ink as described by Hückelhoven and Kogel (1998) and Hückelhoven et al. (1999). Conidial germination rates and interaction phenotypes were recorded according to Koga et al. (1990) by evaluating 100 interaction types per leaf segment (two biological and five technical replicates).

In vitro mycelial growth tests were performed by incorporating the extract solutions into the respective nutrient medium (1,000 ppm final extract concentration). For controls, an equal volume of the plant extract solvent (distilled water) was added to the nutrient medium. Test plates were inoculated with a mycelium agar plug measuring 0.28 cm² by placing the mycelial disk centrally on the plate. After incubation at 22°C for 1 to 3 weeks, the diameter of the grown mycelium was measured crosswise and compared with the control without the extract addition ($\geq$ three biological or technical replicates).

For field trials, the experimental field represented a randomized plot design with four replicates per treatment group of about 10 m² each (seed rate: approximately 350 seeds/m²). The severity of the infection with brown rust was evaluated by sampling a representative number of flag leaves ($\geq$ 150 technical replicates) from several locations inside the test field. Disease symptoms were examined using scales ranging from 0 to 100% (pustules per leaf area).

DNA-microarray and qRT-PCR analyses. For each treatment, three *Arabidopsis* leaves were sampled from three individual plants at 3 and 9 h after treatment (HAT) for the DNA-microarray analyses, and at 3 and 9 HAT or 8 and 16 h after inoculation (HAI) for the qRT-PCR analyses (two independent tests each). DNA-microarray experiments were performed using two biological and one technical replicates (mixed leaf sample). qRT-PCR experiments were performed using two biological and $\geq$ three to five technical replicates. mRNA isolation (RNeasy Plant Mini Kit; QIAGEN GmbH, Hilden, Germany) and cDNA synthesis (SuperScript First-Strand Synthesis System; Life Technologies GmbH, Darmstadt, Germany) were conducted in accordance with the manufacturer's instructions. Microarray analyses were performed using a processing kit (Affymetrix GeneChip [AraGene 1.0 ST Array]; Ambion WT Expression Kit; Thermo Fisher Scientific, Darmstadt, Germany). Sample processing and Affymetrix microarray hybridization were carried out at a genomics core facility: Center of Excellence for Fluorescent Bioanalytics (KFB, University of Regensburg, Germany). The qRT-PCR analyses focused on *PR1*, *PR2*, and *PR5* using a QuantiTect SYBR Green PCR Kit (QIAGEN GmbH) and primers specific to the investigated *PR* genes (primer pairs *At-PR1*, *At-PR2*, and *At-PR5*) (Lindermayr et al. 2010). Ubiquitin and actin were examined for suitability as reference genes. *At-Actin* (primer pair *At-Act1*) (Hermann et al. 2013) repeatedly failed to provide reproducible cycle threshold (Ct) values with a high deviation over 0.5 Ct. Therefore, the reference gene *At-Ubiquitin* (primer pair *At-Ubi*) (Shi et al. 2013) was used for normalization. Primers were synthesized by Metabion International AG, Planegg/Steinkirchen, Germany. Relative transcript abundances were quantified using the $\Delta\Delta$ Ct method (Livak and Schmittgen 2001).

High-performance LC-MS analyses. Three methods were applied to qualitatively and quantitatively evaluate extract ingredients:

(i) the LC-diode array detection (DAD) method (peak area determination), (ii) the full-scan method for substance identification (semi-quantification), and (iii) the quantification method using multiple reaction monitoring, information-dependent acquisition, enhanced product ion (MRM-IDA-EPI) and library matching.

The HPLC method was performed using a Synergi Hydro-RP 80 Å column (250 by 2.0 mm, 4 μ m; Phenomenex Ltd., Aschaffenburg, Germany) with 0.1% (vol/vol) formic acid in water and 100% methanol as mobile phase (gradient elution from 0 to 10% methanol for the first 5 min, then increasing to 100% after 57.6 min, and held at 100% methanol for another 7.4 min), 80.1 min total run time, 0.25 ml min⁻¹ flow rate, 190 to 950 nm DAD detection spectrum, and 10 μ l injection volume, with 4 and 40°C auto-sampler and column temperature, respectively. The combined full-scan method (quadrupole Q1 scan method) was performed using a 4000 QTRAP mass spectrometer from Sciex GmbH, Darmstadt, Germany, which provided a total ion current (source: electrospray ionization, polarity: negative, duration: 80 min, full-scan range: 100 to 1,300 Da, time per cycle: 3 s).

The MRM experiment was used in the quantitative analysis, which also served as a survey scan for the EPI. External calibration was obtained by applying 16 standard substances typical for *Rheum* spp. at concentration levels ranging from 5 to 1,000 ng ml⁻¹, with correlation coefficients $r^2 > 0.99$. The five flavan-3-ols—(+)-catechin, (–)-epicatechin, (–)-epigallocatechin, ECG, and (–)-epigallocatechin gallate—as well as three procyanidin standards (procyanidin B1, B2, and C1) and the standard substances 3,4',5-trihydroxystilbene-3-O- β -D-glucoside/-glucopyranoside (piceid), rhaponticin, and RES were purchased from Extrasynthese, Genay, France. The standard substance piceatannol was purchased from BIOTREND Chemikalien GmbH, Wangen, Switzerland; desoxyrhaponticin from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany; and three phenolic acids (p-coumaric acid, ferulic acid, and chlorogenic acid) from Carl Roth GmbH + Co. KG, Karlsruhe, Germany. The EPI scan (scan rate: 1,000 Da s⁻¹) used for substance confirmation was programmed and added through IDA criteria with dynamic background subtraction of the survey scan. Samples were measured in duplicate.

Statistical analyses. Statistical analyses were conducted using SigmaPlot (version 13.0; Systat Software, Inc., San José, CA, U.S.A.) to determine variances between different treatment groups (one-way analysis of variance and Dunnett's and Dunn's methods). Whereas the Dunnett's test was used for comparisons between experimental treatment groups and a single control group, the Dunn's test served as the statistical method for pair-by-pair comparisons and comparisons versus a control group. The differences in the mean and median values of $P < 0.05$ between treatment groups were set as the significance threshold. The t test (Mann-Whitney rank sum test) was applied to comparisons comprising only two groups. Technical and biological replicates varied depending on the experimental design (see Materials and Methods).

Results

***R. rhubarbarum* extract Rrha_E inhibits mycelial growth of plant-pathogenic fungi and oomycetes.** Infection assays were used to analyze the effect of *Rheum* root extracts in controlling plant diseases. Barley leaves were treated with extracts from *R. rhubarbarum* (Rrha_E), *R. officinale* (Roff_E), and *R. obtusifolius* (RUo_E), dissolved in 0.1% B-T S 240, along with 0.1% B-T S 240 water controls. For LSAs, the pretreated leaves were excised, inoculated with *B. graminis* f. sp. *hordei* at about 1.1 conidia/mm², and incubated until approximately 90% of the leaf surface of the control plants was covered with mycelium. The inhibitory effect of all extracts on *B. graminis* f. sp. *hordei* was macroscopically evident and statistically significant (Fig. 1A and B). Intriguingly, at dosages of 125 and 250 ppm, Rrha_E completely inhibited fungal growth (Dunn's method versus control group, $P < 0.001$). Roff_E and RUo_E reduced *B. graminis* f. sp. *hordei* disease severity in a statistically significant dose-dependent manner by an average of 15 to 5% and 4 to 1%, respectively (Fig. 1A).

The inhibition of the *B. graminis* f. sp. *hordei* infection by Rrha_E in LSAs was analyzed in more detail through microscopy by using

Fig. 1. Antifungal effects of *Rheum* and *Rumex* root extracts. **A** and **B**, *Blumeria graminis* f. sp. *hordei* (*Bgh*) pustule formation (pustules/leaf area) 2 weeks after *Hordeum vulgare* 'Lawina' infection. Three-week-old plants were sprayed with different *Rheum* (*Rheum officinale* [Roff_E]) and *R. rhabarbarum* [Rrha_E] and *Rumex* (*Rumex obtusifolius* [RUo_E]) root extracts followed by inoculation with *B. graminis* f. sp. *hordei* at 48 h after treatment (HAT). For controls, an equal volume of the solvent was sprayed. Significant differences in disease severity assessed using Dunn's method versus control group ($P < 0.001$) are marked with an asterisk (A). Graph bars represent mean values of ≥ 2 biological and 15 technical replicates, error bars represent standard error of the mean (SEM). Images show representative symptom developments (B). **C**, Conidial germination rate and **D**, haustoria formation rate of *B. graminis* f. sp. *hordei* 2 days after *H. vulgare* 'Lawina' infection. Two-week-old plants were sprayed with Rrha_E followed by inoculation with *B. graminis* f. sp. *hordei* at 48 HAT. For controls, an equal volume of the solvent was sprayed. Significant differences in *B. graminis* f. sp. *hordei* germination rate assessed using Dunnett's method ($P \leq 0.009$) and haustoria rate using *t* test ($P \leq 0.031$) are marked with an asterisk. Graph bars represent mean values of two biological and five technical replicates; error bars represent SEM. **E**, Mycelial growth rates of hemibiotrophic and necrotrophic fungi and oomycetes after 1 week of incubation. Images show mycelial growth on solvent-supplemented (control) and Rrha_E-supplemented potato dextrose (*Alternaria brassicicola*, *Fusarium* spp., and *Magnaporthe oryzae*), V8 juice (*Phytophthora capsici*), or malt extract glucose (*Venturia inaequalis*) media. Graph values were measured as average diameter (in centimeters) and given as relative growth versus control (percentage). Growth rates of *M. oryzae* and *V. inaequalis* were determined after 2 and 3 weeks, respectively. Plant pathogens were grown on nutrient agar supplemented with the extracts as described (1,000 ppm). For controls, an equal volume of the solvent was added to the nutrient medium. Significant differences in mycelial growth assessed using Dunnett's method or Dunn's method versus control group ($P \leq 0.032$) are marked with an asterisk. Graph bars represent mean values of ≥ 3 biological or technical replicates, error bars represent SEM.

highly susceptible Lawina barley. In concordance with the extract treatments, the number of conidia that formed appressoria was significantly lower (Dunn's method, $P \leq 0.009$) depending on the dosage (Fig. 1C). Correspondingly, haustoria were formed at significantly lower rates (t test, $P \leq 0.031$) compared with the water-treated controls (Fig. 1D).

It is conceivable that assays using obligate biotrophs are unable to differentiate between direct antifungal activity and resistance-inducing effects in the host plant. Therefore, only the effects of the extracts on mycelial growth of economically relevant hemibiotrophs and necrotrophs could be examined in axenic cultures. Whereas *A. brassicicola*, which causes leaf spots, and the three *P. capsici* isolates exhibited retarded growth, with growth rates of 18 to 35% and 12 to 40%, respectively, compared with the water controls, the hemibiotrophic rice blast fungus *M. oryzae* showed a significant reduction in growth (26 to 29% relative growth versus control) when treated with Rrha_E and Roff_{E39} (Fig. 1E). Due to the Rrha_E treatment, the apple scab fungus *V. inaequalis* exhibited significantly reduced growth rates, with inhibition of up to 100% (Dunn's method, $P < 0.001$). Rrha_E was also effective against *F. culmorum* and *F. oxysporum* f. sp. *lycopersici*, even at lower dosages of 250 to 63 ppm (growth rates of 34 and 41%, respectively, versus control; data not shown). Although Rrha_E and Roff_{E39} provided moderate to complete inhibition of all tested plant pathogens, Roff_{E18} and RU_O_E were effective in only two of eight isolates with reduced impact (30 to 50% relative growth versus control; Fig. 1E).

Stilbenes are the major compounds in the *R. rhubarbarum* extract Rrha_E. The chemical composition of the extracts was determined by LC-MS. Rrha_E was primarily composed of the stilbene derivatives rhaponticin (RHA), desoxyrhaponticin (DRHA), and RES (Fig. 2A). Minor components were the flavonoids catechin, ECG, and procyanidin B1 (PRO B1) (Fig. 2B). These compounds were purified and concentrated as defined fractions using preparative HPLC. RES was highly concentrated in fraction 5 (Fig. 2C and E), while RHA appeared in fraction 4 and DRHA in fraction 7 (Fig. 2C and F). ECG was primarily found in fractions 2 to 4 (Fig. 2D and G). Other stilbenes such as piceatannol glucopyranoside and rhapontigenin in Rrha_E are presented in Supplementary Fig. S1. Compared with the other crude extracts (Supplementary Fig. S2), the quantitative distribution of compounds identified in Rrha_E was similar, with only minor differences from Roff_{E39}. In contrast to Rrha_E and Roff_{E39}, the composition of ingredients in Roff_{E18} and RU_O_E differed remarkably. Large amounts of catechin (approximately 25-fold) and an enrichment of ECG (8-fold) were found in these extracts. Additionally, the content of PRO B1 was seven to eight times higher in Roff_{E18} and RU_O_E than in the other analyzed extracts.

Polyphenolic compounds from stilbenes and flavonoids mediate *B. graminis* f. sp. *hordei* control. To determine the active components in Rrha_E that suppress *B. graminis* f. sp. *hordei* infection, barley LSAs were conducted with *B. graminis* f. sp. *hordei* and distinct fractions of Rrha_E. Flavonoids from fractions 1 and 2 as well as stilbenes from fraction 5 showed strong activity in reducing mildew disease, with only a 2 to 5% disease severity compared with the water-treated controls (Fig. 3A). Other fractions exhibited moderate to low activity (Fig. 3A). Fractions 2 and 5, as well as the major flavan-3-ol and stilbene in these fractions (i.e., ECG and RES by the use of standard substances), significantly reduced conidial germination rates of *B. graminis* f. sp. *hordei* (Dunn's method, $P \leq 0.020$; Fig. 3B). Haustoria formation was also significantly inhibited in leaf segments (t test versus control, $P = 0.011$ or 0.031) treated with fraction 5 (containing the main compound RES) and pure standard ECG (Fig. 3C).

To investigate possible synergistic effects, barley LSAs were performed with commercially available ECG and RES as well as PRO B2 and RHA, and protectively applied individually and in combination. ECG and RES application showed significantly less disease than control application when applied in combination (100% *B. graminis* f. sp. *hordei* efficiency) (Fig. 3D). *B. graminis* f. sp. *hordei* symptom development was not significantly different from control infection (Dunn's method versus control group, $P \geq 0.142$) in the

case of single-dose applications of 8 ppm. Combined treatments resulted in significant (Dunn's method versus control group, $P < 0.001$) and considerably better mildew control than the single applications (Fig. 3D). These inhibitory effects were not additional but synergistic (100% observed versus 70% calculated *B. graminis* f. sp. *hordei* efficiency with the use of 8 + 8 ppm). Similar effects were observed when plants were pretreated with PRO B2 and RHA (Fig. 3E).

***R. rhubarbarum* extract Rrha_E exhibited strong efficacy in field tests.** Due to inhibition of spore germination and mycelial growth after Rrha_E application and because of its plant-protecting potential, Rrha_E was applied in a field trial with Pamier winter wheat in 2015. Flag leaves of 700 individual plants per treatment group were rated for disease incidence. Field sampling revealed severe leaf rust (*Puccinia triticina*) disease pressure. When formulated Rrha_E (Rrha_{Ef}) and fenpropimorph (Corbel) were used (Fig. 4A), average growth rates of brown rust pustules converged depending on Rrha_{Ef} dosage, with disease severities of 18 to 13% (Dunn's method, $P < 0.001$; 200 versus 800 g ha⁻¹; t test, $P < 0.001$). Corbel reduced brown rust infestation by an average of 76% (Dunn's method, $P < 0.001$). Approximately 94% pustule inhibition was achieved through combined treatments with Rrha_{Ef} and Corbel (Dunn's method, $P < 0.001$). The field experiment was independently repeated with Julie E winter wheat in 2017 at the same experimental site (Fig. 4B), yielding similar results (Dunn's method, $P \leq 0.028$). In 2017, the disease pressure differed (2015 versus 2017: Dunn's method, $P \leq 0.033$). However, Rrha_{Ef} significantly reduced brown rust symptoms in flag leaves (Dunn's method, $P \leq 0.028$), especially with the higher dosage of 800 g ha⁻¹ (approximately 20% disease severity). Although fenpropimorph at 94 g ha⁻¹ achieved less control of brown rust (an average of 33% inhibition), combined treatments with Rrha_{Ef} and Corbel inhibited the emergence of brown rust by an average of 70%. Through combined applications, significantly increased brown rust effects could be demonstrated (Corbel or Rrha_{Ef} versus Corbel + Rrha_{Ef}; Dunn's method, $P < 0.001$), even at lower fungicide dosages.

***R. rhubarbarum* extract Rrha_E induces resistance mechanisms in *Arabidopsis*.** In order to discriminate between direct antifungal and local acquired resistance- or SAR-inducing effects of Rrha_E, an *Arabidopsis* mutant screening was performed with mutants that were deficient in accumulating or responding to key regulators within induced resistance (IR) signaling. These plants were inoculated with *E. cruciferarum* at 1.2 spores/mm². By studying the efficacy of 0.5% Rrha_E in protecting *Arabidopsis* from disease, indications for appropriately activated host resistance pathways were detected in the case of the SA-impaired mutant *eds5-1*. The disease severity of Rrha_E-treated *eds5-1* leaves was much higher than the level in Col-8 plants (Dunn's method, $P < 0.001$; Fig. 5A and B). This pointed to an SA-dependent type of resistance induced by Rrha_E. LSAs with Rrha_E fractions 2 and 5 (0.1%) as well as with standard compounds ECG and RES (0.1%) showed (Fig. 5A and B) that the observed IR activation is related to stilbene agents (fraction 5 or RES; 73 to 88% disease severity versus control in the case of *eds5-1*).

To survey the possibility of priming effects that are solely due to extract treatment, *Arabidopsis* Col-8 plants were sprayed with a 0.1% Rrha_E solution and sampled at 3 and 9 HAT for transcript abundance analyses using Affymetrix DNA-microarray technology and qRT-PCR. Microarray data showed various upregulated gene transcripts (up to a 16.90-fold in both tests) with a clear relationship to genes involved in pathogen-resistance mechanisms (Supplementary Table S3). Among the genes considered were those which fit categories for incompatible interaction (response to accumulation of hydrogen peroxide [oxidative burst]), signal transduction (e.g., WRKY transcription factors), mitogen-activated protein kinase cascades, the phenylpropanoid metabolism (e.g., phenylalanine ammonia lyase), as well as SAR-related genes. Upregulated transcripts involved in cell wall fortification (lignin metabolic or biosynthetic process and callose deposition) could be linked to the rate of *B. graminis* f. sp. *hordei* papillae formation as part of the histochemical studies (Fig. 6A, B, and C). The *B. graminis* f. sp. *hordei* papillae formation rate was affected significantly by Rrha_E fractions 2 and 5 due to infection

(Dunn's method, $P < 0.001$). As a result of the Rrha_E treatment (microarray analyses), up to fivefold higher transcript abundances of the *PR* genes *PR1*, *PR2*, and *PR5* were determined compared with the untreated controls. qRT-PCR analyses substantiated the microarray results and *PR* transcript abundances were also significantly increased by extract treatment (Dunn's method versus control group [0 H], $P \leq 0.022$) at 3 HAT ($\geq$ ninefold) and 9 HAT ($\geq$ fivefold) (Fig. 6D).

qRT-PCR was used to analyze *PR1*, *PR2*, and *PR5* transcript abundances after infection. For this purpose, Rrha_E-treated *Arabidopsis*

Col-8 plants were inoculated with *E. cruciferarum* at 3.4 spores/mm² and sampled at 8 and 16 HAI. qRT-PCR analyses showed overall increased transcript abundances of these markers due to the Rrha_E treatment at the defined points in time after inoculation (Fig. 6E). Compared with uninfected but pretreated controls, significant differences could be detected at 8 and 16 HAI (t test, $P \leq 0.005$). Although *PR1* and *PR2* transcript abundances decreased with time, induction of *PR5* was greatest at 16 HAI (t test versus 48 HAT, $P < 0.001$). With respect to all investigated *PR* genes, no significant differences between treatment groups could be observed at 48 HAT (t test,

Fig. 2. Liquid chromatography mass spectrometry (MS) analyses of polyphenols in *Rheum rhabarbarum* crude extract (Rrha_E) and fractions of Rrha_E. Quantification of **A**, stilbenes and **B**, procyanidins and flavan-3-ols in Rrha_E; and **C** and **D**, fractions (frc) of Rrha_E. Samples were measured in duplicate. 3,4',5-Trihydroxystilbene-3-O-β-D-glucopyranoside is abbreviated as ths-glucopyranoside. Representative MS spectra of **E**, resveratrol (RES); **F**, rhaponticin (RHA); and **G**, epicatechin gallate (ECG), identified by the main ion (marked in red) and several fragments (colored).

$P = 0.480, 0.765$, and 0.617 for $PR1$, $PR2$, and $PR5$, respectively; Fig. 6E).

Discussion

Rheum roots contain various bioactive flavonoids, stilbenes, and complex polyphenolic substances. The aim of this study was to investigate the potential of standardized *Rheum* root extracts that include these substances for controlling fungal and oomycete plant pathogens. In contrast to most studies, the investigated *Rheum* root extracts (i.e., $Rrha_E$) were examined in experiments ranging from in vitro assays to large-scale field trials using different dosages, including chemical structure elucidation and the application of isolated single compounds. The data collectively showed that polyphenols from *Rheum* roots exhibited pronounced antifungal activity. Root extracts from *Rheum* such as $Roff_{E39}$ and $Rrha_E$ clearly demonstrated protective disease-controlling effects against a wide range of necrotrophic, hemibiotrophic, and biotrophic phytopathogens that are agriculturally important. These included fungi (e.g., *Fusarium* spp. and *A. brassicicola*) and oomycetes (*P. capsici*) in vitro and, in

particular, the fungus *B. graminis* f. sp. *hordei* in vivo, which also causes major crop losses (Dean et al. 2012; Ridout 2009). The data strongly suggested that reduced nutrient uptake by haustoria from plants treated with ECG and stilbenes may be responsible for the reduction in incidences of mildew disease. $Rrha_E$, which was most effective against *B. graminis* f. sp. *hordei* conidial germination as well as haustoria and pustule formation, also exhibited positive effects against the apple scab pathogen *V. inaequalis*, which can nowadays only be effectively controlled by high-efficacy fungicides (Cox 2015). The current study revealed substantially reduced disease severities in barley leaves infected with *B. graminis* f. sp. *hordei* as well as complete growth inhibition of *V. inaequalis* sustainable in vitro. Throughout Europe, the majority of apple cultivars is susceptible to scab and powdery mildew (*Podosphaera leucotricha*), which requires intensive fungicide applications that are known to have a negative ecological impact (Lespinasse et al. 2000) such as adverse effects to beneficial organisms (e.g., pollinators). Rice, on the other hand, is one of the fifth-largest cash crops and feeds one-third of the human population (Head 2017). In view of the strong effects

Fig. 3. Protective and synergistic effects of $Rrha_E$ fractions and compounds typical for *Rheum*. **A**, *Blumeria graminis* f. sp. *hordei* (*Bgh*) pustule formation (pustules/leaf area) 2 weeks after *Hordeum vulgare* 'Lawina' infection. Three-week-old plants were sprayed with 250 ppm concentrated fractions (frc) of *Rheum rhabarbarum* extract ($Rrha_E$) followed by inoculation with *B. graminis* f. sp. *hordei* at 48 h after treatment (HAT). For controls, an equal volume of the solvent was sprayed. Significant differences in disease severity assessed using Dunnett's method ($P \leq 0.003$) are marked with an asterisk. Graph bars represent mean values of 2 biological and 14 technical replicates; error bars represent standard deviation (SD). **B**, Conidial germination rate and **C**, haustoria formation rate of *B. graminis* f. sp. *hordei* 2 days after *H. vulgare* 'Lawina' infection. Two-week-old plants were sprayed with 250 ppm $Rrha_E$ fractions (frc) 2 and 5 and 125 ppm of the standard compounds epicatechin gallate (ECG) and resveratrol (RES), followed by inoculation with *B. graminis* f. sp. *hordei* at 48 HAT. For controls, an equal volume of the solvent was sprayed. Significant differences in *B. graminis* f. sp. *hordei* germination rate assessed using Dunnett's method ($P \leq 0.020$) and haustoria rate using *t* test ($P \leq 0.031$) are marked with an asterisk. Graph bars represent mean values of two biological and five technical replicates; error bars represent standard error of the mean. **D** and **E**, *B. graminis* f. sp. *hordei* pustule formation (pustules/leaf area) 3 weeks after *H. vulgare* 'Lawina' infection. Three-week-old plants were sprayed with the standard substances ECG and RES (**D**) as well as procyanidin B2 (PRO B2) and rhaponticin (RHA) (**E**) followed by inoculation with *B. graminis* f. sp. *hordei* at 48 HAT. For controls, an equal volume of the solvent was sprayed. Significant differences in disease severity assessed using Dunn's method versus control group ($P \leq 0.002$) are marked with an asterisk. Graph bars represent mean values of two biological and ≥ 9 technical replicates; error bars represent SD. Representative box values demonstrate synergisms obtained by comparing observed (e_o) versus calculated (e_c) efficiencies against *B. graminis* f. sp. *hordei*.

against the rice blast fungus *in vitro*, defined *Rheum* root extracts with well-characterized ingredients may represent a promising prospect for developing chemical leads from these botanicals in future.

The profound *in vivo* *B. graminis* f. sp. *hordei* effects of various *Rheum* root extracts had a high probability of being accompanied by synergistic interactions between minor substances such as ECG, catechin, or PRO B1 or B2 (flavonoids) and major components such as RES, DRHA, or RHA (stilbene derivatives), present in the crude extracts. The hypothesis that heterogeneous polyphenols are bioactive compounds that inhibit *B. graminis* f. sp. *hordei* pustule formation was supported by the barley LSAs with various standard substances typical for *Rheum* (Supplementary Fig. S3). Except for a comprehensive description of polyphenols typical for *Rheum* spp., such synergisms against *B. graminis* f. sp. *hordei* pustule formation have not been described before. Interestingly, the Rrha_E fractions which actively prevented *B. graminis* f. sp. *hordei* pustule formation in barley LSAs contained galloylated (ECG in fraction 2) and hydroxylated (catechin and PRO B1 in fraction 1) flavonoids as well as

hydroxylated (RES in fraction 5), glucosylated (RHA and DRHA in fractions 4 and 7; ths-glucoside in fraction 2), and galloylated (rhaponticin gallate in fraction 5) stilbenes. Flavonoid-dominated Rrha_E fraction 1 and stilbene fraction 5 were also validated by LSAs using tomato leaves infected with *Phytophthora capsici* isolate LT263. The results correlated with large amounts of galloylated (e.g., ECG) and hydroxylated compounds, especially RES in fraction 5 (unpublished). The antifungal activity of gallic acid (e.g., reduced spore germination) was already demonstrated by Dix (1979) and Nguyen et al. (2013). The same applies to the antimicrobial and resistance-inducing potentials of flavonoids (Chakraborty and Chakraborti 2010; Hirasawa and Takada 2004; Tao et al. 2010) and stilbenes in general (Hart 1981; Kumar and Nambisan 2014; Pociecha et al. 2014; Sergent et al. 2014). The results presented here demonstrate the multiconstituent-based antifungal potential of these *Rheum* root extracts, with the advantage of low risks for the development of pathogen resistance. Importantly, the content of anthraquinones in the crude extracts could be sharply reduced by using petroleum benzene

Fig. 4. Effects of *Rheum rhabarbarum* root extract in field trials. Brown rust pustule formation (pustules/leaf area) in infected plots of *Triticum aestivum* A, 'Pamier' and B, 'Julie E' (flag leaves) of a field trial in 2015 (A) and the experimental repeat in 2017 (B). Plants were sprayed with formulated *R. rhabarbarum* extract (Rrha_E) and the chemical Corbel. For controls, an equal volume of the solvent was sprayed. Significant differences in disease severity assessed using Dunnett's method ($P < 0.001$ and ≤ 0.028 for A and B, respectively) are marked with an asterisk. Brackets symbolize significant differences among the treatment groups of individual and combined applications (Dunn's method, $P < 0.001$). Graph bars represent mean values of 700 (A) and 150 (B) technical replicates per treatment group; error bars represent standard error of the mean.

Fig. 5. Effects of *Rheum rhabarbarum* crude extract and fractions in *Arabidopsis*. A, *Erysiphe cruciferarum* pustule formation (disease severity) 1 week after *Arabidopsis thaliana* Col-8 and eds5-1 infection. Eight-week-old plants were sprayed with 0.5% concentrated *R. rhabarbarum* extract (Rrha_E), 0.1% Rrha_E fractions (frc) 2 and 5, and 0.1% of the standard compounds epicatechin gallate (ECG) and resveratrol (RES) followed by inoculation with *E. cruciferarum* at 48 h after treatment (HAT). For controls, an equal volume of the solvent was sprayed. Significant differences in infestation intensity assessed using Dunn's method versus control group ($P < 0.001$) are marked with an asterisk. Bracket symbolizes the significant difference in infestation intensity among the Rrha_E treatment groups (Dunn's method, $P < 0.001$). Graph bars represent mean values of ≥ 3 biological and 16 technical replicates; error bars represent standard error of the mean. B, Images show healthy mycelial growth with dense networks of secondary hyphae in solvent-treated as well as Rrha_E-treated (eds5-1) and stilbene (frc 5 and RES)-treated (eds5-1) leaves. Less intense and nonvital fungal growth was visible in leaves treated with flavan-3-ols (frc 2 and ECG; $\times 6.3$ and $\times 16$ magnification, respectively).

in the extraction process and preparative HPLC for fractionation, because anthraquinones may no longer be used in plant protection products in the EU (notified under document number C(2008) 8133).

The antifungal effects of Rrha_E were also intense in field trials against brown rust in wheat. This could be achieved by a maximum of two sprayings per season. In addition, no phytotoxic side effects were observed for dosages up to 800 g ha⁻¹. In combined use with the chemical fungicide Corbel, the efficacy of Corbel (and Rrha_E) was significantly improved. In combination with systemically active Corbel, which accelerates the plant uptake of combined agents, Rrha_E or mixed formulations can substantially contribute to a reduced spray rate of chemicals against brown rust in wheat, increased plant protection over single compounds, and, thus, the development of fungicides with a lower resistance risk.

Arabidopsis LSAs with the SA-deficient mutant *eds5-1* (Nawrath et al. 2002) showed that the effect of Rrha_E on mildew appears to be SA dependent. SA correlates with local and systemic expression of PR protein-coding genes and is known to be the signal molecule of

SAR (Fu and Dong 2013; Gaffney et al. 1993; van Loon 1999; van Loon et al. 2006; Vlot et al. 2009). According to Chen et al. (2017), mutant *eds5-1* is SAR deficient and impaired in the accumulation of SA and *PR1*-transcripts (Ferrari et al. 2003; Lu 2009; Nawrath et al. 2002). In the current study, qRT-PCR demonstrated that there were significantly higher transcript abundances of the PR genes *PR1*, *PR2*, and *PR5* after treatment and after infection. This hints at a priming effect of Rrha_E, which was confirmed by DNA-microarray analyses displaying several upregulated SAR-related genes. IR is known to be associated with plant cell priming (Beckers et al. 2009; Kohler et al. 2002), which causes responsiveness and greater sensitivity when there is a pathogen attack (Conrath et al. 2002, 2006, 2015). The higher β -1,3-glucanase 3 gene transcription as a result of the Rrha_E treatment as well as the significant postinfection transcript abundance of *PR2* suggests that Rrha_E possibly acts as an elicitor of plant defense responses similar to β -1,3-glucan or chitin oligomers of fungal cell walls (Cosio et al. 1996; Klarzynski et al. 2000; Oliveira-Garcia and Deising 2013; Shetty

Fig. 6. Defense-related formations in barley and transcript abundances of defense-related genes in *Arabidopsis* activated by *Rheum rhabarbarum* root extract Rrha_E. **A**, Papillae formation rate of *Blumeria graminis* f. sp. *hordei* (Bgh) 2 days after *Hordeum vulgare* 'Lawina' infection. Two-week-old plants were sprayed with *R. rhabarbarum* extract (Rrha_E) fractions (frc) 2 and 5 (250 ppm) followed by inoculation with *B. graminis* f. sp. *hordei* 48 h after Lawina treatment. For controls, an equal volume of the solvent was sprayed. Significant differences in papillae appearance assessed using Dunnett's method ($P < 0.001$) are marked with an asterisk. Flavan-3-ol frc 2 and stilbene frc 5 did not differ (t test, $P = 0.381$). Graph bars represent mean values of two biological and five technical replicates; error bars represent standard error of the mean (SEM). **B** and **C**, Microscopy images show papillae in a representative leaf treated with frc 2 main compound epicatechin gallate (pure standard) 72 h after Lawina infection ($\times 400$ magnification) as a phenotypical result of activated defense-related genes (**B**) compared with the respective control (**C**). **D** and **E**, Relative transcript abundances (relative quantification) of *PR1*, *PR2*, and *PR5* 3 and 9 h after treatment (HAT) with *Arabidopsis thaliana* Col-8 (**D**) as well as 8 and 16 h after infection (HAI) of Col-8 with *Erysiphe cruciferarum* (**E**) detected by quantitative reverse-transcription polymerase chain reaction. Eight-week-old plants were sprayed with 0.1% concentrated Rrha_E followed by leaf sampling at 3 and 9 HAT (**D**) or inoculation with *E. cruciferarum* at 48 HAT and leaf sampling at 8 and 16 HAI (**E**). For controls, an equal volume of the solvent was sprayed. The reference gene *At-Ubiquitin* was used for normalization. In **D**, significant differences in transcript abundance assessed using Dunn's method versus control group (0 H; $P \leq 0.022$) are marked with an asterisk. Differences in transcript abundance versus H₂O group (control) were assessed by t test ($P \leq 0.012$). In **E**, significant differences in transcript abundance assessed using t test (48 HAT; $P \leq 0.005$) are marked with an asterisk. Differences in transcript abundance versus H₂O group (control) were assessed by t test ($P \leq 0.040$). Graph bars represent mean values of two biological and $\geq$ three technical replicates; error bars represent standard deviation (**D**) or SEM (**E**).

et al. 2009). van Loon and van Strien (1999) have reported that the expression of *PR1*, *PR2*, and *PR5* is required for defense responses against the biotrophic *Hyaloperonospora parasitica* in *Arabidopsis* and that the PR-1 family is a marker for SAR. In the current study, the polyphenolic agents of Rrha_E may induce an SA-triggered SAR in *Arabidopsis*, primarily due to stilbene agents.

Therefore, it is possible that the protective effects of Rrha_E depend not only on its direct antifungal activity but also on its induction of SAR. According to Conrath et al. (2015), various natural and synthetic substances can be used in chemical defense priming, some of which are included in agricultural plant protection products (e.g., SA or BTH). However, several crops display a reduced tolerance to SA, which is economically disadvantageous (Ryals et al. 1996). The application of Rrha_E might close this gap because tolerance reduction has yet to be observed. In view of the disadvantages of pure plant activators (plant energy expenditure) or curative fungicides (risk of resistance), polyphenols from the *Rheum* spp. could be a valuable alternative in crop protection against plant-pathogenic fungi and oomycetes. Our future goals include collaborations with crop science companies to economically exploit the new extraction technology and antifungal formulations that include synergistically acting ingredients and innovative adjuvants.

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