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Terpenoid and lipid profiles vary in different *Phytophthora cactorum* – strawberry interactions

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ABSTRACT

Specialized metabolites are essential components in plant defence systems, serving as signalling molecules and chemical weapons against pathogens. The manipulation of plant defence metabolome or metabolites can thus be an important virulence strategy for pathogens. Because of their central role, metabolites can give valuable insights into plant-pathogen interactions. Here, we have conducted nontargeted metabolite profiling with UPLC-ESI-qTOF-MS to investigate the metabolic changes that have taken place in the crown tissue of *Fragaria vesca* L. (woodland strawberry) and *Fragaria* × *ananassa* (Weston) Duchesne ex Rozier (garden strawberry) during 48 h after *Phytophthora cactorum* challenge. Two *P. cactorum* isolates were compared: Pc407 is highly virulent to *F.* × *ananassa* and causes crown rot, whereas Pc440 is mildly virulent. In total, 45 metabolites differentially accumulated between the treatment groups were tentatively identified. Triterpenoids and various lipid compounds were highly represented. The levels of several triterpenoids increased upon inoculation, some of them showing distinct accumulation patterns in different interactions. Triterpenoids could either inhibit or stimulate *P. cactorum* growth and, therefore, triterpenoid profiles might have significant impact on disease progression. Of the lipid compounds, lysophospholipids, linoleic acid and linolenic acid were highly accumulated in the most compatible Pc407 - *F.* × *ananassa* interaction. As lysophospholipids promote cell death and have been linked to susceptibility, these compounds might be involved in the pathogenesis of crown rot disease. This metabolite analysis revealed potential factors contributing to the outcome of *P. cactorum* – strawberry interactions. The information is highly valuable, as it can help to find new breeding strategies and new solutions to control *P. cactorum* in strawberry.

1. Introduction

Specialized metabolites play a central role in the interactions between plants and pathogens. As a response to pathogen attack, extensive changes are triggered in plant metabolism. Carbon resources are redirected from growth and development to defence, and a great diversity of specialized metabolites is produced (Caretto et al., 2015). These metabolites serve as signalling molecules, chemical weapons against pathogens, or both (Maag et al., 2015). Antimicrobial compounds can be divided in two categories: 1) phytoalexins, which are synthesized *de novo* after pathogen attack, and 2) phytoanticipins, which are constitutively produced in plant tissues (Piasecka et al., 2015). Phytoanticipins are often stored as inactive, e.g. glycosylated, precursors but are processed to biologically active forms upon pathogen challenge (Morant et al., 2008). All plant species and cultivars possess a unique but dynamic repertoire of specialized metabolites, which varies depending on the environmental conditions and intrinsic factors, such as age and physiological status of the plant (Verma and Shukla, 2015). Importantly, this repertoire can have a central role in determining, whether a plant is resistant or susceptible to a given pathogen.

The capacity of a phytopathogen to deal with plant defence compounds could be a critical pathogenicity factor. The pathogen could degrade the antimicrobial compounds (Pedras and Ahiahonu, 2005) or actively remove the toxin molecules through membrane transporters (Coleman and Mylonakis, 2009). Lack of toxin targets may help the pathogen to avoid the negative impact of defence compounds; for

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example, the membrane disrupting glycoalkaloid, α-tomatine, is not effective against *Phytophthora*, because the pathogen lacks the membrane 3β-hydroxy sterols (Steel and Drysdale, 1988). Another strategy to deal with plant chemical defence, and inducible defence responses in general, is to impede their activation altogether (Wang et al., 2008). Phytopathogens produce a huge array of effectors that interfere with the induction of defence responses (Wang and Jiao, 2019). Pathogens can adapt by manipulating plant metabolism for their own needs, for example, in promoting nutrient acquisition (Parker et al., 2009). Overall, the capability to tolerate and/or detoxify plant defence compounds and the ability to manipulate plant metabolism can be the key factors determining the host range of a pathogen.

The hemibiotrophic oomycete *Phytophthora cactorum* (Lebert & Cohn) J. Schröt. is able to infect more than 200 plant species, including economically important woody ornamentals, fruit trees, and soft fruits, such as *Fragaria* × *ananassa* (Weston) Duchesne ex Rozier (Rosaceae) (garden strawberry) (Erwin and Ribeiro, 1996). The host range and pathogenicity of *P. cactorum* isolates vary. For example, isolates from birch and apple cause more severe symptoms in their original hosts compared to other isolates (Hantula et al., 2000; Van Der Scheer, 1971), and crown rot of strawberry is usually caused by specialized *P. cactorum* pathotypes (Eikemo et al., 2004). The hallmark of strawberry crown rot disease is brown necrosis within the crown tissue, which eventually leads to stunting or wilting of the plant (Maas, 1998). Different *Fragaria vesca* L. (Rosaceae) (woodland strawberry) accessions/genotypes and garden strawberry cultivars show varying levels of resistance to this disease, and resistance appears to be under polygenic control (Denoyes-Rothan et al., 2004; Eikemo et al., 2003, 2010; Parikka, 2003; Shaw et al., 2008). Several quantitative trait loci (QTL) contributing to the crown rot resistance have been identified from garden strawberry, *F.* × *ananassa* (Mangandi et al., 2017; Nellist et al., 2019), and one major QTL, *RPc-1,* has been identified from woodland strawberry, *F. vesca* (Davik et al., 2015). However, the genes and mechanisms conferring the resistance in *F.* × *ananassa* and *F. vesca* are unknown, and new approaches are needed to gain a more profound understanding of strawberry defence responses and *P. cactorum* virulence mechanisms.

Transcriptome analysis of *F. vesca* suggests that specialized metabolites could play important roles in defence responses against *P. cactorum* (Toljamo et al., 2016). Several genes involved in the biosynthesis of specialized metabolites are upregulated in *P. cactorum* -challenged *F. vesca* roots 48 h after inoculation. In particular, sesquiterpenoid and triterpenoid pathways appear to be strongly upregulated, whereas the biosynthesis of steroids and brassinosteroids is down-regulated. Flavonoid biosynthesis genes are up-regulated as well, in particular, those contributing to flavan-3-ols synthesis. Hence, it seems worthwhile to further analyse the changes on metabolic level and to investigate, if similar defence responses are evoked in *P. cactorum* -challenged *F.* × *ananassa*.

Metabolic profiling is a powerful tool in assessing phenotype-level changes and can give interesting insights into plant-pathogen interactions. Metabolic profiling could also help to understand the virulence strategies of *P. cactorum*. We have previously compared the transcriptomes of two *P. cactorum* isolates, Pc407 and Pc440, which differ in their virulence to garden strawberry (Rytkönen et al., 2012; Toljamo et al., 2019). The transcriptome analysis was made from infected *F. vesca* roots 48 h after inoculation, revealing interesting differences between the isolates (Toljamo et al., 2019). In total, 3995 genes were differentially expressed, including 90 RXLR effectors, 23 elicitin/elicitin-like genes, and 120 carbohydrate-active enzymes (CAZymes). Differences were also observed in the processes related to lipid catabolism, membrane fusion, vesicle organization, and transport. Of the CAZymes, several members of glycosyl hydrolase families GH1, GH3, and GH30, showed higher expression levels in Pc407. Besides degradation of cell wall components, some of these GHs might be able to hydrolyse glycosylated specialized metabolites. The CAZyme gene families in *P. cactorum* genome are expanded, and it has been suggested that GHs together with detoxification enzymes could contribute to the ability of *P. cactorum* to adapt the host defence compounds (Yang et al., 2018). However, the metabolic evidence for this is lacking.

The aim of this study is to increase understanding of the interactions between *P. cactorum* and *Fragaria* species on metabolic level. We challenged hydroponically grown *F. vesca* and *F.* × *ananassa in vitro* plants with *P. cactorum* zoospores and investigated the changes in metabolite profiles after 48 h. The zoospores were from Pc407 and Pc440 isolates, which show different virulence to garden strawberry. Only Pc407 causes crown rot in *F.* × *ananassa*, whereas Pc440 is not able to cause crown rot (Rytkönen et al., 2012; Toljamo et al., 2019). Compared to *F.* × *ananassa* cultivars, the *F. vesca* genotype H4.4 of Hawaii4 accession (PI 551572) used here is more resistant to crown rot, and there are no marked differences in virulence between Pc407 and Pc440 (Toljamo et al., 2019). In this study, Pc407 - *F.* × *ananassa* (abbreviated as Pc407-Fa) was used as the most compatible interaction, whereas Pc440 - *F.* × *ananassa* (Pc440-Fa), Pc407-and Pc440-*F. vesca* (Pc407-Fv and Pc440-Fv) interactions were more resistant. Unexposed samples were used as controls (C-Fa and C-Fv). The interesting question was: how do these interactions differ from each other in terms of metabolic responses?

2. Results

2.1. Overview of metabolic changes caused by P. cactorum inoculations

The metabolite analysis revealed 3952 molecular features from the crown samples. Based on the overall metabolite profiles of the samples, also illustrated by principal component analysis (Fig. 1), *Fragaria* species is the predominant factor influencing the metabolite profiles. In *F.* × *ananassa* (cv. Senga Sengana), inoculation with the *P. cactorum* isolate Pc407 (Pc407-Fa) caused a clear separation in PC2 (squares), whereas the Pc440-inoculated samples (Pc440-Fa) clustered near to the controls (C-Fa). In *F. vesca* (accession Hawaii4, clone H4.4), both *P. cactorum* isolates influenced the metabolite profiles (balls), although the differences were less pronounced than in the Pc407-Fa interaction. The biological replicates of C-Fv, Pc440-Fv, and Pc407-Fa, were tightly clustered within each of these groups, whereas clustering of Pc440-Fa, C-Fa, and Pc407-Fv samples was more loose in the PCA plot (Fig. 1).

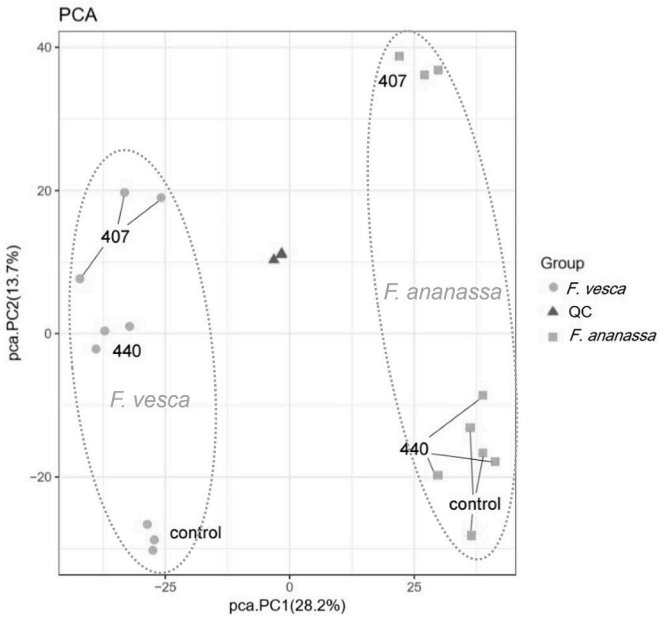

Fig. 1. Principal component analysis of the metabolite profiles of the analysed samples. The two components explaining the largest proportion of the variance (28.2% for component 1 and 13.7% for component 2) in the data are shown. QC = quality control samples pooled from the experimental samples.

This could be attributed to the size variation of the crown samples. The diameters of the crowns were not recorded, but biomass differences between the biological replicates were considerable, particularly among the Pc440-Fa and C-Fa samples (Table S1).

2.2. Differentially accumulated metabolites in *P. cactorum* – *Fragaria* interactions

According to Two-Way ANOVA, the quantity of 1518 molecular features showed significant differences (FDR < 0.05) between the *Fragaria* species (Fig. S1). However, as our main interest was in the metabolic changes in *Fragaria* - *P. cactorum* interactions, our focus here is in the metabolites showing significant differences between the treatment groups (FDR < 0.05). Among the 468 molecular features, we chose the most abundant ones, which were present in all three biological replicates within a treatment group and had the minimum mean intensity of 50 000 at least in one of the treatment groups. In addition, the availability of MS/MS data for the features was taken into account. This left us with 145 molecular features for further analysis (Table S2).

Heatmap visualization of these metabolites and unknown molecular features (Fig. S2) corroborates the results of the PCA plot (Fig. 1); inoculation with the more virulent isolate Pc407 causes more drastic changes in the metabolite profile of *F.* × *ananassa* (Pc407-Fa) compared to the mildly virulent Pc440 (Pc440-Fa). In *F. vesca*, the profiles of Pc407-Fv and Pc440-Fv plants are more alike; however, there is more variation between the three replicates. Some of the observed changes seem to be universal responses, occurring in both *Fragaria* species, while some of the changes appear to be species-specific. In total, 45 compounds were tentatively identified (at level 2 or 3, based on the criteria described by Sumner et al., 2007) among the molecular features that showed differences between treatment groups (Fig. 2, Table S2). Of these, 21 compounds were putatively annotated (identification level 2) and 24 were from putatively characterized compound classes (identification level 3) (Table S2).

2.3. Downregulated metabolites

Among the major, differentially accumulated metabolites, some fell within the clusters showing decreased levels upon inoculation either in *F.* × *ananassa* or in both *Fragaria* species. Of these, ten compounds were tentatively identified as two quercetin glucuronides (neg_m/z_477.07_rt_4.99; pos_m/z_479.08_rt_4.92), flavonoid-*O*-glucuronide ($C_{26}H_{26}O_{16}$ neg_m/z_593.11_rt_4.9), a very long-chain fatty acid (pos_m/z_813.51_rt_10.92), a monogalactosyldiacylglycerol (pos_m/

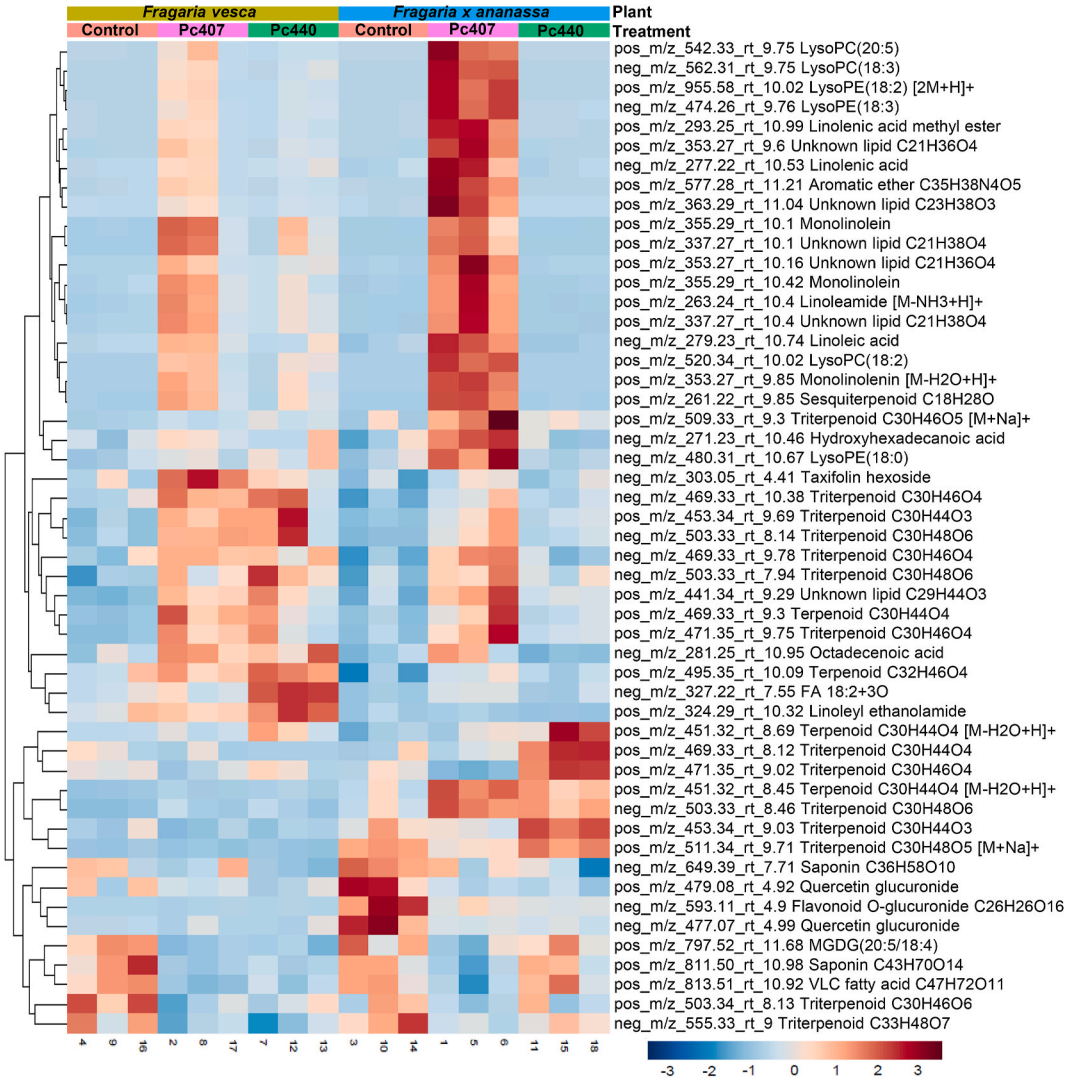

Fig. 2. Heatmap visualization of tentatively identified metabolites differentially accumulated between treatments. Relative accumulation levels of individual samples are shown. Lowest: Blue. Highest: Red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

z_797.52_rt_11.68), two saponins ($C_{36}H_{58}O_{10}$ neg_m/z_649,39_rt_7.71; $C_{43}H_{70}O_{14}$ pos_m/z_811.50_rt_10.98), and three terpenoids ($C_{33}H_{48}O_7$ neg_m/z_555.3_rt_9.00; $C_{30}H_{48}O_4$ neg_m/z_471.35_rt_10.1; $C_{32}H_{46}O_4$ pos_m/z_495.35_rt_10.09) (Fig. 2, Table S2). The levels of two quercetin glucuronides and the flavonoid-*O*-glucuronide were notably lower in inoculated Pc407-Fa and Pc440-Fa samples compared to C-Fa (Table S2). The level of saponin $C_{43}H_{70}O_{14}$ decreased in all inoculated samples, excluding Pc440-Fa, whereas the saponin $C_{36}H_{58}O_{10}$ showed minor decreases compared to controls in the Pc440-Fv and Pc440-Fa samples. As the flavonoid glucuronides and the saponins are glycosylated specialized metabolites, we scanned the masses of the up-regulated metabolites to find potential aglycones of these compounds. A triterpenoid $C_{30}H_{48}O_5$ ([M+Na]+ pos_m/z_511.34_rt_9.71) could be the aglycone of the saponin $C_{36}H_{58}O_{10}$ based on the mass and retention time difference. However, aglycones of flavonoids were not found among the up-regulated metabolites.

2.4. Changes in the terpenoid profiles

Altogether 21 terpenoids were tentatively identified among the differentially accumulated metabolites, including one sesquiterpenoid $C_{18}H_{28}O$ (pos_m/z_261.22_rt_9.85) and several triterpenoids (Table S2). The triterpenoid with formula $C_{30}H_{48}O_4$ (neg_m/z_471,35_rt_10.1), was the most abundant of the identified terpenoids in all *Fragaria* samples even after the negative effects of inoculation (Fig. 3). The levels of some other identified terpenoids were lower but often increased upon inoculation. The terpenoid profiles of the inoculated samples were usually clearly distinguishable from the controls (Fig. 3), the only exception being one Pc440-Fv sample with a profile more similar to that of the controls. Some interesting differences were observed between the two *Fragaria* species and treatments. Compound $C_{30}H_{48}O_6$ (neg_m/z_503.33_rt_8.46) was one of the most abundant triterpenoids in the inoculated *F.* × *ananassa* samples, whereas in *F. vesca* its level was considerably lower. Two other isomers of $C_{30}H_{48}O_6$ (neg_m/z_503.33_rt_7.94; neg_m/z_503.33_rt_8.14) were also highly accumulated in *Fragaria* species after inoculation with Pc407 and/or Pc440. Two of these three isomers, could be myrianthic acid and sericic acid, as their presence in strawberry has been verified by Hirai et al. (2000) and Yang et al. (2016), respectively. The profile of Pc440-Fa was the most distinctive among the inoculation groups, particularly as the abundance of triterpenoid $C_{30}H_{46}O_4$ (neg_m/z_469.33_rt_9.78) was very high. In the other samples, the levels of this compound varied, but were considerably lower compared to Pc440-Fa.

2.5. Changes in lipid composition

Several identified lysophosphatidylcholines (LysoPCs) (LysoPC (18:2) (pos_m/z_520.34_rt_10.02); LysoPC(20:5) (pos_m/z_542.33_rt_9.75); LysoPC(18:3) neg_m/z_562.31_rt_9.75)) and lysophosphoethanolamines (LysoPEs) (LysoPE (18:3) (neg_m/z_474.26_rt_9.76); LysoPE (18:0) (neg_m/z_480.31_rt_10.67); LysoPE (18:2) ([2 M + H]+ pos_m/z_955.58_rt_10.02)) were differentially accumulated in the crown tissue of strawberries upon *P. cactorum* inoculations (Figs. 2 and 4). Dramatic increases were found especially in Pc407-Fa samples, the fold changes (FCs) often being over 100 (calculated as the peak intensity in Pc407-Fa versus the mean peak intensity in C-Fa) (Fig. 4, Table S2). Monolinoleins (pos_m/z_355.29_rt_10.1; pos_m/z_355.29_rt_10.42), monolinolenin (pos_m/z_353.27_rt_9.85), linoleic acid (neg_m/z_279.23_rt_10.74), and linolenic acid (neg_m/z_277.22_rt_10.53) were also highly accumulated in Pc407-Fa; the FCs of monolinoleins and monolinolenin varied from ~20 to 120, whereas the FCs of linoleic and linolenic acid ranged from ~4 to 9. In addition, several unknown lipids accumulated in a similar manner. Similar changes in lipid profiles were observed in some of the inoculated *F. vesca* samples where, in general, the FCs were lower compared to Pc407-Fa and the variation between replicates was high. The most consistent increase in Pc407-Fv and Pc440-Fv plants was seen in octadecenoid acid (neg_m/z_281.25_rt_10.95). In Pc440-Fa, only minor changes occurred in these metabolites. In contrast to the aforementioned compounds, linoleyl ethanolamide (pos_m/z_324.29_rt_10.32) showed a completely different profile: it was accumulated especially in Pc440-Fv (mean FC ~ 2), whereas no such increase was observed in inoculated *F.* × *ananassa*. In addition, the highest level of FA 18:2 + O3 oxylipin (neg_m/z_327.22_rt_7.55) was observed in Pc440-Fv.

3. Discussion

The genetic background is often the most important factor determining the phytochemical profiles of the strawberry plants (Kårlund et al., 2016). This seems to hold true also in the present study, as the *Fragaria* species is the predominant factor influencing the metabolite profiles (Fig. 1). The most distinct profiles were observed in the most compatible interaction Pc407-Fa, whereas the profiles of Pc440-Fa samples were more similar to those in the controls (Fig. 1). Compared to garden strawberries, *F. vesca* genotype H4.4 of accession Hawaii4 (PI 551572) is more resistant to crown rot disease and the virulence difference between Pc407 and Pc440 isolates is less pronounced (Toljamo et al., 2019). Accordingly, the distances between Pc407-Fv and Pc440-Fv samples in the PCA plot are shorter compared to distances

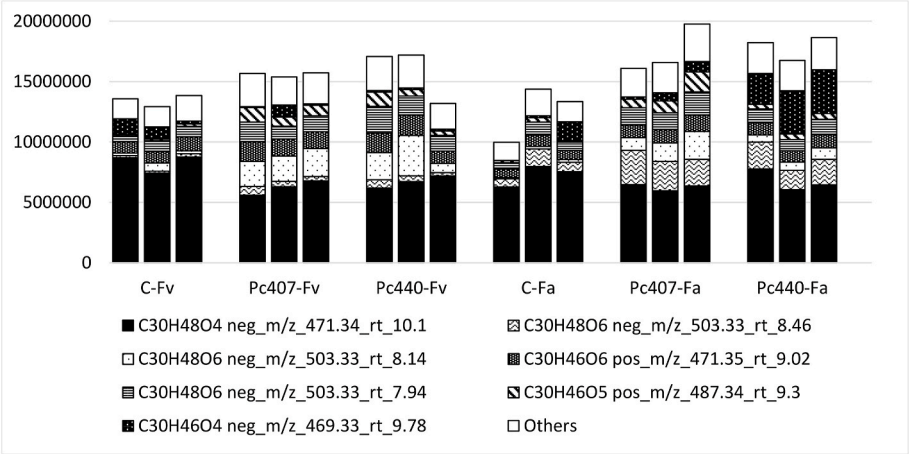

Fig. 3. The profiles of differentially accumulated terpenoids in *F. vesca* and *F. ananassa* samples. The peak intensities of seven most abundant triterpenoid compounds are shown separately, whereas the intensities of other terpenoid peaks are combined.

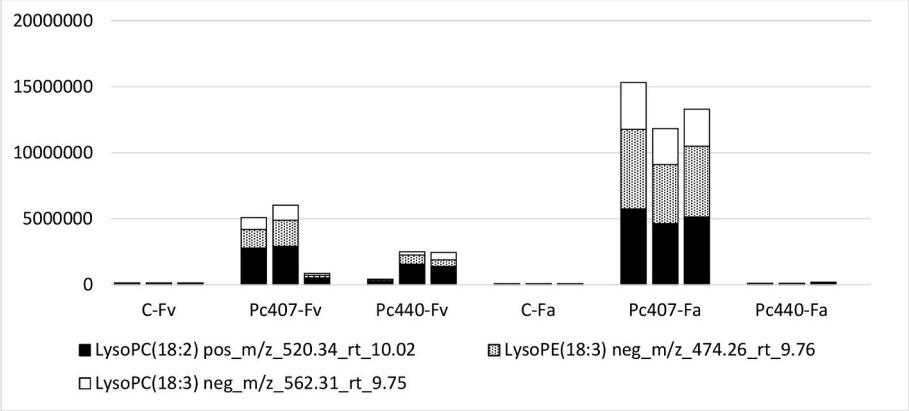

Fig. 4. The profiles of three differentially accumulated lysophospholipids in *F. vesca* and *F.* × *ananassa* samples. The peak intensities of three most highly accumulated lysophospholipids are shown.

between Pc407-Fa and Pc440-Fa samples (Fig. 1). Altogether, the results of the PCA are in line with the previous knowledge of the *Fragaria* - *P. cactorum* interactions (Rytkönen et al., 2012; Toljamo et al., 2019).

We putatively identified 45 differentially accumulated metabolites from strawberry crowns 48 h after *P. cactorum* challenge. Most of the metabolites show increasing levels upon inoculation with Pc407 and/or Pc440. Examples of the metabolites with decreasing levels of abundance compared to controls include two quercetin glucuronides, a flavonoid-*O*-glucuronide, and two saponins. In particular, quercetin glucuronide (neg_m/z_477.07_rt_4.99) is highly accumulated in the C-Fa samples, while the levels in the Pc407-Fa and Pc440-Fa plants are markedly lower. The hydrolysis of glycosylated specialized metabolites by *P. cactorum* has been suggested earlier (Toljamo et al., 2019; Yang et al., 2018), but metabolic evidence for this is still missing. A triterpenoid $C_{30}H_{48}O_5$ could be the aglycone of the saponin $C_{36}H_{58}O_{10}$, but aglycones of flavonoids were not detected among the differentially accumulated metabolites. Therefore, the fate of these compounds is unclear. One possible explanation for the observed decrease in metabolite levels is their conversion to an unidentifiable structure or undetectable form. For example, flavonoid glycosides may be deposited into the secondary cell wall to reinforce the physical barrier against *P. cactorum* (Gunnaiah et al., 2012; Yogendra et al., 2017).

Of the potential defence compounds, triterpenoids are highly represented among the differentially accumulated metabolites. Most of them show increasing levels, while the levels of two triterpenoids and two triterpenoid saponins decrease slightly. These might be converted to other triterpenoids upon inoculation; however, most of the accumulated triterpenoids are probably synthesized *de novo*, as their total levels increase in the inoculated strawberry plants. The synthesis of triterpenoids is in agreement with our previous transcriptomic analysis showing the upregulation of several genes involved in their synthesis in *P. cactorum*-inoculated *F. vesca* roots (Toljamo et al., 2016). The exact identity of the detected terpenoids remains unknown and further characterisation would be needed to resolve their structures. To our knowledge, only few studies have focused on strawberry triterpenoids/saponins and characterized their structures with NMR spectroscopy (Hirai et al., 2000; Song et al., 2013; Yang et al., 2016). Hirai et al. (2000) identified three antimicrobial triterpenoids, i.e., euscaphic, tormentic, and myrianthic acids, from unripe strawberry fruits wounded and inoculated with *Colletotrichum musae* conidia. These triterpenoids showed antimicrobial activity against *C. musae* and may also contribute to resistance against strawberry root pathogen *Phytophthora fragariae* var. *fragariae* (Mussell and Staples, 1971; Hirai et al., 2000).

The effects of some triterpenoids have been tested on *P. cactorum* cultures (Nes and Patterson, 1981; Nes et al., 1980, 1982). As the *Phytophthora* species lack squalene epoxidase, they are unable to synthesise triterpenoids and sterols by themselves (Gottlieb et al., 1978). While sterols have growth-promoting activity on *Phytophthora*, the effect of different triterpenoid compounds may vary from stimulation to inhibition (Nes et al., 1980, 1982; Nes and Patterson, 1981). Therefore, triterpenoid profiles might have an important impact on the outcome of *P. cactorum* – *Fragaria* interaction.

Along with the accumulation of triterpenoids, another striking feature in the metabolite profiles is the strong increase of lysoPCs and lysoPEs in Pc407-Fa samples, which represent the most compatible interaction in this study. The accumulation of lysophospholipids (lysoPLs) and free polyunsaturated fatty acids (e.g. linoleic and linolenic acid) is often observed in plant cells upon pathogen challenge or elicitor treatment (Cho et al., 2012; Scherer et al., 2002; Roy et al., 1995). They are generated from membrane glycerolipids by phospholipase A (PLA) through hydrolysis of ester bonds between the glycerol backbone and fatty acids (Canonne et al., 2011).

LysoPLs appear to function in defence signalling, but they could also have adverse effects on plant cells. Viehweger and collaborators demonstrated that LysoPCs are involved in elicitor-triggered signalling events, leading to phytoalexin synthesis in cultured California poppy cells (Viehweger et al., 2002, 2006). The accumulation of LysoPCs is transient, and they are quickly reacylated to phosphatidylcholine to minimize the cytotoxic effects on plant cells (Schwartze and Roos, 2008). In tobacco, LysoPC(18:1) strongly stimulates the expression of PR (pathogenesis-related) proteins, but also causes cell damage and promotes cell death by inducing the production of reactive oxygen species and ethylene (Wi et al., 2014). Furthermore, LysoPC(18:1) treatment renders tobacco plants more susceptible to *Phytophthora parasitica* var. *nicotianae* infection. Based on these results, Wi et al. (2014) suggested that the effect of LysoPC(18:1) on plants depends on how fast it is removed from the cells to avoid toxicity.

As lysoPLs are highly accumulated, especially in the most compatible Pc407-Fa interaction, it seems plausible that these molecules play some role in pathogenesis of crown rot disease. The cell death -promoting activity of the lysoPLs may contribute to the development of necrotic damage in the crown tissue, which is the most characteristic symptom of this disease. Notably, lysoPLs are generated in other plant species as a response to cold (Li et al., 2014; Skinner et al., 2005; Zheng et al., 2016). As the cold-stored strawberry plants are known to be highly vulnerable to crown rot (Eikemo et al., 2000; Pettitt and Pegg, 1994), it might be worthwhile to investigate, if lysoPLs accumulate in strawberries upon cold storage and if there is a link between lysoPL levels and susceptibility.

Another highly interesting aspect that would deserve further investigation is the effect of triterpenoids on PLA enzymes. According to pharmacological studies, some triterpenoid compounds can bind to PLA_2 enzymes and inhibit their activity (Bernard et al., 2001; Jain et al., 1995; Yap et al., 2018). Therefore, it is tempting to speculate that

distinct triterpenoid profiles observed in Pc407-Fa and Pc-440-Fa samples might be linked to differential PLA activity and lysoPL accumulation. More studies are needed to determine the roles of these metabolites in *P. cactorum* - *Fragaria* interactions. In this respect, triterpenoid $C_{30}H_{46}O_4$ (neg_m/z_469.33_rt_9.78) is of particular interest, as it is highly accumulated in resistant Pc440-Fa interaction.

In addition to LysoPLs, PLA enzymes generate free fatty acids (Canonne et al., 2011). They can be further processed to various oxylipins, of which jasmonic acid is the best-known example. Oxylipins (i.e. oxygenated derivatives of polyunsaturated fatty acids) are important signalling molecules in plant defence, and some of them also possess antimicrobial activities against fungi and oomycetes (Prost et al., 2005; Deboever et al., 2020). In contrast, free fatty acids, especially linoleic acid, has a strong growth-promoting activity in *P. cactorum* cultures (Hohl, 1983). Linoleic acid and linolenic acid were highly accumulated in the most compatible Pc407-Fa interaction, whereas the levels of oxylipin FA 18:2 + O3 and linoleyl ethanolamide were the highest in Pc440-Fv samples. This raises the question, whether the accumulation of free fatty acids and their conversion to oxylipins or other derivatives could contribute to the outcome of the interaction.

Monoacylglycerols are also potential products generated by lipases. Two monolinolein and monolinolenin compounds are highly accumulated in Pc407-Fa, and are present at varying levels in Pc407-Fv and Pc440-Fv samples. To our knowledge, the role of monoacylglycerols in plant immunity has not been studied. However, monolinolein shows strong inhibition potential on *Phytophthora infestans* spore germination (Stoessl et al., 1980). Therefore, it might be worthwhile to test the possible inhibitory activity of monoacylglycerols on *P. cactorum* culture, i.e., if they are able to restrict mycelial growth.

In addition to putatively annotated compounds and compound classes, a number of unknown metabolites were differentially accumulated between treatment groups. As many of these unknown metabolites (and the lipid compounds discussed above) were highly accumulated in the most compatible Pc407-Fa interaction, they can be derived either from the host plant or the pathogen. They may also include plant-derived compounds that have been modified by *P. cactorum*. Under normal conditions, these compounds may be rare in plants and be present only at low concentrations. Perhaps, therefore, they have remained uncharacterised and are missing from the spectral databases we used for compound annotation.

Taken together, this nontargeted metabolite analysis provides interesting insights into strawberry - *P. cactorum* interactions but also raises additional questions. One of the most prominent features in the inoculated plants is the accumulation of triterpenoids. Some of them show distinct accumulation patterns in different interactions; for example, the triterpenoid $C_{30}H_{46}O_4$ (neg_m/z_469.33_rt_9.78) is highly accumulated only in the incompatible Pc440-Fa interaction and is thus an interesting candidate as a resistance marker. The reason for its accumulation and the significance of its selective accumulation are interesting questions to explore. As some of the triterpenoids are potential defence compounds, they may play an important role in crown rot resistance, while others may have opposite effects. Triterpenoids may also have indirect effects on the outcome of this interaction; namely as inhibitors of PLAs. Hence, identification of the accumulated triterpenoids and testing of their activities are important tasks for the future. Another particularly interesting aspect is the role of lysoPLs in pathogenesis of crown rot disease. As demonstrated previously, the generation of lysoPLs is induced by elicitors, and they may have important signalling roles in plant defence. However, excessive, prolonged accumulation of these compounds may be detrimental and thus appears as an inappropriate defence strategy for a plant. It is thus relevant to ask if their accumulation in Pc407-Fa interaction is caused by the virulent *P. cactorum* isolate. Is this pathogen able to manipulate strawberry metabolism, or do *P. cactorum* PLAs make a contribution to lysoPL accumulation? And, what is the role of triterpenoids in this interplay? Knowing the molecular factors behind the resistance is essential, but equally important is to know the key processes leading to susceptibility. This metabolite analysis has revealed potential factors contributing to both of these questions. The knowledge of the roles of the metabolites will help to find new resistance breeding strategies and new solutions to crown rot disease control.

4. Experimental

4.1. General experimental procedures

The system consisted of a 1290 Infinity Binary UPLC (Agilent Technologies, Waldbronn, Karlsruhe, Germany) coupled with a 6540 UHD Accurate-Mass qTOF-MS with Jetstream ESI source (Agilent Technologies, Santa Clara, CA, USA). Reversed phase (RP) separation was carried out with a Zorbax Eclipse XDB-C18 column, 2.1 × 100 mm, 1.8 μm (Agilent Technologies, Santa Clara, CA, USA). Chemicals used: Methanol (CHROMASOLV™ LC-MS Ultra, Riedel-de Haën™, Honeywell, Seelze, Germany) and formic acid (Optima LC/MS, Fisher Chemical, Geel, Belgium).

4.2. Biological material

Micropropagated plants of *Fragaria* × *ananassa* (Weston) Duchesne ex Rozier cv. Senga Sengana were purchased from Agrifood Research Finland MTT, Laukaa Research and Elite Plant Station. The seeds of *Fragaria vesca* L. (National Clonal Germplasm Repository accession PI 551572, Hawaii 4) were provided by Associate Professor Hytönen (Department of Agricultural Sciences, University of Helsinki, Finland). To obtain genetically identical plant material, one seedling of this accession (named as H4.4), was micropropagated as described by Toljamo et al. (2016). *Phytophthora cactorum* (Lebert & Cohn) J. Schröt. isolates Pc407 and Pc440 were obtained from Natural Resources Institute Finland (Rytkönen et al., 2012).

Micropropagated plants were hydroponically grown in modified RITA®-containers in half-strength Hoagland solution as described by Toljamo et al. (2016). After four weeks, the plants were treated by immersing the roots and crowns in *P. cactorum* zoospore suspensions (5000 zoospores/mL) (isolate Pc407 or Pc440) or in water (control) for 30 min. Each group comprised of three replicates. The preparation of zoospore suspensions and inoculation is described in detail in Toljamo et al. (2016). The crown samples were collected for metabolite analysis 48 h after inoculation. The crowns were separated from the roots and leaves with scalpel and immediately flash-frozen in liquid nitrogen. The samples were stored at −80 °C until processed.

4.3. Sample preparation and LC-MS analysis

The metabolite extractions were performed as described by Hanhineva et al. (2008). After homogenizing the frozen crown samples with a TissueLyzer (2.5 min, 30 Hz), aq. methanol (80%) was added (6.5 μl/mg of crown tissue). The weight of each sample is presented in Table S1. The samples were vortexed and sonicated in water bath at room temperature for 15 min. Vortexing was repeated and the samples were centrifuged (3000 g, 10 min). The supernatants were collected and stored at −80 °C overnight. Prior to the analysis, the extracts were filtered through 0.22 μm PTFE filters (Acrodisc ®, PALL). The samples were analysed at Afekta Technologies Ltd. by liquid chromatography–mass spectrometry. Conditions were as decribed by Klåvus et al. (2020). Ionization, after each chromatographic run, was conducted using Jetstream ESI in the positive and negative mode. Collision energies for the MS/MS analysis were 10, 20, and 40 eV.

4.4. Data analysis and compound identification

Peak detection and alignment were performed in MS-DIAL version 2.84 (Tsugawa et al., 2015). For peak collection, all m/z values and

retention times were considered. The peaks were detected using the linear weighted moving average algorithm. For the alignment of the peaks across samples, the retention time tolerance was 0.15 min, and the m/z tolerance was 0.025 Da. To minimize noise and false peak detection, all the peaks had to be detected in all quality control samples.

Principal component analysis (PCA) was performed with in-house scripts using R and was based on the final data set comprising 3592 molecular features. Two-Way ANOVA was conducted with MetaboAnalyst version 3.0 (Xia et al., 2015; Xia and Wishart, 2016). In total, 486 peaks showed significant differences (FDR < 0.05) between treatments. Of these, peaks with available MS/MS data and with minimum mean intensity of 50 000 in at least one of the treatment groups were chosen. This resulted in a set of 145 peaks for identification.

The choromatographic and mass spectrometric characteristics, i.e. retention time, exact mass, and fragmentation patterns, were utilized in compound identification. These characteristics were compared with entries in an in-house standard library, MS-FINDER, publicly available databases, (e.g. METLIN and MassBank of North America (MoNA)), and previously published literature. The annotation of each metabolite and the level of identification was given based on the recommendations published by the Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI) (Sumner et al., 2007).

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Data availability

LC-MS metabolomics data is available on B2SHARE: http://doi.org/10.23728/b2share.026e984896894c48a21218268eb2fc51.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2021.112820.

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