Supplementary Material File 1

Supplementary Results

Production of specific volatiles by *S. plymuthica* HRO-C48 as a response to treatments with fungal VOCs

Seven further substances were emitted due to the contact with one or two of the tested fungi, while not being detected in the control. Four of them were produced only when *Serratia* was confronted with *V. longisporum* VOCs. Among those VOCs, 1-decanol, octanoic acid, ethyl ester and 2-undecanone are known for their antimicrobial properties (Kato and Shibasaki, 1980; Kubo et al., 1995; Togashi et al., 2007; Fialho et al., 2011; Popova et al., 2014; Toffano et al., 2017), while 2-heptanol is mentioned in the literature as a volatile substance that interferes with plants (Kanchiswamy et al., 2015). Incubation of HRO-C48 with *L. maculans* MB and *V. longisporum* ELV 43 VOCs resulted in the production of isoamyl acetate, which was not detected in the untreated control or in the treatment with *R. solani* AG2. Isoamyl acetate is known as the main antimicrobial substance found in the Japanese rice wine sake (Strobel et al., 2001; Ando et al., 2015). Another substance with an unknown ecological function, 2-methyl-3-hexanol, was produced by *S. plymuthica* HRO-C48 only when it was incubated with *R. solani* AG2 (Supplementary Table 2). Contact of *S. plymuthica* HRO-C48 with *V. longisporum* ELV 43 VOCs resulted in a significantly stronger upregulation of VOCs production in the bacterium compared to the other fungi tested. Among 22 upregulated VOCs there were 14 substances with putative antimicrobial function, seven with predicted functions that involve interaction with other microorganisms and plants and two VOCs with unknown function (Supplementary Table 2). Only two VOCs with predicted antimicrobial function were downregulated in *S. plymuthica* HRO-C48 due to *V. longisporum* volatiles when compared to the untreated control (Supplementary Table 2). VOCs produced by *R. solani* AG2 had an intermediate effect on the VOCs production in *Serratia*. Eight VOCs were upregulated, and five were downregulated due to the contact with fungal volatiles. Among the eight upregulated substances, six had a predicted antimicrobial function. For example, 2,3-butanediol, is predicted to be antimicrobial, plant growth promoting and have a positive effect on the biofilm formation in some bacteria (Ryu et al., 2003, 2004; Zhang et al., 2007a; Venkataraman et al., 2014; Audrain et al., 2015). From the downregulated VOCs, two are suggested to have an antimicrobial effect, while the other three may induce plant responses and influence biofilm formation in some bacterial species (Supplementary Table 2). *L. maculans* MB 158 VOCs had the weakest effect on the bacterial VOCs production. Only five substances, three of them predicted to be antimicrobial and two with unknown function were upregulated in the bacterium. No downregulation of VOCs production in *Serratia* due to *L. maculans* volatiles was observed (Supplementary Table 2).

Response of the selected fungi to VOCs emitted by *S. plymuthica* HRO-C48

Three substances were identified in the volatilome of *R. solani* AG2**:** isobutanol, 2-methyl-1-butanol, and dimethyl ether (a substance with unknown function). All three substances were shared with those produced by *V. longisporum* ELV 43 (Fig. 2). Their production was not significantly influenced by the treatment with *S. plymuthica* HRO-C48 volatiles. The production of isobutanol, 2-methyl-1-butanol, and dimethyl ether by *R. solani* AG2 was not significantly influenced by the treatment with *S. plymuthica* HRO-C48 volatiles (Supplementary Table 3). Isobutanol is an antibacterial and plant growth promoting substance (Akpata and Akinrimisi, 1977; Naznin et al., 2013; Farh and Jeon, 2020), while 2-methyl-1-butanol, is mentioned by several authors as a VOC with antimicrobial effects (Saksena and Tripathi, 1987; Linton and Wright, 1993a; Humphris et al., 2001; Toffano et al., 2017). Two further substances, 3-propoxy-1-propene (a substance with unknown function) and ethanol were completely downregulated due to the contact with *S. plymuthica* HRO-C48. Ethanol is mentioned in the literature as an antimicrobial substance that may inhibit fungal spore germination and have a negative effect on biofilm formation in some bacterial species (Linton and Wright, 1993b; Létoffé et al., 2014; Audrain et al., 2015; Lazazzara et al., 2017).

V. longisporum ELV 43 produced the highest number of volatiles among the three tested fungi. Out of 14 VOCs that were detected in the volatilome of *V. longisporum* four were strongly upregulated and 6 downregulated due to the contact with *S. plymuthica* HRO-C48 volatiles (Fig. 2). Three novel substances were produced by *V. longisporum* ELV 43 when it was incubated with *S. plymuthica* HRO-C48: 2-methyl-propanal, 3-methyl-1-butanol acetate, and acetic acid. The last two substances were also produced by *S. plymuthica* HRO-C48 (Table 2), so it cannot be ruled out that only one of the two microorganisms produced one or both substances and cross-contaminated the other one with its volatiles. All three substances are mentioned in the literature as having antimicrobial properties or being able to induce bacterial responses (Strobel et al., 2001; Pimenta et al., 2012; Ando et al., 2015; Kanchiswamy et al., 2015). Six VOCs were either completely switched off due to the contact with bacterial volatiles in *V. longisporum*, or significantly downregulated. From these substances two (3-methyl-pentane and , 3-methyl-3-hepten-2-one) are known for their antimicrobial properties (Kumari et al., 2019) (GROVER and GS, 1980) and one substance 1-butanol has a species-specific effect on biofilm formation in some bacteria and fungi (Létoffé et al., 2014; Audrain et al., 2015). The functions of the other three downregulated VOCs are not mentioned in the current literature.

A common response *S. plymuthica* HRO-C48 to the VOCs of all three tested fungi on the transcriptomic level

Thirty-nine DEGs were common among the genes upregulated due to the exposure to all three fungal VOCs (Supplemental Table S4). Out of these, 11 genes which were with no predicted function were found. Some of the upregulated genes on the other hand (e.g. SOD10_RS23105, SOD10_RS23095,

and SOD10_RS18925) were predicted to code for a phage shock protein. Phage proteins like (Phage shock protein B) belongs to the bacterial phage shock protein stress response system which is activated by events affecting the cytoplasmic membrane. Phage shock protein B was previously shown to prevent lethal cytoplasmic membrane permeability in *Yersinia enterocolitica* and is considered as a sign of a cellular stress (Horstman and Darwin, 2012). Interestingly, the gene (SOD10_RS21985) coding for Type III secretion system lipoprotein chaperone was observed among the upregulated genes upon exposure to volatiles emitted by *S. plymuthica* HRO-C48. The type III secretion system has been previously associated with virulence in some enteropathogenic bacteria like *E.coli* and *Salmonella* spp. (Tosi et al., 2011; Dunstan et al., 2013). A list of all genes which were upregulated in response to confrontation with volatiles from the pathogenic fungi: *R. solani* AG2 (Riz), *L. maculans* MB 158 (Lep) and *V. longisporum* ELV 43 (Ver) have been provided in Supplemental Table S4.

Sixty-three genes were significantly downregulated in *S. plymuthica* HRO-C48 due to the contact with the VOCs of all three fungi. Among them there was one membrane protein (SOD10_RS05170) with peroxide and acid stress response properties. The downregulation of outer membrane proteins, which was also observed, is considered to be a signal of an envelope stress in a 'sigma E dependent' manner (Johansen et al., 2008). It prevents further buildup of misfolded outer membrane proteins in the extra cytoplasmic compartment. Another indication for cellular stress is a downregulation of the two predicted multiple stress resistance proteins BhsA (SOD10_RS16560 and SOD10_RS16565). Disruption of BhsA increases copper sensitivity, induces stress response genes in biofilms, increases aggregation and cell surface hydrophobicity, and decreases indole synthesis. Downregulation of BhsA may also increase biofilm formation by repressing cell-cell interactions and cell surface interactions (Zhang et al., 2007b). A murein hydrolase protein (SOD10_RS19040) is predicted to be involved in regulation of cell wall growth, turnover of peptidoglycan during growth, separation of daughter cells during cell division and autolysis, and in lysis phenomena. A downregulation of the murein hydrolase protein may lead to a slowdown of assembly of large trans-envelope complexes (pili, flagella, secretion systems) and thus could be involved in the decrease of cellular movement and enhance biofilm formation processes (Vollmer et al., 2008). Furthermore, we observed a downregulation of a putative Ferritin-like protein (SOD10_RS01830) in the transcriptome of the *S. plymuthica* HRO-C48 upon the incubation with the volatiles of all three fungi. This class of proteins is involved in iron detoxification as it can protect bacterial cells from iron overload and serve as an iron source when iron is limited. It also protects the bacterial cells against oxidative stress and/or protects DNA against enzymatic or oxidative attack (Smith, 2004). Taken together, this data suggests that the contact of *S. plymuthica* HRO-C48 with VOCs emitted by *V. longisporum, R. solani* and *L. maculans* activates several stress response systems in bacterial cells by up- and downregulation of the transcription of respective genes, thus protecting the bacterial cells from damage by fungal VOCs. Another predicted effect of the VOCs

of all three fungal species on *S. plymuthica*, based on the transcriptome analysis, is the downregulation of cellular motility and enhancement of biofilm formation processes. However, dues to limitations in the database for functional prediction, several genes (COG= no functional prediction) could not be assigned. The list of *S. plymuthica* genes which were down-regulated upon VOCs exposure have been provided in Supplemental Table S4.

Different expression of genes involved in selected functions with the focus on virulence

R. solani VOCs resulted in a much stronger upregulation of predicted virulence-associated genes (3 upregulated). In the samples treated with *V. longisporum* and *L. maculans* VOCs, the expression of virulence-associated genes was often downregulated. Three genes were downregulated, and two were upregulated due to *L. maculans* VOCs, while three genes were downregulated and upregulated due to contact with *V. longisporum* volatiles (Supplemental Table S5). Among the genes upregulated due to treatment with *R. solani* VOCs one gene coding for a predicted enzyme of phenylacetate metabolism was found. Pathways involved in aerobic aromatic compounds metabolism occur in various pathogens, where reactive early intermediates may contribute to virulence (Teufel et al., 2010). Another identified DEG, SOD10_RS08975, codes for a putative HPr-related protein which is involved in the phosphotransferase system. HPr-related proteins are known to interfere with the virulence of some pathogens (Deutscher et al., 2006). The expression of two predicted lipoproteins (coded by genes SOD10_RS05020 and SOD10_RS23345) was significantly downregulated in *S. plymuthica* HRO-C48 due to the contact with *L. maculans* MB158 volatiles. Both of them play roles in bacterial virulence (Ohara et al., 1999; Zückert, 2014). Similarly, the expression of two genes putatively coding for DNA-binding ferritin-like proteins involved in bacterial virulence (Andrews, 2010) was downregulated due to the *L. maculans* MB158 treatment (Supplemental Table S5).

Some virulence-associated genes were differently regulated due to treatments with various fungal species. A gene coding for a predicted lactoylglutathione lyase was significantly upregulated in *S. plymuthica* HRO-C48 due to exposure to *R. solani* VOCs and downregulated in the *V. longisporum* treatment. Lactoylglutathione lyase is involved in methylglyoxal detoxification, and functions as a virulence factor in *Salmonella typhimurium* (Chakraborty et al., 2015). Similarly, we observed an upregulation of one gene putatively coding for a Sec-independent protein secretion pathway component (SOD10_RS00080), while a gene with the same predicted function (SOD10_RS14125) was downregulated due to the *L. maculans* MB158 VOCs treatment.

We found only one DEG putatively involved in the production of secondary metabolites, while two other identified genes were neither downregulated nor upregulated. The DEG was identified as SOD10_RS02040, coding for an acyl carrier protein. The expression of this gene, which is a carrier protein mediating biosynthetic pathways including polyketide and nonribosomal peptide synthases was

downregulated in *S. plymuthica* due to the treatment with *L. maculans* volatiles (Supplemental Table S5).

Different expression of genes involved in selected functions with the focus on biofilm formation and cellular motility

Eight DEGs were identified that are putatively involved in cell motility and biofilm formation (Supplemental Table S5). As previously mentioned, the VOCs of all three fungal pathogens enhanced processes involved in biofilm formation of *S. plymuthica* HRO-C48 as shown by a downregulation of two genes encoding the multiple stress resistance protein BhsA (SOD10_S16560 and SOD10_S16565). The treatment with *R. solani* AG2 VOCs resulted additionally in an upregulation of the P pilus assembly protein, pilin FimA (SOD10_RS04660). Such upregulation may also result in slowing down the rate of movement and enhancement of biofilm formation (De La Fuente et al., 2007).

The effect on the cellular motility was on the other hand not that unambiguous. As mentioned earlier, treatments with the volatiles of all three fungal species resulted in the downregulation of the genes putatively responsible for the effectors of murein hydrolases (SOD10_S19040 and SOD10_S19045) and genes responsible for multiple stress resistance proteins BhsA (SOD10_S16560 and SOD10_S16565). Such downregulation may be associated with the decrease in cell motility (Vollmer et al. 2008; X.-S. Zhang, García-Contreras, and Wood 2007; Romeo 1998). The inactivation of the genes involved in the Cpx response (S08675 and S00410), on the other hand, was shown to adversely affect assembly of some pili and thereby increase cellular motility (Vogt and Raivio, 2012). Two genes involved in the Cpx response were significantly downregulated due to the contact with *L. maculans* MB158 VOCs (SOD10_RS08675 and SOD10_RS00410) and *V. longisporum* ELV 43 VOCs (SOD10_RS08675) (Table 3). *R. solani* AG2 VOCs also resulted in upregulation of a gene putatively coding for the Sec-independent protein secretion pathway components (SOD10_RS00080). This gene is involved in secretion of pili and flagella (Kostakioti et al., 2005) and may thus facilitate an increase in cellular motility. Thus, we conclude that VOCs of all three fungi enhance biofilm formation in *S. plymuthica* HRO-C48, while their effect on the cellular motility is adverse

Different expression of genes involved in selected functions with the focus on stress response

The second strongest response to the fungal volatiles was observed in the expression of genes involved in stress response. Twenty-five genes involved in stress response were significantly up- or down-regulated in *S. plymuthica* HRO-C48 due to the treatment with VOCs of *R. solani* AG2, *L. maculans* MB158 or *V. longisporum* ELV 43 (Supplemental Table S5). We already mentioned the downregulation of specific genes in *S. plymuthica* HRO-C48 coding for predicted multiple stress resistance proteins BhsA (SOD10_RS16565, SOD10_RS16560), outer membrane lipoprotein (SOD10_RS06090),

Ferritin-like protein (SOD10_RS01830) and upregulation of phage shock proteins B (SOD10_RRS23105) in all three treatments with fungal volatiles. These changes in gene regulation are associated with cellular stress. The enhancement of biofilm formation and thus a reduction of cellular motility due to the contact with fungal VOCs is another indicator of cellular stress (Zhang et al., 2007). In addition to the above-mentioned changes in gene expression levels, we also observed the upregulation of three further genes responsible for phage-shock proteins. Such upregulation is associated with the general stress response in various cells (Darwin, 2005; Horstman and Darwin, 2012). Two of the genes (SOD10_RS18925 and SOD10_RS23095) were significantly downregulated following treatments with *R. solani* AG2 and *V. longisporum* ELV 43 VOCs and one (SOD10_RS23110) due to *L. maculans* MB158 VOCs. We also observed a significant downregulation of four further genes coding for predicted outer membrane proteins due to *L. maculans* MB158 VOCs and of three genes due to treatments with *V. longisporum* ELV 43 VOCs. Downregulation of outer membrane proteins is a signal of an envelope stress in a 'sigma E dependent' manner (Johansen et al. 2008). A range of genes involved in stress-induced transcriptional response were significantly up- or downregulated in *S. plymuthica* HRO-C48 due to treatment with fungal volatiles. For example, two genes coding for tRNA-Ser were upregulated in *S. plymuthica* HRO-C48 due to treatments with *R. solani* AG2 volatiles (Supplemental Table S5). Among other functions, tRNA-Ser serves as sensor for cellular stress like nutritional deprivation and regulates apoptosis (Raina and Ibba, 2014). *R. solani* AG2 and *L. maculans* MB158 VOCs induced an upregulation of the expression of the gene SOD10_RS23100 coding for a putative stress-responsive transcriptional regulator, which is another signal of a stress response on transcriptional level. Upregulation of the expression of two universal stress proteins UspA was observed in the treatment with *L. maculans* MB158 VOCs, while one of these genes (SOD10_RS01300) was also upregulated in the treatment with *V. longisporum* ELV 43 VOCs. UspA proteins may have a general protective function related to the growth arrest state. They are also required for resistance to DNA damaging agents (Nyström and Neidhardt, 1994, 1996; Diez et al., 2000). Three genes coding for a predicted bacterial nucleoid DNA-binding protein were downregulated due to treatment with *L. maculans* MB158 VOCs. These proteins help compact the DNA into microdomains and act as global regulators of transcription. They also play crucial roles in the ability of a bacterium to adapt to unfavorable conditions (Hołówka and Zakrzewska-Czerwńska, 2020). Interestingly, while one gene that encodes a cold shock protein was upregulated due to treatment with *R. solani* AG2 volatiles, the gene putatively coding for the Co-chaperonin GroES (HSP10) was downregulated due to the *V. longisporum* ELV 43 and *L. maculans* MB158 VOCs treatments. Cold shock proteins are a general response to a rapid temperature downshift. They are thought to counteract harmful effects of cold by serving as nucleic acid chaperons that may prevent the formation of secondary structures in mRNA at low temperature and thus facilitate the initiation of translation (Keto-Timonen et al., 2016). HSP10 is involved in protection of the cell from diverse stresses such as temperature and phage attack (Liu and Lund, 2007). We observed a significant downregulation of four

genes involved in protection against oxidative stress. A gene coding for a ferritin-like protein was downregulated following treatments with all three fungi, while genes coding for a predicted peroxiredoxin and a DNA-binding ferritin-like proteins were downregulated in the *L. maculans* MB158 VOCs treatment. Thus, we conclude that **v**olatiles of all three fungi induce stress response in *S. plymuthica* HRO-C48 on transcriptional level.

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