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ORIGINAL ARTICLE

Phenolic compound degradation by *Pseudomonas syringae* phylogroup 2 strains

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Abstract

It has recently been shown that *Pseudomonas syringae* strains pathogenic to woody hosts belonging to phylogroup (PG) 2 lack phenolic compound degradation pathways such as the beta-ketoadipate and protocatechuate pathways. The aim of this study was to analyse a selection of *P. syringae* PG 2 genomes, including those used previously to determine if they had other phenolic compound degradation pathways and to determine whether or not they were functional. Twenty-one publicly available genomes of PG 2 strains were analyzed. These strains had previously been isolated from both woody and herbaceous hosts. Phenolic degradation enzymes were present in 5 (23%) of the strains analysed, originating from both woody and herbaceous hosts. Hypothetical pathways were proposed to determine if catechol, anthranilate and benzoic acid were degraded by these strains. Both spectrophotometric and HPLC were used to determine phenolic compound degradation. The five strains with phenolic degradation enzymes were able to metabolize catechol, and HRI-W 7924 and MAFF 301072 could also metabolize anthranilate and benzoate, respectively. The study showed that even though some PG 2 strains lack the beta-ketoadipate and protocatechuate pathways, they still have phenolic compound degrading enzymes that may play a role in virulence.

Keywords Pseudomonas syringae · Phenolic compounds · Spectrophotometer · HPLC-DAD

Introduction

Bacterial species belonging to the *Pseudomonas syringae* complex cause disease on several herbaceous and woody hosts (Lamichhane et al., 2014, 2015). This bacterial species is considered one of the most economically important pathogens and it has been extensively used to model plant-pathogen interactions (Mansfield et al., 2012). Even though most of the research on this pathogen was focused on herbaceous hosts, several recent studies have investigated the molecular basis of virulence on woody hosts (Green et al., 2010; Lamichhane et al., 2014; Nowell et al., 2016; Caballo-Ponce et al., 2017).

Several factors have been identified to be of importance for virulence of *P. syringae* on woody hosts, such as metabolic pathways involved in the degradation of phenolic compounds (Green et al., 2010; Nowell et al., 2016). These pathways include the beta-ketoadipate and protocatechute pathways. Both pathways have been well characterized bioinformatically in the chestnut pathogen, *Pseudomonas syringae* pv. *aesculi,* and are involved in the degradation of protocatechuate and catechol to tricarboxylic acid cycle intermediates, respectively (Green et al., 2010; Harwood and Parales, 2003).

Nine genomospecies were identified in the *P. syringae* complex based on DNA:DNA hybridization (DDH) (Gardan et al., 1999). Average nucleotide analyses (ANI) (Marcelletti and Scortichini, 2014) confirmed the existence of the majority of these genomospecies (gs), with the exception of gs 3 and 8. Recently, Berge et al. (2014) described the presence of 13 phylogroups using Multilocus Sequence Analyses (MLSA) of concatenated housekeeping genes including *cts*, *gapA*, *gyrB* and *rpoD* which largely correspond to the nine genomospecies defined by Gardan et al. (1999).

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Pseudomonas syringae pathogenic on woody hosts falls into one of three phylogroups (PG's) (Nowell et al., 2016). It has been suggested that the presence of metabolic pathways involved in the degradation of phenolic compounds is linked to specific PG's (Nowell et al., 2016; Caballo-Ponce et al., 2017). According to Nowell et al. (2016), the beta-ketoadipate and protocatechuate pathway is exclusively found in PG 1 and PG 3 strains and do not occur in PG 2 strains.

The WHOP region (from woody hosts and *Pseudomonas* spp.) has recently been identified as being of importance for *P. syringae* strains pathogenic on woody hosts (Caballo-Ponce et al., 2017). This region has genes involved in phenolic compound degradation. It consists of four operons and three independently transcribed genes. The two operons, *antABC* and *catBCA*, of which *catBCA* is also part of the beta-ketoadipate pathway, are involved in the catabolism of anthranilate and catechol, respectively; the *ipoABC* operon encodes an enzyme with oxygenase activity and is responsible for providing the aromatic compounds with additional hydroxyl functional groups; the function of the fourth operon is still to be elucidated (Caballo-Ponce et al., 2017). Caballo-Ponce et al. (2017) found that the WHOP region is present in only a few PG 2 strains where its presence is conserved.

Even though phenolic compound degrading pathways have been bioinformatically characterized in several *P. syringae* strains belonging to different PG's, their functionality has yet to be elucidated. Recently, Caballo-Ponce et al. (2017) assessed the functionality of phenolic compound degradation pathways in *Pseudomonas syringae* pv. *savastanoi* NCPPB 3335 and their effect on virulence. They found that the pathways are indeed functional and have a significant influence on virulence.

The aim of this study was to characterize phenolic compound degrading pathways in *P. syringae* PG 2 strains, other than the previously identified gene clusters encoding enzymes in the beta-ketoadipate and protocatechuate pathways (Green et al., 2010; Nowell et al., 2016), and to determine if these pathways are functional.

Materials and methods

***Pseudomonas syringae* strains used for analyses** The genomes of 21 publicly available *P. syringae* strains from seven pathovars belonging to PG 2 were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/). These genomes were analysed together with that of a PG 2 *P. syringae* pv. *syringae* genome of a strain recently isolated from a bacterial canker- infected cherry tree in South Africa (Table 1).

Identification of phenolic compound degradation genes The rapid annotation using subsystem technology (RAST) of *P. syringae* strains in PG 2 was used to detect the presence of the WHOP region and other phenolic compound degrading genes/enzymes known to be involved in the degradation of these compounds. Genes/gene clusters that were identified as being involved in the degradation of these compounds were re-annotated using results from BLAST searches in NCBI (BLAST P) to confirm the RAST annotation.

Functional evaluation of the metabolic pathways The five *P. syringae* strains (HRI-W 7924, HRI-W 2339, HRI-W 2340, MAFF 301072, BRIP 34881) shown to have the phenolic

Table 1 *Pseudomonas syringae* PG 2 strains used for analyses

Pathovar	Strain	Host	Trait (W/H)*
Pseudomonas syringae pv. syringae	PS 126	*Prunus avium*	W
Pseudomonas syringae pv. japonica	MAFF301072	*Hordeum vulgaris*	H
Pseudomonas syringae pv. papulans	CFBP1754	*Malus domestica*	W
Pseudomonas syringae pv. pisi	1704B	*Pisum sativum*	H
Pseudomonas syringae pv. syringae	B728A	*Phaseolus vulgaris*	H
	BRIP34876	*Hordeum vulgare*	H
	BRIP34881	*Hordeum vulgare*	H
Pseudomonas syringae pv. atrofaciens	DSM 50255	*Triticum aestivum*	H
Pseudomonas syringae pv. atrofaciens	LMG 5095	*Triticum aestivum*	H
Pseudomonas syringae pv. panici	LMG 2367	*Panicium miliaceum*	H
Pseudomonas syringae pv. avellanae	ISPaVe037	*Corylus avellana*	W
Pseudomonas syringae pv. avellanae	ISPaVe013	*Corylus avellana*	W
Pseudomonas syringae pv. syringae	1212	*Pisum sativum*	H
Pseudomonas syringae pv. syringae	HRI-W 7872	*Prunus domestica*	W
Pseudomonas syringae pv. syringae	B301D-R	*Pyrus communis*	W
Pseudomonas syringae pv. syringae	NRS 2340	*Pyrus sp.*	W
Pseudomonas syringae pv. syringae	NRS 2339	*Prunus avium*	W
Pseudomonas syringae pv. syringae	HRI-W 7924	*Prunus cerasus*	W
	Cit 7	*Citrus sinensis*	W
	BRIP 39023	*Hordeum vulgare*	H
	642		E

*Trait designation based on host type: H, herbaceous host; W, woody host; E, environment

compound degradation enzymes were further evaluated using assays with pure compounds. Results from assays were evaluated using both spectrophotometer and HPLC analyses.

Spectrophotometric measurements The selected PG 2 *P. syringae* strains were grown on nutrient agar (NA) (Oxoid) for two days at 28 °C. Thereafter, each of the strains was transferred using an inoculation loop to M9 minimal medium consisting of 200 ml M9 salts (16 g Na_2 HPO_4 -$7H_2$ O, 3.75 g KH_2PO_4, 0.63 g NaCl, 1.3 g NH_4Cl), 2 ml 1 M $MgSO_4$, 20 ml 20% glucose, 100 µl 1 M $CaCl_2$ and 800 ml distilled H_2O. Triplicate cultures in 50 ml M9 minimal medium were established in 250 ml Erlenmeyer flasks containing either catechol (300 µM), benzoic acid (500 µM) or anthranilic acid (500 µM). Two negative controls, containing only M9 minimal medium amended with different compounds, were included in this study. All samples were incubated on a shaker (90 rpm) for 24 h at 28 °C. To determine if there was any compound degradation, a Nanodrop 2000 (Thermo Fischer Scientific, USA) was used to measure both the concentration of the compound and bacterial growth every 6 h over a period of 24 h. Catechol, anthranilic, benzoic and salicylic acid were measured at 280 nm, 254 nm, 280 nm and 230 nm, respectively, while bacterial growth was measured at 600 nm. At each of the time points, two mls of each sample were transferred to a sterile Eppendorf tube and subsequently frozen at −80 °C for further high-performance liquid chromatography coupled to an ultraviolet diode array detector (HPLC-DAD) analysis of the phenolic substrates. Phenolic compound degradation was assumed possible if there was a significant difference ($p < 0.01$) between the initial absorbance reading at 0 h and at 24 h. The measurements of all negative controls in this study showed that there was no significant ($p < 0.01$) spontaneous degradation of the compounds between 0 h and 24 h.

HPLC-DAD To confirm the degradation of phenolic compounds, samples that were collected previously for nanodrop analysis were analysed in duplicate at 0 h and 24 h. Negative controls were also included in duplicate. Phenolic compound standards (catechol, benzoic acid, and anthranilic acid) were prepared in 1% (*v*/v) HCl in absolute methanol at concentrations of 100, 75, 50, 25 and 12.5 ppm. These were also filter-sterilized using a syringe with a 0.45 µm PTFE filter (Merck, South Africa) and 20 µl aliquots of the filtered standards were injected in the column and separated singly and in combination to generate standard calibration curves.

HPLC analysis was performed as described by Svensson et al. (2010). Briefly, a HPLC system consisting of a binary pump, DAD detector, autosampler and column oven (Shimadzu, Japan) was used. The samples were filter-sterilized using a syringe with a 0.45 µm PTFE filter and 20 µl of the filtered extract was injected into the HPLC system. The separation of the samples was done on a Kinetex C18 (100 × 4.6 mm, 2.6 µm) reverse phase column (Phenomenex, USA). Separation was carried out at 25 °C with a flow rate of 0.6 ml/min using a 30-min gradient described by Svensson et al. (2010) and monitored at 280 nm, 230 nm, and 254 nm using mobile phases consisting of A (0.1% *v*/v acetic acid in water) and B (0.1% v/v acetic acid in acetonitrile).

Statistical analysis of spectrophotometer and HPLC results To determine if there was a significant difference in the compound

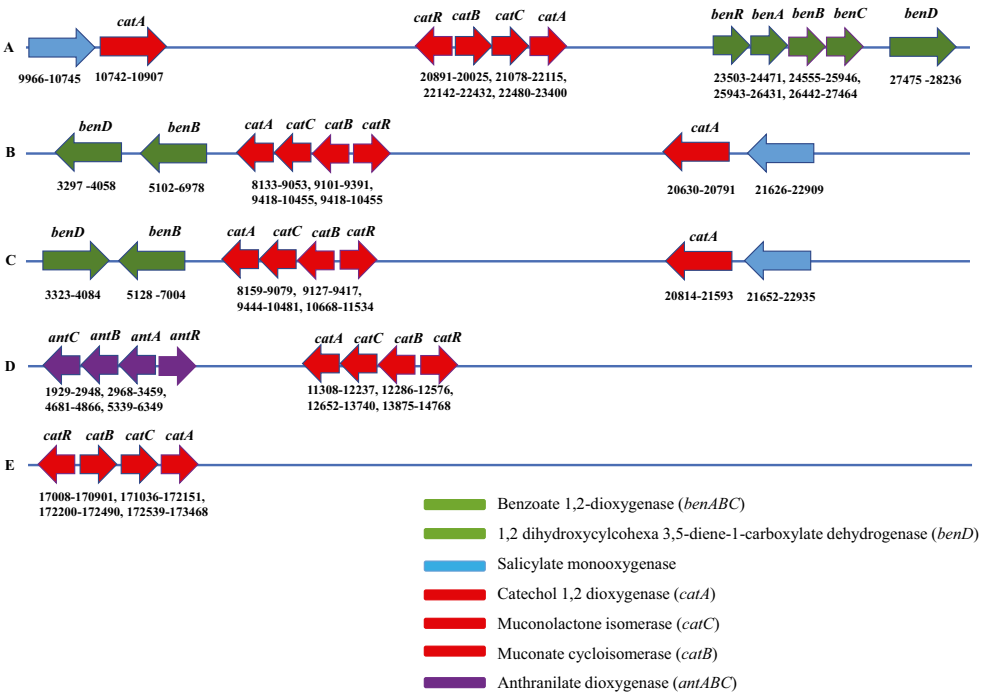

Fig. 1 Phenolic compound degrading genes present in *P. syringae* PG 2 strains; **a** HRI-W 7924, **b** HRI-W 2339, **c** HRI-W 2340, D) MAFF 301072, E) BRIP 34881

concentrations after 24 h, the spectrophotometer and HPLC data were analysed by one-way analysis of variance (ANOVA). Mean separations were performed by Tukey's test using R software (https://www.r-project.org). Differences at $p \leq 0.01$ were considered significant.

Results

Identification of phenolic compound degradation genes Five PG 2 strains, *P. syringae* pv. *syringae* HRI-W 7924, HRI-W 2339, HRI-W 2340, isolated from woody hosts and MAFF 301072 and *Pseudomonas syringae* pv. *avii* BRIP 34881 isolated from herbaceous hosts, were shown to have genes in clusters that could potentially function as a pathway to degrade aromatic compounds. The *catBCA* operon was present in all strains (Fig. 1). The presence of salicylic acid monooxygenase (*sal*), an enzyme responsible for the conversion of salicylic acid to catechol, was present in HRI-W 7924, HRI-W 2339, HRI-W 2340 (Fig. 1). However, this gene lacked an expression regulator (*salR*).

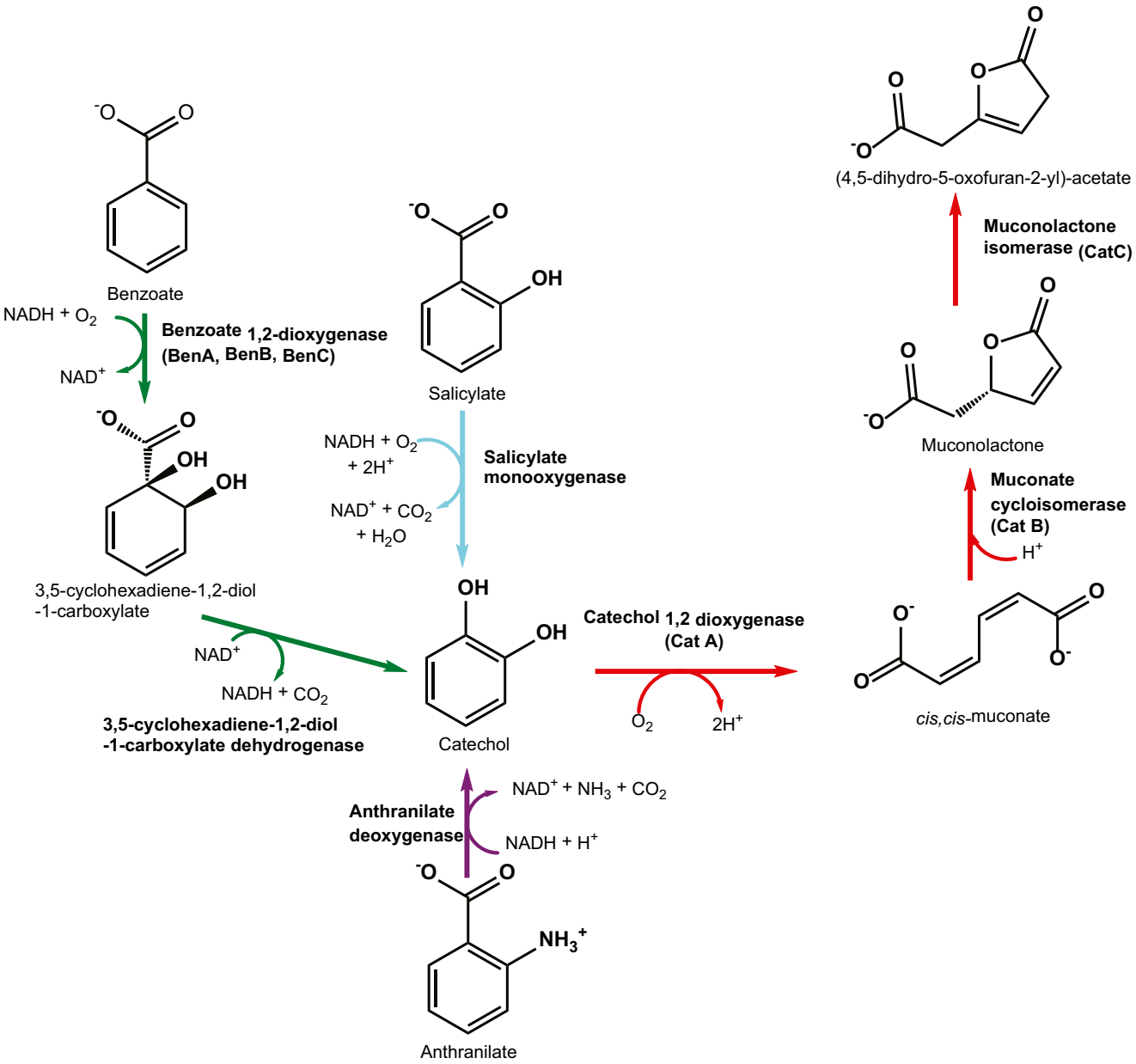

Fig. 2 Proposed metabolic pathways for *P. syringae* PG 2 strains. According to the enzymes present in the PG 2 strains, all strains were hypothesized to be able to metabolize catechol (red) due to the presence of the *catBCA* operon; MAFF 301072 and BRIP 34881 were hypothesized to be able to metabolize anthranilate (purple) and benzoic acid (green) due to the presence of the *antABC* and *benABC* operons respectively; none of the strains were hypothesized to be able to metabolize salicylic acid (blue)

In addition, HRI-W 7924 had the *benABC* operon while MAFF 301072 was the only strain to have the *antABC* operon in its genome (Fig. 1). The operons found in the latter strain corresponded to the findings of Caballo-Ponce et al. (2017). Overall, the findings of this study confirmed those of Caballo-Ponce et al. (2017) as it showed partially conserved WHOP associated phenolic compound degradation enzymes in only a few (20%) of the analysed *P. syringae* PG 2 strains.

Based on the presence of aromatic compound degradation enzymes/operons identified in a few selected PG2 strains, the following metabolic pathways were proposed for these bacteria (Fig. 2): all strains were hypothesized to be able to metabolize catechol as they all have the *catBCA* operon (Figs. 1 and 2).

Even though strains HRI-W 7924, HRI-W 2340 and NRS 2339 show the presence of salicylic monooxygenase (*sal*), they lack the expression regulator for this enzyme, *salR* identified by Sato et al. (2001) in *Pseudomonas putida* S-1. This enzyme was thus considered to be non-functional. Strains MAFF 301072 and HRI-W 7924 would be the only strains able to degrade anthranilic and benzoic acid, respectively, due to the presence of the *antABC* and *benABC* operons (Fig. 1).

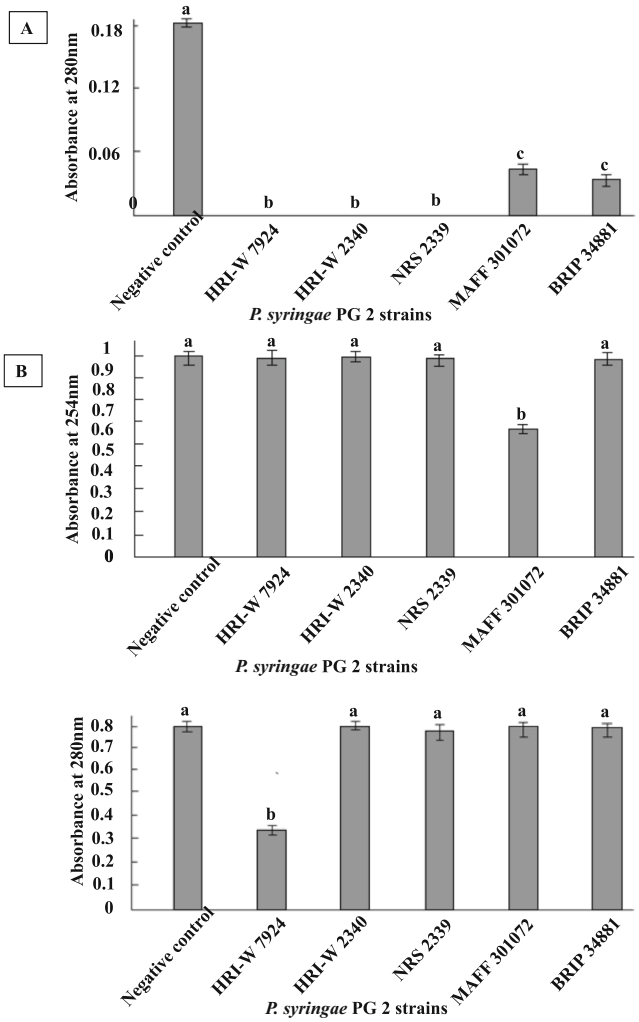

Fig. 3 Spectrophotometric results showing degradation of **a** catechol, **b** anthranilic, **c**) benzoic acid after 24 h. Average values with different letters differ significantly from each other ($p < 0.01$)

Functional evaluation of metabolic pathways. Spectrophotometric evaluation of phenolics degradation All strains were capable of catechol degradation (Fig. 3a) while only one strain, MAFF301072, showed the ability to degrade anthranilic acid (Fig. 3b) and another strain, HRI-W 7924, could degrade benzoic acid (Fig. 3c).

Chromatographic analysis of phenolics degradation HPLC analysis confirmed that all five strains had the ability to degrade catechol (Fig. 4a). Complete degradation of the compound by HRI-W 7924, HRI-W 2340 and NRS 2339 occurred after 24 h. MAFF 301072 and BRIP 34881 were less efficient in degrading catechol than the other strains. However, MAFF 301072 and BRIP 34881 could metabolize anthranilic and benzoic acid, respectively. In the case of MAFF 301072, 357 μM anthranilate (Fig. 4b) and for HRI-W 7924, 184 μM benzoate (Fig. 4c) was degraded in 24 h. The presence of catechol was recorded at a retention time of 6.5 min (Fig. 5).

Discussion

There has recently been a significant increase in reports of diseases caused by *P. syringae* on woody hosts (Lamichhane et al., 2014). Substantial effort has thus been made to understand the molecular basis of pathogenicity of this bacterium on woody hosts (Green et al., 2010; Nowell et al., 2016; Caballo-Ponce et al., 2017). The catabolism of plant derived aromatic compounds for adaption of *P. syringae* strains to woody hosts has been suggested by several authors (Green et al., 2010; Rodríguez-Palenzuela et al., 2010; O'Brien et al., 2011; Ramos et al., 2012).

Our current study confirmed the findings of Nowell et al. (2016) showing that the entire beta-ketoadipate and protocatechuate operons do not occur in any *P. syringae* PG 2 strains. In addition, these strains have a reduced type III secretion repertoire compared to PG 1 and PG 3 strains. It was therefore proposed that they have alternate virulence mechanisms (Nowell et al. 2016). Even though the pathways were not identified, the genes encoding certain phenolic compound degrading enzymes of the recently identified WHOP region, including the *catBCA* and *antABC*, were found to be present in more than 25% of the PG2 strains we analysed. The findings of this study correspond to those of Caballo-Ponce et al. (2017) showing a partial conservation of the WHOP region in these strains. In addition to metabolic pathways encoded by the WHOP region, an additional phenolic compound degrading operon, *benABC,* involved in the catabolism of benzoate to catechol was identified in HRI-W7924. The presence of this operon has not yet been described for any other *P. syringae* strains pathogenic on woody hosts.

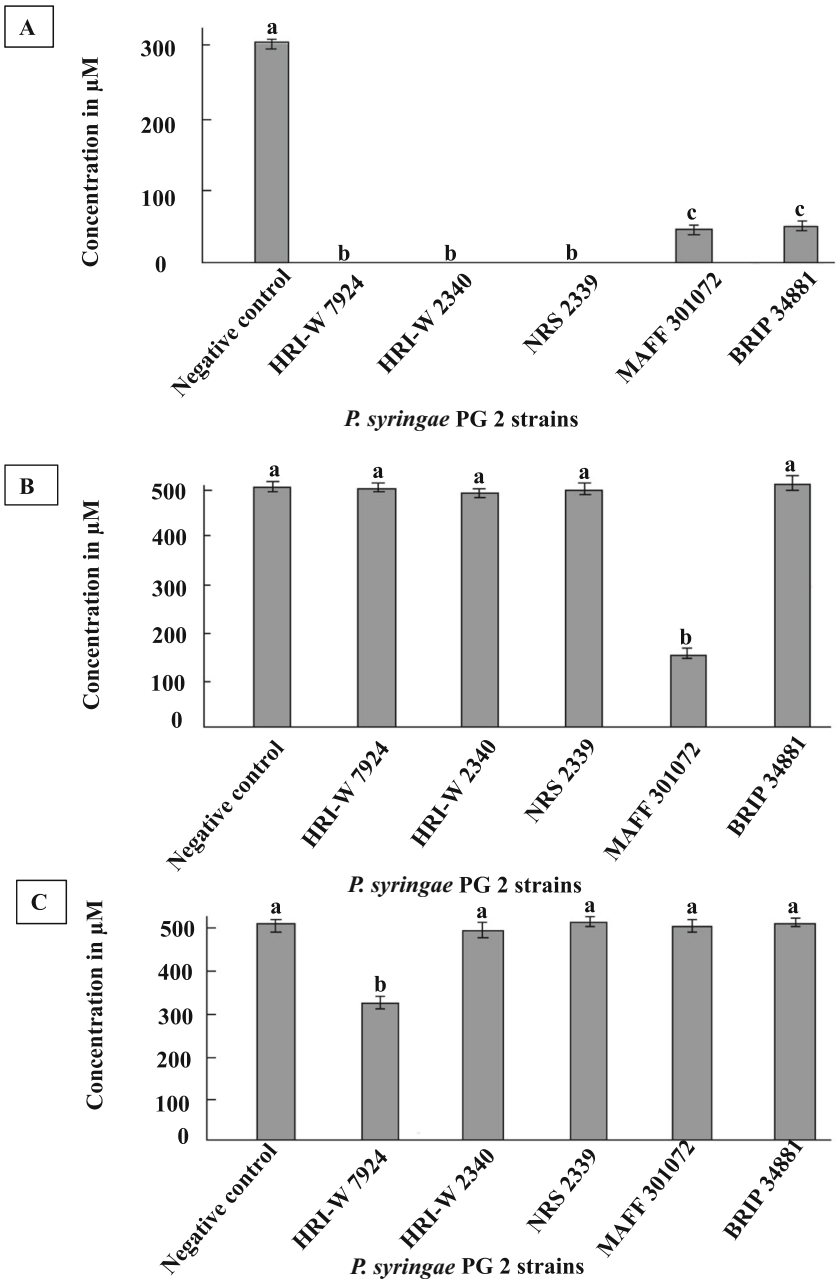

Fig. 4 Quantitative results showing degradation of **a** catechol, **b** anthranilic, **c** benzoic acid after 24 h. Average values with different letters differed significantly ($p < 0.01$)

Besides the identification of metabolic pathways for phenolic compound degradation, this study has also shown the functionality of these pathways. Even though the beta-ketoadipate and protocatechuate pathways have been identified in PG 3 strains such as in *P. syringae* pv. *aesculi* by Green et al. (2010), their functionality has not been assessed. Currently, there is still a lack of functionality assays for phenolic degradation pathways found in *P. syringae* strains pathogenic on woody hosts and only in one strain, *P. savastanoi* pv. *savastanoi* NCPPB 3335, the presence and functionality of the *catBCA*, *antABC* and *ipoABC* operons was demonstrated (Caballo-Ponce et al., 2017).

The degradation of catechol by all strains used in this study is explained by the presence of the *catBCA* operon which is responsible for breaking down catechol into β-ketoadipate enol-lactone via the *ortho* (β-ketoadipate) pathway in *Pseudomonas* spp. (Houghton et al., 1995; Li et al., 2010; Nojiri et al., 2002; Ornston, 1966). Even though *P. syringae* PG 2 strains do not have the full beta-ketoadipate pathway, as do other PG 1 and 3 strains, which would result in the catabolism of catechol to tricarboxylic acid cycle intermediates succinyl-CoA and acetyl-CoA, they can metabolize catechol to (4,5-dihydro-5-oxofuran-2-yl)-acetate. The more rapid degradation of catechol by HRI-W 7924, HRI-W 2340 and NRS 2339 than MAFF 301072 and BRIP34881, as was shown by both spectrophotometer measurements as well as HPLC analysis, could be explained by the presence of a second catechol 1,2-dioxygenase

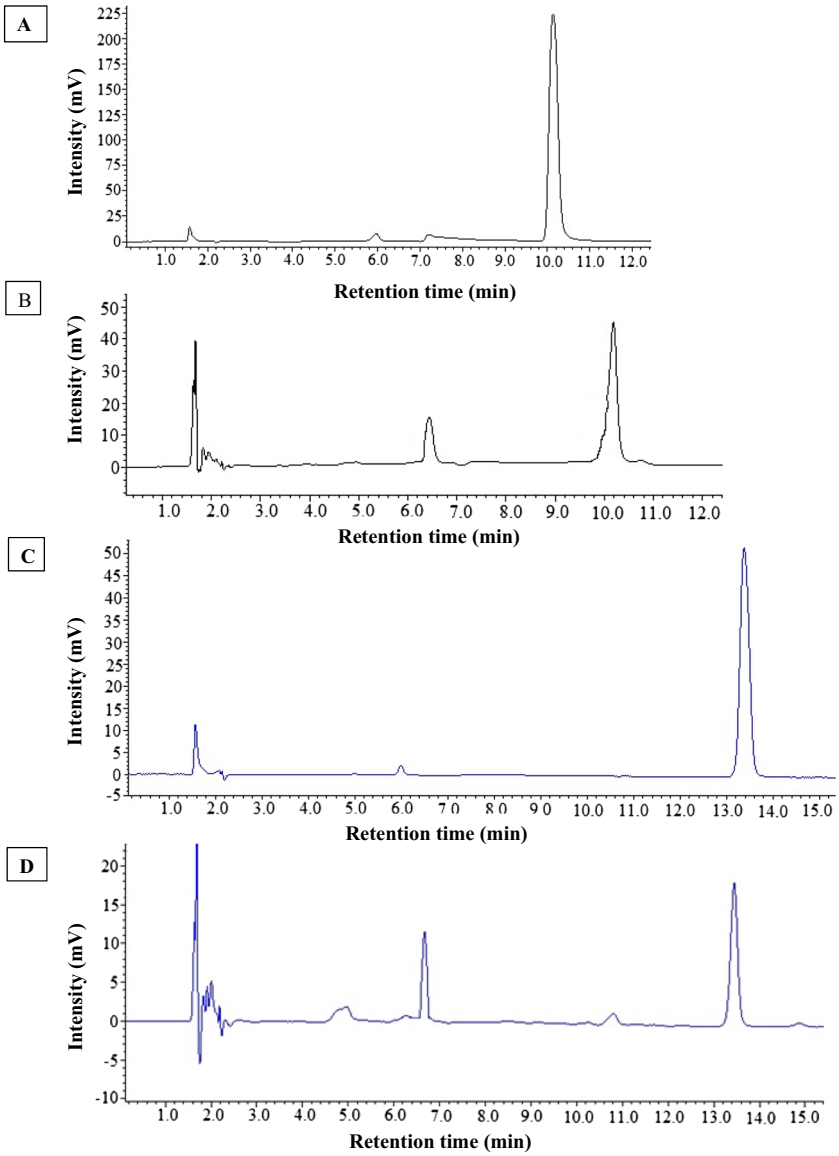

Fig. 5 HPLC chromatograms of anthranilate and benzoate degradation by *P. syringae* PG 2 strains; **a** Anthranilate control; **b** Anthanilate degradation by MAFF 301072; **c** Benzoate control; **d** Benzoate degradation by HRI-W 7924

copy not fond in the other strains. The link between catechol 1,2-dioxygenase copy number and virulence has not yet been demonstrated but van der Nest et al. (2015) found that pathogenic fungi in the Ceratocystidaceae have more copies of carbohydrate-active enzymes (invertases) responsible for hydrolysing glycosidic bonds than closely related saprophytes. Since the presence of the *catBCA* operon has been linked to virulence in strains belonging to the *P. syringae* complex by Caballo-Ponce et al. (2017), it would be interesting to investigate whether additional copies of this operon or genes in this operon have an influence on the degree of virulence in the respective *P. syringae* strains pathogenic on woody hosts.

The ability of MAFF 301072 and HRI-W 7924 to degrade anthranilic and benzoic acid respectively, is due to the presence of the *antABC* and *benABC* operon in these strains. In both cases, there was incomplete degradation of the compounds and the subsequent formation of catechol could be observed. The formation of catechol from the degradation of anthranilate corresponds with the results obtained by Taniuchi et al. (1964) and Caballo-Ponce et al. (2017) for *Pseudomonas resinovorans* CA10 and *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335, respectively. Even though HRI-W 2340 and NRS 2339 showed some elements of the *benABC* operon including the benzoate 1,2 dioxygenase beta subunit (*benB*) and 1,2-dihydroxycyclohexa-3,4-diene-1-carboxylate dehydrogenase (*benD*) they were not able to degrade benzoic acid. This is probably due to the fact that the other elements of this operon including the benzoate 1,2 dioxygenase alpha subunit (*benA*), benzoate dioxygenase, ferredoxin reductase component (*benC*) and *benABC* operon transcriptional activator (*benR*) which are necessary for this pathway to be functional are absent. The partial presence of

these operons can be explained by loss or gain of some elements of this pathway through genetic recombination events such as horizontal gene transfer (Omelchenko et al., 2003).

This is the first study demonstrating the presence and functionality of phenolic compound degradation enzymes of *P. syringae* PG 2 strains. Additional research is required to determine if they play a significant role in virulence on the respective hosts as it was shown for *P. savastanoi* pv. *savastanoi* NCPPB 3335 on olive trees by Caballo-Ponce et al. (2017).

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest. No humans or animals were involved in the execution of this research. All authors have consented to the submission of this manuscript to the Journal of Plant Pathology.

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