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Diverse and strain-specific metabolites patterns induced by fungal endophytes in grape cells of different varieties

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Summary

The potential for endophytes to initiate changes in host secondary metabolism has been well documented. However, the mechanisms underlying endophyte-plant metabolic interactions are still poorly understood. Here, we analysed the effects of fungal endophytes on the metabolite profiles of grape cells from two cultivars: 'Cabernet Sauvignon' (CS) and 'Rose honey' (RH). Our results clearly showed that co-culture with endophytic fungi greatly modified the metabolic profiles in grape cells of both varieties. Treatments with endophytic fungal strains caused the numbers of detected metabolites to vary from 10 to 19 in CS cells and from 8 to 14 in RH cells. In addition, 5 metabolites were detected in all CS cell samples, while 4 metabolites were detected in all RH cell samples. Some endophytic fungal strains could even introduce novel metabolites into the co-cultured grape cells. The metabolic profiles of grape leaves shaped by endophytic fungi exhibited host selectivity and fungal strain specificity. In this assay, the fungal strains RH32 (*Alternaria* sp.) and MDR36 (*Colletotrichum* sp.) triggered an increased response of the detected metabolites, including the greatest increase in the metabolite contents in grape cells of both cultivars. No obvious effects in terms of metabolite numbers and contents in grape cells when co-cultured with fungal strains RH7 (*Epicoccum* sp.) and RH48 (*Colletotrichum* sp.) were observed. The results of this experiment suggest that endophytic fungi could be used to control the metabolic profiles of grapes and thus increase grape quality.

K e y w o r d s : endophytic fungi; grape cells; co-cultivation; secondary metabolites; high pressure liquid chromatography (HPLC).

Introduction

The term 'endophyte' was introduced by De Bary (1866), and was later defined as symbiotic organisms that live within healthy plant tissues or organs without causing any overt symptoms (Stone *et al.* 2000). In general, endophytes comprise fungal, bacterial, archaeal, and protist taxa (Hardoim *et al.* 2015). These microorganisms establish communities in the plant and engage in intimate associations with their hosts (Hardoim *et al.* 2015, Kusari *et al.* 2012). The interplay between plant and endophytes has mostly been acknowledged as a mutualistic interaction that benefits both partners. Specifically, the host plant provides habitation and nutriment for endophytes, and endophytes produce bioactive constituents (plant growth regulatory, antibacterial, antifungal, antiviral, insecticidal constituents) 'in return' to enhance host adaptability to natural environments (Marks and Clay 1996, Silvia *et al.* 2007, Kuldau and Bacon 2008, Ownley *et al.* 2008, Rodriguez *et al.* 2009).

Endophytes did not receive much attention until the detection of taxol (also known as paclitaxel) and related compounds produced by the endophytic fungus *Taxomyces andreanae* that had been isolated from *Taxus brevifolia* (Stierle *et al.* 1993, 1995). Bioactive compounds from endophytic fungi have been traditionally classified according to their reported anticancer, antioxidant, antifungal and antibacterial properties. The biochemical nature of endophytes confers the ability to synthesize bioactive metabolites similar to those found in their host plant, which could be used as therapeutic drugs against numerous diseases (Strobel *et al.* 2004, Staniek *et al.* 2008, Aly *et al.* 2010, Kharwar *et al.* 2011, Kusari and Spiteller 2012). Grapevines harbour diverse fungal endophytes that are the source of the 'terroir' of grape wine qualities and its associated characteristics. During the life of grapevines, endophytic fungi play im-

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portant roles as beneficial microorganisms or pathogens. Many studies on the fungal grapevine community have been conducted, but exactly how the fungal community coexists within the plant and influences the 'terroir' of grapes is unknown. Because of the complexity of endophytic communities within grapevines, determining which fungi control grape metabolism *in vivo* is difficult. In our previous work, the impacts of different endophytic fungal strains on the metabolite profiles of grape cells of 'Cabernet Sauvignon' (CS) were studied in dual culture. However, whether there are genus-specific differences in the response to endophytic fungal infection between different varieties of grape cells is unclear. Here, 14 strains of endophytic fungal isolated from grapevine leaves of RH were co-cultured with grape cells of CS and 'Rose honey' (RH), and variation in the metabolites of grape cells were analysed by high-pressure liquid chromatography (HPLC).

Material and Methods

Grape cells: Grape cells induced from grape flesh (*V. vinifera* cv. CS and *V. vinifera* L. × *V. labrusca* L. RH) were sub-cultured in a growth chamber at 25 °C on G5 solid medium. The medium for grape cell sub-culture was composed of macro- and microelements (MURASHIGE and SKOOG 1962) supplemented with 3 % (m/v) sucrose, 0.75 % (m/v) agar, 0.93 μM cytokinin, 0.54 μM naphthylacetic acid, and vitamins (0.56 mM myo-inositol, 4.56 μM pantothenic acid, 2.97 μM thiamine HCl, 4.91 μM pyridoxine HCl, 8.12 μM nicotinic acid, and 0.04 μM biotin). The pH was adjusted to 5.8 with 1M NaOH. After autoclaving, 30 mL of solid medium was distributed in Petri dishes. Both of the two grape cells (approximately 2.0 g of grape cells per dish) were inoculated onto the centre of every Petri dish and recovered for one week at 25 °C.

Endophytic fungal strain: We isolated 14 endophytes (Tab. 1) from the leaves of the grape variety RH in local vineyards (Yunnan Province, China). All strains were identified by their ITS DNA sequences and grown on Petri dishes containing potato dextrose agar (PDA) medium at 26 °C for one week in the dark (PDA medium composed of peeled and diced potato 200 g, dextrose 20 g, and agar 15 $g \cdot L^{-1}$).

Table 1

Endophytic fungal strains used in the experiment

Strain ID	Species	Strain ID	Species
RH7	*Epicoccum* sp.	RH48	*Colletotrichum* sp.
RH12	*Nigrospora* sp.	RH49	*Fusarium* sp.
RH32	*Alternaria* sp.	MDR1	*Nigrospora* sp.
RH34	*Trichothecium* sp.	MDR3	*Fusarium* sp.
RH36	*Fusarium* sp.	MDR4	*Fusarium* sp.
RH44	*Alternaria* sp.	MDR33	*Colletotrichum* sp.
RH47	*Fusarium* sp.	MDR36	*Colletotrichum* sp.

Inoculation of endophytes on grape cell cultures: Each endophytic fungus was fully suspended in 0.9 % (m/v) normal saline (final concentration was 2.5 $g \cdot L^{-1}$), followed by inoculating 4 μL (*i.e.* 10 μg) of the suspension onto the grape cells. The control group had normal saline solution which did not include any fungi. The co-cultures and the control group were dark-cultured at 25 °C for another 2 weeks. Five biological replicates were performed for each experiment, but only 3 biological replicates with relatively uniform traits were performed for the HPLC assay (individuals with contamination or different degrees of callus oxidation were not considered for HPLC assays).

Sample harvest and pre-treatment: Grape cells were harvested after 2 weeks co-culture, and as much fungal mycelia as possible (including some grape cells that were obviously contaminated with fungal mycelia) were carefully removed with tweezers or a surgical knife. For the HPLC assay, the cells were dried at 110 °C for 10 min, followed by 60 °C for 2 or 3 d until a constant weight was achieved. The grape cells were grounded into powder, and 100 mg of dried grape callus powder was accurately weighed and then extracted with 1 mL of methanol (containing 0.1 % of hydrochloric acid) for 12 h, followed by sonication for 60 min. The extracts were centrifuged for 10 min at 4,000 g at 4 °C, and supernatants were then filtered with 0.45-μm filter columns.

HPLC assay: 10 μL of the filtrates were loaded for analysis on a reversed-phase C_{18} column (Thermo) on an HPLC instrument (Agilent, USA). The elution phase was acetonitrile (Sigma, St. Louis, MO, USA): methanol (Sigma, St. Louis, MO, USA): water (A:B:C, 95:0.5:4.5, v/v/v), and detection was completed with a UV detector at 254 nm, with an elution speed of 1 $mL \cdot min^{-1}$ and a column temperature of 30 °C. Samples were eluted with the gradient procedures (suppl. Tab. 1). Contents of metabolites ($mg \cdot g^{-1}$) were quantified using catechin as a standard with an $r^2 = 0.994$.

Statistical analysis: Data were analysed using Microsoft Excel 2013. Values were represented as means of three replicates (mean ± SD) for each treatment and were analysed using SPSS Statistics 22.0 software for Windows. One-way analysis of variance (ANOVA) and Tukey's HSD tests were used to determine the significance of the difference among samples at the $P \leq 0.05$ level. Heat maps were generated in Microsoft Excel 2013 based on the content of detected metabolites. Principal component analysis (PCA) was conducted in R.

Results

The HPLC assay revealed that the basic metabolite profiles of CS and RH grape cells were different (Fig. 1). Overall, 28 metabolites were detected from grape cells, and these metabolites were marked from M1 to M28 based on their subsequent retention times registered in HPLC. Fourteen and eight methanol extracts were isolated in CS and RH grape cells, respectively. The contents of metabolites ranged from 0.36 $mg \cdot g^{-1}$ to 13.26 $mg \cdot g^{-1}$ and 0.84 $mg \cdot g^{-1}$ to 5.78 $mg \cdot g^{-1}$ in CS and RH cells, respectively. Six metab-

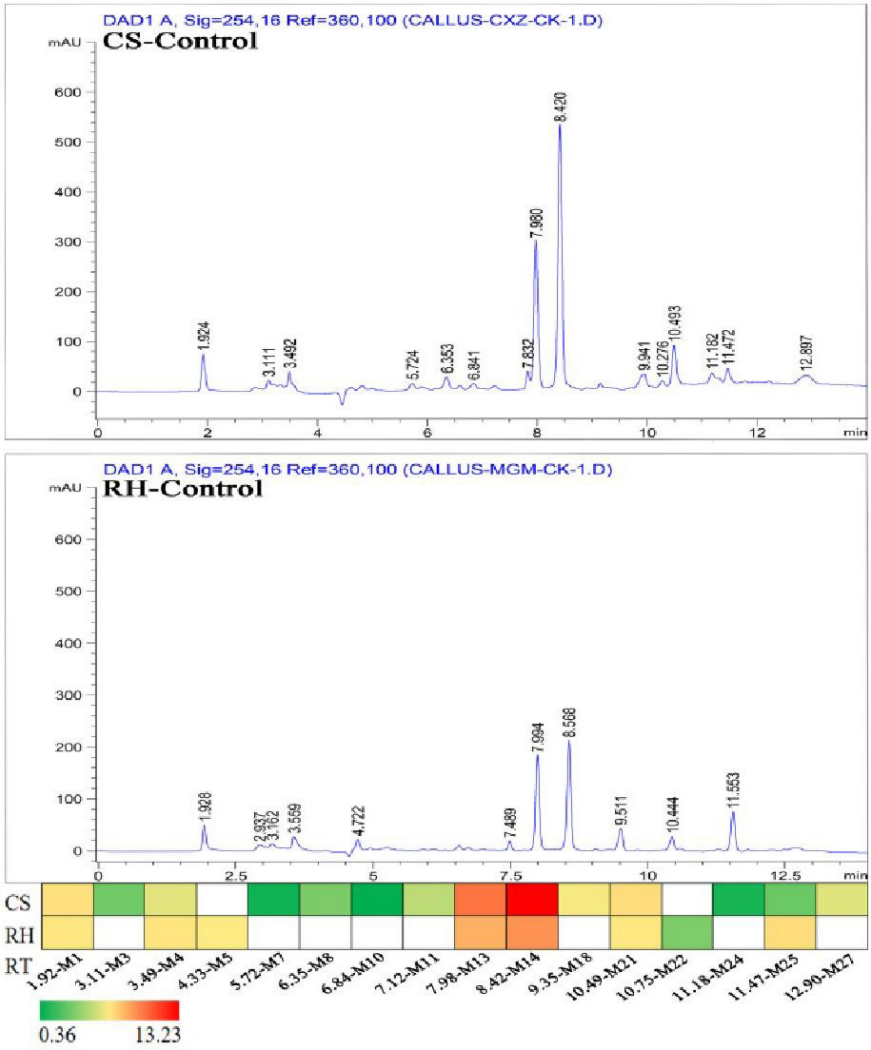

Fig. 1: Basic metabolites profiles from grape cells of CS and RH from the HPLC assay.

olites (M1, M4, M13, M14, M21 and M25) were detected in both CS and RH cells, eight metabolites (M3, M7, M8, M10, M11, M18, M24 and M27) were detected exclusively in CS cells, and 2 metabolites (M5 and M22) were detected exclusively in RH cells.

Aside from the baseline variation in the metabolite profile between CS and RH grape cells, the composition of detected metabolites in grape cells was modified from co-cultivation with fungal endophytes. The detected metabolites in CS cells covered retention times ranging from 1.92 min to 16.52 min, whereas the retention times for the RH cells only ranged from 1.92 min to 11.47 min (Figs 2 and 3; suppl. Figs 1 and 2). Treatments with endophytic fungal strains caused the numbers of detected metabolites to vary from 10 to 19 in CS cells and from eight to 14 in RH cells (Figs 2 and 3; Tab. 2). In CS grape cells, the contents of individual metabolites ranged from 0.27 $mg \cdot g^{-1}$ to 30.40 $mg \cdot g^{-1}$; in RH cells, the detected metabolite contents ranged from 0.39 $mg \cdot g^{-1}$ to 14.62 $mg \cdot g^{-1}$ (suppl. Tabs 2 and 3). According to the chromatograms, co-culture with endophytic fungi reshaped the metabolic profiles in grape cells (Figs 2 and 3; Tab. 2). Replicates of one treatment tended to be clustered together for CS and RH cells based on the presence/absence patterns of the detected metabolites (suppl. Figs 3 and 4).

Figs 2 and 3 show the content of HPLC-detected metabolites as well as the clustering analysis of all treatments in the experiment. For CS grape cells, 26 metabolites were detected, and the numbers of detected metabolites in grape cells of different treatments varied from 10 to 19. The metabolite at a retention time of 5.29 min only appeared in fungal strain RH34 (*Trichothecium* sp.) co-cultured CS grape cells (Fig. 2). Metabolites M11, M19, M22, M23 and M28 were detected in CS cells treated with 2 to 4 fungal strains, while other metabolites could be detected in more than 6 fungal strains co-cultured with CS cells. Fungal strain MDR36 produced the highest concentration of metabolite (M14, 30.40 $mg \cdot g^{-1}$) (Tab. 2, suppl. Tab. 2). Additionally, a higher content (> 16 $mg \cdot g^{-1}$) of metabolite M7 was detected in fungal strains RH12, RH47, MDR3 and MDR4-treated CS grape cells. Co-cultivation with fungal strains RH32 and RH34 also produced higher contents (> 16 $mg \cdot g^{-1}$) of metabolite M14 in CS cells.

For RH grape cells, 17 metabolites were detected, and numbers of the detected metabolites in grape cells of different treatments varied from 8 to 12 (Fig. 3, Tab. 2). Metabolite M15 only appeared in fungal strain MDR36-treated grape cells. Metabolites M8, M10, M12 and M25 could only be detected in 2 or 3 fungal strains. Similarly, fungal strain MDR36 produced the highest content of metabolite M14

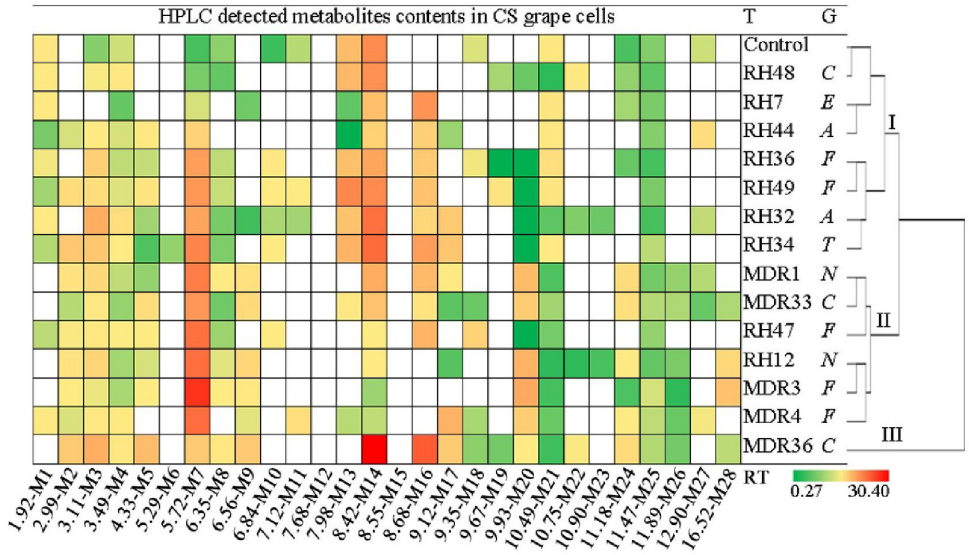

Fig. 2: Heat map and clustering of HPLC detected metabolite contents in CS grape cells. T: treatment (represented as endophytic fungal strain ID and the control). HPLC detected compounds were marked as coloured bricks, and different colours represent the contents of the metabolites. G: genus of the endophytic fungal strains, *C*: *Colletotrichum; E*: *Epicoccum; A*: *Alternaria; F*: *Fusarium; T*: *Trichothecium; N*: *Nigrospora.* RT: retention time displayed when the metabolites appeared in the HPLC assay. M1-M28: metabolites were marked based on their subsequent retention times appearing in HPLC.

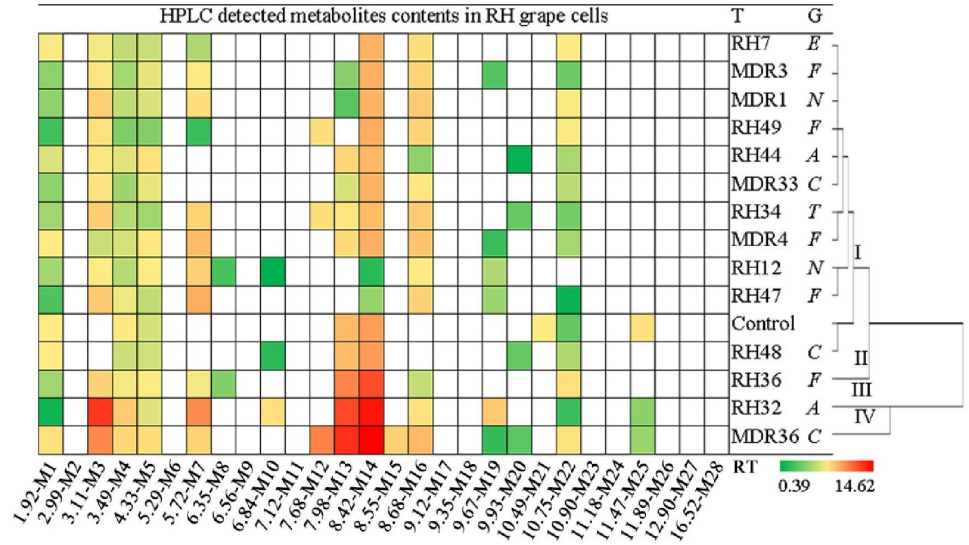

Fig. 3: Heat map and clustering of HPLC-detected metabolite contents in RH grape cells.

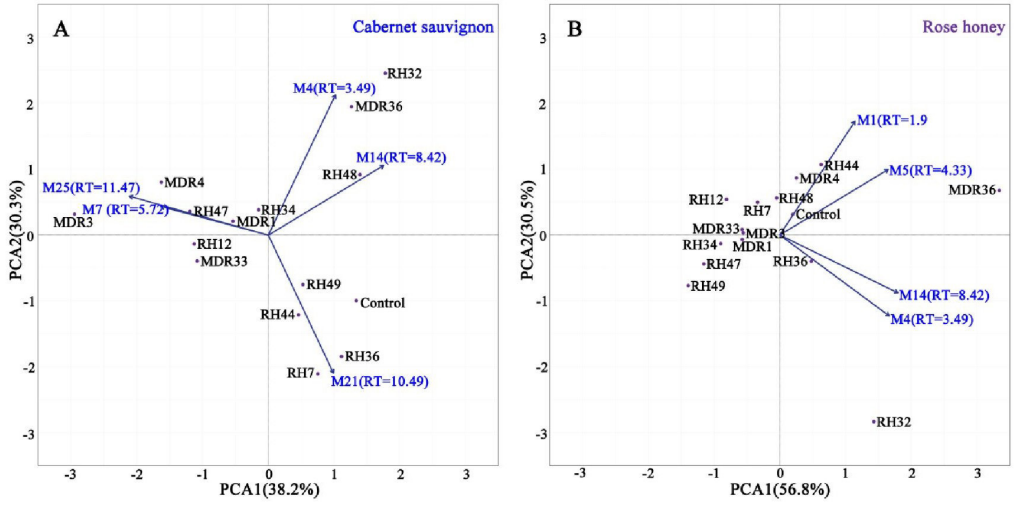

Fig. 4: PCA of the impacts of endophytic fungal inoculation on co-detected metabolites of CS and RH cells.

Table 2

Impacts of endophytic fungal inoculation on special parameters of metabolites

Cells	Treatment	No. of total metabolites detected	No. of novel metabolites detected	No. of suppressed metabolites	Total contents of detected metabolites ($mg \cdot g^{-1}$)	Retention time of maximum metabolites (min)	Contents of maximum metabolites ($mg \cdot g^{-1}$)
CS	Control	14	0	0	32.90	8.42	13.23
	RH7	10	2	6	27.99	8.68	12.15
	RH12	17	9	6	49.27	5.72	16.68
	RH32	18	6	2	64.43	8.42	16.42
	RH34	15	5	4	77.70	8.42	17.16
	RH36	14	2	2	50.54	5.72	10.94
	RH44	12	4	6	27.56	8.68	4.76
	RH47	13	3	4	42.73	5.72	16.45
	RH48	13	3	4	32.09	8.42	12.61
	RH49	15	4	3	66.64	7.98	13.56
	MDR1	16	7	5	60.37	5.72	14.99
	MDR3	14	6	6	52.59	5.72	24.15
	MDR4	17	5	2	50.42	5.72	16.99
	MDR33	19	8	3	48.09	5.72	11.78
	MDR36	19	10	5	102.78	8.42	30.40
RH	Control	8	0	0	19.26	8.42	5.78
	RH7	8	3	3	15.22	8.42	4.68
	RH12	10	6	4	12.93	5.72	3.02
	RH32	12	5	1	56.90	8.42	13.35
	RH34	11	5	2	22.84	8.42	4.12
	RH36	10	4	2	30.73	8.42	10.37
	RH44	9	3	2	16.52	8.42	4.40
	RH47	9	4	3	17.48	5.72	4.95
	RH48	8	2	2	17.00	5.72	5.95
	RH49	9	4	3	17.06	8.42	5.02
	MDR1	9	3	2	19.32	8.42	4.63
	MDR3	10	4	2	17.40	8.42	4.82
	MDR4	10	4	2	22.86	8.42	4.88
	MDR33	8	2	2	14.72	8.42	4.70
	MDR36	14	7	1	63.23	8.42	14.62

(14.62 $mg \cdot g^{-1}$) in RH grape cells (Tab. 2, suppl. Tab. 3). Co-cultivation with endophytic fungal strain RH32 produced higher contents of metabolites M3 and M13 (> 10 $mg \cdot g^{-1}$), and co-culture with fungal strains RH32, RH36 and MDR36 also produced higher contents of metabolite M14 (> 10 $mg \cdot g^{-1}$) in RH grape cells (suppl. Tab. 3). Metabolites M2, M6, M9, M11, M17, M18, M23, M24, M26, M27 and M28, which were detected in CS cells, failed to be detected in RH cells, while metabolites M12 and M15 could be detected in RH cells but were not detected in CS cells.

When clustering analysis was based on the metabolite patterns, all strains could be divided into 3 or 4 groups in CS and RH grape cells, respectively (Figs 2 and 3). For CS grape cells, group 1 included 7 fungal strains that were closely clustered with the control, suggesting that they had fewer metabolomic impacts on CS grape cells (Fig. 2). Except for the control, fungal treatments in group 1 included strains from the genera *Fusarium* (2/5, two of five used in this study), *Alternaria* (2/2), *Colletotrichum* (1/3), *Epicoccum* (1/1) and *Trichothecium* (1/1). In group 2, six strains from the genera *Fusarium* (3/5), *Nigrospora* (2/2) and *Colletotrichum* (1/3) were clustered together. The left group contained only MDR36 (*Colletotrichum*) and had the strongest effect on CS cell metabolomics. In RH grape cells, group 1 included 11 strains that were closely clustered with the control and included strains from the genera *Fusarium* (4/5), *Colletotrichum* (2/3), *Alternaria* (1/2), *Nigrospora* (2/2), *Epicoccum* (1/1) and *Trichothecium* (1/1). Group 2, 3 and 4 included only RH36 (*Fusarium*), RH32 (*Alternaria*) and MDR36 (*Colletotrichum*) respectively and had stronger effects on the metabolomics of RH grape cells (Fig. 3).

Based on HPLC-detected metabolites, treatment of CS grape cells with fungal strains RH32, MDR33 and MDR36 produced the most metabolites (18 or 19), whereas RH7, RH44, RH47 and RH48-treated CS grape cells produced fewer metabolites (10-13) (Fig. 3). Compared with the control, 1 to 10 novel metabolites were introduced in CS grape cells because of the presence of fungal strains (Tab. 2). Co-cultivation with fungal strains RH12, MDR3 and MDR36 introduced the highest number of novel metabolites (8-10), whereas RH7, RH36, RH47, and RH48 introduced fewer novel metabolites into CS grape cells. However, co-culture with fungal strains suppressed the production of 2 to 6 metabolites, compared with the basic metabolite

profile of CS grape cells. Among these fungal strains, RH7, RH12, RH44 and MDR3 suppressed the production of most of the metabolites (6). In RH grape cells, treatments of RH32 and MDR36 produced most metabolites (12 or 14), whereas in RH7, RH44, RH47, RH48, MDR1 and MDR33, fewer metabolites (8-9) were detected in co-cultured RH cells (Tab. 2). Cultivation with fungal strains produced 2 to 7 novel metabolites in RH cells compared with the control. Fungal strains RH12 and MDR36 introduced the most novel metabolites (6 or 7) into RH grape cells, whereas fungal strains RH7, RH44, RH48, MDR1 and MDR33 produced the fewest (2 or 3) novel metabolites in grape cells. Co-cultivation with fungal strains, 1 to 4 metabolites were also suppressed compared with the basic metabolites profile of RH grape cells. RH12 suppressed the highest number of metabolites (4) in RH grape cells.

For both CS and RH grape cells, fungal strains RH32 and MDR36 initiated the highest numbers of metabolites, and strains RH12 and MDR36 introduced the most novel metabolites (Tab. 2). Fewer metabolites were observed in RH7, RH44, RH47 and RH48-treated grape cells. Fewer novel metabolites were detected in RH7 and RH48-treated cells, and RH12 suppressed the highest number of metabolites in both CS and RH grape cells. Additionally, MDR36 had the strongest effect on grape cellular metabolomics in both CS and RH cells, especially metabolite M14 at a retention time of 8.42 min, as the contents of this metabolite in CS and RH grape cells both were at their highest (30.4 $mg \cdot g^{-1}$ and 14.62 $mg \cdot g^{-1}$, respectively).

In addition to the qualitative effects of fungal endophytes on the metabolism of grape cells, quantitative effects were observed for metabolites that were co-detected in all treatments (Tabs 3 and 4). Five metabolites (M4, M7, M14, M21 and M25) were detected in all CS cell samples, and 4 metabolites (M1, M4, M5 and M14) were detected in all RH cells samples. Metabolites M4 and M14 were co-detected in all grape cell samples in this experiment. Contents of these metabolites and differences in significance among treatments were observed (Tabs 3 and 4). Contents of these metabolites in grape cells varied because of the presence of diverse fungal strains, and some of these changes were statistically significant (Tabs 3 and 4). For CS grape cells, RH32, RH34, MDR4 and MDR36 significantly promoted contents of the co-detected metabolites (3 of the 5 metabolites) compared with the control (Tabs 2 and 3). While co-culturing with MDR36 significantly promoted the contents of all the co-detected metabolites in RH grape cells, RH32 significantly promoted the contents of 2 of the co-detected metabolites (Tabs 3 and 4). Metabolite M7 appeared to be strongly induced by all of the used strains in all CS grape cell samples (Tab. 3).

To visualize the effects of endophytic fungi inoculation on co-detected metabolites, PCA was performed (Fig. 4). PC1 and PC2 of CS explained 38.2% and 30.3% of the total variance, respectively. In the plot, PC1 primarily separated the effects of co-detected metabolites based on the contents of metabolites M7, M14 and M25. Metabolite M7 made the largest contribution to PC1, while metabolites M4 and M21 contributed the least. PC2 revealed a contrast between two co-detected metabolites: M4 and M21 (Fig. 4a). The PCA provided a visual representation of the impacts of the inoculation of endophytic fungi on co-detected metabolites. For CS cells, strains RH32 and MDR36 positively contributed to metabolites M4 and M14; strains RH34, RH47, MDR3 and MDR4 contributed positively to metabolites M7 and M25; and strains RH7 and RH36 contributed positively to metabolite M21. However, PC1 and PC2 of RH explained 56.8 % and 30.5 % of the total variance, respectively (Fig. 4b). MDR36 contributed positively to all of the co-detected metabolites; RH32 contributed positively to metabolites M4 and M14; and RH44 contributed positively to metabolites M1 and M5.

Table 3

Comparison of the contents of co-detected metabolites in CS grape cells Values were indicated as 'mean ± standard errors' with different significances marked as '*' or '**', compared to the control. *,** - significant differences between treatments at $P \leq 0.05$ and 0.01, respectively. M: metabolite; T: treatment

M / T	M4 (RT = 3.49)	M7 (RT = 5.72)	M14 (RT = 8.42)	M21 (RT = 10.49)	M25 (RT = 11.47)
Control	1.21 ± 0.01	0.42 ± 0.02	13.23 ± 0.23	2.06 ± 0.05	0.76 ± 0.01
RH7	**0.60 ± 0.03****	1.26 ± 0.15	**7.01 ± 0.33****	2.30 ± 0.02	0.71 ± 0.02
RH12	0.97 ± 0.06	**16.68 ± 0.51****	**1.66 ± 0.01****	**0.28 ± 0.01****	0.59 ± 0.01
RH32	**3.17 ± 0.02****	**10.17 ± 0.01****	**16.42 ± 0.03****	**0.55 ± 0.01****	**0.42 ± 0.00****
RH34	1.55 ± 0.25	**13.92 ± 0.40****	**16.42 ± 0.03****	1.65 ± 0.07	**1.10 ± 0.06***
RH36	1.13 ± 0.08	**10.94 ± 0.82****	**10.04 ± 0.26****	**3.08 ± 0.17***	**0.42 ± 0.07****
RH44	1.26 ± 0.16	**4.33 ± 0.16****	**4.58 ± 0.13****	2.09 ± 0.13	0.78 ± 0.13
RH47	1.56 ± 0.03	**16.45 ± 0.51****	**1.59 ± 0.09****	**0.69 ± 0.00****	0.86 ± 0.09
RH48	**1.92 ± 0.07****	0.71 ± 0.01	12.61 ± 0.16	**0.27 ± 0.00****	0.56 ± 0.01
RH49	1.31 ± 0.00	**11.92 ± 0.12****	13.32 ± 0.73	2.29 ± 0.01	0.73 ± 0.02
MDR1	1.12 ± 0.29	**15.00 ± 0.12****	**9.23 ± 0.13****	**0.48 ± 0.02****	0.68 ± 0.00
MDR3	0.99 ± 0.00	**24.15 ± 0.08****	**0.92 ± 0.00****	**0.41 ± 0.00****	**1.25 ± 0.01****
MDR4	**1.80 ± 0.19****	**16.99 ± 0.27****	**1.13 ± 0.02****	**0.62 ± 0.06****	**1.11 ± 0.18****
MDR33	0.88 ± 0.04	**11.78 ± 0.16****	**6.51 ± 0.06****	**0.96 ± 0.01****	**1.06 ± 0.01***
MDR36	**1.87 ± 0.07****	**5.49 ± 0.28****	**30.40 ± 0.10****	**0.38 ± 0.01****	1.05 ± 0.01

Table 4

Comparison of the contents of co-detected metabolites in RH grape cells (significance: $P \leq 0.05$)

T \ M	M1 (RT = 1.92)	M4 (RT = 3.49)	M5 (RT = 4.33)	M14 (RT = 8.42)
Control	1.60 ± 0.13	1.69 ± 0.16	1.35 ± 0.33	5.79 ± 0.27
RH7	1.51 ± 0.00	**1.26 ± 0.03****	1.29 ± 0.08	**4.68 ± 0.09****
RH12	**1.12 ± 0.01****	**1.20 ± 0.00****	1.53 ± 0.02	**0.59 ± 0.01****
RH32	**0.47 ± 0.02****	**3.38 ± 0.22****	1.39 ± 0.02	**13.35 ± 0.19****
RH34	**1.12 ± 0.07****	**1.20 ± 0.04****	1.11 ± 0.12	**4.12 ± 0.01****
RH36	**1.11 ± 0.02****	1.49 ± 0.01	1.53 ± 0.03	**10.37 ± 0.05****
RH44	**1.39 ± 0.01****	**1.40 ± 0.02****	**2.13 ± 0.02****	**4.40 ± 0.03****
RH47	**0.75 ± 0.00****	**1.47 ± 0.02***	1.25 ± 0.01	**1.08 ± 0.05****
RH48	1.63 ± 0.01	**1.30 ± 0.00****	1.32 ± 0.02	5.95 ± 0.12
RH49	**0.68 ± 0.01****	**0.96 ± 0.00****	**0.98 ± 0.04***	**5.02 ± 0.06****
MDR1	**1.04 ± 0.01****	**1.25 ± 0.00****	1.36 ± 0.01	**4.63 ± 0.16****
MDR3	**1.02 ± 0.01****	**1.13 ± 0.01****	1.43 ± 0.02	**4.82 ± 0.09****
MDR4	1.55 ± 0.00	**1.35 ± 0.01****	**1.70 ± 0.05***	**4.88 ± 0.15****
MDR33	**1.03 ± 0.01****	**1.10 ± 0.01****	1.44 ± 0.02	**4.70 ± 0.02****
MDR36	**2.15 ± 0.02****	**2.70 ± 0.01****	**2.32 ± 0.04****	**14.62 ± 0.05****

Discussion

Endophytic fungi showed strong potential to promote growth, enhance resistance, absorb nutrients, and produce compounds similar to those found in host plants (Hardoim *et al.* 2015, Pacifico *et al.* 2019). As previous studies have shown, endophytes can synthesize bioactive metabolites similar to those found in their host plants, which is considered the most promising source of bioactive compounds for agricultural, industrial and medicinal applications (Aly *et al.* 2011). The environmental factors include both abiotic factors (such as light, temperature, radiation, and soil) and biotic factors (such as environmental microorganisms, pathogens and insect pests), which confer the 'terroir' of grape wine qualities and characters (Meinert 2006). Endophytic fungi are a large group in the environmental microbial community that are most closely related to grape plants; thus, their contribution to grape wine quality and characteristics should not be ignored (Vega *et al.* 2008, Gilbert *et al.* 2014). Based on our results, the contents and composition of the detected metabolites were modified both in CS and RH grape leaves because of the presence of fungal endophytes.

To evaluate the presence of catechin in the sample, we used catechin as a reference and added it into CS cells extract treated with strain RH44; the results showed that the peak height and peak area of the metabolite at a retention time of 11.009 min were greatly increased (suppl. Fig. 5). As expected, the retention time was basically the same as that of the extract of strain RH44 treated CS cells (11.055 min) and that of the reference of catechin (10.930 min), indicating that the metabolite at the retention time of 11.055 min was indeed catechin. Based on the extracts preparation and HPLC method of our study, along with the findings of other studies (Chafer *et al.* 2005, Pomar *et al.* 2005, Masa *et al.* 2007, Novak *et al.* 2008, He *et al.* 2010, Ramirez-Lopez *et al.* 2014, Ribeiro *et al.* 2015, Padilha *et al.* 2017), we speculate that the analysed metabolites consisted of certain types of proanthocyanins, organic acids (such as gallic acid, syringic acid, and caffeic acid), flavan-3-ols (such as catechin, epicatechin, and epigallocatechin), flavonols (such as quercetin, rutin, and kaempferol). All of the aforementioned compounds are important sensory components and contribute to the acidity, bitterness and astringency of grape berry and its wines. Thus, controlling endophyte populations in grapevine tissues may have an important effect on the flavour, colour, hue, and quality of final products.

Previous studies have demonstrated the physiological, biochemical and metabolic changes induced by the dual culture of strains with grape cells through their specific interactions (Huang *et al.* 2017). The aim of this study was to examine the responses of grape cells to different endophytic fungi inoculations: specifically, to determine whether there are any general characters or differences in grape cells from different varieties in response to the infection of certain fungal strains. Based on the HPLC findings, the contents of the detected metabolites as well as the composition of metabolites fundamentally differed between the two varieties of grape cells. The number of metabolites detected in CS grape cells without endophytic fungi inoculations was 14, while only 9 were detected in RH grape cells. Overall, co-culture with the same batch of endophytic fungi introduced higher numbers of novel metabolites in CS grape cells and resulted in greater contents of co-detected metabolites relative to RH grape cells (Tabs 2-4). In addition, the highest contents of total metabolites and novel metabolites were detected in samples of CS and RH grape leaves infected with RH32 (*Alternaria* sp.) and MDR36 (*Colletotrichum* sp.) (suppl. Figs 3 and 4). Fewer total metabolites and novel metabolites were detected in treatments with RH7 (*Epicoccum* sp.) and RH48 (*Colletotrichum* sp.) in both CS and RH cells. Furthermore, RH32 and MDR36 both triggered the greatest response of co-detected metabolites, while RH7 and RH48 treatments triggered the weakest responses of co-detected metabolites in the two types of grape cells (Tab. 2).

The ability of endophytes to secrete and produce novel compounds within their host plants has been widely

documented; however, the mechanisms underlying this metabolic interaction between endophytes and plants are poorly understood. The metabolic impact of endophytes on the host plant has been hypothesized to include endophytes self-metabolizing, endophytes and host co-metabolizing, and signaling (LUDWIG-MÜLLER 2015). Our results confirmed the fact that novel metabolites were introduced as well as that the total contents of the co-detected metabolites in grape cells were modified, suggesting that both self-metabolizing and co-metabolizing pathways worked simultaneously within the host. To confirm this potential implication of our results, the function of endophytic fungi in the host needs to be studied. Additional studies are needed to examine how the functions of endophytic fungi could be optimized to enhance target products. Generally, we suggest that the selection of candidate fungal endophytes could have an important effect on the quality of grapes and should receive increased consideration in the future.

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