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Alterations in grapevine leaf metabolism upon inoculation with *Plasmopara viticola* in different time-points

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ABSTRACT

Grapevines are easily infected by plant pathogens. It was found that resistant grapevines induce a wide range of phenolics upon the pathogen-infection. In this study in order to gain insight into these processes in different time-points the metabolic changes during the interaction of two grapevine cultivars, 'Regent' (resistant) and 'Trincadeira' (susceptible), with the downy mildew pathogen (*Plasmopara viticola*) were investigated. Nuclear magnetic resonance (NMR) spectroscopy on leaf extracts was used at several time points after experimental inoculation. A wide range of metabolites were identified using various two-dimensional (2D)-NMR techniques. Multivariate data analysis characterized both the resistant and the susceptible cultivars and their response against the pathogen. Metabolites responsible for their discrimination were identified as a fertaric acid, caftaric acid, quercetin-3-*O*-glucoside, linolenic acid, and alanine in the resistant cultivar 'Regent', while the susceptible 'Trincadeira' showed higher levels of glutamate, succinate, ascorbate and glucose. This study portrays the analytical capability of NMR spectroscopy and multivariate data analyses methods for the metabolic profiling of plant samples. The results obtained will underline the role of phenylpropanoids and flavonoids in resistance against biotic stresses which in turn provides a firm platform for the metabolic engineering of grapevine cultivars with higher resistance towards pathogens.

1. Introduction

The ability to change defense responses, i.e. metabolic plasticity, is the key for plants survival under different stresses. Naturally plants have to live with a multitude of stress conditions and the biosynthesis of protective chemicals is one of the major strategies [1]. These important phytochemicals involved in defense mechanisms are mainly secondary metabolites, not directly involved in basic processes like growth, development, and reproduction but are necessary in a plants' ecological network [2].

Among the *Vitis* species, *Vitis vinifera* ssp. *vinifera* is presently the most cultivated on a global scale. The phytochemistry of grapevine includes a great variety of compounds known for a vast array of activities. The chemical diversity of grapevine and related activities has been recently reviewed by Ali et al. [3]. Downy mildew is considered as an extremely destructive disease of grapevine, caused by *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni. The disease was introduced from America and is responsible for considerable economical losses to European grapevine growers every year. So far the most effective control is the repeated use of fungicide, which in turn raises other issues related to environmental impact and resistant pathogenic strains [4]. *Plasmopara viticola* has a tendency to colonize both resistant and susceptible cultivars but the development of the parasite is known to be inhibited by resistant cultivars mainly because of the induction of specific stress related metabolites known as phytoalexins [5].

Phytoalexins are generally defined as low molecular weight secondary metabolites of antimicrobial nature. These metabolites attract many plant pathologist and biochemists to investigate their metabolism in plants and pathogenic organisms. Due to their antimicrobial properties, phytoalexins can be used as markers for resistance. As phytoalexins are not as toxic as synthetic fungicides, they locally accumulate within the tissues, to concentrations much higher than those necessary to restrain fungal growth [6].

In Vitaceae family, the phytoalexins seem to comprise a limited number of molecules known as the stilbene family, derived from a compound known as resveratrol. It has been demonstrated that resveratrol and its oxidation products like α-, β-, ε-, and γ-viniferins, are synthesized by grapevine leaves following fungal infection and UV irradiation [7]. Grapevine phytoalexins can

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thus be used as markers for resistance. These phytoalexins have been shown to be produced in different grapevine cultivars when infected with the downy mildew pathogen *Plasmopara viticola* [8]. Schnee et al. [9] showed that upon infection with powdery mildew (*Erysiphe necator*), fungal growth was restricted on leaves in resistant cultivars and the amounts of stilbenes expressed by the infection site allowed discrimination of the resistant and susceptible cultivars.

The high throughput and qualitative metabolic screening to compare and discriminate samples is usually defined as metabolic fingerprinting [10]. Nuclear magnetic resonance (NMR) spectroscopy is most suitable for such analyses as it is fast, reproducible, with easy sample preparation and has been used for the analysis of industrial and natural products [11,12]. NMR is now commonly used in combination with chemometrics methods such as principal component analysis (PCA) and has been broadly used to analyze the various samples, e.g. fruits and beverages [13,14].

In our previous report some characteristic metabolites found more in the leaves of grapevine cultivars resistant to downy mildew pathogen were reported [12] using six different resistant and susceptible cultivars. Continuing the previous study two cultivars were studied in different time-points after inoculation. Two representative *Vitis vinifera* cultivars ('Regent' and 'Trincadeira') were used in this study. 'Regent', bred at the Institute for Grapevine Breeding Geilweilerhof, was chosen as a model since its resistance traits were achieved by multiple crosses introgressing resistance genes from American wild species [15]. Furthermore, it combines high wine quality and resistance to the downy and powdery mildew pathogens. 'Trincadeira' is a highly susceptible Portuguese cultivar of elevated economic interest as used to make important Portuguese wines. The NMR spectroscopy was applied for the metabolomic analysis of 'Regent' and 'Trincadeira' leaf tissues before and at several time-points after inoculation with *Plasmopara viticola*. NMR spectroscopy in combination with chemometrics was used with the aim to identify the major metabolites involved in resistance that cause differentiation between the resistant and the susceptible cultivars used in this study.

2. Materials and methods

2.1. Inoculation of Vitis vinifera cultivars with Plasmopara viticola

Grapevine plants from 'Regent' and 'Trincadeira' cultivars were grown in pots under greenhouse conditions. *Plasmopara viticola* sporangia were collected by incubating symptomatic leaves overnight in a chamber with 90–100% humidity, at room temperature. Sporangia were recovered, stored at −25 °C and checked for their vitality by microscopic examination of their shape and appearance. A 10^4 sporangia/mL suspension was sprayed onto the lower leaf surface in order to challenge the plants. Mock inoculations were done with water. After inoculation, the plants were kept in a moist chamber for 8 h during which time the moisture was 100%. Afterwards it was kept at 40–50%. The temperature ranged between 25 and 30 °C. Plant material (3–5 leaves from the shoot apex) was collected at 0, 6, 12, 24, and 48 h post inoculation (hpi), starting from morning (8:00 am, which is time zero) and immediately treated with liquid nitrogen then grinded with mortar and pestle. Three biological replicates were performed per time-point.

2.2. Extraction of plant material and NMR measurements

Leaves from both *V. vinifera* cultivars sampled at different time points after inoculation were used and extracted and analyzed by NMR according to Kim et al. [16]. Briefly, 50 mg of lyophilized sample was taken in a microtube (2 mL) to which 1.5 mL of methanol-d_4 (750 μL) and D_2O (750 μL) (KH_2PO_4 buffer, pH 6.0) containing 0.005% TMSP-d_4 (trimethyl silyl propionic acid sodium salt-d_4, w/v, Sigma–Aldrich) were added. The mixture was mixed at room temperature for 1 min, with ultrasonication for 20 min (Branson 5510E-MT, Branson Ultrasonics, Danbury, CT, USA), and followed by centrifugation at 17,000 × *g* at room temperature for 5 min. The supernatant (800 μL) was transferred to a 5 mm NMR glass tube and analyzed.

2.3. Data analysis

The AMIX software (Bruker) was used to reduce the ^{1}H NMR spectra to an ASCII file, with total intensity scaling. Bucketing or binning was performed and the spectral data were reduced to included regions of equal width (δ 0.04) equivalent to the region of δ 0.30–10.02. The regions of δ 4.85–4.95 and δ 3.25–3.35 were not included in the analysis because of the remaining signal of D_2O and CD_3OD, respectively. Principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA), and bidirectional orthogonal projection to latent structures-discriminant analysis (O2PLS-DA) were performed with the SIMCA-P+ software (v. 12.0, Umetrics, Umea, Sweden). For PCA, Pareto scaling was used whereas for PLS-DA and O2PLS-DA a Unit Variance (UV) method for scaling was used. The *t*-test for the ^{1}H NMR signals (bucket table) was performed using MultiExperiment Viewer (v. 4.0) and used for the relative quantification of metabolites [17].

3. Results

3.1. Visual analysis of ^{1}H NMR spectra and identification of metabolites

Leaves of resistant ('Regent') and susceptible ('Trincadeira') grapevine cultivars inoculated with *P. viticola* were harvested at different post inoculation time and their extracts were subjected to ^{1}H NMR analysis. A comparison between ^{1}H NMR spectra of different time points of 'Regent' and 'Trincadeira' cultivars were made and considerable differences were observed among the samples from the two cultivars (data not shown). It was found that 'Regent' showed higher accumulation of secondary metabolites while responding to the pathogen challenge. The comparison of ^{1}H NMR spectra at 48 hpi in both cultivars is shown in Fig. 1. The identification of the discriminating ^{1}H NMR resonances along with the characterization of metabolic fingerprints of these resistant and susceptible cultivars will be discussed in Section 3.2. The overlapping of NMR resonances of different metabolites is the major problem for compound identification which is usually overcome by the use of different 2D techniques. Metabolites identified in leaves and grapes using ^{1}H NMR with the help of 2D-NMR techniques [12,18] cover a broad range and include both primary and secondary metabolites.

Signals of gallic acid (δ 7.03, s) and syringic acid (δ 3.89, s; δ 7.39, s) were identified along with the quercetin-3-*O*-glucoside and myricetin in the phenolic part of the ^{1}H NMR spectra. The quercetin signal at δ 6.49 of H-8 was correlated in the ^{1}H–^{1}H COSY spectrum with the signal at δ 6.27 of H-6 and a signal at δ 6.95 of H-5′ with one at δ 7.56 of H-6′. Similar correlations were obtained for the signals of myricetin at δ 6.51 of H-8 with δ 6.28 of H-6 that also showed ^{1}H–^{1}H COSY correlations. Characteristic resonances for (+)-catechin (δ 5.83, *d*, *J* = 2.2; δ 5.95, *d*, *J* = 2.2; δ 6.71, *dd*, *J* = 8.2, 2.0; δ 6.81, *d*, *J* = 8.0; δ 6.88, *d*, *J* = 2.0) and (−)-epicatechin (δ 6.06, *d*, *J* = 2.0; δ 6.96, *d*, *J* = 2.2) were also identified in the same region. The characteristic doublets of 16.0 Hz in the range of δ 6.30–6.50 and δ 7.59–7.70 represent the H-8′ and H-7′ (olefinic protons) of

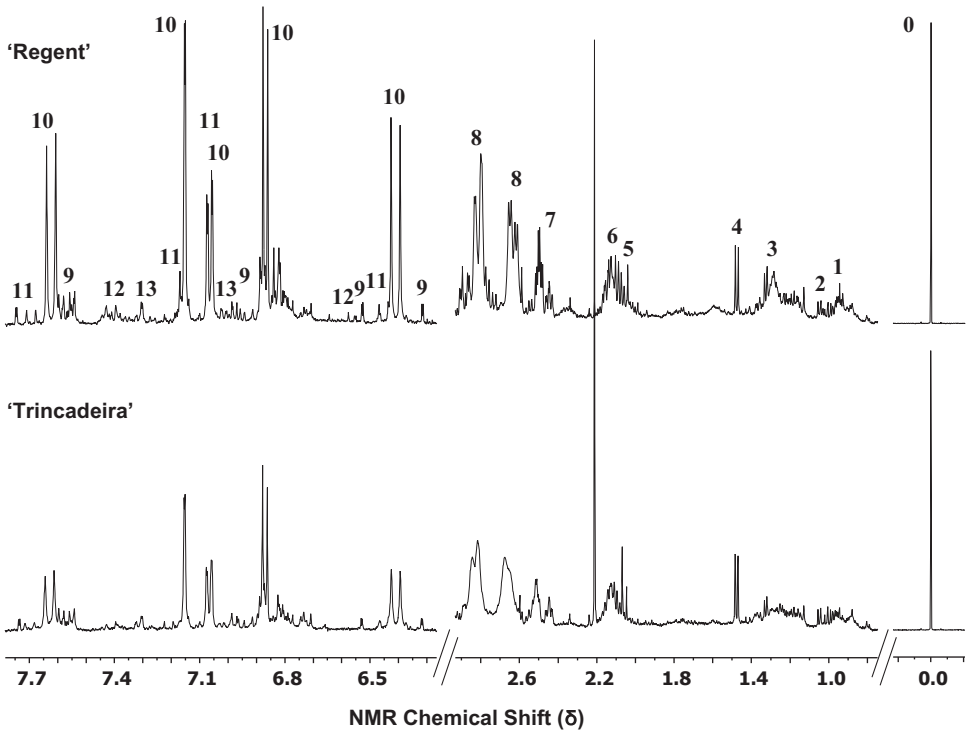

Fig. 1. Comparison of parts of ^{1}H NMR spectra of 'Trincadeira' and 'Regent' cultivars analyzed after 48 h of pathogen inoculation. Numbers indicate assignment of major signals to the metabolites. 0: TMSP (internal standard), 1: leucine, 2: valine, 3: threonine, 4: alanine, 5: glutamate, 6: proline and methionine, 7: glutamine, 8: malate, 9: quercetin glucoside, 10: caffeoyl moiety, 11: feruloyl moiety, 12: myricetin, 13: Tyrosine.

trans-cinnamic acids, respectively. These signals were identified as *trans*-caffeoyl and *trans*-feruloyl derivatives which were found to be linked with tartaric acid with an ester linkage. These compounds were identified as *trans*-caftaric acid (caffeoyl derivative in ester-linkage with tartaric acid) and *trans*-fertaric acid (feruloyl derivative in ester linkage with tartaric acid). The *cis*-forms of these conjugated cinnamic acids were also identified. In comparison with their *trans*-forms, the *cis*-forms showed shifting of the H-8′ and H-7′ signals with the coupling constant of 13.0 Hz. Two clear doublets of 13.0 Hz at δ 5.92 and δ 5.97 were detected for the H-8′ in the *cis*-configuration. The *J*-resolved and COSY spectra for the 48 hpi metabolites of the 'Regent' cultivar are shown in Fig. 2.

The amino acids alanine, threonine, valine, proline, methionine, leucine, tyrosine, glutamine and glutamic acid were also identified. The carbohydrate region showed the signals of the anomeric protons of β-glucose, α-glucose, fructose, and sucrose. Metabolites like choline, α-linolenic acid, and acetic acid with organic acids like succinate, fumarate, formate, ascorbate, malate, and tartarate were also identified. The resonances of few of the above mentioned metabolites are shown in Figs. 1 and 2. All of these assignments were based on our previous reports on grapevine [12,18] and comparison with 1D and 2D NMR spectra from our in-house library.

3.2. Multivariate data analysis

Chemometrics methods were used to identify the ^{1}H NMR resonances which were changed in resistant and susceptible cultivars upon pathogen challenge. The most common method to reduce the dimensionality of multivariate data set is the application of principal component analysis (PCA). The PCA score plot actually shows the variables involved in differentiating the samples and their identification leads to the detection of metabolites responsible for the separation.

Firstly, PCA was applied to the bucketed ^{1}H NMR spectra. As shown in Fig. 3A, samples from 'Regent' and 'Trincadeira', collected at different hours after inoculation, did not exhibit any clear distinction on the score plot. Inoculated samples of the 'Regent' cultivar were found separated from the respective mock inoculations and showed some grouping based on post infection time. But no such separation was observed among the samples of the 'Trincadeira' cultivar and the infected samples were found clustered with their mock inoculations. The very obvious separation among the samples was on the basis of cultivars. In PCA, a clear separation among the groups cannot be expected if a biological variation (among the replicates) is bigger than between the groups because the PCA is an unbiased method and showed maximum variation within the samples. Therefore, it was decided to apply a supervised analytical method to the same bucketed ^{1}H NMR spectra.

The application of supervised methods like partial least squares-discriminant analysis (PLS-DA) and bidirectional orthogonal projection to latent structures-discriminant analysis (O2PLS-DA) are considered to be the next step for the analysis of multivariate data. These analyses can show the correlation between datasets, which correspond in this study to different progressive stages of pathogenesis defense responses and cultivar types. The ^{1}H NMR data (bucket table), from both cultivars, were used as variables for all the supervised chemometrics methods applied in this study.

Since PLS-DA was unable to explain any significant separation among the samples (not shown in figure), it was decided to apply the other supervised method. Bidirectional orthogonal projection to latent structures-discriminant analysis (O2PLS-DA) is a supervised method which we tested to characterize the metabolic responses of the resistant and susceptible cultivars against the downy mildew pathogen. The *Y*-matrix consists of four discrete classes based on the inoculated and mock inoculated cultivars. This O2PLS-DA model was validated using cross validation-analysis of variance (CV-ANOVA) with a *p*-value equal to 6.2×10^{-20}. Fig. 3B shows the score plot of O2PLS-DA, which not only clearly discriminates the 'Regent' cultivar from 'Trincadeira' but also the pathogen inoculated samples from the mock inoculations. Samples from the

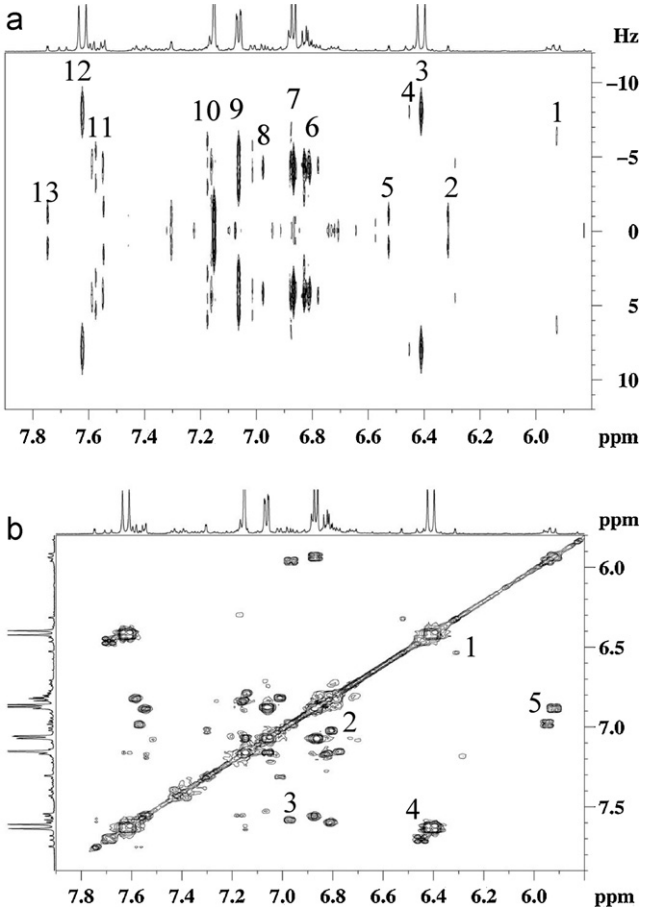

Fig. 2. ^{1}H–^{1}H *J*-resolved spectra (A) shows signals of phenylpropanoids and flavonoids. 1, H-8′: *cis*-feruloyl moiety; 2, H-8: quercetin-3-*O*-glucoside; 3, H-8′; 4, H-8′: *trans*-feruloyl moiety; 5, H-6: quercetin-3-*O*-glucoside; 6, H-5′: caffeoyl moiety; 7, H-3: *trans*-feruloyl moiety; 8, H-5′: quercetin-3-*O*-glucoside; 9, H-6′: caffeoyl moiety; 10, H-6′: *cis*-feruloyl moiety; 11, H-6′: quercetin-3-*O*-glucoside; 12, H-7: caffeoyl moiety; 13, H-2′: quercetin-3-*O*-glucoside. ^{1}H–^{1}H COSY spectra (B) shows correlations among the signals of H-6 with H-8 (1) and H-5′ with H-6′ (3) of quercetin-3-*O*-glucoside; H-5 with H-6 (2) of *trans*- and *cis*-caffeoyl and feruloyl moiety; H-8′ with H-7′ (4 and 5) of *trans*- and *cis*-caffeoyl and feruloyl moiety, respectively.

resistant cultivar were grouped on the positive side of component 1 which can be further categorized by inoculated samples and mock inoculations having negative and positive component 2 scores, respectively. This analysis did not show any clear distinction among the pathogen and mock inoculations in the case of 'Trincadeira'. To search for general differences in the metabolic responses of the two cultivars, again O2PLS-DA was applied with only two classes (*Y*-matrix), 'Regent' and 'Trincadeira'. This regression was validated using CV-ANOVA with a *p*-value equal to 1.06×10^{-29}. The score plot (Fig. 3C) shows nice distinction between the 'Trincadeira' and 'Regent' cultivars. The corresponding loadings plot (Fig. 3D) reveals that the 'Regent' cultivar was higher in phenolics like fertaric acid (feruoyl derivative) and caftaric acid (caffeoyl derivative), quercetin glucoside, along with other metabolites like alanine, proline, threonine, fumaric acid, and gallic acid. Many primary metabolites were found responsible for the separation of the 'Trincadeira' variety including glutamate, methionine, glucose, and sucrose, with other metabolites like succinate and ascorbate.

With the aim of highlighting the time dependent responses of both cultivars, samples from 'Regent' and 'Trincadeira' were separately compared at every time point after inoculation, omitting the other time points. For instance, the samples of 48 hpi of 'Regent' with its mock inoculations were compared with 48 hpi of 'Trincadeira' with its mock inoculations using principal component analysis. Fig. 4 shows the score plots for the PCA of every time point i.e. 6, 12, 24, and 48 hpi, with their respective mock inoculations. It is very clear from this figure that both 'Trincadeira' and 'Regent' reacted differently at the same time points when challenged with the pathogen as they are grouped separately from each other on the PCA score plots. In the case of 'Regent', the mock inoculations are separated from the infected samples at each time point after infection but in the case of 'Trincadeira', samples from the mock inoculations after 24 and 48 hpi are clustered close to their respective infected samples.

By the examining the loadings, the mock inoculations were found different from the fungal inoculated plants. The control sample from both the cultivars, at early hours post inoculation, showed amino acids like glutamate and threonine, with succinic and citric acid in higher levels. This followed by a shift in carbohydrates metabolism as higher glucose and sucrose contents were observed. Malate and fumarate were also detected in higher levels in the control samples after 12 h of inoculations. The mock inoculations, in case of 'Regent', showed less phenolic compounds as compared to their corresponding fungal treated samples at the late hours after inoculation while no significant difference in the phenolics concentrations was observed in the case of 'Trincadeira' cultivar.

Several interesting observations were made by examining ^{1}H NMR spectra along with the loadings plots of the PCA. As the mock inoculations for both cultivars grouped separately, it can be postulated that these two cultivars were inherently different in their metabolic profile as confirmed by inspecting the ^{1}H NMR spectra of both cultivars. The characteristic difference between these two varieties is that the 'Regent' cultivar was found to accumulate more phenolics than 'Trincadeira'. After inoculation with *P. viticola*, 'Regent' showed higher contents of phenolics like fertaric acid (feruoyl derivative), caftaric acid (caffeoyl derivative), and quercetin glucoside, together with the accumulation of valine, alanine, proline, and α-linolenic acid. On the other hand, 'Trincadeira' was characterized by a much lower phenolic content but higher levels of glutamic acid, methionine, succinic acid, ascorbic acid, glucose, and sucrose. Based on these observations, it can be postulated that these cultivars are not only possess distinct metabolic profile but also respond differently when confronted with biotic stress.

3.3. Relative quantification of metabolites

The same bucket table from ^{1}H NMR data was used to perform a *t*-test [19] in order to confirm the statistical significance of the results obtained from multivariate data analyses. The compounds were relatively quantified based on measurement of the average peak areas (bucket value) of the characteristic resonances in comparison with the internal reference, added to each sample in known concentration. The *t*-test confirmed several metabolites discriminating both cultivars with high statistical significance ($p < 0.01$). Fig. 5 shows the compounds' relative quantities at different time points after pathogen challenge for both 'Regent' and 'Trincadeira'.

As shown in Fig. 5, both varieties followed a pattern of appearance of metabolites by the passage of time after being inoculated with the downy mildew pathogen. In 'Regent', phenolics like caffeoyl derivative (*trans*-caftaric acid), and feruloyl derivative (*trans*-fertaric acid), along with inositol, alanine, and α-linolenic acid increased with time after pathogen challenge and their highest concentration was measured at 24 or 48 hpi. Interestingly, quercetin-3-*O*-glucoside concentration was reduced until 12 h after inoculation followed by an increase in the later time points. 'Trincadeira' showed a significantly higher accumulation of glucose, glutamic acid and succinic acid with less phenolic contents as compared to 'Regent'. Fig. 5 also shows that the cultivar 'Regent', in contrast to 'Trincadeira', accumulates higher levels of

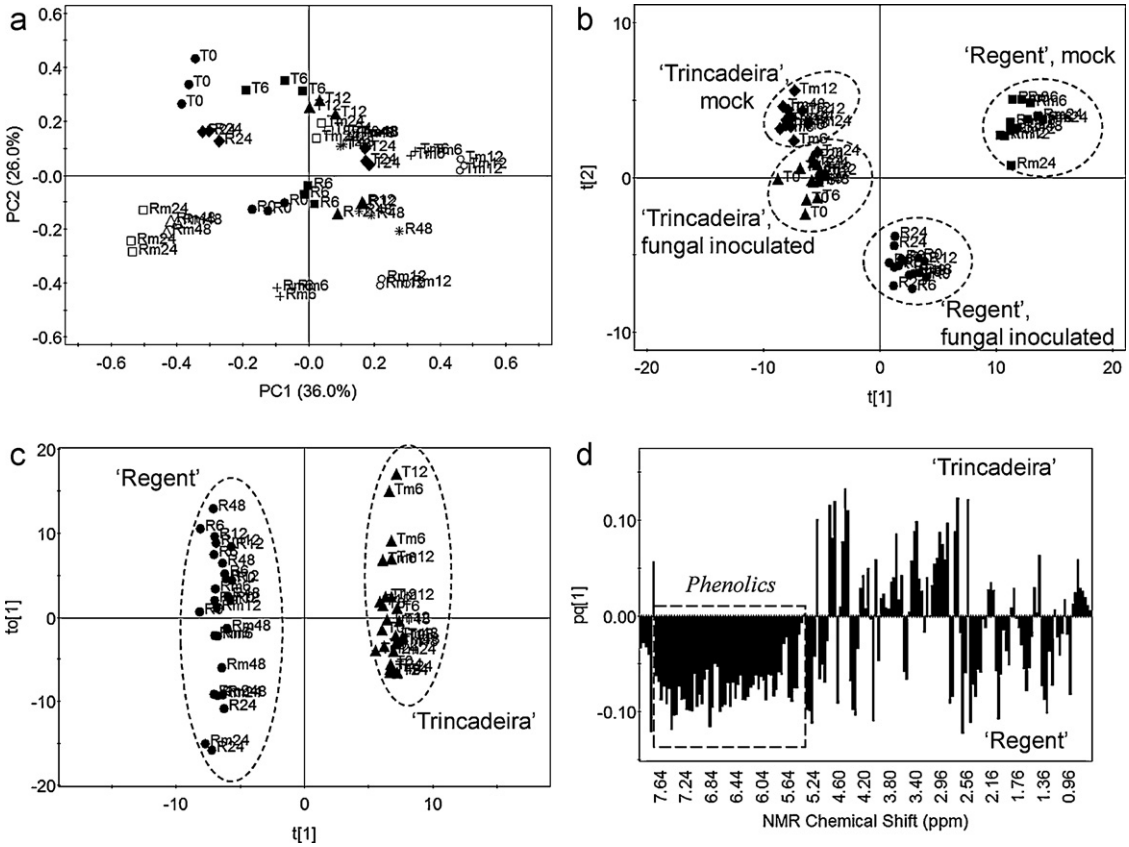

Fig. 3. Score plot of PCA (A), O2PLS-DA based on four (B) and two (C) classes. PCA showing samples from 'Regent' and 'Trincadeira' at all time points after inoculation with *P. viticola* or mock inoculations. T0: 'Trincadeira' at 0 h, T6: 'Trincadeira' at 6 h, T12: 'Trincadeira' at 12 h, T24: 'Trincadeira' at 24 h, T48: 'Trincadeira' at 48 h, R0: 'Regent' at 0 h, R6: 'Regent' at 6 h, R12: 'Regent' at 12 h, R24: 'Regent' at 24 h, R48: 'Regent' at 48 h. Labels with 'm' represent the mock inoculations for that time point. Loading plot (D) of O2PLS-DA based on 'Regent' and 'Trincadeira' classes show higher phenolic contents in the 'Regent' cultivar.

defense-related metabolites as an increase in the concentrations of phenylpropanoids and flavonoids was observed (with the exception of quercetin-3-*O*-glucoside) as early as at 6 and 12 hpi. Another interesting observation is related to glutamic acid and succinic acid. These two compounds showed a decline in their concentration after inoculation in 'Regent' while they were found in significantly increased amounts in 'Trincadeira' at later stages i.e. 24 and 48 hpi.

4. Discussion

Nuclear magnetic resonance spectroscopy with chemometrics methods is an established method for the metabolic characterization cultivars and species [20–24]. Recently metabolic profiling of different grapevine species with varying resistance against *P. viticola* has been done [12]. Previous studies by our group showed the coupling of metabolomics and transcriptomics data to discriminate the two grapevine cultivars (used in this study) based on their resistance towards downy mildew infection [25]. According to this report, these two cultivars are not only different in their pattern of gene expression but also in their metabolic profiles. These examples endorse the vast capabilities of this method which can be helpful in explaining different physiological processes and typical characteristics of plants species.

A considerable part of the plant metabolome consists of phenolic compounds produced by the shikimate pathway. These compounds play important physiological roles like the formation of the cell wall polymer lignin [26], floral and fruit pigments synthesis [27], resistance against microbes [28], and formation of flavor and scent compounds [29]. It is evident from many studies that phenolics like phenylpropanoids and flavonoids are detected as biomarkers of different biotic stresses to the plants. It has been reported that upon infecting tomato with viroid and bacterium [30] and tobacco with virus [31] showed significant increase in the biosynthesis of phenylpropanoids and flavonoids. Similar observations have been made in the case of different *Brassica* [32] and *Arabidopsis* [33] cultivars.

In the present study, different phenolics like caffeoyl derivative (*trans*-caftaric acid), feruloyl derivative (*trans*-fertaric acid), and quercetin-3-*O*-glucoside were identified and found responsible for the separation of the resistant cultivar from the susceptible one. The 'Regent' cultivar showed significantly higher accumulation of these compounds as compared to 'Trincadeira', suggesting their possible involvement in successful defense against pathogens. The results indicate that the 'Trincadeira' and 'Regent' cultivars are inherently different in their metabolic profile as 'Regent' was found to contain higher levels of these stress related metabolites even at 0 hpi. The innate metabolic and transcriptional differences of these two cultivars have been discussed in a previous report [25]. The first 12 h after infection seems to be very critical as 'Regent' showed synthesis of phenolics in this time period. It has been shown by many reports [5,8,34] that grapevine specific phytoalexins can also be produced by the susceptible cultivars upon infection, the metabolic differences at the initial stages of infection, as we have seen during this study, might be acting as the first inducible line of defense and may be the key for resistance in grapevine against fungal pathogens.

Among the metabolites identified, linolenic acid is the precursor to jasmonate in the octadecanoid cascade. Jasmonate functions as a signal molecule and activates defense-related genes [35]. Our

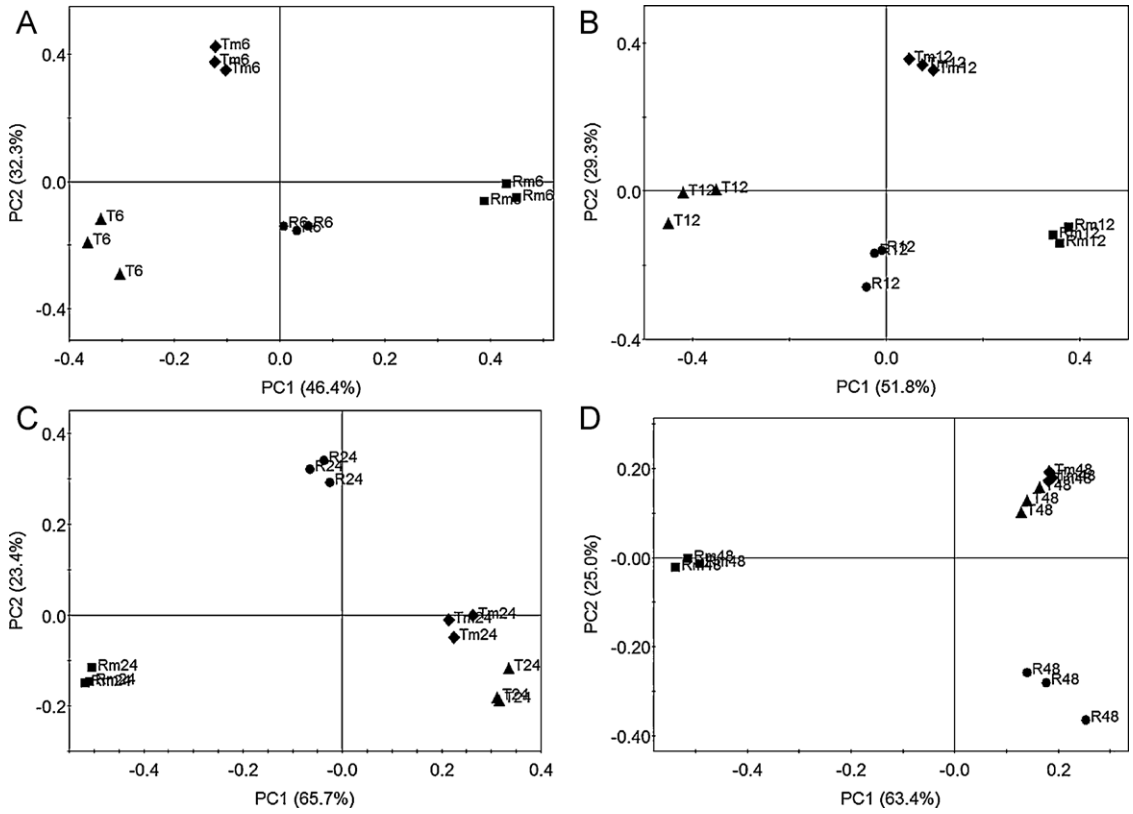

Fig. 4. PCA score plots showing samples at 6 (A), 12 (B), 24 (C), and 48 (D) hours after inoculation along with their mock inoculations. Labels for each sample are the same as in Fig. 3.

results indicate an accumulation of linolenic acid (within 24 hpi) in the 'Regent' cultivar, which could be due to the induction of octadecanoid biosynthesis by *P. viticola* attack and is involved in stress-related signaling to other parts of the plant. Also the elevated levels of phenylpropanoids and flavonoids suggest that in 'Regent' the phenylpropanoid pathway was rapidly induced upon inoculation in comparison with 'Trincadeira'. It is also interesting to know that the phenylpropanoid pathway finally leads to the synthesis of grapevine specific stress related metabolites i.e. viniferins. Our analysis showed no accumulations of viniferins either due to low sensitivity of NMR spectroscopy or mainly because viniferins are known to produce at later stages of infection. Many publications showed viniferins accumulation at least after four to seven days of inoculation [5,8,34]. The results presented here clearly suggest that apart from viniferins, phenylpropanoids also plays crucial role in resistance against pathogen. These observations are also supported by the fact that the transcriptomics analysis of the same set of samples was also performed and several signaling and defense related transcripts were found up-regulated in the 'Regent' cultivar [36].

Essential for plant survival, primary metabolites are also found to be involved in resistance against pathogens [37], insects [38], and herbivores [39]. Many primary metabolites have been identified in this study including amino acids, organic acids, and carbohydrates, and found to discriminate the cultivars in multivariate data analyses including alanine, inositol, glutamic acid, succinic acid, α-linolenic acid, and glucose.

Alanine and inositol are also reported to be involved in resistance as they are known to increase under stress. The accumulation of inositol may result in the rapid synthesis of stress metabolites and for the resistance trait in the 'Regent' cultivar. Inositol has been known to participate in signal transduction and, when accumulated, facilitates the resistant plant to respond quickly to pathogen attack [40]. For alanine the precise function in plant resistance is not yet known but it is known to be induced under several types of stress [41–43]. Our results demonstrate that the resistant cultivar shows a significant elevation in alanine concentration after infection as compared to the susceptible cultivar. An increase in glucose levels was also shown by the cultivars after inoculation. This alteration in carbohydrate metabolism might be associated to the reallocation of nutrients to the non infected parts of the plants and/or provide pool of precursors for the biosynthesis of phenylpropanoids and flavonoids [39,44].

The understanding of resistance mechanism in plants is of utmost importance. While working on the resistance of different grapevine cultivars against *P. viticola*, some key observations were made. As shown by our previous reports [12,25], flavonoids and phenylpropanoids concentrations are the distinctive factors among the resistant and susceptible cultivars. Following to that, it was important to understand the response mechanism of the studied cultivars when challenged with a pathogen. In contrast to our previous report, instead of differentiating the cultivars based on metabolic profiling, this work is designed to observe the response pattern of the studied cultivars against pathogen. As viniferins are supposed to be produced by grapevine in response to the pathogen attack, the induction of phenolics and other stress signaling metabolites biosynthesis is a key finding in order to understand the inducible line of defense in these cultivars. A very recent NMR-based study on *V. vinifera* cv. Alvarinho leaves affected by esca disease [45] authenticates most of the findings presented in this study. This endorsed the fact that regardless of genotype or pathogen differences, the grapevine cultivars share a similar way to respond against pathogen attack.

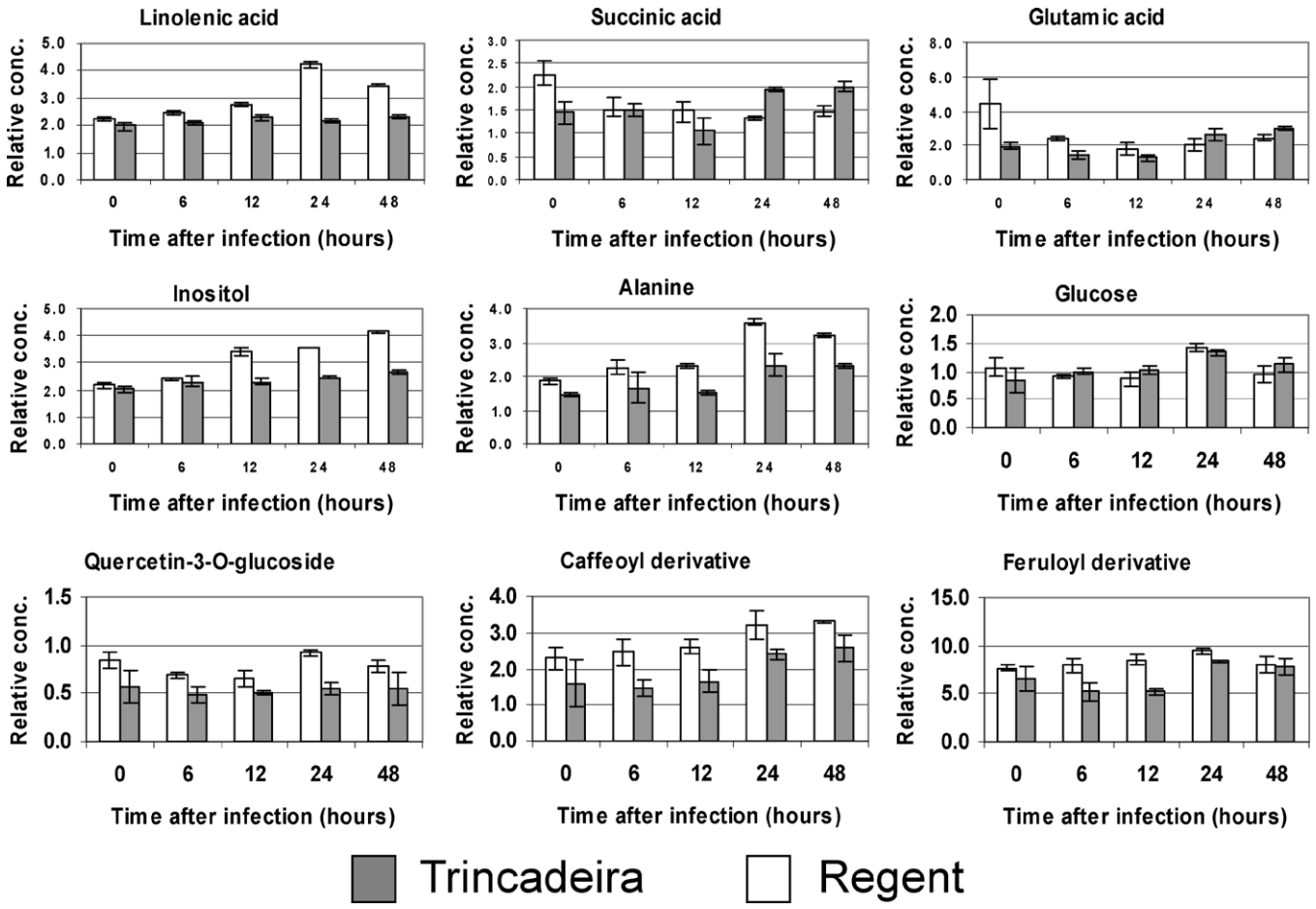

Fig. 5. Relative quantification of metabolites based on the mean area of the resonance peak related with that metabolite ($p < 0.01$) in comparison with the internal reference compound added to each sample in known concentration ($n = 3$).

5. Conclusion

With NMR-based metabolic profiling approach, we traced the metabolic responses of resistant and susceptible grapevine cultivars against infection by *P. viticola*. Multivariate data analyses methods like principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), and bidirectional orthogonal projection to latent structures-discriminant analysis (O2PLS-DA), were used to underscore the genuine metabolic differences between the 'Regent' and 'Trincadeira' cultivars. Based on this, it can be concluded that the cultivar 'Regent' is not only innately different from 'Trincadeira' but also differs in the metabolic responses generated against infection. It was observed that the studied plants alter their metabolism by directing it towards the synthesis of phenylpropanoids and flavonoids, with accumulation of some stress related primary metabolites. The 'Regent' cultivar exhibited a reaction against pathogen stress and is characterized by production and accumulation of stress metabolites like flavonoids and phenylpropanoids together with some amino acids. This work shows the great potential of NMR spectroscopy and similar approaches can be used for the portrayal of different plant samples on the basis of metabolic composition. Furthermore, detailed targeted analysis of these stress related metabolites is of great interest to provide better understanding of their role in resistance against biotic stresses. Moreover analysis of infected grapevine samples of more varieties at shorter time intervals along with even longer exposure time to the pathogen may provide an improved understanding of grapevine physiology related to biotic stresses.

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