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CHAPTER 10

NMR-based study of alterations in grapevine leaf metabolism upon inoculation with *Plasmopora viticola*

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Abstract

Grapevine (*Vitis vinifera* ssp. *vinifera* L.) is one of the most important fruit crops grown in viticultural regions on several continents. Like other crops grapevine are easily infected by plant pathogens. Understanding its defense responses will help to develop improved cultivars for more sustainable plant management. To gain insight into these processes the metabolic changes during the interaction of two differentially susceptible grapevine cultivars, 'Regent' and 'Trincadeira', with the downy mildew pathogen (*Plasmopora 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 cultivar and their response against the pathogen. Metabolites responsible for their discrimination were identified as a fertaric acid, a 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. In addition have more phenolics than 'Trincadeira' upon inoculation with *P. viticola*, 'Regent' was also found more active in identifying the biotic stress and showed more rapid accumulation of phenolics than 'Trincadeira', which might be the key for resistance in this cultivar. This study portrays the analytical capability of NMR spectroscopy in combination with chemometrics methods for the metabolic profiling of plant samples. The results obtained will provide better understanding of the role phenylpropanoids and flavonoids in resistance against biotic stresses which in turn provide a firm platform for the designing of metabolic engineering of grapevine cultivars with higher resistance towards pathogens.

Introduction

Plant species survival depends on their metabolic plasticity, i.e. their ability to diversifying their own defense responses against biotic and abiotic stresses. In their natural environment, plants have to live with a multitude of stress conditions such as drought, flooding, salinity, nutritional deficiency, intense sun light, adverse climatic conditions, pollutants, pathogens and phytophagous insects and animals (Harborne

1999). Since plants are sessile, one of the major strategies for counteracting these adverse conditions is the synthesis of protective phytochemicals that operate on the level of natural selection, biological evolution and biodiversity. 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 (Dixon 2001).

Grapevines (*Vitis* spp) are important fruit species world-wide due to the numerous uses of grapes in the production of wine and grape juice as well as their popularity as fresh table fruits or dried currants and raisins. Among the *Vitis* species, *Vitis vinifera* ssp. *vinifera* is currently the most cultivated around the world. 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. (2010).

Downy mildew, caused by *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni, is one of the most destructive diseases of grapevine. 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 (Kortekamp et al. 2008). *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 (Jean-Denis et al. 2006).

Phytoalexins are low molecular weight antimicrobial secondary metabolites of broad interest. Studies of phytoalexins provide a large field of investigation for plant pathologist and biochemists concerning the aspects of biosynthesis of these compounds in plants and their metabolism by pathogenic organisms. Since phytoalexins have been shown to possess biological activities against a wide range of pathogens, they can be considered as markers for plant disease resistance. Although most phytoalexins are less toxic than synthetic fungicides they can locally accumulate within plant tissues, to concentrations much higher than those necessary to restrain fungal growth (Derckel et al. 1999).

Although phytoalexins display an enormous chemical diversity, in Vitaceae they seem to constitute a rather restricted group of molecules belonging to the stilbene family, the skeleton of which is based on 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 (Langcake and Pryce 1977). Grapevine phytoalexins can 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* (Pezet et al. 2004). Schnee et al. (2008) 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.

Metabolic fingerprinting is the high throughput qualitative screening of the metabolic composition in an organism or tissue with the primary aim of sample comparison and discrimination analysis (Dettmer et al. 2007). The analytical techniques applied to metabolite profiling should be rapid, reproducible, and stable over time, while requiring only simple sample preparation. A technique that potentially meets all the above listed necessities is NMR (nuclear magnetic resonance), which has been widely used as a fingerprinting tool for the analysis and quality assessment of industrial and natural products (Ali et al. 2009; Brescia et al. 2002). NMR is now commonly used in combination with multivariate or pattern recognition techniques such as principal component analysis (PCA) specifically designed to analyze complex datasets. The combination of NMR and multivariate data analyses has been widely applied to the metabolic profiling of various types of samples, e.g. fruits and beverages (Gall et al. 2001; Charlton et al. 2002).

The present research is a continuation of our previous report regarding the metabolic classification of different *Vitis* species (Ali et al. 2009). In this study, two *Vitis vinifera* cultivars ('Regent' and 'Trincadeira') were used. '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 (Eibach and Töpfer 2003). 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 was coupled with multivariate data analyses with the aim to identify the major metabolites involved in resistance that contribute to the discrimination between the resistant and the susceptible cultivars used in this study.

Materials and Methods

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 moist chamber (90-100 % humidity) at room temperature. Sporangia were recovered, stored at -25 °C and checked for their vitality by microscopy. A 10⁴ 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 hours during which time the moisture was 100 %. Afterwards it was kept at 40-50 %. The temperature ranged between 25-30 °C. Plant material (3-5 leaves from the shoot apex) was collected at 0, 6, 12, 24, and 48 hours post inoculation (hpi), starting from morning (8:00 am). Three biological replicates were performed per time-point.

Extraction of Plant Material

Leaves from both *V. vinifera* cultivars sampled at different time points after inoculation were used and extracted according to Kim et al. (2010). Briefly, the freeze dried plant material (50 mg) was transferred to a microtube (2 mL) to which 1.5 ml of methanol-*d*₄ (750 µL) and D₂O (750 µL) (KH₂PO₄ 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, ultrasonicated for 20 min (Branson 5510E-MT, Branson Ultrasonics, Danbury, CT, USA), and centrifuged at 17,000 *g* at room temperature for 5 min. The supernatant (800 µL) was transferred to a 5 mm NMR glass tube.

Data Analysis

The ^1H NMR spectra were automatically reduced to an ASCII file using AMIX software (Bruker). Spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (δ 0.04) corresponding to the region of δ 0.3–10.0 (bucketing). The regions of δ 4.85–4.95 and δ 3.25–3.35 were excluded from the analysis because of the residual signal of D_2O and CD_3OD , respectively. Principal component analysis (PCA) and projections to latent structures-discriminant analysis (PLS-DA), and bidirectional orthogonal projections 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 method for scaling was used. The t -test for the ^1H -NMR signals (bucket table) was performed using MultiExperiment Viewer (v. 4.0) and used for the relative quantification of metabolites (Saeed et al. 2003).

Results

Visual analysis of ^1H NMR spectra

Leaves of resistant ('Regent') and susceptible ('Trincadeira') grapevine cultivars inoculated with *P. viticola* were collected at different time points (0, 6, 12, 24, and 48 hpi) and their extracts were subjected to ^1H NMR analysis. A comparison between ^1H NMR spectra of different time points of 'Regent' and 'Trincadeira' cultivars and it was observed that considerable amounts of phenolics are present even at 0 h and 6 h after inoculation in 'Regent'. A gradual increase in the synthesis of metabolites, particularly phenolics, can be seen as the resonances in the phenolics region are getting higher at later time points after inoculation. Similar analysis has been done for 'Trincadeira' and it also showed a significant accumulation of phenolics at the later time points i.e. 24 and 48 hpi. In order to highlight the differences in the metabolic responses of these varieties, all the time points were compared. It was found that 'Regent' not only showed higher accumulation of secondary metabolites but also was quicker in responding to the pathogen challenge. The comparison of ^1H NMR spectra at 48 hpi in both cultivars is shown in Figure 1. The identification of the discriminating ^1H NMR resonances along with the characterization of metabolic profiles of these resistant and susceptible cultivars will be discussed later.

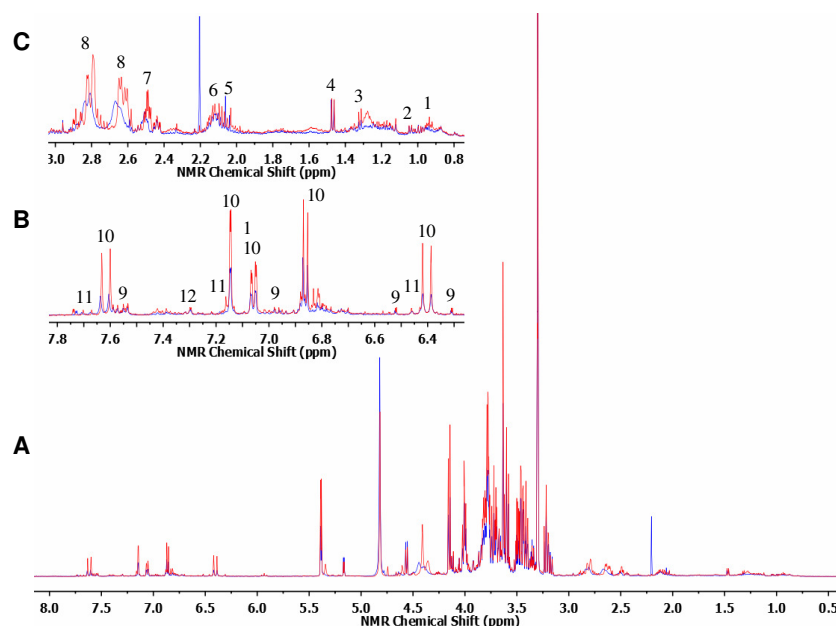


Figure 1. Comparison of full ^1H -NMR spectra (A) of 'Trincadeira' (blue) and 'Regent' (red) cultivars analyzed after 48 hours of pathogen inoculation. The phenolic (B) and amino acid (C) regions are amplified. 1: leucine, 2: valine, 3: threonine, 4: alanine, 5: glutamate, 6: proline, 7: glutamine, 8: malate, 9: quercetin glucoside, 10: caffeoyl moiety, 11: feruloyl moiety, 12: myricetin.

Identification of metabolites

The metabolites identified cover a wide diversity and include amino acids, organic acids, carbohydrates, hydroxycinnamates, hydroxybenzoates, and flavonoids. The aromatic compounds part of the ^1H NMR spectra showed signals of gallic acid and syringic acid. The flavonoids quercetin-3-*O*-glucoside and myricetin were also identified in the aromatic region. The quercetin signal at δ 6.49 of H-8 was correlated in the ^1H - ^1H 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 ^1H - ^1H COSY correlations. Resonances for (+) - catechin and (-) - epicatechin 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 *trans*-cinnamic acids, respectively. In the ^1H NMR spectra, these resonances were assigned to two different hydroxycinnamic acids

moieties that include *trans*-caffeoyl- and *trans*-feruloyl derivatives. These derivatives were found to be conjugated with tartaric acid via an ester linkage. Based on these assignments, these compounds were identified as *trans*-caftaric acid (caffeic acid in ester-linkage with tartaric acid) and *trans*-fertaric acid (ferulic acid in ester linkage with tartaric acid). Along with the *trans*- forms, the *cis*- forms of these conjugated cinnamic acids, i.e. *cis*-caftaric acid and *cis*-fertaric acid, were also detected. When compared to their *trans*- configuration, the *cis*- forms showed an up-field shift of the signals for H-8' and H-7' along with a reduction in the coupling constant from 16.0 Hz to 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 high signal intensities in the amino acids region were helpful to elucidate a number of amino and organic acid signals. The amino acids alanine, threonine, valine, proline, methionine, leucine, tyrosine, glutamine and glutamic acid were identified in leaves by comparison with reference spectra of these compounds. The signals in the carbohydrate regions were highly clustered and overlapped. This region showed the signals of the anomeric protons of β -glucose, α -glucose, fructose, and sucrose. Other compounds including choline, α -linolenic acid, and acetic acid were also identified in this region. A number of signals were assigned to different organic acids like succinic acid, fumaric acid, formic acid, ascorbic acid, malic acid, and tartaric acid. All of these assignments were done by comparing the spectra with 1D and 2D NMR spectra of common plant metabolites in our in-house library and already explain in detail in Chapter 9 of this thesis.

Multivariate data analysis

After visual inspection of ^1H NMR spectra, multivariate data analyses were applied in order to identify the ^1H NMR resonances which were changed in resistant and susceptible cultivars upon pathogen challenge. One of the most common and widely used methods to reduce the dimensionality of multivariate data set is the application of principal component analysis (PCA). The PCA score plot actually shows the differences among the samples and the loadings plot shows the variables (NMR chemical shifts from bucket table) responsible for that discrimination. The identification of these spectral signals leads to the identification of compounds accountable for the separation.

Firstly, PCA was applied to the bucketed ^1H NMR spectra. As shown in Figure 2A, samples from 'Regent' and 'Trincadeira', collected at different hours after infection, 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. Although PCA is undoubtedly a reliable grouping method in metabolomics, a clear separation among the groups can not be expected if variation within the samples of the same group (biological variation) is bigger than the groups themselves as the separation in PCA is achieved from unbiased maximum variation within the tested samples. Therefore, it was decided to apply a supervised analytical method to the same binned ^1H NMR spectra.

The application of supervised methods like projections to latent structures-discriminant analysis (PLS-DA) and bidirectional orthogonal projections to latent structures-discriminant analysis (O2PLS-DA) are considered to be the next step for the analysis of multivariate data. These analyses, unlike the unbiased system used for PCA, are performed with pre-input information regarding the data. The most important information obtained from these analyses is the correlation between data sets which correspond in this study to different progressive stages of pathogenesis defense responses and cultivar types. Like PCA, the differences or similarity among the samples can be detected by using the score plot while the signals responsible for those differences or similarities can be identified by the loadings. The ^1H NMR data (bucket table), from both cultivars, were used as variables for all the supervised multivariate analyses applied in this study.

Since PLS-DA, like PCA, was unable to show any meaningful separation among the analyzed samples (Figure 2B), it was decided to apply another supervised method. Bidirectional orthogonal projections 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 equals to 6.2×10^{-20} .

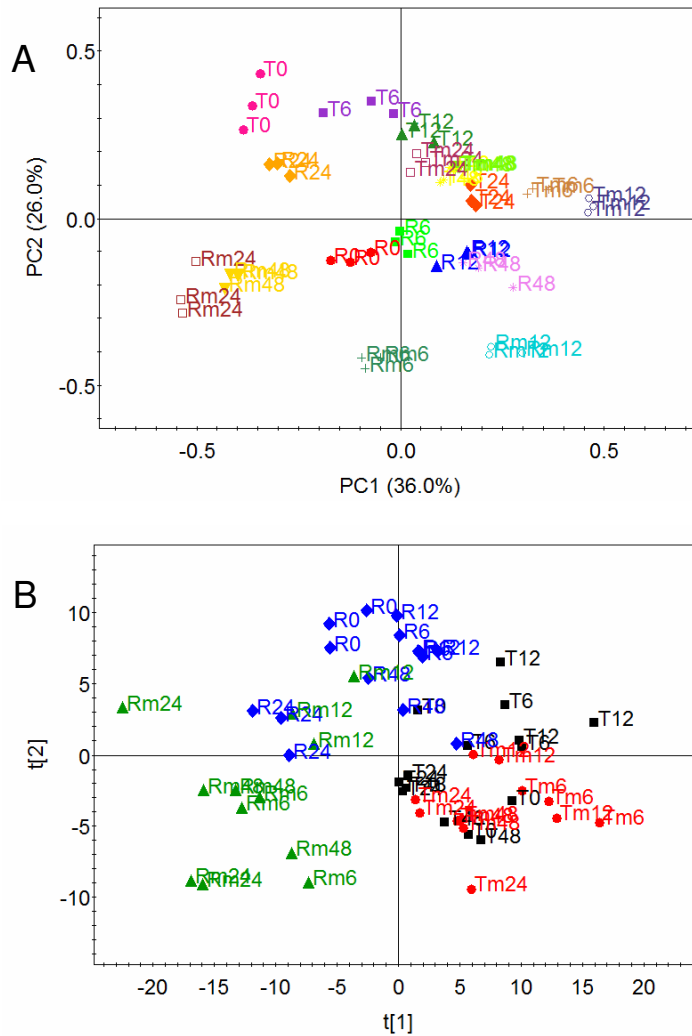


Figure 2. Score plot of PCA (A) and PLS-DA (B) showing samples from ‘Regent’ and ‘Trincadeira’ at all time points after inoculation with *P. viticola* or mock inoculations. T0: ‘Trincadeira’ at 0 hour, T6: ‘Trincadeira’ at 6 hours, T12: ‘Trincadeira’ at 12 hours, T24: ‘Trincadeira’ at 24 hours, T48: ‘Trincadeira’ at 48 hours, R0: ‘Regent’ at 0 hour, R6: ‘Regent’ at 6 hours, R12: ‘Regent’ at 12 hours, R24: ‘Regent’ at 24 hours, R48: ‘Regent’ at 48 hours. Labels with ‘m’ represent the mock inoculations for that time point.

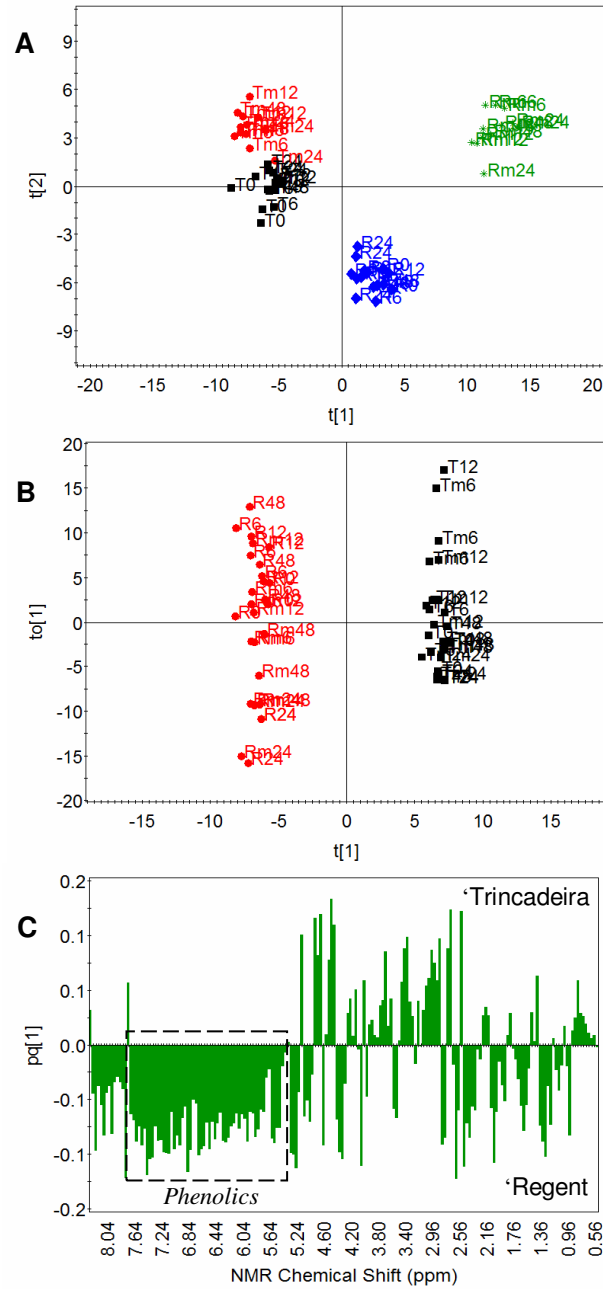


Figure 3. Score plot of O2PLS-DA based on four (A) and two (B) classes. Labels for each sample are the same as in Figure 2. Loading plot (C) of O2PLS-DA based on 'Regent' and 'Trincadeira' classes show higher phenolic contents in the 'Regent' cultivar.

Figure 3A 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 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. Although the samples from the susceptible cultivar were separated from the resistant one, any clear distinction among the pathogen and mock inoculations was not observed in the case of 'Trincadeira'. To search for general differences in the metabolic responses of the two cultivars, again O2PLS-DA was applied. This time the samples were classified into 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 (Figure 3B) shows good separation between the 'Trincadeira' and 'Regent' cultivars with positive and negative component 1 values, respectively. By inspecting the loading plot many metabolites were found to participate in this grouping. The corresponding loading plot (Figure 3C) reveals that the 'Regent' cultivar was higher in phenolics like fertaric and caftaric acid, 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, together with some organic acids like succinate and ascorbate.

With the aim of high-lighting 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. Figure 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. It is also worthy to note that 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.

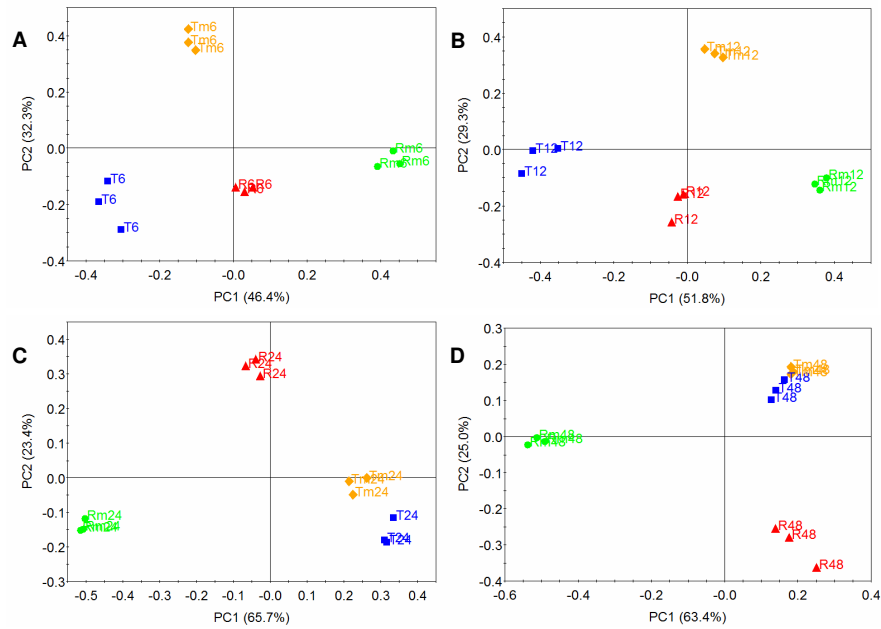


Figure 4. PCA score plots showing samples at 6 (A), 12 (B), 24 (C), and 48 (D) hours after inoculation along with their mock inoculations. Labels for each sample are the same as in Figure 2.

Several interesting observations were made by examining ^1H NMR spectra along with the loading plots of the PCA. As the mock inoculations for both cultivars grouped separately at every time point, it can be postulated that these two cultivars were inherently different in their metabolic profile as confirmed by inspecting the ^1H 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 a gradual increment in its phenolic contents like fertaric acid, caftaric acid, 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 suggested that these cultivars are not only naturally

different in their metabolic profile but also respond differently when confronted by biotic stress.

Relative quantification of metabolites

The ^1H NMR data set (bucket table) was subjected to a *t*-test (Student 1908) 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 mean peak areas of the characteristic resonance signals of these compounds. The *t*-test confirmed several metabolites discriminating both cultivars with high statistical significance ($p < 0.01$). Figure 5 shows the graphs representing the compounds with their relative quantities at different time points after pathogen challenge for both ‘Regent’ and ‘Trincadeira’.

As shown in Figure 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 *trans*-caftaric acid, *trans*-ferric acid and quercetin-3-*O*-glucoside, along with inositol, alanine, and α -linoleic acid increased with time after pathogen challenge and their highest concentration can be measured at 24 or 48 hpi. ‘Trincadeira’ showed a significantly higher accumulation of glucose, glutamic acid and succinic acid with less phenolic contents as compared to ‘Regent’. It is also interesting to observe that ‘Trincadeira’, although susceptible, not only showed the presence of small amounts of phenolics at 0 hpi but they also increased at the later stages, especially the caftaric and ferric acid. Figure 5 also shows that the cultivar ‘Regent’, in contrast to ‘Trincadeira’, not only accumulates higher levels of defense-related metabolites but is also faster in responding to pathogen attack as a rapid increase in the concentrations of phenylpropanoids and flavonoids was observed 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.

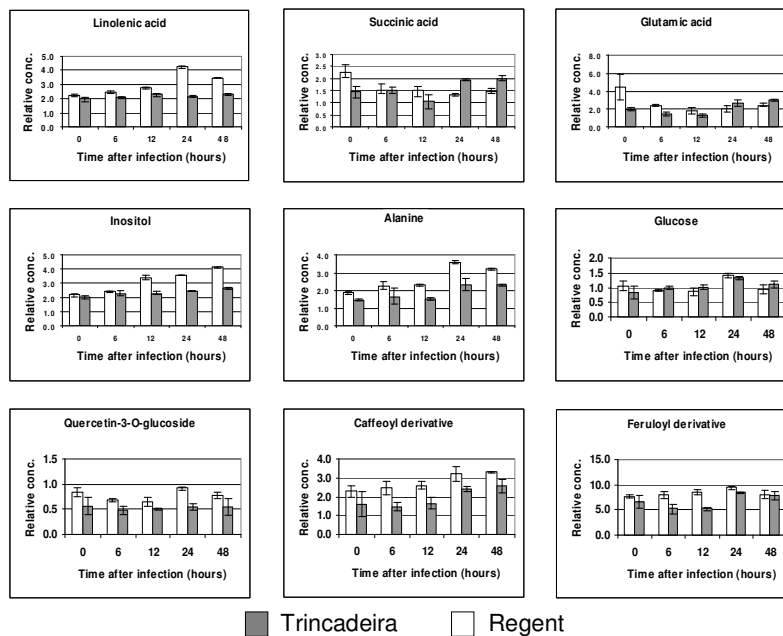


Figure 5. Relative quantification of metabolites based on the mean area of the resonance peak related with that metabolite ($p < 0.01$).

Discussion

Nuclear magnetic resonance spectroscopy in combination with multivariate data analyses has been widely used as a fingerprinting tool for plants. The system has proved very effective for the metabolic characterization of not only cultivars but species as well (Choi et al. 2005; Choi et al. 2004b; Kim et al. 2005). Recently metabolic profiling, using NMR and multivariate data analyses, of different grapevine species with varying resistance against *P. viticola* has been done (Ali et al. 2009). Another report showed the coupling of metabolomics and transcriptomics data to discriminate the two grapevine cultivars on their resistance capacity towards downy mildew infection (Figueiredo et al. 2008). These reports clearly suggest the enormous potential of this approach in metabolic characterization of plants which in turn can be very useful in explaining different physiological reactions and distinctive characteristics of plants species.

Since plants are sessile in nature, to survive and contest different stresses they have a unique ability to diversify their responses against these (Harborne 1999). When challenged by pathogens, plants exhibit several biochemical defense responses including enzyme synthesis, cell wall deposition of lignin, and accumulation of specific

metabolites (Daayf et al. 2000). Among these mechanisms, the metabolites-involved mechanism is usually coupled with plant 'secondary metabolites' biosynthesis, which are mainly involved in plant ecological diversification (Harborne 2001). 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 (Li et al. 2008), floral and fruit pigments synthesis (Tanaka et al. 2008), resistance against microbes (Shadle et al. 2003), and formation of flavor and scent compounds (Schwab et al. 2008). 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 infection tomato (viroid and bacterium) (Lopez-Gresa et al. 2010) and tobacco (virus) (Choi et al. 2006) plants showed significant increase in the biosynthesis of phenylpropanoids and flavonoids. Similar observations have been made in the case of different *Brassica* (fungus) (Abdel-Farid et al. 2009) and *Arabidopsis* (fungus) (Liang et al. 2006) cultivars.

In the present study, different phenolics like fertaric acid, caftaric 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 (Figueiredo et al. 2008). The first twelve hours after infection seems to be very critical as 'Regent' showed rapid synthesis of phenolics in this time period. It has been shown by many reports (Jean-Denis et al. 2006; Slaughter et al. 2008; Pezet et al. 2004) 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 line of defense and can 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 (Farmer and Ryan 1990). Our results indicate a rapid 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 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 (Jean-Denis et al. 2006; Slaughter et al. 2008; Pezet et al. 2004). The results presented here clearly suggest that apart from viniferins, phenylpropanoids also plays crucial role in resistance against pathogen.

Primary metabolites are essential for plant survival as they play a vital role not only in growth, reproduction, and energy generation but also in resistance against pathogens (Lokvam et al. 2006), insects (Berenbaum 1995), and herbivores (Rostas et al. 2002). 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. In this study many primary metabolites were also found induced by fungal infection 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 have been shown to increase under stress. The accumulation of inositol may account for the rapid synthesis of stress metabolites and for the resistance trait in the 'Regent' cultivar. For alanine the precise function in plant resistance is not yet known but it is known to induce a stress protein synthesis in mammalian kidney (Monselise et al. 2003). Our results demonstrate that the resistant cultivar shows a significant elevation in alanine concentration after infection as compared to the susceptible cultivar. Inositol has been known to participate in signal transduction and, when accumulated, facilitates the resistant plant to respond quickly to pathogen attack (Hamzehzarghani et al. 2005). 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 (Rostas et al. 2002; Hendrawati et al. 2006).

Conclusion

By means of NMR-based metabolomics approach, we were able to trace the metabolic responses of resistant and susceptible grapevine cultivars against infection by *P. viticola*. Multivariate data analyses methods like principal component analysis (PCA), projections to latent structures-discriminant analysis (PLS-DA), and bidirectional orthogonal projections to latent structures-discriminant analysis (O2PLS-DA) were used to underscore the genuine metabolic differences between the 'Regent' and 'Trincadeira' cultivars. Several diverse classes of metabolites were identified by comparing the spectra of samples to a library of NMR spectra of standards run under identical conditions. 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. The 'Regent' cultivar exhibited a quick reaction against pathogen stress and is characterized by relatively rapid production and accumulation of stress metabolites like flavonoids and phenylpropanoids together with some amino acids. This works 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.