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ORIGINAL ARTICLE

Towards developing a metabolic-marker based predictive model for *Phytophthora nicotianae* tolerance in citrus rootstocks

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Abstract
Root rot of citrus trees caused by *Phytophthora nicotianae* is responsible for severe economic losses in citriculture. Use of resistant rootstocks is an effective method of managing this problem, however, breeding and selection of new citrus rootstocks is a time-consuming undertaking. The objective was to develop a method for the rapid assessment of rootstocks for *P. nicotianae* tolerance, using a metabolomics approach to identify metabolic markers for the phenotypic trait of tolerance. Sixteen citrus rootstocks were inoculated with *P. nicotianae* in the greenhouse for determination of relative tolerance/susceptibility. Healthy citrus roots from four tolerant and four susceptible rootstocks were used for metabolite analysis with the objective of identifying potential biomarkers. Organic solvent extractions of the roots were prepared and analysed by mass-spectrometry based liquid chromatography, which produced 367 ion features (retention time and m/z). Orthogonal partial least squares discriminant analysis of peak abundance using MarkerLynx software allowed for the identification of ion features that differentiate tolerant and susceptible rootstocks. Using descriptive and inferential statistics based on the ion features of uninoculated tolerant vs. susceptible rootstocks, applying logistic regression, 14 top markers were identified and two of them (22.03_259.0975 and 22.21_313.1445: retention time (rt) and mass to charge ratio (m/z) were accepted as potential metabolic markers. A model that can potentially predict tolerance in citrus rootstocks with >98% accuracy is presented.

Keywords Biomarker · Metabolite abundance; Phytophthora root rot · Plant metabolomics

Introduction

Phytophthora species, particularly *P. citrophthora, P. nicotianae*, and *P. palmivora*, remain important soil and water borne pathogens affecting citrus production worldwide (Boava et al. 2011; Graham and Feichtenberger 2015; Panabières et al. 2016), impacting negatively on the profitability of citriculture (Matheron et al. 1998; Adaskaveg et al. 2014; Meitz-Hopkins et al. 2014). No commercial citrus rootstocks are 100% immune to *Phytophthora* root rot (Castle 1987; Siviero et al. 2006) resulting in rootstocks with varying tolerance to these pathogens (Boava et al. 2011). Citrus rootstock tolerance is defined by Graham (1990) as the capacity of infected rootstocks to withstand infection, however, the innate mechanisms by which plants defend themselves against pathogen invasion is one that is yet to be fully elucidated (Bednarek 2012; Pérez-Clemente et al. 2013; Matsukawa et al. 2017). In South Africa *Phytophthora nicotianae* Breda de Haan (syn. *P. parasitica* Dastur) is the predominant causal agent of fibrous root rot and tree decline of citrus (Thompson et al. 1995; Meitz-Hopkins et al. 2014).

The use of citrus rootstocks with greater tolerance to *P. nicotianae* is considered the most effective and affordable long-term method of managing citrus root rot (Castle 2010; Adaskaveg et al. 2014). Citrus breeding programs aim to replace existing stocks with rootstocks of increased disease tolerance whilst maintaining favourable agronomic qualities (Grosser et al. 1995; Castle 2010; Schinor et al. 2013; Adaskaveg et al. 2014). Pathogenicity screening is the common method used to determine tolerance/susceptibility of rootstocks. However, the breeding and selection of new citrus

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rootstocks is an arduous, decades long undertaking (Castle 2010; Curtolo et al. 2017). Fernie and Schauer (2008) illustrate that metabolomics-based approaches can reduce the time for development of elite lines in crop improvement strategies. While metabolomics allows for greater insight into biological systems (Saito and Matsuda 2010), it is important to remain aware of the challenges, limitations and bottlenecks associated with its application (Matsuda 2016; Matsukawa et al. 2017). Notwithstanding the challenges, it remains essential to explore what possibilities plant metabolomics technologies can reveal for the citrus rootstock-*P. nicotianae* problem. Our objective was to develop a method for rapid assessment of rootstocks for *P. nicotianae* tolerance, using a metabolomics approach to identify metabolic markers for the phenotypic trait of tolerance based on metabolite abundance. Plant metabolomics tools have been used to demarcate citrus genotypes in phenotyping studies (Arbona et al. 2009) and in diagnostic studies, for example, to identify potential citrus Huanglongbing (HLB) tolerance biomarkers (Cevallos-Cevallos et al. 2009). Albrecht et al. (2016) used plant metabolomics applications to identify metabolic profiles associated with disease response and disease tolerance while investigating citrus HLB. The burgeoning prominence of applying metabolomics technology in systems biology enables greater capacity to develop powerful diagnostic and predictive tools for biomarker discovery (Schudoma et al. 2012; Fernandez et al. 2016) as investigated here.

The identification of resistance related metabolites as potential biomarkers for tolerance traits was investigated in barley (Bollina et al. 2011; Kumaraswamy et al. 2011) and in wheat (Hamzehzarghani et al. 2008; Paranidharan et al. 2008). Resistance related metabolites are small molecules or secondary metabolites, which are detected in higher abundance in uninoculated resistant or tolerant plants as opposed to susceptible plants (Hamzehzarghani et al. 2008; Kumaraswamy et al. 2011). These metabolites, in particular from disease free plants are constitutive, and have potential uses as biomarkers for rapid screening of plant genotypes for disease tolerance (Kumaraswamy et al. 2011). Biomarkers are organic indicator compounds that can be used as tracers of a given biological trait (Simoneit 2005; Schudoma et al. 2012; Menard et al. 2013). Metabolic markers are therefore sub-categories of biomarkers and can be either diagnostic, prognostic, or predictive markers (Fernandez et al. 2016; Kumar et al. 2017).

For the study presented here, citrus rootstocks were categorized as being either tolerant, moderately tolerant or susceptible to *P. nicotianae* root rot based on greenhouse assessments. Subsequently root extracts of selected tolerant and susceptible rootstocks were analysed using ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC/MS-MS) aiming to identify metabolites that best distinguish between the two groups, for use as potential metabolic markers. Computational statistics yielded potential biomarkers based on metabolite abundance. The predictive model was developed by selecting markers corresponding with tolerance, as identified in MarkerLynx S-plot of orthogonal partial least squares discriminant analyses (OPLS-DA) and verified through descriptive and inferential statistics. We propose that the current study, on the development of such a model has the potential application towards the rapid identification of tolerant citrus rootstocks based on metabolite abundance. However, necessary follow-up is required to better satisfy the complexities faced when applying metabolomics approaches to trait discovery and predictive model formulation (Fernandez et al. 2016; Kumar et al. 2017; Matsukawa et al. 2017).

Materials and methods

Plants The following citrus rootstocks were included in the study: Australian trifoliate (*Poncirus trifoliata*) (AT); Benton citrange [*Citrus sinensis* (L.) Osbeck. x *P. trifoliata*] (BC); Cairn rough lemon [*Citrus jambhiri* (Lush.)] (CRL); Carrizo citrange [*C. sinensis* (L.) Osbeck. x *P. trifoliata*] (CC); C35 citrange [*C. sinensis* (L.) Osbeck. x *P. trifoliata*] (C35); Cleopatra mandarin (*C. reticulata*) x Swingle citrumelo (*C. paradisi* Macf. x *P. trifoliata*) (C + S); Esselen rough lemon (*Citrus* spp.) (ERL); Flying dragon (*P. trifoliata*) (FD); Minneola tangelo (*C. reticulata* Blanco x *C. paradise* Macf.) x trifoliate orange (*P. trifoliata*) (MxT); Sunki mandarin and Benece trifoliate (SxB); Swingle citrumelo (*C. paradisi* Macf. x *P. trifoliata*) (SwC); Terra Bella citrumelo [*C. sinensis* (L.) Osbeck. x *P. trifoliata*] (TB); Troyer citrange [*C. sinensis* (L.) Osbeck. X *P. trifoliata*] (TC); Volkamer lemon (*Citrus volkameriana*) (VOLK); X639-hybrid [*C. trifoliata* x *C. reticulate* (Blanco)] (X639); Yuma citrange [*C. sinensis* (L.) Osbeck. x *P. trifoliata*] (YC). Seeds of these rootstocks were obtained from Citrus Research Internationals' Citrus Improvement Scheme (Uitenhage, South Africa). Seeds were germinated in concrete containers (44 × 190 × 20 cm) filled with sterile vermiculite at 28 °C with a 12 h light dark cycle in growth cabinets (Conviron- Winnipeg, Montabo, Canada). The growth medium was moistened by watering with heat-sterilized water. After germination, a water-soluble fertilizer (Folifeed® 6–1-4, N-P-K, 1 g/l, Hygrotech, South Africa) was applied to the plants fortnightly. Two months post sowing, all plants were moved from the growth cabinets to a greenhouse. Seedlings were transplanted singly into 16 cm diameter plastic pots containing steam pasteurized sand/peat potting mixture (3:1 *v*/v) and maintained under greenhouse conditions for use in pathogenicity experiments. Plants were watered twice weekly with inclusion of the water-soluble fertilizer once a fortnight.

Pathogen inoculum *P. nicotianae* was isolated from citrus orchard soils (Mbombela, Limpopo province, South Africa) by the citrus leaf-disk method (Grimm and Alexander 1973). Pure cultures were obtained by transferring hyphal tips to fresh V8 juice media plates and incubating in the dark at 25 °C according to Maseko et al. (2007). Isolate pathogenicity was confirmed by infecting healthy citrus seedlings and re-isolating the pathogen, thereby fulfilling Koch's postulates. Morphological and molecular characterisation of the isolate was conducted at the Plant Protection Research Institute, Pretoria, South Africa. Sequence analysis on the isolate of the internal transcribed spacer (ITS) spacers 1 and 2 confirmed the isolate as *P. nicotianae*. The isolate was grown in pure culture on clear V8 juice agar plates for 10 to 12 days prior to bulking to ensure sufficient inoculum quantities to augment soils for pathogen inoculated treatments. Bulk inoculum was prepared as previously described by Fourie (2004). Briefly, autoclave bags containing 200 g millet seed plus 150 ml distilled water were triple autoclaved, inoculated with twenty, 6 mm diameter *P. nicotianae* plugs excised from V8 juice agar plates and incubated for four weeks at room temperature in the dark. Mock treatments were prepared in the same way by excluding the pathogen.

Plant inoculation and experimental design Five greenhouse experiments were conducted over three seasons to evaluate the tolerance of citrus rootstocks to *P. nicotianae*. Two experiments were conducted over two seasons in a sand/peat potting mixture. In experiment 1, season 1 (2011), and experiment 3, season 2 (2012), seven-month-old seedlings were used. Three experiments were conducted over three seasons in a soil/sand potting mixture. In experiment 2, season 1 (2011), nine-month-old seedlings were used, whereas in experiment 4, season 2 (2012), 11-month-old seedlings were used and in experiment 5, season 3 (2013), nine-month-old seedlings were used. It was not always possible to acquire sufficient numbers of seedlings of all rootstocks throughout all the experiments. However, each of the test rootstocks was evaluated at least over two seasons. At the time of inoculation, citrus seedlings were transplanted into either sand/peat (3:1 *v*/v) or soil/sand (2:1 v/v) medium augmented with either *P. nicotianae* millet seed inoculum (5% v/v) or sterile millet seed (5% v/v) i.e. controls. For each rootstock cultivar, an uninoculated control was included as well as a pathogen inoculated treatment, with one pot containing one plant representing a replicate. The experimental design was a completely randomized block design laid out in a greenhouse with five replicates per treatment. Greenhouse temperature was maintained at ±28 °C. Plants were watered twice a week with heat-sterilised tap water.

Disease assessment Plants were harvested eight weeks after inoculation by gently removing them from their pots and rinsing the growth medium from the roots under running tap water. Root rot was assessed by a rating scale of 0 to 4 where 0 = no root rot; 1 = 25% root rot; 2 = 50% root rot; 3 = 75% root rot and 4 = 100% root rot (Fourie 2004). Percentage root rot data were rank transformed prior to one-way analysis of variance (ANOVA) using JMP Pro 11 (SAS Statistical package). Post ANOVA means separations were made with Tukey-Kramer HSD procedure at $P < 0.05$. Plant roots were excised from the stems and stored as frozen samples at −20 °C for biochemical analysis of root extracts.

Metabolite extraction and analysis Five replicates per rootstocks from Experiment 4 were individually frozen in liquid nitrogen and crushed to a fine powder using a mortar and pestle. One gram powdered root material was transferred to glass tubes and 3 ml cold ethyl acetate-ethanol (1:1) (Merck, HPLC grade) mixture was added. Tubes were capped, vortexed for 30 s and allowed to settle for extraction overnight in the dark at room temperature. After 24 h, extracts were recovered from the tubes and transferred to clean, labelled glass tubes before being evaporated to dryness in a fume hood. Resultant residues were suspended in 0.5 ml methanol (Merck, HPLC grade) to produce crude extracts. Aliquots of 50 μl for each rootstock extract were then transferred to Eppendorf tubes and stored at −20 °C until biochemical analyses. UPLC/MS-MS analyses of the 124 samples was performed by the Central Analytical Facility at the University of Stellenbosch, South Africa. The samples were randomised in the sample manager and analysed over a period of five consecutive days to minimize process variance. Two cocktail mixtures of commercially (Sigma, USA) available flavonoid standards were prepared. The cocktails were injected at the beginning and after every eight samples, as technical repeats to confirm the stability of the UPLC/MS-MS system. Metabolites were separated using the Central Analytical Facilities standard procedure, i.e. 0.1% formic acid (solvent A) to acetonitrile (solvent B) gradient, at flow rate of 0.4 ml/min on a Waters BEH C18,2. 1 × 100 mm column for a 30 min run time. Mass spectrometry readings were generated on a Waters SYNAPT™ G2 MS (Manchester, UK) instrument using electron spray ionization (ESI) in positive mode with a cone voltage of 15 V.

UPLC/MS-MS output processing For the development of a predictive model data from four uninoculated tolerant (Benton citrange, Flying dragon, Swingle citrumelo and Terra Bella citrumelo) and four uninoculated susceptible (Cairn rough lemon, Carrizo citrange, Volkamer lemon and X639-hybrid) rootstocks were selected for analysis. This approach would yield resistance related constitutive metabolites (Kumaraswamy et al. 2011). After parameter selection for peak alignment using MarkerLynx software (Waters, MA, USA), advanced multivariate algorithms for orthogonal partial

least squares discriminant analyses (OPLS-DA) were automatically generated. The multivariate analysis was performed using MarkerLynx XS from MassLynx software version 4.1 to identify markers that clearly distinguish between tolerant and susceptible rootstocks. MarkerLynx was used for assignment of putative names for the potential biomarkers through online database searches on Chemspider (http://www.chemspider.com/).

Data reduction and model development To verify potential metabolic markers identified through OPLS-DA by signal strength, i.e. greater abundance in tolerant as opposed to susceptible rootstocks, m/z data from MarkerLynx were exported to MS-Excel. Data were further analysed in SAS (SAS/STAT software v9.3. SAS Institute) for the calculation of descriptive and inferential statistics based on a test hypothesis of the ion features for tolerant vs. susceptible rootstocks using logistic regression. The markers to enter the model were selected using a three-step variable reduction method. The rules were: (1) if the number of missing values for the tolerant group is less than five in the 20 samples; (2) if the number of missing values for the susceptible group is less than five in the 19 samples; (3) if the average tolerant signal is 10% higher than that of the average susceptible signal (fold-change approach; Kumar et al. 2017). The ion features that met conditions 1 to 3 were accepted as top markers according to signal strength from the top 10% of the signal strength in rootstock extracts. Missing values (less than 5) were then imputed by the minimum value observed for particular ion features (variable), per group, i.e. tolerant/susceptible. The numbers of missing values per observation were counted, and a weight variable created so that observations that had fewer missing values carried more weight. A stepwise logistic regression procedure was performed on 39 samples and top markers in order to select the best possible variables for a 1, 2, 3, or 4 variable model using Firth's penalised maximum-likelihood estimation method in order to circumvent quasi separation of the data points. The probability modelled was GroupOriginal = "Control-Tolerant".

Results and discussion

Disease assessment From the results of the greenhouse inoculation experiments, it was possible to categorize the rootstocks as tolerant, moderately tolerant or susceptible according to percentage root rot (Table 1). Trifoliate orange (*P. trifoliata*) cultivars Australian trifoliate (AT) and Flying dragon (FD) and the trifoliate orange hybrids Terra Bella citrumelo (TB) and Yuma citrange (YC) were categorized as tolerant in all experiments (root rot below 20%), over three seasons. Swingle citrumelo (SwC) and Benton citrange (BC) also consistently showed tolerant responses (below 20% root rot), but this increased to 33% root rot for SwC in experiment 3 and to 25% for BC in the same experiment (Table 1). Trifoliate orange and their hybrids are known to be *P. nicotianae* resistant rootstocks (Boava et al. 2011; Graham and Feichtenberger 2015). Under greenhouse conditions, Timmer et al. (1991)

Table 1 Response of citrus rootstock seedlings to inoculation with *Phytophthora nicotianae* in the greenhouse

Rootstocks	Expt. 1 Root rot (%)*	Expt. 2 Root rot (%)	Expt. 3 Root rot (%)	Expt. 4 Root rot (%)	Expt. 5 Root rot (%)	Category
CC – Carrizo citrange	87.5 ^{a}	87.5 ^{a}	91.6 ^{a}	85 ^{ab}	–	S
TC – Troyer citrange	84.3 ^{a}	82.5 ^{a}	87.5 ^{a}	–	–	S
X639 – X639 hybrid	80 ^{a}	85 ^{a}	87.5 ^{a}	85 ^{ab}	–	S
CRL – Cairn rough lemon	75 ^{a}	72.5 ^{ab}	83.3 ^{a}	87.5 ^{a}	72.5 ^{a}	S
VOLK – Volkamer lemon	75 ^{a}	59.5 ^{bc}	83.3 ^{a}	80 ^{ab}	–	S
SxB – Sunki x Benece	55 ^{b}	60 ^{bc}	83.3 ^{a}	75 ^{ab}	62.5 ^{a}	S
C35 – C35 hybrid	30.5 ^{c}	31.5 ^{def}	37.5 ^{b}	45.8 ^{cd}	–	M
MxT – Minneola x Trifoliate	27.5 ^{c}	40 ^{d}	33.3 ^{bc}	53.1 ^{cd}	–	M
ERL – Esselen rough lemon	–	35 ^{de}	–	35.7 ^{de}	–	M
C + S – C. mandarin x S. Citrumelo	–	18.7 ^{efg}	–	64.2 ^{bc}	–	M
BC – Benton citrange	20 ^{cd}	10 ^{g}	25 ^{bcd}	21.8 ^{ef}	12.5 ^{b}	T
YC – Yuma citrange	17.5 ^{cd}	–	16.6 ^{cd}	–	–	T
AT – Australian trifoliate	15 ^{cd}	–	16.6 ^{cd}	–	15 ^{b}	T
SwC – Swingle citrumelo	15 ^{cd}	17.5 ^{fg}	33.3 ^{bc}	14.2 ^{f}	22.7 ^{b}	T
TB – Terra Bella citrumelo	10 ^{d}	7.5 ^{g}	8.3 ^{d}	8.3 ^{f}	9.3 ^{b}	T
FD – Flying dragon	8.3 ^{cd}	12.5 ^{g}	–	18.7 ^{ef}	7.5 ^{b}	T

*Root rot (%) determined according to a rating scale of 0–4. Within column mean values followed by the same letter are not significantly different (Tukey-Kramer test $P < 0.05$). - = no rootstocks. S = susceptible; M = moderately tolerant; T = tolerant

documented that Swingle citrumelo was more susceptible to *P. nicotianae* root rot than Trifoliate orange rootstocks. However, in the current study, there was no statistically significant difference between these two rootstocks. A moderately tolerant response was observed for trifoliate hybrids C35 citrange (C35) and Minneola tangelo x trifoliate orange (MxT) as well as Esselen rough lemon. These rootstocks developed between 27 and 53% root rot in both potting mixtures across all experiments (Table 1). Disease severity was highest for Cairn rough lemon (CRL), Carrizo citrange (CC), Troyer citrange (TC), Sunki mandarin x Benece trifoliate (SxB), Volkamer lemon (Volk) and X639 hybrid, developing root rot of more than 70%. These rootstocks have been documented as susceptible to root rot under greenhouse conditions (Timmer et al. 1991; Burger 2001). Post ANOVA means separation indicated a significant difference between the tolerant and susceptible groups of rootstocks (Table 1). In South African orchards, the main commercial rootstocks include Carrizo and Troyer citrange, Cairn rough lemon and Swingle citrumelo (Meitz-Hopkins et al. 2014). The current study provides up-to-date information to the citrus industry regarding tolerant citrus rootstocks that can be considered as replacements for susceptible rootstocks.

Multivariate data analysis MarkerLynx software selected 367 ion features in the 124 citrus root extracts. The selected markers across the 124 sample injections were aligned by retention time and base peak m/z. The peak areas of an early, middle and late eluting peak were plotted against chromatogram sequence number to test the reproducibility of the peak area. No correction was made for peak area of the citrus samples, as this change, in the standards, was less than 20% for the late eluting peak and less than 10% for the middle and early eluting peaks, and the Coefficients of Variance (CV%) were low. The markers identified by MarkerLynx with OPLS-DA multivariate analysis are displayed in Fig. 1. The markers at

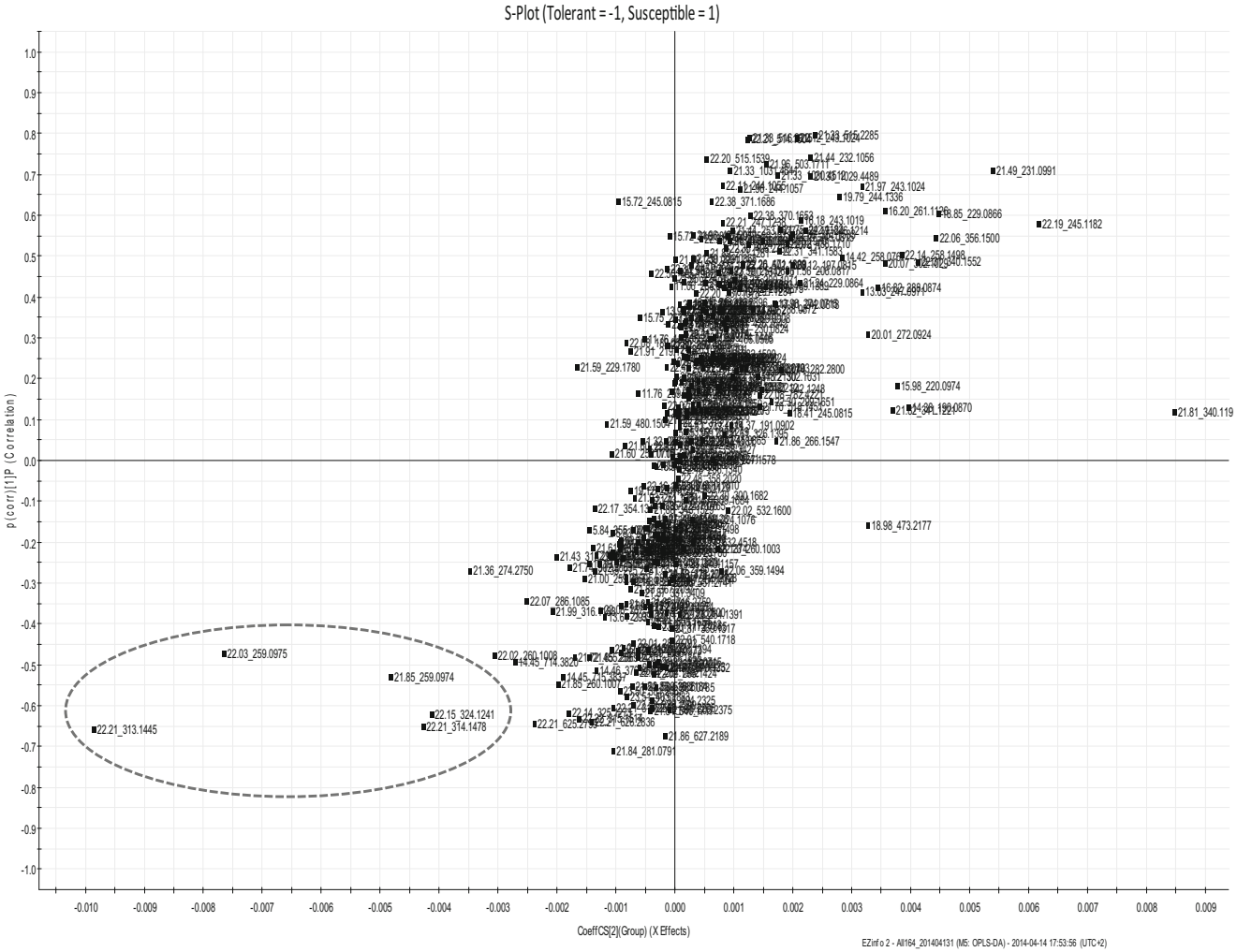

Fig. 1 MarkerLynx S-plot for the markers that differentiate tolerant (lower left quadrant) and susceptible (top right quadrant) cultivars. The markers at the bottom left and top right of the curve, with p corr[1] < −0.5 and > 0.5, occur predominantly in the tolerant and susceptible cultivars respectively. Each feature is identified by retention time underscore accurate mass e.g. 22.03_259.0975. Dash line = highlights top tolerance markers for MarkerLynx putative identification

the bottom left and top right of the curve, with p corr[1] < −0.5 and > 0.5, occur predominantly in either the tolerant or susceptible cultivars, respectively. The circled metabolites (top markers demarcating for tolerance (Fig. 1) where uploaded to online metabolite database Chemspider from MarkerLynx for putative identification of the markers, yielding Wyerone for marker 22.03_259.0975 and 4′-prenyloxyresveratrol for marker 22.21_313.1445.

Data reduction and model development Table 2 shows potential markers derived from the three-rule data reduction procedure developed to accept features with specific characteristics regarding resistance/tolerance related constitutive metabolites. The three-rule procedure was based on signal strength and selected markers that are unique for the two test groups (tolerant or susceptible). It was valuable to select features on this basis to exclude features that would provide conflicting information relating to the overall purpose of the model (Fernandez et al. 2016). The markers were the same as those identified through OPLS-DA (Fig. 1). The two features selected were 22.03_259.0975 and 22.21_313.1445 (retention time and m/z) which appeared as predominant features for tolerant citrus rootstocks as indicated by multivariate OPLS-DA (Fig. 1). A two-variable model was decided upon, since the score χ^2 value's increase flattened out after adding more variables (Table 3). The combination was decided upon after evaluating the three- and four-variable models and observing the variables that appeared most frequently. Firth's penalized maximum likelihood procedure was then employed for fitting a two-variable, logistic regression model (Table 5). All *p*-values were less than 0.01 (Table 4; Table 5) for separating the two classes and were therefore statistically significant.

Table 2 Potential marker compounds highlighting signal strength of top 14 tolerance ion features for input in stepwise logistic procedure (Group Original = "Control Tolerant")

Retention time/mass *(Min_m/z)*	New name	Average signal strength
22.03_259.0975	var2	964
22.15_324.1241	var4	548
22.21_313.1445	var24	659
22.21_314.1478	var27	122
22.14_325.1273	var29	102
22.02_260.1008	var30	144
21.97_243.1024	var40	237
21.85_259.0974	var43	727
22.17_354.1347	var49	141
21.85_260.1007	var75	107
21.59_251.0686	var76	110
21.59_229.0868	var79	2783
21.59_230.0901	var82	385
21.81_340.1190	var125	113

Table 3 Best models resultant from stepwise logistic regression

Regression models selected by score criterion		
Number of variables	Score chi-square	Variables included in model
1	12.3027	var24
1	11.8962	var27
1	10.0857	var75
2	27.5811	var24; var30
2	27.5281	var2; var24
2	26.9825	var27; var30
3	29.0697	var2; var24; var27
3	29.0291	var24; var27; var30
3	28.9949	var24; var30; var125
4	30.9668	var24; var30; var49; var125
4	30.8906	var2; var24; var49; var125
4	30.4867	var27; var30; var49; var125

This yielded the following model:

$$Ln\left(\frac{p}{1-p}\right) = -6.2214189 + 0.00266\,\text{var}2 + 0.01119\,\text{var}24$$

$$\therefore p = \frac{1}{1 + e^{-(-6.2214189 + 0.00266\,\text{var}2 + 0.01119\,\text{var}24)}}$$

The decision criterion was therefore:

$$\text{Decision} = \begin{cases} \text{Tolerant if} & p \geq 0.5 \\ \text{Susceptible if} & \text{otherwise.} \end{cases}$$

Rootstocks with $p > 0.5$ may thus be classified as *P. nicotianae* tolerant. The importance of *p* value and the fold change approach make up conventional methods of ranking influential metabolites (Kumar et al. 2017). Table 6 summarises the fit of the model indicating a 98% concordance with the predictive capacity of the model to select for tolerant citrus rootstocks. Biomarkers can be identified through untargeted metabolite fingerprinting approaches to compare the patterns between the metabolome of tolerant genotypes versus susceptible genotypes (Kumaraswamy et al. 2011; Monteiro et al. 2013; Wolfender et al. 2013).

Biomarkers are important for their uses in mapping quantitative trait loci (QTL) (Fernie and Schauer 2008) and have significant application as predictive tools in marker-assisted plant breeding (Steinfath et al. 2010; Falke and Mahone 2013;

Table 4 Testing global Null hypothesis: BETA = 0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood ratio	29.1262	2	<.0001
Score	27.3772	2	<.0001
Wald	11.5249	2	0.0031

Table 5 Analysis of penalized maximum likelihood estimates

Parameter	DF	Estimate	Standard error	Wald chi-square	Pr > ChiSq
Intercept	1	−6.2214	1.6723	13.8412	0.0002
var2	1	0.00266	0.00006	17.8785	<.0001
var24	1	0.0111	0.00324	11.7381	0.0006

Monteiro et al. 2013; Ernst et al. 2014; Fernandez et al. 2016). An advantage in using ion features as metabolic markers for phenotypic traits is that there is no requirement for annotation (Arbona et al. 2009; Cevallos-Cevallos et al. 2009). However, through online metabolite database searches using MarkerLynx, putative assignments are presented here. Wyerone is a phytoalexin associated with conferring greater tolerance in broad beans (*Vicia faba* L.) following infection by *Botrytis fabae* (Letcher et al. 1970; Fawcett et al. 1971). 4′-prenyloxyresveratrol is a secondary metabolite previously reported to have anti-microbial properties and has been extracted from mulberry (*Morus spp.*) and bread fruit (*Artocarpus incisus*) plants (Likhitwitayawuid and Sritularak 2001). It is associated with Stilbenoid biosynthesis via the mixed phenylpropanoid/polyketide biosynthetic pathway (Likhitwitayawuid and Sritularak 2001). In plants, the defense response to pathogens is increasingly better linked or associated with the production and accumulation of phytoalexins, through activation of the general phenylpropanoid pathway (shikimate-phenylpropanoids-flavonoids pathways) (Bennett and Wallsgrove 1994; Shulaev et al. 2008; Pérez-Clemente et al. 2013). The association of these compounds with plant self-defense, renders these findings significant, however further research is required to confirm the putative annotation of these compounds.

Upon further investigation, statistical work and annotation to complement these initial findings, the metabolic markers and prediction model outlined here have the potential to be applied as the basis for citrus rootstock breeders to identify *P. nicotianae* tolerant rootstocks prior to pathogenicity screening. This may be achieved by including only those rootstocks that contain the proposed tolerance biomarkers from breeding lines. Destructive sampling of a sub-sample of test rootstock population (root material) analysed for these markers may shed light on the overall population. In cases where the markers are prominent, the likelihood of *P. nicotianae* tolerance is high for the whole population. A further advantage is the constitutive nature of the potential markers, rendering them functional for tolerance determination in diseased or healthy plants. Their higher metabolite abundance in the tolerant rootstocks opposed to the susceptible rootstocks is concomitant with Kumaraswamy et al. (2011) descriptions for metabolite markers.

Table 6 Association of predicted probabilities and observed responses

Test			
Percent concordant	98.4	Somers'D	0.968
Percent discordant	1.6	Gamma	0.968
Percent tied	0.0	Tau-a	0.497
Pairs	380	C	0.984

In conclusion this has the potential to help plant breeders assess large numbers of test citrus rootstocks for *P. nicotianae* tolerance more rapidly through the proposed pipeline. The metabolic markers can be used to predict the selected trait of *P. nicotianae* tolerance prior to time consuming greenhouse screening by including rootstocks found to constitutively contain these markers (Menard et al. 2013). Although the model is specific for *P. nicotianae* tolerance biomarkers a similar approach may be used to develop models for other *Phytophthora* species. In this study, we report on a metabolomics approach for rapid assessment of citrus rootstocks for tolerance against root rot caused by *P. nicotianae*. By identifying ion features that correspond with the phenotypic trait of tolerance and applying descriptive and inferential statistics, we were able to develop a model that can potentially predict for this trait in citrus rootstocks with >98% accuracy. This model can conceivably speed up the screening of citrus rootstocks for root rot tolerance if integrated into a rootstock breeding and selection program. The potential application of such a model does however require further development and assessment of a wider range of rootstocks. This study is part of a larger project to utilise plant metabolomics approaches to better elucidate the biochemical mechanisms of tolerance in citrus rootstocks against *Phytophthora nicotianae*.

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