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Metabolic Profiling to Phenotype Potato Genotypes Varying in Horizontal Resistance to Leaf Infection by *Phytophthora infestans*

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ABSTRACT

Screening potato breeding lines, based on disease severity, for horizontal resistance to *Phytophthora infestans* is slow, time consuming and the results under field conditions are often inconsistent over years. This study reports a potential application of metabolic phenotyping as an additional tool for screening potato genotypes for disease resistance. Three potato cultivars 'Libertas', 'Caesar' and 'Russet Burbank' with high, moderate and low resistance to *P. infestans*, respectively, were inoculated with the pathogen or water, and metabolites were analyzed using GC/MS. Ninety-four metabolites were putatively identified, of which 89 had significant treatment effects. Based on univariate analysis, 24 metabolites were identified as Resistance Related Constitutive (RRC) and 32 as Resistance Related Induced (RRI) metabolites. A canonical discriminant analysis of the 89 metabolites identified metabolic phenotypes comparable to disease severity phenotypes. The first three CAN-vectors explained 97% of the total variance. A total of 23 were RR metabolites associated with resistance in 'Caesar' and 'Libertas' (CAN1 = 65%), while 33 were RRI metabolites in 'Libertas' (CAN2 = 23%). In the latter, 25 metabolites were RRI-metabolites and mainly belonged to amino and organic acids group. Among these RRI-metabolites, the abundances of phenylalanine, tyrosine, shikimic acid, malonic acid and benzoic acid significantly increased following pathogen inoculation. These metabolites were previously reported to activate plant secondary defense metabolism particularly the phenylpropanoid and malonic acid pathways that produces several antimicrobial compounds. The potential application of the metabolic profiling technology for high throughput screening of potato breeding lines against late blight is discussed.

Keywords: canonical discriminate analysis, GC/MS, horizontal resistance, *Phytophthora infestans*, RR-metabolites, *Solanum tuberosum*
Abbreviations: C, 'Caesar'; **CDA**, canonical discriminant analysis; **GMD**, Golm metabolome database; **HPI**, hours post inoculation; **KEGG**, Kyoto encyclopedia of genes and genomes; **L**, 'Libertas'; **R**, 'Russet Burbank'; **RR**, resistance related; **RRC**, resistance related constitutive; **RRI**, resistance related induced

INTRODUCTION

Late blight caused by *Phytophthora infestans* (Mont.) de Bary is one of the major diseases of potato (*Solanum tuberosum*) in Canada, particularly after the appearance of A2 mating type (Goodwin *et al*. 1998). This aggressive mating type interbreeds with the A1 mating type producing new races (Fry *et al*. 1993; Peters *et al*. 1999; Stromberg *et al*. 2001). In spite of the high cost and harmful effects on the environment, fungicides are being extensively used to control this disease (Daayf and Platt 1999; Daayf *et al*. 2002). The annual worldwide losses caused by *P. infestans* including the cost of control measures exceed US$ 3 billion (Duncan 1999). Breeding for the vertical resistance has been the most obvious choice of breeders to control this pathogen. Unfortunately, this type of resistance is not durable and can be broken down easily by the emergence of new races of this pathogen. On the contrary, horizontal resistance is considered to be more durable in the field (Peters *et al*. 1999; Haynes *et al*. 2002).

Although high levels of horizontal resistance have been detected in non-cultivated and wild *Solanum* spp., the progress made in transferring horizontal resistance to cultivated potatoes has been very limited because of the difficulty in breeding for polygenic traits (Evers *et al*. 2003). Horizontal resistance can be measured based on multiple epidemiological disease parameters such as infection efficiency, latent period, lesion expansion, sporulation, etc. (Miller *et al*. 1998; Carlisle *et al*. 2002), however, these tests are time consuming. Field evaluation based on disease severity is often inconsistent over years. Accordingly, the plant breeders are looking for effective tools to phenotype cultivars varying in quantitative resistance and also would aid in better understanding of the mechanism of resistance. Metabolic phenotyping of partial resistance could be a potential alternative.

Metabolic profiling and fingerprinting have been used to study genetically modified traits (Roessner *et al*. 2001; Munger *et al*. 2005), single gene mutation in *Arabidopsis thaliana* (Fiehn *et al*. 2000b), salt-stress in tomato (Johnson *et al*. 2003), primary metabolism in rice leaves (Sato *et al*. 2004), transgenics in *Nicotiana tabacum* (Mungur *et al*. 2005), biotic and abiotic stress in *Medicago truncatula* (Broeckling *et al*. 2005), differentiate wild and cultivated tomatoes (Schauer *et al*. 2005), discriminate wild-type and transgenic lines of *Populus* spp. (Robinson *et al*. 2005), effect of thio-disulfide on *Arabidopsis thaliana* metabolism (Kolbe *et al*. 2006), metabolic phenotype resistance in wheat against *Fusarium graminearum* (Hamzehzarghani *et al*. 2005, 2008a, 2008b), temporal dynamics of pathogenesis related metabolites in potato (Abu-Nada *et al*. 2007) and effect of deoxynivalenol on resistance in wheat against

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Fusarium head blight (Paranidharan *et al.* 2008). Moreover, Individual or set of metabolites have been linked to specific genomic locations and metabolic quantitative trait loci (QTLs) have been linked to traits (Schauer and Fernie 2006; Keurentjes *et al*. 2006). The objective of this study was to explore the potential of metabolic profiling to classify potato cultivars varying in horizontal resistance to *P. infestans*, for potential application in screening for disease resistance.

MATERIALS AND METHODS

Potato plant production

Elite seed tubers of three potato cultivars 'Caesar' (C), 'Libertas' (L), and 'Russet Burbank' (R) were obtained from the Potato Research Center, Agriculture and Agri-Food Canada, New Brunswick. 'Russet Burbank' was reported to be susceptible (Porter *et al.* 2004; CFIA 2007; ECDP 2007), 'Caesar' to be moderately resistant (CFIA 2007; ECDP 2007) and 'Libertas' to be highly resistant to *P. infestans* (Colon *et al.* 1995; Douches *et al.* 2004; CFIA 2007; ECDP 2007). A single tuber was planted in a 16 cm diameter pot containing a mixture of 1:1 ratio of soil and PRO-Mix BX® (Premier Horticulture Ltd, Riviere-du-Loup, QC) and maintained at 20°C, 16 h photoperiod and around 70% relative humidity in a growth bench. Plants were fertilized weekly with 200 ml solution (1.5 g L^{-1}), per pot, of Plant-Prod® 20: 20: 20 containing trace elements (Plant Products Co. Ltd., Ontario, Canada). For each pot, up to three stems were maintained.

Pathogen

Phytophthora infestans (Mont.) de Bary (clonal lineage US-8, A2 mating type, isolate 1661) was obtained from AAFC, Charlottetown, PEI. The pathogen was sub-cultured on V-8-Agar media (Caten and Jinks 1968) at 15 C. After 2-3 weeks a water sporrangial suspension was prepared using sterilized water containing 0.02% Tween 80. The sporangial concentration was adjusted to 1.0×10^5 sporangia ml^{-1}.

Inoculation and incubation

Three days before inoculation, 5-6 week old plants grown in a growth bench were transferred to a growth chamber maintained at 20°C, 16 h photoperiod and 90% relative humidity. Fully-grown leaflets were inoculated on their lower surfaces, at either sides of midrib, with 5 µl of the pathogen (P) sporangial suspension or a water aqueous solution of 0.02% Tween 80 (W). The plants were misted with sterile water, covered with transparent plastic bags to maintain high humidity, and returned to the growth chamber. The bags were removed 48 HPI.

For the disease severity assessment, at 4 d post inoculation (DPI), the leaflets were detached, placed on plastic racks covered with moist paper towels and placed in plastic tray-incubators with transparent covers (28 × 54 cm). Water was added to the bottom of the trays to maintain high humidity. The leaflets were misted with sterile water and the trays were covered and returned to the growth chamber. The diameters of diseased lesions were measured at 6 DPI and used to calculate areas of the diseased lesions.

Metabolite extraction

At 48 HPI the leaflets were harvested, discs containing the inoculated lesions were cut using a 15 mm cork borer, frozen in liquid nitrogen, freeze dried for 48 h and stored at –80°C until extraction. The polar and non-polar metabolites were extracted following the methods developed by Roessner *et al.* (2000) and Fiehn *et al.* (2000a, 2000b) with minor modifications. The freeze dried leaf discs were crushed in liquid nitrogen and a 15 mg sample was used for metabolite extraction. 0.7 ml methanol and 175 µl double distilled water were added, vortexed and to this 25 µl ribitol (0.2 mg ml^{-1} of water) and 50 µl nonadecanoic acid methyl ester (2 mg ml^{-1} of chloroform) were added as internal standards. The sample was heated at 70°C for 15 min, centrifuged for 3 min at 13,500 rpm and the supernatant was transferred to a glass tube provided with a screw cap with teflonized inlays, to which 0.7 ml double distilled water was added. For the remaining pellets, 0.75 ml chloroform was added, vortex, heated at 37°C for 5 min with continuous shaking and centrifuged for 3 min at 13,500 rpm. The supernatant chloroform portion (non-polar) and the previously obtained water/methanol portion (polar) were transferred to a 15 ml Millipore Ultrafree®-CL Filters (Amicon, Bioseperation) tube, vortexed and centrifuged for 15 min at 3,800 rpm.

The upper polar phase was separated, dried using a Speed Vac (SAVANT DNA110, Thermo Electron Co.), 60 µl methoxyamine hydrochloride (20 mg ml^{-1} pyridine) was added, heated at 30°C for 90 min, derivatized with 96 µl MSTFA and heated for 30 min at 37°C. 40 µl of the sample was transferred to GC screw top amber glass vial, 25 µl each of the three (Naphthalene, Phenanthrene and Chrysene) Lee's retention time index standards (Eckel 2000) were added and the end volume of the sample was adjusted to 1 ml by adding 885 µl of hexane. 1 µl of the sample was injected into GC/MS in splitless mode.

The non-polar chloroform-phase was transferred to a new vial and 0.90 ml of chloroform and 1 ml of methanol containing 3% (v/v) H_2SO_4 was added. Sample was heated for 4 h at 100°C to transmethylate lipids and free fatty acids. Each sample was cleaned twice by adding 4 ml of pure water, vortexed and centrifuged for 15 min at 3,800 rpm. The water phase was discarded and anhydrous sodium sulfate was added to the remaining non-polar extract to remove excess of water. The supernatant was dried using a Speed Vac, 80 µl of chloroform was added and derivatized with 10 µl MSTFA and 10 µl pyridine at 37°C for 30 min. 33 µl of the end sample was transferred to GC screw top amber glass vial, 25 µl of each of the three Lee's retention time index standards were added and the end volume was adjusted to 1 ml by adding 892 µl of hexane. 1 µl of the sample was injected into the GC/MS in splitless mode.

GC/MS analysis

The leaf extract samples were transferred to an auto sampler (model 8400, Varian®, Canada) connected to a GC/MS (GC Varian®, Saturn 3900 with 2100T MS Detector, Varian® Saturn, Canada). The GC was equipped with a capillary column (30 m DB-5MS column with 0.25 mm diameter, 0.25 µm thick film, Supelco, Canada). The initial injector temperature was 230°C. Helium was used as a carrier gas with a flow rate of 1 ml s^{-1}. For the methanol-water samples, the initial oven temperature of 70°C for 5 min was increased at a rate of 4 C min^{-1} to 280°C followed by 20°C min^{-1} until 290°C at which temperature it was held for 5 min. For the chloroform extract the temperature was ramped from 70°C to 290 at the rate of 5°C min^{-1}. The mass spectra from 50 to 600 *m/z* were recorded using an ion trap mass analyzer. The GC/MS outputs were scans and abundances of mass ions (ion current from ion trap detector, which is proportional to compound concentration).

Mass spectral data processing

The GC/MS output on abundances of mass ions at different scans were imported into a spreadsheet and organized using the Pivot Table operation of the MS-EXCEL® program. The abundances of metabolites were normalized using the abundance of the internal standards, Ribitol for the water-methanol samples and Nonadecanoic acid methyl ester for the chloroform samples. The mass ion spectra of peaks with about the same retention time in five replicates of each treatment was inspected using SATURN workstation version 5.52 and the most probable choice of a name was selected for the compound using NIST Library Version 2.0 (National Institute of Standards and Technology, MD, USA). In addition, the spectra were further compared with the Golm Metabolome Database, GMD (Kopka *et al.* 2005). The low probability match peaks were considered as unidentified and the relative abundances of their mass spectra (m/z) in descending order were given in place of names. Metabolites that were not consistent among replicates were excluded from the analysis.

The Lee Retention Index (RI) was calculated for the metabolites (Lee *et al.* 1979; Eckel 2000):

$RI = \{100 \times (RT_{Unknown} - RT_n)/ (RT_{n+1} - RT_n)\} + 100 (n)$

where $RT_{unknown}$ is the Retention Time of the unknown metabolite; the RT_n and RT_{n+1} are the retention time of the standards that eluted before and after the unknown, respectively. The number of rings of the aromatic standards represents (n), i.e. n=2 for naphthalene, n=3 for phenanthrene and n=4 for chrysene. The Lee RI was also used to confirm or reject the metabolites names proposed by search libraries (NIST and the GMD database) based on the boiling point in degrees Celsius, where the derivatized metabolites with boiling points (obtained from SciFinder® Scholar Version 2002, American Chemical Society) less than their RI were considered as miss identified (Eckel 2000).

Experimental design and statistical analysis

Two experiments were conducted: i) Disease severity assessment: The experiment was designed as completely randomized with three cultivars inoculated with the pathogen and five replicates. The experimental units consisted of 15 inoculated leaflets pooled from three different plants. The data on disease severity (average lesion area in mm^2) were subjected to GLM using SAS program (8.02, Windows Version 5.1) and Duncan's multiple range tests at ($P \leq 0.05$) was used to compare the means of different treatments (Khattree and Naik 2000). ii) Metabolite profiling: The experiment was a factorial with two factors consisting of three cultivars ('Libertas', 'Caesar' and 'Russet Burbank') and two inoculations (pathogen and water), designed as a randomized complete block. The blocks were conducted 5 times (about weekly intervals). Each experimental unit consisted of 20 leaflet-discs collected from five leaves from each of two inoculated plants.

The data on abundances of 94 metabolites were subjected to ANOVA using GLM procedure of SAS to identify the compounds significantly different among treatments using the Duncan's multiple range tests at $P \leq 0.05$ level. The 89 metabolites with significant treatment effects were further subjected to canonical discriminant analysis using CANDISC of SAS to classify the treatments. The position of an observation (a replicate of a treatment) in a three-dimensional scatter plot depends on the scores of each observation for all the three significant CAN-vectors. A positive CAN-vector score of an observation results from metabolites with positive loadings, and a negative CAN-score from metabolites with negative loadings (Comrey and Lee 1992). Accordingly, a metabolite with high abundance would result in high positive CAN-scores if the CAN-loading is positive and vice-versa. For a better visualization of classification of observations the CAN-scores were subjected to hierarchical cluster analysis to obtain a classification tree or a dendrogram. The metabolic phenotypes classified by a CAN-vector were related to disease severity phenotypes to identify the underneath biological functions. The set of metabolites associated, high positive or negative loadings, with the CAN-vector that identified the function was used to putatively explain the function.

RESULTS

Disease severity

The average lesion areas, at 6 DPI, for 'Libertas', 'Caesar' and 'Russet Burbank' were 57.4, 87.3, and 149.9 mm^2, respectively. The three cultivars tested here significantly varied in their disease severity according to Duncan's multiple range tests. Based on the disease severity, the cultivars were grouped into: i) highly resistant ('Libertas'); ii) moderately resistant ('Caesar'); and iii) susceptible ('Russet Burbank').

Metabolic profiles

More than 300 peaks were detected, of which only 94 peaks with abundances >2,000 and present in all the replicates were subjected to statistical analysis. Among the 94 peaks 89 varied significantly among treatments, including 72 from methanol-water and 22 from chloroform extracts, and these were designated as metabolites and retained for further analyses (**Appendix 1**).

Multivariate analysis to identify metabolic phenotypes

The abundances of 89 metabolites, with significant treatment effects, were subjected to canonical discriminant analysis to classify different treatments. The first three CAN-vectors explained 97.4% of the total variance, with 65.2, 22.9, and 9.3% by CAN1, CAN2, and CAN3-vectors, respectively (**Fig. 1**). All the five replicates within each of the six treatments were classified in separate clusters (**Figs. 1, 2**), meaning the variances among replicates were minimal. The pathogen and water-inoculated treatments were clustered close to each other but the cultivars were well separated. The CAN1-vector discriminated susceptible (positive scores) from both the resistant cultivars (negative scores), and accordingly, it was considered to discriminate constitutive resistance in 'Libertas' and 'Caesar' (**Fig. 1**). A total of 23 metabolites [5, 7, 11, 21, 23-28, 32, 33, 63, 64, 66, 72-74, 81, 86, 88 and 89, **Appendix 1**, blue bold scores] were associated with resistance. The CAN2-vector discriminated the pathogen inoculated highly resistant cv. 'Libertas' (positive scores) from rest of the cultivars (negative scores), including water inoculated 'Libertas' which had low positive score. Accordingly, the CAN2-vector was considered to discriminate induced resistance in 'Libertas'. A total of 33 metabolites had high positive loadings (≥0.5) to CAN2 (red bold scores, **Appendix 1**). The CAN3-vector failed to discriminate resistance among cultivars.

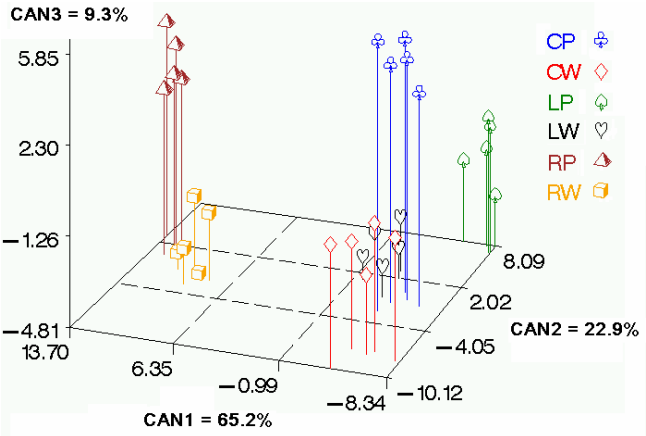

Fig. 1 Scatter plot using the scores for the first three significant CAN-vectors based on the canonical discriminant analysis of the abundances of 89 metabolites with significant treatment effects (3 cultivars, 2 inoculations, 5 replicates = 30 observations). Cultivars: C = Caesar, L = Libertas, and R = Russet Burbank. Inoculations: W = Water, P = Pathogen. A total of 65.2, 22.9 and 9.3% of the variances were explained by CAN1, CAN2 and CAN3 vectors.

Resistance related (RR) metabolites

The metabolites were considered Resistance Related (RR) (Hamzehzarghani *et al.* 2008a) if the abundance in a resistant cultivar, 'Libertas' or 'Caesar', was significantly ($P \geq 0.05$) higher than in a susceptible cv. 'Russet Burbank' (**Table 1**). These RR-metabolites were further grouped into constitutive, based on water inoculated (RRC), and induced, based on pathogen inoculated (RRI): 1) RRC_C = CW>RW; 2) RRC_L = LW>RW; 3) RRI_C = CP> CW and CP>RP; 4) RRI_L = LP> LW and LP>RP, where L = 'Libertas', C = 'Caesar', R = 'Russet Burbank', W = water inoculated, P = pathogen inoculated. Among the 89 metabolites with significant treatment effects, 46 were RR-metabolites (**Table 1**), including 35 RRC-metabolites and 34 RRI-metabolites (**Figs. 3A, 3B**).

***RRC-metabolites*:** Among the 35 RRC-metabolites, 24 and 23 were identified in 'Libertas' and 'Caesar', respectively, with 12 common to both cultivars [21, 23, 24, 27, 28, 33, 60, 66, 72, 74, 88, and 89; **Fig. 3A**. Eight of these meta-

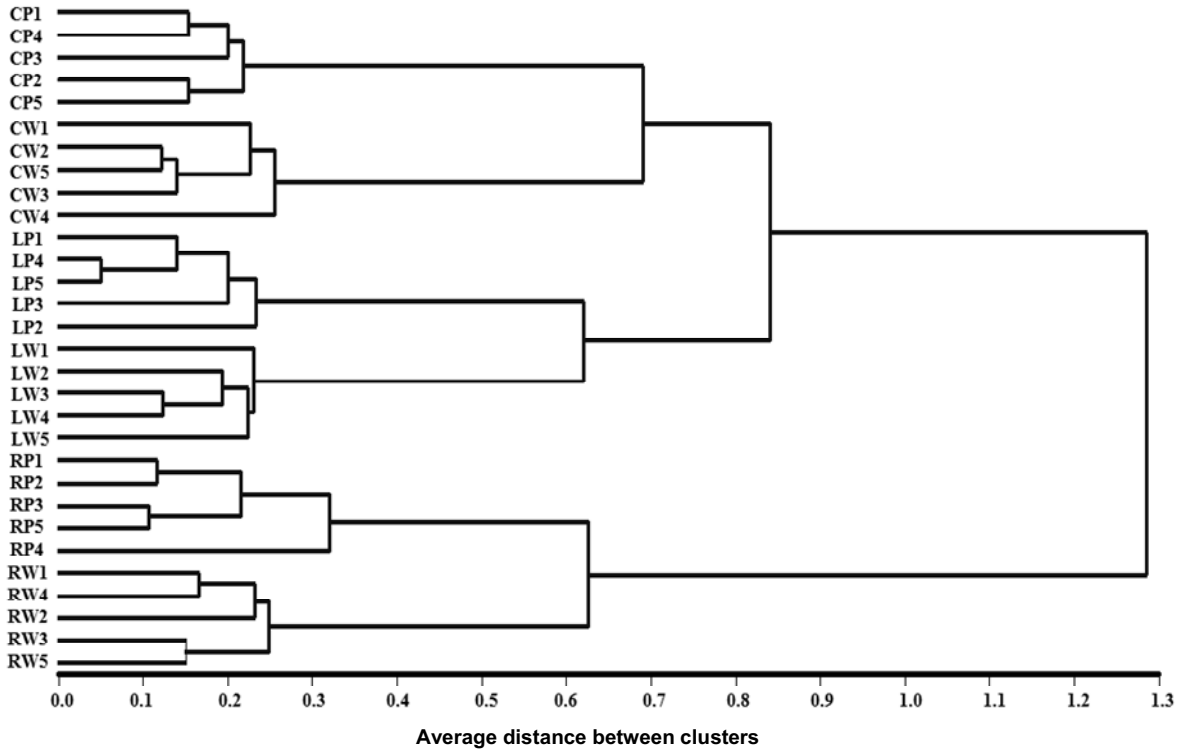

Fig. 2 Cluster tree created based on hierarchical cluster analysis, using the significant canonical values of canonical discriminant analysis. The scale shows the Euclidean distance in canonical space. Cultivars: L=Libertas, C=Caesar, R=R. Burbank; inoculations: W=water, P=pathogen; the numbers 1-5 are replicates.

bolites belonged to the following chemical groups: Organic acids: malonic acid [21], isocitric acid [23], methyl, ethyl malonate [24], fumaric acid [27], butanoic acid [28], γ-aminobutyric acid [60], succinic acid [72], and glucaric acid [74]; Amino acids (AAs): alanine [66]; Ethanolamine group: nor-epinephrine [33]; Unknown: furanone [88] and the unidentified metabolite [89]. Twelve RRC-metabolites were unique to the most resistant cultivar 'Libertas' include: isoleucine [1], valine [2], phenylalanine [5], glutamine [7], glycine [11], stearic acid [12], oleic acid [14], shikimic acid [18], propenoic acid [19], xylitol [30], xylose [31], and dopamine [32].

RRI-metabolites: Among the 34 RRI-metabolites, 32 and 24 were identified in 'Libertas' and 'Caesar', respectively, including 22 common to both cultivars (**Fig. 3B**). In 'Libertas', the 32 RRI-metabolites included 14 organic acids [OAs = 15, 16, 18, 20, 21, 22, 23-28, 63, and 73]: 11 AAs = 1, 2, 4-11, and 66; 2 sugars [SR = 64, 82]; 1 fatty acid [FA = 12]; 2 ethanolamine [EA, norepinephrine = 33, octopamine = 86]; 1 catecholamine [CA, dopamine = 32]; 1 fatty acid [FA, stearic acid = 16]; 1 unidentified [UD, 89]. Of the 14 OAs, four were members or derivatives of the citric acid cycle including malic acid [15], isocitric acid [23], fumaric acid [27] and a succinic acid derivative [26 = butanedioic acid]. Other organic acids were shikimic acid [18], benzoic acid [20], malonic acid [21], and methyl, ethyl malonate [24]. These OAs are known to be the primary blocks for the production of several plant defense secondary metabolites. Of the 11 AAs, two belong to the serine family: serine [4] and glycine [11]; two to the aromatic AAs: phenylalanine [5] and tyrosine [10]; three to aspartic acid family: isoleucine [1], threonine [6] and aspartic acid [9] and 2 belong to the Glutamine family: glutamine [7] and proline [8]; 2 belonged to the Alanine family: valine [2] and alanine [66]. On the other hand, 10 of the 32 RRIL-metabolites were unique to the most resistant cv. 'Libertas' including: isoleucine [1], serine [4], stearic acid [12], malic acid [15], gluconic acid [16], shikimic acid [18], isocitric acid [23], phosphoric acid [25], glucopyranoside [82], and an unidentified metabolite [89].

Among the 32 RRI-metabolites in 'Libertas', 16 were also RRC-metabolites, whose abundances further increased following pathogen inoculation (**Fig. 3C**). Of these metabolites, 6 were OAs including: shikimic acid [18], malonic acid [21], isocitric acid [23], methyl malonate [24], fumaric acid [27] and butanoic acid [28];6AAs including: isoleucine [1], valine [2], phenylalanine [5] and glutamine [7] and glycine [11] and alanine [66]; 1 catecholamine, dopamine [32]; 1 ethanolamine, norepinephrine [33], 1 FA [12= stearic acid], and UID [89] (**Fig. 3C**).

DISCUSSION

The present study reports the potential application of metabolic profiling as a tool for phenotyping potato cultivars varying in horizontal resistance to *P. infestans*. The three potato cultivars used here varied significantly in resistance based on disease severity and were classified as susceptible 'Russet Burbank', moderately resistant 'Caesar' and highly resistant 'Libertas', and these resistance rankings were similar to those reported earlier (Colon *et al*. 1995; Douches *et al*. 2004; Porter *et al*. 2004; CFIA 2007; ECDP 2007), though the classification was based on comparison with different sets of cultivars.

A canonical discriminant analysis identified metabolic phenotypes that were parallel to those based on disease severity phenotypes. The two resistant cultivars ('Caesar' and 'Libertas'; negative CAN1-scores) were separated from the susceptible cv. 'Russet Burbank' (positive CAN1-scores), which identified the resistance function. The resistance function was associated with 23 metabolites that loaded negatively to CAN1-vector. The highly resistant cv. 'Libertas' (pathogen inoculated) was discriminated from rest of the treatments by CAN2-vector, which identified the partly induced resistance function with high loading of 33 metabolites. However, the CDA was not efficient in discriminating 'Libertas' from 'Caesar'.

The data was further subjected to univariate analysis to clearly identify RR metabolites. Univariate analysis identified: a) RRC-metabolites = 24 in 'Libertas' and 23 in 'Caesar' (**Fig. 3A**), with 12 common to both; b) RRI-metabolites = 32 in 'Libertas' and 24 in 'Caesar' (**Fig. 3B**), with

Table 1 Tentatively identified Resistance Related metabolites and their average abundances (x10^6) in three potato cultivars[a] 'Caesar', 'Libertas', and 'R. Burbank', inoculated with water (control) or pathogen (*P. infestans*).

M[b]	Name[c]	GR[d]	RW	RP	CW	CP	LW	LP	RR-Metabolites[e]			
1	Isoleucine (G,N)	AA	0.27	0.34	0.22	0.41	0.37	0.86		RRC_L		RRI_L
2	Valine (N,G)	AA	0.34	0.39	0.29	0.51	0.45	0.88		RRC_L	RRI_C	RRI_L
4	Serine (G,N)	AA	1.28	1.36	0.92	1.28	1.02	1.62				RRI_L
5	Phenylalanine (N)	AA	0.14	0.17	0.15	0.26	0.35	0.79		RRC_L	RRI_C	RRI_L
6	Threonine (N,G)	AA	0.59	0.72	0.52	0.87	0.55	1.24			RRI_C	RRI_L
7	Glutamine (G,N)	AA	3.16	3.56	3.28	5.07	4.06	9.10		RRC_L	RRI_C	RRI_L
8	Proline (G,N)	AA	0.10	0.12	0.10	0.15	0.10	0.20			RRI_C	RRI_L
9	Aspartic acid(G,N)	AA	3.21	3.64	3.09	4.45	2.99	5.92			RRI_C	RRI_L
10	Tyrosine (N,G)	AA	0.11	0.16	0.12	0.23	0.11	0.38			RRI_C	RRI_L
11	Glycine (N,G)	AA	0.43	0.46	0.5	0.58	0.56	0.66		RRC_L	RRI_C	RRI_L
12	Stearic acid (N,G)	FA	0.56	0.61	0.43	0.43	0.67	0.79		RRC_L		RRI_L
14	Oleic acid (N)	FA	0.16	0.17	0.14	0.15	0.22	0.22		RRC_L		
15	Malic acid (N,G)	OA	27.39	28.48	24.01	26.24	29.07	33.01				RRI_L
16	Gluconic acid (N,G)	OA	0.10	0.13	0.07	0.09	0.09	0.15				RRI_L
18	Shikimic acid (G,N)	OA	1.30	1.45	1.25	1.61	1.54	3.42		RRC_L		RRI_L
19	Propenoic acid (N)	OA	0.12	0.12	0.10	0.12	0.19	0.21		RRC_L		
20	Benzoic acid (N)	OA	0.04	0.04	0.03	0.05	0.03	0.07			RRI_C	RRI_L
21	Malonic acid (N,G)	OA	0.24	0.29	0.28	0.32	0.41	0.52	RRC_C	RRC_L	RRI_C	RRI_L
22	Lactic acid derivative (N,G)	OA	0.09	0.11	0.09	0.12	0.10	0.13			RRI_C	RRI_L
23	Isocitric acid (N)	OA	1.56	1.97	2.03	2.06	3.72	4.61	RRC_C	RRC_L		RRI_L
24	Methyl, ethyl malonate (N)	OA	0.06	0.08	0.07	0.10	0.10	0.12	RRC_C	RRC_L	RRI_C	RRI_L
25	Phosphoric acid (N,G)	OA	3.50	3.50	3.71	4.28	4.13	5.56				RRI_L
26	Butanedioic acid (G,N)	OA	0.05	0.05	0.05	0.06	0.05	0.08			RRI_C	RRI_L
27	Fumaric acid (G,N)	OA	0.09	0.10	0.13	0.15	0.23	0.27	RRC_C	RRC_L	RRI_C	RRI_L
28	Butanoic acid (N)	OA	0.02	0.02	0.03	0.04	0.05	0.06	RRC_C	RRC_L	RRI_C	RRI_L
30	Xylitol (N)	SR	1.20	1.22	1.02	1.19	1.56	1.72		RRC_L		
31	Xylose (G)	SR	0.29	0.30	0.23	0.23	0.41	0.42		RRC_L		
32	Dopamine (N)	CA	0.04	0.08	0.03	0.16	0.25	0.33		RRC_L	RRI_C	RRI_L
33	Norepinephrine (N,G)	EA	0.06	0.11	0.15	0.43	0.22	0.51	RRC_C	RRC_L	RRI_C	RRI_L
60	γ-Aminobutyric acid (G,N)	OA	1.26	1.57	1.44	1.58	1.43	1.51	RRC_C	RRC_L		
61	Acetic acid (N)	OA	0.04	0.07	0.08	0.10	0.05	0.05	RRC_C		RRI_C	
62	Malic acid derivative (N,G)	OA	0.05	0.05	0.06	0.07	0.05	0.05	RRC_C		RRI_C	
63	Glyceric acid derivative (G,N)	OA	5.22	5.83	6.44	7.22	5.18	7.86	RRC_C		RRI_C	RRI_L
64	Xylulose (G,N)	SR	0.72	0.85	0.83	0.99	0.80	1.03	RRC_C		RRI_C	RRI_L
66	Alanine (G,N)	AA	0.24	0.31	0.36	0.43	0.28	0.49	RRC_C	RRC_L	RRI_C	RRI_L
72	Succinic acid (G,N)	OA	0.38	0.44	1.03	1.02	0.55	0.57	RRC_C	RRC_L		
73	Trihydroxybutyric acid (N)	OA	1.10	1.10	2.19	2.57	1.41	2.01	RRC_C		RRI_C	RRI_L
74	Glucaric acid (G,N)	OA	0.08	0.11	0.24	0.27	0.33	0.34	RRC_C	RRC_L		
77	Turanose (N)	SR	0.35	0.32	0.44	0.43	0.13	0.18	RRC_C			
78	D-Glucose (N)	SR	49.28	42.76	69.61	54.09	38.01	21.08	RRC_C			
79	Galactose met- (G)	SR	10.97	8.32	15.07	11.32	7.17	4.68	RRC_C			
81	Glucopyranoside (G,N)	SR	0.37	0.35	0.77	0.83	0.46	0.52	RRC_C			
82	Glucopyranoside (N)	SR	11.56	10.92	18.74	20.68	13.49	18.23	RRC_C			RRI_L
86	Octopamine derivative (N,G)	EA	0.11	0.09	0.15	0.21	0.12	0.16	RRC_C		RRI_C	RRI_L
88	Furanone (N)	NA	0.06	0.05	0.12	0.12	0.08	0.09	RRC_C	RRC_L		
89	UID (345,346,73,255,347)	NA	4.68	5.09	11.92	12.52	9.45	11.55	RRC_C	RRC_L		RRI_L

[a] Cultivars: L = 'Libertas'; C = 'Caesar'; R = 'Russet Burbank'; P = pathogen-inoculated; W = water-inoculated; superscripted letters beside the total abundances (average of 5 replicates for each treatment) indicate significance among the 6 treatments (CW, CP, LW, LP, RW, RP) at $P \leq 0.05$ using Duncan's multiple range test.

[b] M = Metabolite reference number.

[c] Shortened names according to NIST Library or GOLM Metabolome Database; UID, unidentified metabolites (in parenthesis) = mass ions (*m/z*) were arranged according to their relative abundances.

[d] GR = Chemical groups of compounds: AA = Amino Acid; CA = Catecholamines; EA = Ethanolamine; FA = Fatty Acid; NA = Not Applicable; OA = Organic Acid; P = Phenolic; SR = Sugar.

[e] RR-metabolites, RRC_C; RRC_L; RRI_C; RRI_L, where L = 'Libertas', C = 'Caesar', R = 'Russet Burbank'.

22 common to both. Thus, in general more RR metabolites were identified in 'Libertas' than in 'Caesar'. Even among the RR metabolites common to both 'Libertas' and 'Caesar', the abundances of all except for three metabolites [22, 64 and 86] were higher in 'Libertas', according to Duncan's multiple range test (**Appendix 1**).

The abundances of OAs, such as fumaric, malic and isocitric acids were the highest in the most resistant cv. 'Libertas'. These metabolites are intermediates of the Krebs Cycle that are directly involved in the production of different AAs belonging to glutamic and aspartic acid families (**Fig. 4**). The abundances of amino acids such as isoleucine [1], threonine [6], and aspartic acid [9] of the aspartic acid family, and glutamine [7] and proline [8] of the glutamic acid family increased, following pathogen inoculation, to a higher level in 'Libertas' (RRI-metabolites) than in 'Caesar' and 'Russet Burbank'. These AAs are also the primary building blocks in the production of different PR-proteins, cell-wall structural proteins and enzymes. Glutamine [7] is a shuttle for carrying nitrogen in many essential intermediate reactions in plant cells, and by itself is a primary precursor for the production of prophyrin ring of chlorophyll (Coruzzi and Last 2000; Taiz and Zeiger 2002). Moreover, Glutamine is a precursor for γ-Aminobutyric acid [60] through the activity of the enzyme 4-aminobutyrate aminotransferase (KEGG 2009). The later was found to be an RRC-metabolite in both cvs. 'Caesar' and 'Libertas'. γ-Aminobutyric acid was reported to be increased in concentration in tomato fruits under salinity and water stress (Zushi and Matsuzoe 2006). Moreover, the AA alanine [66] was found to be an RRC and RRI-metabolite in both 'Caesar' and 'Libertas'. This AA was reported in addition to γ-Aminobutyric acid

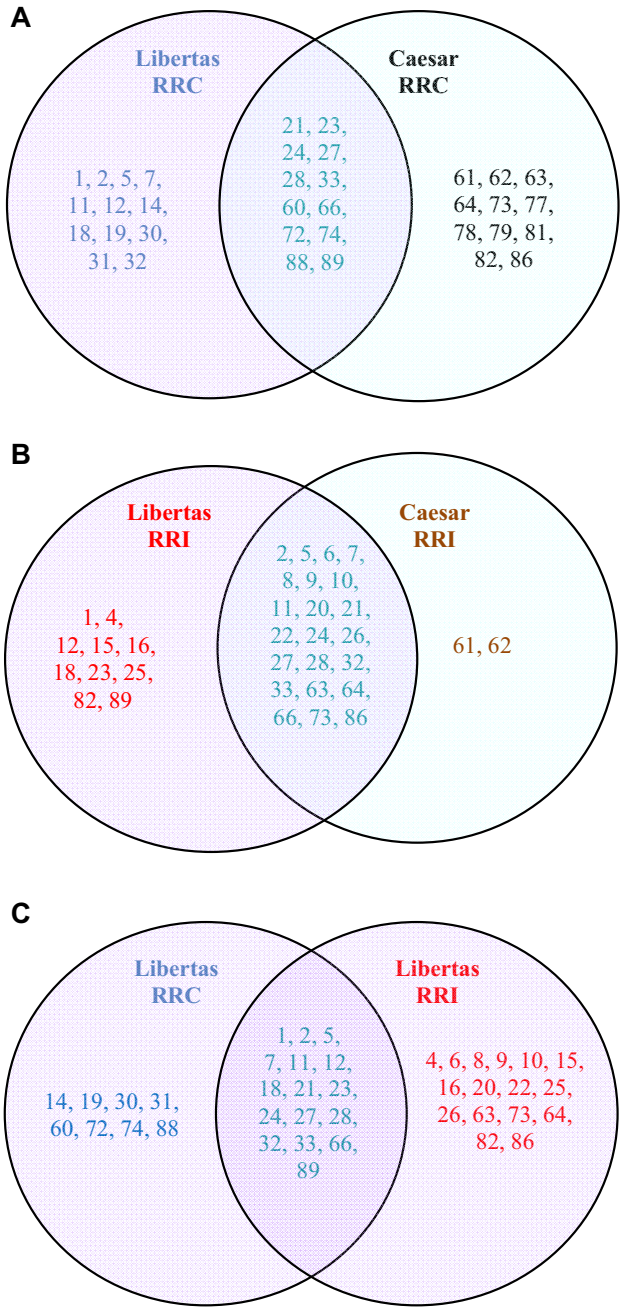

Fig. 3 Venn diagram showing the distribution of 89 Resistance Related (RR) metabolites. a) Resistance Related Constitutive (RRC); b) Resistance Related Induced (RRI); c) Libertas RRC and RRI-metabolites with the highest abundance relative to the susceptible cv. Russet Burbank. The serial numbers of metabolites included here are presented in **Table 1** and **Appendix 1**.

[60] to be accumulated rapidly in soybean leaves in response to rapid decrease in temperature, darkness or mechanical injuries (Wallace *et al.* 1984). Proline is a precursor for the production of extensin, a sub-group of hydroxyl-proline-rich glycoproteins (HRGPs) family, which has the ability to increase the rigidity of the plant cell walls by cross-linking different cell wall components such as pectin (Showalter 1993; Jackson *et al.* 2001). Aspartic acid is a precursor for the production of other AAs such as methionine, lysine, isoleucine and threonine (Azevedo *et al.* 2006; **Fig. 4**). In water stressed tomato leaves, an increase in glutamine, glutamate and asparagine AAs have been reported (Bauer *et al.* 1997). Similarly, high levels of AAs of the glutamate and aspartate families were found *in vitro*-grown potato tubers compared to soil-grown tubers (Roessner *et al.* 2000). The abundance of AA threonine increased significantly following treatment with cell cultures of *Medicago truncatula*, by methyl jasmonate or yeast elicitors (Broeckling *et al.* 2005).

The abundance of OA, shikimic acid [18], significantly increased following pathogen inoculation (RRI-metabolites in the resistant cv. 'Libertas'; **Figs. 3B, 3C, 4**). High levels of fumaric and shikimic acids were linked to elicitors in cell cultures of *Medicago truncatula* (Broeckling *et al.* 2005). Shikimic acid is a precursor of aromatic AAs phenylalanine [5] and tyrosine [10] (**Fig. 4**), which are identified here as RRI-metabolites in 'Libertas', and these are the primary precursors of the phenylpropanoid pathway that produce many plant secondary defense metabolites including: phenolics, lignins and hydrolyzable tannins (Dixon *et al.* 2002; Nakane *et al.* 2003). Phenylalanine [5] is a precursor for the production of benzoic acid [20] via phenylpropanoid pathway and its abundance increased following pathogen-inoculation in 'Libertas'. Benzoic acid [20] is a primary precursor for the production of the signal metabolite salicylic acid and its derivatives that provoke several defense responses in infected plants (Hammond-Kosack and Jones 1996; Mertraux 2002). Tyrosine, on the other hand, is a precursor for the production of dopamine [32] and norepinephrine [33] and both were increased in abundance following the pathogen inoculation of 'Libertas'. Norepinephrine has been reported to produce different alkaloids and also has antimicrobial activity (Kuklin and Conger 1995). Gluconoic acid [16] and the sugar, xylulose [64] were identified here as RRI-metabolites in 'Libertas'. These metabolites are associated with the Pentose Phosphate Pathway (PPP) that produces erythrose-4-phosphate, an essential precursor for the production of aromatic amino acids tyrosine [10] and phenylalanine [5] (**Fig. 4**). An increased rate of respiration, accumulation of CO_2, and the activation of the PPP were reported in parsley plants following inoculation with *Phytophthora megasperma* (Norman *et al.* 1994).

Malonic acid [21] and its derivative methyl malonate [24] were identified here as RRI-metabolites in 'Libertas'. Malonic acid [21] and ρ-coumaroyl-CoA of the phenylpropanoid pathway combined to produce several flavonoids (i.e. coumarines, flavones, isoflavones, isoflavanones) and condensed tannins, which are known to have antimicrobial activities (Croteau *et al.* 2000; Taiz and Zeiger 2006). In addition, malonic acid is a precursor of the octadecanoic acid [12 = stearic acid] identified here as a RRI_{CR}-metabolite. Stearic acid [12] is a precursor that produces linoleic acid and linolenic acid with the help of 12-desaturase and 15-desaturase enzymes, respectively (KEEG 2009; **Fig. 4**). These FAs are the primary blocks of the oxylipin pathway that produces the signal metabolite jasmonic acid and its derivatives (Soulie *et al.* 1989; Somerville *et al.* 2000; Weber 2002). In this study, the abundances of both linoleic and linolenic acids decreased after the pathogen inoculation in both 'Caesar' and 'Libertas'. Since we were unable to detect any jasmonic acid or its derivatives in any of the 6 treatments, more studies are needed to clarify if there is any correlation between the reduction in the abundances of both linoleic and linolenic acids and the production of jasmonic acid.

In this study, from the modest number of RRI-metabolites from the moderately resistant cv. 'Caesar' and RRI-metabolites from the highly resistant cv. 'Libertas' detected following pathogen inoculation, it can be hypothesized that part of the defense responses were mainly associated with an increase in the abundances of many OAs and AAs, indicating high activation of PPP, shikimic acid, malonic acid and phenylpropanoid pathways that are known to produce several antimicrobial metabolites. The highly resistant cv. 'Libertas' appears to be more efficient than 'Caesar' in the activation of the phenylpropanoid and malonic acid pathways. These two pathways are known for their role in the production of a wide range of defense related secondary metabolites in response to both abiotic and biotic stresses.

Metabolic profiling, a large scale metabolite identification and quantification, is an evolving field of systems boilogy. There is no single extraction method to extract thou-

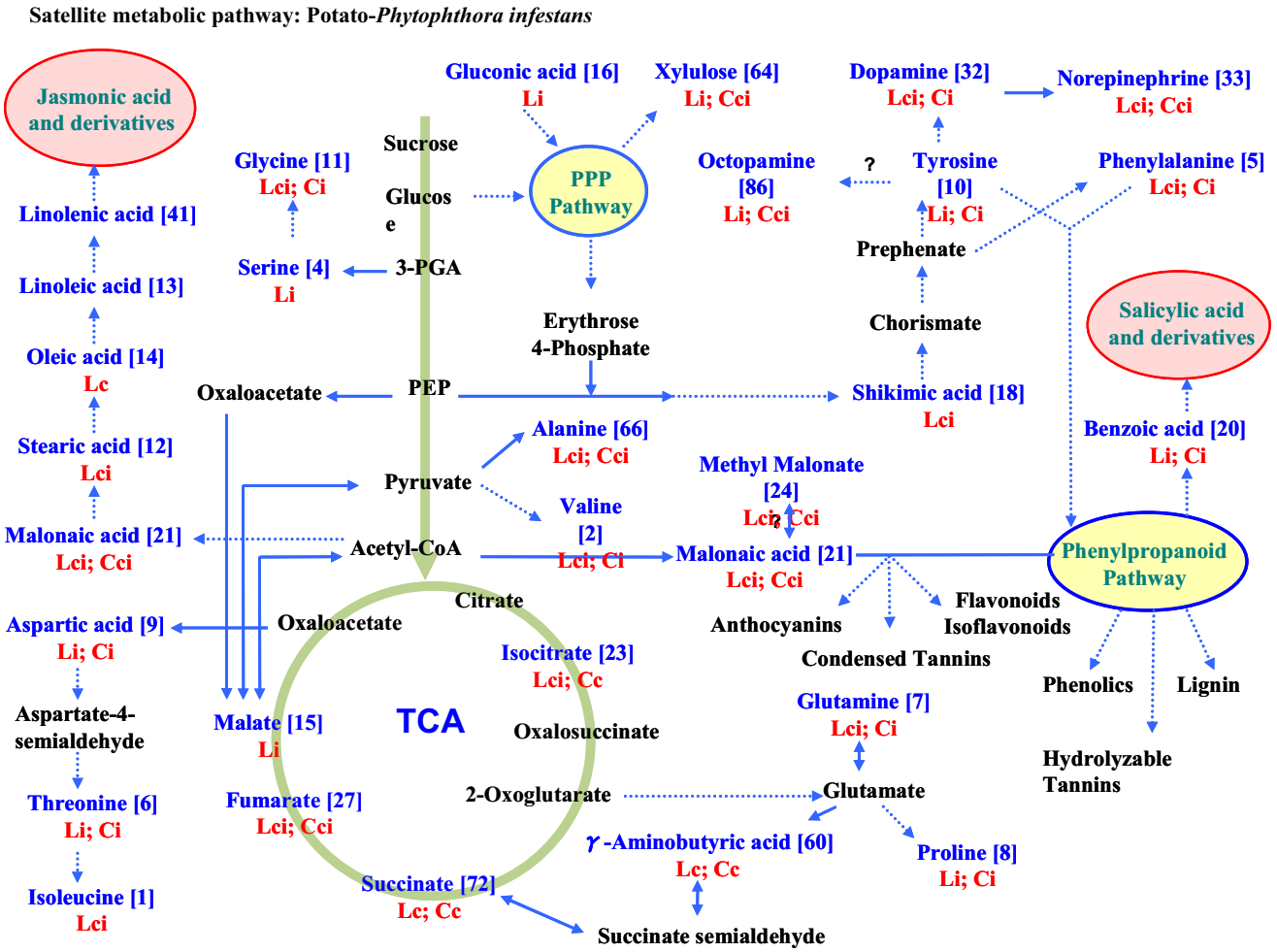

Fig. 4 Simplified metabolic pathway showing the position of RR-metabolites detected in three potato cultivars. The numbers refer to the reference number of metabolites (see **Table 1**; **Appendix 1**); the upper case letters are cultivars: L = Libertas, and C = Caesar, and the lower case letters are resistance related (RR) metabolites, either constitutive (c) or induced (i), relative to the susceptible cultivar. Metabolites with blue colors are reported in this study.

sands of metabolites produced by plants, and due to the large diversity in the chemical structures and the concentration of metabolites in plant tissues, it is impossible to select a single platform to analyze all metabolites in one run (Bino *et al.* 2004). GC/MS is still considered to be the one of the cheapest and best platforms available (Sumner *et al.* 2003; Dunn *et al.* 2004). In spite of these limitations, we were able to detect and tentatively identify close to a hundred metabolites, and discriminate resistance in potato to late blight based on metabolic profiling using GC/MS. On the other hand, it is possible that several metabolites better related to resistance functions were not detected or identified in this study. Many metabolites are not volatiles at GC temperature and thus could not be detected in our study. A study based on liquid chromatography and hybrid mass spectrometry can provide a wider range of potato metabolites (Kumaraswamy *et al.* 2011). On the other hand several metabolites detected here were not significant and accordingly excluded from the analysis.

In conclusion, the technology developed here to discriminate three cultivars with different levels of resistance to late blight disease based on metabolic profiling can be used for practical screening of potato breeding lines against late blight. Although canonical discriminant analysis was able to identify the resistance function, the univariate analysis was important to classify the levels of significance of these metabolites across the cultivars tested. In potato tubers, several plant defense responses were activated after *P. infestans* inoculation including the accumulation of phenylpropanoid metabolites, and the sesquiterpenoid phytoalexins lubimin and rishitin (Nakane *et al.* 2003). Even though we have not detected any of the well reported potato phytoalexin compounds such as rishitin, rishitinol, lubimin and solavitivone (Bostock *et al.* 1981; Zook *et al.* 1987; Jadhav 1991), we have detected several RR-metabolites that are precursors for the production of antimicrobials, signal molecules or cell wall enforcement compounds. Thus, there is potential to use metabolic profiling as a tool for high throughput screening of potato cultivars to discriminate resistance against late blight. The RR-metabolites, especially the 32 RRI-metabolites and even the 24 RRC-metabolites in 'Libertas', identified here can be used as biomarkers, following further validation using more cultivar, for more secure screening of potato cultivars against the late blight pathogen, as an additional or alternative method to those based on disease severity.

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Appendix 1 Average abundances ($\times 10^6$) and the loadings to CAN-vectors of metabolites detected in leaf-extracts of three potato cultivars[a] Caesar, Libertas, and R. Burbank, inoculated with water (control) or pathogen (*P. infestans*).

M[b]	RI[c]	Name[d]	GR[e]	RW	RP	CW[f]	CP	LW	LP	RRI-metabolites[f]				CAN[g]	CAN2	CAN3
1	218.6	Isoleucine (G,N)	AA	0.27^{CD}	0.34^{BC}	0.22^{D}	0.41^{B}	0.37^{B}	0.86^{A}		RRC_L		RRI_L	-0.43	0.80	0.04
2	203.9	Valine (N,G)	AA	0.34^{D}	0.39^{D}	0.29^{E}	0.51^{B}	0.45^{C}	0.88^{A}		RRC_L	RRI_C	RRI_L	-0.47	0.81	0.08
3	220.6	Glycine (G,N)	AA	0.12^{B}	0.13^{AB}	0.11^{B}	0.11^{B}	0.11^{B}	0.16^{A}					0.00	0.55	0.04
4	230.8	Serine (G,N)	AA	1.28^{B}	1.36^{B}	0.92^{D}	1.28^{B}	1.02^{C}	1.62^{A}				RRI_L	0.13	0.79	0.22
5	274.1	Phenylalanine (N)	AA	0.14^{D}	0.17^{D}	0.15^{D}	0.26^{C}	0.35^{B}	0.79^{A}		RRC_L	RRI_C	RRI_L	-0.53	0.76	-0.11
6	235.1	Threonine (N,G)	AA	0.59^{D}	0.72^{C}	0.52^{D}	0.87^{B}	0.55^{D}	1.24^{A}			RRI_C	RRI_L	-0.35	0.72	0.29
7	273.4	Glutamine (G,N)	AA	3.16^{D}	3.56^{D}	3.28^{D}	5.07^{B}	4.06^{C}	9.10^{A}		RRC_L	RRI_C	RRI_L	-0.54	0.74	0.09
8	219.2	Proline (G,N)	AA	0.10^{C}	0.12^{C}	0.10^{C}	0.15^{B}	0.10^{C}	0.20^{A}			RRI_C	RRI_L	-0.38	0.67	0.34
9	257.8	Aspartic acid(G,N)	AA	3.21^{CD}	3.64^{C}	3.09^{CD}	4.45^{B}	2.99^{D}	5.92^{A}			RRI_C	RRI_L	-0.39	0.65	0.30
10	321.4	Tyrosine (N,G)	AA	0.11^{C}	0.16^{C}	0.12^{C}	0.23^{B}	0.11^{C}	0.38^{A}			RRI_C	RRI_L	-0.43	0.66	0.28
11	242.7	Glycine (N,G)	AA	0.43^{D}	0.46^{D}	0.5^{CD}	0.58^{B}	0.56^{BC}	0.66^{A}		RRC_L	RRI_C	RRI_L	-0.71	0.51	0.08
12	364.9	Stearic acid (N,G)	FA	0.56^{C}	0.61^{BC}	0.43^{D}	0.43^{D}	0.67^{B}	0.79^{A}		RRC_L		RRI_L	-0.02	0.81	-0.40
13	344.8	Linoleic acid (N,G)	FA	4.41^{A}	4.56^{A}	3.45^{C}	3.91^{B}	4.05^{B}	4.48^{A}					0.49	0.70	-0.04
14	340.4	Oleic acid (N)	FA	0.16^{B}	0.17^{B}	0.14^{B}	0.15^{B}	0.22^{A}	0.22^{A}		RRC_L			-0.15	0.54	-0.30
15	252.9	Malic acid (N,G)	OA	27.39^{BC}	28.48^{B}	24.01^{D}	26.24^{C}	29.07^{B}	33.01^{A}				RRI_L	-0.06	0.86	-0.21
16	331.3	Gluconic acid (N,G)	OA	0.10^{C}	0.13^{B}	0.07^{D}	0.09^{C}	0.09^{C}	0.15^{A}				RRI_L	0.16	0.79	0.06
17	262.9	Amino isobutyric acid (N)	OA	0.11^{ABC}	0.11^{C}	0.09^{D}	0.1^{CD}	0.12^{AB}	0.12^{A}					0.03	0.64	-0.32
18	301.0	Shikimic acid (G,N)	OA	1.30^{CD}	1.45^{BC}	1.25^{D}	1.61^{B}	1.54^{B}	3.42^{A}		RRC_L		RRI_L	-0.45	0.77	-0.07
19	332.9	Propenoic acid (N)	OA	0.12^{B}	0.12^{B}	0.10^{B}	0.12^{B}	0.19^{A}	0.21^{A}		RRC_L			-0.31	0.59	-0.30
20	232.2	Benzoic acid (N)	OA	0.04^{CD}	0.04^{C}	0.03^{D}	0.05^{B}	0.03^{CD}	0.07^{A}			RRI_C	RRI_L	-0.26	0.67	0.32
21	202.3	Malonic acid (N,G)	OA	0.24^{E}	0.29^{D}	0.28^{D}	0.32^{C}	0.41^{B}	0.52^{A}	RRC_C	RRC_L	RRI_C	RRI_L	-0.59	0.74	-0.14
22	173.1	Lactic acid derivative (N,G)	OA	0.09^{C}	0.11^{B}	0.09^{C}	0.12^{A}	0.10^{C}	0.13^{A}			RRI_C	RRI_L	-0.32	0.65	0.54
23	301.9	Isocitric acid (N)	OA	1.56^{D}	1.97^{C}	2.03^{C}	2.06^{C}	3.72^{B}	4.61^{A}	RRC_C	RRC_L		RRI_L	-0.56	0.68	-0.30
24	175.1	Methyl, ethyl malonate (N)	OA	0.06^{D}	0.08^{C}	0.07^{C}	0.10^{B}	0.10^{B}	0.12^{A}	RRC_C	RRC_L	RRI_C	RRI_L	-0.67	0.69	0.14
25	215.0	Phosphoric acid (N,G)	OA	3.50^{C}	3.50^{C}	3.71^{BC}	4.28^{B}	4.13^{BC}	5.56^{A}				RRI_L	-0.59	0.61	0.03
26	269.6	Butanedioic acid (G,N)	OA	0.05^{C}	0.05^{C}	0.05^{C}	0.06^{B}	0.05^{C}	0.08^{A}			RRI_C	RRI_L	-0.52	0.61	0.27
27	229.7	Fumaric acid (G,N)	OA	0.09^{E}	0.10^{E}	0.13^{D}	0.15^{C}	0.23^{B}	0.27^{A}	RRC_C	RRC_L	RRI_C	RRI_L	-0.66	0.61	-0.22
28	239.8	Butanoic acid (N)	OA	0.02^{E}	0.02^{E}	0.03^{D}	0.04^{C}	0.05^{B}	0.06^{A}	RRC_C	RRC_L	RRI_C	RRI_L	-0.78	0.52	-0.10
29	281.4	Ribose (N,G)	SR	0.56^{BC}	0.75^{A}	0.35^{D}	0.51^{C}	0.60^{B}	0.73^{A}					0.35	0.83	0.06
30	278.2	Xylitol (N)	SR	1.20^{B}	1.22^{B}	1.02^{B}	1.19^{B}	1.56^{A}	1.72^{A}		RRC_L			-0.28	0.74	-0.27
31	279.2	Xylose (G)	SR	0.29^{B}	0.30^{B}	0.23^{C}	0.23^{C}	0.41^{A}	0.42^{A}		RRC_L			-0.16	0.74	-0.45
32	341.7	Dopamine (N)	CA	0.04^{DE}	0.08^{D}	0.03^{E}	0.16^{C}	0.25^{B}	0.33^{A}		RRC_L	RRI_C	RRI_L	-0.55	0.75	-0.13
33	352.6	Norepinephrine (N,G)	EA	0.06^{E}	0.11^{DE}	0.15^{D}	0.43^{B}	0.22^{C}	0.51^{A}	RRC_C	RRC_L	RRI_C	RRI_L	-0.72	0.51	0.30
34	349.5	Octadecanoic acid (G,N)	FA	7.92^{A}	8.22^{A}	6.96^{C}	7.23^{BC}	7.33^{BC}	7.38^{B}					0.77	0.29	0.05
35	347.9	Hexadecanoic acid (N,G)	FA	0.18^{A}	0.20^{A}	0.09^{D}	0.13^{B}	0.10^{CD}	0.13^{BC}					0.78	0.24	0.16
36	335.0	Heptadecanoic acid (N,G)	FA	0.19^{A}	0.21^{A}	0.14^{C}	0.16^{B}	0.17^{B}	0.17^{B}					0.70	0.38	0.06
37	318.7	9-Hexadecenoic acid (N)	FA	0.43^{A}	0.39^{AB}	0.34^{C}	0.25^{D}	0.35^{BC}	0.27^{D}					0.69	-0.23	-0.34
38	337.0	Hexadecanoic acid (G,N)	FA	0.76^{A}	0.79^{A}	0.30^{C}	0.47^{BC}	0.46^{BC}	0.62^{AB}					0.64	0.46	-0.03
39	314.3	7,10-Hexadecadienoic (N)	FA	0.20^{A}	0.18^{AB}	0.17^{B}	0.14^{C}	0.17^{B}	0.13^{C}					0.61	-0.37	-0.40
40	315.2	7,10,13-Hexadecatrienoic (N)	FA	2.24^{A}	1.87^{BC}	1.64^{CD}	1.39^{D}	1.98^{AB}	1.44^{D}					0.57	-0.14	-0.48
41	345.9	9,12,15-Octadecatrienoic (N,G)	FA	10.95^{A}	10.17^{B}	8.02^{D}	8.18^{D}	10.62^{AB}	9.35^{C}					0.55	0.35	-0.54
42	296.9	Methylcitric acid (G,N)	OA	4.89^{A}	5.35^{A}	3.92^{B}	3.85^{B}	3.93^{B}	3.93^{B}					0.84	0.07	0.05
43	325.6	Galactonic acid (N,G)	OA	1.48^{AB}	1.62^{A}	1.3^{B}	1.32^{B}	1.35^{B}	1.33^{B}					0.50	0.10	0.12
44	328.6	Galactonic acid (N,G)	OA	1.88^{A}	2.03^{A}	0.55^{C}	0.46^{C}	1.10^{B}	0.97^{B}					0.87	0.24	-0.19
45	291.8	Ribonic acid (G,N)	OA	0.28^{A}	0.31^{A}	0.14^{C}	0.19^{B}	0.15^{C}	0.20^{B}					0.81	0.24	0.16
46	319.5	Pentadecanoic acid (N)	OA	6.13^{AB}	6.49^{A}	5.10^{D}	5.57^{CD}	5.30^{CD}	5.68^{BC}					0.68	0.28	0.18
47	296.5	L-Gluconic acid (N)	OA	0.59^{A}	0.55^{AB}	0.48^{C}	0.50^{BC}	0.52^{BC}	0.51^{BC}					0.60	0.19	-0.21
48	293.4	2-Keto-l-gluconic acid, p- (G,N)	OA	1.24^{B}	1.43^{A}	0.75^{C}	0.83^{C}	1.19^{B}	1.15^{B}					0.62	0.49	-0.19
49	294.1	Methylcitric acid, tetrak- (N,G)	OA	0.42^{B}	0.49^{A}	0.39^{B}	0.38^{B}	0.22^{C}	0.24^{C}					0.68	-0.40	0.45
50	345.2	Glucose (N)	SR	1.88^{A}	1.99^{A}	1.37^{B}	1.45^{B}	1.42^{B}	1.43^{B}					0.89	0.11	0.11
51	290.0	D-Xylofuranose (N)	SR	2.90^{A}	2.99^{A}	2.37^{B}	2.47^{B}	2.37^{B}	2.31^{B}					0.70	-0.02	0.08
52	348.5	Galactose1 (G,N)	SR	0.38^{A}	0.38^{A}	0.23^{B}	0.24^{B}	0.19^{B}	0.20^{B}					0.80	-0.08	0.07
53	349.3	Galactose2 (G,N)	SR	0.54^{A}	0.53^{A}	0.31^{BC}	0.34^{B}	0.25^{C}	0.25^{C}					0.89	-0.13	0.09
54	356.8	D-Glucose (N)	SR	3.61^{A}	3.74^{A}	2.73^{BC}	2.99^{B}	2.46^{C}	2.85^{BC}					0.73	0.05	0.24
55	249.4	Xylo-hexos-5-ulose (N)	SR	0.32^{B}	0.41^{A}	0.18^{D}	0.20^{D}	0.25^{C}	0.29^{B}					0.75	0.44	-0.01
56	251.2	Arabino-Hexos-2-ulose (N)	SR	0.46^{B}	0.55^{A}	0.37^{C}	0.41^{BC}	0.41^{BC}	0.46^{B}					0.56	0.41	0.18
57	309.8	D-Fructose1 (N)	SR	61.01^{A}	56.87^{AB}	52.23^{BC}	48.13^{C}	36.37^{D}	34.48^{D}					0.68	-0.48	0.17
58	379.0	Gulonolactone (N)	N/A	2.01^{A}	1.97^{A}	1.37^{B}	1.31^{B}	1.37^{B}	1.42^{B}					0.84	0.05	-0.11
59	352.7	UD (327,97,75,111,83)*	N/A	0.77^{A}	0.67^{B}	0.39^{C}	0.28^{D}	0.42^{C}	0.42^{C}					0.86	0.06	-0.30
60	259.2	γ-Aminobutyric acid (G,N)	OA	1.26^{C}	1.57^{A}	1.44^{B}	1.58^{A}	1.43^{B}	1.51^{AB}	RRC_C	RRC_L			-0.21	0.26	0.69
61	176.7	Acetic acid (N)	OA	0.04^{D}	0.07^{C}	0.08^{B}	0.10^{A}	0.05^{D}	0.05^{D}	RRC_C		RRI_C		-0.27	-0.46	0.83
62	232.2	Malic acid derivative (N,G)	OA	0.05^{CD}	0.05^{CD}	0.06^{B}	0.07^{A}	0.05^{D}	0.05^{C}	RRC_C		RRI_C		-0.49	-0.31	0.56
63	225.4	Glyceric acid derivative (G,N)	OA	5.22^{E}	5.83^{D}	6.44^{C}	7.22^{B}	5.18^{E}	7.86^{A}	RRC_C		RRI_C	RRI_L	-0.61	0.31	0.50
64	266.9	Xylulose (G,N)	SR	0.72^{C}	0.85^{B}	0.83^{B}	0.99^{A}	0.80^{BC}	1.03^{A}	RRC_C		RRI_C	RRI_L	-0.52	0.44	0.52
65	213.4	UID (174,73,147,100,175)[3]	NA	0.42^{BC}	0.46^{AB}	0.46^{AB}	0.47^{A}	0.40^{C}	0.40^{C}					0.08	-0.34	0.53
66	181.8	Alanine (G,N)	AA	0.24^{F}	0.31^{D}	0.36^{C}	0.43^{B}	0.28^{E}	0.49^{A}	RRC_C	RRC_L	RRI_C	RRI_L	-0.69	0.37	0.44
67	289.1	Tridecanoic acid (N)	FA	0.25^{AB}	0.29^{A}	0.19^{D}	0.26^{AB}	0.20^{CD}	0.24^{BC}					0.46	0.33	0.43
68	377.0	Eicosanoic acid (N,G)	FA	0.04^{A}	0.04^{A}	0.02^{C}	0.03^{B}	0.02^{C}	0.04^{A}					0.46	0.48	0.27
69	358.8	Heptadecanoic acid (N)	FA	0.05^{A}	0.04^{B}	0.04^{B}	0.04^{B}	0.04^{B}	0.03^{B}					0.37	-0.32	-0.38
70	330.5	Hexadecanoic acid (N)	FA	0.03^{B}	0.04^{B}	0.04^{AB}	0.05^{A}	0.04^{AB}	0.04^{AB}					-0.47	0.04	0.35
71	382.0	Glucuronic acid (N)	OA	0.63^{C}	0.78^{A}	0.64^{BC}	0.72^{AB}	0.66^{BC}	0.67^{BC}					0.27	0.20	0.43
72	223.0	Succinic acid (G,N)	OA	0.38^{C}	0.44^{C}	1.03^{A}	1.02^{A}	0.55^{B}	0.57^{B}	RRC_C	RRC_L			-0.66	-0.54	0.45

Appendix 1 (Cont.)

M[b]	RI[c]	Name[d]	GR[e]	RW	RP	CW[f]	CP	LW	LP	RRI-metabolites[f]				CAN[g]	CAN2	CAN3
73	263.9	Trihydroxybutyric acid (N)	OA	1.10^{C}	1.10^{C}	2.19^{B}	2.57^{A}	1.41^{C}	2.01^{B}	RRC_C		RRI_C	RRI_L	-0.77	-0.18	0.48
74	298.9	Glucaric acid (G,N)	OA	0.08^{C}	0.11^{C}	0.24^{B}	0.27^{B}	0.33^{A}	0.34^{A}	RRC_C	RRC_L			-0.85	0.26	-0.05
75	311.4	D-Fructose2 (N)	SR	48.58^{A}	43.28^{A}	47.71^{A}	44.23^{A}	30.96^{B}	29.96^{B}					0.35	-0.59	0.14
76	286.9	Rhamnose (G)	SR	0.26^{AB}	0.26^{B}	0.23^{B}	0.24^{B}	0.30^{A}	0.26^{AB}					0.08	0.28	-0.31
77	323.7	Turanose (N)	SR	0.35^{B}	0.32^{B}	0.44^{A}	0.43^{A}	0.13^{C}	0.18^{C}	RRC_C				0.12	-0.67	0.45
78	313.7	D-Glucose (N)	SR	49.28^{BC}	42.76^{BC}	69.61^{A}	54.09^{B}	38.01^{C}	21.08^{D}	RRC_C				0.05	-0.86	0.16
79	316.6	Galactose met- (G)	SR	10.97^{BC}	8.32^{CD}	15.07^{A}	11.32^{B}	7.17^{DE}	4.68^{E}	RRC_C				0.01	-0.82	0.05
80	384.5	Mannose (N)	SR	0.13^{B}	0.18^{A}	0.16^{AB}	0.19^{A}	0.16^{AB}	0.18^{A}					-0.23	0.27	0.45
81	320.4	Glucopyranoside (G,N)	SR	0.37^{B}	0.35^{B}	0.77^{A}	0.83^{A}	0.46^{B}	0.52^{B}	RRC_C				-0.64	-0.4	0.40
82	413.0	Glucopyranoside (N)	SR	11.56^{B}	10.92^{B}	18.74^{A}	20.68^{A}	13.49^{B}	18.23^{A}	RRC_C			RRI_L	-0.79	-0.12	0.31
83	306.4	Tetramethyl-2-hexadecen1 (N)	OAA	0.46^{A}	0.45^{A}	0.32^{B}	0.43^{A}	0.44^{A}	0.39^{AB}					0.39	0.31	0.01
84	309.9	Tetramethyl-2-hexadecen2 (N)	OAA	0.49^{ABC}	0.52^{AB}	0.45^{C}	0.55^{A}	0.49^{ABC}	0.47^{BC}					0.17	0.12	0.40
85	211.2	Benzene, m-di-tert-butyl (N)	OAH	0.11^{A}	0.09^{ABC}	0.07^{C}	0.10^{AB}	0.09^{BC}	0.09^{ABC}					0.19	0.12	0.03
86	333.9	Octopamine derivative (N,G)	EA	0.11^{CD}	0.09^{D}	0.15^{B}	0.21^{A}	0.12^{C}	0.16^{B}	RRC_C		RRI_C	RRI_L	-0.72	-0.01	0.44
87	334.5	UID (204,73,319,205,217)	NA	0.60^{A}	0.38^{B}	0.45^{B}	0.42^{B}	0.23^{C}	0.16^{C}					0.48	-0.64	0.04
88	233.1	Furanone (N)	NA	0.06^{C}	0.05^{C}	0.12^{A}	0.12^{A}	0.08^{B}	0.09^{B}	RRC_C	RRC_L			-0.77	-0.39	0.36
89	307.6	UID (345,346,73,255,347)	NA	4.68^{D}	5.09^{D}	11.92^{AB}	12.52^{A}	9.45^{C}	11.55^{B}	RRC_C	RRC_L		RRI_L	-0.95	-0.09	0.25
90	402.4	Dodecanoic acid (N)	FA	0.02	0.03	0.03	0.03	0.02	0.03							
91	277.2	Trihydroxypentanoic acid (G)	OA	0.94	0.96	0.90	0.90	0.89	0.84							
92	342.7	nositol (N)	SR	8.41	6.42	7.04	7.51	8.70	7.54							
93	318.3	Glucitol (N,G)	SR	0.52	0.58	0.45	0.53	0.50	0.57							
94	244.7	UID (172,82,73,75,160)	NA	0.05	0.05	0.05	0.05	0.06	0.07							

[a] Acronyms: L = Libertas; C = Caesar; R = Russet Burbank; P = pathogen-inoculated; W = water-inoculated; superscript letters beside the total abundances (average of 5 replicates for each treatment) indicate significance among the 6 treatments (CW, CP, LW, LP, RW, RP) at $P \leq 0.05$ using Duncan's multiple range test.

[b] M = Metabolite reference number.

[c] RI= Retention time indices calculated according to Eckel (2000)

[d] Shortened names according to NIST Library or GOLM Metabolome Database; UID, unidentified metabolites (in parenthesis) = mass ions (*m/z*) were arranged according to their relative abundances.

[e] GR = Chemical groups of compounds: AA = Amino Acid; CA = Catecholamines; EA = Ethanolamine; FA = Fatty Acid; NA = Not Applicable; OA = Organic Acid; P = Phenolic; SR = Sugar.

[e] The superscript letters indicate that the abundances with similar letters are not significant based on Duncan's multiple range test; the relative abundances of metabolites, among cultivars, were further grouped into: i = Resistant Related Induced (RRI), I = Resistant Related Induced (RRI_{CR}); c = Resistant Related Constitutive (RRC); C= Resistant Related Constitutive (RRC_{CR}) metabolites.

[f] RR-metabolites, RRC_C; RRC_L; RRI_C; RRI_L, where L = 'Libertas', C = 'Caesar', R = 'Russet Burbank'.

[g] CAN-loadings of metabolites to CAN1, CAN2 and CAN3-scores, based on canonical discriminant analysis of abundances of 89 significant metabolites; the loadings can be positive or negative.

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