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Metabolic effects on ‘Zesy002’ (Gold3) leaves of root pruning and associated Actigard™ treatment

Rowan D, McGhie T, Cooney J, Boldingh H, Hedderley D

July 2016















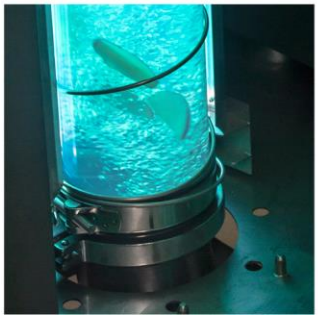


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July 2016

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July 2016

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EXECUTIVE SUMMARY

Metabolic effects on 'Zesy002' (Gold3) leaves of root pruning and associated Actigard™ treatment

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July 2016

Root pruning has been shown to increase the dry matter (DM) content of kiwifruit, and kiwifruit growers are adopting the practice of root pruning, especially in orchards where trunk girdling is less suitable to manage DM. However, we have limited understanding of how root pruning impacts vine susceptibility to Psa. The elicitor, Actigard™, may be an option to help mitigate any negative impacts of root pruning.

Untargeted metabolic profiling (metabolomics) provides a 'broad brush' exploratory tool to look at changes in plant metabolism in response to environmental challenge or stress.
An experimental trial on Gold3 kiwifruit vines was undertaken by Zespri to identify effects on Psa susceptibility and metabolic responses due to root pruning and of Actigard elicitor treatment. Leaf samples from this trial were provided to Plant & Food Research for LCMS-based metabolomics analysis which is the subject of this report.

Increased concentrations of procyanidin polyphenol metabolites were found in leaves from root pruned vines taken 2 days after root pruning but not at subsequent sampling times. This response was associated with root pruning. We found no evidence for an effect of Actigard™ on the procyanidin metabolism in leaves. The procyanidin response to root pruning is similar to that observed by us in previous trunk girdling experiments with 'Hort16A' *Actinidia chinensis* var. *chinensis* 'Hort16A' and *A. chinensis* var. *deliciosa* 'Hayward' vines, and by others studying metabolic responses of plants to stress.

Based on the positive correlation between root pruning and procyanidins, we recommend the further work:

1. Determine the stress hormone levels in these samples to provide independent evidence of the 'stress' status of these plants and the relevance of the observed changes in procyanidins.
2. Establish the generality of the responses seen here to determine if procyanidins may be used as markers of vine 'stress'.

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1 INTRODUCTION

Root pruning has been shown to increase the DM content of kiwifruit, especially when used in conjunction with trunk girdling. Growers are increasingly trialling and adopting the practice of root pruning in orchards where trunk girdling is less suitable. However we have little knowledge of how root pruning impacts on susceptibility to Psa. Does the wound response increase susceptibility? Root pruning is typically carried out around 60 days after full bloom when foliar application of Actigard™ cannot be used, however soil-applied Actigard may be an option to help mitigate any negative impacts of root pruning with respect to Psa susceptibility.

An experimental trial (Project VI1506) was undertaken by Zespri to identify the impacts of root pruning on Psa susceptibility and the use of the elicitor Actigard to mitigate any negative impacts. Leaf samples of kiwifruit (*Actinidia chinensis* var. *chinensis* 'Zesy002' (Gold3)) from control, root-pruned, and Actigard treated coupled with root-pruned vines were harvested 2, 5, 14, 21 and 42 days after root pruning (Appendix 1 and Figure 1 below). Ninety leaf samples in total were collected: six per experimental treatment.

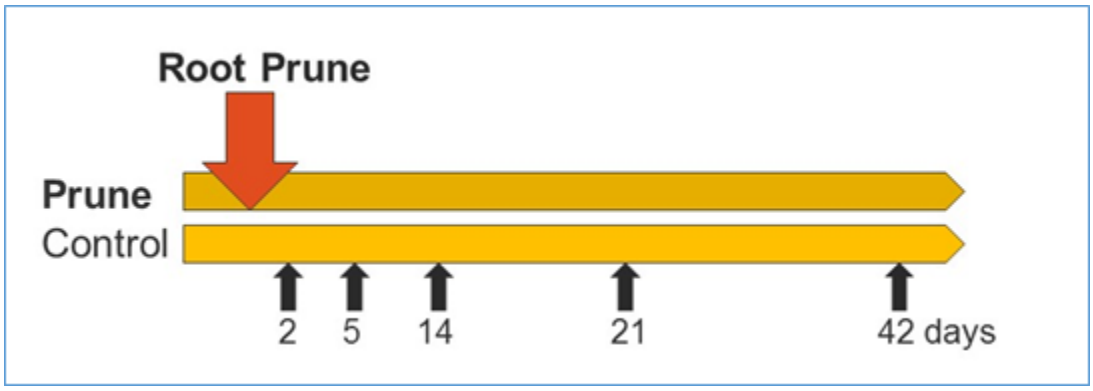

Figure 1: Diagrammatic of root-pruning experiment assessing the metabolic effects on 'Zesy002' (Gold3) leaves of root pruning and associated Actigard™ treatment.

Untargeted metabolic profiling (metabolomics) provides a 'broad brush' exploratory tool to look at changes in plant metabolism in response to environmental challenge or stress. This report describes the metabolomics analysis of leaves obtained from the root pruning/Actigard application experiment described above and the identification of procyanidin polyphenols as the major metabolites found to respond to root pruning.

2 RESULTS

Metabolic profiles were obtained by high mass resolution LC-MS using 80% methanol extracts obtained from the freeze dried leaf samples using methods established by Plant & Food Research. The data analysis followed methods used previously with leaf and stem material from *Actinidia chinensis* var. *chinensis* 'Hort16A' and *A. chinensis* var. *deliciosa* 'Hayward' kiwifruit. The number of metabolites detected was smaller than previously, probably because further sample dilution was made to keep all the metabolites 'on scale'.

Initial principal component analysis (PCA) of the metabolite data indicated the 42 days data was very different from the other days (i.e. it dominated the first principal component; the second principle component appeared to be a time trend over days 2 to 21). Because of this the analysis focused on the day 2–21 data.

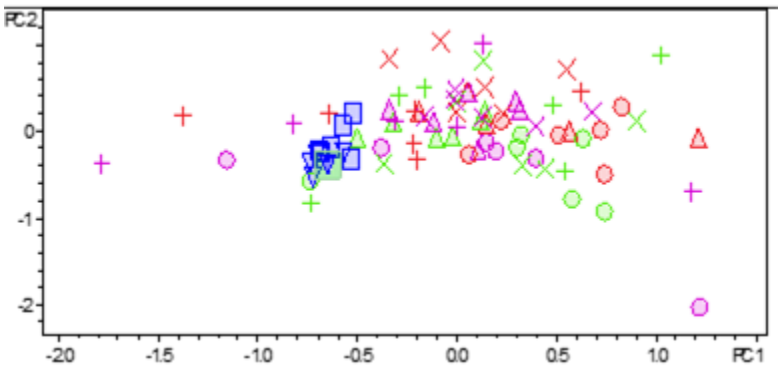

Figure 2. PCA analysis of leaf metabolites as measured by C18-RP LC-MS from samples collected days 2–21 showing no separation of control, root-pruned or of root-pruned and Actigard™ treated samples in PC1 or PC2 of the PCA analysis. Blue symbols are QC samples; different colours of the same symbol represent different treatments.

Data for poorly measured metabolites were removed, resulting in a dataset of 126 mass-tags. This dataset included mass-tags with up to 15% zero readings (not detected in some samples). Supervised analysis using Partial Least Squares-Discriminant Analysis (PLS-DA) was carried out using the 126 best measured metabolites. With PLS, the software attempts to find the most important metabolites to achieve the desired separation of treatments. Only Day 2 data gave a reasonably good separation of the sample classes root pruned v. control with cross-validation misclassification rates of 35–40%. Later days (days 5–21) gave misclassification rates between 60% and 80%, similar to the rate expected by chance (67%). This indicates meaningful differences in metabolite concentrations might only be expected to be found in this data between root pruned and control samples collected 2 days after root pruning.

Sparse Partial Least Squares-Discriminant Analysis (PLS-DA) models were run on the Day 2 data, restricted to picking the best 50 metabolites in two dimensions. Refitting the PLS model to random subsets of data gave 31 metabolites chosen five out of five times for model building (Appendix 2). In the first dimension nine out of 22 metabolites were either procyanidins (six) or related to phenolic biosynthesis (three). In the second dimension five of the nine metabolites were phenolics. The remaining metabolites are mainly of unknown chemical structures.

PLS-DA analysis may perform poorly if, for instance, there were only a small number of changed metabolites and the analysis is then dominated by irrelevant variables. We therefore did Kruskal-Wallis and one-factor analysis of variance (ANOVA) tests on the individual metabolites to see which showed significant differences between the treatment groups (for each

day separately). The ANOVA *P*-values tended to be higher, so we selected the mass-tags (metabolites) which had *P*-values less than 0.10 (Appendix 3). Twenty metabolites were found with *P*<0.1: 8 of unknown structures, six metabolites of the procyanidin pathway, one quercetin glycoside, two phospholipids, coumaric and malic acids, and a threonic acid isomer. The fold changes in these metabolites were consistent whether comparing root pruning or root pruning coupled with Actigard treatments with controls.

2.1 Effect of root pruning on metabolites in Gold3 leaf samples

Consistent differences in metabolites were only seen in those leaf samples collected 2 days after root pruning. These metabolic differences were associated with root pruning rather than with Actigard treatment. The metabolic effect of root pruning on leaf metabolism appears transitory.

Consistent increases ($P < 0.1$) in metabolites of the procyanidin pathway (a specific class of polyphenolics), as well as other metabolites, were found. Increases were found in monomer (gallocatechin), dimer (1), trimer (1), tetramers (2) and in one pentamer (1) member of this pathway. The procyanidin monomers, catechin and epi-catechin (and gallocatechin), were also discriminating metabolites in the PLD-DA analysis.

The fold increases in procyanidins observed were small but consistent for both root-pruned and root-pruned plus Actigard-treated leaves, and across multiple members of this biosynthetic pathway. No search was made in the chemical data for further procyanidins which were not increased by root pruning, however no procyanidins were found which were decreased ($P < 0.1$) by root pruning. Similar increases in procyanidin biosynthesis were observed in leaves of 'Hort16A' and 'Hayward' kiwifruit after trunk girdling (Plant & Food Research, unpublished results).

Procyanidins are oliomeric polyphenols formed by the polymerisation of phenolic metabolites catechin, epi-catechin and epigallocatechin, and are widely distributed in plants, including being found in grapes, apple, blueberries, tea leaves, and present in chocolate. The composition of the procyanidins in kiwifruit pericarp (described only as *Actinidia chinensis*) has been characterised by mass spectrometry and includes oligomers of up to 23 repeating units (Chai, Shi et al. 2014). No detailed composition for procyanidins in kiwifruit leaves appears to have been published. Responses of the procyanidin pathway to bacterial, fungal and herbivore challenge in plants other than kiwifruit are widely reported in the scientific literature (e.g. Iriti et al. 2005, O'May and Tufenkji 2011, Barbehenn and Constabel 2011).

Coumaric acid (a phenolic) and threonic acid were also reduced in the leaves by root pruning ($P < 0.05$). The significance of these changes is unknown. A very small transient increase in trisaccharide (planteose), but not sucrose, observed in the leaves is consistent with previous unpublished trunk girdling results.

2.2 Effect of Actigard on metabolites in Gold3 leaf samples

PLS-DA and ANOVA analysis indicated metabolic differences were associated only with root pruning rather than with Actigard treatment.

3 RECOMMENDATIONS

1. Obtain confirmatory data on stress hormone levels in these samples to provide independent evidence as to the 'stress' status of these plants and the relevance of the observed changes in procyanidin concentrations
2. Establish the generality of the responses seen here to determine whether procyanidins may be used as markers of vine 'stress' or response to challenge.

4 METHODOLOGY

4.1 Samples

Leaf samples were collected by HortEvaluation Limited from an orchard located in Maketu, Bay of Plenty, 2, 5, 14, 21, and 42 days after root pruning according to the Project Description Overview (VI1506). Treatments were controls (Treatment 1), root pruned only (Treatment 2), and root pruned with Actigard treatment (Treatment 3; Actigard (200 g/ha) was applied to the root collar one week prior to root pruning). Roots were pruned by HortEvaluation Limited using a tractor drawn blade at around 60 days post full bloom with the aim of cutting at least 40cm deep at a distance of 60 cm from each trunk. The first leaf sample (day 2) was collected on 10 January 2015. Ninety frozen leaf samples of *Actinidia chinensis* var. *chinensis* 'Zesy002' (Gold3) (Appendix 1) were received from Greg Clark (Zespri) and stored frozen at Plant & Food Ruakura prior to processing.

Sample – Days After Root Pruning	Sample Dates	Treatments Sampled	No. Plots/Treatment Sampled	No.Plots Sampled	No. Leaves/Plot	No. Leaves
2	10/01/2015	1,2,3	6	18	4	72
5	13/01/2015	1,2,3	6	18	4	72
14	22/01/2015	1,2,3	6	18	4	72
21	29/01/2015	1,2,3	6	18	4	72
42	19/02/2015	1,2,3	6	18	4	72

Figure 3. Sampling protocol for collection of leaf samples

Chemical Analysis

Leaf samples were freeze dried, ground and extracted with 80% methanol, and shipped on dry ice to Palmerston North for analysis. Samples in solvent were stored at -80°C prior to analysis.

4.2 Phytohormone Analysis

We have encountered problems with this analysis due to unknown interferences specific to Gold3 leaf material. Data as to concentrations of major stress phytohormones will be provided in a separate report once these analytical problems are solved.

4.3 LCMS based Metabolic Profiling

Metabolic profiles were obtained by HR-TOF-LCMS on 80% methanol extracts obtained from the freeze-dried samples using methods established by Plant & Food Research. Briefly metabolomic analysis used a Brucker micro-TOF Liquid Chromatography-Mass Spectrometer (LC-MS) with negative mode electrospray ionisation operating with a C-18 RP Ultra-High Performance Liquid Chromatograph. Samples were analysed in random sample order interspersed with Quality Control (QC) and authentic standards.

Mass spectral features were extracted from the LCMS chromatographic data using Bruker Profile Analysis software. Peak areas for individual metabolites were normalised to the total metabolite concentration of that sample. Peak areas for 742 normalised mass spectral features were retained and analysed using principal components analysis (PCA). Quality control (QC) and chemical standards (not shown) were well grouped and, together with other measures, indicated data are of good quality. Leaf samples collected on Day 42 (orange circles) are very different from all other samples (Figure 1).

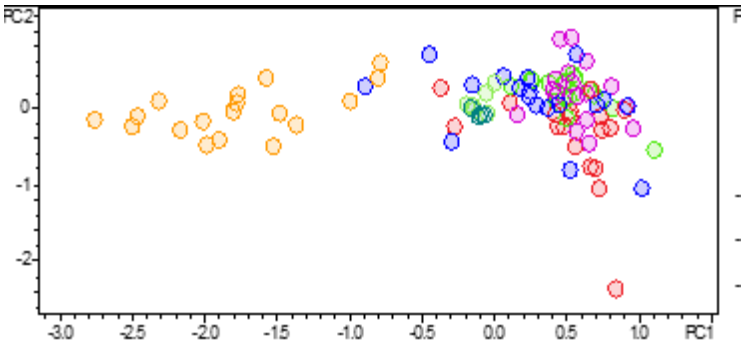

Figure 4. PCA analysis of leaf samples as measured by C18-RP LC-MS showing the different metabolic composition of sample (orange circles) collected 42 days after root pruning.

PCA analysis of this dataset also showed no evidence of any major effect due to root pruning or treatment with Actigard, (Figure 2: blue triangles, crosses and circles) indicating that root pruning and Actigard treatments are not major contributors to the variability of the data.
The data for the Day 42 samples, the last samples collected in the experiment, were therefore removed from the analysis.

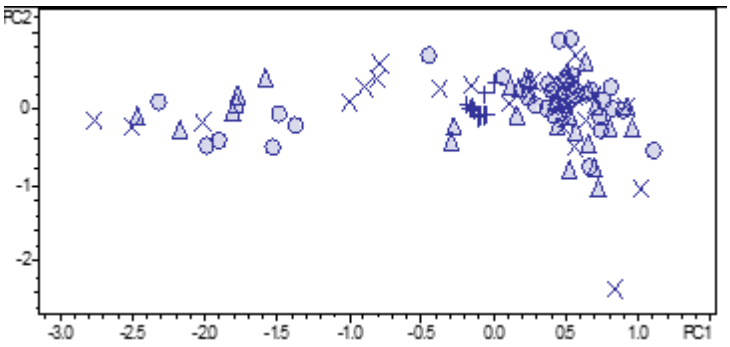

Figure 5. PCA analysis of leaf samples as measured by C18-RP LC-MS showing no separation of control, root pruned or Actigard™ treated samples in PC1 or PC2 of the PCA analysis.

PCA of the remaining samples (days 2, 5, 14 and 21 days after root pruning) plus QC samples and chemical standards (blue symbols) showed the control samples clustering more tightly than the experimental samples – again, data quality is good (Figure 3) but no separation of control, root-pruned or Actigard-treated samples was observed in PC1 or PC2 of the PCA analysis.

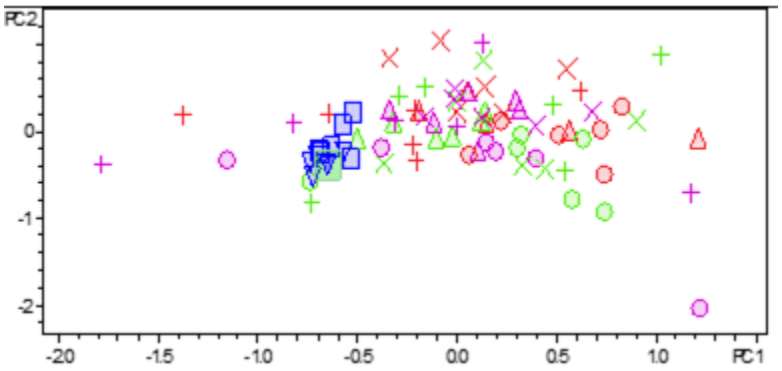

Figure 6. PCA analysis of leaf samples as measured by C18-RP LC-MS showing no separation of control, root pruned or Actigard™ treated samples in PC1 or PC2 of the PCA analysis. Blue symbols are QC samples.

4.4 Statistical Analysis

Measurements of poorer quality were removed from the dataset according to the following criteria:

- metabolites with an overall detection frequency <85% (poorly detected)
- metabolites with a maximum area count of <1500 (weak intensity)
- metabolites with >1 missing value in any treatment (incomplete or biased detection).

Measurements for 126 metabolites (technically mass tags) remained – fewer than expected, but samples were diluted to obtain all peaks on scale based on expectations of differences in concentrations of some sugars.

PLS-DA Analysis

Looking at each day's data separately, using discriminant analysis based on partial least squares (PLS-DA from the mixOmics package in R) to see whether the metabolite profiles of vines treated differently were distinct, there was reasonably good separation with the day 2 data (cross-validated misclassification rates around 35–40%) but not later (misclassification rates between 60% and 80%, similar to the by-chance rate of 67%).

The mixOmics package includes a procedure to pick a subset of the variables which give the best classification. It also includes a procedure to check the stability of that choice, by refitting the data models to random subsets of the data and seeing how often a variable (metabolite) is included in the best-classification subset. Telling the procedure to look for the 50 best metabolites on two dimensions of the PLS-DA using the day 2 data gave the mass-tags selected five times out of five random subsets (Appendix 2).

ANOVA

As PLS-DA is subject to overfitting and results may be dominated by irrelevant metabolites or poorly detected metabolites, a one-factor analysis of variance was also used to select all

metabolites with $P < 0.1$. The chemical structure of these metabolites (n = 20) was identified, where possible, from the MS data, and metabolites were assigned, where possible, to metabolic pathways. Consistent changes in biosynthetically related metabolites from the same biosynthetic pathway were considered as strong evidence for metabolic effects of root pruning or Actigard treatment.

4.5 Metabolite identification

Chemical formulae of selected metabolites (mass tags usually the pseudomolecular ion) were obtained by analysis of individual sample LC-MS data files using Bruker DataAnalysis software. All chemical formulae are within 3 mDa of theoretical accurate mass values and are best matches obtained after consideration of the natural abundance isotope distribution of the selected ions using the Bruker software. Chemical formulae of ions were converted to presumed molecular formula (add H^+) and were searched against compounds in the ChemSpider (http://www.chemspider.com/) and Metlin databases (https://metlin.scripps.edu/). Tentative identifications of metabolites were made based on database matches of the exact mass or derived molecular formula and on prior knowledge of the natural occurrence of plant, microbial and synthetic metabolites.

5 ACKNOWLEDGEMENTS:

Our thanks to Judith Reese and Robyn Wells for processing the leaf samples.

6 REFERENCES:

Barbehenn RV, Constabel, CP 2011. Tannins in Plant-Herbivore Interactions. Phytochemistry 72(13): 1551-1565.

Chai WM, Shi Y, Feng HL, Xu L, Xiang ZH, Gao YS, Chen QX 2014. Structure Characterization and Anti-tyrosinase Mechanism of Polymeric Proanthocyanidins Fractionated from Kiwifruit Pericarp. Journal of Agricultural and Food Chemistry 62(27): 6382-6389.

Iriti M, Rossoni M, Borgo M, Ferrara L, Faoro F 2005. Induction of Resistance to Gray Mold with Benzothiadiazole Modifies Amino Acid Profile and Increases Proanthocyanidins in Grape: Primary versus Secondary Metabolism. Journal of Agricultural and Food Chemistry 53(23): 9133-9139.

O'May C, Tufenkji N 2011. The Swarming Motility of *Pseudomonas aeruginosa* is Blocked by Cranberry Proanthocyanidins and Other Tannin-Containing Materials. Applied and Environmental Microbiology. 77(9): 3061-3067.

LIST OF APPENDICES

APPENDIX 1: LEAF SAMPLES RECEIVED FOR ANALYSIS

Treatments were controls (Treatment 1), root pruned only (Treatment 2), and root pruned with Actigard treatment (Treatment 3; Actigard was applied to the root collar one week prior to root pruning). The mean %DM was higher for treatment samples collected on day 2 (21.2% (Treatment 2, *P* 0.05) and 21.1% (Treatment 3, *P* 0.19) compared to the control sample (20.3% DM). For metabolomics analysis, metabolite concentrations were normalised against the total metabolites in each sample.

Sample ID	Sample #	Plate position	Days after root girdling	Date harvest	Plot	Treatment	% DM	Sample DW/g
RP001	1	A1	2	10/01/2015	11	1	20.0	0.2555
RP002	2	A2	2	10/01/2015	6	1	20.6	0.252
RP003	3	A3	2	10/01/2015	16	1	19.6	0.2551
RP004	4	A4	2	10/01/2015	1	1	21.8	0.2574
RP005	5	A5	2	10/01/2015	20	1	19.9	0.253
RP006	6	A6	2	10/01/2015	26	1	19.7	0.2522
RP007	7	A7	2	10/01/2015	3	2	20.4	0.255
RP008	8	A8	2	10/01/2015	17	2	21.7	0.2514
RP009	9	A9	2	10/01/2015	19	2	21.6	0.2542
RP010	10	A10	2	10/01/2015	5	2	21.3	0.256
RP011	11	A11	2	10/01/2015	12	2	20.5	0.2511
RP012	12	A12	2	10/01/2015	27	2	21.8	0.2551
RP013	13	b1	2	10/01/2015	2	3	23.5	0.2506
RP014	14	b2	2	10/01/2015	4	3	21.5	0.2579
RP015	15	b3	2	10/01/2015	10	3	20.0	0.2574
RP016	16	b4	2	10/01/2015	21	3	20.5	0.2566
RP017	17	b5	2	10/01/2015	18	3	21.2	0.2543
RP018	18	b6	2	10/01/2015	25	3	20.1	0.2514
RP019	19	b7	5	13/01/2015	6	1	24.8	0.2567
RP020	20	b8	5	13/01/2015	9	1	26.5	0.2508
RP021	21	b9	5	13/01/2015	1	1	23.4	0.254
RP022	22	b10	5	13/01/2015	13	1	25.9	0.2554
RP023	23	b11	5	13/01/2015	20	1	25.2	0.2586
RP024	24	b12	5	13/01/2015	30	1	23.4	0.2541
RP025	25	c1	5	13/01/2015	15	2	26.0	0.2506
RP026	26	c2	5	13/01/2015	3	2	25.9	0.2587
RP027	27	c3	5	13/01/2015	19	2	26.8	0.2551
RP028	28	c4	5	13/01/2015	7	2	25.9	0.2554
RP029	29	c5	5	13/01/2015	29	2	22.8	0.2561
RP030	30	c6	5	13/01/2015	24	2	24.7	0.2517
RP031	31	c7	5	13/01/2015	2	3	25.6	0.2568
RP032	32	c8	5	13/01/2015	8	3	26.3	0.252
RP033	33	c9	5	13/01/2015	28	3	22.9	0.2513
RP034	34	c10	5	13/01/2015	14	3	24.4	0.2536

RP035	35	c11	5	13/01/2015	21	3	25.2	0.2567
RP036	36	c12	5	13/01/2015	22	3	25.4	0.2544
RP037	37	d1	14	22/01/2015	20	1	24.6	0.2525
RP038	38	d2	14	22/01/2015	11	1	24.3	0.257
RP039	39	d3	14	22/01/2015	9	1	25.5	0.257
RP040	40	d4	14	22/01/2015	26	1	25.8	0.2547
RP041	41	d5	14	22/01/2015	30	1	26.6	0.2561
RP042	42	d6	14	22/01/2015	13	1	26.9	0.2572
RP043	43	d7	14	22/01/2015	12	2	25.4	0.2528
RP044	44	d8	14	22/01/2015	27	2	25.9	0.2525
RP045	45	d9	14	22/01/2015	15	2	27.1	0.2513
RP046	46	d10	14	22/01/2015	29	2	24.9	0.255
RP047	47	d11	14	22/01/2015	7	2	25.6	0.2542
RP048	48	d12	14	22/01/2015	19	2	27.2	0.2517
RP049	49	e1	14	22/01/2015	28	3	24.5	0.2514
RP050	50	e2	14	22/01/2015	8	3	26.9	0.2523
RP051	51	e3	14	22/01/2015	21	3	26.5	0.2554
RP052	52	e4	14	22/01/2015	10	3	26.5	0.2527
RP053	53	e5	14	22/01/2015	25	3	24.0	0.2539
RP054	54	e6	14	22/01/2015	14	3	26.0	0.2522
RP055	55	e7	21	29/01/2015	6	1	28.6	0.2526
RP056	56	e8	21	29/01/2015	16	1	28.1	0.2568
RP057	57	e9	21	29/01/2015	11	1	24.1	0.2517
RP058	58	e10	21	29/01/2015	13	1	27.2	0.2545
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RP060	60	e12	21	29/01/2015	1	1	26.8	0.2541
RP061	61	f1	21	29/01/2015	12	2	24.2	0.2512
RP062	62	f2	21	29/01/2015	3	2	25.8	0.2595
RP063	63	f3	21	29/01/2015	5	2	27.1	0.255
RP064	64	f4	21	29/01/2015	17	2	25.8	0.2513
RP065	65	f5	21	29/01/2015	24	2	26.0	0.2533
RP066	66	f6	21	29/01/2015	27	2	25.4	0.2569
RP067	67	f7	21	29/01/2015	2	3	27.7	0.2565
RP068	68	f8	21	29/01/2015	22	3	25.9	0.252
RP069	69	f9	21	29/01/2015	10	3	24.9	0.255
RP070	70	f10	21	29/01/2015	25	3	23.3	0.2518
RP071	71	f11	21	29/01/2015	18	3	24.1	0.2585
RP072	72	f12	21	29/01/2015	4	3	27.6	0.2523
RP073	73	g1	42	19/02/2015	16	1	24.2	0.2518
RP074	74	g2	42	19/02/2015	9	1	25.0	0.2535
RP075	75	g3	42	19/02/2015	13	1	25.7	0.2556
RP076	76	g4	42	19/02/2015	26	1	26.3	0.2555
RP077	77	g5	42	19/02/2015	6	1	27.1	0.2569
RP078	78	g6	42	19/02/2015	30	1	30.3	0.2542
RP079	79	g7	42	19/02/2015	7	2	30.5	0.2578
RP080	80	g8	42	19/02/2015	15	2	26.3	0.2514
RP081	81	g9	42	19/02/2015	5	2	28.4	0.252

RP082	82	g10	42	19/02/2015	17	2	28.6	0.2525
RP083	83	g11	42	19/02/2015	24	2	28.6	0.2501
RP084	84	g12	42	19/02/2015	29	2	25.6	0.2529
RP085	85	h1	42	19/02/2015	14	3	27.3	0.2583
RP086	86	h2	42	19/02/2015	28	3	25.8	0.2548
RP087	87	h3	42	19/02/2015	18	3	29.3	0.2517
RP088	88	h4	42	19/02/2015	8	3	29.6	0.2557
RP089	89	h5	42	19/02/2015	4	3	31.6	0.2576
RP090	90	h6	42	19/02/2015	22	3	28.6	0.2513

APPENDIX 2: METABOLITES (MASS TAGS)

Metabolites (mass tags) identified as important in discriminating between root-pruned and control leaves using 'sparse' PLS-DA Analysis.

Dimension 1	Identity	Chosen	Dimension 2		Chosen
0.54min / 509.031m/z	unknown	5/5	0.58min / 439.083m/z	digalloyl hexose	5/5
0.55min / 767.186m/z	unknown	5/5	1.58min / 289.071m/z	catechin	5/5
0.56min / 1187.396m/z	unknown	5/5	2.19min / 281.066m/z	unknown	5/5
0.58min / 533.172m/z	unknown	5/5	2.20min / 163.040m/z	coumaric acid	5/5
0.61min / 133.014m/z	malic acid	5/5	2.31min / 289.071m/z	catechin isomer	5/5
1.34min / 323.134m/z	previously	5/5	3.24min / 463.089m/z	quercetin-glycoside	5/5
1.47min / 355.067m/z	caffeoyl glucuronide	5/5	4.60min / 721.379m/z	previously	5/5
1.90min / 425.087m/z	epigallo-catechin	5/5	14.69min / 367.357m/z	unknown	5/5
1.90min / 577.135m/z	procyanidin dimer	5/5	14.73min / 799.559m/z	PE-lipid	5/5
1.92min / 325.093m/z	coumaric acid glycoside	5/5			
2.20min / 163.040m/z	coumaric acid	5/5			
2.26min / 135.030m/z	threonic acid	5/5			
2.29min / 571.145m/z	unknown	5/5			
2.49min / 865.198m/z	procyanidin trimer	5/5			
2.66min / 1001.212m/z	unknown	5/5			
2.74min / 1153.261m/z	procyanidin tetramer	5/5			
2.76min / 1441.325m/z	procyanidin pentamer	5/5			
3.37min / 1153.259m/z	procyanidin tetramer	5/5			
11.14min / 619.255m/z	unknown	5/5			
12.73min / 815.498m/z	PE lipid	5/5			
13.73min / 838.536m/z	PE lipid	5/5			
14.20min / 840.551m/z	PE lipid	5/5			

APPENDIX 3: KRUSKAL-WALLIS AND ONE-FACTOR ANALYSIS OF VARIANCE (ANOVA) TESTS

Kruskal-Wallis and one-factor analysis of variance (ANOVA) tests on the individual metabolites to see which showed significant differences between the treatment groups for samples collected on day 2 after root pruning.

Day 2	P <0.1	Control mean	Prune mean	Prune +Acti mean	Prune/ control Fold Change	Prune +Acti Fold/ Control Change	Metabolite
0.54min / 509.031m/z	0.095	1927	1564	1375	0.81	0.71	unknown
0.55min / 767.186m/z	0.003	5437	4580	4463	0.84	0.82	unknown
0.58min / 533.172m/z	0.092	77558	63619	59657	0.82	0.77	unknown
0.61min / 133.014m/z	0.092	14365	12488	12182	0.87	0.85	malic acid
1.47min / 355.067m/z	0.031	19005	13922	14300	0.73	0.75	unknown
1.90min / 425.087m/z	0.014	4508	5124	5306	1.14	1.18	epigallocatechin
1.90min / 577.135m/z	0.004	55457	62580	63075	1.13	1.14	procyanidin dimer
2.19min / 281.066m/z	0.057	48228	33097	41664	0.69	0.86	unknown
2.20min / 163.040m/z	0.016	18251	15770	16393	0.86	0.90	coumaric acid
2.26min / 135.030m/z	0.009	7881	6313	3873	0.80	0.49	threonic acid
2.29min / 571.145m/z	<.001	10039	8659	8570	0.86	0.85	unknown
2.49min / 865.198m/z	0.088	54739	57373	59655	1.05	1.09	procyanidin trimer
2.66min / 1001.212m/z	0.026	3731	5124	5206	1.37	1.40	unknown
2.74min / 1153.261m/z	0.001	32997	36603	38892	1.11	1.18	procyanidin tetramer
2.76min / 1441.325m/z	0.005	37735	41047	43663	1.09	1.16	procyanidin pentamer
3.24min / 463.089m/z	0.089	68778	60495	68992	0.88	1.00	quercetin-hexose
3.37min / 1153.259m/z	0.008	5704	6285	6715	1.10	1.18	procyanidin tetramer
11.14min / 619.255m/z	0.022	3200	3966	4273	1.24	1.34	unknown
12.73min / 815.498m/z	0.063	9543	8394	5642	0.88	0.59	PE lipid
14.20min / 840.551m/z	0.048	8514	7813	7162	0.92	0.84	PE lipid

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