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Rotundone and its role in defining terroir in iconic Australian cool climate 'peppery' Shiraz



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1. Abstract

This project lends weight to the concept of terroir and demonstrates how the interplay of site, viticultural management, climatic and environmental effectors can shape aroma compound profiles in cool-climate Shiraz.

The results establish that development of the sought-after 'peppery' aroma from rotundone in Shiraz grapes appears to depend on site characteristics and environmental factors that regulate sesquiterpene biosynthesis, rather than genetic determinants of planted vines. From a practical vineyard management perspective, a late harvest date is key, as extensive formation of the immediate rotundone precursor α-guaiene, and subsequently rotundone itself, typically only commences well after veraison.

2. Executive summary

Previous research has demonstrated that the Grampians and Pyrenees regions can produce wines with substantially higher concentrations of the 'spicy' flavour compound rotundone than other notable Shiraz-producing regions (e.g. Barossa and McLaren Vale). Patterns of rotundone variation appeared to be temporally stable within an individual vineyard across different growing seasons, with large differences in concentrations occurring across individual vineyards and across different growing seasons. In general, cooler ripening periods and limited bunch exposure appeared to favour the synthesis/accumulation of rotundone in Shiraz grapes. Focusing on premium cool-climate Shiraz, this project built on research at the Mount Langi Ghiran 'Old Block' in the Grampians, and expanded that study to other selected premium Shiraz sites across South Australia and Victoria.

In order to explain the magnitude of the observed site-specific differences in rotundone, this research sought to differentiate between genetic features in grapevine planting material (which may be transferable through propagation and between vineyards) and/or environmental features (which are site-specific and might be influenced by management practices) that are contributing to distinctive aroma attributes in wine.

Despite the elusive character of the unique grape aroma compound rotundone in premium Shiraz, detailed concentration-based maps of grape sesquiterpenes including the aroma compound rotundone could be established. Key findings from this project are:

- The broad intra-vineyard variability and distinct spatial structure in grape rotundone is not unique to Mt Langi Ghiran's Old Block and its heritage vines, but appears to represent a common feature found in growing seasons with elevated grape rotundone concentrations and cool-climate vineyards planted with other clones.
- The spatial distribution of grape rotundone at an Adelaide Hills site planted with the Shiraz clones 2626 and 1127 is closely associated with its precursor, α-guaiene, and a range of other sesquiterpenes and is distinct, yet in contrast to Mt Langi Ghiran, may vary from year to year.
- Analysis of grape samples collected across multiple trial sites and growing seasons established that formation of elevated α-guaiene and rotundone are synchronised and typically initiated very late during berry ripening. This means that the biosynthesis of the precursor α-guaiene is key to the concomitant formation of rotundone in grapes. That is, availability of α-guaiene and initiation of α-guaiene biosynthesis appear to be critical for controlling grape rotundone at harvest.
- Analysis of site characteristics, including microbial populations, established that the spatial (intra-vineyard) variability in grape rotundone at the Adelaide Hills site was not related to established measures of vine performance and canopy, such as vine vigour (as measured using remotely sensed imagery). This result was similar to previous observations for Mt Langi Ghiran's Old Block. In respect of other site characteristics, results were equivocal, perhaps due to marked differences in seasonal conditions and, in contrast to Mt Langi, a very limited vineyard area orientated away from north.
- Metagenomics analysis of surface soil samples collected over two seasons from the previously identified high- and low-rotundone zones at Mt Langi Ghiran's Old Block found marked differences in the genetic diversity and composition of the soil bacterial and fungal microbiomes. A few specific taxa/groups of microorganisms were associated with the rotundone-based variation; and bacterial communities in soil from the high-rotundone zone appeared to form a much more complex and connected network than those in the low-

rotundone zone soils. Also, short-term mulching effects did not seem to mask this rotundone zone-based variation in soil microbiology.

- Functional profiling of the soil microbiomes in the low- and high-rotundone zones at Mt Langi Ghiran's Old Block through direct metagenomic sequencing analysis showed significant and distinct dissimilarity between the low- and high-rotundone zone samples at all gene classification levels; that is, for genes, gene ontology (GO) and GO and COG (clusters of orthologous groups of proteins) pathway analysis.
- Studies of the formation of aroma compounds from sesquiterpenes established that sesquiterpene hydrocarbons common to grapes are absent from wine (other than a trace of α-muurolene), because they are not extracted into wine-like hydroalcoholic solutions and/or are removed from juice with solids. This means that sesquiterpene aroma precursors from grapes are most likely not chemically converted or metabolised during fermentation, while at the same time grape marc and wine solids are a potential source of sesquiterpene aroma precursors.
- Model experiments demonstrated that exposure to daylight catalyses the chemical oxidation of sesquiterpene precursors. This means that the formation of potent aroma compounds such as rotundone does not necessarily require the previously described enzymatic reactions by grapevines or microorganisms. In addition, the aroma compound iso-mustakone – formed by oxidation from its precursor α-ylangene – was identified through GC-MS-O analysis for the first time in wine, where it contributes 'celery-like', 'spicy' characters at very low concentrations.

In summary, for the formation of the 'peppery' aroma compound rotundone in Shiraz grapes, site characteristics appear to be more important than genetic determinants of planted Shiraz vines, as similar effects were observed in Shiraz vineyards planted with clonal selections, mass-selected vines, and in vines grafted with low-and high rotundone canes.

Overall, this project provides science-based evidence supporting the concept of terroir, demonstrating how the interplay of site, viticultural management, climatic and environmental/biological effectors (albeit the latter are yet to be identified) shape distinct sensory attributes in Shiraz wine.

Further research and insights into the environmental regulation of sesquiterpene biosynthesis in Shiraz are required to preserve the desirable, but elusive, 'peppery' flavours in existing 'cool-climate' vineyards through preserving the environmental and/or biological effectors. In addition, such knowledge could potentially enable growing Shiraz grapes with 'cool-climate-like' flavour attributes in warmer/hotter regions. Ultimately, the findings of this projects represent an important step towards mitigating risks from climate change, biodiversity loss or viticultural management decisions, which might otherwise lead to unintended flavour consequences in Shiraz.

3. Acknowledgements

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4. Background

The project built on prior research at the Mount Langi Ghiran 'Old Block' in the Grampians (Scarlett et al. 2014, Bramley et al. 2017), but expanded that study to other selected premium Shiraz sites across South Australia and Victoria. Earlier research had demonstrated that the Grampians and Pyrenees regions can produce wines with substantially higher concentrations of the 'spicy' flavour compound rotundone than other notable Shiraz-producing regions (e.g. Barossa and McLaren Vale). Patterns of rotundone variation appeared to be temporally stable within an individual vineyard across different growing seasons, with large differences in concentrations occurring across individual vineyards (~15-fold differences observed) and across different growing seasons (~30 to 40-fold differences). In general, cooler ripening periods and limited bunch exposure appeared to favour the synthesis/accumulation of rotundone in Shiraz grapes. However, it appeared unlikely that seasonal conditions, temperature and bunch exposure alone, could explain the magnitude of the observed site-specific differences in rotundone. Hence this project aimed to identify genetic and/or environmental factors which alone, or in combination, may shape grape composition and wine flavour in Shiraz.

Ultimately, understanding the environmental regulation of rotundone's biosynthesis in Shiraz could potentially enable growing Shiraz grapes with 'cool-climate-like' flavour attributes in warmer/hotter regions. In addition, such knowledge is an important step towards mitigating risks from climate change, biodiversity loss or viticultural management decisions, which might otherwise lead to unintended loss of desirable and/or unique 'peppery' flavour attributes in Shiraz wine.

5. Project aims and performance targets

Australia's unique vineyard sites and terroirs enable producers to craft wines of exceptional quality and finesse. This project targeted cool-climate vineyards and vineyards with old planting material which may be unique to Australia; the research sought to characterise genetic and biophysical factors which are related to observed large differences in grape and wine flavour. The project also targeted insights into how the rotundone element of terroir may be manipulated and managed at a range of scales. A key aspect was to test if desirable flavour attributes of grapes could be transferred from one vine to another, within vineyards and between sites.

The overall objectives for the project were to:

- generate rotundone and α-guaiene maps for three Shiraz vineyards in the Grampians, Pyrenees, and/or Adelaide Hills, in addition to the Mount Langi Ghiran 'Old Block', and characterise their variation in vine performance and the underlying land via EM38, elevation and vigour mapping
- investigate potential genetic determinants for rotundone concentration using reciprocal bud grafts and/or potted vines in greenhouses
- investigate unique key aroma compounds in Shiraz wine from cool-climate terroirs, the role of photochemical oxidation in the formation of aroma compounds from sesquiterpenes, and the kinetics of guaiene and rotundone accumulation/degradation as influenced by the light environment around a grape cluster
- investigate the potential relationship between soil microbes and inherent spatial variation in grape composition and rotundone concentration
- investigate relationships between entomological and vertebrate herbivore activity on the inherent spatial variation in rotundone.

Table 1. 2016-17 (FY1) Outputs and activities

Output	Activities	Activity target date
List of trial sites suitable for the project.	1. Assess cool climate Shiraz vineyards in the Grampians, Pyrenees and Adelaide Hills. 2. Select three sites that complement the Mt Langi 'Old Block' based on records for peppery grapes/wines, soil and site properties.	31/12/2016
Optimised analytical method to quantify α-guaiene and rotundone.	1. Optimise MASE GC-MS method for quantification of α-guaiene and rotundone. 2. Validate analytical method for Shiraz grapes.	31/12/2016
Soil samples for metagenomics testing	1. Sample soil cores at low/medium/high pepper zones at Mount Langi. 2. Extract and purify DNA. 3. Send at least eight DNA replicates per zone to sequencing provider.	30/06/2017
Grape samples for α-guaiene and rotundone analysis.	1. Sample the three additional vineyard sites at harvest, aiming for approximately 30 grape samples/ha (average 150 samples/site). 2. Sample low and high pepper zones at Mt Langi between veraison and harvest, aiming for a minimum of four time points and six biological replicates per zone. 3. Extract α-guaiene and rotundone from grape samples for GC-MS analysis.	30/06/2017
Bud grafted vines in Mt Langi vineyard. Plant material for DNA analysis.	1. Establish reciprocal bud grafts of planting material located in the high, medium and low rotundone zones at Mt Langi 'Old Block'. 2. Obtain plant materials and extract DNA for analysis of sesquiterpene biosynthesis.	30/06/2017

Table 2. 2017-18 (FY2) Outputs and activities

Output	Activities	Activity target date
Maps of α-guaiene and rotundone distribution at the three additional vineyard sites.	1. Analyse extracts from 2017 grapes by GC-MS. 2. Complete EM38, PCD and digital elevation mapping. 3. Prepare maps and statistical analysis.	30/09/2017
Data characterising microbial populations in low/medium/high pepper zones.	1. Obtain amplicon diversity profiling data for bacterial (16S) and fungal (ITS) populations. 2. Undertake bioinformatic analysis of operational taxonomic units (OTUs) to characterise microbial populations in low/medium/high pepper zones.	30/09/2017
Agreed research plan for 2018-19 based on a review and evaluation of results to date.	1. Summarise results to date and discuss at meeting with project team and Wine Australia. 2. Revise project plans for 2018-19 if required.	31/12/2017
Grape samples for α-guaiene and rotundone analysis.	1. Collect grape and/or grapevine samples. 2. Extract α-guaiene and rotundone and other metabolites as required for GC-MS analysis.	30/06/2018
Grapevine samples and soil samples for analysis.	1. Sample grapes and other vine materials for analysis as per project review. 2. Sample soil cores as per project review; extract and purify DNA; send DNA replicates to sequencing provider.	30/06/2018
Qualitative data comparing herbivore activity across the trial sites.	3. Collate observations from 2017 and 2018 across trial sites.	30/06/2018

Table 3. 2018-19 (FY3) Deliverables and activities

Contracted deliverables	This year's activities	Overall target date for these activities
Model experiments conducted based on reciprocal bud grafts and potted vines in greenhouses	1. Maintain potted vines and grafted vines. 2. Sample grapes and analyse for α-guaiene and rotundone. 3. Extract grape DNA for analysis by a collaborator, if warranted by results of sesquiterpene analysis.	June 2019
Maps of α-guaiene and rotundone for multiple trial sites produced over two vintages	1. Analyse extracts from 2018 grapes by GC-MS. 2. Obtain maps from collaborator at CSIRO and design 2019 experiments. 3. Collect vintage 2019 grape and/or grapevine samples. 4. Extract α-guaiene and rotundone and other metabolites as required for GC-MS analysis. 5. Extract grape DNA for analysis by collaborator, if warranted by results of sesquiterpene analysis. 6. Prepare poster for the AWITC in 2019.	June 2019
Knowledge generated of site characteristics, including microbial populations and herbivore activity	1. Obtain data on microbial populations, soil composition and soil attributes for low and high rotundone zones in multiple sites from collaborator at CSIRO, as per 2018 project review.	June 2019
Understanding of the role of photochemical oxidation in the formation of aroma compounds from sesquiterpenes	1. Analyse grapes from 2018 ripening trials at multiple sites for α-guaiene and rotundone. 2. Conduct data analysis, comparing grape composition to data from temperature loggers and light exposure measurements. 3. Design 2019 trials on the role(s) of site characteristics, environmental factors and phenology in rotundone accumulation. 4. Characterise oxidised sesquiterpenes in model experiments and grape samples. 5. Prepare a peer-reviewed publication to summarise studies into the α-guaiene/rotundone relationship.	June 2019

Table 4. 2019-20 (FY4) Deliverables and activities

	Contracted deliverables	This year's activities	Overall target date for these activities
A	Model experiments conducted based on reciprocal bud grafts and potted vines in greenhouses	1. Maintain potted vines and grafted vines. 2. Sample grapes and analyse for α-guaiene and rotundone.	June 2020
B	Maps of α-guaiene and rotundone for multiple trial sites produced over two vintages	1. Analyse extracts from 2019 grapes by GC-MS. 2. Obtain maps from collaborator at CSIRO. 3. Present results at the 17th AWITC 4. Review results to date and design vintage 2020 trial work 5. Collect vintage 2020 grape samples. 6. Extract rotundone and other metabolites as required for GC-MS analysis.	June 2020
C	Knowledge generated of site characteristics, including microbial populations and herbivore activity	1. Summarise and publish data on microbial populations, soil composition and soil attributes for low and high rotundone zones. 2. No work is planned to characterise herbivore activity, as agreed at 2017 project review.	June 2020
D	Understanding of the role of photochemical oxidation in the formation of aroma compounds from sesquiterpenes	1. Analyse grapes from 2019 ripening trial at Mount Langi for α-guaiene and rotundone. 2. Conduct data analysis, comparing grape composition to data from temperature loggers and light exposure measurements. 3. Test the effect of lowered ambient temperature on formation of α-guaiene and rotundone using potted vines. 4. Repeat 2019 ripening trial in the 2020 vintage, if warranted based on seasonal conditions, to validate effects observed from the 2019 extended hang-time experiment at Mount Langi's Sheoak Block; or; characterise the presence of grape-derived sesquiterpenes (other than rotundone) in wine. If a repeat of the 2019 trial is not required, focus efforts on fast-tracking 2019-2020 deliverable B, activities 5 and 6 and bringing forward 2020-2021 deliverable A, activity 1.	June 2020

Table 5. 2020-21 (FY5) Deliverables and activities

	Contracted deliverables	This year's activities	Overall target date for these activities
A	Knowledge of the concentrations of key aroma compounds and aroma-relevant oxidation products of sesquiterpenes in cool-climate Shiraz	1. Finalise analysis and data analysis of samples from 2020. 2. Conduct data analysis of results across all vintages.	February 2021
B	Practical advice and extension materials for grapegrowers and winemakers	1. Review and update relevant roadshow content. 2. Prepare Final Report, presentations and publication(s).	February 2021

This project commenced in 2016-17 under Project Agreement AWR 1602 and continued from 2018-19 under the Investment Agreement between the Australian Grape and Wine Authority (now Wine Australia) and the AWRI. In 2020, the joint Wine Australia-AWRI Management Committee agreed to finalise the work under the 2020-21 Annual Operating Plan (AOP).

6. Methods

6.1 Materials

6.1.1 Trial sites

Sesquiterpene mapping

The following sites were chosen for sesquiterpene mapping:

- The Lane 'Junction Road' vineyard-- 3.21 ha Shiraz, Adelaide Hills, SA (35°00'30.4"S 138°50'16.8"E) consisting of two adjacent blocks separately planted with Shiraz clones, 1127 (1.56 ha) and 2626 (1.65 ha). Samples were collected from 141 vines on the day prior to commercial harvest: 18 April 2017; 22 March 2018; and 8 March 2019. In 2017, 140 grape samples were analysed for rotundone and α-guaiene. In 2018 all 141 samples were analysed for rotundone and α-guaiene and in 2019 all 141 samples were analysed for rotundone and a subset of 18 was analysed for α-guaiene.
- Mount Langi Ghiran 'Sheoak' Shiraz vineyard, Grampians, Vic (37°16'08.0"S 143°06'58.9"E). Samples were collected from 150 sampling points on 22 March 2017, two days after commercial harvest. All samples were analysed for rotundone. This sampling was to be repeated in 2018 and 2019 but this was unfortunately not possible due to conflicting commercial operations.
- See Saw Wines 'Annangrove Park' Shiraz vineyard, Orange, NSW (33°22'14.0"S 148°51'02.4"E). Sampling was conducted immediately prior to commercial harvest from 152 sampling points on 20 March 2017. A subset of 21 samples were analysed for rotundone and, due to the low concentrations of rotundone with an average of just 6.7 ng/kg, the remaining samples were not analysed.
- Selected adjacent sub-blocks of Shiraz at Treasury Wine Estates' 'Woodbury' vineyard, Eden Valley, SA (34°38'14.1"S 139°02'06.9"E). Grape samples from 170 sampling points were collected on 22 March 2019, prior to commercial harvest on 25 March 2019. A subset of 34 samples were analysed for rotundone. The samples were low in rotundone with an average of just 2.8 ng/kg so it was decided not to continue further with analysis.

For all sites, sampling points were selected and georeferenced and grape sampling was conducted as per Scarlett et al. (2014) and Bramley et al. (2017) taking account of the vineyard area, the minimum requirements for variogram estimation prior to map interpolation of 100 sampling points and the finding that 26 samples ha^{-1} (or preferably more) are required for robust characterisation of vineyard variability (Bramley, 2005).

In addition, at both The Lane and Sheoak sites, high resolution electromagnetic induction (EM38) soil surveys were conducted coupled with a real-time kinematic GPS system to generate maps of variation in bulk electrical soil conductivity and elevation. Remotely sensed imagery of vine vigour (PCD – Plant cell density, the ratio of infrared:red reflectance) was also acquired at veraison each year from a commercial provider. These soil, topographic and vine vigour data were acquired for better understanding of underlying vineyard variability (see for example, Bramley et al. 2011 for further detail of these 'precision viticulture' techniques) and to enable comparison of results with those previously obtained at the 'Old Block' site (Bramley et al. 2017). At both 'See Saw' and 'Woodbury' vineyards, similar data obtained during previous projects were sourced, but not used given that full sesquiterpene analysis for mapping did not proceed.

Ripening trials

Three bunches were randomly selected from across each of the target panels on the more shaded side of the vine (i.e. the more eastern-facing sides at The Lane vineyard and the southern facing sides at the Sheoak vineyard). Sampling was conducted either fortnightly or weekly between veraison and harvest with the aim of obtaining a minimum of four timepoints. The grape samples were placed in plastic zip-lock bags and immediately frozen prior to transport back to the AWRI where the grapes were analysed for both rotundone and α-guaiene. The sites chosen for the ripening trials were:

- Mount Langi Ghiran 'Old Block' 6.1 ha Shiraz vineyard, Grampians, Vic (37°18'52.4"S 143°09'04.2"E). Six sampling points were selected from within each of the consistently low and high pepper zones based on the results from Bramley et al. (2017) and the same target panels were sampled for three consecutive years. In 2017, samples were collected from 7 March through to 18 April while the vineyard was selectively hand-harvested for commercial winemaking over a period of four and a half weeks from 31 March, with a final machine harvest on 1 May. In 2018, grapes were sampled from 22 February to 11 April, and commercial harvest of the 'Old Block' vineyard was conducted from 4 April through to 17 April. In 2019 the first sample was taken on 14 March and the final sample on 19 April, and commercial harvest took place from 9 April through to 19 April.
- Mount Langi Ghiran 'Sheoak' Shiraz vineyard (as above). Six sampling points were selected from within the low and high pepper zones based on the results from the 2017 rotundone spatial map. Because the grape rotundone concentrations were very low and the temporal stability of the spatial structure was not confirmed these zones were indicative only. Samples were taken at the same timepoints as 'Old Block' in 2018 and 2019, though in 2018 sampling concluded three weeks earlier than in 'Old Block' due to bird damage to the fruit. Commercial harvest for the 'Sheoak' vineyard was conducted on 7-9 March 2018 and 7-8 March 2019.
- The Lane 'Junction Road' vineyard (as above). Six sampling points were selected from within the low and high rotundone zones based on the results from the 2017 rotundone spatial map. Sampling was conducted from 2 February 2018 to 22 March 2018. A repeat of this sampling was planned for 2019, but could not be conducted due to an unexpectedly early commercial harvest date.

Weather data for the two regions at the relevant times are provided in Tables 6 and 7.

Table 6. Weather data for Mount Langi Ghiran vineyards from the Ararat Prison Australian Bureau of Meteorology station number 089085, located approximately 15.5 km from 'Old Block' and 12 km from 'Sheoak' vineyard.

Year	GSR (mm)	MJT (°C)	MFMT (°C)	GDD FM (°C)	MDS JFM (MJ/m^2)
2017	241.6	20.7	19.4	557.4	21.1
2018	210.8	19.6	18.2	481.0	21.1
2019	187.8	20.8	18.5	499.5	22.2

GSR, growing season rainfall (total rainfall from October through March); MJT, mean January temperature; MFMT, mean February to March temperature; GDD FM, growing degree days February to March; MDS JFM, mean daily solar exposure January to March.

Table 7. Sampling dates, berry data and weather data for the Junction Road vineyard

	berry data*			weather data				
sampling date**	**avg bunch weight (g)**	**avg berry weight (g)**	**rotundone (avg, ng/kg)**	**GSR (mm)**	**MJT (°C)**	**MFMT (°C)**	**GDD FM (°C)**	**MDS JFM (MJ/m^2)**
18 April 2017	194	1.42	93	379.4	21.3	19.8	580.8	20.9
22 March 2018	179	1.26	58	171.6	22.0	20.0	587.8	20.9
8 March 2019	84	0.96	2	168.0	23.3	19.9	583.1	22.4

*Berry parameters calculated from 140 samples. **The sampling date was one day prior to commercial harvest. Weather data obtained from the 'Mount Barker' Bureau of Meteorology station number 023733, located 7.2 km from the vineyard; GSR, growing season rainfall (total rainfall from October through March); MJT, mean January temperature; MFMT, mean February - March temperature; GDD FM, growing degree days February – March; MDS JFM, mean daily solar exposure January to March.

Potted vines

In July 2016 and 2017 grapevine canes were collected from vines within the low, medium and high rotundone zones in the Mount Langi Ghiran 'Old Block' vineyard based on the temporally stable rotundone spatial structure (Bramley et al. 2017). The cuttings were stored at 4°C on-site, then heat-treated prior to being transported to the AWRI.

Grafted vines

Based on the rotundone spatial mapping, 15 vines were selected within each of the consistently low and high rotundone zones in the 'Old Block' vineyard at Mount Langi Ghiran and grapevine cuttings were taken from vines within these zones on 13 July 2016 and stored at 4°C. On 12 November 2016, the tops of the 30 target vines were removed, and two grapevine buds were grafted onto each: one bud originating from the 'low' rotundone zone and one from the 'high' rotundone zone. Although not all grafted buds took, the fruit was harvested from each grafted arm as close to commercial harvest as possible with a total of 20 samples collected on 5 April 2018, 30 samples on 18 April 2019, and 24 samples on 6 April 2020. All grape samples were analysed for both rotundone and α-guaiene.

Microvines

Six Pinot Meunier microvines (Boss and Thomas 2002) with ripe grapes were provided by Dr Paul Boss of CSIRO on 31 January 2019.

6.2 Analytical methods

- Rotundone was quantified as per Geffroy et al. (2014) and Bramley et al. (2017).
- α-Guaiene was quantified based on the method of Zhang et al. (2016) with some modifications: deuterated α-guaiene was obtained for use as internal standard and a calibration function was produced in order to accurately quantify α-guaiene.
- GC-MS analyses of sesquiterpenes and their oxidation products were carried out under similar parameters to Huang et al. (2014).
- GC-MS-O analyses (GC-sniff) of sesquiterpene oxidation products were carried out as above except with an olfactometry set-up as described by Mayr et al. (2014).
- Multidimensional GC-MS-O analyses of α-ylangene oxidation products were carried out as per rotundone quantification except with an olfactometry set-up as described by Mayr et al. (2014).

6.3 Soil sampling and chemical analysis

In order to characterise the microbiome composition, surface soils were analysed from vineyard zones demonstrated to vary in rotundone concentration. Samples were collected immediately prior to harvest from positions adjacent to selected geo-referenced vines from the 'Old Block' vineyard at Mt Langi Ghiran (2017 and 2018), and from The Lane (2018; see above). In the Grampians, individual soil sampling locations in each of the rotundone zones (Bramley et al. 2017) were identified such that seven to ten samples from each zone were collected. A similar approach was followed at the Adelaide Hills site. Air-dried soil samples were analysed using established standard methods in an accredited commercial soil testing laboratory (www.apal.com.au). Soil texture was determined using a combination of micropipette and MIR methods. Total nitrogen levels were measured using a LECO TruMac CN Macro Determinator (www.leco.com). All samples were analysed in duplicate and the results expressed on a per gram dry soil basis for data analysis and interpretation.

6.4 Soil microbial analysis

Microbial activity and catabolic diversity were measured using a community-level physiological profiling method (Gupta et al. 2019). DNA was extracted from 2.5 g samples of soil using the DNeasy PowerMax soil kit 221 (www.qiagen.com) following the manufacturer's protocol. For bacterial and fungal community composition analysis, 16S rRNA and ITS region PCR amplification and sequencing were conducted using the primers for V1-V3 16S region (27F-230 519R) and for ITS1F-2R, respectively. Selected soils from the low- and high-rotundone zones collected during 2017 in the Mount Langi Ghiran vineyard were also deep shotgun sequenced (direct metagenomic sequencing) using Illumina NovaSeq technology. All the sequencing analysis were done through the Australian Genome Research Facility (www.agrf.org.au). Amounts of total fungal and bacterial abundances were quantified using quantitative PCR assays with group specific primers (fungi - FR1 / FF390 and bacteria-F968 /R1378) and the QuantiTect SYBR Green PCR kit (Qiagen, Vic, 288 Australia) and the PCR was carried out on a Strategene Maxpro3000P qPCR system (Agilent, 289 Vic, Australia). Additionally, functional annotation of 16S sequence (prokaryotic taxa) data to know metabolic and ecological relevant functions was done using FAPROTAX database (http://www.loucalab.com/archive/FAPROTAX). Full details of all the methods are available in Gupta et al. (2019). Details of bioinformatic and molecular network analysis for amplicon sequencing results are also provided in Gupta et al. (2019).

The WGS reads were initially assembled by AGRF using metaSpades. The generated contigs were sorted by length and assembled contigs with less than 500 bp were discarded. The assembly summary report was generated using QUAST. Further, these contigs were binned using metaBAT2, MaxBin2 and

CONCOCT and consolidated and refined bins were processed via checkM for bin contamination and completion.

The scaffolds produced were split into genes and annotated using Prokka (Prokka: rapid prokaryotic genome annotation). The .tbl. files produced by Prokka contain entries for each called gene (and other features) and the results of matching these genes against a number of protein reference sets, including UniProt and HAMAP. Each .tbl entry contained a gene code, the name of the product, a UniProtKB identifier, a HAMAP identifier, a COG identifier or an EC number. These per-sample .tbl files were condensed into a table format, and the tables from all 12 assembled samples were merged to produce a gene abundance table. The rows in this table are the gene codes, with their annotations, and the per-sample gene abundance counts are derived from the depth of coverage number given for the contig where the gene was found.

This initial gene abundance table only contained the annotations generated by Prokka, so the UniProtKB and HAMP identifiers were followed back to their reference databases, and GO terms and other database cross-references were retrieved. These additional annotations were used to produce an extended gene abundance table. This extended tables were then summarised over a number of the available annotation types, producing tables of the occurrence of GO terms (components, function and pathway) and COGs (COG, COG category and COG pathway). The GO summary tables were then reduced using the Metagenomic GO Slim produced by EMBL/EBI (https://doi.org/10.1093/nar/gkz1035) to create smaller, metagenomics-focused GO-based abundance tables. The end result of this analysis was a set of abundance tables, with the rows being genes or annotations, such as GO Pathways, with summed abundance numbers for each sample.

Multivariate analysis of community composition and identifying links with soil physico-chemical analysis was conducted using PRIMER-E (Primer 7, Clarke and Gorley 2006) and STAMP software (Parks et al. 2014). Differences in the relative abundances of bacteria and fungi and diversity measures for 356 different zones, soil depths and mulching treatments were compared by ANOVA analysis 357 using Genstat (v18.1.0, VSN International Ltd.).

Table 8. Details of vineyard sites and microbiome analyses conducted using soils from different rotundone zones

Sampling time	Location	Data collection		Output
2017	Old Block	Microbial genetic diversity		
	Low/Medium/High	Amplicon sequencing	16S Bacteria ITS Fungi	Gupta et al. (2019) Vineyard soil microbiome composition related to rotundone 2 concentration in Australian cool climate 'peppery' Shiraz grapes
		Gene abundance	Bacteria Fungi	
		Microbial catabolic diversity		
		Physico chemical analysis		
		Functional profiling	Metagenomic sequencing	Data from metagenomic sequencing showed significant differences between Low and High rotundone zone soils in multiple functional categories.
2018	Old Block	Microbial genetic diversity		Genetic and functional Microbiome composition of vineyards soils.
	Low/High	Amplicon sequencing	16S Bacteria ITS Fungi	
		Gene abundance	Bacteria Fungi	(i) 2017 and 2018 amplicon sequencing data for Old Block (ii) 2018 amplicon sequencing data for the soils from The Lane vineyard. Combined analysis of 2018 data for both vineyards. Functional annotation of 16S sequence (prokaryotic taxa) to known metabolic and ecologically relevant functions, using FAPROTAX database.
		Physico chemical analysis		
2018	The Lane	Microbial genetic diversity		
	Low/High	Amplicon sequencing	16S Bacteria ITS Fungi	
		Gene abundance	Bacteria Fungi	
		Physico chemical analysis		

6.5 Model experiments with vines

Microvines

Three microvines were twice subjected to lowered overnight temperatures of 4°C, whilst the other three remained at room temperature. The grapes were harvested and frozen prior to rotundone analysis.

Potted vines

A total of 210 Shiraz cuttings consisting of low, medium and high cuttings from Mount Langi Ghiran 'Old Block' and four control Shiraz clones were prepared using the Mullins method (Baby et al. 2014, Mullins and Rajasekaran 1981) and maintained at the Plant Accelerator at a daytime temperature of 25°C and night-time temperature of 10°C. Only nine bunches of grapes formed across all potted vines. Those originating from the same type of cutting were combined for rotundone analysis. The vines suffered from salt toxicity originating from the potting soil used by the service provider and as such, only 135 of the initial 210 vines survived. Another set of canes was collected in 2017 and maintained in the same manner. The surviving cuttings from 2016 were repotted in new soil and all vines were maintained in the SARDI shadehouse until the 2019 growing season when they were transported back to the Plant Accelerator where the target temperatures were set to 20°C during the day and 10°C at night-time, though the set temperatures were repeatedly exceeded. The vines suffered from multiple scale and ant infestations, resulting in a high level of powdery mildew. The bunches of grapes that did form were very small with berries not uniform in size or colour. A total of 11 bunches could be harvested and analysed for rotundone. After the 2019 experiments, the vines were maintained in the SARDI shadehouse and were discarded after very little fruit set was observed once again.

6.6 Aroma compound mapping

Non-targeted analysis of volatile metabolites in cool-climate Shiraz grapes

GC-MS scan data of volatile metabolites were acquired for the 2017 and 2018 grape samples from The Lane's 'Junction Road' vineyard in the Adelaide Hills. On 18 April 2017 and 22 March 2018 three bunches of grapes from 140 georeferenced vines were picked, placed in plastic zip lock bags and stored at -20°C until analysis. From each grapevine 150 g of berries were homogenised with a stick blender. For each homogenate, three replicate 5 g subsamples were placed in a 20 mL screw-cap SPME-GC-MS vial with 2.5 ng of d6-α-guaiene standard added. The vials were then capped and placed on a shaking platform for 24 hours. A 2 mL addition of saturated brine solution was made, and the samples were directly analysed by HS-SPME-GC-MS. A 'master mix' was also included which was prepared from mixing equivalents of all the homogenised grape samples. The samples were analysed by EI (70 eV) GC-MS on a 60 m DB-5MS column based on the method of Zhang et al. (2016) with some modifications, including alternating the acquisition mode between scan (*mz* 35 to *mz* 280) and SIM (ions *mz* 105, 133, 147, 153, 161, 189, 195, 204, 209, 210). Non-targeted processing of the raw data files was carried out using the R workflow package MStractor (GitHub 2020). After retention time alignment and feature extraction, signal intensity was normalised using the internal standard d6-α-guaiene and the correlation coefficient between the molecular features of interest and the concentration of (-)-rotundone and α-guaiene was determined. Prior to mapping, signals were subjected to mean normalisation using the 'master mix' as reference.

An additional nine volatile compounds (hexanal, hexenal, hexanol, ethyl hexanoate, phenylacetaldehyde, diethylhexane, nonanal, β-cyclocitral and theaspirane) were tentatively identified based on their relative retention times and mass spectra. Three grape samples from the 2017 vintage, which showed high concentrations of rotundone and α-guaiene, were selected to confirm the identification of hexanol and phenylacetaldehyde by standard addition. The extra samples were prepared as above and analysed using SPME-GC-MS. Immediately after the first GC-MS analysis, reference standards for phenylacetaldehyde and hexanol were then added to each of the vials and the samples were reanalysed by HS-SPME-GC-MS, demonstrating co-elution and symmetrical peak enhancement, thus confirming identification of phenylacetaldehyde and hexanol. For mapping, signals for phenylacetaldehyde and hexanol were normalised based on mean normalisation using the 'master mix' as reference.

Mapping and spatial analysis

All mapping and spatial analysis in this work employed the methods described by Bramley et al. (2017). In brief, maps of sesquiterpenes and other attributes derived from vine sampling were interpolated onto 2 m rasters using global point kriging in VESPER (Minasny et al. 2005) and exponential variograms. In many instances, a log transformation was necessary due to skewness in the data, followed by the back transformation of kriged values of Webster and Oliver (2007). Clustering of map layers was conducted using JMP (v.14.0.0, SAS Institute Inc., Cary, NC, USA) and map display performed using the ArcGIS software suite (v10.4.1; ESRI, Redlands, CA, USA).

Remotely sensed imagery of vine vigour (plant cell density, PCD) was supplied at 40 cm resolution with the non-vine signal removed and was re-sampled/smoothed to the same 2 m raster as for other map layers using local averaging (3 pixels x 3 pixels).

6.7 Formation of aroma compounds from sesquiterpenes

Sesquiterpenes in grapes and wine

A headspace-solid phase microextraction-GC-MS method to quantify sesquiterpene hydrocarbons in wine was developed, based on Cincotta et al. (2015) except using a different phase GC column (DB-5MS), altered oven parameters and a deuterated α-guaiene analogue as the internal standard. A limited number of sesquiterpene references were available commercially. Therefore, (+)-calarene, α-guaiene, α-copaene, valencene and β-caryophyllene were used as representative calibrant compounds. Fourteen commercial Shiraz wines from warm and cool climates across Australia (vintages 2013 – 2018) were purchased and used to determine if any sesquiterpenes identified from the untargeted metabolite analysis (6.6) were present, and if so, they were subsequently quantified. Rotundone was also measured in the wines. Subsequently, a reference standard of α-muurolene (donated) was used to confirm the presence of α-muurolene in Shiraz wine with multidimensional GC-MS analysis (based on the rotundone analytical method).

To determine the extent of transfer of sesquiterpenes from grape solids to wine, a model study was undertaken. Shiraz grape berries were homogenised, a sesquiterpene mix was added (α-copaene, β-caryophyllene, α-guaiene, valencene and α-muurolene) and extracted in parallel with untreated control homogenates. Each sample was centrifuged, and the solids retained. Model wine was added to each of the solids, shaken (24 hr) and centrifuged. The supernatant from the unspiked homogenate was filtered through binder-free glass fibre. From this, six samples were prepared, three of which were spiked with the sesquiterpene mix at an equivalent level and three were left as blank controls. From the supernatant of the original spiked homogenate, three samples were prepared directly and three were filtered (binder-free glass fibre). Internal standard was added to all samples and they were analysed by GC-MS as per the method for sesquiterpenes in wine.

Model oxidation of α-guaiene and α-ylangene

Enriched extracts containing grape berry sesquiterpenes α-ylangene, germacrene D and α-muurolene were prepared from ylang-ylang essential oils. α-Guaiene had been synthesised in-house previously. In a model system, the grape berry sesquiterpenes were applied individually to cellulose filter paper, to mimic the biological structure of the grape berry skin, and then exposed to light sources. Initial experiments used visible light and full sunlight for α-guaiene and then daylight was used for all four sesquiterpenes. The sesquiterpenes and their major oxidation products were monitored by GC-MS for up to four weeks, along with other by-products.

Identification of iso-mustakone in wine

Extracts were prepared using solid phase extraction (SPE) from two cool-climate Shiraz wines (Adelaide Hills 2017 and Canberra District 2017). The presence of the unknown α-ylangene oxidation product was confirmed in both the Shiraz wine extracts by multidimensional GC-MS and by co-injection with iso-mustakone obtained from the aerial oxidation sample α-ylangene. Also, a reference sample of α-copaene was subjected to oxidation using the model system to produce mustakone and a reference sample of mustakone (donated) was used for comparison by multidimensional GC-MS. Subsequently, the Adelaide Hills 2017 Shiraz wine, which contained similar amounts of rotundone and the unknown sesquiterpenoid, was used to prepare a highly enriched and purified extract using SPE followed by semi-preparative HPLC. One HPLC fraction (fraction 49) contained the unknown sesquiterpenoid and was used for multidimensional GC-MS-O which confirmed presence of iso-mustakone.

7. Results and discussion

7.1 Model experiments conducted based on reciprocal bud grafts and potted vines in greenhouses

7.1.1 Reciprocal bud grafts in Shiraz

Reciprocal bud grafts of cuttings taken from the low rotundone zone and grafted onto vines in the high rotundone zone, and vice versa, were completed at Mt Langi Ghiran's 'Old Block' in December 2016. In 2018, 20 grape samples could be obtained from 60 grafted canes, 30 grape samples were obtained in 2019 from low-and high-rotundone grafts and 27 grape samples were collected in 2020. GC-MS analysis of grapes from grafted vines showed small but relevant differences in rotundone concentrations in 2018 and 2019 (Figure 1). In 2020 no differences in α-guaiene were observed in grapes from the pre-assigned low and high rotundone zones and the rotundone results were equivocal.

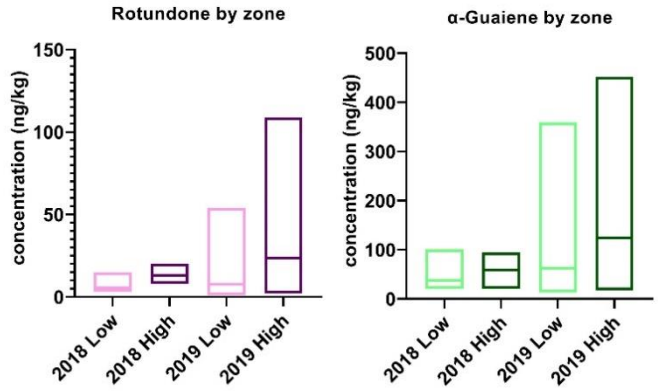


Figure 1. Concentrations of α-guaiene and rotundone in grapes from canes grafted on vines in 'low' and 'high' rotundone zones at 'Old Block'

On closer inspection, analysis of data from the 2018 and 2019 vintage samples demonstrated a significant location effect (Figure 2). The location of the rootstock in the vineyard was key to achieving higher rotundone concentrations, while the scion's phenotype was not significant. This points to a site-specific environmental trigger that is key to sesquiterpene and aroma formation in grapes.

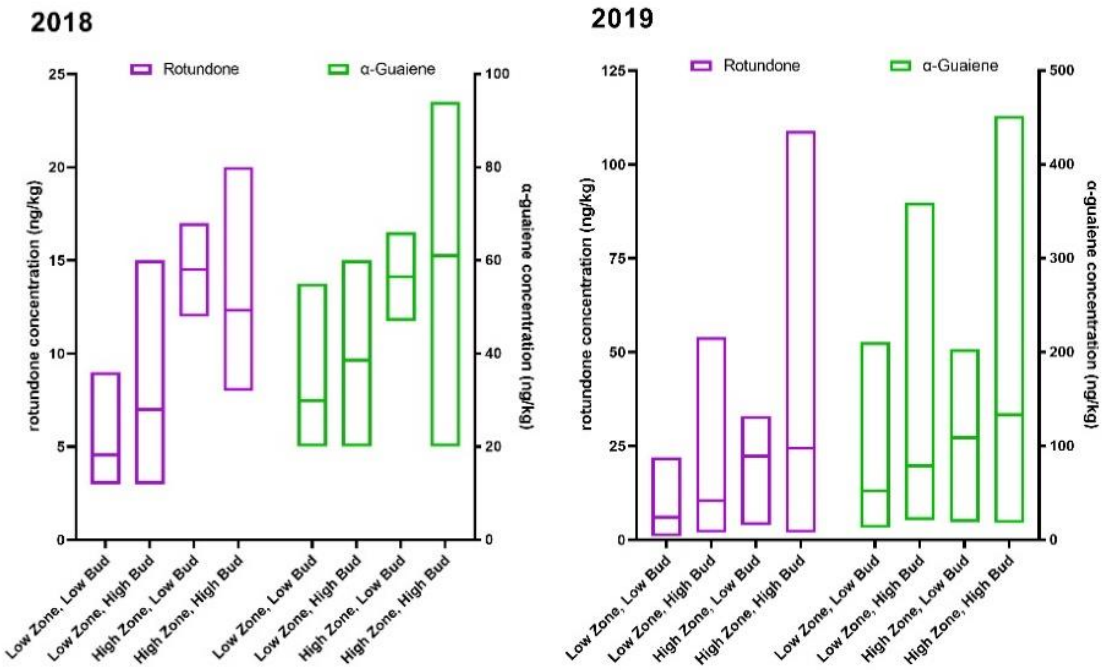


Figure 2. Cane effects on concentrations of α-guaiene and rotundone in grapes from 'low' and 'high' scions grafted onto vines in 'low' and 'high' rotundone zones at Old Block

No attempts were made to characterise α-guaiene biosynthesis at the genome and transcriptome level, as the data from the reciprocal grafts (and the rotundone maps described in 7.2 for the Adelaide Hills vineyard) pointed towards a site-specific rather than a genetic determinant. In addition, quantitative measures of α-guaiene and rotundone were better suited to monitor kinetics in field experiments, given the large and unpredictable seasonable variability of rotundone formation and demanding protocols for grape sampling for DNA and RNA extraction.

7.1.2 Potted Shiraz vines

Initially, 210 potted vines had been established from Shiraz cuttings and were maintained in shadehouses, yielding just nine bunches of grapes in the first year. No grapes could be sourced from potted vines in 2018 and sampling in 2019 yielded only 11 grape samples from approximately 170 potted vines grown in the Plant Accelerator.

While concentrations were low, the analysis of grapes from the 2016/17 growing season confirmed that the grapes from potted vines could contain rotundone, thereby warranting further studies. The vines in the 2018/2019 growing season were severely infected with scale, ants and fungi and once again produced very little fruit. However, the grapes that did form contained rotundone levels approximately 10-fold higher than ever observed in Australia (Figure 3), indicating that vine stressors may trigger rotundone biosynthesis. While the air conditioning system in the greenhouse was set to maintain temperatures chosen to replicate a cool climate vineyard (20°C daytime and 10°C at night-time), the set temperatures were repeatedly exceeded, so growing temperature did not play an obvious role in these experiments. The grape rotundone concentrations from potted vines also appeared independent of the cutting's origin, indicating no apparent genetic effect on rotundone accumulation.

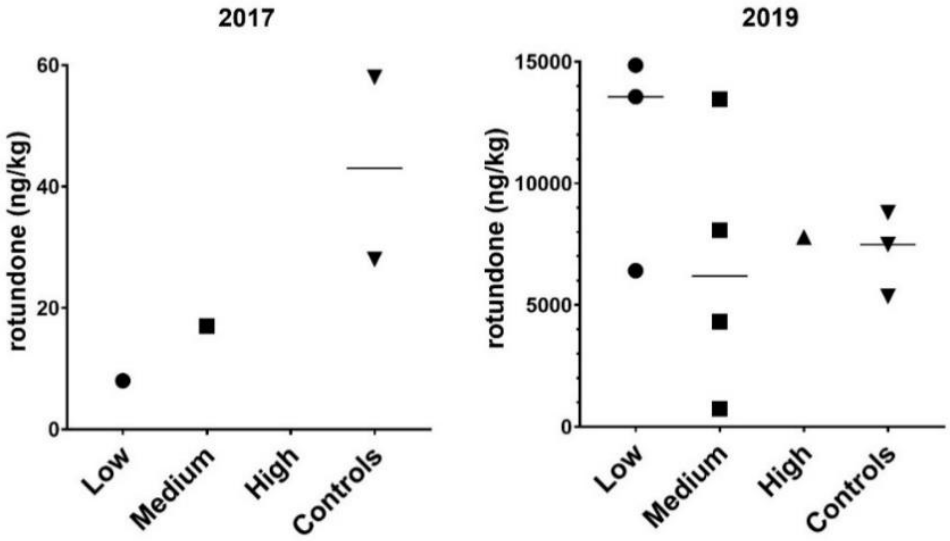


Figure 3. Grape rotundone concentrations in grapes produced in a greenhouse by potted Shiraz vines grown from canes sourced from the 'low', 'medium' and 'high' rotundone zones at 'Old Block' and 'control' canes from a different site of unknown rotundone phenotype

7.1.3 Potted Pinot Meunier microvines

In vineyards, rotundone formation typically occurs late in the growing season in cool-climate regions, indicating a potential temperature impact that might stimulate sesquiterpene biosynthesis in grapes. To test this, CSIRO's microvines were subjected to lowered temperatures of 4°C overnight for two nights while the control microvines were maintained at a constant temperature of approximately 20°C.

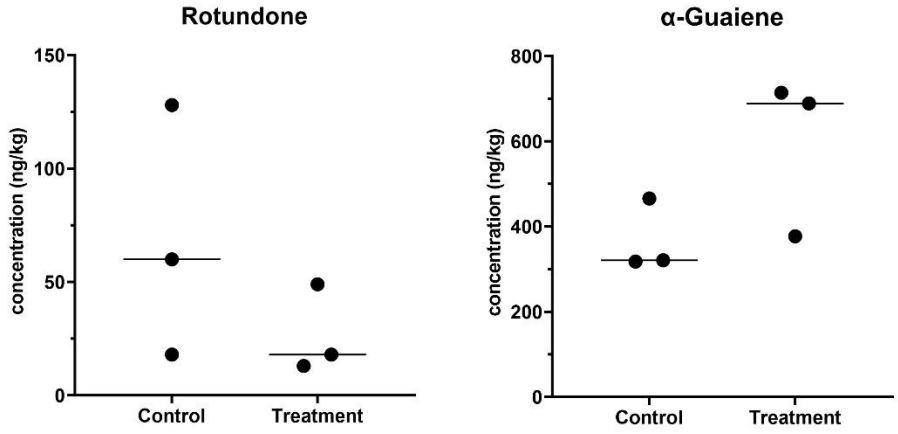


Figure 4. Impact of overnight cold treatment on sesquiterpene grapes from Pinot Meunier microvines

While no significant treatment effects could be observed (Figure 4), this experiment did establish the presence in grapes of sesquiterpenes at reasonably high concentrations and demonstrated the potential of Pinot Meunier microvines as a practical resource for further studies into the biosynthesis of sesquiterpenes and rotundone.

7.2 Aroma compound mapping

7.2.1 Rotundone and its precursor α-guaiene

Variation in berry rotundone concentration at 'Junction Road' vineyard showed marked spatial structure in both 2017 and 2018. But in marked contrast to the previous results from another site in the Grampians (Bramley et al. 2017), there was inconsistency in the patterns of variation between the years. As discussed in 7.2.2, analysis of non-targeted metabolomics data demonstrated also that the profiles of other volatile grape compounds varied between the two vintages. No specific herbivore damage (other than random loss of grapes) was observed on the trial sites and the potential influence of herbivore damage on rotundone formation was not studied further.

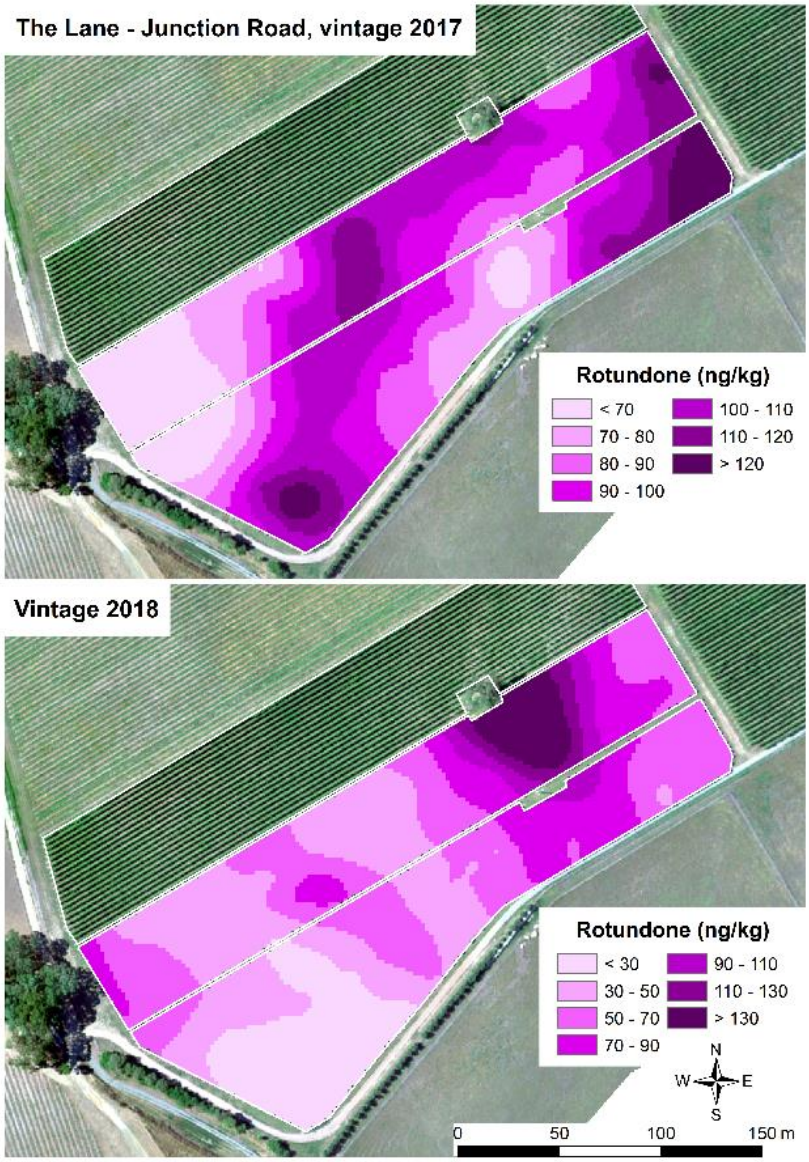


Figure 5. Vineyard-scale maps of rotundone in grape samples from 'The Lane' vineyard in 2017 and 2018

Notably, harvest dates were markedly different between 2017 (18 April 2017) and 2018 (22 March 2018), with 2018 unusually early for a high rotundone year. In addition, comparison of the PCD imagery across the three years of the study did indeed suggest inter-annual inconsistency in patterns of variation in vine vigour that was greater than has typically been seen in other multi-year studies of vineyard variability (e.g. Bramley et al. 2011, 2019). However, both bunch mass and berry mass were notably greater in 2017 than in 2018 and especially 2019 when yields were very low following a very cold flowering period. Of interest here is the fact that this vineyard is cane pruned in contrast to the vast majority of vineyards in previous variability studies which are spur pruned. In this context Bramley et al. (2019) have recently suggested that in cane-pruned vineyards, patterns of variation in vine vigour tend to be stable in time, while patterns of variation in yield are not. Consistent with this are the very different patterns of variation between years in bunch mass, although berry mass does show a stable pattern of variation. The impact of these factors on rotundone formation are unclear. While Trought and Bramley (2011) were able to use the stability in patterns of vine vigour (Bramley et al. 2019) as a predictor of juice quality, when assessed on the basis of Brix, pH and TA, both the present results and those obtained previously in 'Old Block' (Scarlett et al. 2014, Bramley et al. 2017) do not provide evidence of a link between vine vigour and rotundone formation.

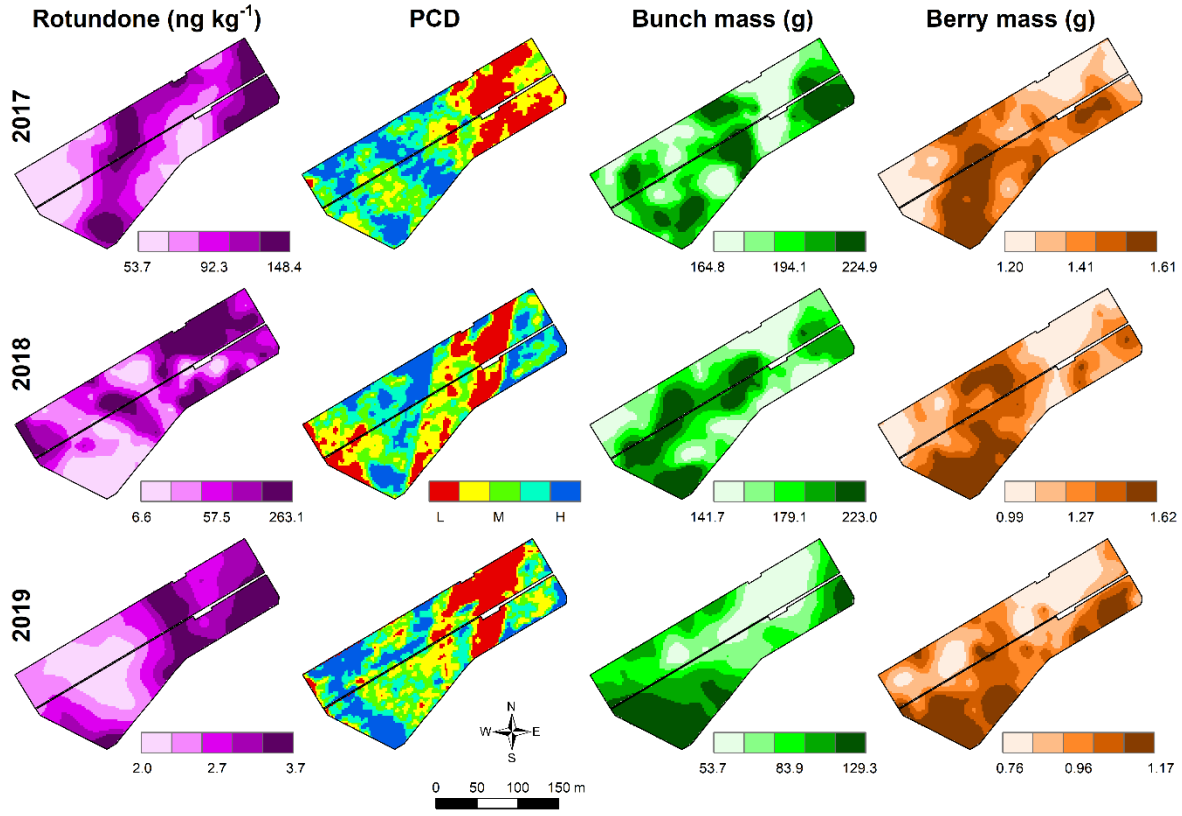


Figure 6. Grape rotundone concentration, bunch mass and berry mass immediately prior to commercial harvest of 141 georeferenced grape samples 'mapped' over the 3.21-ha 'Junction Road' vineyard, and PCD imaging of the vineyard at veraison in 2017 to 2019

Another factor which might explain patterns of variation in rotundone concentration at The Lane, and thus their temporal instability, is the very small proportion of the Adelaide Hills vineyard area that is orientated away from north, which was identified as a key driver of rotundone variation in the 'Old Block' vineyard (Scarlett et al. 2014, Bramley et al. 2019). While the southernmost area of the block

which is orientated away from north was an area of high rotundone in 2017, as might have been expected, it was an area of low rotundone in 2018.

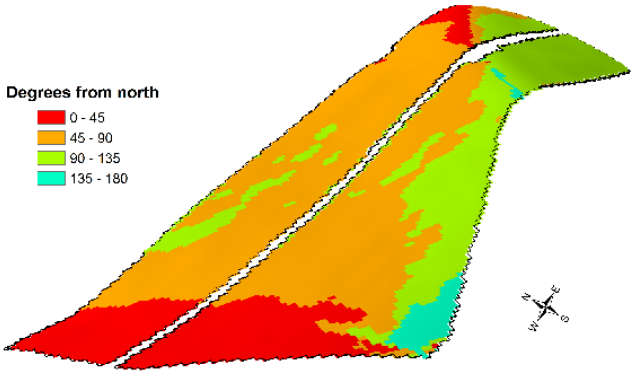


Figure 7. Relative orientation of vines at the 'Junction Road' vineyard

At the 'Junction Road' vineyard, there was no obvious interaction between rotundone and soil variation as assessed by electromagnetic sensing (EM38; data not shown). This was also the case at 'Sheoak', albeit with rotundone levels at 'Sheoak' being too low and with too narrow a range of variation to have confidence in the maps (not shown). Additional cool-climate Shiraz blocks at 'Woodbury' and 'See Saw' vineyards were sampled although it was not possible to investigate site effects given the very low rotundone concentrations observed.

Notwithstanding the significant inter-annual differences in patterns of rotundone variation at The Lane, in both 2017 and 2018, the patterns of rotundone variation closely matched those of variation in α-guaiene and are consistent with the role of α-guaiene as key precursor to rotundone. Berry size – and hence also likely surface area and berry skin area – did not have an obvious effect on grape α-guaiene and rotundone concentrations or the differences between spatial variability in the 2017 and 2018. As shown in Figures 6 and 8, in all years the berry mass maps were relatively similar and smallest berries and bunches were always observed in the north-east corner of the 'Junction Road' blocks, while grape α-guaiene and rotundone concentrations were only at their highest in this particular area in the 2018 vintage.

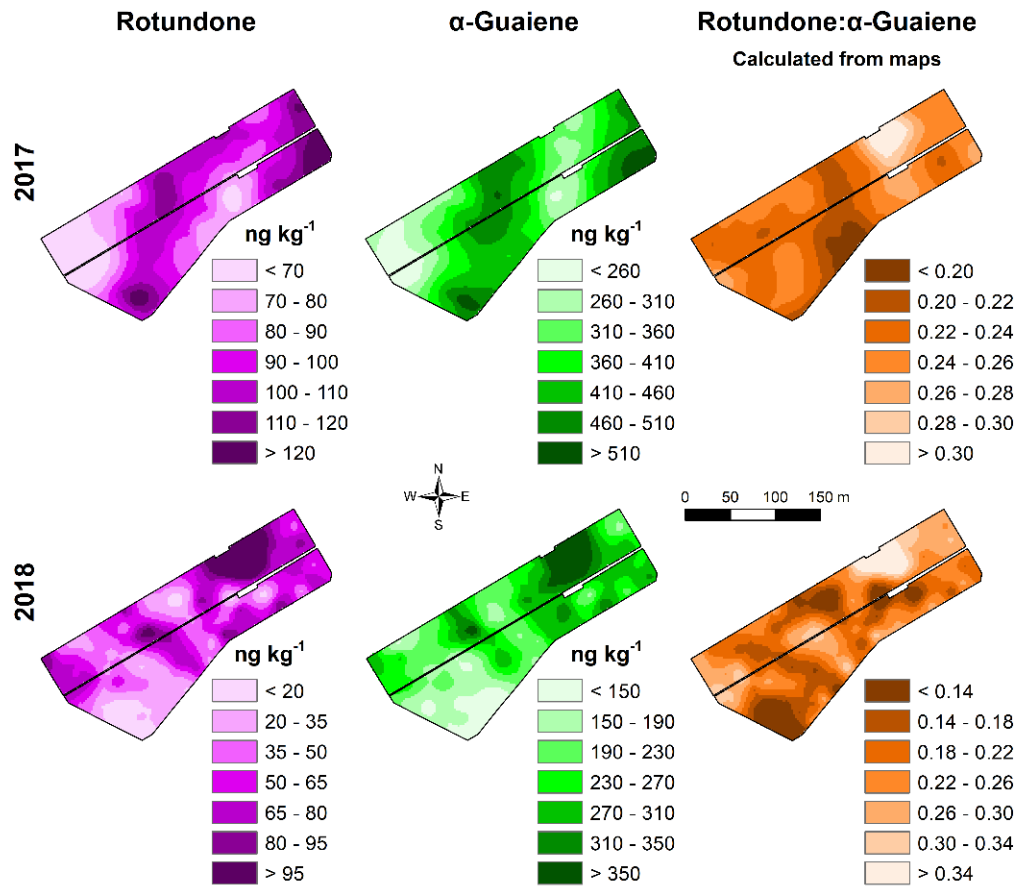


Figure 8. Grape rotundone and α-guaiene concentrations immediately prior to commercial harvest of 141 georeferenced grape samples 'mapped' over the 3.21 ha 'Junction Road' vineyard, in 2017 and 2018

In summary, across the 'Junction Road' vineyard rotundone and its precursor α-guaiene were closely correlated in a given vintage at harvest. Berry size effects or differences in the yield of rotundone formed from its immediate α-guaiene precursor did not explain the variability within this vineyard or differences between spatial patterns for the 2017 and 2018 vintages. This demonstrates for the first time that it is the concentration of α-guaiene that is a key determinant of final grape rotundone concentrations (as opposed to the conversion of α-guaiene to rotundone). In addition, no clone effect was observed for rotundone and its precursor α-guaiene. That is, the absolute grape concentration and spatial patterns of rotundone and other sesquiterpenes varied between vintages, but were independent of the two Shiraz clones present in this vineyard. Together with the results from grape analysis for the reciprocal grapevine graft experiment at Mt Langi Ghiran's 'Old Block' described in 7.1.1, this establishes that site characteristics are more important than genetic determinants of planted vines, as similar effects were observed in Shiraz vineyards planted with clonal selections, mass-selected vines, and in vines grafted with low-and high rotundone canes.

7.2.2 Grape sesquiterpenes and other volatile metabolites

In order to explore whether additional sesquiterpenes and other volatile grape metabolites beyond rotundone and α-guaiene are affected in a similar way, grape samples from the 141 georeferenced vines at The Lane vineyard were subjected to non-targeted metabolite analysis using headspace GC-MS. Bioinformatics data analysis of full mass spectrometry scan data resulted in the identification of 264 molecular features (i.e. putative metabolites) for the 2017 vintage and 203 features for the 2018 vintage. Exploratory data analysis using principal component analysis (PCA) demonstrated that the profiles of volatile grape compounds varied between the two vintages, yet no clone-related effects were obvious. Among those 200+ molecular features, fourteen sesquiterpene hydrocarbons and a number of other major volatiles could be identified tentatively based on a comparison of their mass spectra and retention times with reference data. Concentrations of the additionally identified sesquiterpenes were closely correlated with α-guaiene or rotundone (correlation coefficients ranging from 0.59 for α-copaene to 0.89 for α-ylangene). Five sesquiterpenes, α-ylangene, α-copaene, guaia-6,9-diene, γ-cadinene and δ-cadinene, which are endpoints in metabolic pathways via germacrene-B, -C, and -D were selected for mapping (Figure 9). Amongst the other major volatiles, only hexanol and phenylacetaldehyde showed distinct spatial structure (Figure 9), albeit this was different from that of the sesquiterpenes.

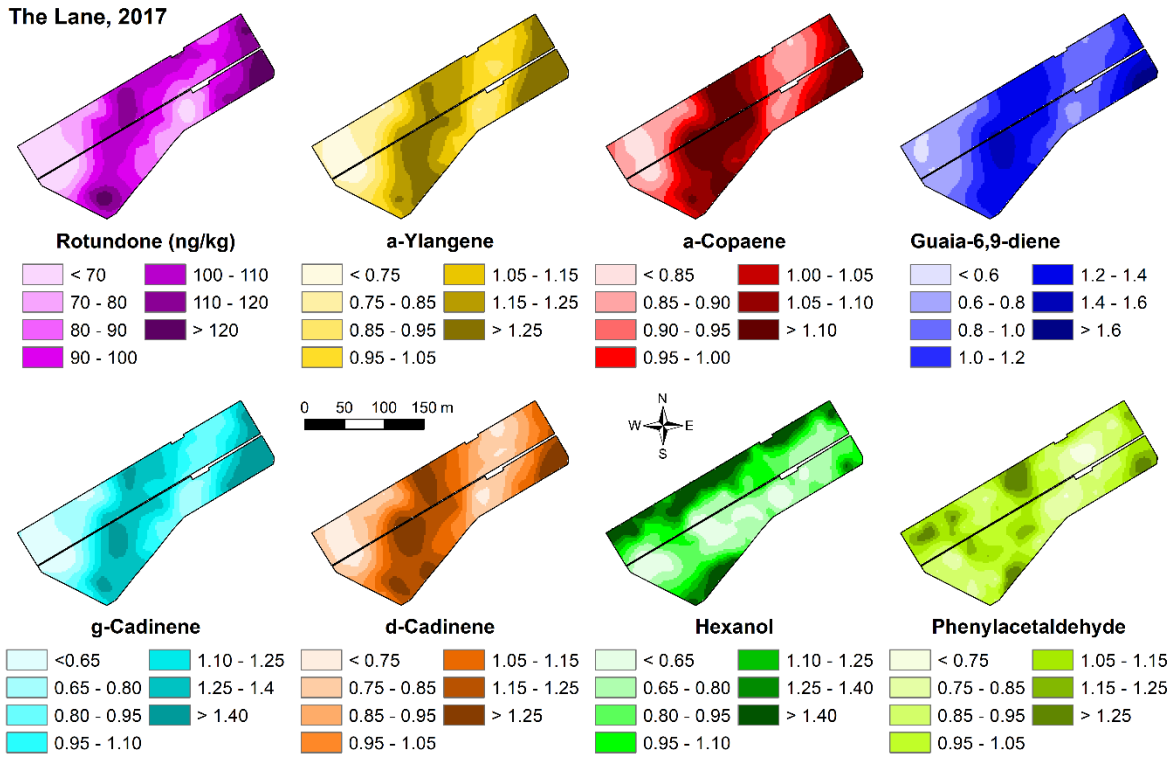


Figure 9. Vineyard-scale maps of key sesquiterpenes and other volatile metabolites in 140 georeferenced grape samples from 'The Lane' vineyard 2017; concentration of rotundone in ng/kg by isotope dilution 2D-GC-MS, relative concentrations of other sesquiterpenes, hexanol and phenylacetaldehyde obtained by non-targeted metabolomics and mean normalisation of GC-MS scan data

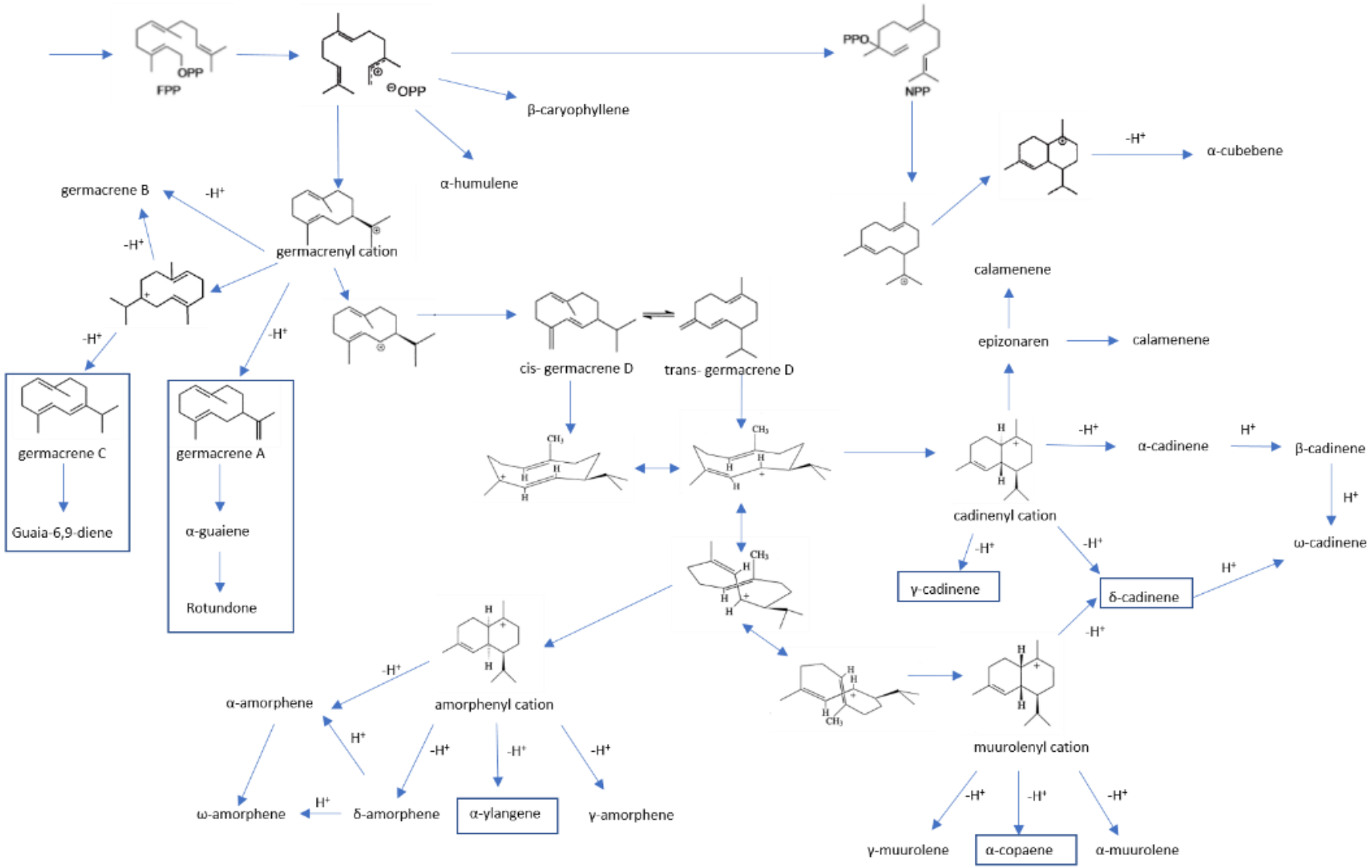


Figure 10. Biosynthesis of sesquiterpenes from farnesyl pyrophosphate (FPP), and key metabolites mapped in this study

In summary, these non-targeted metabolomics experiments demonstrated that out of more than 200 volatile grape metabolites detected by GC-MS only products of the germacrene A – D pathways (Figure 10), and α-humulene were affected in a spatially distinct manner similar to rotundone and α-guaiene. In other words, control of rotundone formation involved a yet to be identified environmental event which triggered formation of germacrenes from the universal precursor farnesyl pyrophosphate (FPP), upstream from the formation of α-guaiene (Figure 10). When compared to the sesquiterpene maps, the different spatial profiles observed for hexanol (in 2017 and 2018) and phenylacetaldehyde (in 2017) reveal that further metabolites amongst the 200+ grape volatiles may also show a degree of spatial structure across a vineyard, which can be distinctively different to the sesquiterpene profiles.

7.3 Site characteristics, including microbial populations

7.3.1 Microbial populations

Soil habitat characteristics are known to modulate the diversity of the soil microbiome and the dynamics of plant-microbe interactions which play an important role in plant growth, abiotic and biotic stress tolerance, nutrition, productivity and product quality. The vineyard microbiome has been suggested to play specific roles in the productivity and disease resistance of the host plant. In order to characterise microbiome composition in surface soils, samples from vineyard zones demonstrated to vary in rotundone concentration were analysed.

7.3.1.1 Microbial communities in soils from rotundone zones in Old Block vineyard – 2017 study

Results from the 16S rRNA and ITS region-amplicon sequencing analysis of microbial communities in the surface soils collected from rotundone-based zones indicated marked differences between zones in the genetic diversity and composition of the soil bacterial and fungal microbiome. Soils in the high-rotundone zone exhibited higher diversity of bacteria, but lower diversity of fungi, compared to the soils in the low-rotundone zone. In addition, the network analysis of the microbial community in the high-rotundone zone soils appeared well structured, especially with respect to the bacterial community, compared to that in the low-rotundone zone soils. The key differences in the microbial community structure between the rotundone zones are obvious for taxa/groups of both bacteria and fungi, particularly for bacteria belonging to Acidobacteria-GP4 and GP7, Rhizobiales, Gaiellaceae, Alphaproteobacteria and the Nectriaceae and Tremellaceae families of fungi (Figure 11).

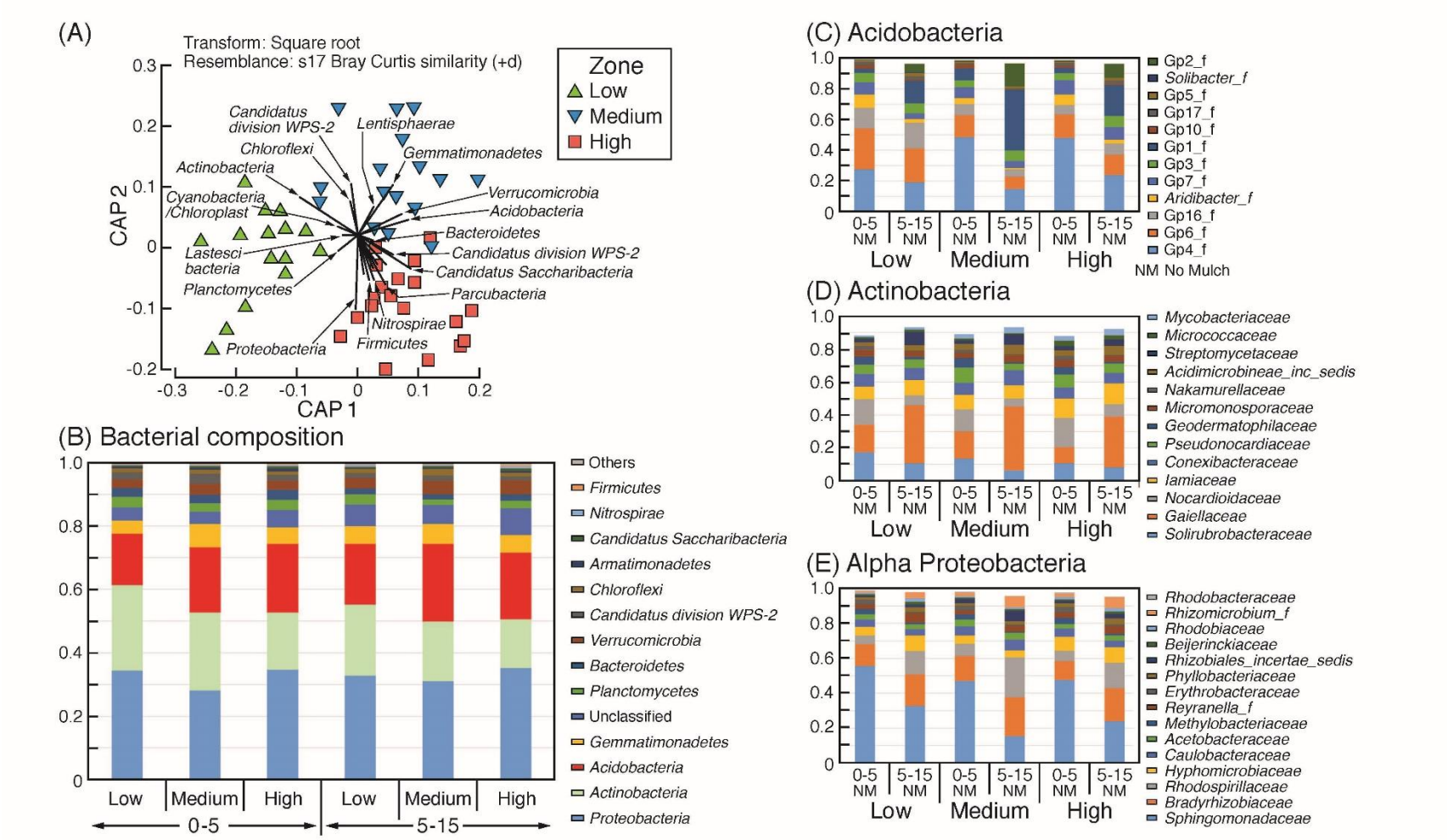


Figure 11. Composition of bacterial communities in the surface soils in the different rotundone-based soil zones. (A) Canonical analysis of principle (CAP) ordination, constrained by zone; relative abundances (B) at phylum level, (C) at family level within selected phyla showing significant variation between zones (Gupta et al. 2019)

Soil and management factors such as ground cover management, organic manure, herbicide and fungicide application and cultivation have been shown to significantly influence bacterial and fungal communities in vineyard soils. Although mulching in some parts of the vineyard caused changes in bacterial and fungal composition and overall microbial catabolic diversity and activity, its effects did not mask the rotundone zone-based variation. Results from the soil microbial catabolic diversity analysis as assessed for the 'Old Block' soils in 2017 showed significant differences between the rotundone zones, in particular in the 0-5 cm depth samples. Mulching generally increased microbial activity responses to C substrate addition in both the zones, but the grouping remained similar between the zones and depths (Figure 12). These results suggest that either the mulching-induced changes in microbial community structure had no direct link to the microbiome variation between the different rotundone zones or that the effect of mulching was yet to be established, although given that mulch was applied 7-8 months prior to soil sampling during the wetter part of the year, this seems unlikely.

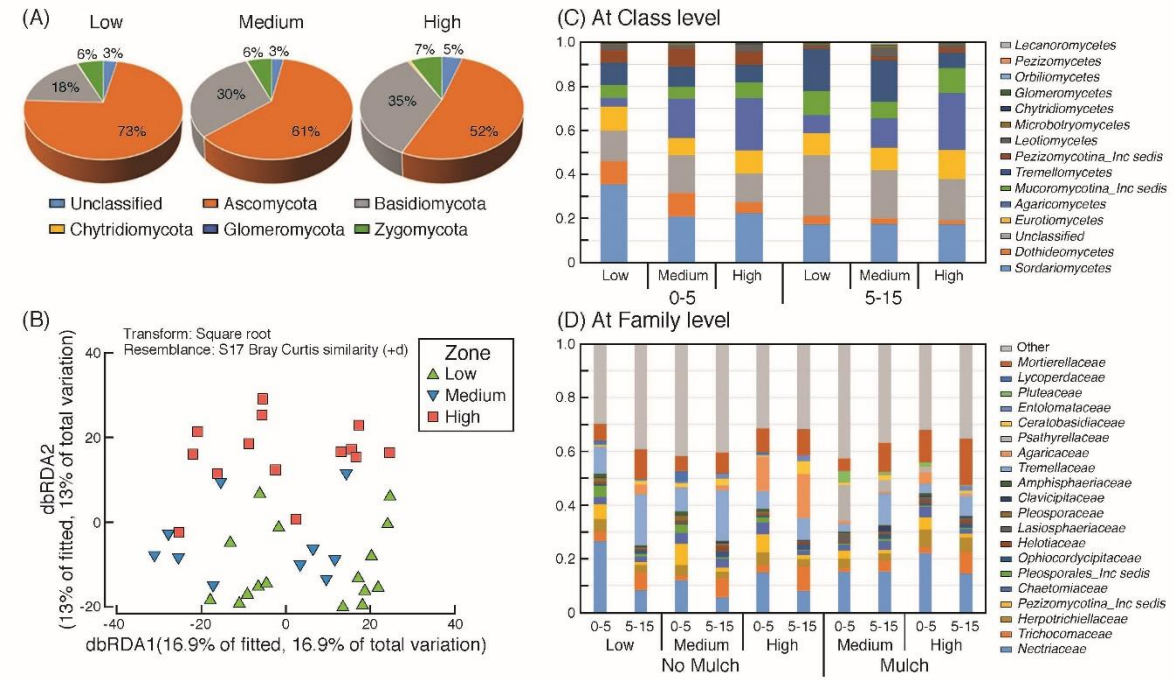


Figure 12. Composition of fungal communities in the surface soils in the different rotundone-based soil zones. (A) at phylum level, (B) distance based redundancy analysis (dbRDA); relative abundances (C) at class level and (D) at family level showing variation between zones and depths (Gupta et al. 2019).

7.3.1.2 Microbial communities in soils from rotundone zones in Old Block – 2018 study

In surface soils collected during the 2018 growing season from the low- and high-rotundone zones microbiological properties (bacteria and fungal communities) were observed that were similar to 2017 samples. Comparison of bacterial and fungal community composition from diversity analysis of data from both seasons using the Bray–Curtis distance metric showed significant dissimilarity between the two rotundone zones (Figure 13). ANOSIM and PERMANOVA analysis results revealed that zone-based variation was the primary factor separating the microbiome composition in the two years and season-based variation was a secondary factor. Also, rotundone zone-based variation in the microbial community composition was similar among samples collected at 0-5 and 5-15 cm depths.

Diversity of fungal communities as indicated by diversity indices was significantly higher in the low-rotundone zone soils compared to that in the high-rotundone soils. For example, species richness values at the 'Old Block' vineyard were 151 and 115 for low- and high-rotundone zone soils and Shannon index values were 5.39 and 4.93 for the low and high rotundone zone soils, respectively. However, diversity indices from the combined analysis of 2017 plus 2018 data did not reflect the trends seen in the individual seasons (i.e. no significant difference in species richness or Shannon index values was observed that could be attributed to the seasonal variations commonly seen in bacterial communities in agricultural soils). Results from the combined analysis of two-year data confirmed previous observations that the key differences in the bacterial community structure between the rotundone zones were not related to any single genus or family of microbes, but related to differences in multiple taxa and/or groups of both bacteria and fungi, particularly bacteria belonging to Acidobacteria-GP4 and GP7, Rhizobiales, Xanthomonadales, Alphaproteobacteria (Figure 14) and the Nectriaceae and Tremellaceae families of fungi. These results demonstrate that a community-based involvement of soil microbes might play a role in a grapevine's sesquiterpene biosynthesis and potentially could affect rotundone concentration of grapes.

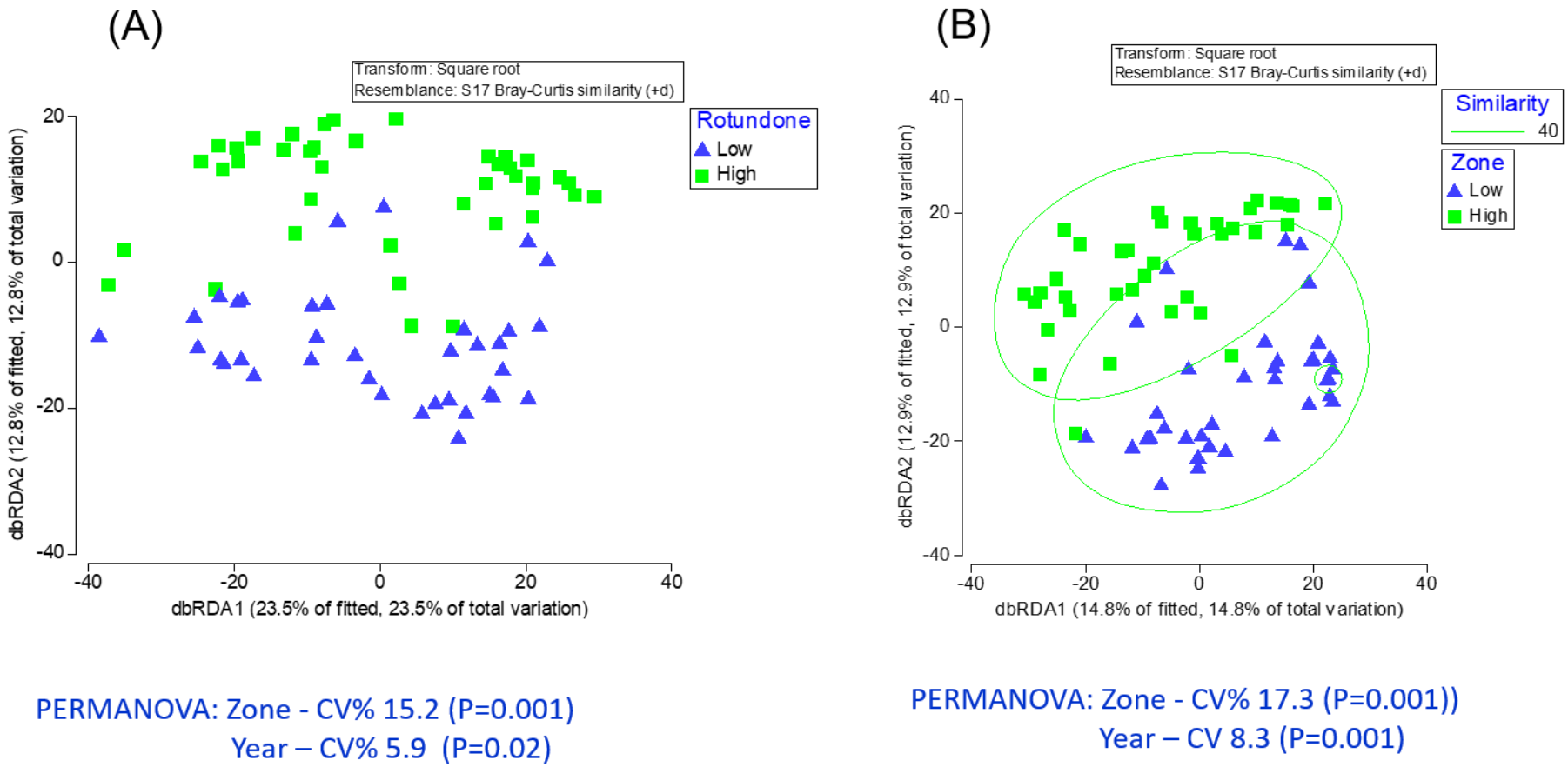


Figure 13. Genetic composition of soil bacterial and fungal communities in the surface soils collected during the 2017 and 2018 seasons. Graphs show results from the Canonical analysis of principle (CAP) ordination, constrained by zone.

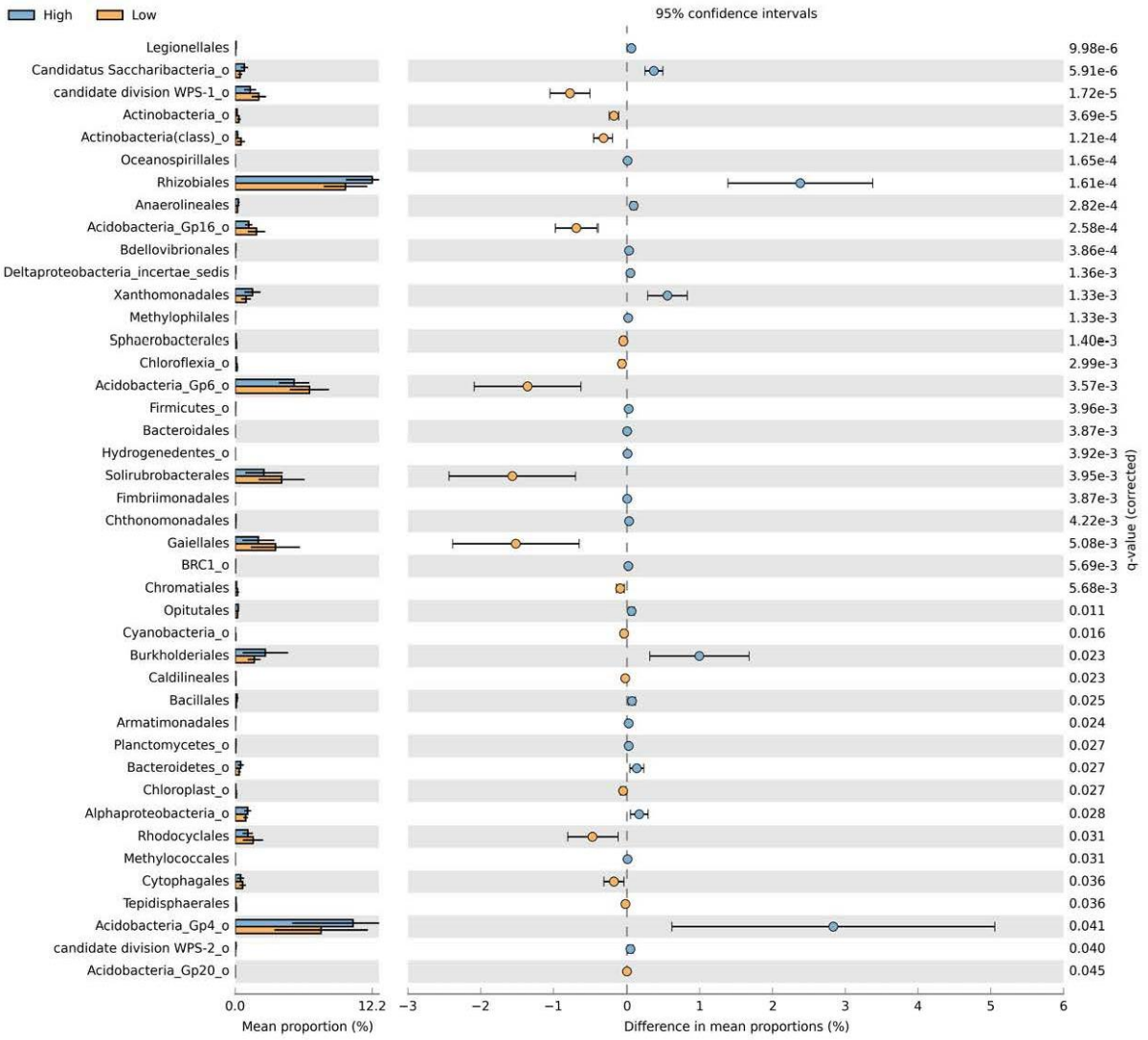


Figure 14. Key indicator bacterial groups significantly different between the low- and high-rotundone zones in the 'Old Block' vineyard, identified from the combined analysis of 2017 and 2018 data

7.3.1.3 Microbial communities in soils from rotundone zones at the 'Junction Road' vineyard

Results for the soil samples from the 'Junction Road' vineyard collected during 2018 indicated trends in bacterial and fungal community composition and diversity similar to those observed at the 'Old Block' vineyard. Briefly, the genetic composition of bacterial and fungal communities was distinctly different between soils from low- and high-rotundone zones and the differences were observed in both the 0-5 and 5-10 cm depth soils (Figure 15). While the diversity indices for bacterial community were higher in soils from high-rotundone zone soils compared to those in the low-rotundone zone soils, there was no significant difference in the diversity indices, species richness and Shannon index of fungal communities between the low- and high-zone soils (Figure 15).

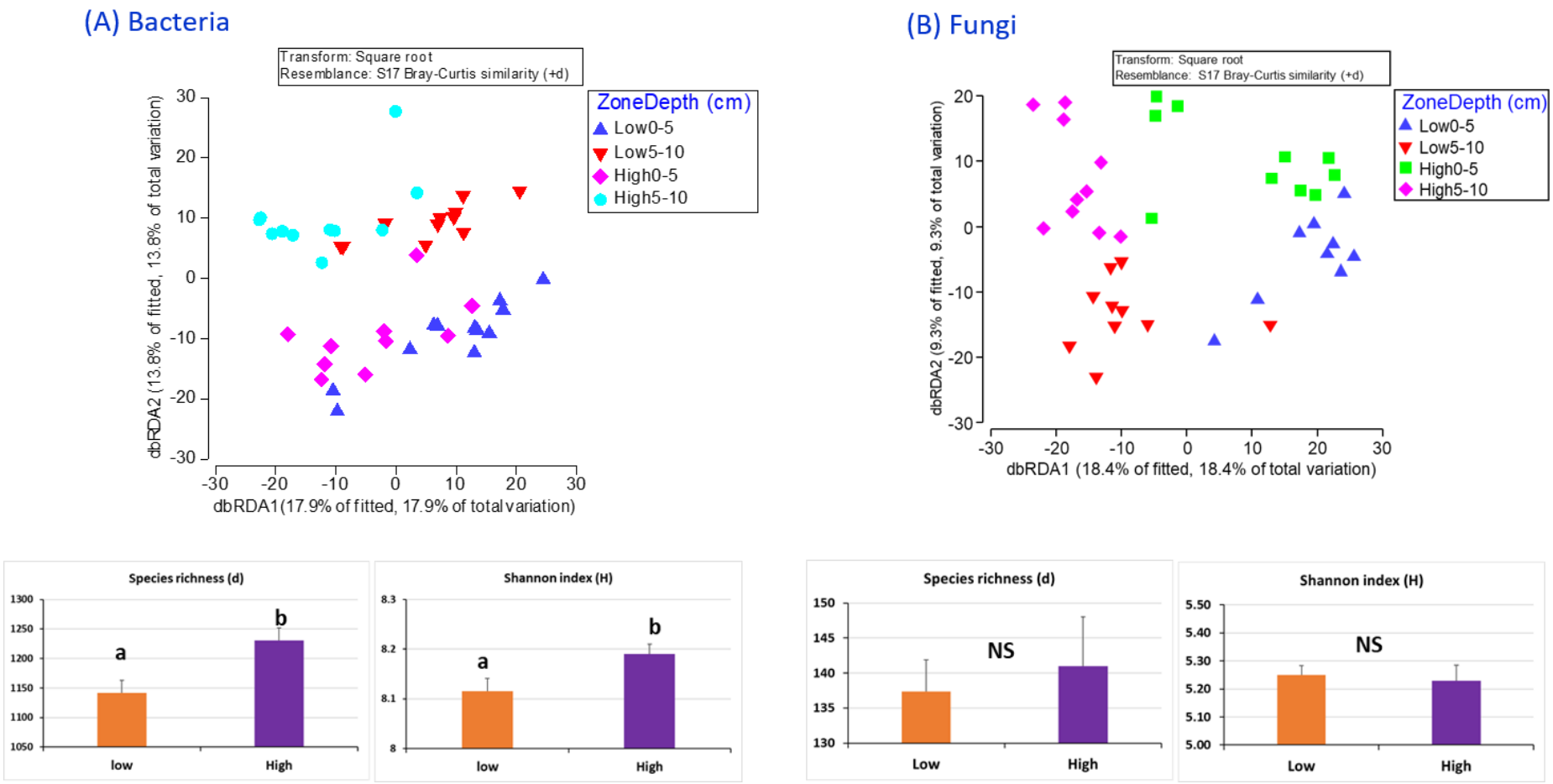


Figure 15. Genetic composition (top) and diversity indices (bottom) of bacterial (A) and fungal (B) communities in soil samples collected in 2018 in the different rotundone-based soil zones in the 'Junction Road' vineyard. Top graphs show results from the Canonical analysis of principle (CAP) ordination, constrained by zone. PERMANOVA – Bacteria: Zones CV = 11.2%, P=0.001; Depth CV=13.5 %, P=0.001. Fungi: Zones CV = 12.9%, P=0.001; Depth CV=18.8 %, P=0.001

In general, a significant variation was observed in the abundance and composition of soil bacteria and fungi in both vineyards, between seasons and between the rotundone zones. However, the differences were not consistent across the two vineyards and microbial groups. While bacterial groups belonging to the phylum Acidobacteria showed significant variation between rotundone zone samples, the total abundance of Acidobacteria did not show a clear and significant trend between rotundone zones.

7.3.1.4 Direct metagenomic and functional profiling analysis of soil microbial communities from 'Old Block' vineyard soils

Building on the findings from descriptive metagenomics of the soil bacterial and fungal communities, attempts were made to characterise the likely functional importance of specific members of microbial communities which varied between zones. It has been suggested that vineyard soil can act as a reservoir of bacteria during the vegetative period ready to colonise the above-ground endophyte communities and the external aerial part of the plants including berries (Zarraonaindia et al. 2015). For example, a diverse and responsive soil microbial community in the high-rotundone zones may provide the vines with opportunities to exploit beneficial factors such as nutrient availability or tolerance to biotic and abiotic stresses.

For metagenomic analysis, soil DNA from the surface (0-5 cm) samples from low- and high-rotundone zones collected in 2017 at the 'Old Block' vineyard was analysed using direct metagenomic sequencing to determine the genetic composition of microbial communities. Results for the taxonomic profiling of the metagenomic sequencing data showed distinct differences in the soil microbiomes between the low- and high-rotundone zone soils (PERMANOVA – Zones CV=9.2%; Depth CV-4.2%, P=0.005). Microbial diversity indices such as Shannon Index and Pilou's evenness were significantly higher for the high-rotundone zone soils compared to those for the low-rotundone zone soils, confirming the findings from the amplicon sequencing analysis described in 7.3.1.1 and 7.3.1.2. Results from the BioEnv and dstLM analysis indicated that the factors driving the dissimilarity in the metagenome data are mostly similar to those seen in the amplicon sequencing data. For example, soil physio-chemical properties such as pH, sand content and exchangeable Ca, K were found to be key factors driving the variation in metagenomic composition.

For functional profiling of the soil microbiomes in the low- and high-rotundone zones, metagenomic sequence data was interrogated and annotated to various functional categories. Comparison of metagenomic data using beta-diversity analysis (generated using the Bray-Curtis distance metric) showed significant and distinct dissimilarity between the low and high rotundone zone samples for all gene classification approaches (e.g. gene names, gene ontology (GO) functions, and GO and COG (clusters of orthologous groups of proteins) pathways) (Figure 16).

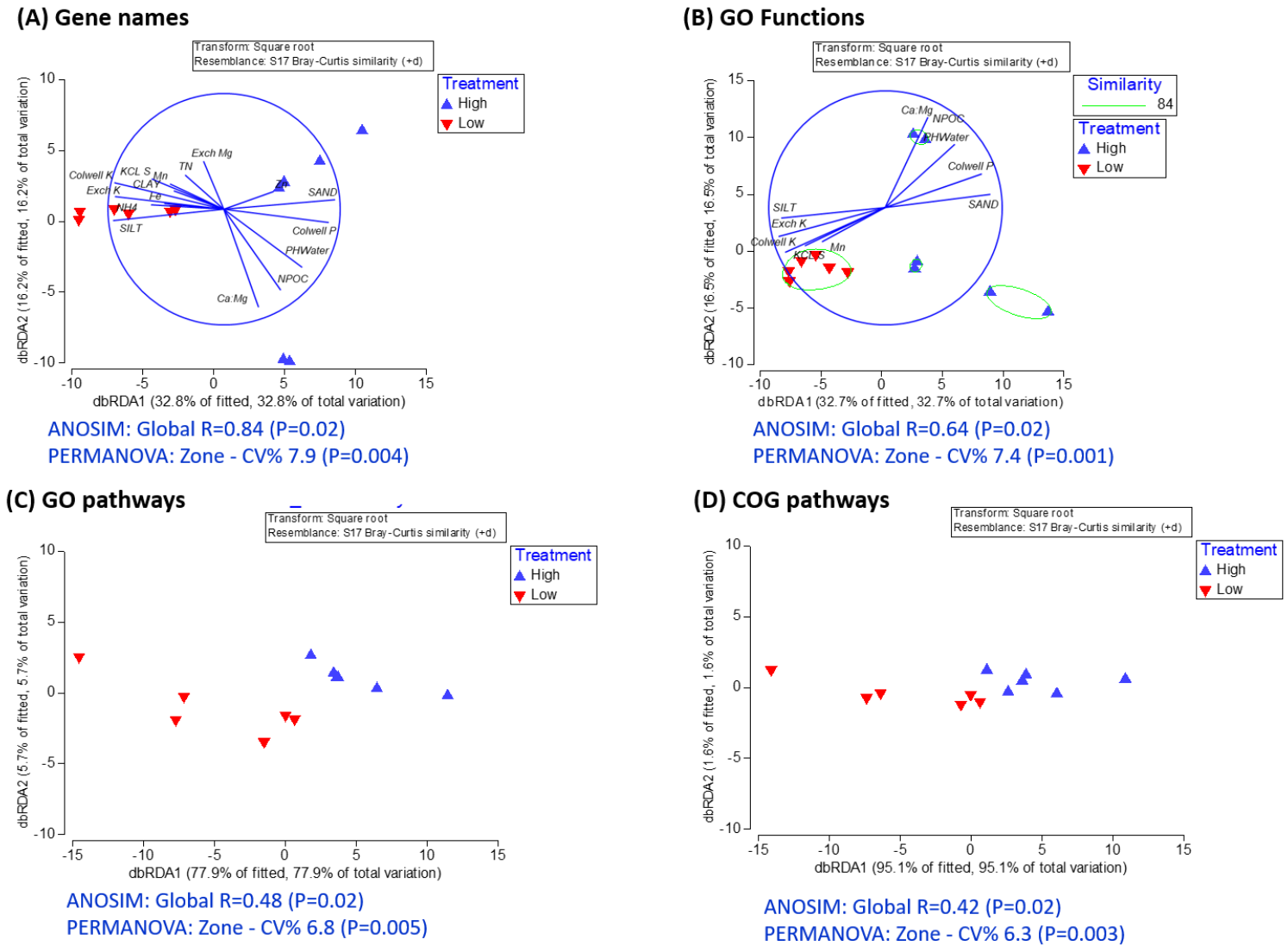


Figure 16. Differences in functional profiles of soil microbial communities in samples from the low- and high-rotundone zones at the 'Old Block' vineyard

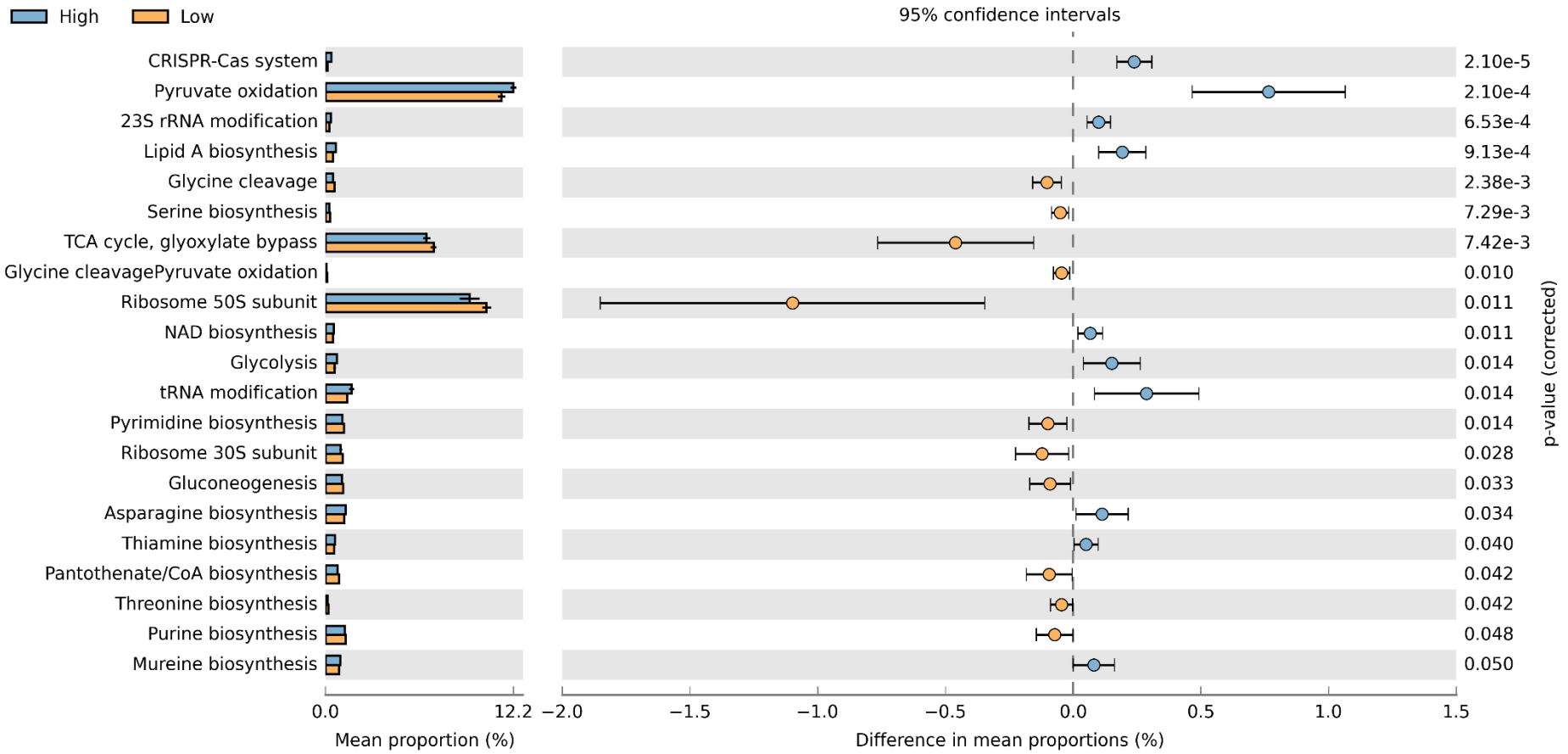


Figure 17. Differences in functional profiles of soil microbial communities expressed as genes related to various COG pathways in samples from low- and high-rotundone zones at the 'Old Block' vineyard collected during the 2017 season

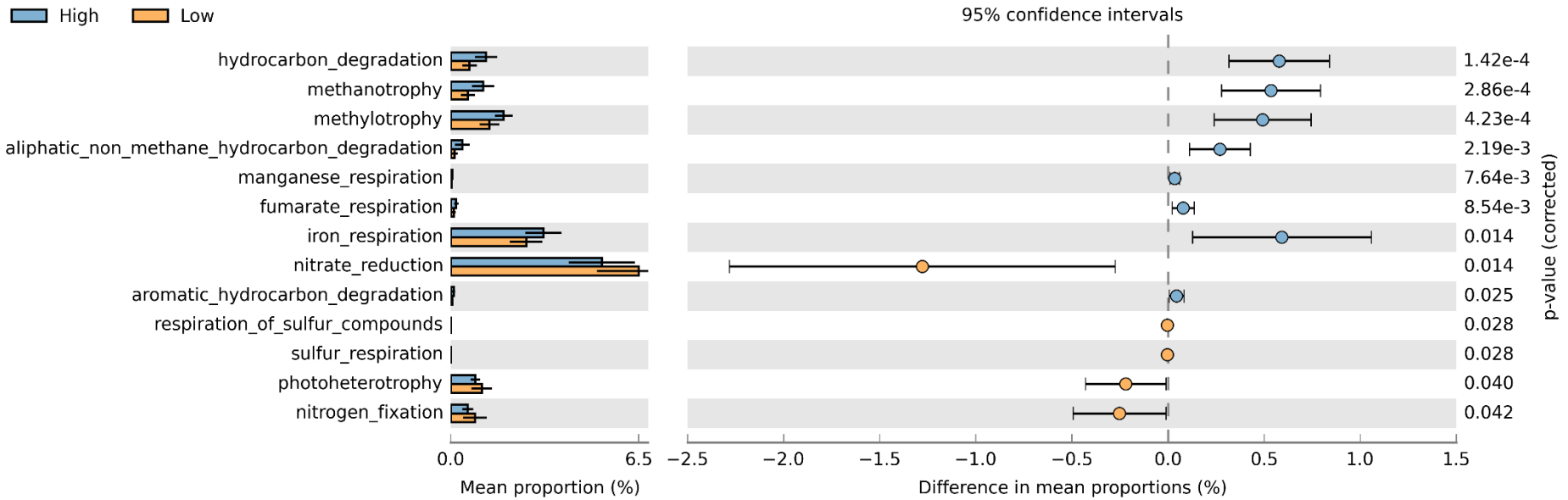


Figure 18A. Differences in functional profiles of soil bacterial communities in 2017 samples from the different rotundone zones, based on known metabolic and ecologically relevant functions from the FAPROTAX database

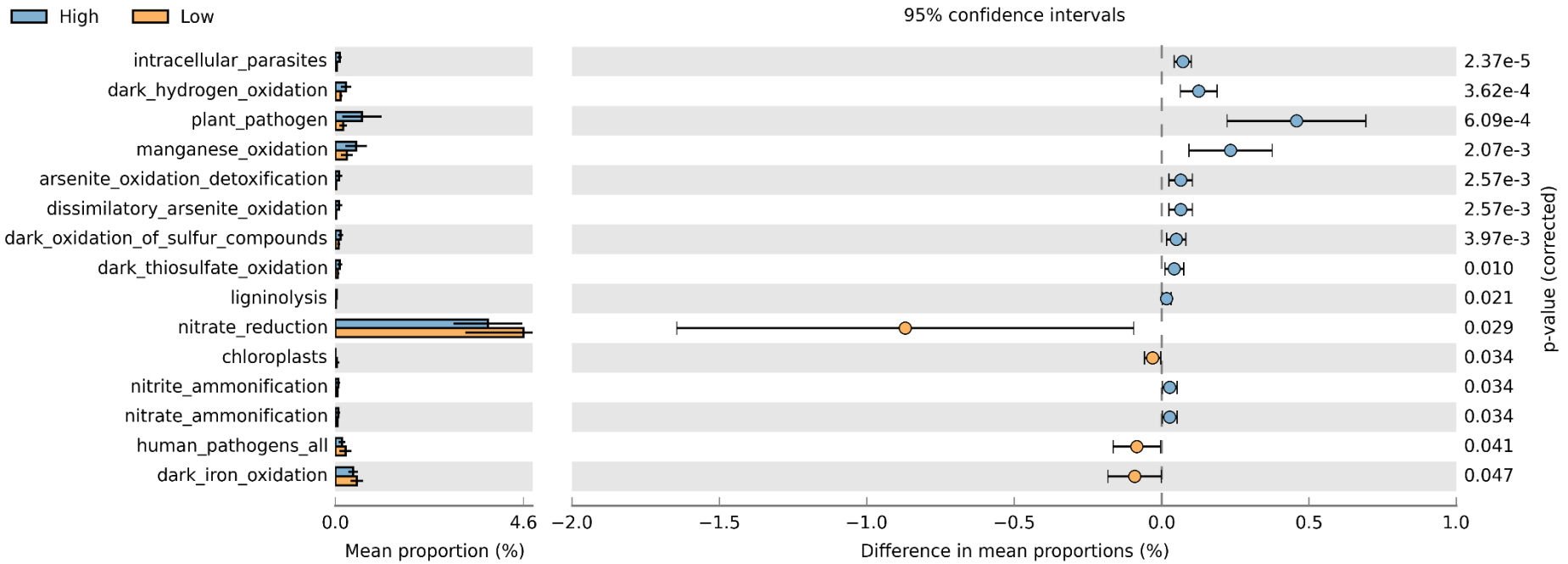


Figure 18B. Differences in functional profiles of soil bacterial communities in 2018 samples from the different rotundone zones, based on known metabolic and ecologically relevant functions from the FAPROTAX database

The variation in the functional profiles for the soils from the two zones was found to be caused by variation in the relative abundances of a community of functional genes related to multiple COG pathways (Figure 17). For example, relative abundances of genes related to COG pathways for pyruvate oxidation, CRISPR-Cas system, tRNA modification, biosynthesis of asparagine, purine and lipid A and glycolysis were higher in the high-rotundone zone soils compared to the low-rotundone zone soils.

When associating the functional profiling of microbial communities from the rotundone zones (Figure 16) with soil physico-chemical properties it becomes apparent that there are significant links between soil properties and community composition at individual gene level or gene function level, and that soil pH, % sand content, Colwell P, exchangeable potassium and calcium:magnesium ratio are predictors for the observed variation (Figure 16 A, B). Additionally, application of DistLM analysis (Distance based linear models) indicated that soil variables including pH, exchangeable Zn and Mn, plant-available (Colwell) P and K, dissolved organic C contributed significantly (R^2=0.514; P=0.001) to the zone-based variation in functional profiles.

In addition to the direct metagenomic analysis, bacterial community composition data was studied through functional annotation of 16S sequence data (prokaryotic taxa) using known metabolic and ecologically relevant functions and the FAPROTAX database. This analysis also revealed significant differences between rotundone zone soils in the 'Old Block' vineyard (Figures 18A, B) for both years.

7.3.2 Soil physico-chemical properties at the 'Old Block' vineyard

In general, there were few significant differences between the different rotundone zones in soil physico-chemical properties such as soil pH, organic carbon and total nitrogen (Appendix 5: Supplementary data). Systematic and significant variation between the rotundone zones was only observed for soil physico-chemical properties such as % sand, Colwell P, DTPA-zinc and DTPA-copper (DTPA: diethylenetriamine pentaacetate). Briefly, Colwell P was significantly higher and nitrate N, KCl extractable S and EC were lower in the high-rotundone zone soils compared to that in the low-rotundone zones. There were some significant differences in the % sand and % clay levels in soils from high- and low-rotundone zones. Surface (0–5 cm) soils generally exhibited significantly higher organic carbon, total nitrogen, calcium:magnesium ratio and higher pH values compared to the 5–15 cm soils.

Mulching generally increased exchangeable potassium, DTPA-extractable manganese and dissolved organic carbon, but showed no significant effect on total soil organic carbon, nitrogen and pH levels and increased exchangeable potassium, DTPA-extractable manganese and dissolved organic carbon. Soil organic carbon, the chief source of energy for the heterotrophic microorganisms, did not show significant relationships with either the abundance or diversity of bacteria and fungi in the non-mulched soils, suggesting that soil carbon variation may not be driving the observed differences in microbial diversity within this vineyard.

In the grapevine soils tested in this study, soil pH, exchangeable calcium, manganese and sodium, and sand content were the major predictors of bacterial composition in the surface soils. Similarly, variation in the fungal composition was influenced by the above factors and exchangeable copper and plant available Colwell P. For example, results observed showed that soils from the mulched areas showed significant changes in some of the fungal groups, such as Nectriaceae, Tremellaceae and Pezizomycotina incertae sedis. Some of the members of the above groups are known decomposers and so can be expected to respond to addition of an organic carbon source such as mulch, but these did not modify or mask the rotundone zone associated differences in overall fungal community structure. While biogeography in the berry microbial community structure has been reported for Chardonnay and Cabernet Sauvignon musts in California (Bokulich et al. 2014), edaphic factors within vineyards played a greater role in determining the bacterial and fungal communities in vineyards in

Long Island, USA (Zarraonaindia et al. 2015), highlighting the importance of soils for microbial community structure under homogeneous climatic conditions.

Overall, these results suggest that either the mulching-induced changes in microbial community structure had no direct link to the microbiome variation between the different rotundone zones, or that the effects of mulching were yet to be fully established. However, given that mulch was applied seven to eight months prior to soil sampling during the wetter part of the year, this lack of mulch expression seems unlikely.

7.3.3 Summary of results from studies into soil microbial diversity and its association with grape rotundone concentrations

Overall, the results from studies of taxonomic and functional composition (through amplicon sequencing and direct metagenomic sequencing) clearly indicate that microbiomes in soils from different rotundone zones at the 'Old Block' and 'The Lane' vineyards were distinctly different. Also, high-rotundone zone soil exhibited a well-connected microbial community network by comparison with the low-rotundone zone soil. Distinct differences in soil bacterial and fungal community composition and structure in different zones within the same vineyard were associated with zonal differences in grape berry rotundone concentration.

The results from Australian vineyard soils indicate that Proteobacteria, Actinobacteria, Acidobacteria, Gemmatimonadetes and Verrucomicrobia are the top five most abundant groups accounting for 84% of the total bacterial community. For fungi, Ascomycetes and Basidiomycetes were the predominant fungal phyla in both the surface (0-5 cm) and (5-15 cm) soils and accounted for 84% of the identified species. Reports by Liu et al. (2020) and Zhou et al. (2021) found similar groups of bacteria and fungi as dominant in vineyards in different wine-growing regions in Victoria and South Australia. Key differences in the microbial community structure between the rotundone zones were apparent for some taxa/groups of both bacteria and fungi, particularly for bacteria belonging to Acidobacteria-GP4 and GP7, Rhizobiales, Gaiellaceae, Alphaproteobacteria and the Nectriaceae and Tremellaceae families of fungi. A diverse and responsive soil microbial community as observed in the high-rotundone zone gives the plants a more diverse and responsive community with potentially beneficial functions such as nutrient availability and tolerance to biotic and abiotic stresses.

While the present results do not infer a cause and effect relationship between grape rotundone concentration, wine 'pepperiness' and soil microbial composition, they do lend further weight to a possible role of the microbiome in contributing to unique grape metabolites and an expression of a wine's terroir. This highlights opportunities for further research seeking to explore functional consequences from differences in the microbial composition and associated differences in characteristics of wine from specific vineyards and zones within a vineyard.

Host plant phenology and seasonal variables can also affect the phyllosphere and composition and abundance of active microbial communities in a vineyard soil. In this study, soil organic carbon, the chief source of energy for the heterotrophic microorganisms did not show significant relationships with either the abundance or diversity of bacteria and fungi in the non-mulched soils, suggesting that variation in soil carbon may not be driving the observed differences in microbial diversity within this vineyard.

It has been suggested that soil microbial community structure may be different between wine-growing regions and that environmental factors may affect wine terroir through changes to microbial community structure (Zarraonaindia et al. 2015). However, these studies are generally based on

investigations looking at the phylogenetic composition of microbial communities only and do not address the functional structure of communities (Liu et al. 2020, Zhou et al. 2021). Results from this project's limited analysis of functional microbiome composition indicate significant variation between different rotundone zones. Thus, to effectively manipulate the vineyard microbiome to achieve distinct wine sensory attributes, a better understanding of specific functional consequences of the distinct microbiomes above and below ground is necessary.

Overall, the observation of a systematic rotundone zone-based variation in soil microbiomes paves the way to bring together understanding of microbial ecology and viticultural management for improved grape composition and wine flavour (terroir). Future research should investigate the direct links between the soil microbiome, the microbiome in wine ferments and ultimately, wine quality. Furthermore, manipulation of soil microbial communities could also be an avenue worthy of exploration once a better understanding of the functional consequences of the distinct microbial communities has been achieved.

7.4 Formation of aroma compounds from sesquiterpenes

7.4.1. Sesquiterpenes in grapes and wine

Sesquiterpenes have been known to be present in grape berries for many years (Schreier et al. 1976), with reported concentrations of α-ylangene up to 15 µg/kg (Parker et al. 2007), but their presence in wine is not well understood. In 14 Shiraz wines, only α-muurolene was detected and tentatively identified by mass spectrum and retention index. The concentration range was 0.08 – 0.36 µg/L, calculated as equivalents of α-guaiene. However, the concentrations of α-muurolene and rotundone in wine did not correlate. Following that work, the identification of α- muurolene was confirmed by co-injection with a reference standard using multidimensional GC-MS and the corresponding second dimension (200 ms phase) peaks gave identical mass spectra and retention times.

Regarding the transfer of sesquiterpenes from grape solids into wine, only a small amount of the sesquiterpenes (0.1 – 0.3%) were transferred from the spiked grape homogenate to the model wine. This is likely due to the lipophilic nature of the sesquiterpene hydrocarbons. Whereas, around 10% of the more polar sesquiterpene ketone rotundone is transferred from grape to wine during fermentation (Caputi et al. 2011). Thus, it is unlikely that sufficient sesquiterpene hydrocarbons from grapes would be present in wine to act as an ongoing precursor for any aroma-active oxygenated sesquiterpene, such that any oxygenated sesquiterpenes in wine must come directly from the grape. Nonetheless, a similar amount of α-muurolene was found in both the blank control extract (0.23%) and the spiked filtered sample (0.28%).

7.4.2 Model oxidation of α-guaiene and α-ylangene

Sesquiterpene hydrocarbons are known to be easily oxidised to aroma compounds such as rotundone by fungal oxidases, grapevine enzymes and chemical oxidation (Huang et al. 2014, Takase et al. 2016). Given their location in the grape skin (Caputi et al. 2011, Siebert and Solomon 2011) the time scale and kinetics of the chemical oxidation from α-guaiene to rotundone was tested. A clear effect of visible light was seen with a faster initial formation of rotundone with exposure to light and a faster decline in α-guaiene compared to no exposure to light. With full sunlight, α-guaiene decreased rapidly and rotundone formed quickly but then also decreased quickly after three days. With 50% shade, rotundone formed more slowly, plateaued, and then decreased slowly over three weeks. In the absence of light, α-guaiene decreased much more slowly, the rotundone formed slowly and then remained stable for

several weeks. Overall, the chemical oxidation of α-guaiene in presence of some daylight proceeded at a rate similar to the rate of formation of rotundone in grapes during berry ripening see section 7.4.4).

With exposure to daylight, different rates of aerial oxidation were found for the four grape sesquiterpenes studied. Overall, α-guaiene reacted the fastest and α-ylangene the slowest and the other two, germacrene D and α-muurolene, were in between. The major oxidised products from α-guaiene, α-ylangene and germacrene D were relatively stable over the experiment, but not the products from α-muurolene.

GC-MS-O analysis of the α-ylangene oxidation products revealed a strong 'celery-like' and 'herbal' aroma-active zone coinciding with an unknown sesquiterpene ketone from wine. The GC-MS peak had a mass spectrum and retention index similar to mustakone. However, mustakone was also present in the model experiment due the oxidation of α-copaene, an impurity from the original essential oil. No further aroma-active zones were detected during GC-MS-O analysis of the products of the aerial oxidation of α-ylangene (beyond rotundone) or α-muurolene.

7.4.3 Identification of iso-mustakone in wine

When quantifying rotundone in red grape and wine samples, occasionally a co-eluting peak was observed that had a very similar retention time and mass spectrum and could only be separated from rotundone using two-dimensional GC-MS (Geffroy et al. 2014). Using multidimensional GC-MS, a mass spectrum similar to that of the unknown sesquiterpenoid and rotundone was initially observed amongst the closely eluting peaks of interest in the α-ylangene and α-copaene oxidation samples. Next it was established that the unknown sesquiterpenoid was not the α-copaene oxidation product, mustakone, by comparison with the reference compound and a sample of oxidised α-copaene. Pure mustakone gave a similar mass spectrum to the unknown sesquiterpenoid and but a different retention times in the second dimension by multidimensional GC-MS during co-injection. Yet from the similarity in mass spectrum and retention index in single dimension GC-MS, it appeared plausible that the unknown sesquiterpenoid was iso-mustakone (Mahmout et al. 2002). As no reference compound was available for iso-mustakone, the unknown sesquiterpenoid isolated from wine was compared to a sample of oxidised α-ylangene by multidimensional GC-MS-O. After separation on the second dimension (200 ms GC column) the eluting peaks gave identical mass spectra and retention times plus matching 'celery-like' aroma. Furthermore, co-injection experiments gave symmetrical peak enhancement and a matching mass spectrum on the second dimension GC column when an addition of the aerial oxidation of α-ylangene sample was made to the enriched wine SPE extract. Thus, it was possible to confirm the identity of the unknown sesquiterpene in cool-climate Shiraz wine as another potent aroma compound, iso-mustakone (Mahmout et al. 1997).

7.4.4 Formation of α-guaiene and rotundone during grape ripening

The grapes from the 'Old Block' vineyard produce Mount Langi Ghiran's most premium Shiraz, a wine that is highly regarded as a superb representation of Australian cool-climate wines. The winemaker describes the wine produced from the 'Sheoak' vineyard as similar to that from the 'Old Block', though not as peppery and lacking the same intensity and range of flavours. Though only six kilometres apart and at the same elevation, the grapes in the 'Sheoak' vineyard ripen earlier than the 'Old Block' and are, therefore, typically harvested earlier. The objective of the ripening trials was to establish the influence of harvest date on grape rotundone and α-guaiene concentrations, to see if formation of both sesquiterpenes was synchronised, and to study the yield of rotundone formed from α-guaiene (i.e. the ratio of rotundone to α-guaiene) as an indirect assessment of the oxidative reaction pathways involved.

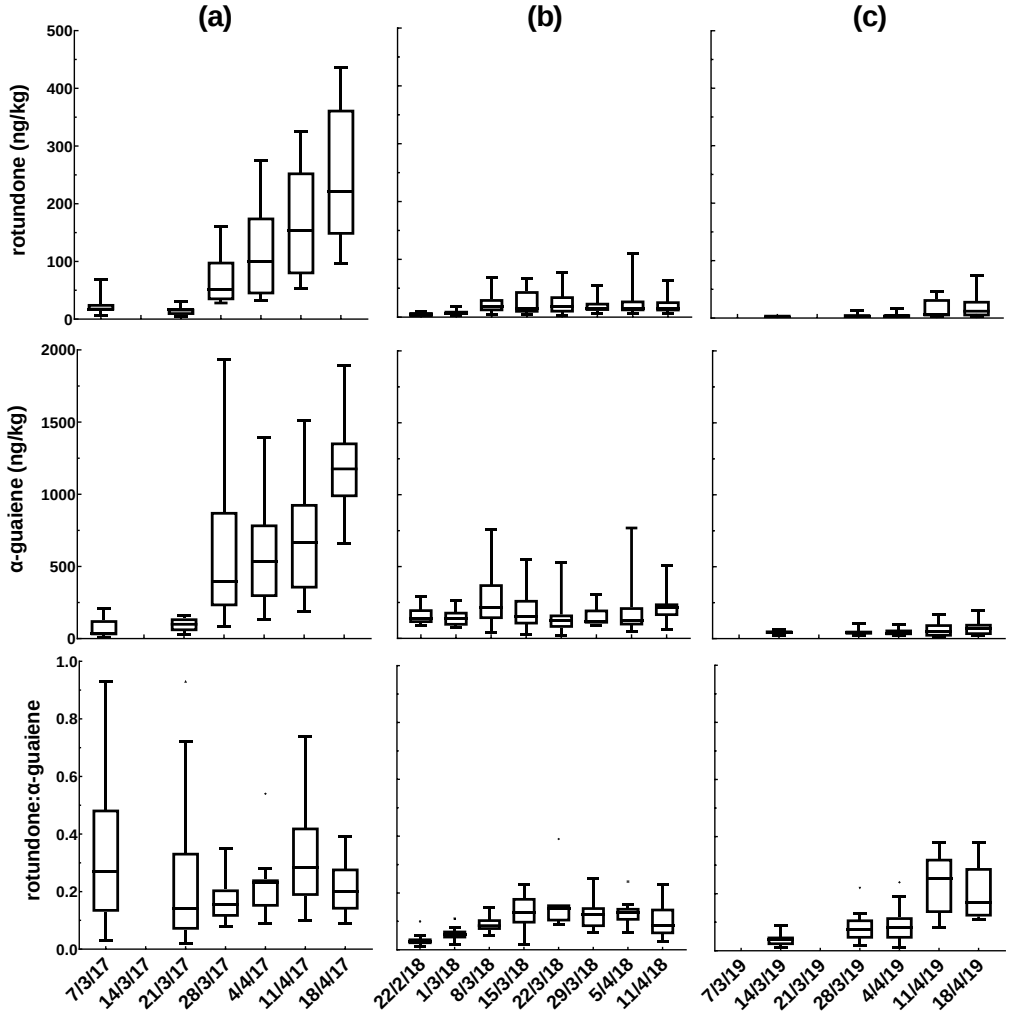


Figure 19. Formation of rotundone and α-guaiene throughout ripening in grapes from the 'Old Block' vineyard, and ratio of rotundone to α-guaiene, in (a) 2017, (b) 2018, and (c) 2019

In 2017, the Old Block vineyard had the highest final rotundone and α-guaiene levels of all three seasons (Figure 19), reaching an average rotundone concentration of 209 ng/kg and an average α-guaiene concentration of 1,066 ng/kg at the final sampling point. In the first three weeks of sampling, there was little-to-no rotundone or α-guaiene present in the grapes, followed by a clear initiation of sesquiterpene biosynthesis between 21 March and 28 March 2017 for both rotundone and α-guaiene. The highest concentration of rotundone at the final sampling point was nine times higher than that of the lowest at the same timepoint. The average ratio of rotundone to α-guaiene was remarkably consistent across all sampling dates with individual ratios ranging from approximately 0.10 to 0.35; in the low rotundone years 2018 and 2019 the highest yield of rotundone was only achieved in the more advanced ripening stages.

In 2018, the 'Old Block' produced much lower levels of the two sesquiterpenes than in 2017 with an average final rotundone concentration of 22 ng/kg and average final α-guaiene concentration of 218 ng/kg. Rotundone only increased in concentration slightly by the third timepoint (8 March), whereas α-guaiene remained relatively consistent across the sampling period. The ratio of rotundone to α-guaiene was also lower, starting at just 0.04 on 22 February and increasing over four weeks to a maximum average of 0.15.

In 2019, the concentrations of both sesquiterpenes were lower again. Their biosynthesis was synchronised, increasing slightly in the last two weeks of sampling to a final average rotundone concentration of 19 ng/kg and an average α-guaiene concentration of 75 ng/kg. Not all grape samples increased in concentration though, with half remaining under 35 ng/kg α-guaiene and under 5 ng/kg rotundone at the final timepoint. The ratio of α-guaiene to rotundone increased from just 0.04 to approximately 0.10 to 0.35 in the final two weeks.

The sesquiterpene profiles in grapes from the 'Sheoak' vineyard were very similar to those in the 'Old Block' at the same timepoints in both 2018 and 2019, although sampling concluded three weeks earlier in 'Sheoak' in 2018 as the grapes were lost to bird damage. The ratio of rotundone to α-guaiene increased throughout ripening from an average of 0.05 at the first timepoint to 0.30 at the final timepoint in 2018 (Figure 20A).

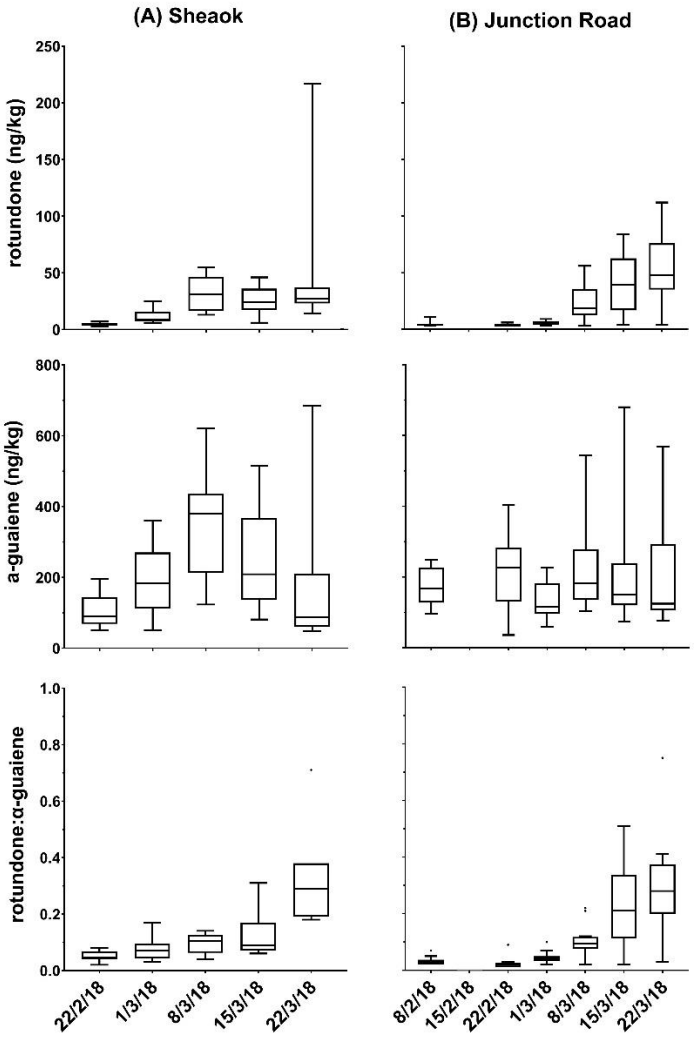


Figure 20. Formation of rotundone, α-guaiene and ratio of rotundone to α-guaiene throughout ripening in 2018 for grapes from the 'Sheoak' vineyard (A), and the 'Junction Road' vineyard (B)

After distinct rotundone concentrations had been observed in 2017 for grapes from the 'Junction Road' vineyard, a ripening trial was conducted in 2018 with a subset of target vines. There was a clear initiation point for rotundone formation in early March 2018 in the 'Junction Road' vineyard (Figure 20B). One target panel did not produce a rotundone berry concentration above 4 ng/kg at any

timepoint, suggesting that the 'trigger' for α-guaiene and rotundone formation was not synchronised across the entire block.

The ratio of rotundone to α-guaiene started off very low at an average of 0.03 at the first timepoint on 8 February 2018 and increased to an average of 0.30 at the final timepoint on 22 March (Figure 20B). Notably, the average rotundone to α-guaiene ratio from the entire set of 141 target vines sampled for mapping on the day prior to commercial harvest (18 April 2017; 22 March 2018) was very similar, 0.28 in 2017 and 0.23 in 2018.

In summary, analysis of data from ripening trials with grape samples collected across multiple trial sites and growing seasons established that formation of elevated α-guaiene and rotundone were synchronised and typically initiated very late during berry ripening. This means that the biosynthesis of the precursor α-guaiene is key to the concomitant formation of rotundone in grapes. That is, availability of α-guaiene and initiation of α-guaiene biosynthesis appear to be critical for controlling grape rotundone at harvest; differences in the efficacy of the oxidation reaction from α-guaiene to rotundone has no apparent major impact on final grape rotundone concentrations. In summary, a late harvest date is a pre-requisite for growers aiming for elevated rotundone concentrations in grapes.

8. Outcomes and conclusions

Despite the elusive character of rotundone, this project has generated a wealth of valuable new knowledge that is of significant value for Shiraz growers and may guide future research direction. Key findings from this project are summarised below.

The results of the model experiments:

- established that in a year with sufficiently high grape rotundone concentrations, such as 2018 in the Grampians, significantly more rotundone was observed in all grapes from vines in the high zone irrespective of the low or high phenotype of a cane, compared to samples from vines in the low pepper zones. This points towards a site effect at the grapevine scale that is responsible for elevated grape rotundone concentration.
- confirmed difficulties with obtaining grapes from potted vines grown in greenhouses. Interestingly, in 2019 potted vines that suffered from extensive insect (scale, ant, mite) infestation and some concomitant fungal damage yielded grape samples with extremely high rotundone concentrations. This points towards insect and/or fungal effectors which trigger sesquiterpene biosynthesis in grapes and may make an important contribution to the formation of desirable aroma compounds.
- demonstrated that short-term exposure of grape-bearing vines to reduced ambient temperatures overnight alone is not sufficient to trigger rotundone formation in grapes.

The results of the aroma compound mapping component in commercial cool-climate vineyards:

- confirmed the elusive nature of rotundone as a unique grape aroma compound in premium Shiraz, which is only produced in some vineyards and growing seasons.
- established that the broad intra-vineyard variability and marked spatial structure in grape rotundone is not unique to Mt Langi Ghiran's 'Old Block' and its heritage vines, but appears to represent a common feature found in growing seasons with elevated grape rotundone concentrations and vineyards planted with other clones. However, it was not possible to establish consistent drivers of the patterns of rotundone variation across sites; at the 'Old Block' vineyard, orientation away from north and soil effects were key, but at the 'Junction Road' vineyard, the results were equivocal.
- established that the spatial distribution of grape rotundone at the Junction Road vineyard in the Adelaide Hills
    - is closely associated with its precursor, α-guaiene, and a range of other sesquiterpenes, but no other grape metabolites
    - is spatially distinct yet may vary from year to year – this indicates that it is unlikely that mobile vectors such as birds or kangaroo damage might trigger sesquiterpene biosynthesis.

The results of analysing site characteristics, including microbial populations:

- established that the spatial (intra-vineyard) variability in grape rotundone at the Adelaide Hills site was not related to established measures of vine performance and canopy, such as plant cell density and other site characteristics. This result was similar to previous observations for Mt Langi Ghiran's 'Old Block'.
- demonstrated through metagenomic (amplicon and direct sequencing) analysis of surface soil samples collected from the previously identified high- and low-rotundone zones at Mt Langi Ghiran's 'Old Block', marked differences in the genetic diversity and composition of the soil bacterial and fungal microbiomes. This trend was also demonstrated in soils from the 'Junction Road' vineyard. A few specific taxa/groups of microorganisms were associated with the rotundone-based variation; and bacterial communities in soil from the high-rotundone zone appeared to form a much more complex and connected network than those in the low-rotundone zone soils. Also, short-term mulching effects did not seem to mask this rotundone zone-based variation in soil microbiology. This work has clearly demonstrated, as a first report of this type, that distinct differences in soil bacterial and fungal community composition and structure in different zones within the same vineyard are associated with different propensities for grape berry rotundone concentration.
- demonstrated the presence of distinct variation in the functional microbiome composition, measured through direct metagenomic sequencing, between high- and low-rotundone zone soils at Mt Langi Ghiran's 'Old Block'. The variation in the functional profiles for the soils from the two zones was found to be due to differences in the relative abundances of a community with functional genes related to multiple biosynthetic pathways. Developing a systematic rotundone zone-based variation in soil microbiomes, both in terms of taxonomic composition and functional capacities, paves the way for bringing together understanding of microbial ecology and viticultural management for improved grape composition and wine flavour attributes, such as expression of terroir.

The results of studies of formation of aroma compounds from sesquiterpenes:

- identified the aroma compound iso-mustakone through GC-MS-O analysis for the first time in wine, where it contributes 'celery-like', 'spicy' characters at very low concentrations.
- established through model experiments that oxidation by air of the grape sesquiterpene precursors, α-guaiene and α-ylangene, can lead to potent aroma compounds rotundone and iso-mustakone, and that exposure to daylight catalyses the chemical oxidation of the sesquiterpene precursors. This means that the formation of both potent aroma compounds may occur under typical grapegrowing conditions and does not necessarily require the previously described enzymatic reactions by grapevines or microorganisms.
- established in grape samples collected across multiple trial sites and growing seasons that formation of elevated α-guaiene and rotundone are synchronised and typically initiated very late during berry ripening. This means that the biosynthesis of the precursor α-guaiene is key to the concomitant formation of rotundone in grapes. That is, availability of α-guaiene and initiation of α-guaiene biosynthesis from FPP and germacrene-A appear to be the critical steps for controlling grape rotundone at harvest.
- established that sesquiterpene hydrocarbons common to grapes are absent from wine (other than a trace of α-muurolene) because they are not extracted into wine-like hydroalcoholic solutions and/or are removed from juice with solids. This means that

sesquiterpene aroma precursors from grapes are most likely not chemically converted or metabolised during fermentation, while at the same time grape marc and wine solids are a potential source of sesquiterpene aroma precursors.

The findings about the genetic and catabolic diversity and composition of microbial communities in soils from vineyard zones varying in rotundone concentrations have already been published (Gupta et al. 2019). In addition, a number of peer-reviewed manuscripts of key results are under preparation, covering:

- sesquiterpenes in grapes and wine
- the oxidation of sesquiterpenes and identification of iso-mustakone as new wine aroma compound
- the kinetics of α-guaiene and rotundone accumulation in grapes, the role of α-guaiene as precursor of rotundone and functional soil microbiomes in zones within a vineyard varying in rotundone concentrations.

Further publications demonstrate results from a number of collaborative projects which were supported through input into experimental designs and data analysis, exchange of reference compounds and analytical support to further research into environmental, oenological and sensory effects of rotundone. These are listed in Appendix 1.

9. Recommendations

Overall, this project provided science-based evidence to support the concept of terroir; that is, how the interplay of site, viticultural management, climatic and environmental/biological effectors (albeit the latter are yet to be identified) shape distinct sensory attributes in premium Shiraz wine. However, its relatively short duration, coupled with the ability to study rotundone being dependent on seasonal conditions which favour its formation, meant that establishing the causative role of site factors (soil, topography, vigour) was not possible. Nonetheless, the results do suggest that rotundone variation is in some way an expression of terroir and point to a possible role for the soil microbiome in terroir expression. Whether the latter is pursued in work focused on rotundone or on other elements of grape and wine composition, the present work does suggest that role of soil microbiology in terroir expression is worthy of further work, with potential implications for management of wine style in the vineyard.

Further viticultural research is warranted to identify the underlying genetic and biochemical regulation of sesquiterpene biosynthesis in *Vitis vinifera*, together with required environmental and biological effectors. A detailed understanding of specific biosynthetic pathways and environmental triggers in the ripening season could potentially enable the growing of Shiraz grapes with 'cool-climate-like' flavour attributes in warmer/hotter regions, and/or preservation of the desirable, but elusive, peppery flavours in existing cool-climate vineyards.

Further oenological and flavour research should focus on establishing and exploiting the unique flavour-modulating and enhancing effects of rotundone and related compounds such as iso-mustakone (as described recently for other fruit aromas) when present at sub-, peri- and supra-threshold concentrations in typical wine aroma preparations.

Overall, the observation of systematic rotundone zone-based variation in soil microbiomes paves the way to bring together understanding of microbial ecology and viticultural management for improved grape composition and wine flavour (terroir). Future research should investigate the direct links between the soil microbiome, the microbiome in wine ferments and ultimately, wine quality. Furthermore, practical ways to manipulate soil microbial communities could also be an avenue worthy of exploration once a better understanding of the functional consequences of these distinct microbial communities is available.

10. Appendices

Appendix 1: Communication

Conference presentations:

- Siebert, T., Barter, S. Determination of the potent flavour compound rotundone in grapes and wine using MDGC-MS and membrane assisted solvent extraction. Invited speaker at Gerstel 50th Anniversary Conference, 7 June 2017, Marina Bay Sands, Singapore.
- Barter S, Herderich M, Bramley R, Siebert T, Krstic M. α-Guaiene is a key precursor of the impact aroma compound, rotundone, in Shiraz grapes. Crush 2018. 25-26 September 2018, Waite Campus, Adelaide, Wine Innovation Cluster.
- Gupta, V.V.S.R., Bramley, R. Microbial metagenomics of vineyard soils. Crush 2018. 25-26 September 2018, Waite Campus, Adelaide, Wine Innovation Cluster.
- Barter, S.R. 2019 Rotundone and the inability to smell it. 17th Australian Wine Industry Technical Conference workshop program, Adelaide, 21 July 2019.
- Bramley, R.. Digital approaches to winegrowing. Invited presentation to the conference on science, technology, engineering and economics for Digital Agriculture (steeDA), University of Sydney, 3 December 2019.
- Bramley, R. Grapes, sugar, myths (?) and some other stuff. Presentation at E. & J. Gallo Winery, Modesto, CA, USA, 12 December 2019.
- Gupta, V., Bramley, R., Greenfield, P., Yu, J., Herderich, M. Microbial metagenomics of vineyard soils and wine terroir. XIIIth International Terroir Congress, 18 November 2020, Adelaide.

Posters:

- Barter S, Bramley R, Krstic M, Siebert T, Herderich M. 2019. Unravelling the complex pattern of 'pepperiness' in cool-climate Shiraz. Available from: https://awitc.com.au/wp-content/uploads/2019/07/75-2019_AWITC_75Pepper_2-FINAL.pdf

Publications:

- Pepper flavour in wine. AWRI fact sheet, updated: February 2019. Available from : https://www.awri.com.au/wp-content/uploads/2019/02/fact-sheet-pepper-flavour-in-wine.pdf
- Gupta, V.V.S.R., Bramley, R.G.V., Greenfield, P., Yu, J., Herderich, M.J. 2019. Vineyard soil microbiome composition related to rotundone concentration in Australian cool climate 'peppery' Shiraz grapes. *Front. Microbiol.* 10: 1607.
- Gupta, V., Bramley, R., Greenfield, P., Yu, J., Herderich, M. 2020. Microbial metagenomics of vineyard soils and wine terroir. Proceedings of the XIIIth International Terroir Congress, 17-19 November, Adelaide (submitted)

Appendix 2: Intellectual Property

Nothing to report.

Appendix 3: References

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Appendix 5: Supplementary data

Table S1. Physico-chemical properties of soils sampled in 2017 from 'Old Block' vineyard

Property	Low	Medium	High	F-test	LSD (P<0.05)
pH(Water)	6.9	6.7	7.0	NS	
Organic Carbon (%)	2.0	2.3	1.8	NS	
TN (µg/g)	8.6	5.0	5.3	NS	
DOC (µg/g)	26.0	47.9	19.5	0.00	15.1
MinN (µg/g)	6.3	2.0	3.7	0.003	3.2
Colwell P (µg/g)	11.3	15.5	26.9	0.001	7.1
Colwell K (µg/g)	171.0	249.6	130.0	0.001	1.3
KCL Sulfur (µg/g)	7.5	8.9	5.6	NS	
DTPA-Cu (mg/kg)	6.9	7.2	12.0	0.014	4.7
DTPA-Zn (mg/kg)	2.1	3.7	5.9	0.007	2.1
DTPA-Mn (mg/kg)	1.8	2.7	1.7	0.046	0.7
DTPA-Fe (mg/kg)	30.6	37.4	26.1	NS	
Exchangeble CEC	9.0	9.6	7.7	NS	
Clay (%)	5.3	6.2	3.6	0.006	1.7
Sand (%)	42.6	39.7	61.1	0.001	6.7
Silt (%)	52.1	54.1	35.4	0.001	5.8

Table S2. Physico-chemical and chemical properties of soils sampled in 2018 from 'Old Block' vineyard

Soil property	Low 0-5	Low 5-10	High 0-5	High 5-10	Low	High	Zone F-test	Zone LSD (P<0.05)
pHw	7.113	6.685	7.298	6.819	6.90	7.06	NS	
EC	0.0602	0.0638	0.0802	0.059	0.06	0.07	NS	
Org C (%)	1.93	1.382	2.07	1.311	1.66	1.69	NS	
Total N								
NO3 (ug/g)	12.64	10.66	10.8	5.71	11.65	8.26	0.021	3.987
NH4 (ug/g)	2.14	1.525	1.7875	1.64	1.83	1.71	0.044	0.513
Total Min N (ug/g)	14.8	11.9	11.2	4.9	13.35	8.05	0.017	4.26
Colwell P (ug/g)	21.6	13	28	21.6	17.3	24.8	0.041	7.17
Colwell K (ug/g)	186	110.6	154.4	72.5	148	113	0.022	29.3
KCl-S (ug/g)	6.15	7.6	6.28	5.09	6.88	5.69	NS	
DTPA Cu (mg/kg)	7.13	3.418	11.83	5.539	5.27	8.68	0.057	3.522
DTPA Zn (mg/kg)	2.68	1.02	7.712	2.231	1.85	4.9715	0.012	2.389
DTPA Mn (mg/kg)	2.23	1.67	2.14	1.45	1.95	1.795	NS	
DTPA Fe (mg/kg)	19.73	25.9	16.05	24.6	22.82	20.33	NS	
Boron (mg/kg)	0.651	0.346	0.618	0.269	0.499	0.444	NS	
Ex-K	152.6	74.6	149.2	64.4	113.6	106.8		
Ex-Ca	1224	907.5	1425.9	788.1	1066	1107	NS	
Ex-Mg	161.4	138.1	168.6	121.6	149.75	145.1	NS	
Ex-Na	33.68	33.14	53.11	51.59	33.41	52.35	<0.001	7.62
Exchangeable CEC	12.6	10.8	12.4	11.1	11.7	11.7	NS	
Ca:Mg ratio	5.2	5.4	3.5	3.5	5.3	3.5	0.037	0.563
Clay	9.69	11.17	4.76	4.8	10.43	4.78	<0.001	1.6
Sand	61.3	58.6	76.5	74.3	59.95	75.4	<0.001	3.3
Silt	29	30.3	18.8	21	29.65	19.9	NS	

Table S3. Physico-chemical and chemical properties of soils sampled in 2018 from the 'Junction Road' vineyard.

Soil property	High 0-5	High 5-10	Low 0-5	Low 5-10	Low	High	Zone F-test	Zone LSD (P<0.05)
pHw	6.689	6.785	7.128	7.169	7.15	6.74	<0.001	0.18
EC	0.1717	0.1	0.187	0.1108	0.15	0.14	NS	
Org C (%)	3.13	2.59	2.43	2.02	2.23	2.86	<0.001	0.277
Total N								
NO3 (ug/g)	27.58	17.32	12.02	5.09	8.6	22.5	<0.001	6.1
NH4 (ug/g)	5.51	3.67	2.74	2.07	2.4	4.6	<0.001	1.16
Total Min N (ug/g)	33.1	21	14.8	7.2	11	27.05	<0.001	6.3
Colwell P (ug/g)	115.5	93.8	51.3	29.2	40.3	104.7	<0.001	16.6
Colwell K (ug/g)	388	290	456	341	398.5	339.0	0.023	50.8
KCl-S (ug/g)	23.5	14.4	23.6	16.87	20.2	19.0	NS	
DTPA Cu (mg/kg)	8.87	6.1	8.37	4.82	6.60	7.49	NS	
DTPA Zn (mg/kg)	6.18	4.1	6.33	4.08	5.21	5.14	NS	
DTPA Mn (mg/kg)	18.38	6.27	13.32	5.85	9.59	12.33	0.051	
DTPA Fe (mg/kg)	78.4	82.5	49.3	47	48.2	80.5	<0.001	11.47
Boron (mg/kg)	1.039	0.799	0.903	0.662	0.783	0.919	0.012	0.104
Ex-K	401.3	261.5	327.9	227.1	278	331	<0.001	28.6
Ex-Ca	1910	1692	1701	1546	1624	1801	0.027	156
Ex-Mg	228.6	197.3	310.4	289.4	300	213	<0.001	36
Ex-Na	30.43	17.89	104.15	82.98	93.6	24.2	<0.001	25.8
Exchangeable CEC	8.0	6.0	9.1	5.3	7.24	7.01	NS	
Ca:Mg ratio	4.63	3.95	5.43	4.35	4.89	4.29	<0.001	0.78
Clay	6.48	6.53	5.1	5.19	5.1	6.5	NS	
Sand	67.7	66.3	67.3	64.6	66.0	67.0	NS	
Silt	26.1	27.2	27.6	30.1	28.9	26.7	NS	

Appendix 6: Budget reconciliation

Not applicable.