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A Review of Trimen's False Tiger Moth, *Agoma trimenii* (Lepidoptera: Agaristidae): Seasonal Biology, Potential Monitoring and Control Techniques

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Trimen's false tiger moth, *Agoma trimenii* (Lepidoptera: Agaristidae), has recently been found to occur in vineyards in the Northern Cape and Limpopo (Groblersdal area) provinces of South Africa. As little is known about the biology and behaviour of the moth, no official monitoring methods or economic thresholds relating to it exist. Consequently, management and registered control options still require development. The first aim of the current review was to gather and critically discuss all the available information on *A. trimenii* in the context of the information gained from field observations conducted in the Northern Cape during the 2016/2017 and 2017/2018 seasons. The paper also includes reporting on field observations made with regard to various aspects of the seasonal life cycle and ecology of *A. trimenii*, with a view to investigate, in future research, the potential biological control options available. Potential monitoring strategies of *A. trimenii* in the field were investigated. Various life stages of *A. trimenii* were identified, peak flight times were established, overlapping generations were determined, and the behavioural traits of all life stages were documented. Ultraviolet blue light traps proved to be the most promising potential monitoring strategy, with the prospect for an *A. trimenii* pheromone lure holding potential as an alternative monitoring strategy in the future. In summarising all current information on *A. trimenii*, recommendations for growers to monitor and control *A. trimenii* are presented towards the development of an integrated pest management system for the moth.

INTRODUCTION

South Africa is a major contributor to the worldwide export of table grapes, having produced an estimate of 59.4 million cartons during the 2018/2019 season (SATI, 2019). Table grapes are prone to outbreaks of various pest arthropods, and damage may occur directly to the fruit or indirectly by weakening the plant. Both types of damage negatively affect grapevine production.

Agoma trimenii (Felder) (Lepidoptera: Agaristidae), Trimen's false tiger moth, was first described in 1874 in subtropical Africa (De Prins & De Prins, 2012) and has recently been found to occur in parts of South Africa, following reports of moth presence in vineyards in the summer rainfall areas (Pretorius *et al*., 2012). Members of the Agaristidae exhibit great diversity in other parts of the world, where they are also known to occur on vines. The painted vine moth, *Agarista agricola* (Donovan) (Beutenmüller), and the grapevine moth, *Phalaenoides glycinae* (Lewin), are both common insects that occur on vines in Australia (Australian Museum, 2010).

Knowledge relating to the biology and life cycle of *A. trimenii* is limited, and consequently there is a need for a monitoring system for this pest. Improved knowledge of *A. trimenii* would assist in decision-making on and the application of control options towards developing and implementing an integrated pest management (IPM) strategy for the pest. The aim of the current review and preliminary research study was to gather and critically discuss all the available information on *A. trimenii*, in the context of new information gained from field observations conducted in the Northern Cape province, South Africa, during the 2016/2017 and 2017/2018 seasons (Morris, 2018). To improve our understanding of the seasonal cycle, biology and behaviour of *A. trimenii*, field observations included visual scouting, recording behaviour and damage, morphological observations of the different life stages, and small-scale testing of trapping techniques. The paper reports on the first of such observations done on *A. trimenii* in South Africa.

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GEOGRAPHICAL RANGE

Both the introduction of *A. trimenii* to South African vineyards and its origin are uncertain, as no previous record of the presence of this moth species on any crop exists prior to 2012 (Pretorius *et al*., 2012). In South Africa, *A. trimenii* is present in the Northern Cape and Limpopo provinces. Beyond the South African borders, *A. trimenii* is known to occur in the Democratic Republic of the Congo, Ethiopia, Gambia, Kenya, Malawi, Mozambique, Niger, Nigeria, Senegal, Sierra Leone, Somalia, Sudan, Togo, Uganda, Zambia and Zimbabwe (De Prins & De Prins, 2012; Pretorius *et al*., 2012). Reports limit the presence of *A. trimenii* to the African continent.

HOST RANGE

The feeding of the larvae of *A. trimenii* is described as being host-specific (Picker *et al*., 2002). Known host plants are wild grapes (Vitaceae) belonging to the *Cissus* and *Rhoicissus* genera (Pretorius *et al*., 2012). Reports of damage to the leaves of *Cyphostemma cirrhosum* (Thunb.) (Vitaceae) have also been recorded (De Prins & De Prins, 2012). Within the Limpopo and Northern Cape, damage to the leaves of table grapes, wine grapes and raisin grapes has been reported (Pretorius *et al*., 2012).

LIFE AND SEASONAL CYCLE

Pretorius *et al*. (2012) reported that, in the Northern Cape province, moths are visible in vineyards between October and March, predominantly during the harvesting of wine grapes during January. Field observations conducted during the growing season and harvest time of the table grapes (from October 2017 to May 2018 – week 41 to week 19), comprising a 33-week period, indicate that *A. trimenii* can have four overlapping generations during that time. This was determined by visual scouting and recording the presence/absence of adult moths, larvae on the vines and pupae in the soil in demarcated sites on two table grape farms in the Northern Cape every day over the 33-week period (Fig. 1): site 1 with table grape variety Thompson Seedless (THS); and site 2 with table grape variety Sugraone (SSD). The demarcated areas on both of the farms were not treated with pesticides or fungicides for the duration of the observational study. Within each month during the 33-week period, counts of adult moths and larvae were carried out for a seven- to 10-day period according to the system developed and described by De Villiers and Pringle (2008) (Figs 2 and 3).

Peak flight times were recorded in mid-October and mid-December, as well as at the beginning of February, and finally towards the end of March (Fig. 1). Female moths deposit their eggs singly on the surface of grape leafs, from which the eggs begin to hatch after approximately three days (Pretorius *et al*., 2012). Visual scouting indicated that the single eggs are laid on the adaxial surface of the leaves in the top parts of the canopy of the vines. The length of time for larval development (from hatching to pupation) ranged from five to six weeks, according to the time interval between the first presence of the larvae after first moth flight to pupation before the next moth flight (Fig. 1). The first instar larvae disperse and feed on vegetative tissue, particularly on new shoots and leaves. The larvae continue to feed, and undergo numerous moults (exact number not yet determined), until they reach their final instar stage. Final instar larvae drop from the plant, via a silken thread, and burrow into the soil to a maximum depth of 4 cm in the soil mounds surrounding the vine. They remain in a soft-bodied prepupal state until they develop into fully formed, hardened pupae. Burrow holes (diameter of ± 2 cm) are visible in the soil. The pupae were found in the soil around the vines within a radial range of 15 to 70 cm. Most were located closer to the middle of the vineyard block than to the perimeter. During the season, pupal development in the soil took ± three weeks before the adult moths emerged. The last generation enters diapause and overwinters as pupae in the soil, only to emerge as adult moths when temperatures become favourable again and new vegetative tissue grows on the vines.

MORPHOLOGY

As described by Pretorius *et al*. (2012), adults of *A. trimenii* are of medium size, with a wingspan of approximately 54 mm. A black-grey border outlines the black forewings,

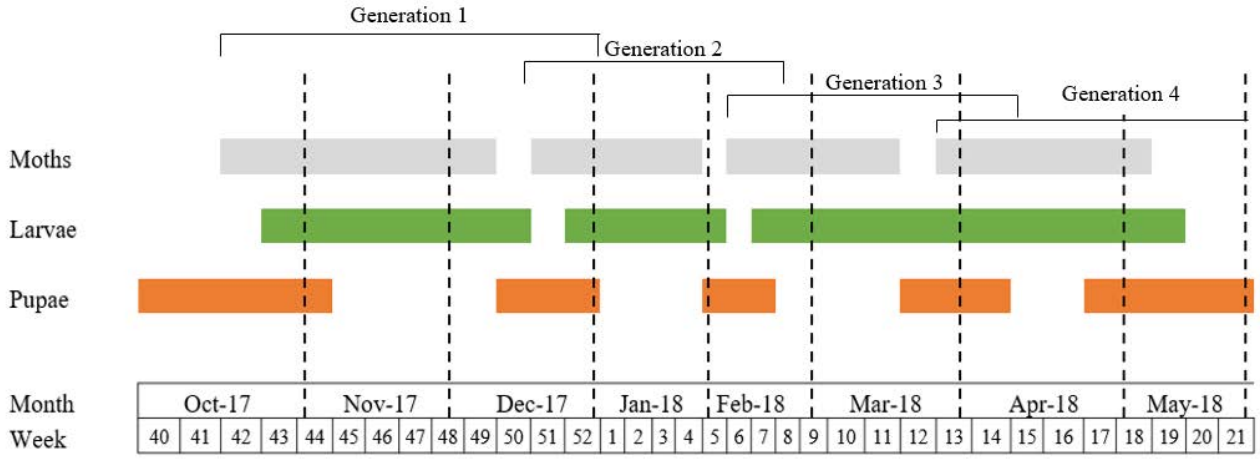

FIGURE 1

Presence of different life stages of *Agoma trimenii* from October 2017 to May 2018. Horizontal bars represent the presence of moths (grey), larvae (green) and pupae (orange)

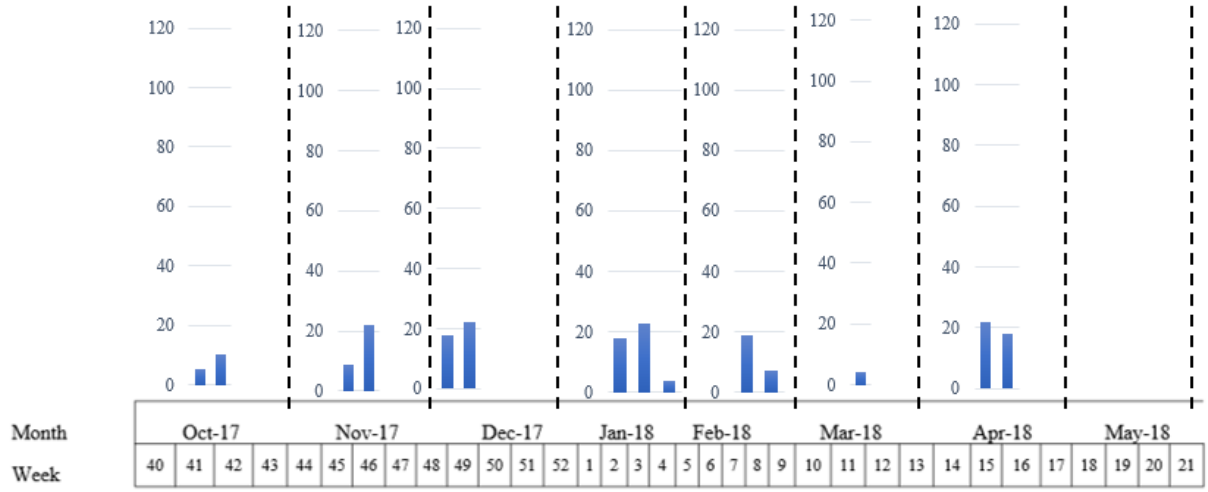

FIGURE 2

Agoma trimenii adult moth counts during monitoring period October 2017 to May 2018. Vertical bars represent moth counts over the seven- to 10-day periods per month

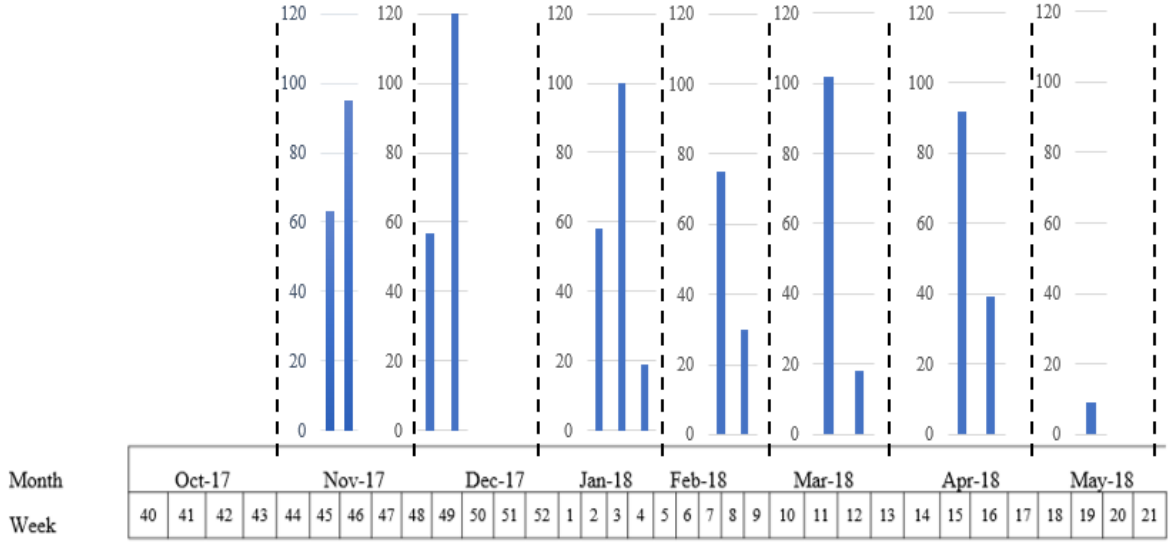

FIGURE 3

Agoma trimenii larval counts during the monitoring period October 2017 to May 2018. Vertical bars represent larvae counts over the seven- to 10-day periods per month

with a cream-coloured oval patch near the tip and a triangular cream-coloured patch near the base. The bright orange hindwings are outlined by a black border. The moth's orange-coloured abdomen matches the hindwings, with a single longitudinal black stripe running down the centre of the abdomen.

Field observations and collection of larvae and pupae in the field were used to further describe the different life stages of *A. trimenii*. The singly laid eggs are circular, with their light-yellow colour against the vine leaves making them almost impossible to see with the naked eye. The eggs become cream-coloured, with irregular brown markings, as they mature. When the first instars eclose, the larvae are off-white to yellow/orange in colour, with an orange head capsule, and ± 2 mm in length. The larvae darken as they feed, later appearing orange in colour. The subsequent larval instars increase in length and develop a distinct colouration of black stripes against the orange body. The head capsule is a red/orange colour, and the length of the body is covered in small hairs/bristles. The hairs along the length of the body are more striking in the relatively large later instar larvae. The rear end of the abdomen has a reddish hump, with two black dots (a possible defence mechanism against predators). The final instar measures ± 4.5 cm in body length, with the entire body darkening, excluding the red head capsule and the red hump at the rear end of the abdomen. It was also noted that larval instars, irrespective of age, become darker (even black) when under stress.

The number of instars is currently unknown. However, the head capsule width of 10 differently sized larvae was measured using a stereomicroscope fitted with a camera and microscope computer software (ZEISS, ZEN, Oberkochen,

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Germany). Such measurements provide some indication of the different larval instars, and can contribute to the understanding of the developmental process from instar to instar. The larval head capsule measurements ranged from 1 900 to 4 519 mm. Larvae of different sizes, collected from the field, are shown in Fig. 4 to give a general idea of the appearance of the different instars.

When final instar larvae stop feeding, they migrate to the ground via a silken thread and burrow into the soil, where they form soft-bodied prepupae which have a greenish-brown colouration. The prepupae develop into pupae protected by a hardened outer shell/cuticle, which is reddish-brown in colour, typical of most noctuids. The pupae darken as they age, eventually becoming dark brown. The approximate length of the pupae was measured at 1.9 cm, with a width of 1.3 cm. Pretorius *et al*. (2012) reported approximate pupal length as 2.5 cm. Male and female pupae can be differentiated by characteristic markings on the ventral abdominal terminal segments. Male pupae have triangular markings, while female pupae have rounded markings (Van den Berg, personal communication, 2017).

BEHAVIOUR AND DAMAGE

Observations in the field show that the early larval instars feed on the new shoots and leaves of the vines and, as they grow and develop, they feed on the older and larger leaves. Larval feeding is quite severe and, when population numbers are high, can result in complete defoliation of vines. The larvae expel a green droplet from the mouth when disturbed and, if disturbed, all the larval stages, excluding the final instar, drop from the leaves and hang suspended by a silken thread. The later instars don a characteristic pose when threatened, with the head flung back so that the thoracic legs are directed forwards. The larvae also display violent curling and uncurling, as well as rapid twisting of their body, when disturbed. The adult moths are nocturnal and most active during the night, although a few of the moths are, sporadically, visible during the day. The moths rest on vine leaves within the canopy during the day, with a few seen resting on the soil mounds surrounding the vines. Diurnal moth mating was observed.

Foliar damage is most visible from November to January. All the larval instars, excluding the final instar level, consume most of the young leaf material, including the succulent tissues, veins and midrib. Larvae measuring ± 3 cm in length tend to cause the most severe foliar damage. No alternative host plants were found in the surrounding vegetation during field observations.

TRAPPING AND MONITORING

Monitoring for insect pests is the first fundamental step to be taken in developing an effective integrated pest management (IPM) programme (Prasad & Prabhakar, 2012). The forecasting of the presence of insect pests and determining pest population density and distribution in the field are crucial components of IPM, enabling farmers to make cost-effective and environmentally sound decisions to minimise crop losses and optimise pest control (Binns & Nyrop, 1992; Prasad & Prabhakar, 2012). Pests are monitored by means of various physical trapping tools, such as pheromone, pitfall and light traps, and by visual scouting procedures. Scouting procedures include vine inspection at set time intervals and the classifying of each vine as infested or not. This is done by means of inspecting a predetermined number of leaves and/or bunches of grapes, as was done during the field observations described above (De Villiers *et al*., 2006; De Villiers & Pringle, 2008).

Pheromone traps are reliant upon a sex pheromone-based lure to attract specific insect species (Witzgall *et al*., 2010). Lepidopteran species populations, like those of the false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), and the codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae) – both significant pests of various crops in South Africa, are monitored using pheromone traps as standard practice. Although the pheromone of the Australian grapevine moth, *Phalaenoides glycinae* Lewin (Lepidoptera: Noctuidae), has not been produced for use in trapping, the semiochemicals that constitute the sex pheromone have been identified as a combination of 2-phenylethanol and phenethyl acetate (Edgar *et al*., 1979). Since *A. trimenii* and *P. glycinae* both belong to the family Agaristidae, the possibility of the two species sharing the same or similar semiochemicals was considered. A lure, based on the semiochemical ratio identified from *P. glycinae* and manufactured by Chempak (Pty) Ltd (Paarl, South Africa) was tested in the field during the 2017/2018 season

FIGURE 4
Larval stages of *Agoma trimenii*, from final instar (left) to earliest instar (right)

in yellow delta traps. Additionally, live bait traps consisting of virgin female moths of *A. trimenii* were tested in the field. Although this method of monitoring is an old practice, it still proves useful in certain monitoring situations. Further illustrations of its usefulness are described in numerous studies of different moth species, including cabbage looper moths, *Trichoplusia* (Lepidoptera: Noctuidae), and spruce budworms, *Choristoneura* (Lepidoptera: Tortricidae) (Miller & McDougall, 1973; Birch, 1977). However, neither of these attractants resulted in the capture of *A. trimenii* in baited traps in preliminary trials (data not shown).

The application of ultraviolet (UV) light traps has proven to be a promising method of surveying nocturnal moth populations. This method exploits the attraction of such moths to artificial light (Jonason *et al*., 2014). Awareness of contributing factors should increase the likelihood of selecting the right time for the setting of traps to their best advantage, so as to minimise the 'non-productive' effort exerted in the deploying of light traps when no moths are likely to be trapped (Steinbauer, 2003). As *A. trimenii* displays high nocturnal activity, the potential use of light traps was tested in the field as a possible monitoring option. A light trap emitting blue UV light with a water trap, and a solar-powered inflorescent light in the vine canopy (± 2 m high), with white sticky pads in close proximity to the emitted light so that light was reflected onto the sticky pads, were tested in the field on the two table grape farms in the Northern Cape. Traps were checked each morning for a period of three weeks during the growing season, the number of trapped moths were recorded, and the water traps or sticky pads were replaced. Observations indicated that trapping adult moths via a UV blue light trap was three times more effective than the influorescent light during the testing period, attracting and trapping up to 42 moths in one night compared to 12 moths caught by the solar-powered inflorescent light.

CONCLUSION

What we now know about *A. trimenii* sheds some light on the biology and ecology of this pest and can be implemented to improve control. Since female moths lay eggs in the top parts of the vine canopy, it is important that this part of the vine canopy is monitored closely for early signs of feeding damage. Control carried out in the early stages of infestation will minimise damage and subsequent population increase. In the absence of a pheromone lure for *A. trimenii*, light traps can be used to monitor moth activity, as well as to act as a mass-trapping control measure to reduce population numbers. As the pupae of *A. trimenii* are easy to locate and remain immobile once burrowed into the soil, testing possible control options against them is fairly easy in relation to the other life stages. The pupae cause no damage to the vines, and the overwintering period may be an ideal time for the application of potential biological control agents. However, the protective cuticle of the pupae may inhibit the efficacy of control, and targeted control at the soft-bodied prepupae may be a more viable option. However, ensuring that the control measures act during the short prepupal stage could be challenging. Since control measures against this pest are lacking, it is recommended that an IPM strategy be developed, and the information presented here provides a platform from which an integrated approach for the control of *A. trimenii* can be developed.

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Evaluation of South African Chenin Blanc Wines Made From Six Different Trellising Systems Using a Chemical and Sensorial Approach

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Key words: Trellising systems, wine, sensory evaluation, chemical composition, check-all-that-apply (CATA), high-resolution mass spectrometry (HRMS), Chenin blanc

There are many ways of manipulating the components of grape berries and one of these is the implementation of a specific trellising system. This will affect light exposure in the bunch zone, air flow through the canopy, crop load, etc., and consequently the primary metabolites that contribute to the production of secondary compounds in wine. The aim of the present study was to characterise the grape musts and wines of Chenin blanc made from grapes of different trellising systems, using chemical fingerprinting of the samples and the check-all-that-apply method, combined with a quality scoring test. The results indicate that, while the wines could not be separated according to treatment from an aroma point of view, the grapes produced by different trellis systems resulted in wines characterised by differences in taste and mouthfeel. The present study shows that trellising systems can influence amino acids, yeast assimilable nitrogen, phenolic content and aroma compounds, as well as sensory characteristics. In this case study, wine quality was not affected by the trellising systems, with one exception. Fingerprinting using high-resolution mass spectrometry proved to be a successful tool to separate the samples according to the systems.

INTRODUCTION

Chenin blanc belongs to a group of grape varieties defined as 'neutral' (Augustyn & Rapp, 1982); for this reason, the style of the resulting wines is dictated by the winemaking process, as well as the manipulation of the vines' microclimate. Young Chenin blanc wines exhibit a fruitlike aroma as a result of volatile esters formed during fermentation, but additional or different aromas can be induced by canopy management practices aimed at modifying the physiology of the grapevine, and therefore some of the derived grape precursors (Reynolds & Vanden Heuvel, 2009). Furthermore, the choice of yeast strain (Reynolds *et al*., 2001), yeast strain nutrition (Van Rooyen & Tromp, 2017), skin-contact time (Marais & Rapp, 1988) and pressing (Somers & Pocock, 2015) are amongst the oenological practices that alter the content and concentration of volatiles and non-volatiles in juices or wines.

One of the significant ways of manipulating the canopy, and subsequently the grape, must and wine composition and sensory profile, is modifying the architecture of the vines with different trellising systems. Generally, training systems make a difference in maintaining a balance between the fruit-producing parts and the energy-producing structure, different degrees of exposure to light in the bunch zone (Marais *et al*., 1992), as well as proper air flow through the canopy to avoid conditions favourable for fungal infections (Van Zyl & Van Huyssteen, 1980b). In the specific case of sun exposure, such factors affect the content of vine metabolites constituting the grape volatile profile and aroma reservoir (Reynolds *et al*., 1996), and consequently wine aroma (Zoecklein *et al*., 2008). Many of these influences occur in aromatic varieties such as Riesling (Reynolds *et al*., 1996), Viognier (Zoecklein *et al*., 2008), Sauvignon blanc (Marais *et al*., 1999) and, to some extent, in Chardonnay grapes (Zoecklein *et al*., 1998). It has been demonstrated that training systems influence grape quality components such as sugars, acids, phenols and primary aroma compounds (Reynolds *et al*., 2004; Ji & Dami, 2008; Zoecklein *et al*., 2008). However, assessing the evolution of these components through to wine is not as complete.

The overall quality of wine is determined by several properties, including colour, aroma and taste perceptions, which are all equally important for consumer acceptance (Charters & Pettigrew, 2006). The aroma profile of a wine results from a combination of various compounds present in the grapes or derived from the fermentation and ageing processes (Ribéreau-Gayon *et al*., 2006). A lot of volatile compounds are present in low concentrations (µg/L and lower); however, they play a significant role in nuances in

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wine aroma. These compounds emerge from heterogeneous classes such as alcohols, esters, acids, terpenes, phenols and aldehydes, as well as sulphur compounds (Ferreira *et al*., 2000; Jeffery *et al*., 2012).

Neutral varieties like Chenin blanc obtain aromas from the fermentation process (Du Plessis & Augustyn, 1981; Augustyn & Rapp, 1982), and thiols have also been demonstrated to contribute to wine aroma (Wilson, 2017). Moreover, thiol precursors are one of the classes of molecules influenced by various vineyard practices (Kobayashi et al., 2011). Practices such as leaf removal have a significant influence on grape composition and wine quality (Marais et al. 1992, 1999). The ability of the trellis to expose canopies to sunlight and eventually to the impact of the surrounding environment, and the accumulation of organic compounds (Van Zyl & Van Huyssteen, 1980a, 1980b) also influence wine quality (Volschenk & Hunter, 2001).

To assess the quality of food and beverages, a number of sensory evaluation methods have been used (Lawless & Heymann, 2010). In the evaluation of wines, the judgment of quality is consigned to winemakers or experts. Quality-judging systems have been applied based on points, a popular method being the 20-point scale developed by the University of California Davis to evaluate wine sensory properties for quality control and commercial purposes. In this method, points are assigned in sensory categories such as appearance, aroma, taste and overall quality, with a possible total of 20 points. Most studies have used this system to assess the quality aspects of wine. However, although this method is suitable for general quality assessment, it may not distinguish among the group of wines of high quality, since it is based on penalisation for defects from the typicality of the wine style. Also, points allocation for each category gives a different weight to the sensory characteristics, which may or may not reflect their importance in the overall quality.

To address these problems, descriptive analysis (DA) can be coupled with this system to characterise sensory differences in wines across multiple attributes, as has been demonstrated for Cabernet Sauvignon and Chardonnay wines (Niimi *et al*., 2018). DA uses both qualitative and quantitative methods in the evaluation of a product, but also has some drawbacks: panellists need training, so it is time-consuming and leads to additional costs. Therefore, researchers have developed alternative applicable methods for the characterisation of wines that produce similar results but use rapid techniques (Valentin *et al*., 2012).

One of the alternative methods that have gained popularity is check all that apply (CATA), originally used in marketing (Rasinski *et al*., 2002) and subsequently proposed as an alternative method in the food industry to gather information about consumers' perceptions (Adams *et al*., 2007). CATA is a rapid sensory-profiling technique that uses a questionnaire consisting of a list of attributes (in the form of words or phrases), from which trained or untrained panellists can select all the descriptors they consider appropriate to characterise each sample (Valentin *et al*., 2012). In the South African wine industry, CATA has been used to characterise the aroma profile of Chenin blanc in both experimental (Botha, 2015) and commercial wines (Buica & Panzeri, 2018), as well as in Pinotage commercial wines (Panzeri *et al*., 2019).

The main analytical technique applied for the investigation of volatile compounds in wine is gas chromatography (GC), coupled with FID or mass spectrometry (MS). Although this technique is applicable for targeted and untargeted analysis, it has limitations and disadvantages, such as the inability to directly identify non-volatiles and the high cost of the equipment.

In cases where a list of analysis is incomplete, and also when wine matrices have a significant effect on suppressing or enhancing aromatic expression, it is worthwhile exploring additional methods. The most viable approach is untargeted metabolomics, a comprehensive analysis of metabolites that reveals a chemical fingerprint. Metabolic profiling has been successful in characterising grape and wine typicality and quality (Atanassov *et al*., 2009), and profiling wine according to variety (Vaclavik *et al*., 2011) and phenolic compounds (Salvatore *et al*., 2013). Fingerprinting allows the extraction of hidden information from the acquired multidimensional data, for instance to authenticate wine using LC-HRMS (Rubert *et al*., 2014) or attribute wine styles to commercial Chenin blanc (Buica *et al*., 2017).

The aim of this study was to investigate the effects of canopy microclimate manipulation through trellising systems on the chemical and sensory profiles of Chenin blanc wines. From a chemical point of view, and despite more readily available advanced analytical methods for the identification and quantification of the chemical composition of wines together with multivariate analysis, no work has been done on the characterisation of wines made from different trellising systems to date. To achieve the profiling of the products, wine fingerprinting by LC-HRMS was done, coupled with principal component analysis (PCA) and hierarchy cluster analysis (HCA). From a sensory point of view, the hypothesis proposed was that aroma, taste and mouthfeel are affected by changes in the canopy. While previous studies have assessed the impact of oenological and other viticulture aspects from a chemical point of view, there is no study that has evaluated the sensory profile of wines from different trellising systems. Given the previously outlined advantages of the method, CATA was chosen for this study. In addition, quality rating with industry experts was investigated to evaluate the effect of yield variation on the marketable characteristics of the wines produced.

MATERIALS AND METHODS

Experimental vineyard

Grapevines (*Vitis vinifera* L. cv. Chenin blanc clone SN 24B grafted onto 110R rootstock) were planted in a single block in 2010 and trained to six different systems, namely: Santorini (S), Ballerina (B), Smart Dyson (SD), T-Frame (TF), Lyre (L) and 'Stok-by-Paaltjie' (P), also known as 'staked vines' or 'Echalas'. Each system was planted in a different row. The vineyard is located on one of the oldest Cape Dutch farms in the Franschhoek valley region, Western Cape, South Africa (33°49'23.4''S latitude and 18°55'29.4"E longitude). The experiment was conducted over two vintages, namely 2017 and 2018, as listed in Table 1. All vineyard practices, including irrigation and pruning, were applied uniformly to all treatments by the estate.

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TABLE 1
Codes used for the trellising systems, number of vines per system, winemaking repeats, vintages included in the project, and basic oenological parameters for musts and wines. All wines made in triplicate, with the exception of P (2017).

		Vintage											
		2017											
		Must			Wine			Must			Wine		
Trellis	No of vines	Brix	pH	TA (g/L)	Alcohol (%)	pH	TA (g/L)	Brix	pH	TA (g/L)	Alcohol (%)	pH	TA (g/L)
Santorini (S)	28	17.8	3.6	8.4	10.2	3.3	5.3	21.1	3.7	5.0	12.7	3.4	3.8
Ballerina (B)	27	22.6	3.6	8.1	12.7	3.3	4.6	23.0	3.9	4.6	13.8	3.4	4.0
Smart Dyson (SD)	27	23.1	3.5	8.3	13.6	3.4	4.8	24.0	3.8	5.5	14.1	3.6	3.9
T-Frame (TF)	24	23.9	3.7	6.9	14.6	3.7	4.1	23.5	3.9	5.9	13.9	3.4	3.9
Lyre (L)	20	23.2	3.6	6.3	14.1	3.8	4.3	23.6	3.9	5.8	13.8	3.6	3.9
Stok-by-Paaltjie (P)	10							-	-	-	-	-	-

Vinification process and wines

Grapes were harvested at 22 ± 0.5°B in both vintages. Harvested grapes from the vineyard were transported to the experimental cellar of the Department of Viticulture and Oenology (DVO) of Stellenbosch University. The grapes were weighed and the yield of each type of trellising system was recorded, after which the grapes were refrigerated overnight at 4°C. The following day, the grapes were destemmed and crushed with the addition of 40 mg/L SO_2 and 0.03 g/kg of pectolytic enzyme (Lafazym Extract enzyme, Laffort, South Africa). Skin contact was allowed for two to three hours. Pressing was done by vertical hydro-press at one cycle up to 1 bar. Rapidase® Clear Enzyme, at 4 mL/100 L (Laffort, South Africa), was added to the juice and left overnight in a 4°C refrigerated room to help juice settling and clarification. Biological repeats were separated in the cellar before inoculation. The must was treated with 50 mg/L SO_2, inoculated with *Saccharomyces cerevisiae* strains Vin7 and Vin13 (ANCHOR YEAST Zymasil®, AEB Group SpA, Bologna, Italy) in a ratio of 50:50, previously rehydrated according to the manufacturer's instructions, and then transferred into 20 L stainless-steel tanks for vinification. All wines were made in triplicate, except for the P treatment in the 2017 season, as there was only enough crop to make two replicates. Fermentation was carried out at 15°C until completion (about 14 days). Wines were racked into 20 L stainless-steel tanks and placed at 15°C. All wines were left in contact with the fine lees for three months prior to bottling and were gently stirred twice a week without opening the canisters to avoid oxidation. After this period, the wines were racked off and 50 g/hL of bentonite was added prior to cold stabilisation. The tanks were placed in a -4°C refrigeration room for two weeks. The cold-stabilised wines were bottled and stored at 15°C for six months until the chemical and sensory analyses were performed. Wine evaluations are summarised in Fig. 1.

Chemical analyses

Oenological parameters

Grape berries were monitored before harvesting and analysed after crushing for sugar concentration (Brix), using a hand-held refractometer (PAL1, Atago). pH and TA were measured with a potentiometric titrator (702 SM Titrino, Metrohm). Wine ethanol was quantified by infrared spectroscopy using the Winescan FT120 spectrometer (FOSS Analytical, Denmark), and in-house calibrations were done as described by Nieuwoudt *et al*. (2004).

Nitrogen composition

Racked juice samples were analysed for ammonium and free amino nitrogen (FAN), the sum of which gives yeast assimilable nitrogen (YAN). The analysis was done at VinLab (Stellenbosch) for both the 2017 and 2018 harvest years, using enzymatic methods according to ISO 17025 standards.

Twenty amino acids were quantified for the 2017 juice samples as described in Petrovic *et al*. (2019), using a derivatisation method based on labelling with AccQTag© (Waters), with Norvaline (Nvl) as internal standard, followed by determination by LC-UV/Vis at the Mass Spectrometry

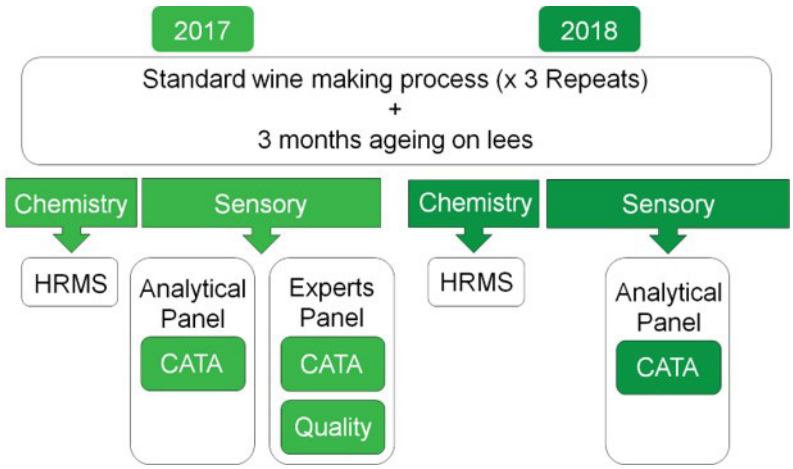

FIGURE 1
Workflow indicating winemaking repeats, the stage at which wine evaluation took place, and the chemical and sensory tests performed

Unit of the Central Analytical Facility of Stellenbosch University. Alanine, arginine, asparagine, glutamic acid, glutamine, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, valine, gamma-aminobutyric acid (GABA) and ornithine were quantified.

Major volatiles

Wines samples for 2017 and 2018 were quantified for major volatiles using the GC-FID method described by Louw *et al*. (2010). In brief, 5 mL wine samples were spiked with methyl-pentanol as internal standard, and extracted with 1 mL ether. The extract was dried over anhydrous Na_2SO_4 and injected in duplicate into a GC-FID (HP-6890, Agilent).

Thiols

The volatile thiols, 3-mercaptohexan-1-ol (3MH), methyl-4-mercaptopentan-2-one (4MMP) and 3-mercapto-hexylacetate (3MHA), were quantified following the method of Mafata *et al*. (2018), using DTDP derivatisation, SPE sample clean-up, and injection into a convergence chromatography-tandem mass spectrometry instrument (UPC2-MS/MS, Waters).

High-resolution mass spectrometry (HRMS)

HRMS coupled with liquid chromatography (LC-HRMS) was used for wine fingerprinting. The samples were analysed by UPLC (Waters Corporation) equipped with a Synapt G2 quadrupole time-of-flight mass spectrometer (Waters Corporation). The separation was done on an Acquity UPLC HSS T3 column (1.8 µm internal diameter, 2.1 mm x 100 mm, Waters Corporation) using 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B), and a scouting gradient. The flow rate was 0.3 mL/min and the column temperature was 55°C. The injection volume was 2 µL. The software is integrated directly with SIMCA-P (Umetrics) and the statistical algorithms are applied directly to the processed datasets (Buica *et al*., 2017).

Sensory evaluation

Panels for sensory evaluation

Two separate groups of participants were selected for the project: a panel of thirty industry experts and a panel of ten analytical (trained) panellists. Experts were recruited on the basis of their experience, interest and availability. The age group varied from 26 years to 45 years old (six women and 24 men, 30 answers in total). Experts only assessed wines of the 2017 vintage. The analytical panellists were selected based on their experience in wine profiling using multiple sensory methods and were often recruited by the Department of Viticulture and Oenology (Stellenbosch University) for sensory evaluations. Their ages varied from 26 to 66 years old; in 2017 there were 10 women , whereas in 2018 the panel consisted of eight women and two men.

Sensory sessions

The evaluation was done six months after bottling. Prior to sensory evaluation by experts, the wines were screened and wines from the same treatment were blended. Blending was done to meet the volume of wine required, and also for quality-assessment reasons. Therefore, the experts evaluated six wines (blended) representing six trellising systems, while the analytical panel evaluated the six wines with their biological repeats.

The sensory tests were carried out in two separate sessions. The first session involved the industry experts and was carried out at the Paul van der Byl Laboratory (Stellenbosch University) in a well-ventilated, naturally lit room kept at ± 20°C. Experts were tasked with evaluating aroma, taste and mouthfeel using the CATA method and, secondly, evaluating the quality using the 20-point scale method. The second session involved the analytical panellists and was carried out in the sensory laboratory of the Department of Viticulture and Oenology of Stellenbosch University. The laboratory is designed specifically for sensory analysis (ISO 8589) and contains individual tasting booths in which the temperature and humidity are controlled. For the CATA method, both experts and analytical panellists used black glasses, and wine

samples were poured 20 minutes prior to testing and covered with Petri dishes. In addition, the expert tasters were served a supplementary set of the same wines in clear ISO glasses for quality scoring in order to allow them to evaluate the appearance of the samples. Twenty millilitre samples were dispensed using a measuring device and maintained at a temperature of 20°C.

CATA aroma terms used in this study were selected from the South African Chenin blanc aroma wheel. The taste and mouthfeel attributes were chosen by a focus group after a preliminary screening of the wine samples. The total list of descriptors used for this exercise comprised 40 words. The analytical and expert panels were instructed to evaluate aroma as well as taste, and to check all the terms they considered appropriate for describing each sample. The samples were coded with individual three-digit codes and randomised across panellists according to a William Latin square design. With the experts, the exercises were conducted in one day. They evaluated two flights: in the first flight, they were asked to evaluate aroma and taste, and in the second flight wine quality based on the three aspects (appearance, aroma and taste) using the 20-point scorecard. The analytical panel was only tasked with evaluating aroma and taste, done in three flights over three days (three technical repeats, resulting in 30 answers).

Statistical analysis

Chemical data

Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were applied in order to find natural configurations in the data according to treatments and samples by grouping/clustering (SIMCA 14.1, Umetrics, Sweden). Additional data analysis and graphical representations were performed using Microsoft Excel 2013.

Sensory data

Data was captured using Compusense® at-hand software (West Guelph, Ontario, Canada) and analysed on XLStat 2018.5 (Microsoft, www.xlstat.com). Correspondence analysis (CA) was performed on a contingency table using Statistica® 13.3 software. Graphical representations of the sensory profiles, including aroma, taste and mouthfeel characteristics, were provided as bi-plots by plotting the mean values for the sensory descriptors. Least significant differences (LSD) were calculated between wines by analysis of variance (ANOVA) using the Statistica ®13.3 program, and the results were evaluated at the 95% confidence level for quality scores.

RESULTS AND DISCUSSION

Results of chemical evaluation

Nitrogen composition of the must

YAN levels of the musts were higher in 2018, ranging from 270 mg N/L to 353 mg N/L, compared to 2017, with a range of 173 mg N/L to 267 mg N/L. All values were above the "critical level" of 140 to 150 mg N/L (Table 2). The concentration differed significantly between the systems in 2017, whereas there were no significant differences in 2018. The juices from the L system had the highest level of YAN concentration on average, at 247 mg N/L and 353 mg N/L in the two seasons, respectively, while the lowest YAN concentration was found in the SD system, at an average of 173 mg N/L, and the S system, at 270 mg N/L, for 2017 and 2018, respectively.

The current results are in agreement with the average concentration of free amino nitrogen and ammonia concentration in South African Chenin blanc must (Petrovic *et al*., 2019). S had the highest concentration, at 70 mg N/L, while SD had the lowest in the 2017 season. In the second season, 2018, L had the highest concentration, at 90 mg N/L, whereas S had the lowest, at 70 mg N/L, although this remained the same as in the previous year. Overall, the ammonia concentrations for 2018 increased from those of the previous year, with the exception of the S system.

It can be hypothesised that vintage effect could have played a role in this instance. The concentration of free amino acids varied between systems, with an average of 143 mg N/L (B) and 207 mg N/L (L) for 2017 and 200 mg/ N/L (S) and 260 mg N/L (L) for 2018. Previously, from the point of view of canopy manipulation (shoot positioning, defoliation, topping and suckering), no variation was observed between the concentrations of FAN in the must of Chenin blanc from different seasonal practices (Volschenk & Hunter, 2001). Other than that, there are no other reports on the effects of trellising systems on free amino nitrogen.

ANOVA showed statistical differences between the juices based on specific amino acids (Table 3). Amino acids (AA) can be grouped according to the order in which yeast (*Saccharomyces* spp.) metabolises them. The group of yeast-preferred amino acids consists of individual amino acids

TABLE 2
The yeast assimilable nitrogen (YAN), free amino nitrogen (FAN) and ammonia concentrations in the must.

Trellis	FAN (mg N/L)		NH_4 (mg N/L)		YAN (mg N/L)	
	2017	2018	2017	2018	2017	2018
S	177	200	70	70	247	270
B	143	230	50	80	193	310
SD	133	240	40	80	173	320
TF	173	250	50	80	223	330
L	207	260	60	90	267	353

TABLE 3
The concentration in mg N/L of the 20 amino acids measured in the musts of the six trellising systems in 2017. Significant differences ($p < 0.05$) are designated by different letters.

Amino acid	**Trellis**				
Yeast preferred	**S**	**B**	**SD**	**TF**	**L**
Alanine	96.2bc	79.3c	77.4c	113.5b	180.7a
Arginine	353.6ab	285.2bc	242.7c	305.0bc	424.1a
Aspartic acid	101.2a	66.2bc	66.6bc	52.3c	85.5ab
Glutamic acid	104.7c	111.9bc	112.8bc	128.3b	169.3a
Glutamine	80.4b	60.8c	48.9c	76.4b	100.8b
Serine	58.5b	57.6b	58.4b	77.9a	85.3a
Branched chain and aromatic amino acids					
Valine	24.6cd	29.3bc	22.2d	39.4a	34.1b
Leucine	26.9b	25.3b	21.1b	36.1a	27.3b
Isoleucine	12.5b	15.7ab	12.1b	19.2a	14.9ab
Phenylalanine	25.5b	36.5ab	25.7b	43.3a	32.6ab
Tryptophan	95.6b	132.6 ab	82.1b	188.8a	101.2b
Others					
Hydroxyproline	2.4b	8.3a	6.4ab	8.3a	9.4a
Proline	170.6d	297.5c	306.6c	534.1b	676.4a
Methionine	1.0a	1.3a	0.1a	1.7a	0.1a
Lysine	2.6a	3.0a	2.7a	4.0a	4.1a
Threonine	101.3b	112.9ab	95.3b	121.5a	124.6a
Glycine	2.5a	3.1a	2.8a	3.8a	4.3a
Histidine	32.9a	22.2b	22.2b	30.4a	31.0a
Ornithine	1.9a	0.3b	0.0b	0.4b	1.9a
GABA	34.8c	38.8c	48.9bc	64.9ab	70.5a

such as alanine (ALA), arginine (ARG), aspartic acid (ASP), glutamic acid (GLU), glutamine (GLN) and serine (SER) (Ljungdahl & Daignan Fornier, 2012). This group was found to have the highest concentration in the L system must, with glutamic acid, glutamine and alanine significantly higher than in the other systems.

Another subgroup of AA is branched chain and aromatic amino acids (BCAAs, valine (VAL), leucine (LEU), isoleucine (ILE), phenylalanine (PHE) and tryptophan (TRP)). These amino acids play an important role as precursors of certain aroma compounds (Bell & Henschke, 2005). The TF trellis produced juices with a significantly higher concentration of valine and leucine, and they were also higher in the other three BCAAs, although not significantly so, whereas the SD trellis was found to have the lowest concentration of BCCAs.

The data shown in Table 3 illustrates that concentrations of the secondary amino acids proline (PRO) and hydroxyproline (HYP) were significantly higher in the musts of the L system (676 mg N/L), while S had the lowest concentrations (170 mg N/L). Even if the concentration of proline is the highest among amino acids, secondary AA are usually not metabolised by yeast. However, proline is seen as an indicator of stress in the vineyard, as found by Ashraf and Foolad (2007). A similar trend is seen in other amino acids (GABA, ornithine (ORN), and threonine (THR)). Notably, the juices from the S system were significantly higher only in histidine (HIS), while the SD and B systems were recorded to have the lowest average value.

The PCA generated using the AA concentrations (Fig. 2, PC1 39.4% and PC2 21.2%, respectively) showed that the samples belonging to one system tended to group together, but samples from different systems were also interposed, as demonstrated by cluster analysis.

Amino acid concentration varies according to cultivar (Kliewer, 1970); it is also known that trellis type can influence grape amino acid composition and concentration through differences in either bunch or leaf exposure to light (Kliewer *et al.*, 1991). In the present study, it can be

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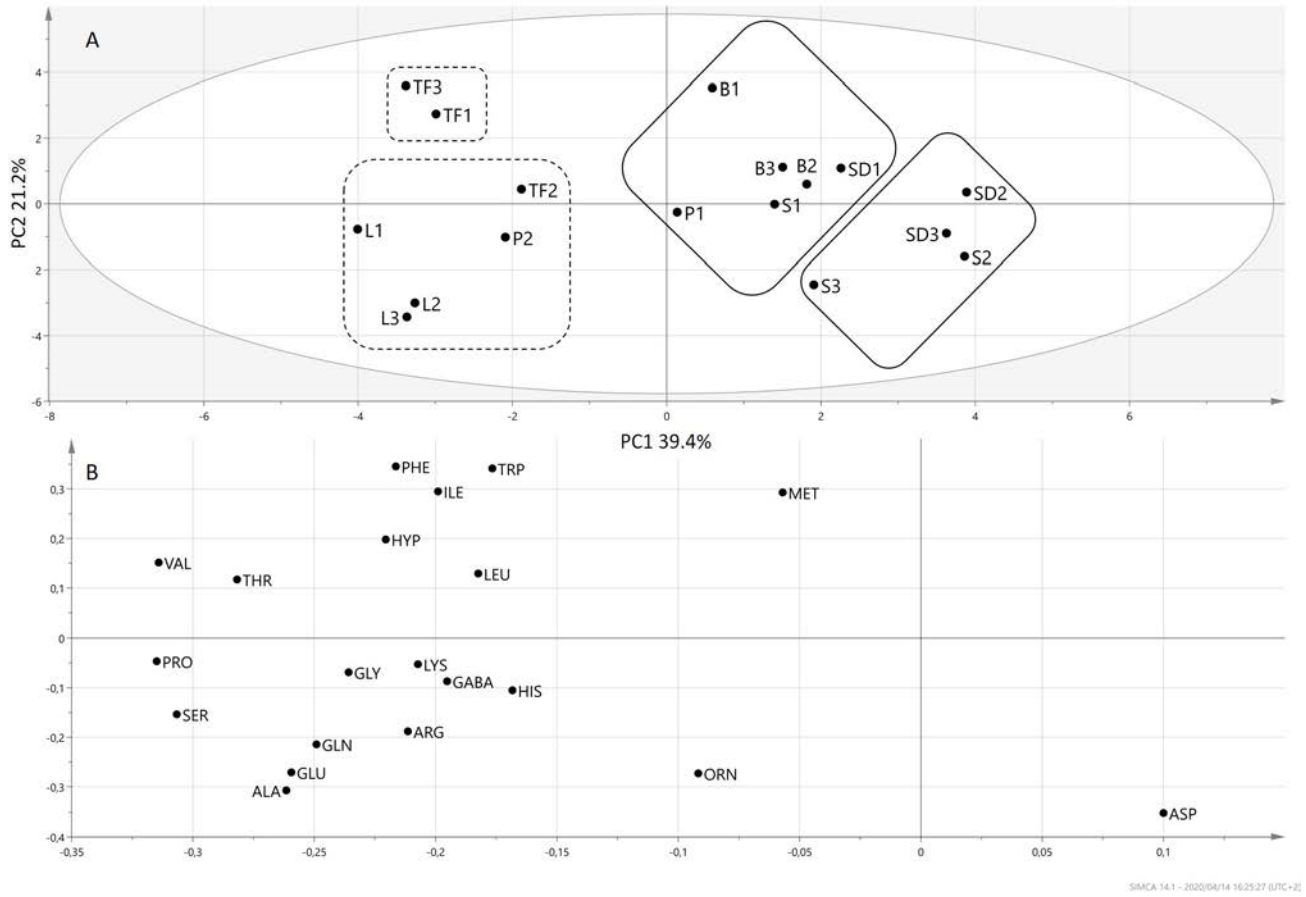

FIGURE 2

Plots of principal component analysis scores (top) and loadings (bottom) for the amino acid composition of the 2017 must. Grayscale in the scores plot codes according to trellis system. The groupings in the scores plot are designated according to the hierarchical cluster analysis (HCA) of the same data, and the different/similar outlines indicate level of distances between groups based on the HCA dendrogram

speculated that a particular trellis also resulted in specific leaf and bunch exposure, which possibly influenced the concentration of individual amino acids. Looking at the similarities in multivariate and ANOVA, it could mean that the effect of the trellising on the concentration of amino acids played a consistent role only in the L and S systems. Pereira *et al*. (2006) found higher amino acid concentrations in the juice of unshaded berries of Merlot compared to shaded berries. Other studies (Friedel *et al*., 2015) have also provided evidence that sun exposure through leaf and bunch removal increases the amino acids of Riesling grapes. However, the variety itself could play a role in its response to factors such as light; for example, Gregan *et al*. (2012) found a reduction in total amino acids in berries exposed to sunlight in Sauvignon blanc. However, other studies, such as that of Šuklje *et al*. (2016), have confirmed that the differences in amino acid concentration were also due to the different clones' responses to bunch exposure. As mentioned earlier, TF musts specifically were significantly higher in BCAAs. TF trellises have open canopies, which may result in the berries being exposed to better light interception, consequently affecting amino acid metabolism. Similarly, Pereira *et al*. (2006) also found a higher level of BCAAs (valine and leucine) in sun-exposed berries of Merlot.

Aroma composition

A total of 25 major volatile compounds were identified and quantified in the Chenin blanc wines using GC-FID; for discussion, they have been classified into five groups: esters, acetates, ethyl esters, acids and alcohols, according to their functional groups and metabolic formation (Tables 4A and 4B). Even though differences were found between the systems for the individual compounds and classes of the major volatiles, none of them were statistically significant. Another class of odour compounds measured were thiols (Tables 4A and 4B). Again, the samples did not differ significantly in 2017; ANOVA indicated significant differences between the systems for 3MHA in 2018, but only for the highest concentration (Table 4B).

PCA was performed using all the data on the 2017 volatiles (major volatiles and thiols, PC1 37.9% and PC2 18.8%, respectively), to see if there was any grouping based on these compounds (Fig. 3 top). PCA showed a pattern in which the P and L samples were grouped together (with two of the TF samples), separate from the rest of the system samples along PC1. Furthermore, the loading plots (Fig. 3 bottom) showed no discriminant factor to produce clearer groupings based on the HCA results. As shown by ANOVA, the two treatments were the highest in total volatiles

(Table 4A).

PCA was also performed on the volatiles using major volatile compounds and thiol concentrations for 2018 (results not shown). No separation of wines was observed according to the treatment (PC1 31.6% and PC2 24.7%, respectively) Despite the significant differences for 3MHA in the L wines, this was not a strong enough discriminant factor to separate these samples in the PCA. Similarly, the cluster analysis and loadings showed no specific compound to be highly associated with specific wines or groupings based on trellising system.

Aroma compounds come from either grapes, the fermentation processes or ageing. Those derived from grapes are likely to be influenced by environmental conditions, including as a result of training system (Zoecklein *et al*., 2008). Most importantly, light or sunlight exposure is one of the factors that affect the accumulation and synthesis of aroma-related compounds (Ford, 2007). From previous work, it can be seen that UV-C light irradiation amplifies the thiol precursors (Kobayashi *et al*., 2011); however, it is not fully understood how there is a correlation between thiol precursors and wine thiols. Parish-Virtue *et al*. (2019) reported a positive response of light on Sauvignon Blanc from grapes to the corresponding wines.

It is hypothesised from the literature that a similar design exposes the fruit zone to light intensity and influences the type and level of chemical compounds synthesised, consequently affecting the aroma profile (Šuklje *et al*., 2016). This only applies to compounds directly affected by sun exposure, in this case thiols. Light (for example driven by trellising systems) may have induced variations in the concentration of 3MHA. However, this effect may be revised in the case of the current study, because 3MHA was not detected in the wines from the L system in the first season.

Looking at other treatments, there was an increase in the concentration of 3MHA from the first season to the second, which is similar to the findings of Drenjančević *et al*. (2018) and Louw *et al*. (2010), who demonstrated that vintage is the

TABLE 4A

Concentrations of major volatiles (μg/L) and thiol compounds (ng/L) in wines made from the six trellising systems (2017 vintage). 4MMP was not detected in the 2017 wines. Significant differences indicated by different letters.

Compounds	**S**	**B**	**SD**	**TF**	**L**	**P**
Ethyl acetate	29.17	36.45	41.52	48.86	68.31	48.97
Ethyl lactate	6.19	4.86	5.15	3.74	4.64	3.74
Ethyl caprylate	1.61	1.46	1.16	1.28	1.60	1.16
Ethyl caprate	1.67	1.42	1.37	1.54	2.0	1.95
Ethyl phenylacetate	1.03	1.07	1.18	1.36	1.40	1.67
Ethyl hexanoate	6.80	6.80	9.46	6.63	1.08	9.50
2-Phenylethyl acetate	4.88	4.26	3.38	3.59	4.05	3.41
Diethyl succinate	2.62	2.94	2.59	3.06	3.21	3.08
Isoamyl acetate	5.13	5.27	4.88	5.18	6.05	5.28
Isobutanol	21.91	19.90	25.86	31.29	32.59	35.60
Pentanol	5.74	6.10	5.74	7.57	7.45	6.29
Isoamyl alcohol	170.12	157.27	183.06	173.88	176.49	204.26
Hexanol	1.40	4.01	6.73	5.94	8.22	6.94
Butanol	4.34	7.40	8.12	4.11	1.60	1.21
Propanol	21.25	18.67	18.75	30.80	52.47	33.64
2-Phenyl ethanol	4.08	3.83	3.13	2.38	1.78a	2.04
Propionic acid	1.37	1.49	1.73	2.23	2.62	2.29
Isobutyric acid	1.59	1.15	1.45	1.46	1.70	2.25
Butyric acid	1.61	1.16	1.39	3.97	1.32	2.05
Isovaleric acid	5.79	3.91	1.24	1.40	1.55	1.47
Valeric acid	5.84	5.34	5.98	1.65	2.35	2.05a
Hexanoic acid	24.36	25.33	19.55	26.55	22.17	31.6
Octanoic acid	4.69	6.14	6.70	5.42	5.61	5.13
Decanoic acid	4.69	6.14	6.70	5.42	5.61	5.13

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TABLE 4A (CONTINUED)

Compounds	S	B	SD	TF	L	P
Major volatile groups						
Total volatiles	341.22	337.52	371.66	381.13	421.60	425.84
Esters	62.46	69.69	75.53	77.06	98.06	83.86
Acetates	39.18a	45.98	49.78	57.63	78.41	57.66
Ethyl esters	52.45	60.16	67.27	68.29	87.96	75.17
Acids	49.94	50.65	44.75	48.11	42.93	52.01
Alcohols	228.82	217.18	251.38	255.96	280.61	289.97
Thiols						
3MH	150	354	112	340	338	135
3MHA	0.2	34	17.6	16	n.q.	1.52

n.q. – not quantified

TABLE 4B
Concentrations of major volatiles (µg/L) and thiol compounds (ng/L) in wines made from the six trellising systems (2018 vintage). Significant differences indicated by different letters.

Compounds	S	B	SD	TF	L
Ethyl acetate	57.24	84.78	69.19	78.22	81.30
Ethyl lactate	3.35	9.13	2.01	2.15	2.24
Ethyl caprylate	2.28	2.40	1.97	2.05	2.19
Ethyl caprate	3.25	3.84	3.69	3.83	4.00
Ethyl phenelthylacetate	1.27	1.34	1.50	1.33	1.39
Ethyl hexanoate	1.14	1.15	1.13	1.15	1.17
2-Phenylethyl acetate	5.00	6.95	4.43	5.51	5.03
Diethyl succinate	4.14	2.22	3.69	3.85	3.13
Isoamyl acetate	6.17	7.95	6.70	7.98	8.32
Isobutanol	31.18	40.71	34.85	34.95	32.04
Pentanol	8.63	1.15	1.02	4.01	8.97
Isoamyl alcohol	203.93	219.43	211.97	203.56	209.54
Hexanol	8.82	7.55	7.86	7.52	7.31
Butanol	7.09	1.13	3.82	3.76	4.01
Propanol	42.43	63.96	63.95	71.20	79.47
2-Phenyl ethanol	35.26	38.96	28.55	26.58	22.88
Propionic acid	2.07	2.94	2.88	2.64	2.90
Isobutyric acid	1.69	1.86	1.65	1.66	1.57
Butyric acid	1.33	1.48	1.37	1.33	1.42
Iso-valeric acid	2.37	4.01	3.65	6.42	8.95
Valeric acid	3.30	3.62	3.58	4.05	3.47
Hexanoic acid	3.29	4.35	3.93	4.29	4.50
Octanoic acid	4.12	4.17	3.91	4.36	4.62
Decanoic acid	8.82	8.96	8.63	1.02	3.58

TABLE 4B (CONTINUED)

Major volatile groups					
Total volatiles	454.06	530.23	480.81	489.98	510.53
Esters	89.72	125.95	99.20	112.63	115.31
Acetates	68.40	99.68	80.31	91.71	94.64
Ethyl esters	78.56	111.05	88.08	99.14	101.97
Acids	26.99	31.39	29.60	25.78	31.00
Alcohols	337.34	372.89	352.01	351.58	364.22
Thiols					
3MH	334	390	307	298	292
3MHA	37.2b	38.4b	29.5b	37.3b	63.4a
4MMP	2.41	2.50	2.54	2.38	2.56

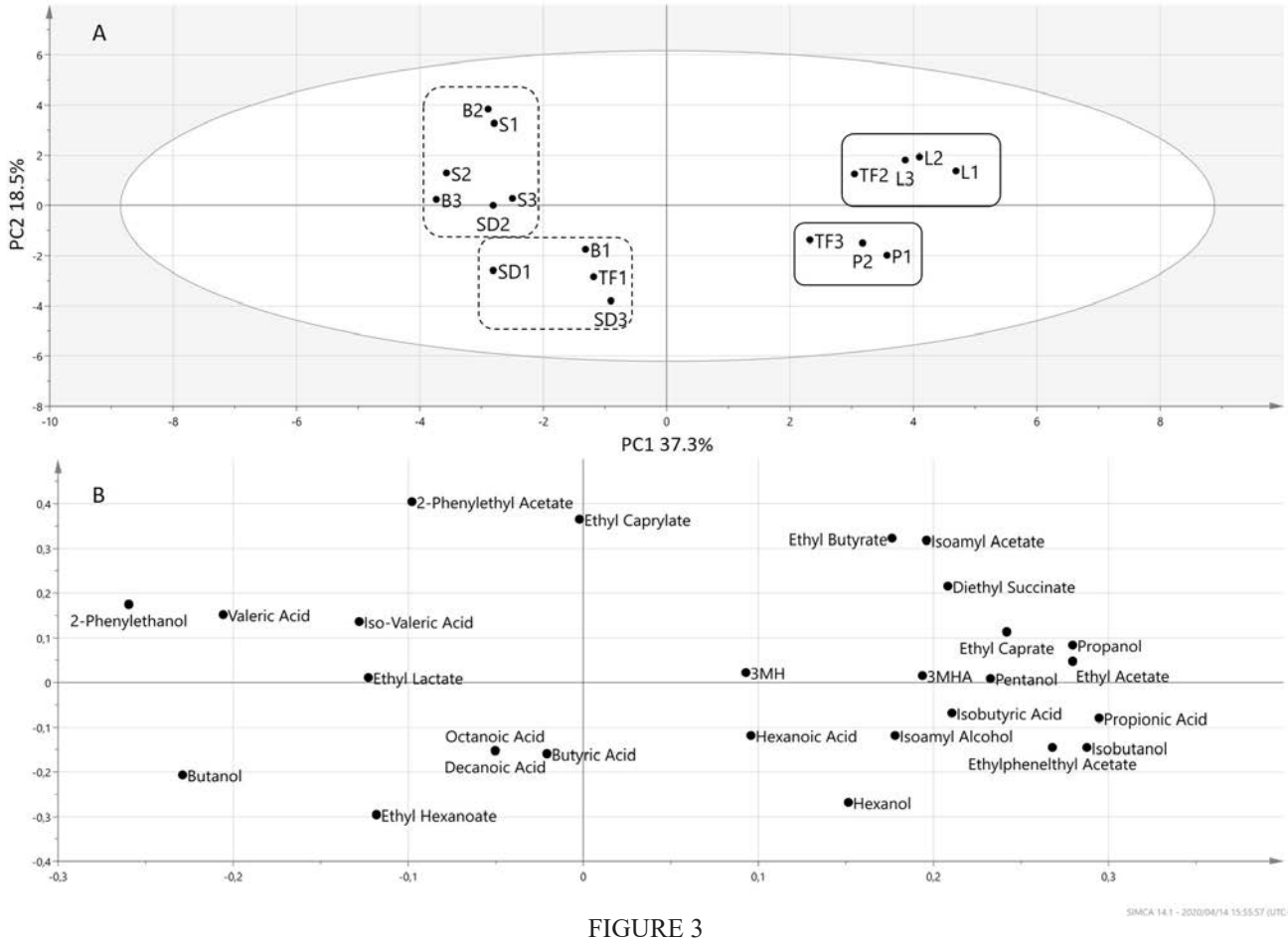

FIGURE 3

Principal component analysis scores (top) and loading plots (bottom) for the major volatile and thiol data from wines from the 2017 vintage. Grayscale in the score plot codes according to trellis system. The groupings in the score plot are designated according to hierarchical cluster analysis (HCA) of the same data, and the different/similar outlines indicate level of distances between groups based on the HCA dendrogram

source of variation in the volatile composition of Cabernet Sauvignon, Sauvignon blanc, Chardonnay, Pinotage, Merlot and Shiraz wines. Microclimatic conditions were not measured in the current study but, based on previous research, moderate cluster exposure to sunlight increases flavour compounds in Traminette grapes (Ji & Dami, 2008). Common compounds that are documented as being influenced by light are the C_6 compounds (Zoecklein *et al*.,

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2008), and these are among the potential precursors involved in 3MH and 3MHA formation (Harsch *et al*., 2013).

Marais *et al*. (1981) found a correlation between amino acids in the must and ester formation. Because of this phenomenon, the hypothesis would be that a significant difference in certain amino acids in the must should correspond to a significant difference in the resulting esters' concentrations in the wine. For the major volatiles, as derived from AA metabolism, a configuration similar to the AA results was found, with the L, P and TF samples grouped together in both cases (Figs 4 and 5).

In addition, some trellising systems, such as Lyre, optimise leaf surface area, which may lead to the optimisation of sunlight use. It is possible that other chemical compounds like terpenes were influenced in the current study. Marais (1983) generated data that suggests that Chenin blanc leaves are rich in terpenes, and Lawrence (2012) and Bruwer (2018) later confirmed the presence of monoterpenes in wines.

Therefore, there is a chance that compounds other than major volatiles and thiols (*viz.* terpenes) may have contributed to the aroma profile of the wines because it has been demonstrated that sunlight influences the concentration of terpenes in wine (Marais *et al*., 1992). The manner in which the canopies in the L system are opened up permits good interception of light, which improves fruit exposure and may lead to an increase in the concentrations of thiol precursors in the berries. It has been confirmed that there is an increase in the Gluy-3SH level in the grapes and must of Sauvignon blanc as a result of the effect of nitrogen status on 3MH content (Helwi *et al*., 2016). A study by Lloyd (2013) reported the enhancement of green characters in Sauvignon blanc wines as a result of changes in light exposure.

Untargeted analyses

The untargeted LC-HRMS analysis was used to evaluate the effect of trellising systems on the chemical characteristics of the corresponding wines. PCA was used to explore the samples' grouping according to the positive and negative ionisation dataset generated for the two seasons (2017 and 2018, Fig. 4).

Interestingly, the separation corresponded with the taste and mouthfeel profile trend in the sensory results. The hypothesis could be that some compounds (for example polyphenols that give an MS signal in the negative ionisation mode) have been affected similarly by certain types of trellising. Polyphenols make a relevant contribution to

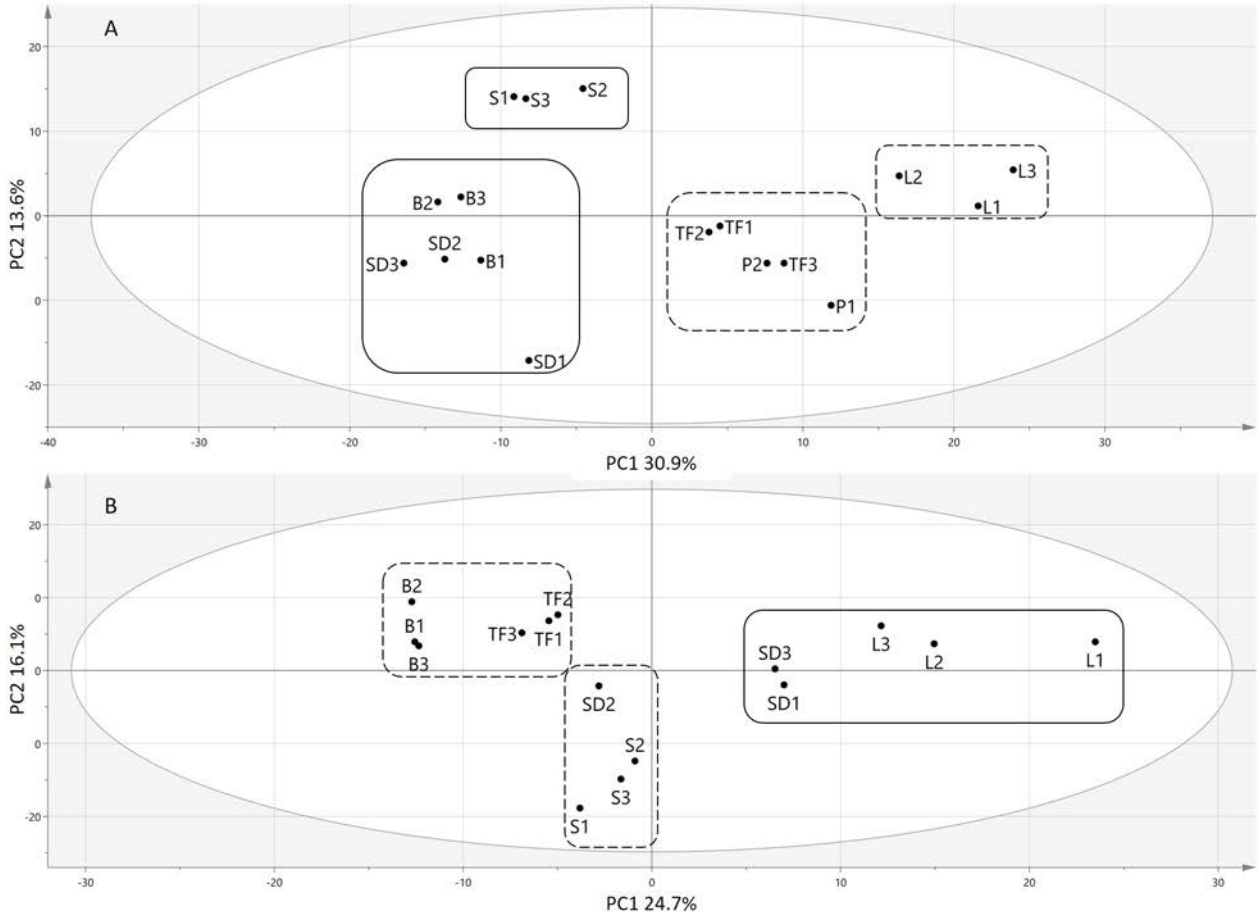

FIGURE 4

Plot of principal component analysis scores and the hierarchical cluster analysis dendrogram for the high resolution-mass spectrometry data for the 2017 (top) and 2018 (bottom) vintage wines. Grayscale in the score plot codes according to trellis system. The groupings in the score plot are designated according to the hierarchical cluster analysis (HCA) of the same data, and the different/similar outlines indicate distances between groups based on the HCA dendrogram

sensorial properties such as taste and mouthfeel (Gawel *et al*., 2018).

The results from the two vintages illustrate that trellising systems have an effect on the chemical composition of wines, mostly on compounds responsible for the taste and mouthfeel of wine. Although this study could not identify the compounds responsible for the configuration due to the untargeted nature of the analysis, they are the factor that leads to the grouping according to trellising systems. Phenolics are responsible for the taste and texture characters in wine in interaction with other components, are influenced by practices in the vineyard, and there is a correlation between phenolics and sunlight (Šebela *et al*., 2017). Because the differentiation of wines according to trellising systems by fingerprint is based largely on phenolics, it can be hypothesised that sunlight exposure played a role in the samples' configuration.

Considering the canopy structure, SD and B systems are parent and variant of each other, therefore the exposure of their foliage and/or berries to sunlight would be comparable. As a result, they could produce wines with analogous fingerprints, as seen in the close placement on the PCA score plots (Fig. 4). Following the same reasoning, TF and L systems with open horizontal canopies would result in a similar light distribution and interception by the berries, hence the corresponding wines were positioned next to each other on the PCA score plots (Fig. 4). Santorini architecture places the grape berries in the shade, and for each season this system produced wines with a different fingerprint from the other systems. To date, no published work has evaluated the LC-HRMS profile of wines made from different trellising systems to be able to compare to the results of this study.

Results of sensory evaluation

Aroma profile of Chenin blanc wines

For the 2017 expert panel, the biplot obtained from the CATA results showed an overlapping trend for a set of wines from different trellising systems along dimension 1 (Dim 1) and dimension 2 (Dim 2), which explained 33.8% and 26.0% of the variance, respectively, and totalling 59.8% (Fig. 5). All wines were grouped, except for the wines from the L system. The raw data (frequency of citation) from experts in the first season (2017) shows that the most used attribute overall was 'passion fruit'. This attribute also had the highest citation frequency for the wine from the L system. Overall, it appears that the differences were small with regard to the number of terms used per treatment to characterise the wines. To further investigate the individual attributes used, compiling a 'top five' and a 'top ten' list based on frequency counts helped explain the groupings of the correspondence analysis. According to these lists, the term 'pineapple' was used frequently across all treatments, while 'lemon' 'melon' and 'peach' appeared in four to five treatments. 'Honeysuckle' and 'hay'/'straw' also appeared in the top five frequently cited notes associated with SD and TF, respectively, as unique features compared with the rest. In spite of the unique features in some treatments, it is suggested that there was no significant impact on odour threshold perception, as there was no clear separation between samples. The overall visualisation shows that all treatments had a common fermentative origin, as 'fruity' and 'floral', and no discriminant attribute or compound was identified.

L system wines were characterised by 'grapefruit' and 'passion fruit' descriptors, which are typically associated with 3MHA, which is formed by the esterification of 3MH with acetic acid during fermentation (Tominaga *et al*., 1998). 3MHA levels were the highest in the first season, and were significantly higher in the L system wines compared to the rest of the systems, as seen from the chemical results of this study (Table 4A). The architecture of the L systems opens up for good light interception and enhances fruit exposure, which might have led to increased concentrations of precursors of this class of aroma compounds. This can be correlated with ripening at harvest induced by light exposure (Lloyd, 2013).

Also, non-volatile precursors found in the berries and the must can be increased. Helwi *et al*. (2016) found an increase in Gluy-3SH levels in the grapes berries and must of Sauvignon blanc as a result of a positive effect of nitrogen status on 3MH content. Additionally, L wines were significantly higher in yeast-preferred amino acids, namely glutamine and alanine, the same trend seen with FAN, which plays a vital role in ester production (Tables 2 and 3), although the difference was not reflected in the aroma descriptors of the resulting wines.

Unlike the experts, the analytical panel used the term 'pineapple' frequently and across all treatments. In addition, 'passion fruit' was common in all the sample wine treatments, and 'guava' and 'lemon' appeared in the top five notes in four out of five treatments. Another attribute that was prominent was 'grapefruit'; although cited the least compared to the other top five notes, it appeared in all six treatments. Similarly to the experts, the analytical panel characterised all wines with 'fruity' and 'floral' attributes, in addition to certain attributes that were associated with particular treatments, although these did not contribute significantly. An indication of similarities between the wines' perceived aroma could already be based on this raw data and was confirmed by multivariate analysis (Fig. 5B). Correspondence analysis obtained from the analytical panel for the same 2017 vintage using the CATA results shows a total of 48.4% explained variance for the first two dimensions (Fig. 5B).

Even though the grouping was similar by the analytical panel and the experts, and the panels used the same CATA list, the two panels described the wines differently. The experts profiled wines from the L systems as 'baked bread', 'vanilla' and 'stewed fruits', whereas the analytical panel perceived them as having 'oak' and 'fynbos' characters. Despite the terminology used by the two types of panels being different – which is to be expected given the nature of their background knowledge – both groups of descriptors implied a certain degree of 'toasted', 'woody' and 'sweet associated' characters.

In the second season, the most frequently cited term was 'pineapple'. Unlike in the previous season, this attribute was highly associated with the L system; however, it was present in all treatments. Other terms that were frequently cited were 'passion fruit', 'apple' and 'orange'. Moreover, 'lemon' was commonly used in all treatments as part of the top ten most-

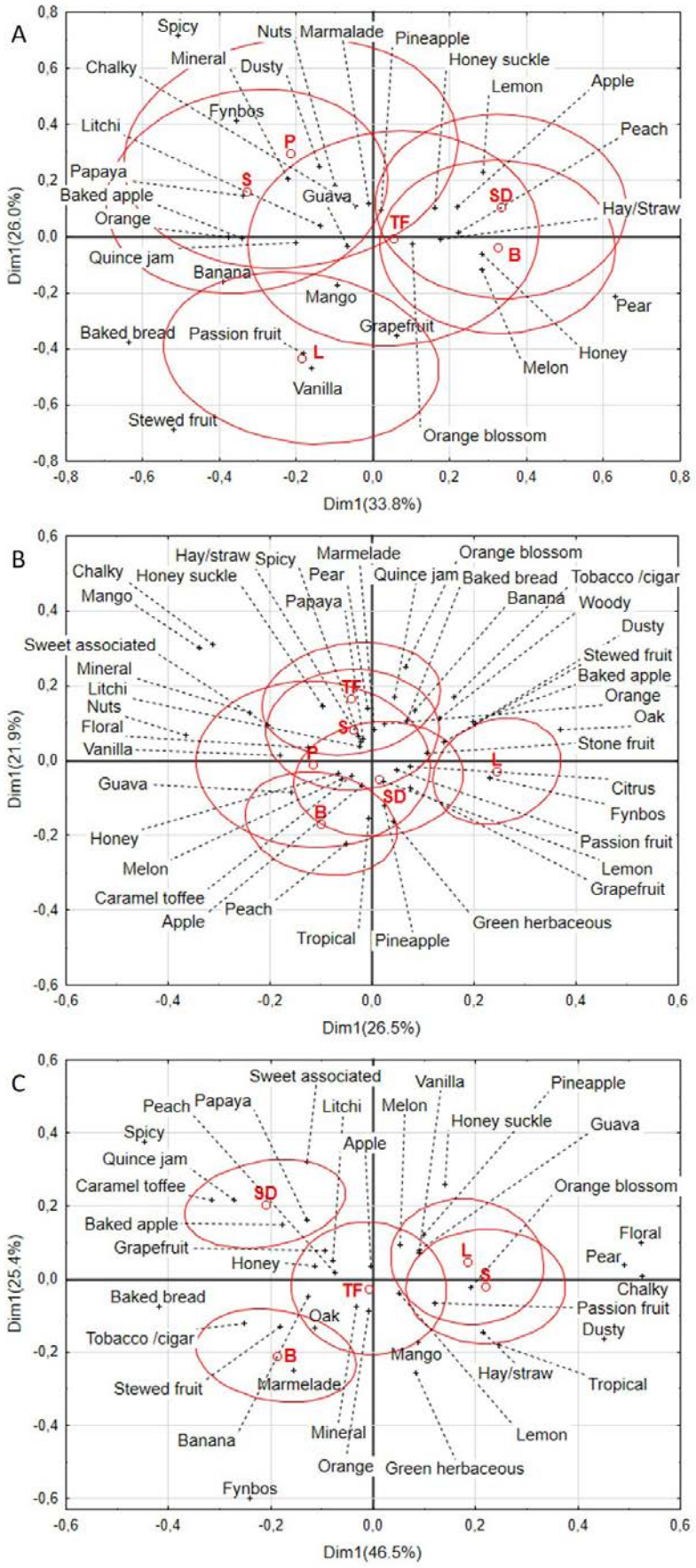

FIGURE 5

Correspondence analysis biplot for the results of the aroma evaluation for: A. 2017 expert panel (n = 30 judges, 30 answers); B. 2017 analytical panel (n = 10 judges, 30 answers); and C. 2018 analytical panel (n = 10 judges, 30 answers). Ellipses indicate 95% confidence

used terms, and 'peach' was the least-used attribute among the top ten. Overall, three of the top five attributes used were the same ('pineapple', 'apple' and 'passion fruit') in both vintages across all treatments.

Furthermore, a similar trend was observed in the panels regarding the raw data from 2017 and 2018, namely that all wines were described as having 'fruity', 'guava', and 'floral' notes, which relate to esters, thiols and possibly other aromatic chemical compounds (terpenes) not analysed in the current study.

The 2018 vintage aroma profiles in the bi-plot obtained from correspondence analysis of the CATA data showed a better separation than in the previous vintage (Fig. 5C). The separation is derived from Dim 2, which captured 25.4%, while Dim 1 captured 46.5% of the explained variance, totalling 71.9% of the explained variance.

The attributes associated with the groups that were formed could be linked to the volatile and non-volatile composition of must and wine. The descriptors for wines from the SD system, namely 'caramel' and 'sweet associated', may be linked with the sugar level at harvest, which was higher than in any of the other systems. Equally important, the sugar content of grape juice influences the alcohol content of the wine, which is known to impart a sweet flavour to wine (Peynaud & Blouin, 1996). In 2018, the berries from the SD system were exposed to sunlight, which led to sunburn in most of the berry bunches. Furthermore, Marais *et al*. (1992) found faster sugar accumulation in sun-exposed grapes than in shaded grapes. This could have further affected the flavour of the resultant wines based on the aroma profile.

Taste and mouthfeel of Chenin blanc wines

The CA results of the evaluation of the taste and mouthfeel of the 2017 vintage wines by experts explained 92.5% of the total variance among the wines samples, with the first dimension mainly responsible for the separation, with 84.6% (Fig. 6A). The first dimension shows the trend among wines based on the body, projecting, from left to right, a separation from full body to medium through to light body. The TF, L and P wines were associated with the 'full body' and the 'long aftertaste driven by alcohol'. The SD and B wines were associated with the 'medium body', with the 'medium aftertaste' driven by 'bitterness' and 'flavours'. The S system wines were associated with the 'unbalanced light body', with a 'short aftertaste' that was driven by 'acidity'.

The taste and mouthfeel profiles obtained from the analytical panel data and that of the experts showed a similar trend and configuration (Fig. 6B). However, there is a visible separation between wines in the analytical panel data, notably forming three groups: wines from the TF, L and P systems (complex wines, 'full body', and 'long aftertaste' driven by 'alcohol' and 'flavour'), wines from the B and SD systems ('medium body', 'medium aftertaste'), and wines from the S system ('light body', 'short aftertaste' driven by 'acidity').

The biplot obtained from the analytical panel data for the taste and mouthfeel in 2018 showed a trend along Dim 1, which explained 60.5% of the variance, whereas Dim 2 explained 31.1% of the variance (Fig. 6C). The body and aftertaste influenced the pattern of wines described by 'complex full body'-'alcohol'-'flavour'-'balanced', through to 'medium body'-'medium aftertaste', and to 'water light body'-'unbalance'-'acidy', which were discriminated in Dim 2. Wines from the L and TF systems were described by 'full body' and 'long aftertaste', although the aftertaste of the TF wine was driven by 'alcohol' while L was driven by 'flavour'. Wines from the SD system were associated with 'medium body', 'medium aftertaste', and were driven by 'acidity' and 'flavour'. B system wines were described by 'medium body', 'medium complexity', 'unbalance' and 'bitterness'. The S system wines were characterised by 'short aftertaste', 'light body', and 'watery', with the latter attribute potentially linked to the sugar level at harvest, which was significantly lower than that of any other system and resulted in a lower percentage of alcohol (Table 1).

The raw data already made reference to the S treatment being associated predominantly with negative attributes like 'acidity', 'water', 'light body' and 'unbalance', possibly related to the shaded bunches. Multivariate analysis then confirmed the trend of S being separated from the rest of the samples in both vintages. Other samples could not clearly be ascribed simply by looking at the raw data; however, multivariate analysis was able to separate them. The TF treatment, perceived to have a taste driven by flavour, could be linked to the optimal interception of light by the system, as well as the alcohol percentage, which was higher than for the other systems.

Grape berries exposed to sunlight are generally higher in sugars and phenolic compounds compared to shaded berries (Morrison & Noble, 1990). The systems with open canopies and canopies that allow sunlight exposure (TF, L and P) are expected to produce wines higher in sugars, therefore full-bodied wines. Not all wines are expected to be big, full-bodied wines. However, they should present a pleasing entry into the palate and finish. The three aspects in wines, namely balance, body and astringency, are categories that are always in the consumer's mind when consuming wine. Good balance in wine complements the aroma, and highly acidic wines tend to be thin and watery with a dry perception (Conde *et al*., 2007). It is possible that the wines produced from the S system may be acidic due to the canopy architecture, which caused the berries to be in the shade. The contribution of sugars can counteract the acidity and build body in a wine. The S system's wines started with the lowest sugar, therefore there was nothing to counteract the acidity.

As part of canopy management, the temperature around the canopy could be the cause of a lower sugar level and consequently affect the organoleptic properties of wines from the S system. Other wines, such as those from the B and SD systems, produced similar wines with the same taste and mouthfeel, and again the design could play a role here because these two systems are similar (the B system being a variant of the SD system). Also, the TF and L systems are quite similar (vertical divided canopies). The wines from these systems had desirable characters, such as a 'long aftertaste' and an 'aftertaste driven by flavour', and had a 'full body'. Opening the canopy makes room for good light interception and also prevents sunburn and controls sugar levels.

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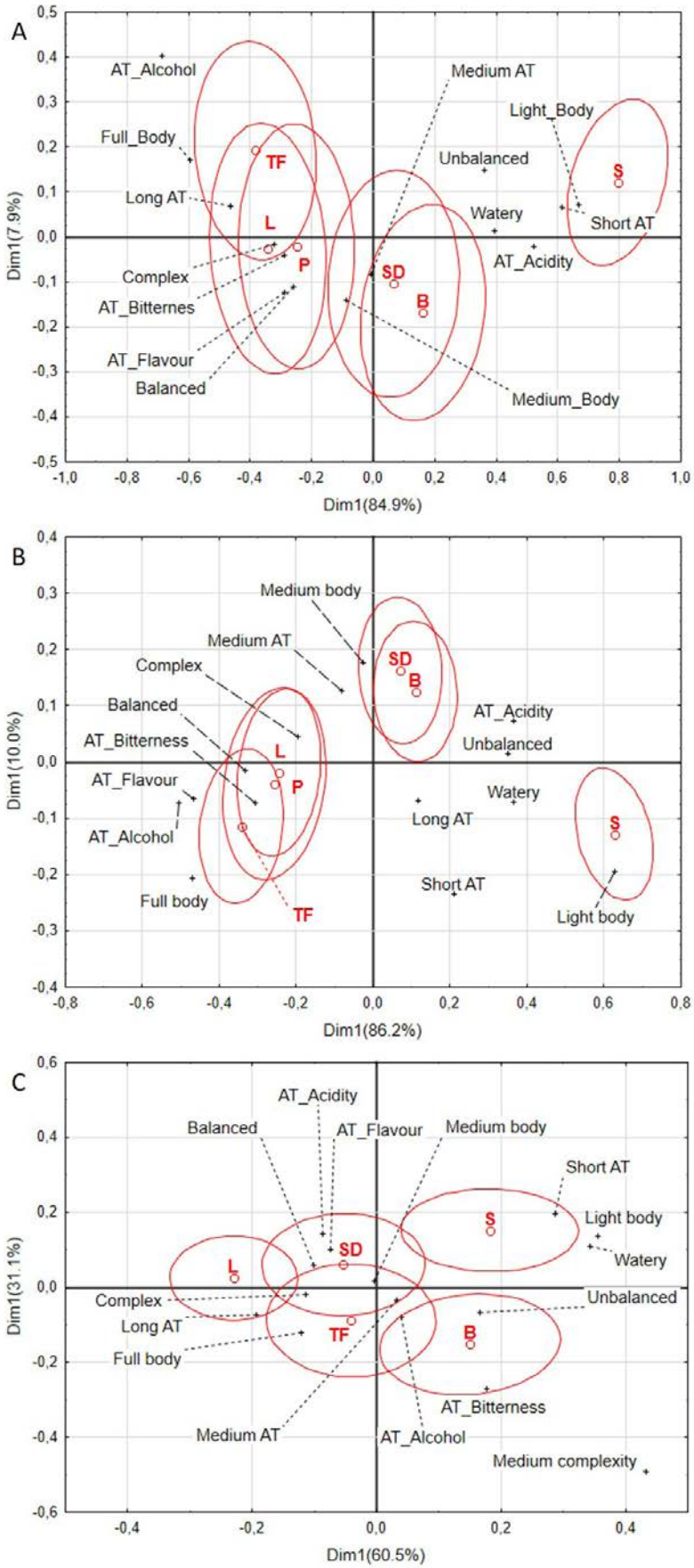

FIGURE 6

Correspondence analysis biplot of the results from the taste and mouthfeel evaluation for: A. 2017 expert panel (n = 30 judges, 30 answers); B. 2017 analytical panel (n = 10 judges, 30 answers); and C. 2018 analytical panel (n = 10 judges, 30 answers). Ellipses indicate 95% confidence.

Overall quality assessment

Differences between the wines based on appearance, aroma, taste and overall quality were obtained from one-way ANOVA results at $p < 0.05$ (results not shown). Among the sets, the TF and L wines scored the highest and the S wines scored the lowest for overall quality, with significant differences. Taste scoring showed the same trend as for overall quality; aroma and appearance had similar trends, although no significant differences were observed.

Considering that bush vines and S systems have a similar canopy architecture, it may be expected that they produce wines with similar characteristics. However, the quality scoring for S wines in the current study and in that of Van Zyl and Van Huyssteen (1980b) are conflicting. In the investigation by Van Zyl and Van Huyssteen (1980b), the Chenin blanc wines from bush vines were rated the highest based on colour, whereas in the current study, the wines from similar canopy architecture (S) systems scored the lowest among all the systems.

Aroma, taste and mouthfeel played a part in the quality-assessment scores in the present work, whereas appearance (colour) had no significant influence. Similar findings by Valentin *et al*. (2016) show that colour was not the major contributor to the overall quality of Sauvignon blanc and Pinot noir wines in a study comparing Burgundy wines to New Zealand wines. In contrast, Van Zyl and Van Huyssteen (1980b) found colour to be a determinant in Chenin blanc wine quality differences; however, the cause of colour differences were the result of grapes infected by fungus, rather than driven by canopy microclimates.

CONCLUSIONS

It is significant for winegrowers to make the right choice of trellising system that can maintain or even improve wine organoleptic properties and overall quality. One way to evaluate the improvement of sensory characteristics and quality aspects is to profile the resultant wines. The current work evaluated the effects of wines made from grapes grown on six different trellising systems regarding sensory characteristics and quality rating using CATA for profiling and quality assessment.

Regardless of the panel (experts or analytical), it was shown that the different trellising systems in this study did not effect the aroma perception of the Chenin blanc wines. One possible major factor could be that Chenin blanc grapes are neutral – which means they lack a typical character and hence their aroma is highly dependent on the winemaking process rather than on viticultural practices.

On the other hand, taste and mouthfeel were affected more prominently, as the systems have an impact on the phenolic compounds responsible for mouthfeel and on other compounds, as discussed in the text (sugar levels at harvest correlated with alcohol levels in the resulting wines). The differences in taste and mouthfeel further played a role in the wines' quality scores. Wines from the S system were associated with negative attributes, such as 'acidity', 'light body', 'watery' and 'short aftertaste' and scored the lowest. In brief, with regard to trellising system there is no one size that fits all. Under equitable conditions in the same vineyard, there were differences in aspects related to taste, mouthfeel and quality, but not aroma. These results should be considered carefully before extrapolating them to wines from a vineyard with a different terroir and, more specifically, to wines from a different cultivar. This is particularly also so in the light of climate change, drought and consumers requiring lower-alcohol wines. Choosing a trellis should be based on the objectives of the winemaker, but should not underrate the significance of consumer preferences and economic factors.

The chemical composition of grapes is an important aspect in winemaking, as it determines the characteristics of the wine, in addition to the fermentation and ageing processes. The content, concentration and level of certain compounds in grapes can be modified by several factors, including vineyard practices. One possible way of evaluating the impact of vineyard practices, including trellising systems, on wine characteristics is through analytical methods. Chemical composition entails volatile and non-volatile compounds that contribute to defining wine typicality and style. Vines on similar types of trellises were found to have similar responses to nitrogenous compounds like amino acids and the yeast assimilable nitrogen components of their juice. The amino acid profile made it possible to differentiate the musts from the TF and L systems, which were different from the that of the rest of the systems. Even if individual and groups of amino acids varied between systems, the differences did not reflect in the major volatile composition of the wines, in particular in the amino acids that are involved in ester production.

Differences were not apparent between the systems based on the volatile composition of the wines. Again, vines on the horizontal dividing or open canopy type of trellis (L and TF) in this study had higher concentrations of thiol compounds and major volatiles than the rest of the systems, although they were comparable in practical terms.

According to the profiles provided in this study, the trellising systems may influence other wine aspects, as revealed by HRMS fingerprinting. Other than that, factors such as variety, vineyard location, vintage, water usage and yield could be enough tools for wine growers to make decisions on the type of system to use. The results of this study have to be considered carefully before extrapolation to other regions or cultivars, because of the genetic makeup of a vine or cultivar and its response to external factors, or because of other aspects such as clones.

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Pinking in White Wines – A Review

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In the late 1960s, a phenomenon was discovered in white wines. It was noted that certain white wines turned pink in the bottle. This phenomenon was dubbed as pinking. Research was done on the pinking to establish its cause and effect. Analysis of SO_2, pH and polyvinyl polypyrrolidone (PVPP) showed that a minimum of 45 mg/L of SO_2 were needed for the wine not to be susceptible to pinking. Tests on the decrease in pH showed that there was no increase in pink colour with a decrease in pH, which meant that monomeric anthocyanins were not the cause of pinking. Recent research claims that malvidin-3-O-glucoside is the most abundant monomeric anthocyanin found in pinked wines and could be the cause of pinking. This led to the theory that phenols contribute to pinking susceptibility, and this was accepted as fact in recent years. The establishment of a pinking assay in 1977 made the testing for pinking easier and cheaper for winemakers. The sales of PVPP increased as winemakers worked preventatively with their wine to decrease susceptibility to pinking. This review attempts to describe the history of pinking, the establishment of the assay, as well as to describe factors that could lead to pinking susceptibility in white wines.

INTRODUCTION

The first incidence of pinking in white wines was reported by Singleton and Esau in 1969. This led to a series of research articles on pinking from 1977 to 1983 by an Australian researcher, Dr Bob Simpson (1977a, 1977b, 1980a, 1980b; Simpson *et al*. 1982, 1983). This was followed by a research article on the use of polyvinyl polypyrrolidone (PVPP) by Lamuela-Raventós *et al*. (2001) and two articles on the presence of anthocyanins by Andrea-Silva *et al*. (2014) and Cosme *et al*. (2019). This literature review reports on the findings of Simpson and other researchers that investigated pinking susceptibility in white wines

Simpson (1977a) defines pinking as "the troublesome discolouration" that develops during the storage of white wines. He later adds that it develops over several days, but most likely after vinification or when the wine is no longer protected by a CO_2 blanket. This led to the discovery that pinking occurs after contact with air (Simpson, 1980a). In 1982, Simpson stated that "white wines develop a pink colouration on exposure to air". Andrea-Silva *et al*. (2014) define pinking as "the appearance of a salmon-red blush in white bottled wines produced exclusively from white varieties". Therefore, a comprehensive definition of pinking could be established, as follows: Pinking, or oxidative pinking, is the slight discolouration of white wines from a pinkish to a salmon-red blush colour, affected by certain oenological processes before and after fermentation until storage during which the wine could come into contact with air.

Different cultivars have been reported to show some degrees of pinking susceptibility. In America, the white cultivars reported are Thompson Seedless, Semillon, Sauvignon blanc, Chardonnay and Chenin blanc (Tobe, 1983; Jones, 1989). In Australia, the cultivars reported to be prone to pinking are Muscat Gordo Blanco, Sultana, Palomino, Riesling, Doradillo and Crouchen (Simpson, 1977a). In Spain, the cultivars Sauvignon blanc, White Riesling, Chardonnay, Albariño, Macabeo, Xarel·lo, Parellada, Garnatxa blanca (Grenache) and Verdejo were reported to have the potential to pink (Lamuela-Raventós *et al*., 2001). In Portugal, the cultivar reported was Síria (Andrea-Silva *et al*., 2014), and in the Czech Republic it was Pinot blanc, Pálava, Pinot Gris, Sauvignon blanc, Grüner Veltliner and Chardonnay (unpublished data). This is an extensive list of white cultivars. According to Simpson (1977a, 1977b), Tobe (1983), Jones (1989), Lamuela-Raventós *et al*. (2001) and Andrea-Silva *et al*. (2014), the

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predominant cultivar that shows susceptibility to pinking is Sauvignon blanc. Winemakers must take note of this when producing Sauvignon blanc. Although these cultivars showed a tendency to pink, regional variations and yearly differences also influence the potential of these wines to pink (Simpson, 1977a; Andrea-Silva *et al*., 2014).

Wines made by winemaking practices such as cooling of the must, cold fermentation and the use of inert gasses (Ar, N_2 and CO_2) show higher susceptibility to pinking (Singleton & Esau, 1969; Simpson *et al*., 1982). This led to the assumption that air contact or O_2 increases pinking susceptibility (Simpson, 1980b). Other factors, such as storage temperatures, the presence of light, free SO_2 content and the pH of the wine also play a role in pinking susceptibility (Simpson, 1977; Simpson *et al*., 1982). With the influence of light came the suggestion that wine must not be bottled in clear glass bottles, but rather in green or dark green bottles (Lamuela-Raventós *et al*., 2001). Anecdotal evidence also suggests that pinking does not affect the aroma or taste of the white wines (Simpson, 1980b; Lamuela-Raventós *et al*., 2001), but this has never been proven scientifically.

Simpson (1980b) states that there is "good evidence" that the compounds causing pinking have their origin in phenolics. This led to a worldwide belief that phenols cause pinking in white wines (Jacobson, 2006; Jackson, 2014, 2016), and that polyvinylpolypyrrolidone (PVPP) should be used for their removal (Lamuela-Raventos *et al*., 2001).

MEASUREMENT OF PINK SUSCEPTIBILITY

Development of an assay

Simpson (1977a, 1980b) did extensive studies on the pinking susceptibility of white wines and the analysis thereof. Spectrophotometric studies on normal white wines and wines with a visible pinking showed a distinctive bump over the 500 nm absorbency range (Figs 1 & 2). Therefore, because the greatest differences occurred at an optical density of 500 nm, this wavelength was chosen as a suitable wavelength for testing for pinking susceptibility.

The absorbance of a normal white wine therefore will have a smooth curve at 500 nm, but a white wine with a visible pink colouration will show an absorbency at 500 nm. Thus, when white wine is tested for pinking susceptibility, two samples of the wine are taken. One will be the control and the other the treatment. The control sample is determined first at 500 nm, followed by the pink induced sample. The difference between the two samples will show the pinking susceptibility of the wine. Simpson found that light-colour wines will show a pinking susceptibility of 5 (0.005 AU x 10^3), and darker coloured wines will have a pinking susceptibility above 10 (0.01 AU x 10^3). With darker coloured white wines, Simpson meant wines that border on a more yellowish colour.

Wines that show a tendency to browning rather than pinking will show a greater absorbency at 420 nm. At the wavelength of 420 nm, there will be no interference from the pink colouration. Both pinking and browning therefore can be measured in white wines.

Simpson (1977a) prepared a 0.3% (w/v) solution (1 mL in 100 mL distilled water) of 30% (w/v) hydrogen peroxide (H_2O_2). He used increments of 0.05 mL, starting from 0.05 mL in a 10 mL wine sample, and ending with 0.40 mL from this 0.3% (w/v) H_2O_2 solution to end up with concentrations ranging from 15 mg/L to 120 mg/L H_2O_2. For each wine tested, there were two samples, one of which was the control and the other one that received the H_2O_2 addition. These two samples of each wine were then kept in the dark for 24 hours before being analysed spectrophotometrically.

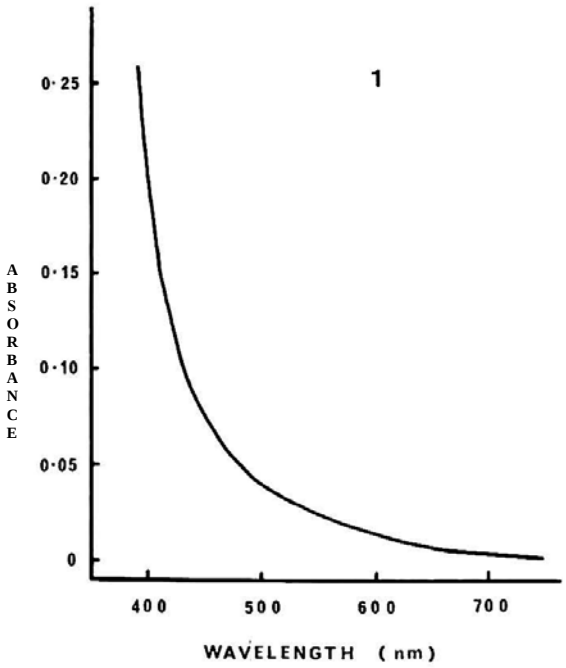

FIGURE 1
Spectrum of a wine showing no pinking (from Simpson, 1977).

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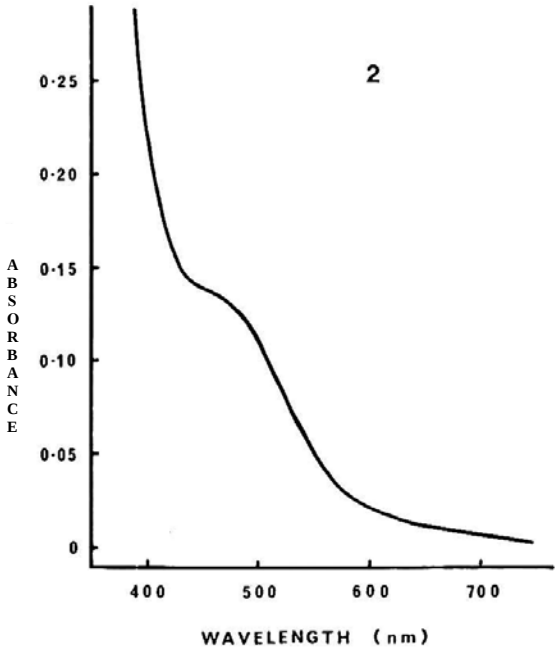

FIGURE 2
Spectrum of a wine showing pinking (from Simpson, 1977).

Simpson (1977a) found that the increase in pinking was linear up to 24 hours, reaching a peak at three days and then decreasing to 14 days. However, the reason for the shorter than three-day assay periods for pinking susceptibility used in the practice today could be that winemakers need to make a decision as quickly as possible and waiting three days for results is too long.

Simpson (1977a, 1980b) reports that, at a concentration of 75 mg/L (0.25 mL), H_2O_2 shows the most consistent results. At 75 mg/L (0.25 mL) H_2O_2, Simpson also found that SO_2 did not influence the outcome, but at a concentration of 45 mg/L (0.15 mL) H_2O_2, the lack of SO_2 or low concentrations of SO_2 could influence the values and therefore give a false negative to the winemaker.

Assays used in Australia, South Africa, America and Europe

The assays for pinking and various approaches used in different parts of the world are described below. Although laboratories in winemaking countries use the assay established by Simpson (1977a), there are variations in different countries adapted to best suit their final objectives.

Australia

A 100 mL clear glass screw cap bottle is labelled as 'control' and another as 'test'. The 'control' bottle is filled with wine. Forty mL of the same wine is measured into the 'test' bottle, to which 0.5 mL of 0.3% (w/v) hydrogen peroxide is added and mixed well. The 'test' sample is then placed in a dark cupboard at approximately 25°C overnight (about 12 hours). The degree of pinking of the 'test' wine is compared to that of the 'control'. In addition to visual assessment, spectral measures of the 'test' and 'control' wines can be performed at 520 nm, which gives a quantitative comparison. In this case, the wines are filtered through a 0.45 µm filter for assessment. A change greater than 0.050 at 500 nanometres (nm) between the control and treated sample indicates significant susceptibility to pinking (Australian Wine Research Institute [AWRI], 2020).

South Africa

According to the SASEV Methods of Analysis for Wine Laboratories (2002), a 0.072% (w/v) H_2O_2 solution (1.2 mL of 30% (w/v) H_2O_2 in 500 mL volumetric flask with distilled water) is used. A set of 5 x 25 mL sample bottles are filled with wine and additions of 0, 0.5, 0.75, 1.00 and 1.25 mL of the 0.072% (w/v) solution are done. The sample bottles are mixed gently and left for at least eight hours (the temperature and whether in a dark place or not are specified in the method). After eight hours, the samples are measured on a spectrophotometer at 500 nm, zeroed with the control sample (0 mL of H_2O_2 added) and, if the optical density (OD) is above 0.05, the wine is susceptible to pinking.

America and Europe

In both America and Europe, the method described by Simpson (1977a) is roughly followed (personnel communications). In America, 250 µL of a 0.3% (w/v) H_2O_2 solution is added to the wine sample, while in France 125 µL of the 30% (w/v) H_2O_2 is used. In both countries, the samples are kept in a dark cupboard for 24 hours. The specific method is not revealed by the laboratories and the personnel were not willing to part with all aspects of the methods. The spectrophotometer is zeroed with distilled water and both the control and treated samples are measured. The difference between these two is given as AU and, when the value is ≥ 0.05, the wine is seen as having a pinking susceptibility.

Concluding remarks

Simpson (1977a) reports that if the AU is above 5, the wine shows potential for pinking susceptibility. In his research work, he multiplied the absorbance unit (AU) by 1 000 (x 10^3) to get to a whole number. In all the methods, an AU of 0.05 (10 times higher than the 0.005 Simpson used in his original work) is used. It is not sure when this decision was made and for what reason, and this discrepancy has never been questioned.

The use of a 0.3% (w/v) H_2O_2 solution in Australia and America has been reported, while in South Africa it is 0.072% (w/v). In France, a 30% (w/v) undiluted solution is added to the wine sample (Table 1). Different volumes of the H_2O_2 concentrations are used by the different countries (500 µL in 40 mL of wine sample, 250 µL in 10 mL of wine sample, 1 000 µL in 25 mL of wine sample and 125 µL in 10 mL of wine sample, respectively). The final concentrations of H_2O_2 in the treatment sample differ, but could easily be worked out with the formula $C_1V_1 = C_2V_2$. This will lead to different sensitivity measurements and different absorbency units, and possibly different conclusions on whether or not a wine shows pinking susceptibility.

Another difference between the countries is that Australia uses a wavelength of 520 nm, while South Africa and France use 500 nm, as stated by Simpson (1977a). The reason for this could be that some countries scan through a wide range of spectra, i.e. 400 to 650 nm, to determine the wavelength of maximum absorbance.

Simpson (1977a) originally used a 10 mL sample bottle for his experiments. This is also the case in America and France, while in Australia a 40 mL sample bottle is used (Iland *et al*., 2012). In South Africa, a 25 mL sample bottle is used (SASEV, 2002). The question arises if the addition of H_2O_2 from the different stock solutions will have an impact on the values for pinking susceptibility and regarding the pinking sensitivity of the wines. Would it then not be better to standardise to the original concentrations set by Simpson (1977a).

The above shows that there is no real standard for the testing of pinking susceptibility. In some cases, the waiting period is up to 24 hours, whereas in countries like South Africa it is reduced to eight hours of waiting. It is evident that there is a need to develop a standardised, shorter and more reliable method for the testing of pinking susceptibility. There is also no protocol prescribed by the International Organisation of Vine and Wine (OIV) on an assay for pinking.

FACTORS INFLUENCING PINKING IN WHITE WINES

In a series of articles published from 1980 to 1983, Simpson and co-workers discussed different factors that could contribute to the pinking susceptibility of white wines. The factors influencing pinking in white wine are discussed on the basis of these abovementioned articles and supplemented by other authors.

The role of phenols

Singleton and Esau (1969) discussed the possibility of colourless plant phenols turning pink in an acidic medium, like wine, when the colourless anthocyanogens turn into anthocyanidins. In 1977, Simpson also stated that the spectral and chemical properties of the pink wines tested indicated that the precursors could be phenolic in origin. This started the reasoning that the oxidation of phenols could lead to pinking.

Phenolics in wine are divided into two groups. These are the flavonoids, of which the flavan-3-ols are part, and the non-flavonoids, of which the hydroxycinnamic acids and hydroxybenzoic acids are part. The flavan-3-ols consist of catechin, epicatechin, epigallocatechin and epicatechin-gallate and are found mainly in the skins and pips of grapes (Monagas *et al*., 2005; Aron & Kennedy, 2008; Piñeiro *et al*., 2012). The hydroxycinnamic and hydroxybenzoic acids are normally found in the fleshy parts of the grapes (Garrido & Borges, 2013; Nel, 2018). Compounds that have an ortho-diphenol grouping are highly reactive with dissolved oxygen (Garrido & Borges, 2013) to form an ortho-quinone:

$$\text{Ortho-diphenol} + O_2 \rightarrow \text{ortho-quinone} + H_2O_2$$

These ortho-quinones are very unstable because of their highly electrophilic nature and can react in a further three ways. Firstly, the ortho-quinone can form dimers or polymers if reacting with the nucleophilic parent. Secondly, the ortho-quinone can undergo further nucleophilic additions with other nucleophiles (amino acids, glutathione, and other phenols). Thirdly, the ortho-quinone can be reduced by other reducing species, like ascorbate and other phenols, to form ortho-diphenols (Fulcrand *et al*., 2006). All these non-enzymatic reactions are catalysed by Fe^{3+}/Fe^{2+} or Cu^{3+}/Cu^{2+}. The oxidation of these phenols leads to the browning of white wines (Fulcrand *et al*., 2006; Garrido & Borges, 2013; Rustioni, 2017). The cause of pinking is still speculative when it comes to phenols as causative agents.

TABLE 1
The differences in the assays used in South Africa, France, America and Australia

	H_2O_2 from 30% (w/v) stock solution	Sample volume (mL)	H_2O_2 added (µL)	Waiting period (hours)
South Africa	0.072%	25	1 000	8
Australia	0.3%	40	500	12
United States of America	0.3%	10	250	24
France	30%	10	125	24

The H_2O_2 solutions are all in % (w/v)

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The attribution of several possible phenols (protocatechuic acid, catechin, epicatechin, caffeic acid, gallic acid, ethyl gallate, p-hydroxybenzoic acid, quercetin-3-rutinoside, quercitin-3-glucoside, quercetin-3-L-rhamnoside, quercetin-3-D-galactoside, cis-coutaric acid, trans-coutaric acid, m-coutaric acid, p-coutaric acid, caftaric acid, ferulic acid, fertaric acid and coumaric acid), the implication of unstable flavonoid phenols (astilbin and engeletin) and the chemical degradation of some of these procyanidins in the presence of oxygen to form anthocyanogens may lead to the appearance of a pink colour (Tobe, 1983).

The role of temperature

Simpson (1977b) states that the development of the pink colour can generally be linked to the ingress of oxygen during winemaking processes. These processes are normally critical points in which oxygen uptake plays a role, such as pump-overs, filtration, bottling, etc. The solubility of oxygen increases with a decrease in temperature (Simpson, 1980b). Oxygen solubility in wine at room temperature and atmospheric pressure is about 6.0 mL/L (8.6 mg/L) (Castellari *et al*., 2004; Waterhouse & Laurie, 2006). This solubility increases by about 10% with a decrease in temperature (Waterhouse & Laurie, 2006). Winemakers therefore should be careful about practices in which wine temperature is kept low and the chances for oxygen uptake are high, like crushing and destemming, pressing and pumping of wine at low temperatures.

Light

Simpson (1980a) suggests that when a wine pinks in the bottle it can be exposed to direct sun or UV light for about 10 minutes to reduce the pink discolouration. UV exposure might lead to reduced pinking, but can have other negative effects. To explain the chemistry behind UV exposure, Clark *et al*. (2011) show that Fe^{3+} and light have the potential to degrade wine compounds, like tartaric acid, 3-mercaptohexanol (3MH) and 3-mercapto hexylacetate (3MHA). Different coloured glass bottles have different degrading properties and protection against UV light and the degradation of compounds. The different coloured glass bottles, with their protective abilities in increasing order, are Flint < Arctic Blue < French Green < Antique Green glass (Dias *et al*., 2012). Light can also catalyse free radical reactions that are involved in the peroxidation step of autoxidation (Simpson, 1980b). The UV light furthermore promotes the browning of phenols in wine (Clark *et al*., 2011; Parish-Virtue *et al*., 2019). UV light has the ability to excite singlet oxygen, which is then able to diffuse over a large distance of 270 nm (2.7×10^{-4} mm). The singlet oxygen molecule is electrophilic, as it has a completely vacant $2p\pi$ orbital. Therefore, the singlet oxygen molecule can react with high e-density double bonds via a six-membered ring. This results in the formation of hydroperoxide radicals ($HOO\cdot$) that again assist in autoxidation (Choe & Min, 2009).

Trace metals

During non-enzymatic oxidation or chemical oxidation, H^+ ions are transferred from a diphenol to an O_2 to form H_2O_2, but these reactions can only take place in the presence of metal ion catalysers like Fe^{3+} and Cu^{2+}. This process is mediated by the redox cycle, in which Fe^{3+}/Fe^{2+} and Cu^{2+}/Cu^{+} reduce oxygen to hydrogen peroxide (Oliveira *et al*., 2011). For this reason, knowledge of the iron and copper concentration of the wine is of utmost importance, as it can have a significant impact on the autoxidation of the wine.

SO_2 concentration of the wine

During a study done by Simpson (1977a) on the effect of SO_2 on pinking susceptibility, he reduced the pH of a range of wines to pH 1. Sparging of the wine with nitrogen expelled the SO_2 from the wine. Simpson then adjusted the pH of the wines back to their normal states and added SO_2 in potassium metabisulphite form up to a free SO_2 of 60 mg/L. Two concentrations of H_2O_2, viz. 15 mL/L and 75 mL/L, were added to the wines and the pinking susceptibility was tested. With the lower concentration (15 mL/L) of H_2O_2, the pinking susceptibility was reduced proportionally with the increase in free SO_2. With the higher concentration of H_2O_2 (75 mL/L), there was a reduction in pinking at a free SO_2 of 40 mg/L. The amount of free SO_2 (concentration of about 40 mg/L) in the wine was sufficient to react with H_2O_2 to form an HSO_3^- anion. This will also be sufficient to prevent pinking in white wines (Simpson, 1977a).

Ascorbic acid addition as an antioxidant

Ascorbic acid is a very strong antioxidant as it reacts effectively with O_2 in the wine (Simpson, 1980a, 1980b). The H_2O_2, formed from transferring an H^+ ion to an O_2 from ascorbic acid, is also a very strong oxidising agent (Bradshaw *et al*., 2004, 2011; Barril *et al*., 2016) and therefore the concentration of free SO_2 in the wine needs to be at least 40 mg/L (Simpson, 1977a). Ascorbic acid is one of the agents that works very well in preventing pinking in white wines. The addition of ascorbic acid prior to bottling may also keep the wine safe against oxidative browning in the bottle (Gibson, 2006). But there could also be a downside to the addition of ascorbic acid as it may also decrease the shelf life of the wine, with the risk of oxidative browning and even pinking (Bradshaw *et al*., 2011; Barril *et al*., 2016). Bradshaw *et al*. (2004) found that the molar ratio of ascorbic acid to SO_2 must be 1:1.7 to prevent oxidative browning and pinking in wines.

Wine pH

The equilibrium of molecular SO_2, bisulphite and sulphite ions in wine is pH dependent. A sulphite anion attached on the C-4 position of the anthocyanin transforms it into a colourless form. This means that, at a lower pH, more molecular SO_2 is available for the protection of the wine against oxidation (Simpson, 1980b; Abramovič *et al*., 2015). Simpson (1977a) tested the influence of pH on pinking. Wine with a known pinking susceptibility was used to provide a pH range from 2.75 to 4.00. Pinking values were then obtained four hours after the addition of 75 mg/L H_2O_2. In a second test, samples were acidified to a pH of 1 and assayed for pinking. No significant differences were obtained in the pH range, as well as for the acidification test. This led Simpson to believe that the compound causing pinking is not a flavylium salt or its glucosides (anthocyanins). This

was confirmed by Tobe (1983), who used seven cultivars made from grapes in an experimental wine cellar in 1981, and Jones (1989), who made wine in three consecutive years (1985 to 1987). The wines that were made were treated specially for the experiments planned. Although true for monomeric anthocyanins, polymeric anthocyanins are more resistant to SO_2 bleaching and pH changes (Somers, 1971; Andrea-Silva *et al*., 2014). During ageing and/or maturation, a polymerisation of anthocyanins takes place at the C-8 and C-6 positions, forming anthocyanin-tannin condensation reactions (Monagas *et al*., 2005). These reactions lead to a stable polymeric anthocyanin, which therefore is resistant to decolouration by SO_2 and to pH changes (Somers, 1971).

TREATMENT OF PINKING SUSCEPTIBILITY OF WHITE WINES

Lamuela-Raventós *et al.* (2001) did a series of experiments to find the best product to remove the precursors for pinking susceptibility in white wines. Wines were divided into four lots, control wine; wine with 1 g/L PVPP, wine with 1 g/L PVPP + 0.5 g/L bentonite and wine with 1 g/L PVPP + 15 mg/L ascorbic acid. Wine treated with 1 g/L PVPP reduced pinking by 74%, the wine with 1 g/L PVPP + 0.5 g/L bentonite reduced pinking with 90%, and the wine with 1 g/L PVPP + 15 mg/L ascorbic acid reduced pinking with 98%. However, after 20 days the capacity of ascorbic acid + PVPP to reduce pinking decreased to the same levels as that of PVPP + bentonite. Tobe (1980) investigated the removal of precursors by bentonite and PVPP. He found initially observed decreases in total phenols by bentonite fining, to be ineffective after applying the Freundlich equation. PVPP was more favourable in removing the total phenols. Lamuela-Raventós *et al.* (2001) added various concentrations of ascorbic acid to a wine, i.e. 0, 15, 30, 45 and 100 mg/L. At 30 mg/L pinking was reduced, however, at 45 mg/L pinking was completely prevented. This showed that ascorbic acid is a good agent to prevent pinking susceptibility in white wine, but it could lead to oxidative browning after an extended period (Lamuela-Raventós *et al.*, 2001).

ALTERNATIVE EXPLANATIONS TO PINKING

Andrea-Silva *et al*. (2014) report the compound to cause pinking susceptibility in Siria wines to be malvidin-3-O-glucoside, which was the most abundant anthocyanin tested. Siria is a Portuguese cultivar, a widely planted Iberian variety also known as Roupeiro, Doña Blanca and Cigüente. The wine is aromatic but oxidises easily (Robinson *et al*., 2012).

Andrea-Silva *et al*. (2014) mention that the wine used for their experiments pinked naturally. After pinking, 0.8 g of PVPP was added. The suspension was then filtered through a cheesecloth and washed with 100 mL of water and 100 mL of ethanol (95%). Thereafter, the PVPP was loaded into an empty SPE cartridge and eluted with acetonitrile and acetone, an aqueous solution of 1% HCl, ethanol and 0.1 M NH_3 in ethanol. Each fraction was kept separate. After evaporation and reconstitution with 0.2 mL of methanol and water, the samples were loaded onto an HPLC. The main compound found was malvidin-3-O-glucoside. Andrea-Silva *et al*. (2014) also established that the minimum amount of total anthocyanin must be 0.3 mg/L (300 µg/L) to turn the wine a visible pink colour. This experiment was repeated in 2019 (Cosme *et al*., 2019). Arapitsas *et al*. (2015) analysed grapes of Sauvignon blanc, Chardonnay and Riesling using a UPLC-MS/MS. They found measurable amounts of malvidin-3-O-glucoside, as well as carboxypyranomalvidin-3-O-glucoside (A-type vitisin) and pyranomalvidin-3-O-glucoside (B-type vitisin). The amounts were 55.44 µg/kg, 37.05 µg/kg and 38.99 µg/kg, respectively, for Sauvignon blanc, Chardonnay and Riesling (Arapitsas *et al*., 2015).

In genetic analyses for anthocyanins in red and white grapes, six genes were determined in the flavonoid biosynthetic pathway. Some genes were expressed in all grapes, even where little or no anthocyanins accumulated, but an expression of the gene encoding a UDP glucose-flavonoid 3-o-glucosyl transferase (UFGT) was only detected in red grapes that synthesised anthocyanins. The analysis of the white grapes indicated that the UFGT gene was present but was not expressed (Boss *et al*., 1996). External environmental conditions and vineyard practices therefore can switch on these genes to start the anthocyanin metabolic pathways (Boss *et al*., 1996).

The original researcher on pinking, Dr Bob Simpson, reported that phenols (flavonoid and non-flavonoids) and not anthocyanins are the causative compound. Research on pinking in wine is thus far from over and more evidence is needed to find the colour-forming compound.

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Modulation of Aroma and Sensory Properties of Prokupac Wines by a *Bacillus*-based Preparation Applied to Grapes Prior to Harvest

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Modern viticulture requires the replacement of hazardous agrochemicals with eco-friendly, bio-based products such as microbial preparations that enhance grape and wine quality while protecting the grapevine from pest and disease attacks. This study investigated the effects of a commercially available *Bacillus*-based preparation on the volatile and sensory properties of wines made from *Vitis vinifera*, cv. Prokupac grapes. Three different concentrations of preparation based on *Bacillus subtilis* Ch-13 were applied to grapevines two weeks prior to harvest. The total soluble solids in the grapes was affected by the application of *B. subtilis* Ch-1 and the alcohol content of the wine made from these grapes was greater. Wines made from the *B. subtilis* Ch-13-treated grapes showed an average increase in total phenolic compounds of about 27%, compared to the wine made from the untreated control grapes. The colour intensity of wines from the treated grapes, independently of the concentration, was higher by more than 30% than for the wine from the control grape sample. The *B. subtilis* Ch-13 treatment also affected the content of 3-methyl-1-butanol, ethyl decanoate and ethyl octanoate in the wine, at about 35%, 40% and 20%, respectively. The latter compounds are responsible for floral and fruity aromas. Generally, wines made from the treated grapes showed similar sensorial characteristics but scored better overall than the control. Principal component analysis showed a clear differentiation between wine made from the control and that from the *B. subtilis* Ch-13-treated grapes. The results suggest that the application of *B. subtilis* Ch-13 to grapevines two weeks prior to harvest has a positive effect on wine quality.

INTRODUCTION

Traditional agricultural practices are based on the use of large quantities of different agrochemicals (fertilisers, fungicides, pesticides, hormones), which lead to pest, weed and disease resistance, the accumulation of hazardous residues in food and a negative impact on the environment. Consequently, there is an increasing interest in modern agriculture to replace hazardous substances with alternative yet innovative practices using natural, eco-friendly and bio-based preparations (Alori & Babalola, 2018; Damalas & Koutroubas, 2018; Abbey *et al*., 2019). Bearing in mind that healthy and high-quality grapes are one of the cornerstones of wine production, finding alternative techniques to control diseases in viticulture is also increasingly gaining importance.

One of the prospective solutions that meet these global demands is to replace agrochemicals with microbial-based preparations. Microbial-based preparations consist of different beneficial microorganisms that have both phytostimulatory and biopesticidal effects. Such a method is concurrently harmless to the environment, human health or the agro-ecosystem (Pertot *et al*., 2017), without a negative influence on the grape and wine microbiota (Escribano-Viana *et al*., 2018). Microbial-based preparations can be applied during the preharvest period harmlessly when the grapes are highly susceptible to fungal diseases (Otoguro & Suzuki, 2018). Using beneficial microorganisms in microbial products should not only prevent or protect grapevines from diseases and pests (Kim & Hwang, 2007), but it can also help to enhance aroma and polyphenol compound biosynthesis, increase anthocyanin content in the berry skin (Otoguro & Suzuki, 2018), add or mobilise nutrients from the soil (Alori & Babalola, 2018) and improve the growth and yield of grapevines (Rolli *et al*., 2017). So, besides reducing the economic losses in vineyards, the application of microbial-based preparations also improves grape berry quality and positively affects wine quality (Otoguro & Suzuki, 2018).

Different microorganisms (fungi, bacteria, yeast, actinomycetes) or their active ingredients have been used for the formulation of some commercially available microbial preparations (Abbey *et al*., 2019; Calvo-Garrido *et al*., 2019).

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Among them, *Bacillus* spp. have been used in many *in vitro* and field studies, and its antagonistic efficacy against various plant pathogens has been confirmed (Shafi *et al*., 2017; Calvo-Garrido *et al*., 2019). Aside from their antifungal effect, *Bacillus* spp. act as a plant growth promoter. It has been proven that this bacterium has the potential to stimulate the synthesis of grape polyphenols, aroma compounds and precursors, improve anthocyanin accumulation (Otoguro & Suzuki, 2018) and synthesise antimicrobial secondary metabolites and fungal cell wall-degradation enzymes (Shafi *et al*., 2017; Alori & Babalola, 2018; Stamenković *et al*., 2018). Although it is clear that using this species in viticulture opens up new possibilities for the safe production of quality grapes, and therefore wine with enhanced flavour and aroma, the exact impact that a particular microorganism might have has not been studied sufficiently.

For this reason, the aim of this study was to investigate the effect of commercial *Bacillus*-based preparations on grape and wine quality, as well as on the aroma and sensory profile of wines produced from an indigenous Serbian *Vitis vinifera* red grape cultivar, Prokupac.

MATERIALS AND METHODS

Grape treatment

The experiments were carried out during the 2018 season in a six-year-old vineyard (Central Serbia wine region, Tri Morave wine subregion, 43°37' N, 21°34' E, single Royat cordon vine training system) planted with *Vitis vinifera* (cv. Prokupac), which is a Serbian autochthonous red grape variety. Two weeks before the grapes reached full technological maturity, a commercially available preparation based on *Bacillus subtilis* Ch-13 (Ekstrasol®, minimum 10^7 CFU/ml, BioGenesis d.o.o., Serbia) was applied. Three different volume percent concentrations (v/v), 0.3% (1.35 L/ha), 0.5% (2.25 L/ha) and 0.7% (3.15 L/ha) (chosen according to the manufacturer's recommendations), were applied directly onto vine leaves using a hand-held compressed-air sprayer. Each treatment was performed in triplicate (three consecutive rows of approximately 100 vines for each experiment, one row per replicate). Three rows of untreated grapevines served as controls.

Winemaking processes

Grapes from each row were harvested manually (when total soluble solids reached approximately 20 to 21°Brix) and further processed separately (de-stemming, crushing with the addition of potassium metabisulphite (50 mg/L) and enzyme EXV (3 g/hL, Lallemand, Canada). Yeast nutrient Fermaid E (Lallemand, Canada) was added later during fermentation (30 g/hL). Musts were inoculated with *Saccharomyces cerevisiae* strain ICV D254 (25 g/hL, Lallemand, Canada) and small-scale fermentation (50 L) on skins was performed at 18°C to 20°C for all the samples. In order to improve maceration, the cap (solid grape parts) was punched down every three hours 24 h after yeast inoculation and during the active fermentation phase. Delastage (rack-and-return of fermenting juice) was performed daily until the sugar content dropped below 7°Brix. When alcoholic fermentation was finished (residual sugar level under 4 g/L), wine samples were separated from the skins, pressed gently and sulphited (25 mg/L), and then racked off from the primary yeast lees after 72 h. Before bottling, wine samples were again racked off and filtered using Seitz filter plates K 100 (Pall Seitz, Germany). Finally, twelve wines were produced (triplicate of the three treatments and control) and stored in bottles for six months at 15°C until chemical and sensory analysis.

Standard oenological parameters

Official methods recommended by the International Organisation of Vine and Wine (OIV, 2019) were used to analyse the standard oenological parameters of wine samples. The total phenol index (TPI) was evaluated by measuring absorbance at 280 nm (González-Rodríguez *et al*., 2002), while total anthocyanins, flavonoids and flavan-3-ols were determined by the methods described by Nedelkovski *et al*. (2017).

Extraction of volatile organic compounds in wines by headspace-solid phase microextraction (HS-SPME)

The SPME manual holder and fused silica fibre coated with Carboxen®/Polydimetilsiloxane (CAR/PDMS) stationary phase (85 µm thickness) were used for aroma compound extraction. The fibre was preconditioned before the first use (1 h at 300°C). Twenty millilitres of wine sample, 3 g of NaCl and a magnetic stirrer bar were placed in a 30 ml amber glass bottle, closed with a rubber septum and sealed with parafilm. The samples were heated to 55°C and agitated using a magnetic stirrer for 15 minutes (pre-extraction). The volatiles were extracted for 35 minutes maintaining the same heating and stirring conditions. The fibre was than desorbed for 10 minutes in split/splitless inlet set at 250°C in 20:1 split mode and analysed by GC/MS and GC/FID.

Volatile organic compounds from the wine samples were analysed using an Agilent Technologies 7890B gas chromatograph, coupled with inert, selective 5977A mass detector. Components were separated using a HP-5MS column (30 m × 0.25 mm, 0.25 µm film thickness; Agilent Technologies, USA). Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The GC oven temperature was held for 2 min at 40°C, increased to 250°C at a rate of 7°C/min, and finally held at 250°C for 2 min. The temperatures of the MSD transfer line, ion source and quadruple mass analyser were set at 300°C, 230°C and 150°C, respectively. The ionisation voltage was 70 eV and mass detection was done in the scan mode, in *m*/*z* range from 25 to 550. GC-FID analysis was performed under the same experimental conditions. The FID detector temperature was set at 300°C.

Data processing was performed using MSD ChemStation (revision F.01.00.1903) in combination with AMDIS (revision 2.70) and NIST MS Search (version 2.0g) software (Agilent Technologies, USA). The retention indices of the components were determined experimentally using a homologous series of *n*-alkanes from C_8 to C_{20} as standards, and the identification of the compounds was done using the Adams 2007 as well as Willey 6, NIST11 and RTLPEST 3 libraries. The content (%) of a particular component in the samples was determined on the basis of the area percent report generated by Agilent ChemStation software. The Area% report gives the area of each peak as a percentage of the total area of all peaks.

Sensory analysis

Descriptive sensory analysis was conducted by the officially certified members of the sensory panel authorised for wine sensory analysis by the Serbian Ministry of Agriculture, Forestry and Water Management (six females and five males, from 29 to 51 years old). The wine samples were evaluated in duplicate in random order, while the intensity of each attribute was rated by using a 10-point scale (0 – not detected, 10 – very intense). For each wine sample, the panellists evaluated olfactory (spice, colour intensity, vegetable, red fruit berries, black fruit berries, floral, complexity, intensity, typicality and toasted) and taste (harmony, acidity, astringency, fulness, complexity, duration, structure, intensity, typicality) attributes.

Statistical analysis

Results from the standard oenological parameters of wine were analysed using one-way analysis of variance (ANOVA), followed by Tukey's HSD post-hoc test (significance $p < 0.05$). Principal component analysis (PCA) was used to determine the contribution of identified volatile organic compounds to the differences observed between wine samples. PCA was performed using Statistica 10 Software (StatSoft Inc., Tulsa, U.S.A., trial version).

RESULTS AND DISCUSSION

Basic must and wine parameters

The initial quality parameters of the must made from the control grape samples and grape samples treated with different concentrations of *B. subtilis* Ch-13 preparation are shown in Table 1. An increase in the total soluble solids of about 8% was observed independently in the preparation concentration, without a statistically significant effect on the juice pH. Total acidity (g/L tartaric acid) was slightly different and ranged from 5.7 for the control grape to 6.2 for the musts from the treated grapes.

It is well known that sugar accumulation in grapes depends primarily on the cultivar, stage of maturity and environmental conditions (temperature, light, humidity) (Jackson, 2008; Jordão *et al*., 2015), but some authors indicate that viticultural practices (defoliation, cluster thinning, irrigation) could also affect the sugar content in grape berries (Esteban *et al*., 2002; Bogicevic *et al*., 2015), as well as fungal and bacterial infections (Jordão *et al*., 2015). Sivčev *et al*. (2005) confirm that the application of a biological preparation based on the *Azotobacter chroococcum* and *Bacillus megaterium* strain on the Riesling grape variety also positively affects sugar and acid accumulation in the grape juice. More recently, Escribano-Viana *et al*. (2018) reported that a biofungicide based on the *Bacillus subtilis* QST 713 had no significant impact on the pH, sugar and acid content of Tempranillo grapes, while Chebotar *et al*. (2009) showed that the *Bacillus subtilis* Ch-13 strain has the ability to stimulate plant growth and act as biofungicide due to the extracellular synthesis of phytohormone, lytic enzymes and other antifungal metabolites.

Although the increase in the sugar content of grapes is undesirable in the context of warmer ripening periods and a changing climate (Mozell & Thachn, 2014), it could be useful for northern and cool-climate regions or for some varieties in which an insufficient sugar level is a common occurrence in the grapes (Yadav *et al*., 2011). The increase in grape sugar and acid levels in our experiments caused by the use of *B. subtilis* Ch-13 preparations should be considered a positive phenomenon, since the grape variety Prokupac is known to produce only satisfactory (not high) sugar levels and shows a rapid decrease in acid concentration in the final stages of ripening. The work of Marković *et al*. (2017), which included twenty-five different Prokupac clones, showed that only five of them reached s sugar level above 20% at the time of technological maturity. Bearing in mind that the optimal sugar content for the production of red wine is 21% to 24%, it can be considered that the use of the *B. subtilis* Ch-13-based preparation has a positive effect on the quality of the Prokupac grape and potentially has an effect on wine quality.

The effects of the application of the *B. subtilis* Ch-13 preparation on the basic quality parameters of Prokupac wine samples are summarised in Table 2. The application of the *B. subtilis* Ch-13 preparation significantly affected the quality parameters of Prokupac wines (Table 2). The wines obtained from the Prokupac grapes treated with different concentrations of this biopreparation had a higher alcohol content than the wine obtained from the untreated (control) grapes, which is in accordance with the initial sugar content of the grapes. Better grape sugar accumulation, and consequently higher wine alcohol content, was also observed in Riesling wine when grapes were treated with a microbiological preparation based on the *A. chroococcum* and *B. megaterium* bacterial cultures (Sivčev *et al*., 2005), and in Merlot wines obtained from grapes treated with a preparation based on cultures of *A. chroococcum*, *B. megaterium* and *Bacillus circulans* (Raicevic *et al*., 2004).

Wine samples obtained from grapes treated with different concentrations of *B. subtilis* Ch-13 preparation contained a

TABLE 1
Quality parameters of control grapes and grapes treated with different concentration of *B. subtilis* preparation

		Concentration of *B. subtilis* preparation, %		
Parameter	Control	0.3	0.5	0.7
Total soluble solids, ˚Brix	19.8 ± 0.2a	21.13 ± 0.4b	21.57 ± 0.2b	21.36 ± 0.3b
Total acidity, g/L tartaric acid	5.7 ± 0.2a	5.8 ± 0.1a	5.9 ± 0.2ab	6.2 ± 0.2b
pH	3.25 ± 0.01a	3.23 ± 0.01a	3.22 ± 0.01a	3.22 ± 0.01a

Means followed by the same letter within a row are not significantly different at $p \leq 0.05$ by Tukey's HSD test

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TABLE 2
Quality parameters of wines obtained from control grapes and grapes treated with different concentrations of *B. subtilis* Ch-13 preparation

Parameter	Control	Concentration of *B. subtilis* preparation, %		
		0.3	0.5	0.7
Alcohol, %	11.5 ± 0.01a	12.3 ± 0.00b	12.7 ± 0.02c	12.6 ± 0.05d
Total dry extract, g/L	25.9 ± 0.44a	27.6 ± 0.20b	27.4 ± 0.40b	27.1 ± 0.19b
Total titratable acidity (as tartaric acid), g/L	5.5 ± 0.20a	5.7 ± 0.10ab	5.7 ± 0.10ab	6.0 ± 0.20b
Volatile acids (as acetic acid), g/L	0.50 ± 0.09a	0.32 ± 0.05b	0.36 ± 0.03b	0.33 ± 0.05b
Residual sugars, g/L	2.0 ± 0.22a	1.5 ± 0.09b	1.6 ± 0.09b	1.8 ± 0.07a
Total polyphenols, g/L	1.23 ± 0.07a	1.39 ± 0.04b	1.39 ± 0.02b	1.38 ± 0.02b
Total anthocyanins, g/L	0.34 ± 0.01a	0.40 ± 0.03b	0.41 ± 0.04b	0.39 ± 0.01b
Colour intensity	0.44 ± 0.002a	0.60 ± 0.03b	0.62 ± 0.01b	0.54 ± 0.04c
Wine hue	0.568 ± 0.04a	0.511 ± 0.04b	0.500 ± 0.02b	0.501 ± 0.02b
Flavan-3-ols, mg/mL	0.26 ± 0.02a	0.30 ± 0.02ac	0.30 ± 0.01ac	0.33 ± 0.03bc
Total flavonoids, mg/mL	0.68 ± 0.02a	0.81 ± 0.01b	0.87 ± 0.02c	0.81 ± 0.01b
Total phenolic index	27.6 ± 0.25a	34.7 ± 0.24b	36.4 ± 0.19b	34.7 ± 0.67b
Free SO_2, mg/L	24.76 ± 1.08a	24.74 ± 1.14a	24.04 ± 0.86a	23.68 ± 0.92a
Total SO_2, mg/L	51.96 ± 1.16a	53.92 ± 0.96a	53.58 ± 1.06a	52.90 ± 0.98a

Means followed by the same letter within a row are not significantly different at $p \leq 0.05$ by Tukey's HSD test

higher content of total phenolic compounds, anthocyanins, flavonoids and flavan-3-ols, at about 13%, 18%, 22% and 19%, respectively when compared to the control wine sample. A statistically significant difference was observed between the control wine and wines from treated grapes ($p < 0.05$). The total phenolic index (TPI) values for wine samples from treated grapes indicate that treatment with *B. subtilis* Ch-13 preparation causes an average increase in total phenolic compounds of about 27%. The TPI value gives a reliable estimation of wine polyphenolic content and is considered better and more useful than other routine spectrophotometric methods for the quick and easy screening of wine quality (González-Rodríguez *et al*., 2002).

Recently published results show that the content of total phenolic compounds (323.2 mg/L and 307.2 mg/L) in Prokupac wine (2013 and 2014 vintages) was four times lower (Lakićević *et al*., 2019) than the contents in the wine samples analysed in this study, while a significantly higher content of total phenolic compounds (about 2.3 g/L) and lower levels of total anthocyanins (about 0.28 g/L) were observed in another sample of Prokupac wine (Malićanin *et al*., 2017). Such a large difference can be explained by differences in climatic factors, soil type, sunlight exposure, vineyard altitude or vinification process, which are all crucial for the wine phenolic content (Cosme *et al*., 2017).

Since wine phenolic compounds are extracted mainly from grape berry skins, our results indicate that the treatment with *B. subtilis* Ch-13 preparation improves the synthesis of phenolic compounds in grape berry skins, with a moderate impact on soluble solids. Therefore, it can be concluded that *Bacillus* treatment could enable simultaneous technological and phenolic maturation. Such a feature might facilitate the decision on the harvest date and enhance the polyphenol composition of the corresponding wines. Knowing that polyphenolic compounds strongly affect wine quality, mainly by contributing to its sensory (colour, astringency, bitter, aroma) or ageing characteristics (Niculescu *et al*., 2018), a higher content of these classes of compounds in wine made from treated grapes is also consistent with the fact previously reported in the literature that biopreparates based on microorganisms could improve grape and wine quality (Otoguro & Suzuki, 2018).

More recently, it was revealed that the application of *Bacillus cereus* strain NRKT improves the synthesis of stilbene synthase, which is the most important enzyme for resveratrol synthesis, and consequently leads to an increase in resveratrol in the grape berry (Aoki *et al*., 2017). Also, our results are in line with the findings according to which sugars such as glucose, fructose and sucrose serve as signalling molecules affecting the flavonoid pathway and promoting anthocyanin synthesis (Zheng *et al*., 2009; Shi *et al*., 2018). It is safe to assume that the observed higher sugar content in treated grapes enhances the polyphenolic content in grapes and in the produced wines.

Although the content of polyphenolic compounds in grapes is considered to be dependent mainly on the grape cultivar and environmental and climatic conditions (Guerrero *et al*., 2019), it is worth noting that viticultural practices, such as the application of microbiological preparations, could promote the accumulation of these classes of compounds.

Treatment with *B. subtilis* Ch-13 preparation had no statistically significant effect on the free and total SO_2 content

in the wine samples, while a significant reduction in volatile acids was observed compared with the control sample (Table 2). Although the amount of volatile acid detected in all samples is in accordance with European legislation (1.2 g/L of acetic acid), high concentrations are undesirable and would give an unpleasant aroma of acetic acid to wine (Vilela *et al*., 2013). Comparing the effect of three microbiological preparations based on different microorganism strains (*A. chroococcum*; mixture of *A. chroococcum* and *B. megaterium* or mixture of *A. chroococcum, B. megaterium* and *B. circulans*) on the total acids in Riesling wines, the authors highlighted that the total acid content depended on the type of microorganisms on which the preparation was based. The highest total acids (8.21 g/L) were observed in wine from grapes treated with preparations based on *A. chroococcum*; lowest total acids (6.32 g/L) were found in wines from grapes treated with preparations based on *A. chroococcum* and *B. megaterium,* while the control wine sample contain 6.45 g/L total acids (Sivčev *et al*., 2005).

The residual sugar in the wine samples did not exceed 2 g/L, which indicates that, regardless of the treatment, all wines should be considered dry. The chromatic parameters of the wine samples (colour intensity, hue) indicated that the *B. subtilis* Ch-13 preparation treatment significantly affects the colour of Prokupac wine samples. The colour intensity of wines from the treated grapes, independently of the concentration, was higher by more than 30% than for the wine from the control grape sample. Colour hue represents the ratio between the yellow/orange and the red wine pigments (Coradini *et al*., 2014), so the higher values for the control wine sample indicate a smaller percentage of red pigments. The correlation between values for the colour intensity and the hue of wine colour, where higher colour intensity corresponds to a lower hue value, was also observed for Merlot, Vranac, Prokupac, Cabernet Sauvignon, Game (Babincev *et al*., 2016), Pinot Noir and Burgund (Coradini *et al*., 2014) wines. As the anthocyanins are mainly responsible for red wine colour, the values for the chromatic parameters were expected and in accordance with the improved accumulation of this class of compounds in the treated grape samples (Table 2). Since the wine colour is an important wine characteristic that highly affects overall consumer acceptance, and bearing in mind that wines produced from the Prokupac grape variety typically have a lower colour intensity than red wines produced from varietals such as Cabernet Sauvignon, Vranac, Merlot and Game (Babincev *et al*., 2016), these research findings can be considered very valuable, especially in the production of quality wines from poorly coloured grape varieties.

The content of total dry extract in all wine samples was within the range of 25 to 30 g/L, which is typical for light-bodied red wines (Castilhos *et al*., 2013), such as Prokupac wines are traditionally. However, significant differences were observed in total dry extract between the control sample and the wine samples produced from treated grapes, with the control wine sample having the lowest content. The alcohol content and total dry extract observed in this study were significantly higher compared with the results obtained in previous studies (Lakićević *et al*., 2018, 2019), in which alcohol content and total dry extract for Prokupac wine (vintage 2013 and 2014) were in the ranges 10.26% to 10.49% and 16.10 g/L to 17.45 g/L, respectively. This may be related to the weather conditions during the growing season, different vinification procedures or yeasts, as well as the initial quality of the grape and must. A positive effect on total dry extract content has also been reported for Riesling wine obtained from grapes treated with the microbiological preparation based on *A. chroococcum* + *B. megaterium* compared to the wine obtained from the control sample (Sivčev *et al*., 2005). Moreover, a recent study demonstrated that treating the plant with microbial fertilisers can enhance the content of macro- and microelements in different grape varieties (Tangolar *et al*., 2019). Knowing that the total dry extract depends directly on the content of minerals in the grapes and wine, it can be assumed that the use of *B. subtilis* Ch-13 preparation improves the uptake of macronutrients and micronutrients and positively affects the mineral content in the treated grapes and produced wine.

However, it is also important to emphasise that the application of a microbial preparation based on *B. subtilis* QST713 had no significant impact on the berry and wine microbiota (Escribano-Viana *et al*., 2018), thus it does not affect spontaneous alcoholic fermentation.

Volatile composition of wine samples

A total of 26 different volatile aroma compounds were identified by HS-SPME-GC-MS in Prokupac wines obtained from a control and from grapes treated with different concentrations of *B. subtilis* Ch-13 preparation. All compounds were subdivided, according to their chemical structure, into five groups (alcohols, ethyl esters, acetates, volatile fatty acids, aldehydes), among which the most abundant were higher alcohols, followed by the ethyl esters (Table 3).

The majority of the wine higher alcohols are the by-products of alcoholic fermentation, while some could be found in the grapes (Jackson, 2008; Pineau *et al*., 2009). At an optimal concentration, they contribute positively to fruity characters and the complexity of wine (Lambrechts & Pretorius, 2000). The dominating compound in the Prokupac wines analysed in our study, independently of the grape treatment, was 3-methyl-1-butanol (isoamyl alcohol), while treatments of the grapes with *B. subtilis* Ch-13 preparations increased the content of this compound in wines by about 35% compared to the control sample. It has been reported that isoamyl alcohol has a positive effect on wine aroma (Bleve *et al*., 2016), while other researchers have associated the presence of this compound with vegetal/pepper (De-la-Fuente-Blanco *et al*., 2016), herbaceous, fruity, alcohol or cheese odour characteristics (Chambers IV & Koppel, 2013). However, although the effect of the *B. subtilis* Ch-13 preparation treatments on the aromatic profiles of the resulting wine is not negligible, there is no clear relationship between preparation concentration and the content of wine volatile organic compounds.

Ethyl octanoate (range 13.8% to 17.8%) and ethyl decanoate (range 5.9% to 8.5%) were detected as the dominating esters in Prokupac wines. Generally, the application of the *B. subtilis* Ch-13 preparation positively affected the presence of esters in wines. In particular, the

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TABLE 3
Identified volatile organic compounds[a] and their relative peak areas[b] (in %) in Prokupac wine analysed by HS-SPME-GC-MS

	Wine sample				
		Concentration of *B. subtilis* Ch-13 preparation, %			
Compound	Control	0.3	0.5	0.7	**Aroma descriptor**
Alcohols					
Isobutyl alcohol	3.2	1.1	2.6	-	Alcohol, solvent[1]
3-Methyl-1-butanol	22.9	31	31.4	30.4	Floral, malt, sweet[1]
2-Methyl-1-butanol	14.2	11.5	9.6	10.9	Floral, fruity, sweet[3]
1-Pentanol	10.3	12.4	11.8	12.7	Fruity, balsamic[2]
(S)-2-Methyl-1-butanol	3.3	3.5	3.2	1.4	-
1-Hexanol	1.8	0.6	0.6	0.6	Herbaceous, woody[2]
2-Methyl-1-pentanol	1.4	-	-	-	-
2-Phenylethanol	2.7	3	3.1	4.1	Rose, honey [2]
4-Methyl-1-pentanol	-	0.9	0.5	0.8	Almond, toasted[2]
2-Butanol	-	-	2.1	-	Sweet, fruty[2]
Total area (%)	59.8	64	64.9	60.9	
Acetates					
2-Phenyl acetate	1.1	-	-	-	Rose, honey[1]
2-Methylbutyl acetate	-	0.3	0.1	0.3	-
3-Methylbutyl acetate	-	-	-	0.4	Banana, apple, estery[1]
Ethyl acetate	4.2	1	1.5	1.1	Sweet, fruity [2]
Total area (%)	5.3	1.3	1.6	1.8	
Esters					
Ethyl butanoate	0.2	-	0.1	-	Strawberry, apple[2]
Ethyl hexanoate	1.1	0.8	0.7	1.1	Apple, fruity, sweetish[1]
Ethyl 3-methyl pentanoate	2.2	2.8	2.7	3.1	-
Ethyl octanoate	13.8	15.3	16.7	17.8	Sweet, fruity, pear[2]
Ethyl-(4E)-decanoate	0.6	0.6	0.9	1.1	-
Ethyl decanoate	5.9	8.4	8.5	9.4	Fruity, grape[2]
Ethyl dodecanoate	0.5	0.8	0.8	0.8	Sweet, floral, fruity[3]
Diethyl succinate	-	0.4	0.5	0.6	Fruity[2]
Total area (%)	24.3	29.1	30.9	33.9	
Volatile fatty acids			1.3	1.5	
Octanoic acid	1.6	1.4	0.3	0.2	Cheese, fatty acid, sweet[1]
Decanoic acid	-	0.3	-	0.6	Fatty, unpleasant[2]
Hexanoic acid	-	-			Cheese, fatty acid[2]
Total area (%)	1.6	1.7	1.6	2.3	
Aldehydes					
Benzaldehyde	0.9	0.2	0.3	0.3	Almond, cherry[3]
Total area (%)	0.9	0.2	0.3	0.3	

1. Vilanova *et al*. (2010); 2. Welke *et al*. (2014); 3. Yilmaztekin (2014)
[a] Compounds identified by matching to the NIST MS library spectra and comparison of the Kovats retention indices
[b] Relative amounts of compounds (in %) were calculated by dividing the peak area of each component by the total peak area of all components

content of ethyl decanoate increased greatly (by about 40%) with an increase in preparation concentration, while the content of ethyl octanoate increased in wines from treated grapes (by about 20%), independent of the preparation concentration. The two esters designated as most abundant in the produced Prokupac wines have been characterised previously as responsible for the dominant red-berry and fresh-fruit aromas of Merlot and Cabernet Sauvignon wines (Pineau *et al*., 2009) and as aroma-enhancing compounds (Zhu *et al*., 2016). Since fresh fruity aromas are generally desirable for red wines, these results may be of interest to red wine producers, not just when making wine from the Prokupac grape variety, but also from other grape varieties. The low level of free fatty acids should be associated with their esterification in the presence of alcohol during fermentation. Confirmation of this fact may be the existence of all corresponding ethyl esters, which are derived from the present free fatty acids.

Although wine volatile aroma compounds are mainly secondary yeast metabolites, their concentration depends directly on the chemical components of the grapes (Lambrechts & Pretorius, 2000; McKay & Buica, 2020). Based on the results presented, we can hypothesise that the application of *B. subtilis* Ch-13 preparation to the Prokupac grapevine increases the content of the neutral grape compounds (sugars, amino acids), which are the precursors for the formation of higher alcohols or esters and therefore can be considered responsible for the improvement in the aromatic profile of the wines.

Sensory profile of wine samples

Olfactory and gustatory characteristics, apart from the chemical composition and aromatic profile, represent one of the most essential attributes of the wine that contribute to consumer acceptance. The results of the sensory evaluation (Fig. 1) indicate that treatment by a *B. subtilis* Ch-13 preparation exhibits an influence on the sensory characteristics of the produced wines. All wine samples from the treated grapes, independent of the applied concentration, show similar sensorial characteristics and scored better overall results than the wine produced from the control grape sample. However, the difference is more noticeable in the gustatory than in the olfactory sensations. In fact, the wine produced from the control grape sample had the lowest value for all analysed olfactory characteristics, while for the difference was most observable for "harmony", "fulness", "complexity", "duration" and "colour intensity". This observation is supported by and based on the observed differences in basic chemical characteristics, such as the higher content of total dry extract and polyphenolic compounds (total phenolic compounds, flavonoids, anthocyanins, flavan-3-ols), the lower content of acids, and the different chromatic parameters of wines produced from the treated grapes. The impact of different viticultural practices on the sensory characteristics of wine was revealed previously (Biarnès *et al*., 2009; Thiollet-Scholtus *et al*., 2014), while wine produced from organically grown grapes compared to wine from conventionally grown grapes also demonstrated a change in taste (sour and bitter) and astringency sensations (Pagliarini *et al*., 2013).

Wines produced from the Prokupac grapes in this study had the highest intensity of 'red fruit berries', followed by 'black fruit berries' flavour. In terms of spice attributes, white pepper and cinnamon flavours were the most common descriptors, while linden, acacia and rose flavours were recognised as the floral attributes in all wine samples. Besides the minimal differences in 'red fruit', colour intensity and 'astringency' attributes, wines obtained from Prokupac in this study are sensorily similar to recently published results on wines from the same grape variety (Malićanin *et al*., 2017).

Principal component analysis (PCA)

Principal component analysis (PCA) was used to obtain additional information about the effects of the treatment by *B. subtilis* Ch-13 preparations on the aromatic profile of the

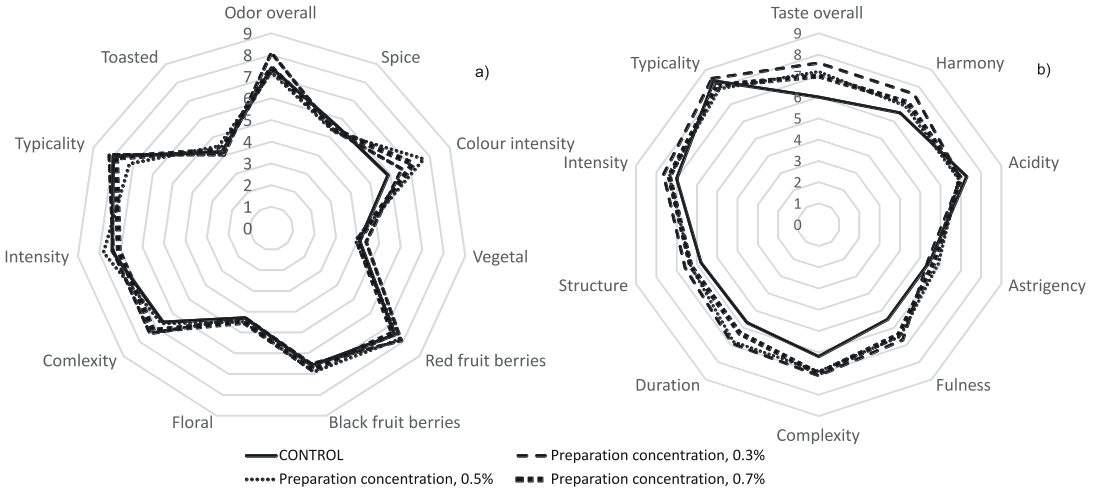

FIGURE 1
Visual appearance, olfactory (a) and gustatory (b) characteristics of Prokupac wines obtained from control grapes and grapes treated with different concentration of *B. subtilis* Ch-13 preparation

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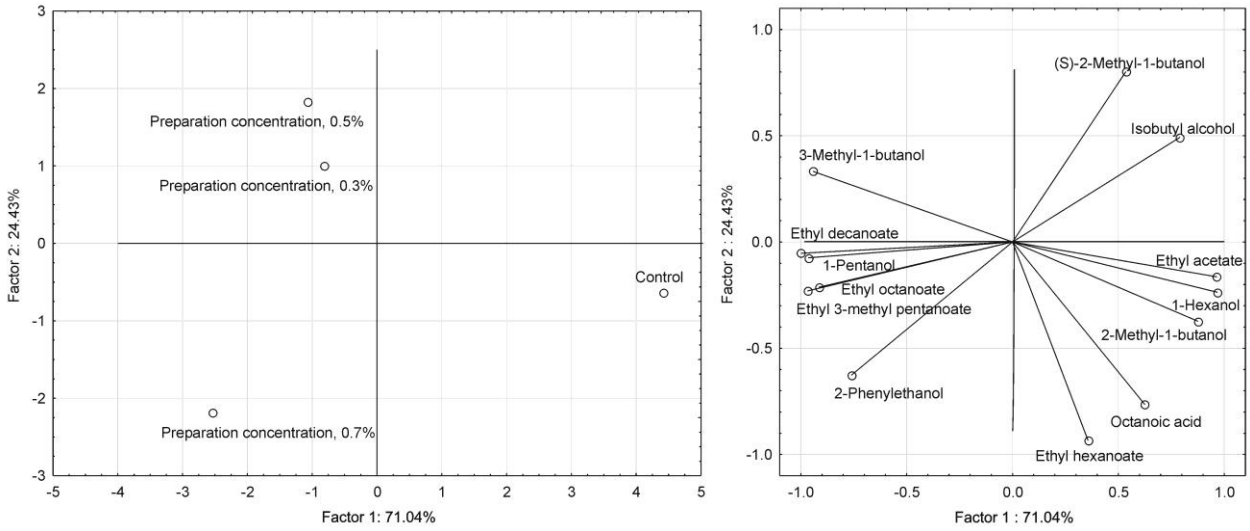

FIGURE 2

Principal component analysis of the relative chromatographic areas (considering only the volatile compounds with a relative peak area higher than 1% of the volatile organic compounds identified in the Prokupac wines obtained from control grapes and grapes treated with different concentrations of *B. subtilis* Ch-13 preparation

produced Prokupac wines. PCA explained more than 90% of the total variability among wine samples (Fig. 2), while the first principal component (PC1) explained more than 70% of the total variability.

PCA showed a clear differentiation between the control wine sample and the wine samples produced from the treated grapes. It was observed that the wines samples produced from the grapes treated with *B. subtilis* Ch-13 preparations showed strong and positive correlations with the ethyl ethers, while the wine produced from the control grape sample showed a strong correlation with 2-methyl-1-butanol and ethyl acetate, which are responsible for sweet and floral aromas. However, it was observed that the wines produced from the grapes treated with higher preparation concentrations (0.5% and 0.7%) were grouped in the negative quadrant of PC2, which indicates that higher *B. subtilis* Ch-13 preparation concentrations resulted in wines with more 3-methyl-1-butanol and ethyl dodecanoate, which are responsible for floral and fruity aromas.

CONCLUSIONS

To date, agricultural producers have become accustomed to using agrochemicals to protect crops and enhance yields. This has had unintended consequences on human health and the environment. Finding an alternative to hazardous agrochemicals has become a major focus in modern agriculture. Microbial-based preparations are natural pesticides that have great potential to replace agrochemicals because they are not harmful for either the environment or the plant. In contrast, they can even promote plant growth and enhance fruit quality. This knowledge is especially important in viticulture because of the susceptibility of grapes to disease attacks in the pre-harvest period, when agrochemicals cannot be applied due to withholding periods. The application of microbiological preparations is an ideal alternative because, in addition to having a biofungicidal effect and protecting the grapes, they have the effect of improving grape quality. This represents great potential for improving wines quality, especially wines prepared from grape varieties with certain undesirable properties that are sure to affect the final product's characteristics. In particular, the Prokupac grape, studied in this work, is a grape variety that typically gives wines with lower colour intensity, which can be perceived negatively by consumers because of the prejudice that wines that are not intensely coloured are not of superior quality.

In this paper, we have shown that the application of microbiological preparation based on *B. subtilis* Ch-13 to the grapes in the pre-harvest period significantly improves the coloration of wines made from the treated grapes. So, these research findings can be considered very valuable, especially for the production of quality wines from poorly coloured grape varieties. In addition, the results of our study show that *B. subtilis* Ch-13 positively influences the sugar content of grapes, which could be useful for northern and cool climatic regions or for varieties in which an insufficient sugar level in the grapes is a common occurrence. Also, the application of this preparation can improve the accumulation of polyphenolic compounds in grapes and wine, and increase the content of higher alcohols and esters, which are all responsible for the sensorial characteristics of the wine. Since the content of polyphenolic compounds has thus far been associated with the grape cultivar and environmental and climatic conditions, it is valuable to know that viticultural practices, such as the application of microbiological preparation, could promote the accumulation of these classes of compounds. The established potential of particular microbiological preparations represents valuable

information for grape and wine producers, and it should also serve as a stimulus for further research in this field.

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Investigating the Concept of South African Old Vine Chenin Blanc

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Although South African vineyards are still young by European standards, there is a belief in the industry that vines aged 35 or more years produce grapes and wines with specific characteristics ("old vine wines"). The aim of this study was to investigate the existence of the concept of old vine Chenin blanc wines using a typicality rating and sorting tasks. Chenin blanc wines were made from grapes harvested from vines aged five to 45 years old. Winemaking was standardised, with no wood contact. Typicality rating and sorting tasks were performed on young (first-stage) and two-year bottle-aged (second-stage) wines. Principal component analysis (PCA) on rating data demonstrated judge consensus, but no correlation was found between vine age and typicality rating. Sorting results were submitted to agglomerative hierarchical clustering (AHC) performed on the correspondence analysis (CA) and multidimensional scaling (MDS) results for grouping and attributes resulting from the sorting task. The clusters were different for the young wines and two-year bottle-aged wines. The verbal aspect of the sorting demonstrated the judges' agreement on the concept of old vine Chenin blanc, shown by the annotation of the *old vine* group as 'complex', 'balance', 'rich' and 'good mouthfeel'. However, because the judges did not sort the wines according to vine age, the perceptual aspect of the concept could not be confirmed, its features could not be tested further, and the sensory space could not be built.

Abbreviations: RV (regression vector); PCA (principal component analysis);
MDS (multidimensional scaling); CA (correspondence analysis);
AHC (agglomerative hierarchical clustering); DA (descriptive analysis);
CATA (check all that apply)

INTRODUCTION

In comparison to the long history of European and Middle Eastern vines (Stevenson, 2005), South African vineyards are young, with the first vines planted in the 17th century. According to recent statistics, 64% of the Chenin blanc planted (by area under vine) is less than 20 years old and 36% is older than 20 years (SAWIS, 2018). The "old vine" designation has been used as a heritage mark to support the conservation of these vines and was established by the South African Old Vine Project (OVP) in 2017. The OVP demarked South African "old vines" as being 35 years or older, based on information gathered from years of collaborative input from industry experts, including viticulturists and winemakers (Crous, 2016).

Old vines (vineyards, grapes and wines) tend to receive special treatment with regard to viticultural and winemaking practices, documented by several surveys and interviews with industry experts. This special treatment is actively encouraged by the OVP, as it is believed that it will harness the full potential of the old vine and impart the character to the resulting wine. Some of the guidelines include a "holistic approach to weed control", "movement from inorganic fertilisers to organic fertilisers", "a minimalistic approach towards winemaking" for the wines to "be given the chance to reflect their specific terroir", etc. (Old Vine Project [OVP], n.d.). Worldwide, it has been shown that any special treatment of a product (wine or other foodstuff) creates an emotional attachment to the product, along with expectations (Schouteten *et al*., 2015; Niimi *et al*., 2019).

The agreement among experts, which is reinforced through the OVP and its experience, is that old vine wines are less intense in fruity attributes but have more complex sensory attributes focused on mouthfeel; additionally, the full potential of the wine is reached after some years in the bottle, with the wines not being released in the harvest year (SASEV, 2018). Anecdotal evidence collected by the authors concerning old vine character (SASEV 2018) has created an interest in substantiating these ideas. In defining and testing the concept of "old vine character", evidence needs to be collected and hypotheses have to be formulated and tested.

Currently, there is little scientific support for the

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anecdotal evidence, as only one study profiled 16 Chenin blanc wines from vines older than 40 years using descriptive analysis (Crous, 2016). The study evaluated multiple sensory modalities, namely odour and in-mouth sensations, with a focus on mouthfeel. It also used calibrated standards and, where standards were not available, conceptual consensus was established based on discussions among judges. The reasoning for the mouthfeel approach was based on the anecdotal evidence mentioned above (OVP, n.d.; SASEV, 2018) . In the work by Crous (2016), when panellists described old vine Chenin blanc wines, the terms *body*, *concentration*, *complexity*, *length*, *acidity*, *heat*, *balance* and *integration* featured prominently. Since the samples were commercial wines made using different protocols, Crous (2016) noted that the effects of winemaking outweighed any possible correlations with the vine age.

One approach to studying old vine character is through establishing its associated typicality features. Wine typicality refers to a group of sensory attributes that, together, become the defining features describing a concept; typicality may be categorised under cultivar, winemaking style, regionality (appellation) or, in this case, old vine character. In this context, typicality is defined as the level (or "degree of representativeness") of a sample to a category, measured against a prototype (Chrea *et al*., 2005). In the case of a sensory concept, the prototypes or "established references" (Perrin & Pagès, 2009) can be different for each assessor due to differences in experience and exposure; hence, typicality judgments may differ among experts. Consistency among assessors suggests the homogeneity of the prototypes, or even the existence of a common prototype and possible conditions for demonstrating a typicality concept (Casabianca *et al*., 2006). In practice, it was demonstrated that wines that are less representative of the prototype belong to neighbouring categories (Perrin & Pagès, 2009) and it is possible for instances of borders between categories to arise (Ballester *et al*., 2005).

There are four stages to testing concepts of typicality and, according to the methodology proposed by Perrin and Pagès (2009), these have to be followed in sequence. Firstly, *panel agreement* has to be established, followed by *conceptual agreement*, *perceptual agreement* and, finally, *measuring the feature/drivers* can be considered. Each step is dependent on the previous one. If at any point agreement is not achieved, the investigation cannot be continued and the methods or panels have to be revisited.

Typicality can be investigated sensorially in different ways using verbal and/or non-verbal methods (Perrin & Pagès, 2009). The reasoning behind this is that the differences between wines considered to be most and least representative of the concept under investigation should manifest both intuitively (as seen in non-verbal methods) and through verbal cues. It is important to understand when to use which type of method (verbal, non-verbal or a combination), how to choose the mode of assessment (gustatory, olfactory or global) and which type of panel to use (experts or trained). Elements to consider when making these decisions are whether or not the concept has been well established previously, whether there are known features that contribute to the concept, and whether these features have standards that can be used for calibration (Perrin & Pagès, 2009).

Verbal methods used for typicality studies include descriptive analysis (DA) for the colour of Provence Rosé wines (Coulon-Leroy *et al*., 2018) and check all that apply (CATA) for the minerality of Burgundy Chardonnay (Ballester *et al*., 2013). Non-verbal methods include sorting for demonstrating the existence of a Chardonnay wine concept (Ballester *et al*., 2005), typicality and hedonic rating for minerality in French vs New Zealand Sauvignon blanc (Parr *et al*., 2015), and other various combinations.

As mentioned previously, the evaluation can be used to investigate the contributions of the features to the concept through gustatory, olfactory or global assessment. Studies have found the differences in the success of the mode of assessment to be based on the dominant features related to the concept. If, for example, the prominent features are known to manifest in the aroma, then the assessment will be on the olfactory stimuli. If, however, a concept has not previously been annotated with features, then a global assessment is used. This type of systematic investigation is illustrated by Ballester *et al*. (2008) in testing the concept of Chardonnay by both expert and consumer panels. The study found a clear distinction between Chardonnay wines and Melon de Bourgogne (used as a non-Chardonnay example to establish the borders of the concept) by an expert panel. The borders of representativeness were then tested in two ways using rating (to look at the degree of representativeness) and sorting (to look at the membership in the designated groups).

The use of trained and expert panels has also been investigated in the literature. If a concept has features that can be calibrated for using standards and/or definitions, a trained panel may be used (Ballester *et al*., 2008). Concepts that include features that could not be calibrated, and thus rely on experience, favour expert panels. In this case, it is possible that the conceptual agreement when defining terms and the perceptual agreement when consistently assessing the features in wine are not unified, as was the case with the minerality of Burgundy Chardonnay (Ballester *et al*., 2013); although the investigation achieved both panel consensus and conceptual agreement on minerality, perceptual agreement could not be reached and hence the features could not be verified.

In this context, the aim of the current study was to investigate the concept of old vine Chenin blanc using typicality rating, sorting, and free word association. Compared to the previous study by Crous (2016), in which the intrinsic features of each wine were measured by DA using a bottom-up approach that is experimentally directed (Lindsay & Norman, 1977), the current work proposes a top-down approach in which the understanding of the concept is first developed before trying to measure its features (Lindsay & Norman, 1977; Brochet & Dubourdieu, 2001). A combination of non-verbal (rating and sorting) and verbal (the added annotation of sensory attributes in the sorting exercise) methods was used. The sensory panel was constituted of industry professionals. Since the previous study noted the potential influence of winemaking (Crous, 2016), the same winemaking protocol was used in this study for all the grapes sourced from vineyards aged five to 44

years. In addition, the wines were evaluated young (first evaluation stage approximately three months after bottling) and after two years of ageing in the bottle (second evaluation stage).

MATERIALS AND METHODS

Grape sources and winemaking

Chenin blanc grapes were sourced from 23 vineyards across the Western Cape province of South Africa. Grapes were harvested in 2017 at commercial maturity according to the growers, ranging from 23°Brix to 25°Brix, with two exceptions at 17.3°Brix (sample 765) and 19.2°Brix (sample 769). Twelve young vines (< 35 years old) and 11 old vines (≥ 35 years old) were included in the project; vine ages ranged from five to 45 years. Grapes were treated with 30 mg/L sulphur dioxide (SO_2) at crushing. The juice was settled overnight at 4°C, racked and allowed to come to room temperature. Juice was inoculated with Vin7 yeast (Zymasil, AEB Group SpA, Bologna, Italy) according to the manufacturer's instructions. The fermentation was allowed to proceed in a temperature-controlled room at 15°C to 18°C. The SO_2 levels were adjusted to 50 mg/L post-alcoholic fermentation, and 50 mg/L bentonite was added before cold stabilisation, which took place over two weeks at -4°C. The wine was then racked and bottled without filtration in 750 mL screw cap green bottles (Consol, South Africa). The wines were stored in the vinoteque under controlled temperature and humidity conditions until their evaluation: first as young wines (three months after bottling), then as bottle-aged wines (two years after bottling). Grape juice and wine oenological parameters (Table 1) were measured on a Metrohm 862 compact titrosampler (Herisau, Switzerland) using chemicals (sodium hydroxide, potassium iodide/potassium iodate and sodium thiosulfate) purchased from Cameron Chemical Consultants (Cape Town, South Africa).

Sensory evaluation

The approach used in this study is based on the methodology published by Ballester *et al*. (2008). The analysis was performed in a quiet, well-ventilated and odour-free room with the temperature set at 20 ± 2°C. Samples were presented in black ISO glasses, covered with a Petri dish and labelled with a three-digit code. Samples were randomised across judges prior to analysis according to a William's Latin square design. An expert panel of 32 judges in 2018 and 14 in 2019 assessed the 23 wines; the judges were industry professionals with more than five years' experience in the production and evaluation of old vine Chenin blanc. The experimental design was done using Compusense cloud (Compusense, Guelph, Canada).

Two sensory tasks, namely rating and sorting (Valentin *et al*., 2012), were performed in one session with a 15-minute break and a free word association exercise between them. The first task was a typicality rating on a 100 mm unstructured line scale, ranging from "very bad example" anchored at 0 to "very good example" anchored at 100 (Garrido-Bañuelos *et al*., 2020) and samples were presented monadically. The experts were instructed to rate each sample on the scale according to their judgement for an old vine Chenin blanc wine. Before beginning the second task, judges were asked to list three to five words that came to mind when "typical old vine Chenin Blanc wine" was mentioned. The second task was a flexible sorting exercise with all 23 wines presented at once. This was considered a flexible sorting since the judges were instructed to sort the wines into two groups, namely "young vine CB" or "old vine CB", but they were allowed to create a third group if the samples did not fit either of the two groups. Judges were also asked to give three to five attributes associated with each group. The terms generated during the sorting task were consolidated based on their semantic and synonymous relationship by agreement among the researchers.

Statistical analysis

Rating data was captured as a judge vs wines correlation matrix. Principal component analysis (PCA) was performed on the correlation matrix to evaluate judge consensus (Perrin & Pagès, 2009). The data was averaged over the judges and PCA was performed on the resulting correlation matrix to investigate correlations between the different wines (Perrin & Pagès, 2009). Data groupings on the basis of the sorting were captured as a co-occurrence matrix and the attributes used to describe the groups were captured as a correlation matrix of wines and attributes. Multidimensional scaling (MDS) was performed on the co-occurrence matrix and correspondence analysis (CA) on the correlation matrix (Salkind, 2012). Regression vector (RV) coefficients were calculated among the CA and MDS biplot co-ordinates for each year, and between the young and the two-year bottle-aged wines (Abdi, 2007). Unweighted pair-average agglomerative hierarchical clustering (AHC), using a similarity-based, Pearson correlation coefficient, was performed on the MDS and on the CA for both the wines' and the attributes' correlation matrices. Statistical analyses were performed in *X*LSTAT2018 (Addinsoft, Paris, France).

RESULTS

Judge consensus

In order to evaluate panel consensus, PCAs were conducted on the rating scores for both the young and bottle-aged wines (Fig. 1). The results for the young wines show a cumulative explained variance of 16% for the first three dimensions. Full explained variance (100%) was achieved over 22 dimensions, with all dimensions contributing almost equally (from PC1 with 5.8% to PC22 with 3.7%). Results from the bottle-aged wines showed a cumulative explained variance of 17% for the first three dimensions of the PCA, with the full explained variance being achieved over 21 dimensions (from PC1 with 6.1% to PC21 with 3.7%).

Although the cumulative explained variance for both years of the evaluation was less than 20% for the first three dimensions (Fig. 1), the linear correlation across the first dimension was an indicator of good consensus between the judges. The correlation between judges varied linearly along the first dimension, with judges 12 and 24 being the exception for the first evaluation stage (young wines) and judge 10 for the second (bottle aged). The judges who were not in consensus with the rest of the panel were not excluded from further analyses, because they were within the 95% confidence interval and thus not statistical outliers.

TABLE 1
Oenological parameters of Chenin blanc grapes (mass, NOPA, ammonium, YAN, and ˚Brix) harvested from old and young vines in 2017 and their resulting wines (pH and TA).

Sample code	Vine age (years)	Class	Mass (kg)	NOPA (mg N/L)	NH_4 (mg N/L)	YAN (mg N/L)	˚Brix	pH	TA (mg/L)
YV751	29	Young	17	180	50	230	21.7	3.25	6.57
OV752	**n/s	Old	18	160	30	190	22.2	3.30	5.51
YV753	5	Young	20	200	60	260	21.8	3.35	5.66
OV754	**n/s	Old	20	170	30	200	23.6	3.34	6.56
OV755	**n/s	Old	22	180	60	240	22.4	3.42	4.59
OV756	39	Old	20	170	50	220	24.1	3.46	5.53
YV757	34	Young	19	130	30	160	24.6	3.34	6.24
YV758	34	Young	19	150	40	190	21.8	3.36	6.99
YV759	28	Young	34	-	-	-	20.0	3.41	6.06
OV760	39	Old	24	-	-	-	24.6	3.53	5.22
YV761	34	Young	18	120	30	150	24.2	3.50	6.53
YV762	**n/s	Young	18	150	50	200	23.8	3.64	4.71
YV763	6	Young	19	140	30	170	23.9	3.60	5.59
YV764	24	Young	20	130	50	180	23.0	3.43	5.70
OV765	39	Old	21	210	80	290	17.3	3.17	10.35
YV766	33	Young	17	160	50	210	21.6	3.57	4.38
OV767	37	Old	23	210	50	260	22.1	3.68	5.88
OV768	41	Old	20	190	150	340	21.5	3.75	4.34
YV769	31	Young	38	160	50	210	19.2	3.35	8.16
OV770	37	Old	18	150	40	190	22.7	3.46	5.03
OV771	35	Old	19	220	50	270	22.5	3.63	5.58
YV772	27	Young	21	140	40	180	24.5	3.55	5.03
OV773	44	Old	17	-	-	-	23.0	3.54	6.03

Young – vines 34 years and younger; Old – vines 35 years and older. Mass means the mass of grapes as measured before crushing. NOPA – nitrogen by *o*-phthaldialdehyde assay; NH_4 –ammonium; YAN – yeast assimilable nitrogen; TA – titratable acidity; n/s – not specified

Non-verbal typicality assessments

Typicality rating

In order to see if there was a correlation between vine age and the typicality rating, the average scores per sample were plotted against the vine age. If the old vine concept was to be observed, the old vine wines should have been rated higher on the typicality scale than the young vine wines, according to their degree of representativeness of the concept. This was not the case, as linear regression analysis showed no correlation between the average rating score and the vine age for either young wines or bottle-aged wines.

The results for both evaluation stages show a wide distribution of the average typicality scores. Judges used the entire scale (from 0 to 100), with the average scores ranging from 20 to 66 for young wines and 29 to 67 for bottle-aged wines. This result indicates that the judges did not have a unified perception of the wine typicality with regard to the old vine status. Statistically, the score distribution of each sample was not always normal, as some samples had a bimodal distribution whereas others had a random distribution (Fig. 2). For young wines, the wine rated the lowest was OV765, which was made from a 39-year-old vine. Surprisingly, the wine made from the oldest vines (OV773, 44 years old) and youngest vines (YV753, five years old) were rated similarly (56 and 49 for OV773 and YV753, respectively). For bottle-aged wines (second stage), the sample with the lowest rating was the wine from the oldest vines in the set, OV773, which was rated even lower than the wine made from the youngest vines in the sample group (YV753, five-year-old vines).

In order to investigate any relationship between the two years' results, the average scores for each year were plotted against each other. The regression coefficient ($R^2 = 0.5852$) indicated only a trend between the young and bottle-aged

wines. This means that any changes that occurred during ageing could neither be correlated with vine ageing nor typicality rating. Given the random distribution of samples, no borders could be imposed based on vine age, and thus no classifications could be made according to age. This means that there was no perceptual agreement between judges when it came to old vine South African Chenin blanc typicality as measured by the rating task.

Multidimensional scaling (MDS) on typicality sorting data

The second non-verbal assessment of the typicality of old vine Chenin Blanc wine was the sorting task. Unlike the rating task, in which the presentation of the samples is monadic, in this second task wines were judged together and grouped according to their similarity under the groups *old vine* and *young vine*.

The first three dimensions of the MDS were considered enough for assessing significant relationships between samples based on Kruskral's stress indices (results not shown) for both evaluation stages (young wines and two-year bottle-aged wines). MDS and agglomerative hierarchical clustering (AHC) were then performed on the first three dimensions, and the results are shown in Figs 3 and 4 for the two evaluation stages.

Cluster analysis of the MDS gave three main clusters and showed no grouping of samples according to vine age for either evaluation stage. The wine from the oldest vine (wine OV773, 44-year-old vine) and the youngest vine (YV753, five-year-old vine) were in two separate clusters. For both stages, the distribution within each cluster was random, the distances between the members of each cluster (i.e. samples or branches) was also random and not related to vine age. It can be concluded that clustering was related to neither the categories "old vine"/"young vine" nor to any observable trends according to vine age.

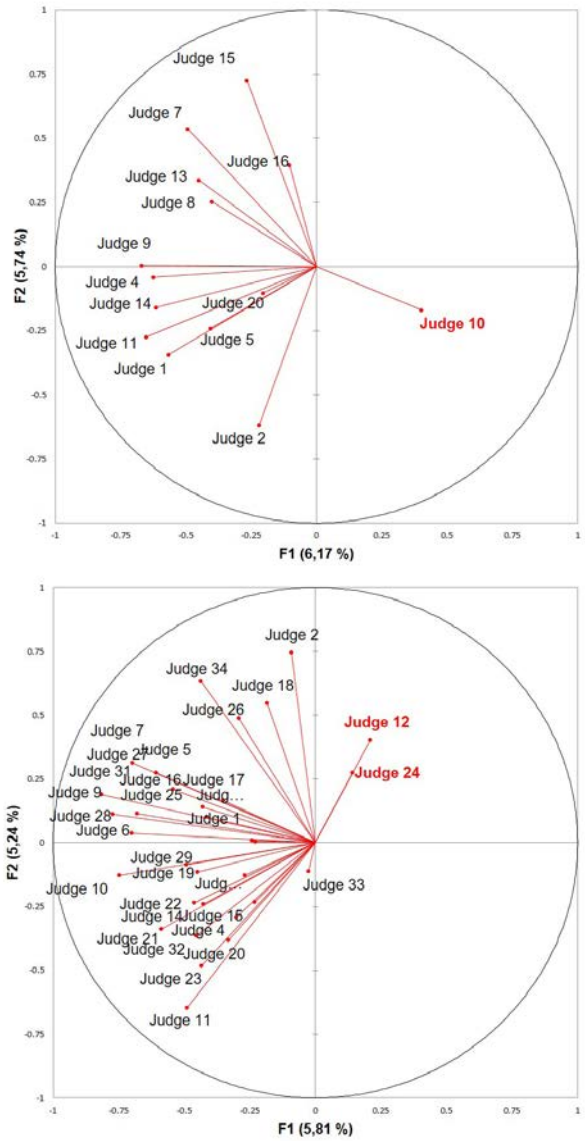

FIGURE 1
Principal component analysis (PCA) of rating data collected from young wines (top) and wines aged for two years in the bottle (bottom).

Correspondence analysis (CA) on typicality sorting

Correspondence analysis of the sorting data provided a biplot that showed the correlation between samples (presented in this section) and between attributes (presented under Verbal assessments below).

CA showed the distribution of the total inertia (0.327 and 0.494 for the first and second evaluation stage, respectively) over 22 and 21 dimensions, respectively. The first three dimensions had cumulative percentages of 61% and 64% of the inertia respectively for the two stages. AHC was done only on these first three dimensions (Figs 5 and 6). Three clusters were formed in each case; the clusters contained samples from different vine ages. The clustering of samples was related neither to the "old vine"/"young vine" categories, nor to vine age. Unlike in the MDS, the wine from the oldest vines (OV773, 44 years old) and the wine from the youngest vines (YV753, five years old) belonged to the same cluster for the first evaluation stage and to the same cluster for the second.

Comparison of sample configurations

RV coefficients were calculated in order to assess any differences or similarities between sample configurations generated in the two stages through MDS and CA. The comparison was two-fold: within a stage, MDS to CA, and between the stages, CA to CA and MDS to MDS configurations. The data captured from the rating task also generated one PCA for each evaluation stage that contained sample configurations. However, as one of the samples was not included in the second-stage evaluation, RV coefficients could not be calculated for the rating results.

MDS and CA plots were generated for the verbal and non-verbal aspects of the sorting data. The main difference in these analyses is that the MDS relies only on the associations between samples, whereas the CA uses the attributes to generate the correlation between samples. Since these were done within one task, although looking at different aspects, they should result in a similar relationship between samples. As such, RV coefficients were used to measure the configurational similarity between the CA and MDS plots.

For sorting, the results for young wines showed CA vs

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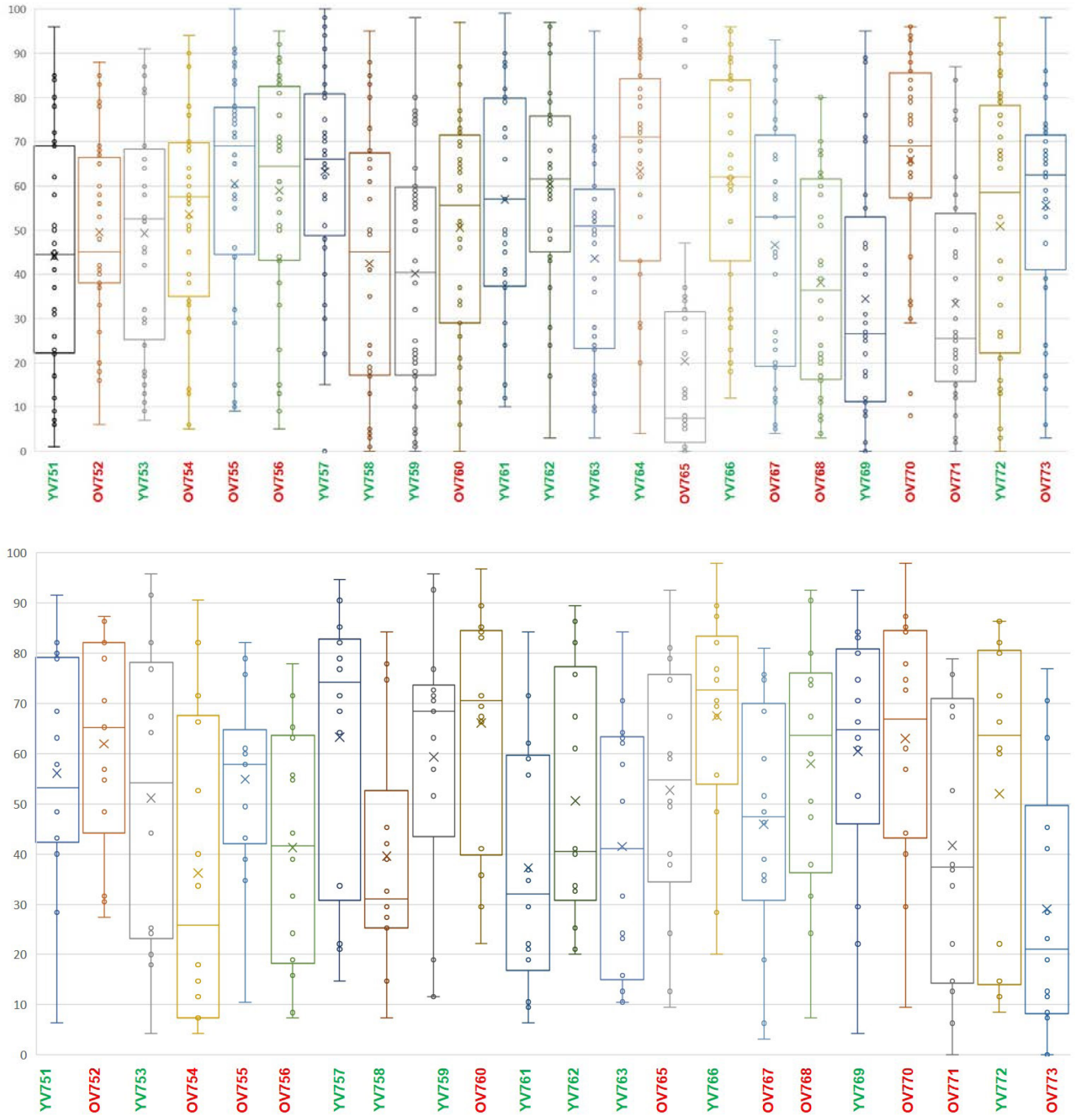

FIGURE 2

Box-and-whisker distribution plot of typicality rating scores for young wines (a) and two-year bottle-aged wines (b) from old vine Chenin blanc grapevines of different ages. Young vines are coded with YV (green) and old vines with OV (red) before the unique three-digit code.

MDS RV coefficients of 0.68 and 0.60 for the first two and three dimensions, respectively. The second stage (bottle-aged wines) results showed CA vs MDS RV coefficients of 0.68 and 0.71 for the first two and three dimensions, respectively. Looking at correlations between the two years of evaluation, RV coefficient were calculated for MDS vs MDS (0.37 and 0.34, first two and three dimensions, respectively) and CA vs CA (0.47 and 0.39, first two and three dimensions, respectively). These values were low, meaning that the samples were sorted differently for the different evaluation stages. Although three clusters were formed for both the evaluation stages, the members belonging to each of the clusters were different.

Looking for any similarity between the two datasets (rating and sorting), the configurational space was assessed using RV coefficients. The wine samples were considered observations in the rating data and modelled by PCA; the resulting configuration was used to generate the RV coefficients against the CA and MDS results.

In the case of the evaluation of the young wine, the

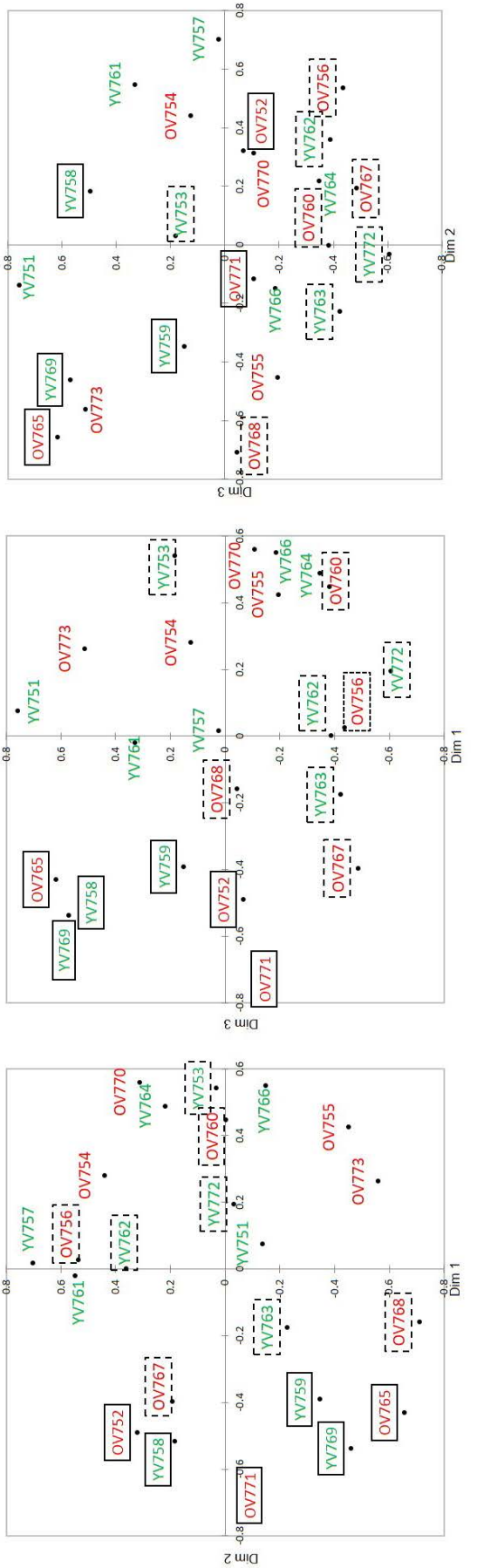

FIGURE 3

Multidimensional scaling (MDS) of sorting task of old (red) and young (green) vine Chenin blanc wines analysed in the first year. Different shadings indicate the groups according to agglomerative hierarchical clustering (AHC) performed on the first three dimensions of the MDS.

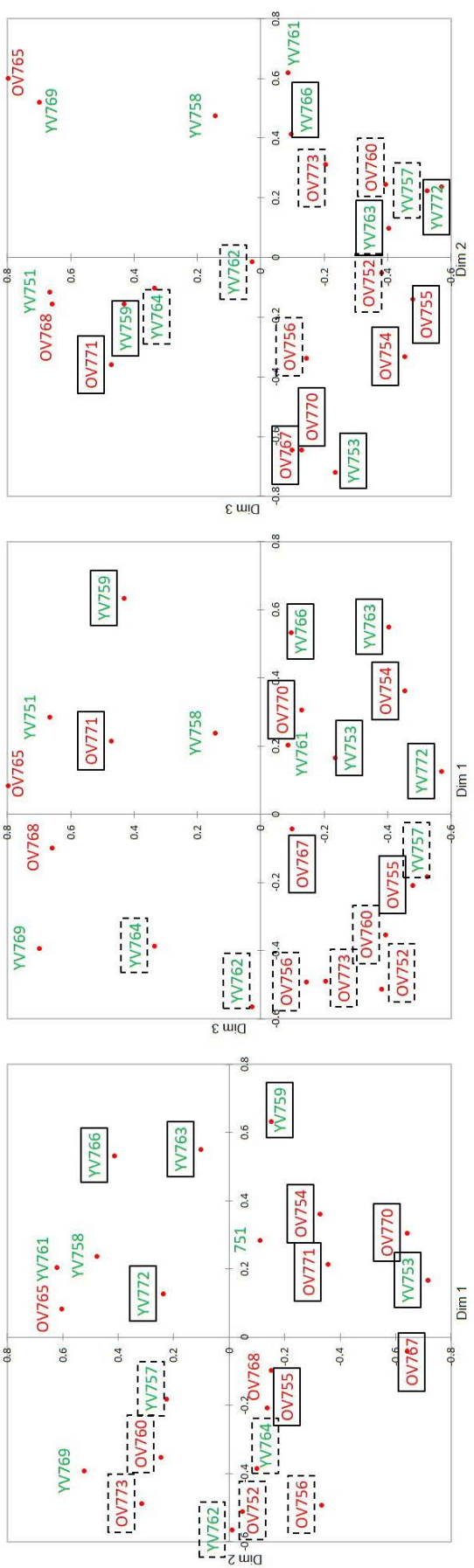

FIGURE 4

Multidimensional scaling (MDS) of sorting task of old (red) and young (green) vine Chenin blanc wines analysed after two years of ageing in the bottle. Different shadings indicate the groups according to agglomerative hierarchical clustering (AHC) performed on the first three dimensions of the MDS.

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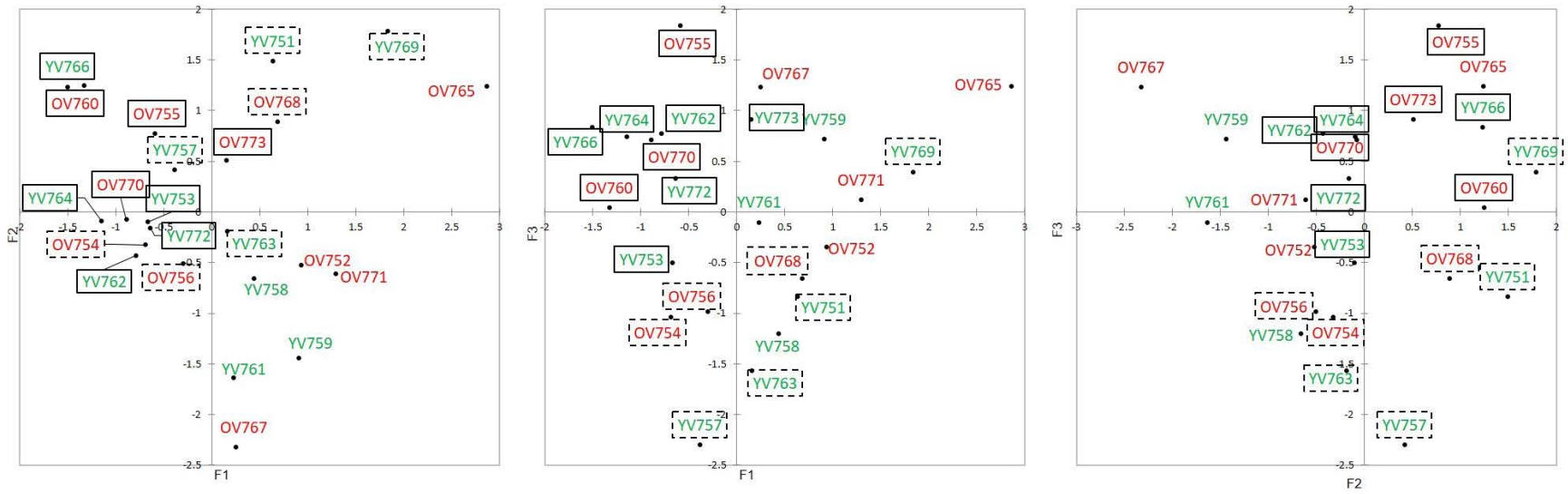

FIGURE 5

Correspondence analysis (CA) of sensory analysis of old (red) and young (green) vine Chenin blanc wines analysed in the first year. Samples with the same box shading belong to the same cluster, analysed using agglomerative hierarchical clustering (AHC) on the first three dimensions.

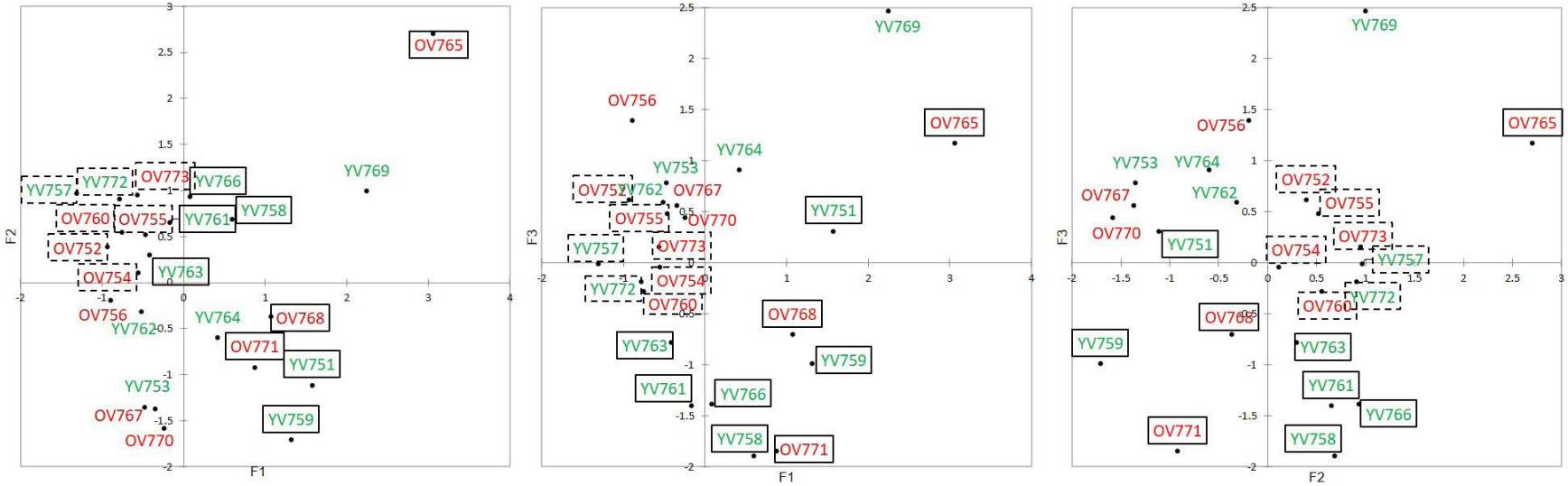

FIGURE 6

Correspondence analysis (CA) of sensory analysis of old (red) and young (green) vine Chenin blanc wines analysed after two years of ageing in the bottle. Samples with the same box shading belong to the same cluster, analysed using agglomerative hierarchical clustering (AHC) on the first three dimensions.

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results showed poor correlation between the configurations for rating by PCA and sorting by MDS (first two dimensions, RV = 0.44; first three dimensions, RV = 0.41) and between rating by PCA and sorting by CA (first two dimensions, RV = 0.52; first three dimensions, RV = 0.474). This could be because of the non-normal distribution of the rating scores for each sample, as discussed above. The membership of the same sample to different groups (*young vine* and *old vine*) in the sorting could also contribute to the differences in configurations (i.e. low RV coefficient values). Since sample 764 was excluded from the rating of the bottle-aged wines, the RV coefficients for the second evaluation stage could not be calculated.

Verbal assessment of typicality

Verbal aspects of the sorting task

The sorting resulted in three groups for both the young wines and the two-year bottle-aged wines. The groups *young vine* and *old vine* were allocated to them, but the judges collectively generated the *teenager* and *outlier* group identities for the first and second evaluation stages, respectively. The consolidation of attributes resulted in 46 terms for young wines and 68 for bottle-aged wines, which were used to generate the CA. The first three dimensions of the CA contained 61% and 64% of the explained variance for the two evaluation stages, respectively. AHC done on the three-dimensional space resulted in the formation of two

TABLE 2
AHC groups for the first three dimensions of the CA for the wines analysed in the first year.

		41.88%	11.19%	8.46%	61.53%
Attribute	Weight (relative)	F1	F2	F3	Sum
CLUSTER 1					
Old	0.123	0.108	0.013	0.003	0.124
Textured	0.027	0.030	0.025	0.004	0.059
Robust	0.008	0.011	0.000	0.002	0.013
Rich	0.028	0.032	0.021	0.000	0.054
Nutty	0.016	0.020	0.031	0.020	0.070
Complex	0.027	0.010	0.000	0.005	0.014
Crispy	0.010	0.010	0.000	0.097	0.106
Stone fruit	0.010	0.012	0.016	0.000	0.027
Good mouthfeel	0.009	0.011	0.002	0.009	0.022
Warm mouthfeel	0.005	0.000	0.013	0.004	0.017
Long AT	0.040	0.036	0.002	0.036	0.075
Full bodied	0.019	0.012	0.000	0.005	0.017
Faulty	0.013	0.031	0.086	0.033	0.150
Mineral	0.029	0.000	0.059	0.024	0.084
Acidic	0.024	0.170	0.206	0.045	0.422
Bitter	0.005	0.030	0.053	0.001	0.083
Natural	0.004	0.003	0.012	0.073	0.089
Premium quality	0.004	0.005	0.001	0.004	0.011
CLUSTER 2					
Young	0.105	0.092	0.020	0.014	0.126
Wood	0.002	0.001	0.039	0.006	0.046
Low fruitiness	0.009	0.005	0.019	0.001	0.026
Fresher	0.052	0.024	0.044	0.003	0.071
Medium intensity	0.003	0.003	0.076	0.010	0.089
Citrus	0.024	0.011	0.033	0.007	0.050
Tropical	0.034	0.002	0.006	0.001	0.009
Peach	0.013	0.000	0.024	0.121	0.145

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TABLE 2 (CONTINUED)

		41.88%	11.19%	8.46%	61.53%
Attribute	Weight (relative)	F1	F2	F3	Sum
CLUSTER 2					
Short AT	0.014	0.005	0.006	0.021	0.032
Linear	0.013	0.011	0.004	0.011	0.025
Medium bodied	0.003	0.000	0.021	0.005	0.026
Teenager	0.017	0.035	0.001	0.006	0.042
Low flavour	0.005	0.002	0.003	0.001	0.006
Fruity	0.057	0.027	0.030	0.013	0.070
Green fruit	0.007	0.017	0.005	0.057	0.078
Subtle/ delicate	0.019	0.000	0.007	0.016	0.023
Unbalanced	0.016	0.023	0.003	0.000	0.026
Sweet	0.017	0.021	0.038	0.045	0.104
Light bodied	0.042	0.044	0.001	0.006	0.051
Vegetative	0.004	0.002	0.041	0.013	0.056
Easy drinking	0.005	0.023	0.002	0.004	0.028
Vibrant/ lively	0.010	0.001	0.011	0.024	0.036
CLUSTER 3					
Structured	0.010	0.009	0.008	0.094	0.112
Ripe	0.034	0.014	0.002	0.024	0.039
Concentrated	0.014	0.009	0.002	0.001	0.011
Yellow fruit	0.008	0.023	0.000	0.041	0.065
Aggressive	0.004	0.001	0.000	0.000	0.001
Balanced	0.028	0.035	0.000	0.027	0.062
Well rounded	0.013	0.007	0.000	0.011	0.019
Straw	0.008	0.011	0.010	0.048	0.069
Elegant	0.013	0.013	0.005	0.005	0.023

TABLE 3
AHC groups for the first three dimensions of the CA for the wines aged in the bottle for two years.

		38.18%	15.37%	10.88%	64.43%
Attributes	Weight (relative)	F1	F2	F3	Sum
CLUSTER 1					
Old	0.077	0.046	0.002	0.000	0.048
Less fruity/ subtle fruit	0.012	0.008	0.016	0.011	0.035
Lime	0.005	0.009	0.003	0.009	0.020
textured	0.019	0.000	0.006	0.008	0.014
Rich mouthfeel	0.014	0.017	0.009	0.013	0.039
Full/ Full body/ Full mouthfeel	0.040	0.018	0.009	0.003	0.030
Well-rounded	0.014	0.013	0.009	0.007	0.028
dense palate	0.012	0.007	0.011	0.004	0.022

TABLE 3 (CONTINUED)

		38.18%	15.37%	10.88%	64.43%
Attributes	Weight (relative)	F1	F2	F3	Sum
CLUSTER 1					
broad palate	0.026	0.021	0.001	0.000	0.022
Smooth	0.011	0.013	0.002	0.016	0.031
Length	0.051	0.036	0.003	0.007	0.047
Structure	0.019	0.002	0.001	0.000	0.004
Complex	0.019	0.027	0.003	0.021	0.051
Savoury	0.006	0.008	0.001	0.011	0.020
Herbal	0.006	0.008	0.001	0.011	0.020
Flint	0.014	0.001	0.004	0.009	0.014
Mineral	0.016	0.020	0.003	0.016	0.038
Earthy	0.004	0.007	0.000	0.002	0.009
Oily	0.010	0.013	0.000	0.004	0.017
Elegant	0.006	0.006	0.000	0.002	0.009
CLUSTER 2					
Young	0.074	0.073	0.020	0.010	0.104
Less intense aroma/ subtle nose	0.020	0.020	0.001	0.001	0.022
Fruity	0.056	0.002	0.042	0.010	0.053
Fresh	0.011	0.004	0.008	0.014	0.026
Less ripe	0.002	0.044	0.015	0.050	0.110
Banana	0.005	0.003	0.040	0.045	0.088
Litchi	0.005	0.003	0.040	0.045	0.088
Citrus	0.005	0.003	0.040	0.045	0.088
Peaches	0.032	0.003	0.011	0.000	0.014
Granadilla	0.007	0.003	0.005	0.005	0.012
Floral	0.023	0.000	0.001	0.001	0.002
Bitter	0.007	0.002	0.010	0.002	0.014
Crisp acidity	0.006	0.008	0.004	0.014	0.026
Acidic	0.018	0.115	0.002	0.002	0.119
Light texture	0.011	0.009	0.004	0.002	0.014
Watery	0.012	0.013	0.065	0.018	0.095
Thin body/ Low body	0.026	0.035	0.006	0.060	0.101
Thin/ Thin mouthfeel	0.018	0.057	0.001	0.022	0.080
Unbalanced	0.022	0.053	0.014	0.000	0.066
Short AT	0.004	0.001	0.050	0.003	0.055
Low alcohol	0.002	0.044	0.015	0.050	0.110
high alcohol	0.013	0.000	0.001	0.000	0.001
Small yield	0.005	0.000	0.038	0.009	0.047
Mature	0.005	0.000	0.038	0.009	0.047
Vibrant	0.005	0.007	0.003	0.006	0.016

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TABLE 3 (CONTINUED)

		38.18%	15.37%	10.88%	64.43%
Attributes	Weight (relative)	F1	F2	F3	Sum
CLUSTER 3					
Outlier	0.012	0.107	0.177	0.111	0.394
Ripe	0.027	0.001	0.033	0.007	0.042
Yellow fruit	0.008	0.008	0.004	0.000	0.012
Guava	0.021	0.014	0.042	0.019	0.075
Tropical	0.018	0.001	0.001	0.029	0.031
Quince	0.010	0.009	0.016	0.027	0.052
Pineapple	0.012	0.002	0.016	0.015	0.033
Sweet	0.030	0.001	0.003	0.052	0.056
Balanced/ balanced acidity	0.041	0.042	0.006	0.002	0.049
Creamy	0.011	0.000	0.039	0.050	0.090
Tannic	0.008	0.008	0.004	0.000	0.012
No mid-palate	0.003	0.003	0.000	0.051	0.054
Concentrated	0.012	0.003	0.022	0.011	0.036
Tension	0.005	0.001	0.006	0.032	0.040
Faulty	0.006	0.018	0.072	0.016	0.107

main clusters (Fig. 7). The members of each cluster, their weight and their contributions to the explained variance in the first three dimensions are listed in Tables 2 and 3. The *old vine* cluster had associated terms that are mouthfeel-related and support the findings of Crous (2016). Some examples are 'robust', 'texture, 'good mouthfeel' and 'complex' for the young wines, and 'structure', 'dense palate', 'texture' and 'rich mouthfeel' for the bottle-aged wines.

DISCUSSION

The original idea of the project was to explore the sensory space typical of the OV Chenin blanc wines. As required by the methodology used when testing a typicality concept, the process was laid out in steps in such a way that multiple checks were put in place. The systematic approach taken in establishing and understanding an oenological concept requires a reliable panel (judge consensus), as well as conceptual and perceptual agreement (Perrin & Pagès, 2009; Maitre *et al*., 2010). The establishment of a sensory space unique to a concept (in this case the OV Chenin blanc) would constitute the final step in the process, which can be reached *only* once all the previous stages have been demonstrated.

In the current study, the panel agreement was proven from the rating results, even if the explained variance was distributed almost equally over a large number of dimensions. Scalar data with a single measurement has an approximately equal distribution of the explained variance across the multiple dimensions of the PCA; in other words, all dimensions have an almost equal input into the distribution of data (Granato & Ares, 2014), as observed for the results of the current work. Conversely, even if the explained variance is high, the experiment stops if panel consensus is not reached. This was the case in the study by Ballester *et al*. (2013), in which no correlations were observed in the agreement between judges assessed by PCA; in that case, there was no consensus and the investigation did not proceed further.

Only after the reliability of the panel was confirmed could the perceptual agreement be tested. The borders of the perceptual agreement can be gradual, referred to as the "degree of representativeness", and are tested using rating tasks (Ballester *et al*., 2005; Chrea *et al*., 2005). These borders can also be categorical, referred to as membership in the concept group, and are tested using sorting (Ballester *et al*., 2005). This means that the samples selected to test the concept need to cover the range of representativeness, including their borders (Ballester *et al*., 2005; Chrea *et al*., 2005).

The focus of a sorting task is the grouping of samples according to the given criteria (Valentin *et al*., 2012), in this case *old vine*/*young vine*. The instruction to describe the groups provided a secondary (verbal) aspect to the task. The flexible sorting task, as designed in this study, had both bottom-up and top-down elements to it (Lindsay & Norman, 1977; Brochet & Dubourdieu, 2001). To decide whether a sample belonged to the *old vine* group, a judge had to think first of the characteristics that qualify the sample for that category (top-down thinking). To describe the group based on the samples included, the judge had to consider the attributes of the wines themselves (bottom-up thinking).

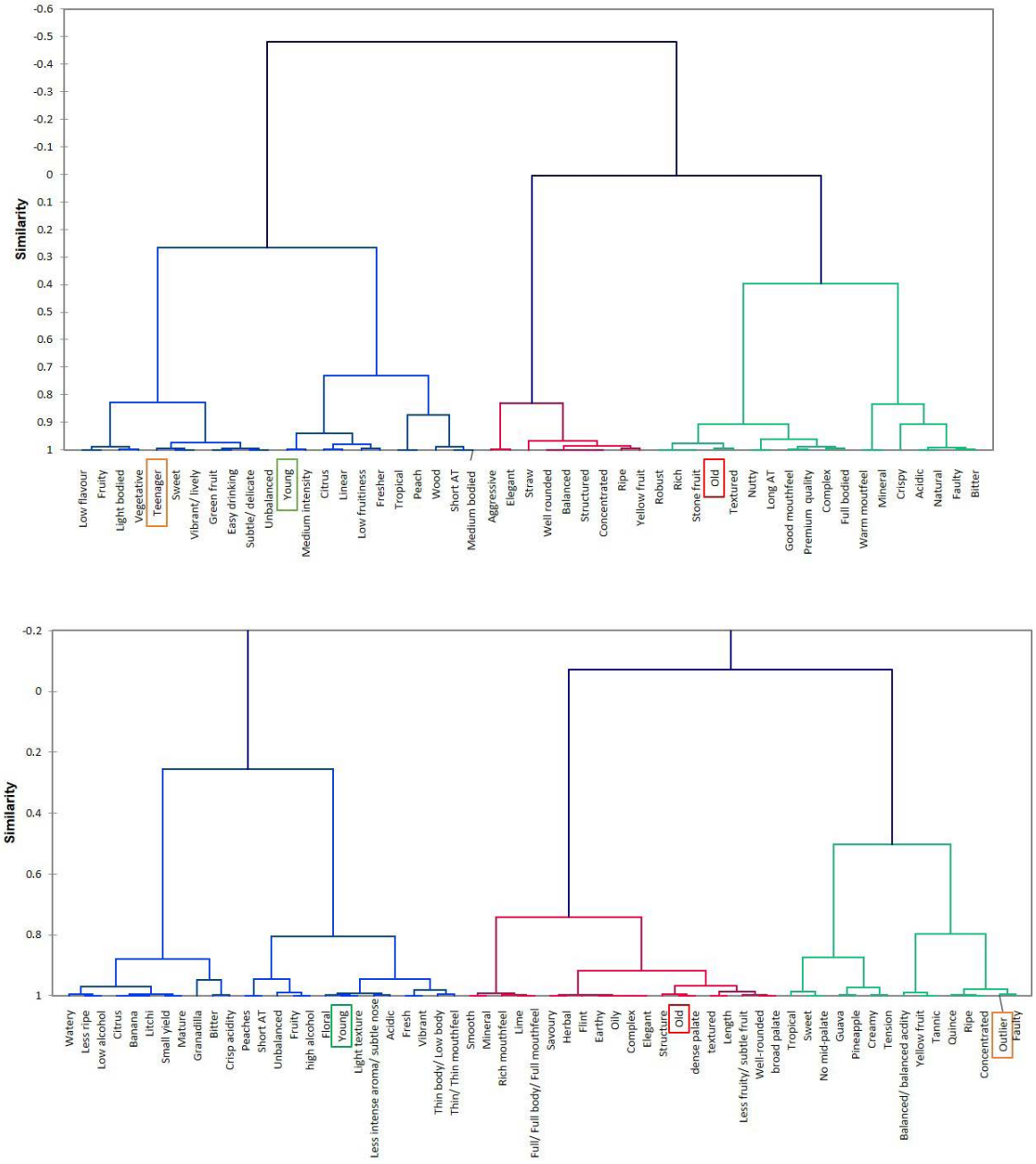

FIGURE 7
Agglomerative hierarchical clustering (AHC) on the first-year results for CA attributes for the first-year (top) and two-year bottle-aged (bottom) wines.

Since these two aspects are intertwined, both the grouping and the descriptors were used to give an indication of the conceptual space related to old vine Chenin blanc typicality. The values of the RV coefficients supported the hypothesis that the verbal and non-verbal aspects of the sorting task were in agreement.

In line with the idea related to the origin of the old vine character coming from the grapes, this study covered sample variability in terms of vine age, but limited variability from a winemaking perspective. The wines were tested as young and bottle aged. Although the same number of clusters resulted from the analysis of the sorting results for both evaluation stages, the members belonging to each of the clusters were different. Using vine age as the single source of variability may have resulted in wines being too similar to each other for the judges to be able to distinguish between them. Unlike in this study, the previous study by Crous (2016) included variability in winemaking, but not in vine age. This may have created a greater variability between the wine samples but, as often seen, highly involved winemaking practices may outweigh other factors (in this case, vine age).

Conceptually, the experts agreed on the attributes associated with the OV concept. Perceptually, the experts could not agree on a set of wines whose only variable was vine age. At this point, the process could not be taken further.

It is only once the perceptual agreement and the borders

are elucidated that the attributes associated with the concept can be tested (Perrin & Pagès, 2009). This would have resulted in building and describing a sensory space unique to OV Chenin blanc wines. The correct samples have to be consistently associated with the attributes in order for them to be considered features of the tested concept. This was not the case in the current study, where the last stage in the investigation could not be carried out due to the lack of perceptual agreement. As such, the features and the drivers of the concept could not be identified. In addition to the possible lack of variation in the resulting wines coming from a standardised winemaking, one other possible cause for the lack of perceptual agreement could be linked to the "expertise" and "exposure" factors related to the expert judges, factors highlighted in the literature in similar cases of testing complex concepts (Chrea *et al*., 2005; Perrin & Pagès, 2009). Even though the industry professionals participating in this experiment were experts in the topic, their reference (or "prototype", as described by Chrea *et al*. (2005)) most probably was built on repetitive exposure to a variety of old vine wines, with common but also very different characters. This aspect is one of the most difficult ones in relation to ensuring consistency in concepts, in contrast to attributes or features for which the researchers can use standards and calibrate analytical panels or even experts.

Previous studies have used predictive models, such as partial least squares (PLS) (Coulon-Leroy *et al*., 2018) and multiple linear regression (MLR) (Ballester *et al*., 2005; Parr *et al*., 2015), to explore the relationship between the rating and sorting data in the case of typicality. These models work when there is both panel consensus and perceptual consensus, so that the features of the typicality concept can be correlated or predicted. Since perceptual agreement on vine age or the categories of *old vine*/*young vine* was not reached in the current study, predictive or linear regressions could not be used.

CONCLUSIONS

The South African old vine Chenin blanc typicality was tested perceptually and conceptually. The perception of a Chenin blanc wine as having "old vine character" was evaluated using a typicality rating and a flexible sorting task. The conceptual understanding of old vine Chenin blanc was investigated by allowing judges to describe the *old vine* and *young vine* sorted groups.

As shown by the results, a unique sensory space of the OV Chenin blanc could not be demonstrated because the results indicated a lack of perceptual consensus among the industry professionals during the sorting task. However, the industry professionals did demonstrate a conceptual alignment/agreement, as demonstrated by the rating results, which was the foundation on which the rest of the work was built.

If similar work were to be repeated with commercial wines (from YV and OV), the existence of a unique sensory space of commercial OV wines could be demonstrated. However, such an experiment would still not answer the question: where is this character coming from? Researchers could get closer to answering the question by finding the features/drivers of the concept and maybe backtrack them to the origin. However, the source of the OV character could be multiple – interactions between the vineyard conditions, winemaking techniques, and vineyard and cellar flora. Even if experiments were to be designed around these factors, excluding them one by one, the interaction aspect would be lost.

The sensory space characteristic of OV Chenin blanc wines can also be re-created by better understanding the opinions of the wine industry professionals. Qualitative approaches such as interviews and surveys would be insightful.

These results show that, conceptually, the experts agreed on the attributes of old vine Chenin blanc wines, although they could not align perceptually. Since variability in winemaking was factored out, the unique properties gained by the wine during winemaking and the inclusion of viticultural and microbiome elements (wild fermentations) have been lost. However, if the guidelines of the OVP to take the minimalistic approach are to be followed, it is put into perspective how the various approaches taken in winemaking practices influence the final product.

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RESEARCH NOTE
Laboratory Bioassays on the Susceptibility of Trimen's False Tiger Moth, *Agoma trimenii* (Lepidoptera: Agaristidae), to Entomopathogenic Nematodes and Fungi

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Trimen's false tiger moth, *Agoma trimenii* (Lepidoptera: Agaristidae), recently developed as a pest of grapevine in the Northern Cape and Limpopo (Groblersdal area) provinces of South Africa. Little is known about the biology of *A. trimenii* and control options are lacking. The aim of this study was to test the susceptibility of *A. trimenii* larvae and pupae to two locally isolated entomopathogenic nematodes (EPNs), *Steinernema yirgalemense* and *Heterorhabditis noenieputensis*, and two commercially available entomopathogenic fungi (EPF), *Metarhizium anisopliae* and *Beauveria bassiana*, under laboratory conditions. Larvae and pupae were screened for pathogenicity of the two nematode species, using a concentration of 100 infective juveniles (IJs)/50 µl of water. After 48 h, 100% mortality of the larval stage was found. However, no pupae were infected with EPNs. Larvae and pupae were screened for pathogenicity of the two EPF isolates by means of a dipping test, at a concentration of 0.2 ml/500 ml water and 0.5 g/500 ml water, respectively. Five days post-treatment, 100% larval mortality was recorded in comparison with no deaths in the controls. Overt mycosis was only observed in the case of *M. anisopliae*. However, in the case of pupae, no mortality was observed for both the nematode and the fungal applications. In future studies, the prepupal soil stage of *A. trimenii* should be screened for susceptibility to EPNs and EPF. The results of this study indicate the excellent potential of EPNs and EPF as biological control agents against the larvae of *A. trimenii*, especially for application to small areas with high infestation, without disrupting an integrated pest management programme.

INTRODUCTION

Trimen's false tiger moth, *Agoma trimenii* Felder, is an indigenous lepidopteran that is classified within the Noctuidae family and the subfamily Agaristidae. *Agoma trimenii* targets the young shoots and leaves of wine, table and raisin grapes in the Northern Cape and Limpopo provinces of South Africa. As challenges associated with *A. trimenii* in the grape-producing regions of South Africa increase, so does the need to reduce populations to below damaging levels. Since the occurrence of *A. trimenii* is sporadic and confined, it is important to be able to control seasonal spikes in an environmentally friendly way, without disrupting natural enemies and the current control measures applied in vineyards (Morris, 2019; Morris *et al*., 2020). Biocontrol agents are a safe alternative, as they can be applied at any time close to or during harvest.

Potential biological control strategies for *A. trimenii* include the application of entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPF). Such biological control agents exhibit many beneficial traits, including offering minimal risk to human health, the absence of toxic residues in crops, minimal risk to beneficial and other non-target insects, and host specificity (Inglis *et al*., 2001; Goettel *et al*., 2005). Testing the above-mentioned biological control options on *A. trimenii* is important, as no such control options currently exist.

EPNs belonging to the genera *Heterorhabditis* and *Steinernema*, which are found in soils throughout most parts of the world, are parasitic to a broad range of insect

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pests (Malan & Hatting, 2015). Each genus is associated with a unique symbiotic bacterium – *Photorhabdus* in the case of heterorhabditids, and *Xenorhabdus* in the case of steinernematids (Ehlers, 2001), and together the EPNs and their associated bacteria are highly successful in parasitizing and killing their insect hosts. Upon encountering a suitable host, the free-living and non-feeding infective juveniles (IJ) enter the host insect through its natural openings, like the mouth, spiracles or anus (Griffin *et al*., 2005). The bacteria rapidly replicate within the nutrient-rich haemolymph of the host, generating various toxins, as well as a variety of primary and secondary metabolites that kill the host by means of inducing lethal septicaemia within 48 h of infection (Griffin *et al*., 2005).

The advantageous attributes of *Heterorhabditis* and *Steinernema* species for effective biological control include high virulence and the ability to actively seek out well-hidden life stages of insects (Lacey & Georgis, 2012). Additionally, they are compatible with commercial rearing and application techniques (Shapiro-Ilan *et al*., 2012). The entomopathogenic activity of both locally isolated steinernematids and heterorhabditids has been documented against a broad spectrum of insect pests in diverse habitats in South Africa (Hatting *et al*., 2009; Malan *et al*., 2011; Hatting & Malan 2017; Malan & Ferreira, 2017). However, their effectivity against *A. trimenii* is yet to be tested.

Entomopathogenic fungi (EPF), including *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) and the *Metarhizium anisopliae* (Metschnikoff) (Hypocreales: Clavicipitaceae) complex, are ubiquitous microorganisms that attack a variety of arthropods by means of inducing acute mycosis (Barta, 2010). The EPF can rapidly disperse horizontally among host populations by means of aerially produced conidia, and by infecting their host through penetration of the cuticle with germ hyphae (Bidochka & Small, 2005; Barta, 2010). Identification of the EPF generally relies on their physical appearance on culture media, combined with molecular identification. Morphologically, *B. bassiana* cultures are a powdery white-cream colour, turning yellow with age, whereas *M. anisopliae* cultures are varying shades of green (Coombes, 2012). The virulence of both *B. bassiana* and *M. anisopliae* is mainly a factor of the ability of the conidia to penetrate the insect's cuticle. Death of the insect host is then brought on as a result of a combination of such effects as toxinosis, general obstruction due to hyphal growth, and nutrient depletion (Wraight *et al*., 2007). Both fungal species are considered safe to vertebrates and, although they are known to have a wide host range, different strains tend to have restricted host ranges, making them suitable for use in biological control programmes (Zimmermann, 2007a, 2007b; Hatting *et al*., 2019). Additionally, the species concerned are easy and relatively inexpensive to cultivate on artificial media, which is an advantageous trait in respect of commercialisation (Kaya & Lacey, 2007). Both fungal species are known to target and successfully infest the larval and pupal stages of numerous lepidopteran pests (Nguyen *et al*., 2007; Coombes, 2012; Oliveira *et al*., 2012). However, their effectivity against *A. trimenii* has not been tested.

The current study investigated the potential of two local EPN species and two commercially available EPF species to infect *A. trimenii* under laboratory conditions and to determine their feasibility for use as part of an integrated pest management (IPM) programme directed at the management of the pest.

MATERIALS AND METHODS

Source of pupae and larvae

Both the pupae and the larvae of *A. trimenii* were collected from demarcated field sites on two table grape farms in the Northern Cape province, South Africa. Both sites consisted of netted blocks of table grapes. A block with table grape variety Thompson Seedless was used on Farm A, and a block with table grape variety Sugraone was used on Farm B. Pupae were collected during the winter months of May 2017 and June 2018 from soil mounds surrounding the vines, which were excavated to a depth of 5 cm and a radius of 60 cm. The pupae were stored in a thin layer of moistened soil in 2 L plastic containers and kept in a growth chamber at 25°C. Larval instars at various stages of development were collected in January 2018 from the top and bottom leaves of the vines. The larvae, which ranged from 1.5 cm to 4.5 cm in length, were stored in 2 L plastic containers, provided with a mesh-covered hole in the lid so as to ensure adequate ventilation, and they were given vine leaves on which to feed. The containers were stored at room temperature.

Source of nematodes and fungi

The local EPN species used in the study, *Heterorhabditis noenieputensis* Malan, Knoetze & Tiedt and *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, were obtained from previous surveys and stored in Stellenbosch University's nematode collection (Malan *et al*., 2006, 2011, 2014). Infective juveniles (IJs) of the two species were cultured *in vivo* at room temperature, making use of the last instar of the greater wax moth larvae, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) (Griffin *et al*., 2005). The rearing, harvesting and quantification of the IJs of both nematode species were conducted according to the methods described by Kaya and Stock (1997). Within the first week of emergence, the IJs were harvested and stored in horizontally positioned, vented 500 ml culture flasks containing approximately 100 ml of distilled water. The nematodes were used within one week after harvesting. The nematode concentrations used against the pupae and larvae were calculated according to the method of Navon and Ascher (2000).

Two commercial fungal isolates, *Beauveria bassiana* (EcoBb strain R444) and *Metarhizium anisopliae* (ICIPE 69), were received from two South African manufacturers, Plant Health Products and Real IPM, respectively. Both products were used at the concentrations stipulated on their respective labels.

Bioassay protocol for nematodes

Pathogenicity screening was conducted using six-well bioassay plates (flat-bottom, Nunce, Cat. No. 144530, Thermo Fisher Scientific [Pty] Ltd, Johannesburg, Gauteng, South Africa), with each well lined with filter paper (25 mm diameter). Each well of the bioassay plates was inoculated

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with 50 µl of an adjusted IJ suspension, using a Pipetman^{â} micropipette. A single insect was added to each inoculated well, which was then covered with a lid. An identical replicate control for each treatment was prepared on the day of screening, with 50 µl of distilled water only added to each well. The bioassay plates were then placed in 2 L plastic containers, lined with moistened paper towels and closed with a lid, to ensure the maintenance of high levels of humidity (RH ± 95%) using iButtons (iButton Link LCC – temperature and humidity data loggers). The containers were kept in the dark at 25 ± 2°C for 48 h. After two days, the insects were removed from the inoculated well plates and examined.

Pathogenicity of entomopathogenic nematodes

Pupae

The pathogenicity of *S. yirgalemense* and *H. noenieputensis* to the pupal stage of *A. trimenii* was tested at a concentration of 200 IJs/50 µl per pupa, using the bioassay protocol described above. For each EPN isolate, five six-well bioassay plates were used ($n = 30$), together with replicate controls for each treatment ($n = 30$). After 48 h, mortality was assessed by holding each pupa against a heated hotplate for 15 sec. The pupae that showed movement in response to the heat were considered alive and uninfected, while those that showed no movement were dissected and examined under a microscope for nematode infection. The experiment was repeated on a different test date with a fresh batch of nematodes, resulting in two replicate tests for each EPN tested.

Larvae

Larvae of *A. trimenii*, varying in length from 0.5 mm to 3.0 mm, were collected from vineyards (Morris, 2019). The pathogenicity of *S. yirgalemense* and *H. noenieputensis* to these larvae was tested at a concentration of 100 IJs/50 µl per larva, according to the described bioassay protocol. For each EPN isolate, four six-well bioassay plates were used ($n = 24$), with a control per EPN isolate being prepared ($n = 24$) on the day of screening. The pathogenicity was recorded by means of evaluating the mortality caused by nematode infection. After the 48 h exposure period, the larvae were rinsed with distilled water and the dead specimens were transferred to clean Petri dishes (90 mm diameter) lined with filter paper, and moistened with 800 µl of distilled water. The Petri dishes were sealed with Parafilm^{Ò} and placed in a dark growth chamber for a further 48 h to encourage nematode development. All dead larvae were dissected and the presence of developing nematodes confirmed visually. The experiment was repeated with a fresh batch of nematodes on a different test date.

Bioassay protocol for fungi

The virulence of *B. bassiana* and *M. anisopliae* to *A. trimenii* pupae and larvae was assessed by means of a dipping bioassay. A conidial suspension of EcoBb (*B. bassiana* formulation) and Met69 (*M. anisopliae* formulation) was prepared by adding 0.5 g EcoBb and 0.2 ml Met69 to 500 ml distilled water, respectively. The insects were dipped in the 500 ml conidial suspensions for 30 sec. Excess suspension was removed by waiting for any surplus droplets to fall.

Six-well bioassay plates lined with filter paper were used to conduct the bioassays. The dipped insects were placed onto the filter paper of each well, and water was added to the filter paper (with no free water being allowed to remain), depending on the level of moisture obtained from each dipped insect. As controls, the insects were dipped in distilled water before being placed in their respective wells. The bioassay plates were placed in 2 L plastic containers lined with moistened paper towels, and closed with a lid to ensure the maintenance of high levels of humidity. The containers were kept in the dark at 25 ± 2°C for five days. After the set period of time had elapsed, the insects were removed from the bioassay plates and cleaned of external fungi by being dipping separately into a series of six Petri dishes containing 5% sodium hypochlorite (NaOCl), distilled water, and a 70% ethanol solution, followed by an additional three Petri dishes containing distilled water. Each dip lasted for 30 s.

Pathogenicity of entomopathogenic fungi

Pupae

For the virulence screening of each EPF isolate, five six-well bioassay plates were used (n = 30), with the control pupae (n = 30) following the bioassay protocol for the fungi described above. After five days of exposure, and subsequent washing, the pupae were transferred to 90 mm diameter Petri dishes containing a selective medium of Sabouraud Dextrose Agar (SDA) to encourage mycosis. After 10 days of possible infection, the pupae were examined visually for fungal growth. The mycosis of both isolates was recorded by observing the characteristic coloration of the conidia on the agar plates (with *B. bassiana* displaying a white mass of conidiophores and *M. anisopliae* displaying a green mass of conidiophores). Mortality was assessed by holding each individual pupa against a heated hotplate and observing any signs of movement. The pupae responding to the emitted heat were considered to be alive and uninfected.

Larvae

The virulence of both EPF isolates to the various sizes of field-collected *A. trimenii* larvae, which varied between 0.5 mm and 3.0 mm in length (Morris, 2019), was evaluated by following the dipping bioassay protocol, as described. Four six-well bioassay plates ($n = 24$) and an identical control ($n = 24$) were used per EPF isolate. After five days of exposure to EPF, dead larvae were removed from the bioassay plates and transferred to Petri dishes containing SDA to encourage mycosis. The Petri dishes were sealed in 2 L plastic containers were lined with moistened paper towels, and then placed in a growth chamber at 25 ± 2°C for a further 10 days. The mycosis of both isolates was recorded by noting the number of dead larvae, overt mycosis and the colour of the conidia.

RESULTS

Pathogenicity of entomopathogenic nematodes

Pupae

In all the bioassays, zero mortality and infection by EPNs were obtained against the pupal stage of *A. trimenii*. The pupae treated with both EPN isolates eventually emerged as adult moths after ± six months, further proving that both of

the isolates had failed to infect the pupae. No mortality was observed in the control group.

Larvae

In all the bioassays, both S. *yirgalemense* and *H. noenieputensis* caused 100% mortality after 48 h of exposure to the different larval instars of *A. trimenii* (ranging from 1.5 cm to 4.5 cm in length), at a concentration of 100 IJs/insect. No control group replicates showed mortality.

Pathogenicity of entomopathogenic fungi

Pupae

Similarly, screening *B. bassiana* and *M. anisopliae* against *A. trimenii* pupae showed zero mortality. Two pupae treated with *M. anisopliae* and one pupa treated with *B. bassiana* were assumed to be dead, as no movement was observed when they were held against the heated hotplate. However, upon inspection under the microscope, all three pupae were found to have been damaged previously, possibly as a result of excavation in the field during sample collection. Therefore, 28 of the 30 pupae treated with *M. anisopliae* were confirmed to be alive after the incubation period, and 29 of the 30 pupae treated with *B. bassiana* were confirmed to be alive after treatment. The characteristic coloration of fungal spores for each isolate was found to be lacking when the SDA plates were examined for mycosis.

Larvae

All the larvae of *A. trimenii* treated with the commercial products of *M. anisopliae* and *B. bassiana* died within five days after inoculation. After transferring the cadavers to SDA plates, the larvae treated with *M. anisopliae* showed overt mycosis after a further five days, with green fungal spores, which are typical of *Metarhizium* infection. No mortality was observed in the control group. However, the *A. trimenii* cadavers of *B. bassiana*-treated larvae showed a red/yellow colouration, with no visible mycosis. No mortality was observed in the control group.

DISCUSSION

A biological assay is the starting point of any investigation in which virulence is of importance, as it allows for the removal of factors that could reduce virulence towards the target host. Both *S. yirgalemense* and *H. noenieputensis* were found to be highly virulent, causing 100% mortality of the larval stages of *A. trimenii*. The results also indicate that inoculation with lower nematode concentrations should be applied in future studies, as this could indicate the difference in virulence between different EPN species. Success in using EPNs to control the soil life stages of other grapevine pests, including false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) (Malan & Moore, 2016; Steyn *et al*., 2019a, 2019b); fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) (James *et al*., 2018); and weevils, *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae) (Ferreira & Malan, 2014; Dlamini *et al*., 2019), has resulted in research being undertaken on mass culturing and the formulation of local entomopathogenic nematodes (Ferreira *et al*., 2016; Dunn *et al*., 2020). The pathogenicity of *S. yirgalemense* to *A. trimenii* larvae further emphasises its potential to control more than one target pest, which should enhance its acceptability by, and usefulness for, farmers.

The screening of both EPF isolates against the larvae of *A. trimenii* resulted in a mortality of 100%. At 10 days post-treatment, the SDA plates containing individuals treated with *M. anisopliae* showed the typical characteristic of overt mycosis with the development of green spores. However, in the case of *B. bassiana*, incubation of insect cadavers on SDA plates did not result in overt mycosis, even though no mortality occurred in the control larvae treated with water only. Further investigation to confirm toxic mycosis due to infection by *B. bassiana* is needed. These results also indicate that the application of these two biologicals in combination, to obtain a possible additive or synergistic effect, is worth investigating (Anbesse *et al*., 2008).-

Although both EPF isolates showed 100% mortality against the larval stages of *A. trimenii*, the pupal stage showed no susceptibility. The initial observations – of external sporulating fungal growth at the location of the conidial applications on the pupae, but no signs of subsequent infection/death – resemble the results obtained by Boucias and Latgé (1988) and Sitch and Jackson (1997) in the case of resistant and susceptible aphids. The pupal cuticle of *A. trimenii* provides a higher level of protection from soil-dwelling fungi than the cuticle of the arboreal larval stage.

Anand *et al*. (2009) established that the pupae of *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) that resisted infection took two to five days longer to emerge than the pupae not exposed to the fungus. Additionally, Hafez *et al*. (1997) found that the pupae of the potato tuber moth, *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae), which emerged successfully despite exposure to *B. bassiana*, showed reduced fecundity. However, the indirect effects of EPF isolates on *A. trimenii* were not assessed in the present study. Despite failure to kill the pupae, the findings of this study provide future scope for testing the indirect effects of both *M. anisopliae* and *B. bassiana* on *A. trimenii* pupae. Control at the pupal stage is desired, because it would potentially significantly reduce the number of egg-laying adults and the size of subsequent *A. trimenii* populations. Testing all the EPN and EPF isolates against the prepupal stage of *A. trimenii* should be the next step in screening. Infection can possibly occur during the short window period that the last larval instar spends in the soil prior to pupation, and during the time taken by the newly eclosed moths to emerge from the soil. During the prepupal period, the cuticle tends to be softer and more malleable than the heavily sclerotized cuticle of the fully formed pupa, which makes it more challenging for the IJs to penetrate and infect the host. Kaya and Hara (1980) showed that the prepupal stage of *G. mellonella*, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) and *Mythimna unipuncta* Haworth (Lepidoptera: Noctuidae) was the most susceptible stage, exhibiting the highest mortality across all tested EPNs.

The results obtained in the present study provide useful information on the potential of EPNs and EPF to control *A. trimenii*. Limitations of the study were that a laboratory culture was not available and specimens could only be hand collected in untreated vineyards during the growing season.

All research was done on site or under quarantine conditions at the Plant Quarantine Station, Stellenbosch, South Africa. However, from the findings it can be concluded that the two local EPN species are good potential biological control agents against the larval stages of *A. trimenii*, but cannot be used to control the pupal stage, which showed zero mortality.

Applying EPNs and EPF to target the prepupal stage in the open soil cocoon (Morris *et al*., 2020), as well as targeting the newly eclosed adults moths, such as found in the case of false codling moth (Malan *et al*., 2011), holds potential for controlling the population at a time when there is no threat of damage to grapes. The ability of the EPN and EPF isolates to perform effectively under field conditions remains to be tested. Future research should be directed at investigating the effect of other EPF isolates, which are available as commercial products, either individually or in combination with EPNs as potential biological control agents against *A. trimenii*. The application of biologicals to hot spots of *A. trimenii* infestations will prevent the negative effect of chemical applications in grapevines and the disruption of a successful IPM programme.

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Cold Hardiness of Primary Buds of Wine and Table Grape Cultivars in Poland

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The aim of this study was to compare the cold resistance of grapevine cultivars in field conditions. Following the winters of 2016/2017 and 2017/2018, an assessment of frost damage was carried out on the vines of 42 wine cultivars and 45 table grape cultivars grown in central Poland (Skierniewice, latitude 51.9627 N, longitude 20.1666 E). The minimum temperature for each of the two winters was recorded on 2017-01-07 (-20.9°C) and on 2018-02-27 (-20.1°C). Among the assessed cultivars, 19 (13 wine grape cultivars and six table grape cultivars) belonged to *V. vinifera* species and 68 were inter- or inter-intraspecific hybrids. Cultivars were divided into five classes of different frost tolerance, with information on the proportion of primary buds frozen given in the brackets: very resistant (below 1.9%), resistant (2% to 24.9%), medium susceptible (2% to 74.9%), susceptible (75% to 95.9%) and very susceptible (above 96%). The number of wine and table grape cultivars in particular classes (mean for two winters) was as follows: very resistant – 20 (wine) and 10 (table), resistant – 15 and 13, medium susceptible – six and 20, susceptible – one and two. Both the *V. vinifera* cultivars and the interspecific hybrids were highly diversified in terms of frost tolerance. In most cultivars, the number of frozen buds after the winter of 2016/2017 was greater than after the winter of 2017/2018. Among *V. vinifera*, 'Riesling', 'Pinot Noir', 'Chasselas Dore' (reference) and 'Tauberschwarz', 'Veltliner Frührot' ('Fruehroter Malvasier'), 'Turan', 'Domina', 'Tressot Panache', 'Blue from Tychy', and 'Irsai Oliver' were very resistant or resistant. Other cultivars of *V. vinifera* were medium susceptible or susceptible.

INTRODUCTION

As a result of climatic changes and progress made in plant breeding, the cultivation of grapevine is being moved north of the traditional areas in Europe and North America (Gustafsson & Mårtensson, 2005; Rayne *et al*., 2011). In the past decades a rapid development of vineyards has been observed in Poland and other countries in the cold climatic zone (Vool *et al*., 2015). This conventional term is used to describe wine-growing regions with a latitude above 50°N and cold winters. The frost tolerance of vines is the basic characteristic determining the usefulness for cultivation in areas where winters are cold, such as the central and northern states of the USA, Canada, central and northern Europe (Baltic region), and eastern Europe (Russia and Ukraine). Grapevine genotypes are characterised by varied frost susceptibility, which is related to their origins. Contemporary cultivation takes advantage of inter- and intraspecific hybridisation and develops cultivars that are satisfactorily resistant to frost and produce good-quality fruit (Kostrikin, 1994). American species, such as *V. riparia*, *V. rupestris*, *V. lincecumii* and *V. labrusca* and the Asian *V. amurensis* are often used as donors of frost resistance in hybrids with *V. vinifera* (Alleweldt *et al*., 1990; Luby, 1991; Kostrikin, 1994; Reisch & Pratt, 1996; Clark & Moore, 1999). Frost susceptibility of wintering buds is assessed under laboratory or field conditions (Bourne *et al*., 1991). Exposure of the scions of one-year-old shoots to low temperature in a cold chamber is a frequently used evaluation method (Cindric & Korac, 1990). From a practical point of view, field assessment is particularly valuable because it includes the effects of all factors relevant to the level of frost damage. The degree of damage depends not only on minimum temperature and the time of its occurrence, but also on fluctuations in temperature, force of the wind, and the age and physiological state of the plants (Plocher & Parke, 2001; Fennel, 2004). Current knowledge of the frost susceptibility of particular cultivars is varied. Data on the frost susceptibility of many cultivars is available in scientific papers (Bourne *et al*., 1991; Clark & Moore, 1999), textbooks (Pospišilova, 1981; Lott *et al*.,

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2010) and even authorised websites on the Internet (HORT 3040, 2016; Bell, 2018; Vinograd.info, 2019). There is a lack of data on some cultivars recently introduced to Poland from regions with less severe winters. The assessment of frost susceptibility in Poland is being conducted, along with the acquisition of new genotypes (Lisek, 2009, 2012).

The minimum critical (bud-damaging) temperatures tolerated by varieties depend on their ecological-geographical and genetic origin. Among the commonly cultivated varieties of *V. vinifera*, the most resistant to cold are 'Riesling', whose buds tolerate temperature drops to -21°C (Cindric & Korac, 1990) or even to -25°C (Pospišilova, 1981), and 'Pinot Noir', tolerating temperatures down to -20°C (Pospišilova, 1981). Both varieties come from Western Europe and belong to *V. vinifera* L. subsp. *sativa* D.C. proles *occidentalis* Negr. Critical temperatures for cultivars from other ecological-geographical groups of *V. vinifera*, proles *pontica* and *orientalis* Negr. are up to -15°C or -16°C (Nikov *et al*., 1983; Cindric & Korac, 1990). The frost resistance of interspecific hybrids varies greatly on the basis of their genetic composition and may exceed -26°C (HORT 3040, 2016). Frost damage to buds occurs even when the critical temperature drops for one day (Nikov *et al*., 1983). In laboratory tests, the critical temperature is maintained for 12 hours (Cindric & Korac, 1990). The value of critical temperatures changes during the winter, along with physiological changes on a biochemical basis (Ferguson *et al*., 2014; Nenko *et al*., 2018). Grapevines acclimate to cold temperatures in the autumn and de-acclimate when warm temperatures return in the spring, and models of bud frost resistance and bud development are variety dependent (Ferguson *et al*., 2014). In the northern hemisphere, varieties belonging to *V. vinifera*, including subproles *occidentalis*, achieve maximum frost resistance in the middle of winter (second half of January), hybrids of *vinifera* x American species in the middle and at the end of winter (second half of February), and hybrids of *vinifera* x *amurensis* at the beginning (December) and in the middle of winter (Cindric & Korac, 1990). More rapid de-acclimation and budbreak occur earlier in hardier genotypes originating from colder climates (Ferguson *et al*., 2014). In areas with cold and long winters at high latitudes, like Poland, damage to the buds occurs most often during deep dormancy (endodormancy), before sap rise, because January and February are the coldest months in the northern hemisphere, including Central Europe (Pospišilova, 1981; data in the present research from Poland). In warmer regions with relatively mild winters and an early spring, critical temperatures may occur at the end of winter, shortly before or after sap rise, and will not be as extremely low as the critical temperatures at the beginning and middle of winter (Ferguson *et al*., 2014; Nenko *et al*., 2018).

The aim of this work was to undertake a field assessment of the winter frost susceptibility of wine and table grape cultivars that could possibly be grown in central and northern Europe and that have not yet been methodically assessed in terms of cold resistance. Vines were tested for cultivation in large, commercial vineyards that produce wine, as well as for home garden or agrotourism farm cultivation, without covering the plants for the winter.

MATERIALS AND METHODS

The assessment of frost damage to the vines was carried out following the winters of 2016/2017 and 2017/2018 in the field collection of the Research Institute of Horticulture in Skierniewice (Poland, latitude 51.9627 N, longitude 20.1666 E), located on a luvisol soil, slightly acidic (pH 6.3) and containing 1.3% organic matter. Each genotype gathered in the collection was represented by three vines, planted at a spacing of 2.5 x 1 m and maintained in the form of a low head with the trunk 0.15 m to 0.2 m high and three to five spurs, pruned into two to three buds (six to 10 young shoots per vine). The young shoots were pruned above the 10^{th} to 12^{th} leaf, past the last cluster of grapes, in July. Fertilisation and plant protection were carried out according to current recommendations for commercial vineyards. Mineral fertilisation – 'Azofoska', a multi-component fertiliser containing macronutrients N, P, K and Mg and micronutrients Cu, Zn, Mn, B and Mo, and triple superphosphate (P) were applied in the spring, at the beginning of April. Half of the necessary nitrogen dose was applied in the first half of June in the form of calcium nitrate. The annual dose of macronutrients was N: 40 kg ha^{-1}, P: 30 kg ha^{-1}, K: 100 kg ha^{-1}. A chemical protection against fungal diseases was carried out, using fungicides containing copper, sulphur (two treatments per season by each agent), mancozeb+metalaxyl, pyraclostrobin+boscalid, cyprodynil+fludioxonil, penconazole (optionally, one to two treatments per season by product, depending on the weather). Vines were covered with cereal straw (mounds of 0.4 m) for the winter (December).

Frost damage to the vines was assessed after the winters, which were characterised by weather conditions as set out in the Table 1. The sum of active temperatures (SAT) – medium daily temperature from April to October, higher than 10°C – amounted to 2 667°C (growing degree days, GDD – 1 098) in 2016, and 2 693°C (GDD – 1 030) in 2017, which did not diverge from the average of the preceding ten years. There were no drops in temperature below -10ºC in November 2016 and 2017, which allowed the plants to become adapted to winter conditions gradually. Extremely low temperatures occurred in the first two weeks of January 2017 (winter 2016/2017), and in last week of February and the first days of March 2018 (winter 2017/2018). The minimum temperature at a height of 2.0 metres recorded on 2017-01-07 was -20.9°C. At a height of 0.2 metres the temperature dropped to -22.3°C. The minimum temperature of the next winter was -20.1°C (-20.6°C at a height of 0.2 metres) and was recorded on 2018-02-27.

Forty-two grapevine cultivars valuable for wine production and 45 grapevine cultivars for table grapes (including reference cultivars) were selected for the study out of the 320 genotypes represented in the collection. From among the wine cultivars, the following were selected as references: 'Marechal Foch' (interspecific hybrid), 'Solaris' and 'Regent' (inter-intraspecific hybrids), 'Riesling' and 'Pinot Noir' (*V. vinifera*). Inter-intraspecific (intra-interspecific) hybrids were isolated into a separate group (Theocharis *et al*., 2010), and classified as *Vitis vinifera* cultivars (Vitis International Variety Catalogue, 2019). 'Muscat Bleu' and 'Chasselas Dore' were chosen as reference

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TABLE 1
Climatic conditions in Skierniewice in the period July 2016 to March 2018

Year	Month	Air temperature at the height of 2.0 m and date on which it occurred (°C)		Average monthly air temperature (°C)	Precipitation (mm)
		Minimum	Maximum		
2016	July	7.2 (22.07)	32.0 (11.07)	18.7	103.8
	August	5.5 (12.08)	30.7 (05.08)	17.9	0.2
	September	-2.0 (27.09)	30.0 (12.09)	14.9	0.8
	October	-1.8 (07.10)	21.5 (02.10)	7.1	32.6
	November	-6.2 (10.11)	14.2 (21.11)	3.0	0.6
	December	-8.1 (12.12)	9.4 (26.12)	1.2	1.6
2017	January	-20.9 (07.01)	3.8 (13.01)	-4.3	9.0
	February	-12.2 (13.02)	13.2 (27.02)	-1.2	34.8
	March	-1.5 (08.03)	20.5 (31.03)	5.9	46.8
	April	-4.0 (17.04)	22.6 (02.04)	7.0	72.2
	May	-2.5 (10.05)	27.6 (20.05)	13.6	54.4
	June	7.2 (02.06)	29.5 (20.06)	17.5	149.8
	July	6.5 (06.07)	32.0 (30.07)	18.2	51.8
	August	6.4 (29.08)	35.0 (01.08)	18.9	71.8
	September	2.1 (29.09)	23.5 (10.09)	13.3	249.4
	October	0.6 (31.10)	21.3 (16.10)	9.6	77.6
	November	-1.5 (22.11)	12.4 (05.11)	4.7	41.4
	December	-3.5 (20.12)	10.9 (12.12)	2.3	67.6
2018	January	-8.6 (16.01)	9.9 (29.01)	0.6	22.2
	February	-20.1 (27.02)	5.5 (01.02)	-3.6	8.0
	March	-19.4 (02.03)	11.7 (30.03)	0.2	15.8

table grape cultivars. Vines of the assessed cultivars were planted in 2012. Information on wine and table grape cultivars, such as origin and skin colour of the berries, is given in Tables 2 and 3, which also contain the results of the study. Frost damage to the buds was assessed at the time of cutting back the woody shoots at the end of March and at the beginning of April. Three samples of cane were taken from each of the plots (cultivars), each with 50 buds from the three plants being assessed. One-year-old completely woody shoots of the evaluated cultivars, with tested primary buds free from mechanical damage and symptoms of disease, were taken from the parts of vines that were not covered with straw or snow during the winter, at a height of 0.5 m to 1.5 m above the surface of the ground. The buds under assessment were cut across, and the colour of the incipient shoots was determined. An overwintering bud was considered to be dead if both the central bud and the lateral buds underneath were dark brown. The obtained results, expressed as a percentage of the buds damaged by frost in relation to the total number of buds, were analysed statistically using the variance analysis method, which was applied to the values after Bliss's transformation. The significance of the means was evaluated using Duncan's test at the 5% level. Cultivars were divided into five classes of different frost tolerance according to the percentage of frozen buds: very resistant (below 1.9%), resistant (2% to 24.9%), medium susceptible (25% to 74.9%), susceptible (75% to 95.9%) and very susceptible (above 96%).

RESULTS AND DISCUSSION

For most cultivars, frost injuries to primary buds were greater after the winter of 2016/2017 than after the winter of 2017/2018 (Tables 2 and 3). During the assessment in the spring of 2017, one wine cultivar, 'Cabernet Mitos', and three table grape cultivars, 'Vienetchnij', 'Philipp' and 'Evita', were included in the very susceptible class. After expanding the results as a mean of a two-winter assessment, the number of wine and table grape cultivars in particular classes was as follows: very resistant – 20 (wine) and 10 (table), resistant – 15 and 13, medium susceptible – six and 20, and susceptible – one and two.

Interspecific hybrids originating from the US – 'E.S. N-5-10', 'Frontenac', 'Kay Gray', 'Marquette', 'Norway Red', 'Sabrevois', 'St. Croix', 'St. Pepin' and 'Troubadour'; from the Ukraine – 'Muskat Odesskij'; and from Canada – 'Vandal Cliché', proved to be the wine cultivars most tolerant to cold damage. During the study, wintering buds of those cultivars were not damaged by frost – the same as in the case of the reference hybrid, 'Marechal Foch', which is widely grown in countries with cold winters (Plocher & Parke, 2001).

Cultivars classified as inter-intraspecific hybrids bred in Germany were characterised by varied frost tolerance. 'Baron' and 'Hibernal' were the most resistant. Among cultivars most often grown in the Baltic region, including Poland, and taking into consideration the mean results of two winters, 'Solaris' was very resistant and 'Regent' was resistant. Some of the inter-intraspecific hybrids, including 'Regent' and 'Calandro', were far inferior in frost resistance

TABLE 2
Cold damage to wine grapevines following the winters of 2016/2017 and 2017/2018, Skierniewice.

Cultivar	Colour of berry skin*	Origin**	Country of breeding	Frozen buds (%)			Class of frost tolerance*** (mean)
				2016/2017	2017/2018	Mean	
Baron	N	IIH	Germany	0.0 a	0.0 a	0.0 a	VR
E.S. N-5-10	R-N	IH	USA	0.0 a	0.0 a	0.0 a	VR
Frontenac	N	IH	USA	0.0 a	0.0 a	0.0 a	VR
Kay Gray	B	IH	USA	0.0 a	0.0 a	0.0 a	VR
Marechal Foch	N	IH	France	0.0 a	0.0 a	0.0 a	VR
Marquette	N	IH	USA	0.0 a	0.0 a	0.0 a	VR
Muskat Odesskij	B	IH	Ukraine	0.0 a	0.0 a	0.0 a	VR
Norway Red	N	IH	USA	0.0 a	0.0 a	0.0 a	VR
Sabrevois	N	IH	USA	0.0 a	0.0 a	0.0 a	VR
St. Croix	N	IH	USA	0.0 a	0.0 a	0.0 a	VR
St. Pepin	B	IH	USA	0.0 a	0.0 a	0.0 a	VR
Troubadour	N	IH	USA	0.0 a	0.0 a	0.0 a	VR
Vandal Cliché	B	IH	Canada	0.0 a	0.0 a	0.0 a	VR
Hibernal	B	IIH	Germany	0.2 ab	0.0 a	0.1 ab	VR
Allegro	N	IIH	Germany	0.9 bc	0.0 a	0.2 a-c	VR
Helios	B	IIH	Germany	2.0 cd	0.0 a	0.5 a-c	VR
Jutrzenka	B	IH	Poland	2.0 cd	0.0 a	0.5 a-c	VR
Riesling	B	*Vin*	Germany	2,6 de	0.0 a	0.7 a-c	VR
Souvignier Gris	R	IIH	Germany	4.0 ef	0.0 a	1.0 a-c	VR
Solaris	B	IIH	Germany	6.6 gh	0.0 a	1.7 a-c	VR
Felicia	B	IIH	Germany	9.3 hi	0.0 a	2.4 a-c	R
Bolero	N	IIH	Germany	10.6 i	0.0 a	2.7 b-d	R
Hybrid from Karpicko	N	IH	Poland	12.0 i	0.0 a	3.1 b-e	R
Villaris	B	IIH	Germany	12.0 i	0.0 a	3.1 b-e	R
Cabernet Cantor	N	IIH	Germany	9.3 hi	0.2 b	3.2 b-e	R
Pinot Noir	N	*Vin*	France	8.6 hi	0.9 c	3.8 c-e	R
Tauberschwarz	N	*Vin*	Germany	5.3 fg	3.3 d	4.2 c-e	R
Veltliner Frührot	R	*Vin*	Austria	26.0 j	0.9 c	9.6 d-f	R
Muscaris	B	IIH	Germany	35.3 mn	0.0 a	9.8 d-f	R
Monarch	N	IIH	Germany	36.0 mn	0.2 b	10.0 ef	R
Calandro	N	IIH	Germany	31.3 k-m	6.6 e	17.2 fg	R
Sirius	B	IIH	Germany	40.7 n	6.6 e	21.5 g	R
Turan	N	*Vin*	Hungary	29.3 j-l	14.7 g	21.5 g	R
Regent	N	IIH	Germany	30.6 j-m	9.3 f	18.7 fg	R
Domina	N	*Vin*	Germany	26.7 jk	18.0 g	22.5 g	R
Bouvier	B	*Vin*	Slovenia	35.3 mn	16.7 g	25.4 g	MS
Merlot	N	*Vin*	France	32.6 lm	23.3 h	27.9 g	MS
Schönburger	R	*Vin*	Germany	68.0 p	16.7 g	41.0 h	MS
Morio Muscat	B	*Vin*	Germany	65.4 op	25.3 h	44.9 h	MS
Syrah	N	*Vin*	France	62.7 o	29.3 i	45.4 jk	MS
Dunaj	N	*Vin*	Slovakia	90.7 q	24.0 h	60.0 i	MS
Cabernet Mitos	N	*Vin*	Germany	99.8 r	76.1 j	92.4 j	S

Means followed by the same letter do not differ significantly at p = 0.05.
* Colour of berry skin: B (blanc) – green-yellow; R – rose; N (noir) – blue-black
** Origin: *Vin – V. vinifera*; IH – interspecific hybrid from crossing *V. vinifera* and species from among *V. riparia*, *V. rupestris*, *V. lincecumii*, *V. amurensis* and *V. labrusca*; IIH – cultivar from crossing interspecific hybrid and *V. vinifera*
*** Class of frost tolerance, with percentage of frozen buds in brackets: VR – very resistant (below 1.9%), R – resistant (2% to 24.9%), MS – medium susceptible (25% to 74.9%), S – susceptible (75% to 95.9%) and VS – very susceptible (above 96%).

TABLE 3
Cold damage to table grapevines following the winters of 2016/2017 and 2017/2018, Skierniewice.

Cultivar	Colour of berry skin*	Origin**	Country of breeding	Frozen buds (%)			Class of frost tolerance*** (mean)
				2016/2017	2017/2018	Mean	
Bluebell	N	IH	USA	0.0 a	0.0 a	0.0 a	VR
Izopan	B	IH	Poland	0.0 a	0.0 a	0.0 a	VR
Kivikrass	N	IH	Estonia	0.0 a	0.0 a	0.0 a	VR
Mars Sdl (seedless)	N	IH	USA	0.0 a	0.0 a	0.0 a	VR
Siewiernij Rannij	B	IH	Russia	0.0 a	0.0 a	0.0 a	VR
Somerset Sdl	R	IH	USA	0.0 a	0.0 a	0.0 a	VR
Trollhaugen Sdl	N	IH	USA	0.0 a	0.0 a	0.0 a	VR
Vanessa Sdl	R	IH	Canada	0.0 a	0.0 a	0.0 a	VR
Venus Sdl	N	IH	USA	0.0 a	0.0 a	0.0 a	VR
Jupiter Sdl	R-N	IH	USA	0.2 a	0.2 b	0.2 ab	VR
Muscat Bleu	N	IH	Switzerland	8.6 b	0.0 a	2.2 a-c	R
Tressot Panache	N (B)	*Vin*	France	5.3 b	2.0 c	3.5 bc	R
Marquis Sdl	B	IH	USA	5.3 b	3.3 cd	4.2 b-d	R
Zodiak	R	IH	Russia	6.0 b	4.6 d	5.3 b-e	R
Zoltaja Lora	B	IH	Ukraine	14.6 c	4.6 d	9.0 c-f	R
Chasselas Dore	B	*Vin*	Unknown	22.0 d	7.2 e	13.8 d-g	R
Pleven Muskatnyj	B	IH	Bulgaria	22.0 d	8.6 ef	14.7 d-h	R
Blue from Tychy	N	*Vin*	Unknown	22.0 d	9.3 e-g	15.1 e-h	R
Charlie (Antracit)	N	IH	Russia	32.5 ef	7.9 e	18.6 f-i	R
Sophie	B	IH	Hungary	30.6 e	9.3 e-g	18.8 f-i	R
Irsai Oliver	B	*Vin*	Hungary	22.6 d	18.8 m	20.6 f-j	R
Krasotka	R	IH	Russia	38.0 f	8.6 ef	21.4 f-j	R
Piesnia	B	IH	Russia	28.5 e	18.0 lm	23.0 f-k	R
Aron	B	IH	Hungary	45.3 g	11.3 f-h	26.5 g-l	MS
Romulus Sdl	B	IH	USA	45.3 g	14.6 i-l	28.7 g-m	MS
Oryginal Tchernij	N	IH	Ukraine	55.3 hi	11.3 f-h	31.0 h-m	MS
Laura	R	IH	Ukraine	60.7 ij	12.7 h-j	34.5 i-n	MS
Saturn Sdl	R-N	IH	USA	62.0 ij	14.7 i-l	36.6 j-n	MS
Stefanie	B	IH	Austria	65.4 j	13.3 h-k	37.4 j-n	MS
White from Leszno	B	*Vin*	Poland	51.3 gh	26.3 n	38.6 j-n	MS
Chryzolit	B	IH	Russia	66.0 j	17.3 lm	40.4 k-n	MS
Katharina	R	IH	Austria	50.7 gh	31.3 o	40.8 k-n	MS
Argo	R	IH	Ukraine	72.7 k	15.3 i-m	42.6 l-n	MS
Elegant	B	IH	Russia	82.0 lm	12.0 g-i	45.8 l-o	MS
Nadiezhda AZOS	N	IH	Russia	79.3 lm	15.9 k-m	46.9 m-o	MS
Sfinks	N	IH	Ukraine	78.7 l	16.6 k-m	47.0 m-o	MS
Talisman	B	IH	Russia	79.4 lm	26.7 n	53.6 n-p	MS
Galbena Nou	B	IH	Russia	80.7 lm	43.3 p	63.0 o-q	MS
Danmarpa Polonia	B	*Vin*	Poland	84.7 m	46.6 p	67.2 pq	MS
Europleven	B	IH	Bulgaria	95.7 o	30.6 no	68.4 pq	MS
Favor	R	IH	Russia	91.4 n	51.3 q	74.0 q	MS
Stout Sdl	B	IH	USA	91.4 n	51.3 q	74.0 q	MS
Vienetchnij	B	IH	Ukraine	99.6 p	30.0 no	74.5 q	MS
Philipp	N	IH	Austria	98.7 p	46.7 p	79.8 qr	S
Evita	B	IH	Austria	99.8 p	68.0 r	89.9 r	S

Means followed by the same letter do not differ significantly at p = 0.05.
* Colour of berry skin: B (blanc) – green-yellow; R – rose; N (noir) – blue-black
** Origin: *Vin* – *V. vinifera*; IH – interspecific hybrid from crossing *V. vinifera* and species from among *V. riparia*, *V. rupestris*, *V. lincecumii*, *V. amurensis* and *V. labrusca*
*** Class of frost tolerance, with percentage of frozen buds in brackets: VR – very resistant (below 1.9%), R – resistant (2% to 24.9%), MS – medium susceptible (25% to 74.9%), S – susceptible (75% to 95.9%) and VS – very susceptible (above 96%).

to most tolerant *V. vinifera* cultivars, such as 'Riesling', 'Tauberschwarz' and 'Pinot Noir'. The *V. vinifera* cultivars 'Dunaj' and 'Cabernet Mitos' were the most susceptible to frost damage.

Hybrids crossbred with *V. labrusca* in the USA – 'Bluebell' and seedless 'Mars', 'Somerset', 'Trollhaugen', 'Venus', 'Jupiter' and 'Marquis', in Poland – 'Izopan', in Estonia – 'Kivikrass', in Canada – 'Vanessa' and the Russian hybrid crossbred with *V. amurensis* – 'Siewiernij Rannij', were table grape cultivars observed to be most frost resistant. The *V. vinifera* cultivar that proved most resistant to frost damage was the chimera, 'Tressot Panache'. Among interspecific hybrids with large, firm berries, 'Zodiak' was relatively resistant; its frost tolerance was similar to the reference hybrid 'Muscat Bleu', and definitely higher that the other reference cultivar, 'Chasselas Dore', which belong to *V. vinifera*. Most interspecific hybrids with attractive table grape berries originating from the Ukraine, Russia, Bulgaria, Hungary and Austria, with the exception of 'Zoltaja Lora' and 'Pleven Muskatnyj', proved to be more susceptible to frost damage than 'Chasselas Dore'. Most susceptible were buds of 'Philipp' and 'Evita'. Damage to the buds of 85% to 90% was accompanied by damage to one-year-old wood. The trunks and basal part of one-year-old shoots were not damaged, as the vines were covered with straw.

Planting frost-resistant cultivars is essential for the successful expansion of grapevine cultivation in central, eastern and northern Europe, Canada and the central and northern parts of the USA (Plocher & Parke, 2001). The results presented here for the most part confirm data available in the existing literature, textbooks and Internet databases. Some of the results, however, differ from the available data, which could be attributed to differences in growing areas, climatic conditions, training system, cultural practices and vine condition (Fennel, 2004).

The present results confirm the high frost tolerance of wine and table grape cultivars grown in Canada and the USA; they meet the needs of central states such as Arkansas, and the northern states such as Wisconsin and Minnesota. 'Mars', 'Marquis', 'Jupiter' and 'Vanessa' are a group of resistant cultivars which, under American conditions, withstand temperatures between -23°C and -26°C. 'Marquette', 'St. Croix', 'Sabrevois', 'Frontenac', 'Kay Gray', 'St. Pepin', 'Bluebell', 'Trollhaugen' and 'Somerset' (HORT 3040, 2016), and 'Vandal Cliché' (Bell, 2018) can all withstand a temperature below -26°C. Our own study confirmed American data that indicated that, among seedless cultivars grown in Arkansas (USA), 'Venus', 'Mars' and 'Jupiter' are more frost resistant than 'Saturn' (Bourne *et al*., 1991; Clark & Moore, 1999).

In the course of the present study, traditional wine cultivars were proven to be medium susceptible to frost ('Merlot') or resistant ('Veltliner Frührot'), the same as in Slovakia (Pospišilova, 1981). Only some cultivars originating from Russia and Ukraine were confirmed to be very or relatively frost resistant under Polish conditions – as was declared in the respective countries of origin. 'Siewiernij Rannij' (from -23°C to -26°C), 'Zodiak' (from -23°C to -26°C), 'Zoltaja Lora' (from -23°C to -26°C) and 'Chryzolit' (-21°C) belonged to this group; the temperature ranges enclosed in the brackets indicate what temperatures the cultivars are supposed to withstand in Russia and Ukraine (Vinograd.info, 2019). Some of the cultivars, e.g. 'Favor' (from -23°C to -26°C), 'Elegant' (-25°C), 'Galbena Nou' (-25°C), 'Vienetchnij' (-23°C) and 'Sfinks' (-21°C), were more susceptible to frost damage than declared in the abovementioned Russian source (the brackets enclose Russian data on the minimum temperatures that the buds of the cultivars can tolerate).

For the most part, our results confirm reference data from Germany, but they do differ in the case of several cultivars. As in Germany, 'Baron', 'Helios', 'Hibernal' and 'Tauberschwarz' were very resistant or resistant (Lott *et al*., 2010). However, 'Sirius' – described by Lott *et al*. (2010) as a cultivar characterised by frost resistance similar to that of 'Riesling', was proven by the research to be far more susceptible than the reference cultivar. 'Cabernet Mitos', assessed by the abovementioned German authors as equally resistant to frost damage as 'Pinot Noir', was also susceptible under Polish conditions. Lott *et al*. (2010) describe 'Solaris' and 'Regent' as resistant cultivars, but this and previous research conducted in Poland (Lisek, 2012) indicates that the buds of 'Regent' were more severely damaged by frost than the buds of 'Solaris'. Considerable differences were observed between the results obtained in our own research and the German results in the case of table grape cultivars. According to Lott *et al*. (2010), 'Romulus', 'Philipp', 'Evita', 'Sophie' and 'Katharina' are characterised by high frost resistance, whereas the results presented in this paper show that the buds of 'Evita' and 'Philipp' were strongly damaged by cold. Differences in defining varieties as cold resistant in Germany and Poland may result from the fact that, in Germany, frost resistance of -16°C to -18°C is sufficient. In Poland, however, temperature drops are greater than in Germany, which causes the conditions of field evaluation to be different and changes the criteria for recognising a variety as cold-resistant. The tendency for the buds of 'Evita' and 'Philipp' to freeze up is probably of genetic origin. The parental forms of both include varieties sensitive to frost: 'Evita' is F1 of 'Perlette', and 'Philipp' is F2 of 'Chaouch Rose' (Pospišilova, 1981; Nikov *et al*., 1983; Vitis International Variety Catalogue, 2019). 'Philipp' is characterised by late fruit ripening and high fertility, which may impede the maturation of the wood and the adaptation of buds in the winter under Polish conditions.

The research allowed us to assess, in terms of frost resistance, the cultivars that have not yet been documented in the methodical research available to the authors. Considering the mean results of the two winters, 'Allegro', 'Jutrzenka', 'Souvignier Gris' and 'Izopan' were very resistant. 'Villaris', 'Bolero', 'Cabernet Cantor', 'Felicia', 'Calandro', 'Muscaris', 'Turan' and 'Charlie' ('Antracit') were resistant, while 'Danmarpa Polonia' showed medium susceptibility.

The practical value of the present two-year study lies in the determination of the relationship between reference varieties and a large group of genotypes with cold resistance that is unknown in Poland. The reliability of these results is confirmed by the compliance of the data on reference varieties obtained in the present and historical studies carried out in the same collection and according to the same methodology

(Lisek, 2009, 2012) and by other authors (Cindric & Korac, 1990). In the present and historical research carried out in Skierniewice, different genotypes were assessed each time, but the reference cultivars of different origins and grape utility remained the same. These differences concern both the relationship between the cultivars and the results obtained within the cultivar, as the data on frost resistance may differ according to year of assessment, the physiological state of the plants, or the minimum temperatures and the time of their occurrence. Following the winter of 2009/2010, the buds of 'Pinot Noir' suffered more damage than the buds of 'Regent' (Lisek, 2012). The reverse relationship between these two cultivars was found after the winter of 2016/2017. Historical data show that the cold damage to the buds of the reference varieties after the winters of 2008/2009 and 2009/2010 was greater than following the winters of 2016/2017 and 2017/2018 (Table 4). The reason was a large drop in temperature found in the historical research, sometimes exceeding the values considered critical for the tested cultivars. The minimum temperature during the winter of 2008/2009 at the height of 2.0 metres as recorded on 2009-01-06 was -23.0°C. The minimum temperature of the next winter was -28.1°C, which was recorded on 2010-01-26. The pattern of cold resistance of the reference varieties after the winters of 2008/2009 and 2009/2010, in decreasing order, was as follows: 'Marechal Foch' (R) > 'Muscat Bleu' (R) > 'Riesling' (MS) > 'Solaris' (MS) > 'Regent' (MS) > 'Chasselas Dore' (MS) > 'Pinot Noir' (S). After the winters of 2016/2017 and 2017/2018, the varieties were ranked in the following cold tolerance pattern: 'Marechal Foch' (VR) > 'Riesling' (VR)' > 'Solaris' (VR) > 'Muscat Bleu' (R) > 'Pinot Noir' (R) > 'Chasselas Dore' (R) > 'Regent' (R). In both patterns, the first four cultivars, with greater frost resistance, and the next three, with lower frost resistance, were the same, although the order was slightly different. Also, Cindric and Korac (1990), who conducted a long-time study (six to nine years) in a cold chamber, with a temperature drop to -21.0°C, ranked the varieties according to decreasing frost resistance as: 'Riesling' > 'Pinot Noir' > 'Chasselas Dore', recognising all tolerant of cold damage. Both in the research by the abovementioned authors and in the present research conducted in Skierniewice, 'Merlot' buds were more severely damaged by frost than 'Riesling', 'Pinot Noir' and 'Chasselas Dore' buds. Although frost resistance is primarily determined by a genetic factor (Alleweldt *et al*., 1990), it is to some extent modified by the physiological state of plants (Cindric & Korac, 1990). A heavy crop load (Evans, 2000) and fungal disease infestation (Plocher & Parke, 2001) may reduce carbohydrate accumulation, weaken the vines and reduce cold hardiness. In the present study, the tendency to over-cropping was demonstrated in the interspecific hybrid 'Muscat Bleu', the inter-intraspecific hybrids 'Regent', 'Monarch' and 'Sirius' and, to a lesser extent, by 'Solaris'. In turn, among the varieties belonging to *V. vinifera*, 'Pinot Noir' was characterised by a greater tendency to over-cropping and susceptibility to fungal diseases compared to 'Riesling'.

The results regarding frost resistance depend on the climate model of the growing area (occurrence of critical temperatures over time) and the genetic pattern of the varieties, which is responsible for the chemical composition of the grapevine tissues and the physiological and biochemical processes taking place in them (Ferguson *et al*., 2014; Nenko *et al*., 2018). However, in practical research on the frost resistance of varieties in a cold climate, the first step is to determine the critical temperature and compare the frost resistance of the tested and reference varieties, which was the aim of the present work. Our results were collected when the mid-winter (January to February) was frosty, which precluded the early termination of endodormancy and reduced the risk of damage to the buds due to late winter frosts.

Even a few decades ago, countries such as Poland, located around 50°N latitude, did not meet the conditions for commercial wine growing, which is recommended for regions where temperatures do not fall below -20°C to -22°C, and they remain at this level for one to two days, not more often than once in five to six years (Nikov *et al*., 1983). Currently, climate changes are noticeable and are changing the regionalisation of grape growing (Rayne *et al*., 2011). As an example of these changes, we can mention unusually mild winters in central Poland (Skierniewice) in 2018/2019 and 2019/2020, when the temperature did not drop below -12°C. Thanks to the progress occurring in the breeding of varieties, valuable interspecific hybrids and inter-intraspecific (intra-interspecific) hybrids are available,

TABLE 4
Historical cold damage to reference wine grapes and table grapes following the winter of 2008/2009 and 2009/2010, Skierniewice.

Cultivar	Frozen buds (%)			Class of frost tolerance* (mean)
	2008/2009	2009/2010	Mean	
Marechal Foch	11.4 a	0.0 a	5.7 a	R
Pinot Noir	75.1 g	85.0 g	80.2 e	S
Regent	58.6 e	60.3 d	59.4 d	MS
Riesling	13.5 b	60.5 e	37.0 c	MS
Solaris	50.5 d	27.0 b	38.6 c	MS
Chasselas Dore	60.1 f	62.1 f	61.3 d	MS
Muscat Bleu	14.4 c	29.0 c	21.7 b	R

Means followed by the same letter do not differ significantly at p = 0.05.
* Class of frost tolerance, with percentage of frozen buds in brackets: VR – very resistant (below 1.9%), R – resistant (2% to 24.9%), MS – medium susceptible (25% to 74.9%), S – susceptible (75% to 95.9%) and VS – very susceptible (above 96%).

for which critical temperatures range from -20°C to -25°C. It is worth determining these varieties precisely in order to select the appropriate ones for cultivation and to minimise potential cold damage.

CONCLUSIONS

Frost resistance of the assessed grapevine cultivars within the *V. vinifera* species, as well as within the groups of interspecific and inter-intraspecific hybrids, varied extensively. Frost resistance of grapevine cultivars should be verified in region of cultivation.

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Complete Genome Sequencing of *Lactobacillus plantarum* UNQLp 11 Isolated from a Patagonian Pinot Noir Wine

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***Lactobacillus plantarum* UNQLp 11 strain was isolated from a Patagonian Pinot noir wine at the oldest commercial winery (110 years old) in General Roca, North Patagonia, Argentina, and has demonstrated its ability to survive during winemaking processes and successfully carry out malolactic fermentation. This work aimed to obtain the whole assembled genome of the UNQLp 11 strain, analysing its architecture and the possible functions of the predicted genes from the oenological properties of this strain. The genome size is 3 534 932 bp, with a mean GC content of 44.2%, 3 412 CDS, 80 transposons and 148 tandem repeats. A comparison between the genome size and gene content of 14 *Lb. plantarum* strains from different origins was performed, and UNQLp 11 exhibited the largest size. The *in silico* genome-wide analysis allowed us to confirm the existence of genes encoding enzymes involved in the synthesis of several metabolites of oenological interest, in addition to bacteriocins and exopolysaccharides. Furthermore, it is possible to speculate on this strain's adaptation to different environments, as it is able to use diverse substrates for its growth. All these features suggest the potential of UNQLp 11 to be a good starter culture for malolactic fermentation.**

INTRODUCTION

Lactobacillus plantarum (recently reclassified in it genus as *Lactiplantibacillus plantarum*, Zheng *et al*., 2020) is a species belonging to the lactic acid bacteria (LAB) group, which is found in different ecological niches such as vegetables, meat, fish and dairy products, as well as in the gastrointestinal, vaginal and urogenital tracts (Siezen *et al*., 2010). This species is a facultative heterofermentative LAB that can utilise a broad range of fermentable carbon sources, hence their application in the production of a variety of foods and beverages, as well as to obtain vitamins, bacteriocins, probiotics, antifungals and potential anticaries agents (Evanovich *et al*., 2019). Genome sequencing and comparative genomics of different *Lb. plantarum* strains have revealed high genomic diversity, versatility and flexibility, which ensure its success in various niches and applications (Siezen & Van Hylckama Vlieg, 2011; Jiang & Yang, 2018; Evanovich *et al*., 2019). This versatility and metabolic capacities make this LAB species highly relevant for industrial applications, and many strains are marketed as starter cultures for fermented foods and beverages (Molin, 2001; Weinberg *et al*., 2004; Luxananil *et al*., 2009).

In general, LAB have reduced genomes, but *Lb. plantarum* presents a larger genome with numerous genes that were acquired by horizontal gene transfer (HGT), mainly via mobile elements (Hubert & Kammerer, 1994; Kleerebezem *et al*., 2003). The habitat diversity of *Lb. plantarum* might be related to abundant gene functions. resulting in increased genome size (Bringel *et al*., 2001; Kant *et al*., 2011). Genomic analysis has facilitated a more comprehensive characterisation of the genetic characteristics of some bacterial strains (Lasek *et al*., 2017). In the past decade, several LAB strains were subjected to genome sequencing to further assess their physiological functions and environmental adaptation mechanisms. To date, more

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than 50 complete genome sequences of *Lb. plantarum* strains are available in the NCBI GenBank database (Yao *et al*., 2020). Most of these complete genomes correspond to strains with probiotic properties (Kleerebezem *et al*., 2003; Zhang *et al*., 2010; Siezen *et al*., 2010; Liang *et al*., 2019). In contrast, only a few genomes of oenological strains have been published (Lamontanara *et al*., 2015; Zhao *et al*., 2016) or are available from the NCBI GenBank database (CP017363.1).

Oenococcus oeni is the main LAB species responsible for the malolactic fermentation (MLF) of wine, which normally follows the alcoholic fermentation (AF) produced by yeasts (Davis *et al*., 1985). Also, it is probably the LAB species best adapted to overcome the harsh environmental conditions of wine, and therefore represents the majority of commercial MLF starter cultures. Due to global warming, the harvesting of grapes of higher maturity has resulted in higher pH of the musts. Under these high-pH conditions, *Lb. plantarum* bacteria have shown especially interesting results for their capacity to induce MLF and for minimising the risk of acetic acid production (Krieger-Weber *et al*., 2020). Some *Lb. plantarum* strains can also tolerate the high alcohol and SO_2 levels normally encountered in wine and are responsible for MLF in various wine regions and cellars (Guzzon *et al*., 2009; González-Arenzana *et al*., 2012; Valdés la Hens *et al*., 2015). Wine pH has been increasing gradually over the past several years, and red wines with a pH of over 3.5 to 3.6 are occurring more frequently (Martínez de Toda & Balda, 2014). At these pH levels, it is possible to observe a very fast growth in various indigenous microorganisms, some of which are spoilage bacteria that can cause a loss of wine quality (Krieger-Weber *et al*., 2020). Among these species, *Lb. plantarum* strains have shown the most interesting results for their capacity to induce MLF under conditions of high pH, their facultative heterofermentative properties that avoid acetic acid production from hexose sugars, and their more complex enzymatic profile and different metabolism compared to *O. oeni*, which could play an important role in the modification of wine aromas (Du Toit *et al*., 2011; Bravo-Ferrada *et al*., 2013; Berbegal *et al*., 2016; Iorizzo *et al*., 2016; Krieger-Weber *et al*., 2020).

Valdés la Hens *et al*. (2015) showed that Merlot and Pinot noir wines from the oldest cellar (more than 100 years old) in the North Patagonian region of Argentina, at different stages of spontaneous MLF, contained both LAB species, namely *O. oeni* and *Lb. plantarum*. In particular, the UNQLp 11 strain of *Lb. plantarum* was isolated from a Patagonian Pinot noir wine (vintage 2012), and has demonstrated its ability to survive AF and successfully carry out MLF (Bravo-Ferrada *et al*., 2013, 2014; Brizuela *et al*., 2017, 2018a, 2018b, 2018c, 2019), suggesting its potential to be employed as a starter culture. In addition, this strain can be grown in a low-cost medium and then used as a starter culture without any previous acclimation treatment (Cerdeira *et al*., 2019).

The aim of this work was to obtain the whole assembled genome of the UNQLp 11 *Lb. plantarum* strain so as to analyse its architecture and the possible functions of the predicted genes. The genome sequence of this native Patagonian strain will provide us with useful information about the metabolic capabilities required to successfully carry out the MLF process and its ability to adapt to the stressful wine environment. Furthermore, a comparison with genomes of other *Lb. plantarum* strains, obtained from different sources, will reveal if UNQLp 11 has undergone specific genetic adaptations that allowed it to be a member of the native bacterial microbiome associated with wine grapes of the Patagonian Pinot noir variety, and to survive during the winemaking process, which would exhibit remarkable performance. The control, improvement and innovation of the MLF process demands detailed knowledge of the possible responses of the bacterium during fermentation and processing conditions.

MATERIALS AND METHODS

Strain information

The UNQLp 11 *Lb. plantarum* strain was obtained from a Patagonian Pinot noir wine (vintage 2012), in which AF and MLF were spontaneous. This wine was produced in a commercial cellar located in General Roca, Río Negro Province, Argentina. UNQLp 11 was chosen for its significant technological and oenological characteristics (Brizuela *et al*., 2017, 2018a, 2018b, 2019).

Bacterial growth and DNA extraction

The UNQLp 11 strain was grown in MRS broth at 28°C for two days. To obtain DNA, 1 mg/ml of lysozyme with 1% sodium dodecyl sulphate was used. Proteins were removed with 0.1 g/ml of proteinase K, followed by phenol-chloroform-isoamyl alcohol (25:24:1) extraction. Sixteen µg of high-quality genomic DNA was used for library preparation and sequencing (Iglesias *et al*., 2019).

Genome sequencing, assembly and bioinformatics analysis

A whole-genome shotgun library was constructed using a 20-kb SMRTbell version 1.0 template prep kit, followed by single-molecule real-time (SMRT) sequencing conducted on an RS II (Pacific Biosciences) sequencer (Macrogen). A total of 1 268 593 327 reads (383,24-fold coverage and a polymerase read N_{50} size of 21 044 bp), with an average length of 14 480 bp and an estimated accuracy of 85.5%, were used as input for de novo assembly with the Canu package v1.8 (Koren *et al*., 2017). The Canu output consisted of a single circular contig without gaps. Prediction of the coding sequences was done with Gene MarkS-2. Replication and terminus origins were identified using GC-skew analysis and ORF orientation shift.

For genome annotation, the NCBI Prokaryotic Genome Annotation Pipeline was used (Chaudhari *et al*., 2016). Protein function prediction and KEGG analysis were done by Blast2GO 5.1.1 (Götz *et al*., 2008). The presence/absence of exclusive genes in specific *Lb. plantarum* strains was evaluated by BPGA (Bacterial Pan Genome Analysis Pipeline) (Chaudhari *et al*., 2016). BPGA determines the core (conserved), accessory (dispensable) and unique (strain-specific) gene pool of a species. COG analysis was done by WebMGA (BMC Genomics 2011, 12:444) (Altermann *et al*., 2005).

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RESULTS AND DISCUSSION

General genome features of UNQLp 11 Lb. plantarum strain

The complete genome of *Lb. plantarum* UNQLp 11 strain (Gene Bank Accession Number CP031140) contains a single, circular chromosome of 3 534 932 bp with an average GC content of 44.2%, and no plasmid structures were found. Its main features are shown in Table 1. *In silico* analysis revealed the presence of 3 412 ORFs, 83% of which showed similarities to classified genes from other organisms, and 17% remained unknown. The UNQLp 11 genome also contains 80 transposons, 148 tandem repeats, five ribosomal RNA operons (rRNA), one clustered regularly interspaced short palindromic repeat (CRISPR) locus, and four ncRNAs. CRISPR loci represent a family of DNA repeats typically composed of short and highly conserved sequences (~30 bp), interspaced by variable sequences, often found adjacent to *cas* genes (CRISPR associated), which are involved in defence systems against the invasion of foreign genetic material, in particular phages (Barrangou *et al*., 2007). They were also observed in 40% of the sequenced bacterial genomes, and their presence may increase the genome stability, and therefore the environmental adaptation, of bacteria (Sorek *et al*., 2008).

Fig. 1 shows the chromosome atlas of the UNQLp 11 strain. Replication and terminus origins were identified and it was observed that they were located in almost symmetric chromosome positions. Genes encoded in the UNQLp 11 genome are predominantly transcribed in the replication direction, a feature observed in many Gram-positive genomes with low GC content (Kleerebezem *et al*., 2003).

Fig. 2 shows a comparison between genome size and gene content in 14 *Lb. plantarum* strains from different origins (plasmid genes present in other strains were not evaluated). UNQLp 11 exhibited the largest size and contains 342 genes more than WCFS1 (control), 405 more than XJ25, 341 more than Lp90, 740 more than Zhang-LL, and 282 more than TMW 1.277 (strains of oenological origin). Higher gene count is not necessarily indicative of transcription followed by a translation into functional proteins.

The UNQLp 11 genome contains 79.3% of protein-encoding genes of the COG class that are involved in the major metabolic pathways and could be assigned to 25 functional categories. Annotation of the UNQLp 11 genome sequence revealed the presence of genes mainly corresponded to the categories transcription, replication, recombination and repair, cell wall/membrane/envelope biogenesis, carbohydrate transport and metabolism. The remaining genes were catalogued as unknown functional proteins. Fig. 3 shows the relative abundance of each COG category of the oenological strains and the control, namely UNQLp 11, Zhang-LL, Lp90, XJ25 and WCFS1.

Furthermore, comparative genomics (gene content) analysis of some *Lb. plantarum* strains isolated from different fermented foods is shown as a cladogram (Fig. 4). It is evident that the information contained in the genomes of the strains of the *Lb. plantarum* species of oenological origin cannot determine a relationship between them. It is currently accepted that properties of potential oenological significance are strain-dependent, and the genomic bases have not yet been elucidated (Spano *et al*., 2006; Capozzi *et al*., 2012; Bravo-Ferrada *et al*., 2013). Further studies on the gene regulation mechanisms of *Lb. plantarum* species are required.

In order to evaluate the similarity between the genomes of *Lb. plantarum* strains isolated from fermented beverages, we compared the protein coding genes of UNQLp 11 with WCFS1 (reference strain from human saliva), XJ25 (from red wine), Lp 90 (from red wine), Zhang-LL (from rice wine) and TMW1277 (from palm wine). This analysis revealed that the compared genomes share only 2 115 core genes (data not shown).

The analysis of unique genes revealed that they belonged to the categories transcription, replication, recombination and repair, and transport and metabolism of carbohydrates. These results allowed us to assume that a greater gene number in these mentioned categories could increase the ability of UNQLp 11 to employ different sources of nutrients, and to better regulate the transcription processes. UNQLp 11 contains 325 unique genes (10% of its genome), and the smallest percentage of exclusively absent genes (0.1%). These data show that, among the compared genomes, UNQLp 11 contains the highest number of single proteins coding genes and the lowest number of exclusively absent genes (Fig. 5A). A COG analysis of the 10% of unique genes showed that 25% of them could not be classified in any of the COG categories (Fig. 5B). Furthermore, 13% of the 325 unique genes correspond to phage-related genes.

Among the strain-specific genes found in the UNQLp 11 genome are those coding for ATP-dependent Clp proteases, alpha-glucosidases, ABC transporters, PTS (sugar phosphotransferase system) sugar transporters, and USP (universal stress protein). ATP-dependent Clp proteases are

TABLE 1
Genome characteristics of the UNQLp 11 *Lactobacillus plantarum* strain.

Genome size, bp	3 534 932
Overall G+C content, %	44.2
Number of genes	3 504
Number of coding genes	3 241
Number of CDS (total)	3 412
Number of coding CDS	3 241
Number of rrn operons	92
ncRNAs	4
CRISPR array	1
Pseudogenes (total)	171
Pseudogenes (ambiguous residues)	0 of 171
Pseudogenes (frameshifted)	101 of 171
Pseudogenes (incomplete)	51 of 171
Pseudogenes (internal stop)	38 of 171
Pseudogenes (multiple problems)	16 of 171

involved in several cellular processes, such as degradation of misfolded proteins, regulation of short-lived proteins and housekeeping removal of dysfunctional proteins (Porankiewicz *et al*., 1999). They are also implicated in the control of cell growth and in the stress response in low-GC Gram-positive bacteria (Fiocco *et al*., 2010; Russo *et al*., 2012). Alpha-glucosidases have been suggested to be involved in the degradation of yeast-derived macromolecules as a nutrient source for cell growth (Guilloux-Benatier *et al*., 1993). The expression of the USP protein is associated

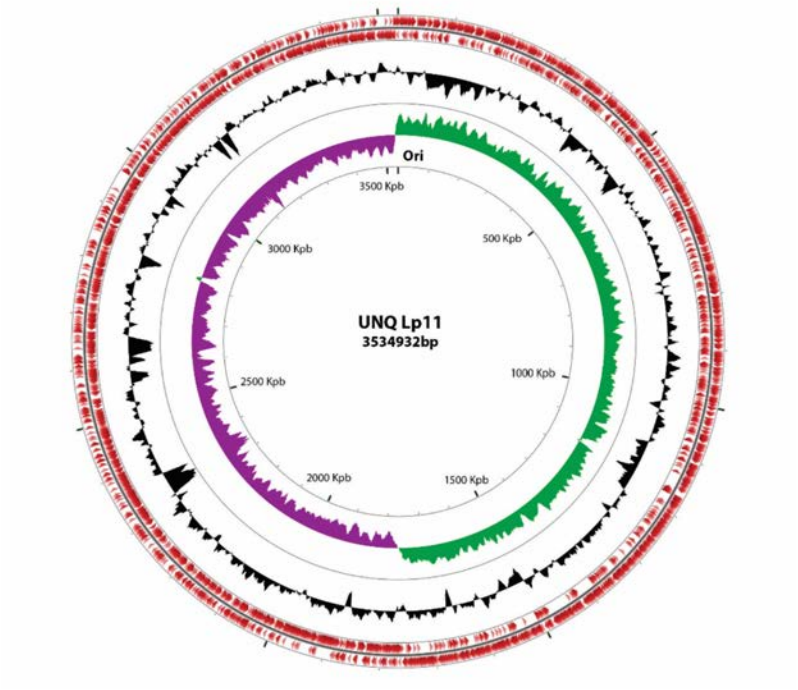

FIGURE 1

Chromosome atlas of *Lb. plantarum* UNQLp 11 strain generated using CGView v1.0 (Grant & Stothard, 2008). The GC content is illustrated in the black circle; positive and negative GC skew in the green and purple circles respectively; and CDS (CoDing Sequence) in the red circle. Genome positions in kbp are also shown in this atlas.

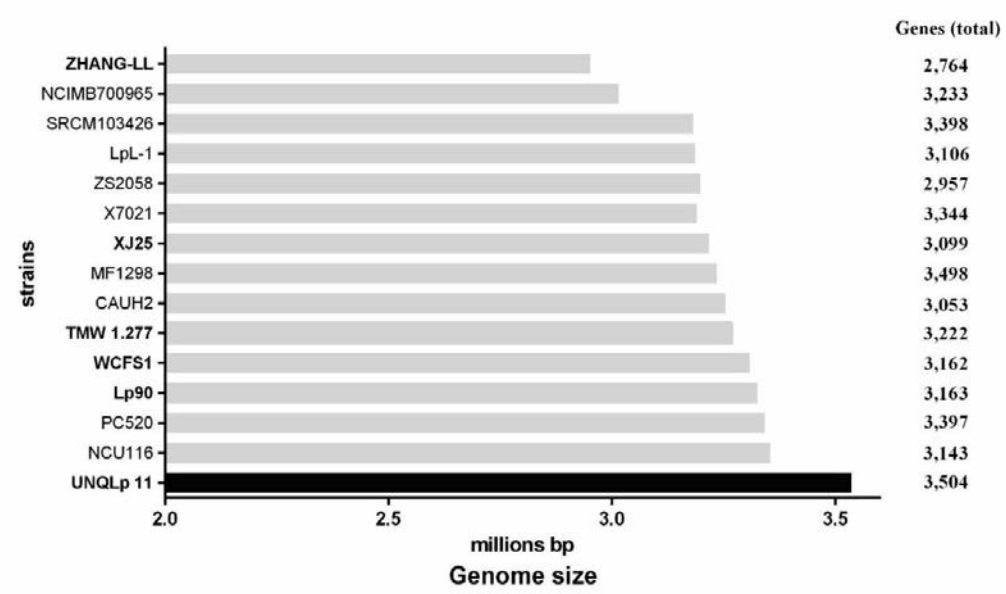

FIGURE 2

Comparison of genome size of 14 *Lb. plantarum* strains from different sources (sequences obtained from NCBI – GeneBank database). The source of each compared strain is: Zhang-LL (rice wine), NCIMB70096 (cheese), SRCM103426 (fermented food), LpL-1 (fermented fish), ZS2058 (sauerkraut), X7021 (tofu brine), XJ25 (red wine), MF1298 (fermented sausage), CAUH2 (vegetable pickles), TMW 1.277 (palm wine), WCFS1 (human saliva), Lp90 (red wine), PCS20 (pickles), NCU116 (pickles), and UNQLp 11 (Patagonian red wine).

with better stress responses by mechanisms still poorly understood (Tkaczuk *et al*., 2013). The PTS and ABC transporters play a central role in the uptake of different sugars, both in *Lb. plantarum* and many other bacteria, and their presence is linked with a better capture of nutrients from the environment. Another strain-specific gene also found in UNQLp 11 was *mutS*, which is involved in the bacterium's repair systems (Garcia-Gonzalez *et al*., 2012).

Finally, we performed a comparative genomic analysis of four complete genomes, namely UNQLp11, Lp90, Zhang-LL and XJ25, and the reference strain WCFS1, which showed that genes found only in the oenological strains belong to the category of transport and metabolism of carbohydrates. An interesting finding, in the UNQLp 11 genome, was the identification of 27 genes belonging to the family of regulatory proteins of the xenobiotic-sensitive element (Xre), 17 more than in the reference strain, WCFS1 (data not shown). Xre is a critical regulator when the bacterium is in stressful environments, e.g. related to acid tolerance in *Lb. acidophilus* (Azcárate-Peril *et al*., 2004) and to ethanol levels in *O. oeni* (Olguín *et al*., 2015).

Genes encoding proteins related to carbohydrate transport and metabolism

The *Lb. plantarum* species, due to its facultatively heterofermentative properties, is heterofermentative for pentoses and homofermentative for hexoses (Dick, 2006). Wine contains many monosaccharides and disaccharides, with glucose, fructose and arabinose being the main sugars metabolised by this LAB species (Hedberg *et al*., 2008). Numerous genes related to carbohydrate transport and metabolism were found in the UNQLp 11 genome, including 67 phosphoenol pyruvate sugar-transferase systems (PTS) and five sugar-ABC transporters. Among the PTS systems, six families of PTS permeases were identified, namely PTS glucose-glucoside (Glc), PTS fructose-mannitol (Fru), PTS lactose-N,N0-diacetylchitobiose-b-glucoside (Lac), PTS glucitol (Gut), PTS galactitol (Gat), and PTS mannose-fructose-sorbose (Man). As expected, the genome analysis of UNQLp 11 revealed the presence of genes corresponding to whole phosphoketolase and glycolysis pathways. This genome contains five transduction sites (locus tags = DVH03_15355, DVH03_16150, DVH03_06425, DVH03_07445, DVH03_11680) for the L-lactate dehydrogenase protein. This observation supports the relevance of pyruvate-dissipating capacity in this strain.

During the catabolism of readily fermentable sugars such as glucose, the synthesis of enzymes involved in the catabolism of other sugars is repressed, a global regulatory phenomenon termed carbon catabolite repression (CCR) (Brückner & Titgemeyer, 2002). The dominant mechanism of global carbon control in low-GC Gram-positive bacteria is one involving HPr, the bifunctional HPr kinase/phosphatase (HPrK), and the catabolite control protein A, CcpA (Henkin *et al*., 1991). CcpA is a member of the LacI-GalR family of bacterial regulator proteins and regulates the transcription of genes at a global level by binding to a *cis*-acting DNA sequence designated a catabolite responsive element (*cre*) (Hueck & Hillen, 1995; Miwa *et al*., 2000). Catabolite control by CcpA involves both transcriptional activation and repression, and the CcpA regulon is commonly scattered throughout the entire bacterial genome, which has been confirmed by the comparative whole-genome transcriptome analyses in the Gram-positive species *Bacillus subtilis*, *Lb. lactis*, and *Lb. plantarum*, and their *ccpA* mutant derivatives (Titgemeyer & Hillen, 2002). Also identified

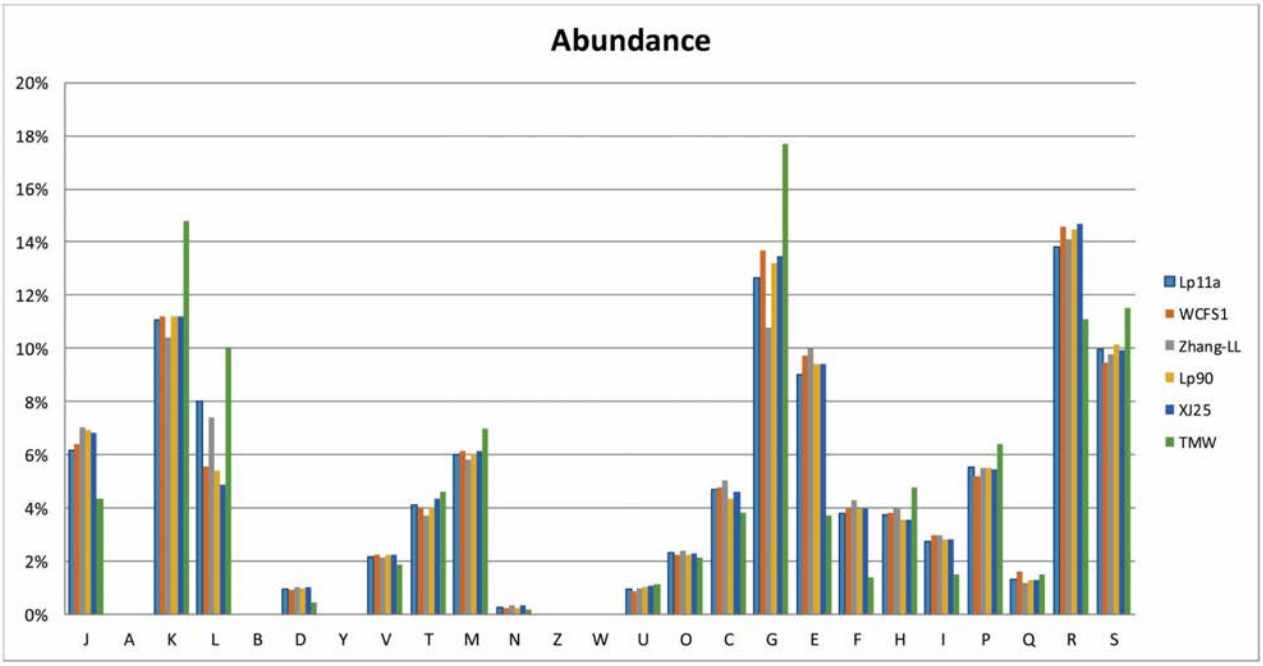

FIGURE 3
Relative abundance of genes associated with general COG functional categories in the UNQLp 11, ZhangLL, Lp90, XJ25 and WCFS1 *Lb. plantarum* strains.

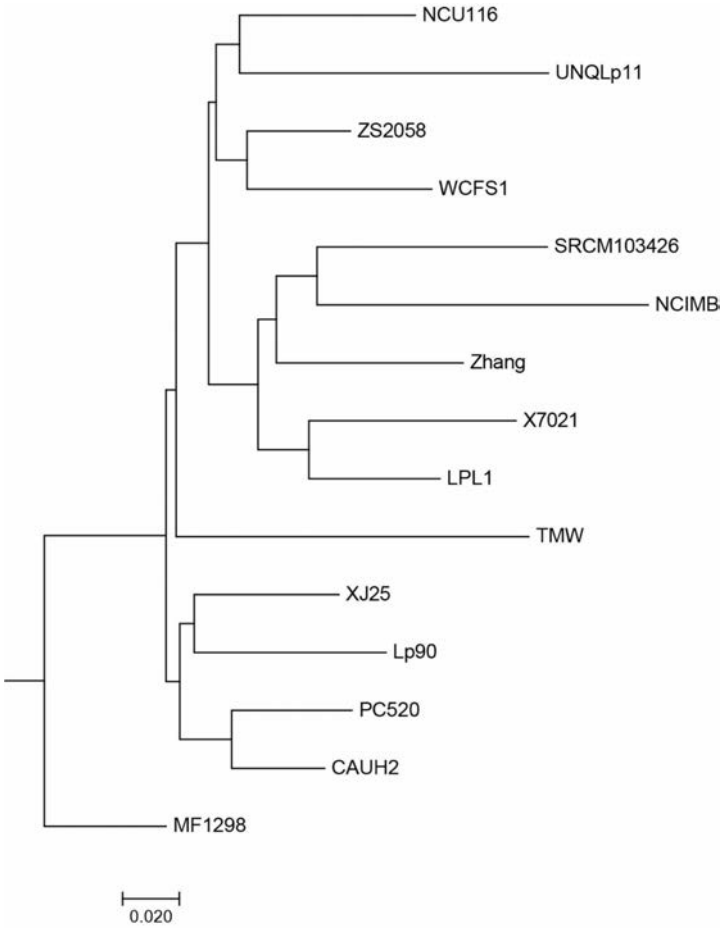

FIGURE 4
Cladogram, obtained by using BGA software, from the gene content analysis of UNQLp 11 (Patagonian red wine), WCFS1 (human saliva), XJ25 (red wine), Lp90 (red wine), Zhang-LL (rice wine), and TMW1277 (palm wine) *Lb. plantarum* strains.

were coding genes for the regulatory proteins HPr, CcpA, and HPrK/P (locus tags = DVH03_11560, DVH03_14365, DVH03_06855).

Genes encoding proteins related to amino acid biosynthesis

LAB generally inhabit protein-rich environments (including milk) and are equipped with protein-degradation machinery to create a selective advantage for growth under these conditions (Kleerebezem *et al*., 2003). *Lb. plantarum* has uptake systems (Opp and Dtp) for peptides and, once internalised, these peptides are degraded by a variety of peptidases, which have been studied extensively in *lactococci* and *lactobacilli* (Ferain *et al*., 1996). Despite this elaborate protein-degradation machinery, the UNQLp 11 genome contains the genes for the whole biosynthetic pathways of 13 amino acids, namely alanine, aspartate, glutamine, lysine, arginine, cysteine, isoleucine, leucine, methionine, serine, threonine, valine and glycine. For the remaining seven amino acids (asparagine, glutamate, histidine, phenylalanine, tryptophan, tyrosine and proline), the biosynthetic pathways were incomplete, according to the KEGG analysis of maps (data not shown).

Genes encoding proteins related to flavour-development enzymes

Lb. plantarum has a diverse array of enzymes that could have positive effects on aromatic and sensorial wine properties (Du Toit *et al*., 2011). Besides the malolactic enzyme itself (locus tags = DVH03_12300, DVH03_12380 in the UNQLp 11 genome), other enzymes involved in flavour development include glycosidases, esterases, phenolic acid decarboxylases and citrate lyases (Matthews *et al*., 2004; Grimaldi *et al*., 2005). Some (polygalacturonase, pectin methyl esterase) are involved in colour improvement in red wines, or reduce problems associated with wine filtration, such as tannase.

An *in silico* analysis was performed to look for genes encoding aminopeptidases, glutamate dehydrogenase and phosphotransacylase, among other enzymes (Table 2). Genes that code for alcohol dehydrogenase (AlcDH) and aldehyde dehydrogenase (AldDH) were identified. These enzymes catalyse the conversion of aldehydes to alcohols (AlcDH) or to carboxylic acids (AldDH), which are involved in the fruit flavour development in red wines (Styger *et al*., 2011). Likewise, the gene coding PAD (phenolic acid decarboxylase) was also found in the UNQLp 11 genome (Table 2, EC: 4.1.1.102). This enzyme participates in the metabolism of phenolic acids from must and wine (Cavin *et al*., 1993; Swiegers *et al*., 2005; Mtshali *et al*., 2010).

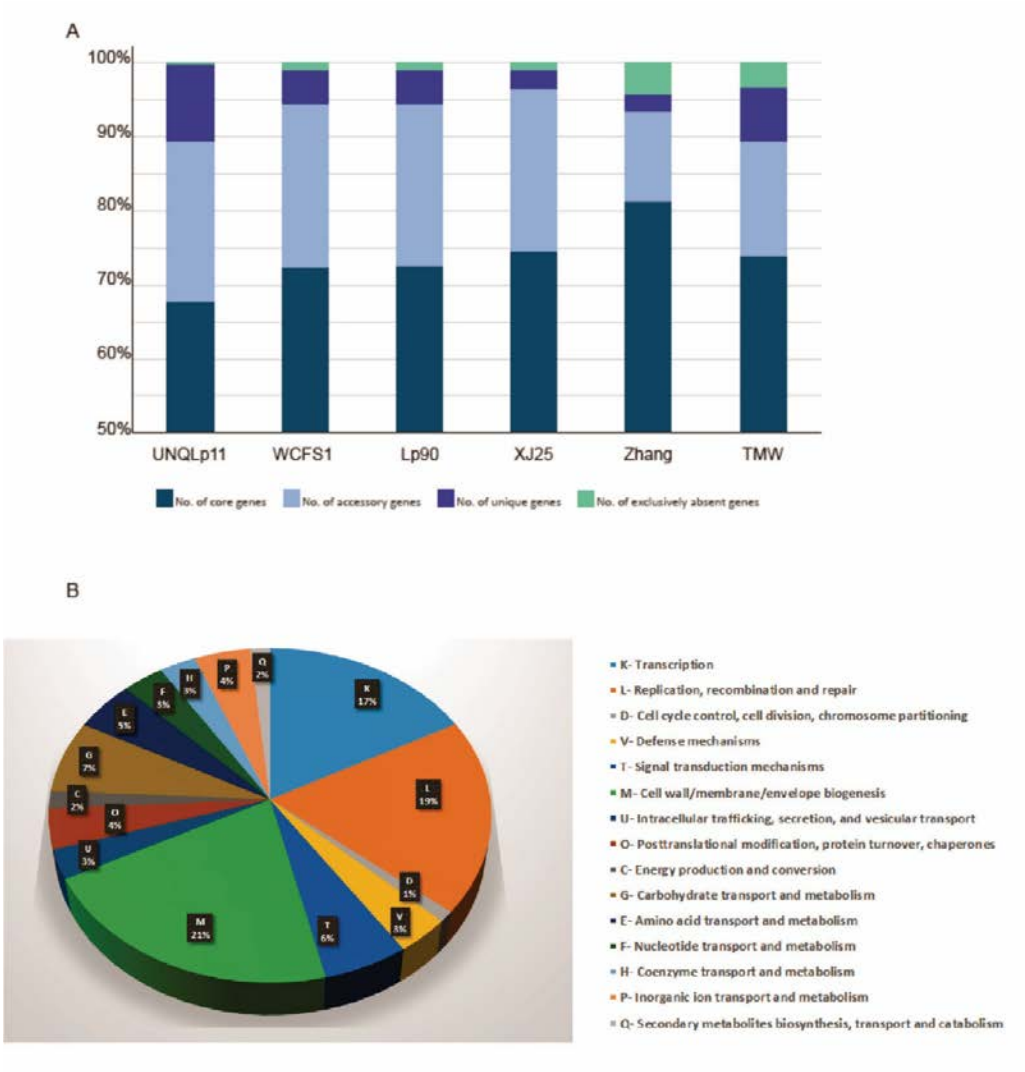

FIGURE 5

A: Ratio comparison of content in core genes (colour), accessory genes (colour), unique genes (colour) and exclusively absent genes (colour) among the *Lb. plantarum* strains UNQLp 11 (red wine), WCSF1 (human saliva), XJ25 (red wine), Lp90 (red wine), Zhang-LL (rice wine) and TMW1277 (palm wine).

B: COGs (clusters of orthologous groups) analysis performed on the 10% of unique genes found in the UNQLp 11 genome.

The beta-glucosidase enzyme catalyses the release of different aroma compounds by glycosidic bound cleavage, transforming terpenes, alcohols, fatty acids, etc. from bound to free forms (Grimaldi *et al*., 2000; Spano *et al*., 2005) (locus tags = DVH03_01055, DVH03_04430, DVH03_04435, DVH03_15725).

Citric acid is present in the wines in concentrations between 0.1 and 1 g/L. The lactic bacteria of the wine cleave it into a molecule of oxalacetate and another of acetate by way of the citrate lyase enzyme. Oxaloacetate is decarboxylated to pyruvic acid, and diacetyl, which is important from an organoleptic point of view because it gives buttery notes to wine, is formed as an intermediate metabolite in the reductive decarboxylation of pyruvic acid to 2,3- butanediol (Bartowsky & Henschke, 2004; Ribéreau-Gayon *et al*., 2006).

A typical citrate lyase gene cluster (*citC*, *citE*, *citF*, *citX*) (locus tags = DVH03_12375, DVH03_12365, DVH03_12360, DVH03_12315) was observed in the UNQLp 11 genome.

The gene coding for tannin acyl hydrolase (tannase) (EC: 3.1.1.20), which catalyses ester bond hydrolysis in hydrolysable tannins, such as tannic acid, releasing glucose and gallic acid (Lekha & Lonsane, 1997), was also found in the UNQLp 11 genome. Microorganisms with tannase activity are currently used in the food industry to remove the tannins responsible for undesirable effects in food processing, such as turbidity in wine or fruit juices (Vaquero *et al*., 2004).

Genes encoding proteins related to stress responses

UNQLp 11 genome analysis allowed the identification of several genes involved in tolerance mechanisms to survive harsh conditions, as previously described in various works (Jobin *et al*., 1999a; 1999b; Beltramo *et al*., 2004; Spano & Massa, 2006; Van Bokhorst-Van de Veen *et al*., 2011). The genes found include *clpX* (locus tag = DVH03_07590), *clpLP* (locus tag = DVH03_14965), and *trxA* (locus tag =

TABLE 2

In silico analysis of the UNQLp 11 genome looking for genes coding for enzymes involved in flavor-forming pathways. A: amino acid degradation; * involved in methionine and cysteine metabolism; G: glucosidases; O: others.

Enzyme (EC)	Name	Pathway	Function	Genes in UNQ Lp11
2.6.1.42	branched-chain aminotransferase	A	Conversion of valine, leucine and isoleucine into ketoacid components. The ketoacids are then further converted into aldehydes, alcohols and esters, which are important aroma compounds (Liu et al., 2008)	1
2.6.1.57	aromatic aminotransferase	A	Conversion of tyrosine, tryptophan and phenylalanine into ketoacid components (Liu et al., 2008)	2
2.6.1.1	aspartate transaminase	A	Conversion of aspartate into ketoacid components	2
1.4.1.2	glutamate dehydrogenase	A	Catalyses the deamination of glutamate to oxoglutaric acid related of the generic amino acid- (branched-chain amino acids, aromatic amino acids and methionine) degradation pathway (Liu et al., 2008)	1
1.1.1.1	alcohol dehydrogenase	A	Catalyses the conversion of aldehydes to alcohols (Liu et al., 2008)	5
1.2.1.10	acetaldehyde dehydrogenase	A	Catalyses the conversion of aldehydes to carboxylic acids (Liu et al., 2008)	2
2.3.1.19	phosphotransbutyrylase	A	Involved in the first step for the conversion of acid keto to esters (Liu et al. 2008).	1
1.1.1.-	D-hydroxyacid dehydrogenase	A	Catalyses the reduction of two keto branched chain acids to hydro acids of interest in flavour formation (Chambellon et al., 2009)	2
3.1.1.1	esterase A	A	Catalyses the biosynthesis of esters derived from short-chain fatty acids (Liu et al., 2008)	1
2.3.1.30	serine acetyltransferase	A*	Is involved in the synthesis of cysteine from L-serine (Liu et al., 2008)	1
4.2.1.22	cystathionine beta-synthase	A*	Lyases that have an O-acetylserinethiol-lyase (cysteine synthase) activity (Liu et al., 2008)	1
2.5.1.48	cystathionine gamma-synthase	A*	Catalyses an α,γ-elimination reaction, converting cystathionine to homocysteine or cysteine (Liu et al., 2008)	1
2.1.1.10	homocysteine S-methyltransferase	A*	Catalyses the methylation of homocysteine in the final stage of the biosynthesis of methionine (Liu et al. 2008)	1
2.1.1.14	homocysteine methylase	A*	Involved in the final stage of homocysteine methylation for methionine synthesis (Liu et al., 2008)	1
1.1.1.3	homoserine dehydrogenase	A*	Has homoserine trans-acetylase activity that is involved in the biosynthesis of methionine (Liu et al., 2008)	2
2.7.1.39	homoserine kinase	A*	Involved in the onset of methionine biosynthesis (Liu et al., 2008)	1
2.5.1.49	O-acetylhomoserine sulphydrolase	A*	Has homoserine trans-acetylase activity involved in the biosynthesis of methionine (Liu et al., 2008)	1
3.2.1.86	6-phospho-beta-glucosidase	G	Hydrolytic activity in glycosylated compounds, acts on the glucosidic bonds β (1-4) (Grimaldi et al., 2005)	4
3.2.1.40	alpha-L-rhamnosidase	G	Hydrolytic activity on terminal non-reducing alpha-L-rhamnose residues in alpha-L-rhamnosides (Grimaldi et al., 2005)	2
3.2.1.20	alpha-glucosidase	G	Hydrolytic activity on terminal, non-reducing (1 -> 4)-linked alpha-D-glucose residues with release of D-glucose (Grimaldi et al., 2005)	7
4.1.1.102	PAD	O	Catalyses the decarboxylation of phenylacrylic acids present in plant cell walls (Mtshali et al., 2010)	1
3.1.1.20	tannase	O	Catalyses the decarboxylation of phenacrylic acids present in plant cell walls (Rodriguez et al., 2008)	1
4.1.1.101	malolactic enzyme	O	Involved in the malolactic fermentation of wine, which results in a natural decrease in acidity and favourable changes in wine flavours	2

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TABLE 2 (CONTINUED)

Enzyme (EC)	Name	Pathway	Function	Genes in UNQ Lp11
2.6.1.42	branched-chain aminotransferase	A	Conversion of valine, leucine and isoleucine into keto acid components. The keto acids are then further converted into aldehydes, alcohols, and esters, which are important aroma compounds (Liu et al 2008).	1
2.6.1.57	aromatic aminotransferase	A	Conversion of tyrosine, tryptophan, and phenylalanine into keto acid components (Liu et al 2008).	2
2.6.1.1	aspartate transaminase	A	Conversion of aspartate into keto acid components.	2
1.4.1.2	glutamate dehydrogenase	A	Catalyzes the deamination of glutamate to oxoglutaric acid related of generic amino acid (branched-chain amino acids, aromatic amino acids, and methionine) degradation pathway (Liu et al 2008).	1
1.1.1.1	alcohol dehydrogenase	A	Catalyze the conversion of aldehydes to alcohols (Liu et al 2008).	5
1.2.1.10	acetaldehyde dehydrogenase	A	catalyze the conversion of aldehydes to carboxylic acids (Liu et al 2008).	2
2.3.1.19	phosphotransbutyrylase	A	It is involved in the first step for the conversion of acid keto to esters (Liu et al 2008).	1
1.1.1.-	D-hydroxyacid dehydrogenase	A	Catalyzes the reduction of two keto branched chain acids to hydro acids of interest in flavor formation (Chambellon et al 2009)	2
3.1.1.1	esterase A	A	Catalyze the biosynthesis of esters derived from short-chain fatty acids (Liu et al 2008).	1
2.3.1.30	serine acetyltransferase	A*	It is involved in the synthesis of cysteine from L-serine (Liu et al 2008).	1
4.2.1.22	cystathionine beta-synthase	A*	Lyase that have an O-acetylserinethiol-lyase (cysteine synthase) activity (Liu et al 2008).	1
2.5.1.48	cystathionine gamma-synthase	A*	catalyze an an α,γ-elimination reaction, converting cystathionine to homocysteine or cysteine (Liu et al 2008).	1
2.1.1.10	homocysteine S-methyltransferase	A*	It catalyzes the methylation of homocysteine in the final stage of the biosynthesis of methionine (Liu et al 2008).	1
2.1.1.14	homocysteine methylase	A*	It is involved in the final stage of homocysteine methylation for methionine synthesis (Liu et al 2008).	1
1.1.1.3	homoserine dehydrogenase	A*	It has homoserine trans-acetylase activity involved in the biosynthesis of methionine (Liu et al 2008).	2
2.7.1.39	homoserine kinase	A*	Involved in the onset of methionine biosynthesis (Liu et al 2008).	1
2.5.1.49	O-acetylhomoserine sulfhydrolase	A*	It has homoserine trans-acetylase activity involved in the biosynthesis of methionine (Liu et al 2008).	1
3.2.1.86	6-phospho-beta-glucosidase	G	Hydrolytic activity in glycosylated compounds, act on the glucosidic bonds β (1-4) (Grimaldi et al 2005).	4
3.2.1.40	alpha-L-rhamnosidase	G	Hydrolytic activity on terminal non-reducing alpha-L-rhamnose residues in alpha-L-rhamnosides (Grimaldi et al 2005).	2
3.2.1.20	alpha-glucosidase	G	Hydrolytic activity on terminal, non-reducing (1->4)-linked alpha-D-glucose residues with release of D-glucose (Grimaldi et al 2005).	7
4.1.1.102	PAD	O	Catalyzes the decarboxylation of phenacrylic acids present in plant cell walls (Mtshali et al 2010)	1
3.1.1.20	tannase	O	Catalyses the decarboxylation of phenacrylic acids present in plant cell walls (Rodriguez et al 2008)	1
4.1.1.101	malolactic enzyme	O	It is involved in the malolactic fermentation of wine, which results in a natural decrease in acidity and favorable changes in wine flavors.	2

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DVH03_14965). In addition, class I heat shock genes (*groES*, *groEL* and *dnaK/dnaJ* operons) (locus tags = DVH03_14475, DVH03_14470, DVH03_08265, DVH03_08260), involved in the disulphide-reducing pathway (*trxA* and *trxB* homologs) (locus tags = DVH03_06790, DVH03_14250), as well as genes codifying proteins implicated in the ethyl stress response (HrcA and CtsR) (locus tags = DVH03_08250, DVH03_12730), were also identified.

Bacteria protect themselves from changes in environmental osmolarity by using stretch-activated (or mechano-sensitive) channels that respond to changes in membrane tension when cells expand. In the UNQLp 11 genome, genes encoding two-channel protein families (Voltage-gated ion channel (VIC) (locus tag = DVH03_06830) and large conductance mechano-sensitive channel (MscL) (locus tag = DVH03_11240) were found, as well as some aquaporins (locus tag = DVH03_01360) of the MIP (membrane intrinsic proteins) family, which may function primarily to protect the bacteria against osmotic stress (Biggin & Sansom, 2003). In the work of Bienert *et al*. (2013), it is observed that the number of genes that encode MIP in *Lb. plantarum* is the largest of those reported in other BAL species, and is suggested to have potential flexibility for different substrates.

Genes encoding proteins related to exopolysaccharide production

Exopolysaccharides (EPSs) are high-molecular-weight carbohydrate polymers secreted extracellularly by many microorganisms (Zhou *et al*., 2019). EPS are thought to protect bacterial cells against extreme environmental conditions, such as biotic and/or abiotic stresses, including temperature, light intensity, pH and osmotic stress (Donot *et al*., 2012). The UNQLp 11 genome contains genes that are associated with surface polysaccharide production (locus tags = DVH03_04750, DVH03_09260, DVH03_15400). It should be noted that EPSs have been reported to be responsible for the high viscosity of musts. Besides, after alcoholic fermentation, EPSs form aggregates that could block the filter system, delay spontaneous clarification by sedimentation and worsen wine filterability (Dimopoulou *et al*., 2017). The amount and type of EPS depends on fermentation conditions, *Lactobacillus* strain and the growth stage of the microorganism (De Vuyst & Degeest, 1999; Zannini *et al*., 2016). EPSs can be degraded by other organisms in a complex microbial consortium of wine (Salazar *et al*., 2009). For these reasons, EPS production of UNQLp 11 in wine requires more studies.

Genes encoding proteins related to bacteriocins

Bacteriocins are ribosomally synthesised antimicrobial peptides or proteins, are ubiquitous in the microbial world and are produced by both Gram-positive and Gram-negative bacteria, have cationic properties and kill target cells by destabilising the integrity of the inner membrane envelope, causing disruption of the membrane potential and/or leakage of cellular solutes that eventually leads to cell death (Diep *et al*., 2009). Different *Lb. plantarum* strains (NC8, WCFS1, J23 and J51) have been found to harbour mosaic *pln* loci in their genomes (Diep *et al*., 2009). In the UNQLp 11 genome, we have identified a region containing some bacteriocin putative genes (plnJ, plnF and plnE, locus tags = DVH03_15860, DVH03_15815, DVH03_15810 respectively) implicated in the synthesis of the EF and JK plantaricins, and plantamicin A. The *pln* locus is widespread among *Lb. plantarum* strains of oenological origin and shows genetic diversity and plasticity (Sáenz *et al*., 2009). The production of these bacteriocins could generate a competitive advantage over other microorganisms, thus favouring successful implantation in the wine environment.

Genes encoding proteins related to undesirable metabolites

Arginine is the major amino acid present in wine, with grape juice concentrations ranging from a few hundred mg l^{-1} to approximately 2.5 g l^{-1} (Liu *et al*., 1995). One of the major concerns about arginine metabolism by wine LAB is the synthesis of ethyl carbamate (EC) precursors (Arena & De Nadra, 2005). Ethyl carbamate, also referred to urethane, is a known animal carcinogen found in fermented foods and beverages, including wine (Araque *et al*., 2011). The *in silico* analysis showed that the *Lb. plantarum* UNQLp 11 genome does not contain enzymes coding genes involved in EC synthesis.

Also, the synthesis of biogenic amines (BA) by LAB during winemaking processes, should be avoided, because these compounds affect wine quality and acceptability (Lonvaud-Funel, 1999; Moreno-Arribas *et al*., 2003), and they can also induce a variety of diseases when they are consumed (and absorbed) in high concentrations. Although the worldwide regulation is not uniform, wines containing high amounts of histamine are rejected in certain markets due to recommendations for or suggested limits of this compound (Smit *et al*., 2008). In our analysis, it was possible to verify that the UNQLp 11 genome does not contain genes encoding the enzymes histidine decarboxylase (EC: 4.1.1.17), putrescine carbamoyltransferase (EC: 2.1.3.6), and tyrosine decarboxylase (EC: 4.1.1.25), which are responsible for the synthesis of the main BA found in wines (histamine, tyrosine, putrescine).

CONCLUSIONS

The analysis of the complete genome of the UNQLp 11 *Lb. plantarum* oenological strain revealed the presence of genes involved in the transport and catabolism of different sugars, the biosynthesis of 13 amino acids, the synthesis of wine aroma compounds, and the production of exopolysaccharides and bacteriocins. When unique genes were analysed, it was observed that they belonged to the categories transcription, replication, recombination and repair, and carbohydrate transport and metabolism. These findings allow us to speculate on their adaptation to different environments, as they are able to use diverse substrates for their growth.

The genome comparison of UNQLp 11 with fourteen other *Lb. plantarum* strains from fermented foods and beverages did not allow us to observe any genotypic relationship among the strains isolated from wine, revealing that the compared genomes shared only 2 115 core genes.

Although gene presence does not guarantee its

expression during winemaking, the genome sequencing and analysis of the native UNQLp 11 strain allowed us to associate phenotypes expressed by this strain under different winemaking conditions (Bravo-Ferrada *et al*., 2013, 2014; Brizuela *et al*., 2017, 2018a, 2018b, 2018c) and will improve our understanding of its performance in future winemaking assays on pilot scale.

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Colour Evaluation of Pinot Noir and Merlot Wines after Malolactic Fermentation Carried out by *Oenococcus oeni* and *Lactobacillus plantarum* Patagonian Native Strains

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Malolactic fermentation is a complex process that involves many reactions aside from the decarboxylation of L-malic acid. But we still have only glimpses of that complexity. It is not clear if the phenolic composition and colour are affected by malolactic fermentation and, if so, to what extent. So, the aims of this study were: 1) to evaluate the behaviour of native Patagonian strains of *Oenococcus oeni* and *Lactobacillus plantarum* in two wine varieties, and 2) to analyse the effect of malolactic fermentation on the colour of these wines. Our results show that the survival of bacteria and L-malic acid decarboxylation is different depending on the lactic acid bacteria strain employed and the wine variety. In addition, we found that *O. oeni* can survive in wine even when L-malic acid is not being consumed. We found some correlations between MLF and colour-related parameters in Pinot noir but not for Merlot. In fact, some of the colour-related parameters measured in Merlot (total polyphenolic index, colour intensity, hue, as well as the CIELAB parameters) were affected even when L-malic acid was not being consumed.

INTRODUCTION

Malolactic fermentation (MLF), which is carried out by lactic acid bacteria (LAB), is desired in most red and some white and sparkling base wines (Bartowsky, 2017). The first three advantages given by this process are widely known: i) it deacidifies wine due to the decarboxylation of L-malic acid into the softer L-lactic acid; ii) it improves wine aroma by the production of secondary metabolites; and iii) it improves microbiological stability due to the consumption of the remaining carbon and energy sources.

During the last few decades, MLF has been proven to be a much more complex process due to the complexity and variability of LAB metabolism (Vivas *et al*., 1994; Bartowsky, 2017). MLF modifies organoleptic parameters and affects the aroma profile (Brizuela *et al*., 2017, 2018) and colour parameters (Hernández *et al*., 2007; Massera *et al*., 2009; Abrahamse & Bartowsky, 2012; Burns & Osborne, 2013; Izquierdo-Cañas *et al*., 2016). Besides colour parameters, we need to consider the astringency and bitterness, as all these wine attributes are related to the grape phenolic composition. While colour is conferred by molecules called anthocyanins, astringency and bitterness are related to polyphenolic compounds known in general as tannins and, more specifically, condensed tannins or proanthocyanidins (Cheynier *et al*., 2006; Garrido & Borges, 2013; Nel, 2018). Information about MLF affecting wine phenolic composition and colour is still scarce. In Argentina, there is only one study confirming this relationship using wines from the Malbec grape variety (Paladino *et al*., 2001). The change in colour parameters is complex and depends on grape variety, *terroir*, viticulture and oenological practices (including the alcoholic fermentation and yeast strain), as well as LAB species and strain (Versari *et al*., 2008; Dobrei *et al*., 2010; Mangani *et al*., 2011). *Oenococcus oeni* is the first species to be recognised as responsible for MLF, since it is the best adapted to the wine conditions and the main bacterium responsible for MLF in many countries and wineries (Lonvaud-Funel, 2015; Lorentzen & Lucas, 2019; Sumby *et al*., 2019). *Lactobacillus plantarum* is starting to

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be used as a starter culture, since it has been proven to be able to carry out MLF as well as *O. oeni* (Du Toit *et al*., 2011; Berbegal *et al*., 2016; Brizuela *et al*., 2019; Krieger-Weber *et al*., 2020). For instance, *Lb. plantarum* is available commercially, as well as in the co-inoculant bacterial starter with both the *O. oeni* and *Lb. plantarum* strains.

As more information is obtained thanks to wine research and new techniques are being applied in this field, more questions arise. There is still little or no information about *O. oeni* and *Lb. plantarum* modifying wine colour and astringency sensations. And even when most winemakers recognise the importance of carrying out MLF, they are still reluctant to perform a deep analysis of their wines in relation to this subject. Due to the need to know more about the interaction of LAB, MLF, grape variety, wine colour and astringency, we decided to contribute to this paradigm. The aim of this research was to evaluate the MLF performance of two Patagonian native strains, one of *O. oeni* and one of *Lb. plantarum,* plus two additional strains (*O. oeni* ATCC 27310 and *Lb. plantarum* ATCC 14917), and to relate their performance to the colour of the wine and the phenolic content using the Pinot noir and Merlot grape varieties.

MATERIALS AND METHODS

Microvinifications and experimental design

Microvinifications were carried out on a laboratory scale using 2 kg of must from two grape varieties, Pinot noir and Merlot, harvested from vineyards located in General Roca, North Patagonia, Argentina in 2018. The must was fermented in one container for each variety by inoculation with the commercial yeast strain, *Saccharomyces cerevisiae bayanus*, Lalvin QA23 (Lallemand BIO S.L.), according to the manufacturer's instructions. The alcoholic fermentation (AF) was monitored every two days by measuring the temperature and density of the must, and a pigeage was performed each time to favour phenolic compounds dissolution. The temperature of the fermenting must was stable, at around 21ºC, and the process took 14 days for Pinot noir and 21 days for Merlot. At the end of AF, the ethanol concentration was 13% (v/v) in the Pinot noir and 14.5% (v/v) in the Merlot. At this point, both wines contained approximately 13 mg/L free sulphur dioxide. After AF, the wines were separated from the skins and then sterilised by filtration through a 0.2 µm pore size filter (Sartorius Stedim Biotech Gmbh, Göttingen, Germany). Then, 50 mL of each wine was poured into each of ten sterile glass flasks before inoculation with lactic acid bacteria.

Growth conditions and MLF

The two *O. oeni* strains (ATCC 27310 (OeATCC 27310) and UNQOe19 (KY561603, CP027431)) and the two *Lb. plantarum* strains (ATCC 14917 (LpATCC 14917) and UNQLp11 (CP031140)) were cultured at 28°C in tubes containing MRS broth medium supplemented with L-malic acid (4 g/L) and fructose (5 g/L) at pH 5.0. After 48 h, cells were harvested by centrifugation and resuspended in the acclimation medium (50 g/L MRS, 40 g/L fructose, 20 g/L glucose, 4.5 g/L L-malate, 1 g/L Tween 80, 0.1 mg/L pyridoxine, pH 4.6) containing 6% (v/v) ethanol (Bravo-Ferrada *et al*., 2014). The acclimation tubes were cultured at 21°C for 48 h, after which cells (approximately 10^8 CFU/mL) were harvested by centrifugation and inoculated into the sterile wines (prepared as described above). From these 10 flasks, two were left without inoculation, as they were the control condition. The other eight flasks were inoculated with each of the four strains by duplicate and then kept at an incubation temperature of 21°C. Cell survival was analysed every two days at the beginning of MLF, and then every five days until approximately the 35th day, by counting colonies on plates of MRS medium supplemented as described above. L-malic acid consumption was measured using the L-malic Acid Enology Enzymatic kit (BioSystems SA, Barcelona, Spain) according to the manufacturer's instructions.

Wine chemical analysis

Both wines were analysed for pH, ethanol and free sulphur dioxide concentrations according to the methods recommended by the OIV (2009). The total polyphenol index (TPI) was determined following the method described by Ribéreau-Gayon *et al*. (2006). Briefly, wines samples were diluted to 1:100 and then absorbance was measured at 280 nm in a cuvette of 1 cm optical path. The results were multiplied by the dilution factor. Total tannins were determined by absorbance measurement at 550 nm, after acid hydrolysis of the samples as described by Elorduy Vidal (2014). Colour intensity (IC) was estimated by summing the absorbance values at 420, 520 and 620 nm (Glories, 1984), and the hue was determined as the ratio between the values obtained at 420 and 520 nm (A420/520). The CIELAB parameters, lightness (*L**), chroma (*C**), hue (*H**), redness (*a**) and yellowness (*b**), were determined according to Ayala *et al*. (1997). The total anthocyanin (TA) content was determined as described by Durán and Trujillo (2008), using a sulphur dioxide bleaching protocol with some modifications according to Pandeya *et al*. (2018). Two tubes were used for each wine sample, both containing 100 µL of wine, 100 µL of HCl in ethanol (0.1%), and 200 µL aqueous HCl (20%). A total of 440 µL of water was then added to tube A and 440 µL of potassium bisulphite (26%) solution was added to tube B. Both mixtures were diluted 1:1 and absorbance was measured at 520 nm after 20 min, against a blank (500 µL HCl in ethanol (0.1 %), and 1 mL aqueous HCl (20%) and 2.7 mL water). The TA content was then quantified using the formula TA (mg/L) = 875 (ΔA_{520}).

RESULTS

Cell survival and MLF performance in wine

The four LAB strains behaved differently depending on the wine (Fig. 1). *O. oeni* survived and maintained its viability in Pinot noir wine (Fig. 1a). A difference in the rate at which L-malic acid was converted by *O. oeni* ATCC 27310 (OeATCC 27310) and UNQOe19 was evident, with the former being more active (Fig. 1c). Both *Lb. plantarum* strains, ATCC 14917 (LpATCC 14917) and UNQLp11, presented similar behaviour in terms of population survival and L-malic acid consumption (Fig. 1a and 1c).

In Merlot wine, both *O. oeni* strains slowly decreased their viability (Fig. 1b), with OeATCC 27310 being the one that was able to keep its viability stable at $1x10^4$ CFU/mL until the end of analysis, despite no L-malic acid

consumption being detected by either *O. oeni* strain (Fig. 1d). *Lb. plantarum* UNQLp11 maintained its viability for at least ten days (Fig. 1b) and then decreased abruptly, but it was the only strain able to consume almost all L-malic acid (Fig. 1d). LpATCC 14917 decreased its population as soon as it was inoculated into wine and, accordingly, no L-malic acid consumption was registered.

Colour parameters in Pinot noir wine with and without MLF

Table 1 shows the different parameters that were measured in the Pinot noir wine with and without MLF, inoculated, or not inoculated (no-MLF). The no-MLF condition (control) corresponds to the wine sample filtrated after alcoholic fermentation but not inoculated. The pH was higher only in the wine samples in which MLF fermentation was successful, i.e. samples inoculated with *O. oeni* strains. Tannins (TAN) were significantly reduced in the wine inoculated with LpATCC 14917, whereas colour intensity (CI) was significantly increased in the wine inoculated with strain UNQOe19 compared to all other conditions. The hue values, calculated as the ratio of the absorbances obtained at 420 and 520 nm (A420/520), were significantly higher in wines inoculated with the *Oenococcus* strains and with the LpATCC 14917 strain. Also, the values of redness (a*) were significantly lower than in the control condition, and in the conditions where MLF was not successful (wines inoculated with *Lactobacillus* strains). Although not statistically significant, our results show a decrease in chroma (*C**) and an increase in hue (*H**) in the samples in which MLF was successful (samples inoculated with *Oenococcus*) in comparison with all other conditions. Finally, we found that colorimetric differences (*ΔE**) between the inoculated samples and the control condition (no-MLF) were higher than 2.7 CIELAB units and that these differences were higher in wines in which MLF was successful.

Colour parameters in Merlot wine with and without MLF

Table 2 shows the different parameters that were measured in Merlot wine with and without MLF, inoculated or not inoculated (no-MLF). The no-MLF condition (control) corresponds to the wine sample filtrated after alcoholic fermentation but not inoculated. In this grape variety, only UNQLp11 was able to complete MLF, which is reflected in the higher modification in pH even when there is also a significant difference in the pH value for samples in which MLF was unsuccessful (UNQOe19 and LpATCC 14917). A significant increase in the total phenolic index (TPI) was observed in the wine inoculated with strain LpATCC 14917, whereas a slight though not statistically significant increase was detected in wines inoculated with *Oenococcus* strains. Only the wine sample with a successful MLF (wine inoculated with UNQLp11) maintained a similar TPI value to the control condition (no-MLF). The colour intensity (CI) was significantly increased in samples inoculated with *Lactobacillus* strains and with OeATCC 27310. The hue values, calculated as the ratio of the absorbances obtained at 420 and 520 nm (A420/520), were significantly higher in wines inoculated with *Lactobacillus*, although a slight increase was also found in wines inoculated with *Oenococcus*, in comparison with the control condition.

Almost all CIELAB parameters presented a significantly different value to that of the control condition (no-MLF). The lightness (*L**) was reduced in all the inoculated wines, but

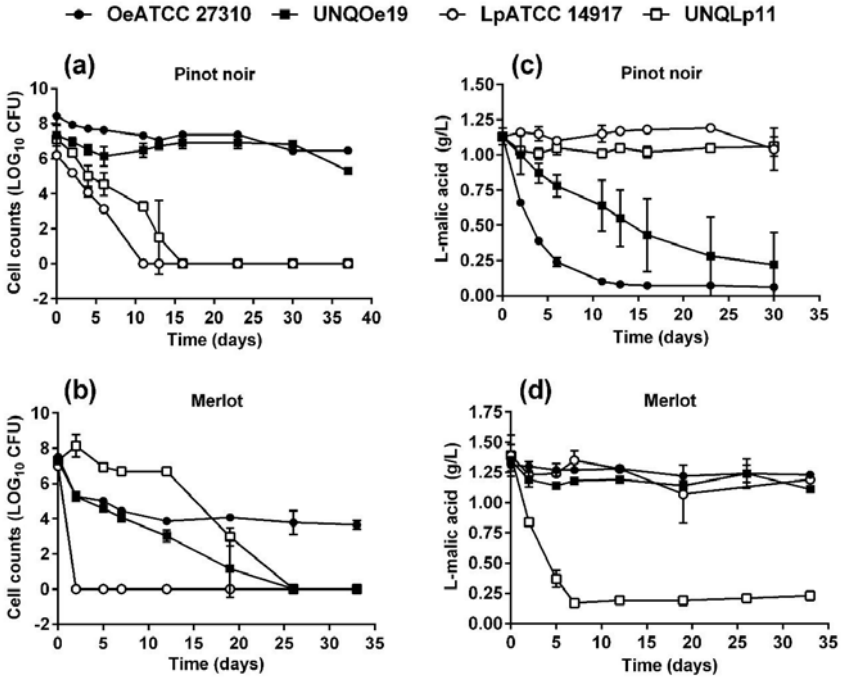

FIGURE 1
Survival and MLF performance of *O. oeni* and *Lb. plantarum* strains in Pinot noir and Merlot wines.

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TABLE 1
Colour parameters of Pinot noir wine samples inoculated with different LAB strains.

	Pinot noir				
	no-MLF	**OeATCC 27310**	**UNQOe19**	**LpATCC 14917**	**UNQLp11**
pH	3.61 ± 0.01	3.82 ± 0.01*	3.79 ± 0.05*	3.59 ± 0.04	3.62 ± 0.01
[1]TPI	32.1 ± 0.55	33.97 ± 0.02	33.06 ± 0.28	32.79 ± 0.1	33.62 ± 1.46
[2]TAN	0.841 ± 0.05	0.812 ± 0.02	0,791 ± 0.01	0.756* ± 0.01	0.798 ± 0.01
[3]TA	67.3 ± 7.67	79.3 ± 3.71	74.9 ± 4.46	61.6 ± 6.69	68.5 ± 3.83
[4]CI	5.06 ± 0.35	5.41 ± 0.17	5.86 ± 0.52*	4.83 ± 0.04	5.04 ± 0.04
[5]A420/520	1.023 ± 0.07	1.184 ± 0.04**	1.147 ± 0.04**	0.944 ± 0.01*	0.97 ± 0.01
L*	65.5 ± 0.99	61.65 ± 5.3	63.25 ± 1.91	68.15 ± 1.34	64.75 ± 0.64
C*	114.25 ± 2.47	107.9 ± 4.81	108.6 ± 1.13	117.55 ± 0.21	115.2 ± 0.14
H*	42.6 ± 0.54	47.17 ± 0.17	45.7 ± 0.98	42.05 ± 0.81	42.92 ± 0.12
a*	84.11 ± 2.52	73.36 ± 3.48*	75.85 ± 0.54*	87.29 ± 1.24	84.37 ± 0.25
b*	77.31 ± 0.85	79.13 ± 3.29	77.73 ± 2.1	78.71 ± 1.12	78.45 ± 0.11
ΔE*		12.03 ± 1.87	8.70 ± 2.73	4.65 ± 3.82	2.71 ± 0.13

[1]TPI, total polyphenolic index; [2]TAN, tannins are expressed in g/L; [3]TA, total anthocyanins are expressed in mg/L; [4]CI, colour intensity; [5]A420/520, hue calculated as the ratio of the absorbances obtained at 420 and 520 nm. The statistical analysis was performed against the control (no-MLF) condition. Asterisks indicate that means differed significantly at * $p < 0.05$, ** 0.01 (or less) from the control condition.

it was lower in wines inoculated with *Lactobacillus* strains, and even lower in the one inoculated with UNQLp11. No change was observed in the chroma (*C**), hue (*H**), redness (*a**) and yellow-blue component (*b**) presented the lower values. The only CIELAB parameter that showed a different trend was the colorimetric difference (*ΔE**). All samples presented higher values than 2.7 CIELAB units, but there were significant differences among them. The *ΔE** values in wines inoculated with OeATCC 27310 and UNQOe19 were statistically equal, whereas the *ΔE** value in the wine inoculated with UNQLp11 was statistically different and higher.

Comparison of changes in colour parameters between wine varieties

Table 3 shows the general increase or decrease in the different colour parameters in wines that were inoculated with the different LAB strains and in which consumption of L-malic acid occurred (MLF+), or where no L-malic acid was consumed (MLF-), based on the results from Table 1 and Table 2. The increase or diminution of values is indicated by arrows (double arrows when values are statistically different) with reference to the control condition (no-MLF), not included in this table. It is difficult to generalise, but it seems that the occurrence of MLF has different effects on some of the colour parameters, depending on the wine variety. For instance, the total anthocyanin (TA) content increased when MLF was successful, but only in Merlot, as it was not clear in Pinot noir. On the other hand, the hue (A420/520) increased with successful MLF in Pinot noir, but not in Merlot. The chroma (*C**) diminished after MLF, whereas it increased when MLF was not successful in Pinot noir. In Merlot, the chroma (*C**) diminished in all samples, as also did lightness (*L**), hue (*H**), redness (*a**) and yellowness (*b**).

DISCUSSION

We evaluated the MLF performance of four strains, two of *O. oeni* and two of *Lb. plantarum*, and found out that they behave differently depending on the wine variety, and thus the wine chemistry. *O. oeni* consumed L-malic acid in Pinot noir but not in Merlot, and one strain of *Lb. plantarum* consumed L-malic acid in Merlot but not in Pinot noir. We also found that *O. oeni* can survive in wine even when L-malic acid is not being consumed.

Our second goal was to find out if there was a relationship between the MLF conducted by each strain and the wine colour, but we were unable to do so due to the uneven results in viability and L-malic acid consumption. However, our results show that some colour parameters can change even when MLF is not successful.

The strains used in this work were selected according to previous studies (Brizuela *et al*., 2017; Olguin *et al*., 2019), except for LpATCC 14917. The latter strain was selected for comparison purposes and it was the first time it was used in our studies, so its performance was new to us. It was surprising to discover that OeATCC 27310 was able to maintain a higher viability than the selected native UNQOe19 strain in Merlot wine, even when none of the employed *O. oeni* strains consumed L-malic acid in this wine variety. Renouf *et al*. (2007) found viable LAB –most of them *O. oeni* – in aged Bordeaux wines. These results again highlight the ability of *O. oeni* to survive under stressful conditions (Lonvaud-Funel, 2015; Sumby *et al*., 2019). It might be useful to search for additional acclimation

TABLE 2
Colour parameters of Merlot wine samples inoculated with different LAB strains.

	Merlot				
	no-MLF	**OeATCC 27310**	**UNQOe19**	**LpATCC 14917**	**UNQLp11**
pH	3.53 ± 0.01	3.55 ± 0.0	3.6 ± 0.01**	3.58 ± 0.01*	3.67 ± 0.0**
[1]TPI	46.32 ± 0.18	47.34 ± 0.01	47.56 ± 0.47	48.74 ± 0.7*	46.72 ± 0.42
[2]TAN	1 140 ± 0.02	1 161 ± 0.04	1 192 ± 0.04	1 110 ± 0.09	1 086 ± 0.02
[3]TA	108.8 ± 13.8	98.3 ± 16.58	123.7 ± 10.95	103.5 ± 12.37	131.6 ± 1.05
[4]CI	8.29 ± 0.04	9.04 ± 0.53*	8.82 ± 0.17	9.29 ± 0.33**	9.54 ± 0.09**
[5]A420/520	0.772 ± 0.0	0.830 ± 0.0	0.834 ± 0.0	0.857 ± 0.1*	0.857 ± 0.0*
L*	50.55 ± 1.91	37.85 ± 0.35**	37.4 ± 0.14**	34.2 ± 0.14**	32.35 ± 0.07**
C*	120.9 ± 4.38	97.53 ± 0.7**	96.02 ± 0.08**	90.09 ± 0.33**	85.88 ± 0.04**
H*	43.34 ± 0.25	41.14 ± 0.08**	41.17 ± 0.08**	40.09 ± 0.07**	39.69 ± 0.1**
a*	87.94 ± 2.86	73.45 ± 0.43*	72.28 ± 0.03*	68.92 ± 0.17*	66.09 ± 0.13**
b*	83 ± 3.4	64.16 ± 0.57*	63.2 ± 0.15*	58.02 ± 0.3**	54.85 ± 0.09**
ΔE*		28.74 ± 3.08[a]	30.24 ± 2.47[a]	37.18 ± 1.93	41.78 ± 2.36[b]

[1]TPI, total polyphenolic index; [2]TAN, tannins are expressed in g/L; [3]TA, total anthocyanins are expressed in mg/L; [4]CI, colour intensity; [5]A420/520, hue calculated as the ratio of the absorbances obtained at 420 and 520 nm. The statistical analysis was performed against the control condition. Asterisks indicate that means differ significantly at * $p < 0.05$, **0.01 (or less) from the control (no-MLF) condition. Letters denote significant difference between *Oenococcus* and *Lactobacillus*

conditions, as different strains may not respond and become activated in the same way.

When analysing the colour parameters, we found that some of them changed significantly in comparison to the control condition (no-MLF), even when no L-malic acid was consumed (MLF-) and particularly in the Merlot wine. But when consumption of L-malic acid occurred (MLF+), the changes in those colour parameters were even higher (UNQLp11). We also observed that some of the CIELAB parameters changed in the samples in which no L-malic acid was consumed (MLF-) in both wines. Some authors suggest that the variation, especially the decrease in wine colour, could be attributed to the absorption of polyphenols by LAB cell walls (Costantini *et al*., 2009; Burns & Osborne, 2013), the increase in pH or the LAB strain involved, which is an ongoing discussion (Costello *et al*., 2012; Burns & Osborne, 2013). In the case of pH variation, we could not explain why there was a significant increase in samples in which L-malic acid was not consumed. As pH and acidity are not only related to MLF (Comuzzo & Battistutta, 2019), we will consider additional analyses in the future.

We agree that the different parameters that determine wine colour, astringency and bitterness may be modified according to LAB species or even strains carrying out the MLF (Hernández *et al*., 2007; Burns & Osborne, 2013; Wang *et al*., 2018). An important point in the selection of novel LAB for use as malolactic starter cultures is to know if the selected strain will affect wine colour and/or astringency.

The fact that only *O. oeni* fermented the Pinot noir wine, and only the Patagonian strain of *Lb. plantarum* fermented the Merlot wine, brought some challenges to find correlations regarding colour parameters. For instance, looking at the CIELAB parameters in Pinot noir, a decrease in redness (*a**) was the only clear and statistically significant change after MLF. This result agrees with the increase in the hue (A420/520), which in this case denotes the relative importance of the yellowness over the redness (Zamora, 2003). And, although not statistically significant, the reduction of the chroma (*C**) and increase of the hue (*H**) after MLF may also be contributing to the colour of this wine variety. In fact, when analysing the *ΔE** component, the higher values correspond to the wines in which L-malic acid was consumed (OeATCC 27310 and UNQOe19). As a brief clarification, when *ΔE** is equal to or more than 2.7 CIELAB units, the wines being compared can be chromatically differentiated by the human eye, even when the variation in colour intensity (CI) is very low (Casassa & Sari, 2006). This comparison was made against the control (no-MLF) condition.

When looking at the results for the Merlot, it is difficult to come up with a general assumption for MLF. With or without L-malic acid consumption, there was a general increase in the values of the total phenolic index (TPI), colour intensity (CI) and hue (A420/520). In contrast, there was a general and significant decrease in the CIELAB parameters, especially in the wine with successful MLF (UNQLp11). Finally, the *ΔE** component showed a significant difference to that of the control (no-MLF) condition, and again was higher in the wine with successful MLF. So, these results highlight that the presence of LAB might be responsible for some of these changes, even when they are not consuming L-malic acid.

We undoubtedly need more colour measurement assays

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TABLE 3
General differences in colour parameters between Pinot noir and Merlot wines, with and without L-malic acid consumption, in comparison with the control condition (no-MLF).

	Pinot noir		Merlot	
	MLF-	**MLF+**	**MLF-**	**MLF+**
[1]TPI	-	↑	↑	-
[2]TAN	↓	↓	nc	↓
[3]TA	nc	↑	↓	↑
[4]CI	nc	↑	↑↑	↑↑
[5]A420/520	↓	↑↑	↑↑	↑↑
L*	nc	↓	↓↓	↓↓
C*	↑	↓	↓↓	↓↓
H*	-	↑	↓↓	↓↓
a*	nc	↓↓	↓↓	↓↓
b*	↑	nc	↓↓	↓↓
ΔE*	↑	↑	↑	↑↑

MLF-, wines that were inoculated but in which no L-malic acid consumption was detected; MLF+, wines with successful MLF. [1]TPI, total polyphenolic index; [2]TAN, tannins are expressed in g/L; [3]TA, total anthocyanins are expressed in mg/L; [4]CI, colour intensity; [5]A420/520, hue calculated as the ratio of the absorbances obtained at 420 and 520 nm; nc, not clear; -, values maintained. Arrows represent ↓, decrease or ↑, increase in each value. Double arrows represent significantly different values in both strains of the same species.

to complement these comparisons, as well as more *O. oeni* and *Lb. plantarum* strains to successfully perform MLF in the same wine variety. We are also considering working with higher volumes of wines and to include analyses after some time of wine evolution (ageing), with or without oak addition, as the presence of wood seems to correlate well with MLF (De Revel *et al*., 2005; Izquierdo-Cañas *et al*., 2016; González-Centeno *et al*., 2017). In addition, we need to consider the inoculation strategy that is being used (simultaneous or sequential yeasts and LAB bacteria, and the compatibility between them), which seems to affect tannin concentration (Abrahamse & Bartowsky, 2012; Massera *et al*., 2009), and/or grape variety (Dobrei *et al*., 2010; Mangani *et al*., 2011), since colour, astringency and bitterness are influenced by the concentration of anthocyanins and other phenolic compounds, and the extent of polymerisation and copigmentation, among other chemical parameters (Versari *et al*., 2008).

In summary, the two *O. oeni* strains used in this study completed L-malic acid consumption in Pinot noir wine but not in Merlot. Only the Patagonian *Lb. plantarum* strain consumed all L-malic acid in the Merlot wine, but not in the Pinot noir. The two *O. oeni* strains, but mostly OeATCC 27310, were able to survive in Merlot wine even when not consuming L-malic acid. From our results, we hypothesise that the phenolic composition of wine may vary depending on the LAB strain, and that a successful MLF will have a higher impact on this variation. Further work is needed to confirm these results and to increase our knowledge on this subject.

CONCLUSIONS

Different strains of *O. oeni* and *Lb. plantarum* behave differently depending on wine variety, and successful MLF modifies wine colour, astringency and bitterness. Our results also demonstrate that sometimes not only *O. oeni*, but also *Lb. plantarum*, can be fastidious and unpredictable bacteria when inoculated into wine. This is exactly the reason why it is so important to keep studying MLF, as we still have a long road ahead to understand how different species and strains of LAB will react to different wine chemistry, and the consequences of such interaction for wine quality.

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A Review of Leaf-mining Insects and Control Options for their Management, with Special Reference to *Holocacista capensis* (Lepidoptera: Heliozelidae) in Vineyards in South Africa

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A leaf-mining pest occurring on commercial varieties of *Vitis vinifera* in South Africa was investigated due to the presence of cocoons on fruit. The leaf miner, *Holocacista capensis*, was reported on grapevines in 2012. Since its discovery on commercial grape varieties, control strategies have consisted solely of insecticide applications. Despite the fact that the leaf-mining habit is taxonomically diverse and considered ancient, little is generally known of leaf-mining larvae. A review was conducted in order to better understand the leaf-mining strategy and amalgamate the current knowledge of other leaf-mining insects. The general evolutionary history within the full complement of taxa that display the leaf-mining habit is discussed. The review focuses on lepidopteran leaf-mining pests and discloses the known information associated with the first report and the impact of *H. capensis* in the Western Cape, South Africa. As no control methods have been identified for *H. capensis* in vineyards, various chemical, biological and cultural control strategies adopted for other leaf-mining pests were investigated. Control options, including the use of entomopathogenic nematodes (EPNs), parasitoids, physical and cultural control measures, were considered and are discussed. Alternate control methods are pertinent for the grape-growing industry to avoid the development of the insecticide resistance that is common amongst leaf miners. This review aims to consolidate the available literature and therefore aid in the development of an integrated pest management strategy to effectively control *H. capensis* in infested vineyards in South Africa.

INTRODUCTION

Grapevines in South Africa are host to more than 35 insect pests, with the key pest orders being Hemiptera (mainly shield bugs, scale insects, mealybugs and planthoppers), Coleoptera (beetles) and Lepidoptera (butterflies and moths) (Allsopp *et al*., 2015), which pose a threat to the industry.

In 2012, an unknown leaf-mining heliozelid was reported, infesting a table grape vineyard in the Western Cape province, South Africa. At the time, the known heliozelid fauna from Africa were limited to three species described in South Africa (Van Nieukerken & Geertsema, 2015). Subsequent field visits indicated high larval/leaf mine abundances and cocoon casings of the leaf miner on the foliage, stems, trellises and grape bunches in vineyards. In 2015, the leaf miner was described by Van Nieukerken and Geertsema (2015) as *Holocacista capensis* Van Nieukerken & Geertsema (Lepidoptera: Heliozelidae). Since the discovery of *H. capensis* in 2012, a concomitant study by Wang *et al*. (2015), using gas chromatography-mass spectrometry, identified the sex pheromone (more accurately, a male attractant) of *H. capensis*. Baseline studies to better understand the bio-ecology of *H. capensis* in the Western Cape were conducted by Torrance (2016).

Sustainable, effective control of the leaf miner is pertinent for the grape-growing industry in South Africa to avoid the development of resistance against commonly used insecticides. This review consolidates the available literature regarding the leaf-mining habit, lepidopteran miners as pests, and the effect of the environment on their infestation levels. Management options for leaf miners with regard to chemical control, the use of entomopathogenic nematodes (EPNs), parasitoids and other control measures were considered in the light of possible future control options for *H. capensis* on grapevines in South Africa.

LEAF-MINING INSECTS

Globally, little is known of leaf-mining insects (Vári, 1961; Auerbach *et al*., 1995; Lees *et al*., 2014). Leaf-mining insects are a taxonomically diverse group of endophagous insects and the larvae of leaf-mining taxa are, in most cases, concealed within the plant tissue of their hosts during larval

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development, or at least for part thereof (Hering, 1951; Kirichenko *et al*., 2018). The duration of the leaf-mining stage varies between species and is not only associated with larval growth, but can also cover the development of pupae and the emergence of adult insects in taxa that pupate within the leaf mine (Connor & Taverner, 1997).

Despite the fact that the leaf-mining habit is ancient, it continues to be lost and acquired by a number of phytophagous insect lineages (Connor & Taverner, 1997) and has evolved independently numerous times (Auerbach *et al*., 1995). The leaf-mining habit is known to occur in at least 57 families within four insect orders, accounting for more than 10 000 leaf-mining species (Connor & Taverner, 1997). The mines originating from the respective orders are classified into specific groups, namely lepidopteronome (Lepidoptera), dipteronome (Diptera), coleopteronome (Coleoptera) and hymenopteronome (Hymenoptera) mines (Hering, 1951).

The geographical distribution of endophagous insects, like leaf miners, is inevitably dependent on the distribution of their larval host plants. In most cases, however, the distribution of a leaf miner is less extensive than that of its host plant (Hering, 1951). Amongst the herbivorous insects, many leaf miners pose a threat to a variety of forest and urban plant species, whilst others are regarded as important pests of agricultural crops and are considered an economically important group globally (Spencer, 1973; Nielsen & Common, 1991; Digweed *et al*., 2009).

Over the last decade, an increase in incidents of leaf-mining insects has attracted the attention of the agricultural and horticultural industries due to their presence in commercial forests, agricultural landscapes and on ornamental plant varieties of high value (Van Nieukerken & Geertsema, 2015; Kirichenko *et al*., 2018).

THE LEAF-MINING HABIT

In the past, the concealed feeding environment of endophagous insects was speculated to provide a competitive advantage when compared to their exophagous counterparts (Hering, 1951; Nielsen & Common, 1991), and to protect feeding larvae from natural enemies (Hering, 1951; Price *et al*., 1987). It also provides a buffer against the physical environment (Connor & Taverner, 1997), and enables the feeding larvae to avoid plant defences (Feeny, 1970) and thus facilitates selective consumption of more nutritious leaf tissue (Cornell, 1989). Price *et al*. (1987) and Connor and Taverner (1997) reviewed some of these hypotheses amongst various endophagous feeders and arrived at similar conclusions. Connor and Taverner (1997) suggest that the selective advantages inherent to the leaf-mining habit are to facilitate: 1) increased feeding efficiencies, which support some of the hypotheses and findings of Cornell (1989); 2) the avoidance of negative effects associated with disease, should it be present within a population or species, by internally feeding larvae; 3) the protection of larvae from the direct and indirect effects of photochemical changes in plant chemistry, for example due to UV radiation; and 4) the reduction of water loss and lessening the risk of desiccation by the presence of a buffered micro-environment within the feeding leaf gallery.

Connor and Taverner (1997) also highlight the disadvantages of the leaf-mining habit. These include: 1) the loss of mobility, which thus cause larvae to be unable to escape parasitoids and predators; this is supported by statements made by Nielsen and Common (1991); 2) decreased species richness within leaf-mining lineages when compared to that of exophagous insects; 3) mortality associated with plant senescence, herbivory and premature abscission of leaves; and 4) reduced fecundity due to the small size of individuals.

From an evolutionary perspective, the disadvantages of the leaf-mining habit outweigh the advantages. The persistence of leaf-mining guilds in various insect orders and environmental niches in the present day, however, proves that, for some taxa, the leaf-mining habit is a feasible means of survival under certain circumstances (Connor & Taverner, 1997).

LEPIDOPTERAN LEAF MINES

Apodal or legless lepidopteran leaf-mining larvae (or "serpentine larvae") consume the mesophyll between the upper and lower epidermal layers of a leaf (Stehr, 1992; Bernardo *et al*., 2015), creating small blotch mines or galleries within the parenchymal tissues of host plants (Hering, 1951). These feeding channels, or cavities, serve as both living and feeding quarters for leaf-mining larvae (Hering, 1951).

The shape of a leaf mine and the presence of voluminous frass often presents a unique feeding pattern within an infested leaf, which can be used as a diagnostic tool for species-specific identification (Hering, 1951; Kirichenko *et al*., 2018). Mines produced by any leaf-mining insect can be used to determine the order, family and, in many cases, the particular genus (Hering, 1951; Vári, 1961). Lepidopteran hyponomology (the study of mines) often provides a clear and more accurate indication of species identity than comparing fine differences in larval and adult morphology.

LEPIDOPTERAN LEAF-MINING PESTS

Lepidoptera account for the majority of leaf-mining insects (Kirichenko *et al*., 2018). As a result, and due to the destructive qualities of the larval life stages of some of the leaf-mining species, these Lepidoptera are considered to be of great economic importance (Nielsen & Common, 1991). At least 40 lepidopteran families exhibit leaf-mining habits, with considerable variation between species. These lepidopteran leaf miners account for approximately 70% of all known insect families associated with leaf-mining activities (Connor & Taverner, 1997; Kirichenko *et al*., 2018). Within the Lepidoptera, the leaf-mining families of economic importance include the Gelechiidae, major pests in the forestry and agricultural industries (Lee *et al*., 2009); the Gracillariidae, notorious as invasive leaf-mining pests of woody plants (Kirichenko *et al*., 2018); and the Heliozelidae, predominantly pests on trees and vines (Davis, 1998). A list of lepidopteran leaf-mining agricultural pests is presented in Table 1.

HELIOZELIDAE – THE "SHIELD BEARERS"

The Heliozelidae (Lepidoptera: Adeloidea) are a group of widely distributed, cosmopolitan, minute, diurnal micro-Lepidoptera (Davis, 1998; Powell, 2003, Van Nieukerken

TABLE 1
A (non-exhaustive) summary of agriculturally important leaf-mining lepidopteran pests

Family	Leaf-mining species	Common name	Commercial host	Native range	Region of invasion	Source
Bedelliidae	*Bedellia somnulentella*	Sweet potato leaf miner	Sweet potato	Eurasia	Cosmopolitan	Visser (2015a); Dos Santos *et al.* (2018)
Heliozelidae	*Holocacista capensis*	NA	*Vitis vinifera*	Southern Africa	Southern Africa	Van Nieukerken & Geertsema (2015)
	Holocacista rivillei	European grapevine leaf miner	*V. vinifera*	Europe	Southern Europe, Western Asia	Van Nieukerken *et al.* (2012)
	Antispila oinophylla	Grapevine leaf miner	*V. vinifera*	Eastern North America	Northern Italy	Van Nieukerken *et al.* (2012)
	Antispila uenoi	Grapevine leaf miner	*V. vinifera*	Japan	Japan	Van Nieukerken *et al.* (2012)
	Antispila nysaefoliella	Tupelo leaf miner	Black gum	Southeastern United States	United States	Low (2012)
	Coptodisca splendoriferella	Resplendent shield borer	Apples, cranberries	Unknown	North America	Boush & Anderson (1967)
Incurvariidae	*Protaephagus capensis*	Blotch leaf miner	Protea, *Leucadendron* sp.	Southwestern Cape (South Africa)	Southern Africa	Wright (2015)
Gelechiidae	*Aproaerema modicella*	Groundnut leaf miner	Ground nut, soybean	South-South-East Asia	South, South-East Asia	Shanower *et al.* (1993)
	Aproaerma simplexella	Groundnut leaf miner	Groundnut, soybean, possibly lucerne	Africa and Australia	Africa, Australia	Buthelezi *et al*. (2012)
	Bilobata subsecivella	Groundnut leaf miner	Groundnut, soybean, lucerne	South-East Asia	Africa	Du Plessis (2015)
	Tuta absoluta	Tomato leaf miner	Tomato, potato	Western neotropics	South America, Afro-Eurasia	Siqueira *et al.* (2001); Biondi *et al.* (2018)
	Phthorimaea operculella	Potato tuber moth	Potato, tomato, gooseberry, brinjal, chilli, tobacco	South America	All tropical, subtropical potato-growing regions	Kroschel & Zegarra (2013); Visser (2015b)
	Symmetrischema tangolias	Andean potato tuber moth	Potato and tomato	South America	South America, New Zealand, Australia, United States	Kroschel & Zegarra (2013); Sporleder *et al.* (2017)
Gracillariidae	*Phyllocnistis vitegenella*	American grape leaf miner	*Vitis vinifera*	North America	Europe	Ureche (2016)
	Phyllocnistis citrella	Citrus leaf miner	Citrus	South-east Asia	Worldwide (all citrus-producing areas)	Kirichenko *et al.* (2018)
	Acrocercops bifasciata	Cotton leaf miner	Cotton and okra	Unknown	Southern Africa	Bennett (2015)

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TABLE 1 (CONTINUED)

Family	Leaf-mining species	Common name	Commercial host	Native range	Region of invasion	Source
	Acrocercops gossypii	Cotton leaf miner	Cotton	Unknown	Southern Africa	Bennett (2015)
	Spulerina sp.	Mango twig miner	Mango	Unknown	Southern Africa	Grové *et al.* (2015)
	Phyllocnistis sp.	Thin line leaf miner	Protea	Unknown	Southern Africa	Wright (2015)
Lyonetiidae	*Leucoptera caffeina* and *Leucoptera meyricki*	Coffee leaf miner	Coffee	Central, East, Southern Africa	Africa	Fragoso *et al.* (2002); Schoeman (2015)
	Leucoptera coffeella	Coffee leaf miner	Coffee	Africa	Neotropics, Mexico	Fragoso *et al.* (2002); Lomelí-Flores *et al.* (2010)

et al., 2011; Regier *et al*., 2015; Milla *et al*., 2018), present in all major faunal realms, with no representatives in New Zealand and Antarctica. One hundred and twenty-five described species comprise the Heliozelidae, placed in 12 genera (Van Nieukerken *et al*., 2011; 2012; Van Nieukerken & Geertsema, 2015). The family is taxonomically poorly studied, although taxonomic revisions associated with heliozelids have been conducted by Van Nieukerken *et al*. (2011), Regier *et al*. (2015), Van Nieukerken and Geertsema (2015) and Milla *et al*. (2018) (Fig. 1) in recent years.

Heliozelid moths are typically small, with their forewings ranging from 1.7 mm to 7.0 mm in length (Regier *et al*., 2015). Due to their small size, most heliozelids are rarely seen or collected, even when population abundances are high (Powell, 2003; Regier *et al*., 2015). Most adult moths within the Heliozelidae possess fundamentally dark wing colouration with iridescent scaling (Scoble, 1992; Powell, 2003).

Larval instars are obligate leaf miners, with the exception of the final instar (Stehr, 1992; Regier *et al*., 2015). A flat, lenticular case is constructed by the penultimate instar from the epidermal layers of a mined leaf, lined and bound with silk to form a firm, cocoon-type covering (Holloway *et al*., 1987; Stehr, 1992; Regier *et al*., 2015). The vernacular name, "shield bearers", refers specifically to the oval, lenticular shape of the crafted casing (Scoble, 1992; Davis, 1998). The casing is either suspended by means of a silken thread,

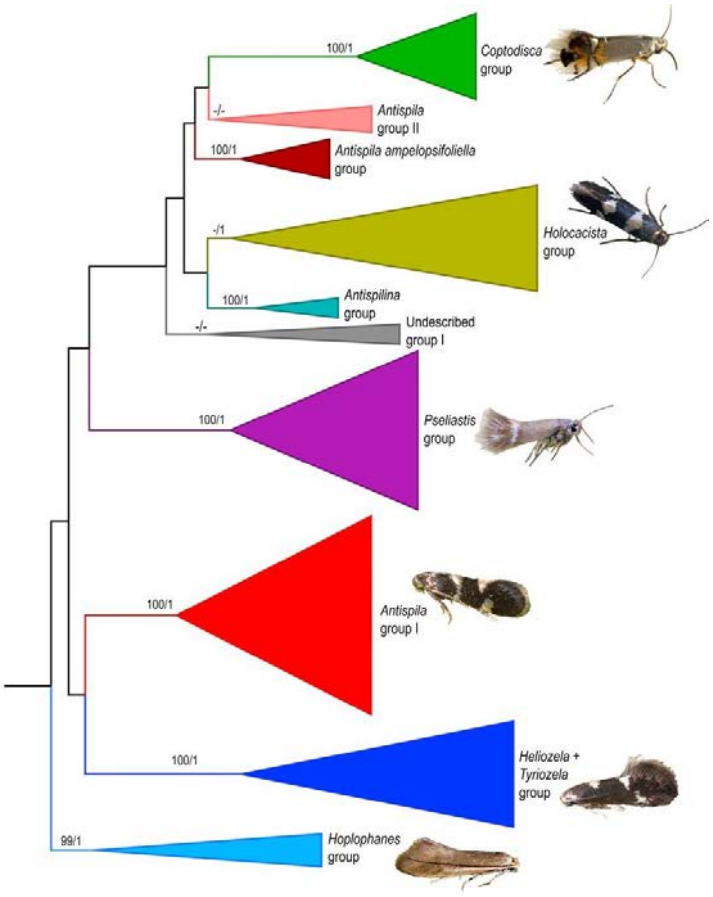

FIGURE 1
The maximum likelihood tree compiled by Milla *et al*. (2018) that represents the major genera within the Heliozelidae.

or carried or dragged from the infested leaf by the encased larvae (Scoble, 1992; Regier *et al*., 2015). The larvae anchor themselves by weaving a silken mat onto objects with which they come into contact.

Detailed accounts of the morphology of all the life stages of the Heliozelidae have been documented by Bourgogne (1951), Hering (1951), Holloway *et al*. (1987), Scoble (1992), Davis (1998), Powell (2003) and Patočka and Turčáni (2005). Keys in Mey (2011) and Patočka and Turčáni (2005) enable the identification of some genera and species within the Heliozelidae.

Almost all individual heliozelid species are hostplant-specific, confined to genus level or, at least, the plant family level (Regier *et al*., 2015), which may lead to gregarious behaviour, depending on local plant assemblages. Within the agricultural context, a number of heliozelids are considered to be of economic importance (Table 1). Over the last three decades, four heliozelid species have unexpectedly been encountered on commercial grapevines. These are *Antispila oinophylla* Van Nieukerken & Wagner (reported in Northern Italy in 2007, one of the two species of North American origin), *Antispila uenoi* Kuroko (a pest native to Japan, reported on commercial vineyards (Kuroko, 1987; Ueno *et al*., 1987) and *H. capensis* (a pest thought to be a native species, presently reported on commercial vineyards in South Africa) (Van Nieukerken & Geertsema, 2015).

HOLOCACISTA CAPENSIS

Holocacista capensis is a multivoltine (having several generations per year) pest present throughout a grapevine growing season (Van Nieukerken & Geertsema, 2015; Torrance, 2016).

Morphology and known biology

The adults are small, diurnal moths with a wingspan of ca. 3.9 mm to 4.9 mm (Van Nieukerken & Geertsema, 2015). The black ground colour of the wings is ornamented by silvery-white spots or fascia. The head and face are covered by silvery-white metallic, appressed scales. Male and female moths can be differentiated based on the colour of the posterior abdominal segments (lead-coloured in males, jet black in females) and the markings on their forewings (in females the first costal and dorsal spots are joined to form a contiguous band) (Fig. 2). The adults of *H. capensis* closely resemble *Holocacista salutans* (Meyrick) and *Holocacista varii* (Meyrick). Eggs are laid singly in leaves by females after mating (Van Nieukerken & Geertsema, 2015).

The larvae develop through four feeding instars (Van Nieukerken & Geertsema, 2015). These larvae are unable to move to other leaves upon damage to the natal leaf or mine (Torrance, 2016). The heads of feeding larvae are usually characterised by dark, prognathous head capsules. Their bodies are yellow or whitish (Van Nieukerken & Geertsema, 2015). The larvae feed on leaf tissue only (Torrance, 2016) and completed mines reach 12 mm to 15 mm in length (Van Nieukerken & Geertsema, 2015). The fifth, final instar is non-feeding and constructs the cocoon casing in which it will pupate.

Symptoms of damage

The mining activities of larvae cause physical damage to the infested grapevine leaves. The effect of the mines on the photosynthetic ability of a grapevine is not yet known, although it appears to be limited (Van Nieukerken & Geertsema, 2015). The mines are predominantly present along the leaf margin. When high infestations are reported, it is usually later in a growing season, usually after harvest, or when leaf miner populations were left unmanaged.

The final instar larva in its cocoon casing descends from the leaf by means of a silken thread (similar to most other leaf-mining heliozelids) (Torrance, 2016). Upon landing on an object in its immediate surroundings (e.g. leaf, trellis post or grape bunch), the larva will move to an appropriate location and firmly attach itself to the object (Van Nieukerken & Geertsema, 2015; Torrance, 2016). It therefore is undesirable if the cocoon casings are present on fruit intended for consumption.

Bio-ecology

Larval and adult abundance tends to increase throughout a season, coupled with a rise in the prevailing temperatures (Torrance, 2016). February and March mark the peak in adult

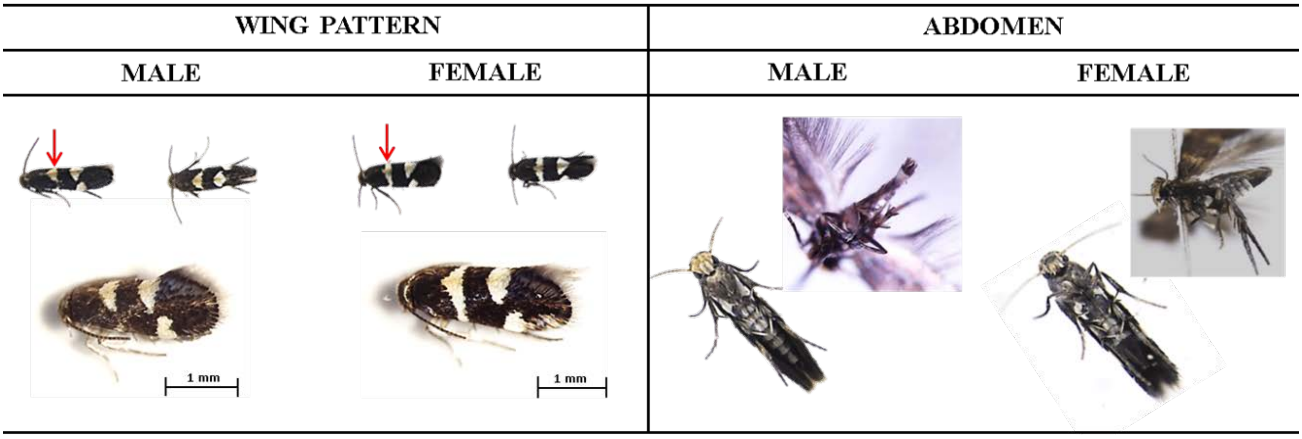

FIGURE 2
Different wing (indicated by red arrows) and abdominal patterns of male and female *Holocacista capensis* adults. Adapted from Torrance (2016).

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and larval abundance (Van Nieukerken & Geertsema, 2015; Torrance, 2016). According to Torrance (2016), temperature plays a vital role in leaf miner population abundance. Other variables (including trellis angle and block aspect) affecting leaf miner infestation were also investigated, but definite conclusions regarding their specific effect on population numbers could not be drawn (Torrance, 2016).

It is estimated that the life cycle of the moth takes at least seven weeks to complete, and a minimum of four generations can be present within a growing season (Torrance, 2016). The leaf miner overwinters in the larval or pupal life stage within the cocoon casing that is sheltered from the elements (e.g. under the bark of a grapevine stem, in leaf litter or in the crevices of trellising posts) (Torrance, 2016). These individuals will eclose in the ensuing growing season and will produce the first generation in the new season (Van Nieukerken & Geertsema, 2015; Torrance, 2016).

Holocacista capensis is widely distributed throughout the Western Cape, South Africa and has established itself in relatively high abundances in some of the major table grape-producing regions in southern Africa (Fig. 3) (Torrance, 2016). Synonymy amongst populations (molecular identifications) has not yet been confirmed.

VARIABLES AFFECTING LEAF MINER INFESTATION

Auerbach *et al*. (1995) state that the dominant cause of mortality or absence of leaf miner populations in suitable habitats can be attributed to vertical (interactions between miners, host plants and natural enemies) and horizontal interactions (including inter- and intraspecific interactions between miners and herbivores). This does not, however, account for environmental and abiotic factors affecting leaf miner infestation.

Little is known of the direct effects of abiotic factors or variables on leaf miner abundance and survival (Auerbach *et al*., 1995). Pereira *et al*. (2007) identified rainfall as an important factor affecting mortality of *Leucoptera coffeella* (Guérin-Méneville & Perrottet) (Lepidoptera: Lyonetiidae), and also considered that weather conditions could affect egg mortality. However, their study concentrated on the environmental factors operative between the two seasons (rainy vs. dry) and not necessarily on the factors influencing population abundances within a particular season. Potter (1992) excluded shade as an important factor affecting the abundance of *Phytomyza ilicicola* Loew (Diptera: Agromyzidae). A study by Johns and Hughes (2002) indicated a negative association between emergence success and adult weight of *Dialectica scalariella* Zeller (Lepidoptera: Gracillariidae) in Paterson's curse, *Echium plantagineum* (Boraginaceae), and elevated CO_2 as a result of reduced foliar quality of *E. plantagineum*. The invasion ecology of the horse chestnut leaf miner, *Cameraria ohridella* Deschka & Dimić (Lepidoptera, Gracillariidae), on the other hand, has been found to be affected by long-distance dispersal and increased human population densities (increasing the probability of accidental transport of leaf miners as a result) (Gilbert *et al*., 2004).

In the case of *H. capensis*, the average adult male abundance has been strongly correlated with the average minimum humidity (and thus also the average maximum temperature) (Torrance, 2016). Edge effects, the difference between externally located plots and internally located plots, did not affect leaf miner abundance. Spatial distribution and abundance in grapevine blocks have not been assessed,

FIGURE 3
Recorded distribution of *Holocacista capensis* (Van Nieukerken & Geertsema, 2015; Torrance, 2016).

however, and require further investigation. Human-mediated means of dispersal have also been speculated (Torrance, 2016).

PEST MANAGEMENT

On a global scale, most commercial vineyards are protected against leaf-mining pests (as with a number of other pests) by the use of insecticides (Maier, 2001). However, various other control strategies have also been used to control pest populations. A summary of these strategies and their respective leaf-mining insect targets is given in Tables 2 to 4.

Chemical control

Chemical control can be achieved through the use of synthetic chemical insecticides or botanical insecticides (Isman, 2006) (Table 2). In terms of environment-friendly pest management, botanical insecticides pose an attractive alternative to the use of synthetic insecticides, being less of a threat to human health or to the environment. Generally speaking, synthetic pesticides exhibit some adverse effects, such as their toxicity for non-target organisms, contamination of groundwater and the development of pesticide resistance in pest populations (Isman, 2006).

Short-term (seasonal) control of *H. capensis* has been achieved in vineyards where dichlorvos and spinosad were applied (Torrance, 2016). Extensive research has been conducted on insecticide use and the corresponding insecticide resistance of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) (Biondi *et al*., 2018), raising concern about long-term control strategies for other leaf-mining pests with similar generation times, including *H. capensis*.

Entomopathogenic nematodes

Of the various beneficial, parasitic groups within the nematode complex, entomopathogenic nematodes (EPNs) are used to control insect pests (Stock & Hunt, 2005; Stock, 2015). The genera within this group include members of the genera *Steinernema* Travassos (Steinernematidae: Rhabditida) and *Heterorhabditis* Poinar (Heterorhabditidae: Rhabditida) (Kaya *et al*., 1993). Together with their associated pathogenic bacteria (from the genus *Xenorhabdus* and *Photorhabdus* for steinernematids and heterorhabditids respectively), EPNs kill their hosts within a few days (Dillman *et al*., 2012, Lewis *et al*., 2015).

For all EPNs there is a free-living, non-feeding stage known as the infective juvenile (IJ) or dauer (Griffin *et al*., 2005). When an appropriate host is located, an IJ will enter through any natural opening (e.g., mouth or anus), the cuticle or spiracles in search of the nutrient-rich haemolymph. Here, the IJs will release their symbiotic bacteria from their intestines; these reproduce and release toxins. The infected insect usually succumbs within 48 h. Within the cadaver, the IJs feed on the bioconverted host tissues (and bacteria), grow and develop into adults. As the food source becomes scant within the cadaver, the nematodes develop in crowded conditions and become arrested as IJs. The new IJs, with their specific symbiotic bacteria, will emerge from the cadaver in search of a new host (Griffin *et al*., 2005).

In a study by Steyn *et al*. (2019), high mortality of

TABLE 2
A (non-exhaustive) summary of chemical control strategies used against leaf-mining pests.

Order/Family	Scientific name	Common name	Active agent/species	Success	Source
Diptera/ Agromyzidae	*Liriomyza trifolii*	Celery leaf miner	Abamectin	Yes	Hara *et al*. (1993)
	L. trifolii	Celery leaf miner	Abamectin, cyromazine	Yes[1]	Trumble (1985); Ferguson (2004)
	L. trifolii	Celery leaf miner	Spinosad	Yes[1]	Ferguson (2004)
	L. trifolii	Celery leaf miner	Methomyl	No	Trumble (1985)
	Liriomyza huidobrensis	Potato leaf miner	Abamectin, cyromazine	Yes[2]	Weintraub & Horowitz (1998)
	Chromatomyia horticola	Pea leaf miner	Acetamiprid, methamidophos, imidacloprid, *Bacillus thuringiensis* and abamectin mixture	Yes	Khan *et al*. (2015)
Lepidoptera/ Gelechiidae	*Tuta absoluta*	Tomato leaf miner	Abamectin, chlorantraniliprole	Yes	Pereira *et al*. (2014)
	T. absoluta	Tomato leaf miner	Chlorpyrifos	Yes[1]	Haddi *et al*. (2017)
	T. absoluta	Tomato leaf miner	Diamide	Yes[1]	Roditakis *et al*. (2017)
	T. absoluta	Tomato leaf miner	Indoxacarb, spinosad	Yes[1]	Pereira *et al*. (2014); Roditakis *et al*. (2018)

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TABLE 2 (CONTINUED)

Order/Family	Scientific name	Common name	Active agent/species	Success	Source
	T. absoluta	Tomato leaf miner	Methamidophos, phenthoate, cartap hydrochloride, chlorfenapyr	Yes[3]	Pereira *et al*. (2014)
Lepidoptera/ Gracillariidae	*Phyllocnistis citrella*	Citrus leaf miner	Permethrin, methidathion, fenoxycarb	Yes	Beattie *et al*. (1995a)
	P. citrella	Citrus leaf miner	Petroleum spray oil	Yes	Beattie *et al*. (1995b)
	P. citrella	Citrus leaf miner	Polysaccharides	No	Beattie *et al*. (1995b)
	Cameraria ohridella	Horse chestnut leaf miner	Harpin protein, potassium phosphite, salicylic acid derivative	Yes	Percival & Holmes (2016)
	C. ohridella	Horse chestnut leaf miner	Benzothiadiazole, probanazole, deltamethrin	No	Percival & Holmes (2016)
Lepidoptera/ Lyonetiidae	*Perileucoptera coffeella*	Coffee leaf miner	Chlorpyrifos, disulfoton, ethion, methyl parathion	Yes[1]	Fragoso *et al*. (2002)

[1] Resistance reported; [2] Negative effects on parasitoids; [3] Effect on natural enemies

H. capensis larvae was obtained for *Heterorhabditis baujardi* Phan, Subbotin, Nguyen & Moens (92%), *Heterorhabditis noenieputensis* Malan, Knoetze & Tiedt (85%) and *Heterorhabditis indica* Poinar, Karunakar & David (83%). Almost double the number of *H. noenieputensis* (34 nematodes/insect) penetrated the insect larvae in comparison with the other two EPNs. However, the relative potency of *H. baujardi* was 3.56 times higher than it was for *H. indica*, whilst that of *H. indica* was 2.57 times higher than it was for *H. noenieputensis*. These authors concluded that the results obtained in the laboratory were encouraging, especially with regard to the nematodes' ability to penetrate the leaf-mining galleries and to infect the larvae successfully.

A variety of EPNs have successfully controlled certain leaf-mining pest populations (Table 3). In the case of *T. absoluta*, leaf bioassays conducted on leaves infested with larvae, using concentrations of 1 000 IJs/ml (equivalent to a 60 IJs/cm^2 dose) of *Steinernema carpocapsae* (Weiser, 1955) Wouts, Mráček, Gerdin & Bedding, *Steinernema feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding and *Heterorhabditis bacteriophora* Poinar caused high levels of mortality (88.6%, 92% and 76.3%, respectively) after 72 h of exposure to the respective EPNs (Batalla-Carrera *et al*., 2010). These results revealed that the EPNs were able to find and kill larvae, despite their relative position on or within a leaf (i.e. outside of or within leaf galleries). Field trials conducted by Gözel and Kasap (2015) with the same EPNs on netted plants, using a conventional airblast-sprayer at an application rate of 50 IJs/cm^2, confirmed these results (ca. 46%, 92% and 82% total mortality, respectively). Similar results were obtained by Van Damme *et al*. (2015), who applied a concentration of 27.3 IJs/cm^2 of each of the three EPN species to infested leaves by means of an automated spray boom. Beattie *et al*. (1995b) tested *S. carpocapsae* against the larvae of *Phyllocnists citrella* Stainton (Lepidoptera: Gracillariidae) at concentrations of 5 x 10^6, 10 x 10^6 and 30 x 10^6 IJs/l water. A significant increase in mortality was only obtained at the highest dose, resulting in 35% mortality.

Parasitoids

In contradiction to the inferences made by Ayabe and Hijii (2016) regarding the study by Connor and Taverner (1997), the leaf-mining habit does not allow leaf miners to escape predation. According to Connor and Taverner (1997), the loss of mobility, and thus escape strategies, in leaf-mining insects has led to higher mortality rates associated with hymenopteran parasitoids than in exophagous insects. This has led to the evolution of more species of associated parasitoids than in any other insect-feeding guild. In the case of *H. capensis*, several parasitoids have been found to attack the larval and pupal life stages, although these parasitoids have not yet been identified. The use of parasitoids against leaf-mining insects is a popular alternative to the use of insecticides.

A few case studies with promising results are listed in Table 4. Trichogrammatidae, Encyrtidae and Eulophidae (all of which belong to the superfamily Chalcidoidea) have been found to parasitize lepidopteran leaf-mining pests (Table 4). The species within the Chalcidoidea are generally less than

TABLE 3
A (non-exhaustive) summary of the entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) used as control strategies against leaf-mining pests.

Order/Family	Common name	Scientific name	Species/Active agent	Success	Source
Diptera/ Agromyzidae	Celery leaf miner	*Liriomyza trifolii*	*Steinernema bicornutum*; *Heterorhabditis indica*	Yes	Jacob & Mathew (2016)
	Celery leaf miner	*L. trifolii*	*Steinernema carpocapsae*	Yes	LeBeck *et al.* (1993); Jacob & Mathew (2016)
	Celery leaf miner	*L. trifolii*	*Steinernema feltiae*	Yes**	Hara *et al.* (1993)
	Potato leaf miner	*L. huidobrensis*	*S. feltiae*	Yes	Williams & Walters (2000)
	Potato leaf miner	*L. huidobrensis*	*S. feltiae* - *trichlorfon, *dimethoate	Yes	Head *et al*. (2000)
	Potato leaf miner	*L. huidobrensis*	*S. feltiae* - *trichlorfon, *dimethoate	No	Head *et al*. (2000)
	Tomato leaf miner	*Liriomyza bryoniae*	*S. feltiae*	Yes	Williams & Walters (2000)
	Chrysanthemum leaf miner	*Chromatomyia syngenesiae*	*S. feltiae*	Yes	Williams & Walters (2000)
Hymenoptera/ Tenthredinidae	Amber-marked birch leaf miner	*Profenusa thomsoni*	*S. carpocapsae*	No	Progar *et al*. (2014)
Lepidoptera/ Gelechiidae	Tomato leaf miner	*T. absoluta*	*Heterorhabditis bacteriophora*	Yes	Batalla-Carrera *et al*. (2010); Gözel & Kasap (2015); Van Damme *et al*. (2015); Kamali *et al.* (2017)
	Tomato leaf miner	*T. absoluta*	*Steinernema affine*	Yes	Gözel & Kasap (2015)
	Tomato leaf miner	*T. absoluta*	*S. carpocapsae*	Yes	Batalla-Carrera *et al*. (2010); Gözel & Kasap (2015); Kamali *et al.* (2017)
	Tomato leaf miner	*T. absoluta*	*S. feltiae*	Yes	Batalla-Carrera *et al.* (2010); Gözel & Kasap (2015); Van Damme *et al*. (2015)
	Tomato leaf miner	*T. absoluta*	*Steinernema karii*; *Heterorhabditis* sp.	Yes	Mutegi *et al*. (2017)
	Citrus leaf miner	*P. citrella*	*S. carpocapsae*	Yes	Beattie *et al.* (1995a)
Lepidoptera/ Heliozelidae	NA	*Holocacista capensis*	*Heterorhabditis indica*; *Heterorhabditis noenieputensis*; *Heterorhabditis baujardi*	Yes	Steyn *et al.* (2019)
			Heterorhabditis bacteriophora; *Heterorhabditis zealandica*; *Steinernema jeffreyense*; *Steinernema yirgalemense*	No	Steyn *et al.* (2019)

* Added independently ** Dependent on relative humidity

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TABLE 4
A (non-exhaustive) summary of parasitoids used as control strategies against leaf-mining pests.

Order/family	Common name	Scientific name	Species/Family	Success	Source
Diptera/ Agromyzidae	Celery leaf miner	*Liriomyza trifolii*	*Chrysocharis flacilla* (Eulophidae)	Yes	Muchemi *et al*. (2018)
	Celery leaf miner	*L. trifolii*	*Diglyphus isaea* (Eulophidae)	Yes	Minkenberg & Van Lenteren (1986)
	Potato leaf miner	*L. huidobrensis*	*C. flacilla*	Yes	Muchemi *et al*. (2018)
	Potato leaf miner	*L. huidobrensis*	*D. isaea*	Yes	Maharjan *et al*. (2017)
	Potato leaf miner	*L. huidobrensis*	*Opius dissitus* (Braconidae)	Yes	Wei & Kang (2006)
	Vegetable leaf miner	*Liriomyza sativae*	*O. dissitus*	Yes	Wei & Kang (2006)
	Vegetable leaf miner	*L. sativae*	*C. flacilla*	Yes	Muchemi *et al*. (2018)
	Holly leaf miner	*Phytomyza ilicis*	*Chrysocharis gemma* (Eulophidae)	Yes	Heads & Lawton (1983)
	Holly leaf miner	*P. ilicis*	*Opius ilicis* (Braconidae)	Yes	Kirichenko *et al*. (2018)
Hymenoptera/ Tenthredinidae	Amber-marked birch leaf miner	*Profenusa thomsoni*	*Lathrolestes thomsoni* (Ichneumonidae)	Yes	Soper *et al*. (2015)
	Birch leaf miner	*Fenusa pumila*	*Lathrolestes nigricollis* (Ichneumonidae), *Grypocentrus albipes* (Ichneumonidae)	Yes	Langor *et al*. (2000)
Lepidoptera/ Gelechiidae	Tomato leaf miner	*T. absoluta*	*Trichogramma euproctidis*, *Trichogramma achaeae* (Trichogrammatidae)	Yes	El-Arnaouty *et al*. (2014)
	Tomato leaf miner	*T. absoluta*	*Trichogramma pretiosum* (Trichogrammatidae)	Yes	Parra & Zucchi (2004)
Lepidoptera/ Gracillariidae	Citrus leaf miner	*P. citrella*	*Ageniaspis citricola* (Encyrtidae)	Yes	Hoy *et al*. (2007)
	Citrus leaf miner	*P. citrella*	*Citrostichus phyllocnistoides* (Eulophidae)	Yes	Garcia-Marí *et al*. (2004)

3 mm in length, making it rather difficult to collect and study individuals (Noyes, 2003).

Cover cropping

Ingels and Klonsky (1998) describe a cover crop as a crop (or secondary plants) of little to no economic significance that is grown in intra- and inter-rows of vineyards, the presence of which, however, provides numerous other potential benefits. Parolin *et al*. (2012) provide an extended definition of barrier plants, as "… a plant which is used within or bordering a primary crop for the purpose of disease suppression and/ or interception of pests and/or pathogens". In terms of their potential to harbour pests and pathogens, the effect of barrier plants or cover crops on population numbers of most leaf-mining pests is not known and should be investigated, as the use of different cover crops to reduce pests in vineyards is regarded as an option in the future.

OTHER PEST MANAGEMENT METHODS

Entomopathogenic fungi (EPF) have been used successfully in a variety of integrated pest management (IPM) strategies against many leaf-mining pests of economic importance (Shah & Pell, 2003). Various strains of *Metarhizium anisopliae* (Metschnikoff) (Sorokin) (Hypocreales: Clavicipitaceae) and *Beauveria bassiana* (Balsamo) (Vuillemin) (Hypocreales: Cordycipitaceae) have been used to control the pea leaf miner, *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae) (Migiro *et al*., 2010; 2011); and the tomato leaf miner, *T. absoluta* (Rodríguez *et al*., 2006; Allegrucci *et al*., 2017).

Mating disruption implies the use of a formulated female pheromone to disrupt or regulate the mating habits of a target pest species (Cardé & Minks, 1995). Amongst the leaf-mining Lepidoptera, mating disruption has only been explored and successfully achieved against *P. citrella* (Stelinski *et al*., 2008; Stelinski *et al*., 2010; Willett *et al*., 2015). Mating disruption studies on *T. absoluta* have only proved successful under greenhouse conditions (Vacas *et al*., 2011; Cocco *et al*., 2013).

The practice of bagging grapes (as a physical measure of control) using a bunch cover/bag dates back to 1919 (Signes *et al*., 2007). After ripening, bunches are typically

covered with a cover/bag which is only removed during harvest. Bagging has been used to promote uniform colour development within a bunch, reduce the incidence of blemished fruit, reduce the incidence of disease, delay the ripening process (ideally when harvest needs to be delayed for increased market access), increase hygiene (reduced contact with pesticides sprays and other contaminants), protect grape bunches against adverse environmental variables (e.g. moisture, hail, sunburn and cracking/bursting of fruit), and to provide protection against attack by birds and insects (Signes *et al*., 2007; Sharma *et al*., 2014). Pre-harvest fruit bagging has been used to avoid insect infestation in a variety of crops (Sharma *et al*., 2014).

The use of netting (overhead netting, vineyard layover netting and zone netting) in vineyards has become widespread in recent years (Suvočarev *et al*., 2013). Netting is used to reduce the number of pests (reduced immigrant invasion from surrounds), leading to a reduction in the number of pesticide applications; reduce radiation exposure of plants during hot summer months; and minimise hail and bird damage (Suvočarev *et al*., 2013). Neither bagging of grapes nor netting, however, has been tested to exclude leaf-mining pests.

RECOMMENDATIONS

The discovery of a newly recognised pest in an industry that contributes to a country's economy requires novel and baseline studies to understand the pest's ecology and distribution so that pest populations can be controlled adequately. The use of dichlorvos and spinetoram has been used to reduce the density of *H. capensis* populations in infested vineyards. When studying and considering the various control options, however, it is important to consider the restrictions imposed on growers regarding the use of harmful chemical insecticides and the effect of insecticides on the evolution of insecticide resistance. The investigation of alternative control strategies therefore is pertinent in enhancing IPM strategies. It is clear that there is potential for the use of parasitoid wasps and EPNs to control *H. capensis*. The review highlights the need to commercialise smaller, native EPN species and to test a variety of parasitoids (especially the trichogrammatids, encyrtid and eulophid species identified in this review) against *H. capensis* in the laboratory and field environments. Furthermore, continued research will increase the current knowledge of *H. capensis* and the use of chemical and biological control options that potentially could be used as a reference for studies focused on other emerging leaf-mining pests, such as *T. absoluta*, in South Africa.

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Evolution of Phenolic Composition During Barrel and Bottle Aging

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During red wine ageing, phenolic compounds undergo several reactions that have an impact on wine colour and mouthfeel properties. The evolution of phenolic content is affected during wine ageing. The aim of this study was to investigate the phenolic content and evolution of 82 commercial red wines subjected to barrel and bottle ageing. The phenolic content evolution of wines that underwent an ageing period of 12 months in commercial 225 litre barrels, followed by 12 months in the bottle, was monitored. While the total phenolic content remained stable, the anthocyanin fraction was affected the most, which led to substantial changes in the colour properties of the wines. Differences were found during both ageing regimes, indicating certain phenolic reactions being favoured or compromised under different ageing conditions, with an impact on wine colour properties. This paper provides the first large-scale study on the phenolic evolution of commercial red wine during ageing.

INTRODUCTION

Phenolic compounds contribute an essential part to the organoleptic attributes of red wines. The roles played by this group of compounds in some of the most important quality attributes of wine lead to an increasing demand for phenolic analysis during winemaking. Among phenolic compounds, two groups, viz. anthocyanins and tannins, are thought to play major roles in wine organoleptic properties (Casassa & Harbertson, 2014).

The role of anthocyanin is related mainly to the colour properties of wines, and indirectly to the intensity of astringency. The indirect role is due to the decrease in astringency perception derived from tannin-anthocyanin structures (Casassa & Harbertson, 2014). Anthocyanins initially are found in grapes in monomeric forms; however, due to their reactive nature, a large number of reactions and interactions involving anthocyanins take place during winemaking and ageing (He *et al*., 2012a).

Proanthocyanidins or tannins have the ability to interact with salivary proteins, leading to macromolecular complexes that evolve until they become insoluble and precipitate from solution, causing a drying sensation known as astringency (Barak & Kennedy, 2013). The astringency sub-qualities, as well as the bitter taste, are mainly due to tannins differing in composition and conformation. During winemaking, tannin-cleavage reactions take place together with polymerisation. These reactions, along with tannin insolubility, determine the evolution of the astringency intensity during wine ageing (McRae & Kennedy, 2011).

Once the fermentation is completed, red wines generally undergo an ageing process. Red wines are commonly exposed to barrel ageing, which lasts from a few months to a few years. Subsequently, red wines often are also aged in bottles for periods of variable duration. During ageing in barrels, red wines are often exposed to increased levels of oxygen, due to its incorporation into the wine matrix through the wood vessel. Some of the reactions between phenolic compounds are promoted by binding agents derived from the oxidation of wine components (Fulcrand *et al*., 2006; Oliveira *et al*., 2011). The reactions occurring during this oxidative process are thus different from those occurring in the absence or at low levels of oxygen, i.e. during the bottle-ageing period.

The development of efficient analytical procedures for phenolic analysis, such us the use of spectroscopy calibrations, can contribute to the phenolic information available to scientists and winemakers (Aleixandre-Tudo *et al*., 2018). On the other hand, the interpretation of the results obtained from current analytical practices is often not understood, and the generated information is therefore not fully utilised. A number of recent research studies have investigated the evolution of phenolic compounds during wine ageing (Sun *et al*., 2011; Gambuti *et al*., 2013; Gómez Gallego *et al*., 2013; Bimpilas *et al*., 2015). However, these studies normally only report data during bottle ageing, and very often conclusions are made based on the small number of wines made from the same batch of grapes under experimental conditions. Although relevant intrinsic value is provided to the scientific community, an evident gap between the generated knowledge and the commercial/industrial applicability of the results obtained is also observed.

The main aim of this research study was to investigate

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the evolution of phenolic content and composition of a large number of commercial wines subjected to barrel and bottle ageing through the evaluation of several phenolic and colour parameters. This study might provide a useful contribution to wine producers, potentially extending knowledge of the evolution of phenolic compounds during wine ageing.

MATERIALS AND METHODS

Reagents

Hydrochloric acid, sodium metabisulphite, acetaldehyde, tartaric acid and sodium hydroxide were purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

Wine samples

Eighty-two red wines (2016 vintage) were initially sourced from wineries in the Western Cape region of South Africa after the completion of malolactic fermentation. The sample set included Cabernet Sauvignon (23), Shiraz (19), Pinotage (13), Merlot (11), Ruby Cabernet (2), Cabernet Franc (4), Cinsault (1), Grenache noir (1), Malbec (1), Mourvedre (1), Petit Verdot (4) and Pinot Noir (2), with the number of wines per cultivar shown in brackets. The wines were aged for one year in barrels in an unsupervised approach, i.e. barrels were from different origins, toast intensity and/or number of fills. After barrel ageing, the wines were sampled and bottled under screw cap at the experimental cellar at Stellenbosch University (South Africa), where an additional bottle-ageing period of one year was undertaken under controlled temperature (15°C). Sulphur dioxide addition was also unsupervised. No instructions were given to the collaborating wineries, and oenological additions were done according to their standard procedures. The wines were therefore analysed after the malolactic fermentation process (T0), as well as after a year of barrel ageing (T1) and after a year of bottle ageing (T2) (two years of ageing in total).

Phenolic analysis

The modified Somers assay method was used to quantify chemical age 1 and 2, ionised anthocyanins (mg/L), total anthocyanins (mg/L), SO_2-resistant pigments and the total phenolic content (Mercurio *et al*., 2007). Specifically, the colour corresponding to the yellow (420 nm yellow), red (520 nm red) and blue (620 nm blue) wine components was measured, in addition to the total colour intensity (colour density) from the addition of the aforementioned wine colorations (Aleixandre-Tudo *et al*., 2017). The hue was also calculated as the ratio between the red and yellow wine colours.

Statistical analysis

Mixed-model ANOVA was used to statistically evaluate significant differences between levels of the phenolic measurements during the ageing period. The unsupervised experimental approach followed in this study caused some of the samples to be lost during the ageing process due to the producers blending the wines. One of the advantages of mixed-model ANOVA applied to longitudinal data is that wines with incomplete data over the ageing process can still be included (Gibbons *et al*., 2010). Moreover, the existence of significant differences between the change (increase or decrease) taking place during both ageing regimes was also approached statistically. A less conservative Fisher LSD post hoc test at the 0.05 confidence interval was used. Fisher LSD was selected in favour of detecting findings that might be false, rather than missing important results as non-significant. Data analysis was performed with the statistical software package STATISTICA (version 13, TIBCO Software Inc. 2017, http://statistica.io).

RESULTS AND DISCUSSION

A significant decrease in the total phenolic content (TP) was observed after 12 months of barrel ageing, accompanied by a non-significant decrease between 12 and 24 months of ageing (Table 1). This indicates increased phenolic stability during the bottle-ageing period, with wines being exposed to a less-oxidative environment. Significant differences between the variation in total phenolic content during both ageing regimes were also observed (Table 2), confirming a notable decrease in the total phenolic content during barrel ageing. The majority of the reported studies showed stable total phenolic content during the first few months of ageing, and a slight decrease when wines were evaluated after a few years (Mazza *et al*., 1999; Darias-Martín *et al*., 2007; Gambuti *et al*., 2013; McRae *et al*., 2013; Rodrigues *et al*., 2013; Bimpilas *et al*., 2015). On the contrary, an initial decrease in the total phenolic content, followed by stability over an ageing period of two years, was reported in Tempranillo wines (Revilla & López, 2005). These latter results, in line with our study, might indicate that the total phenolic content of a young wine is initially altered by oxygen exposure during a certain period, after which the phenolic content becomes less prone to participate in oxidation/degradation reactions.

Monomeric forms of anthocyanins are sensitive to changing pH conditions, SO_2 bleaching and water decolouration (Mercurio *et al*., 2007; Aleixandre-Tudo *et al*., 2017). The total anthocyanin content showed significant decreasing levels during the ageing process in our study (Table 1). The results highlight the reactive nature of anthocyanins participating in reactions that span from degradation to self-associations or combinations with other grape and wine components, including other phenolic compounds (He *et al*., 2012b). The Somers assay measures the simple monomeric anthocyanin forms, which include monomeric anthocyanins and copigmented molecules. The studies reported in the literature showed a steady decrease in the total anthocyanin content during bottle ageing for periods up to 42 months (Kwiatkowski, 2003; Darias-Martín *et al*., 2007; Gambuti *et al*., 2013; McRae *et al*., 2013). Moreover, a study on Tempranillo wines showed notable decreases in the anthocyanin content during the first years of barrel ageing (Revilla & López, 2005). A slight decrease was observed during the second year, which is in agreement with this study, bearing in mind that our second year of ageing was in bottles (Revilla & López, 2005). In a similar way as for the total phenolic content, it seems that the magnitude of the anthocyanin reactivity slows down after an initial period in the barrel. However, instead of reaching stability, anthocyanins continue their evolution during the ageing process.

TABLE 1
Values for average and standard deviation of the phenolic parameters and colour measurements of the wines included in the study. The wines were analysed at the end of fermentation, after 12 months in barrels and after another 12 months in bottles (24 months of total ageing time). Significant differences are shown between the time points. N corresponds to the number of wines at the time of evaluation.

	Time 0 months (N = 82)	Time 12 months (N = 69)	Time 24 months (N = 61)
Total phenolics	56.54 ± 12.06 a	53.41 ± 9.50b	52.97 ± 9.61b
Total anthocyanins (mg/L)	471.06 ± 125.65 a	336.84 ± 72.58b	264.12 ± 61.80c
SO_2-resistant pigments	2.78 ± 0.90 c	3.25 ± 0.97b	3.93 ± 1.21a
Ionisation %	22.65 ± 5.49 b	25.17 ± 5.94b	47.63 ± 24.94a
Chemical age 1	0.33 ± 0.05 c	0.47 ± 0.05b	0.56 ± 0.06a
Chemical age 2	0.12 ± 0.03 c	0.19 ± 0.04b	0.29 ± 0.08a
420 nm yellow	4.98 ± 1.51 c	7.26 ± 2.75a	6.51 ± 1.79b
520 nm red	8.50 ± 3.37 b	10.53 ± 3.83a	9.07 ± 2.77b
620 nm blue	1.86 ± 0.69 c	2.59 ± 1.05a	2.22 ± 0.70b
Colour density	15.35 ± 5.50 c	20.38 ± 7.42a	17.80 ± 5.21b
Hue	0.61 ± 0.09 c	0.70 ± 0.12b	0.73 ± 0.06a

Wines made with Cabernet Sauvignon (23), Shiraz (19), Pinotage (13), Merlot (11), Ruby Cabernet (2), Cabernet Franc (4), Cinsault (1), Grenache noir (1), Malbec (1), Mourvedre (1), Petit Verdot (4) and Pinot Noir grapes (2) were used in the study. The number of wines per cultivar is shown in brackets.

TABLE 2
Increase or decrease between the phenolic levels observed during the barrel and bottle ageing periods. Significant differences between the two periods are reported. N corresponds to the number of wines at the time of evaluation.

	T0-T1 (N = 69)	T1-T2 (N = 61)
Total phenolics index	3.20 ± 5.01 a	0.98 ± 2.48 b
Total Anthocyanins (mg/L)	132.88 ± 65.06 a	70.00 ± 28.12 b
SO2 resistant pigments	-0.51 ± 0.41	-0.64 ± 0.51
Ionization %	-2.41 ± 7.74 a	-22.20 ± 21.17 b
Chemical Age 1	-0.14 ± 0.04 b	-0.08 ± 0.05 a
Chemical Age 2	-0.07 ± 0.03 a	-0.10 ± 0.06 b
420 nm yellow	-2.30 ± 2.49 b	0.90 ± 2.14 a
520 nm red	-2.05 ± 3.65 b	1.61 ± 2.55 a
620 nm blue	-0.74 ± 0.94 b	0.41 ± 0.80 a
Colour density	-5.09 ± 6.83 b	2.92 ± 5.28 a
Hue	-0.09 ± 0.12 b	-0.02 ± 0.10 a

Wines made with Cabernet Sauvignon (23), Shiraz (19), Pinotage (13), Merlot (11), Ruby Cabernet (2), Cabernet Franc (4), Cinsault (1), Grenache noir (1), Malbec (1), Mourvedre (1), Petit Verdot (4), and Pinot Noir grapes (2) were used in the study. The number of wines per cultivar is shown in brackets.

Ionised anthocyanins include those with a positively charged flavilium cation and provide an indication of the monomeric structures responsible for wine colour. A non-significant effect in anthocyanin ionisation (%) during the barrel-ageing period was followed by a significant increase during the bottle-ageing process (Tables 1 and 2). Despite the decrease in the total content of anthocyanins during both ageing periods, the percentage of anthocyanins in the ionised form was at the highest levels at the end of the bottle-ageing period. In agreement with our study, an increase in the ionisation percentage during bottle ageing was reported in Cabernet Sauvignon and Listan Negro wines (Kwiatkowski, 2003; Darias-Martín *et al*., 2007). However, in contrast to what was observed in this study, the increase in the ionisation

percentage seemed to be linear during the 24 months' ageing process (Kwiatkowski, 2003). The absence of barrel ageing in the above-mentioned studies limits a direct comparison.

During ageing, simple monomeric forms of anthocyanins lead to an anthocyanin content mostly represented by other, more complex polymeric structures (Waterhouse *et al*., 2016). It is therefore expected that, in combination with anthocyanin oxidation and degradation, the presence of new pigments, as well as the potential co-precipitation with tannins or other wine components, accounts for the decrease in the monomeric anthocyanin content during wine ageing (Cheynier *et al*., 2006; He *et al*., 2012b). The SO_2-resistant pigments, ChA1 and ChA2, parameters that provide the proportion of monomeric to polymeric forms of anthocyanins, are significantly increased during both ageing regimes, as well as during the overall ageing process (Table 1). This may be explained by increased polymeric pigment formation, which includes self-anthocyanin associations, direct and indirect tannin-anthocyanin reactions, pyranoanthocyanin formation or intramolecular copigmented structures (He *et al*., 2012b; Morata, 2019). Moreover, despite anthocyanin degradation occurring to a larger extent during barrel ageing, the polymeric pigment-formation kinetics were not altered during both ageing regimes (Table 2). Several studies have reported an increase in and steady formation of SO_2-resistant pigments during bottle ageing periods of variable duration up to 24 months (Kwiatkowski, 2003; Gambuti *et al*., 2013; Rodrigues *et al*., 2013; McRae *et al*., 2015). Additionally, a decrease in the SO_2-resistant pigment levels after three years of bottle ageing has been reported (Bindon *et al*., 2014), suggesting reversible reactions or net loss of non- bleachable resistant pigments.

A limited number of references were found in the literature reporting on the evolution of the chemical age indexes over time (Kwiatkowski, 2003; Darias-Martín *et al*., 2007). As expected, both these studies showed an increase in the index during ageing. Differently to what was observed here (Table 2), a linear increase was reported over a 24-month ageing period (Kwiatkowski, 2003). However, the ageing process happened in bottles, restricting a direct comparison with the current study.

Due to the wavelength absorbance nature of some of the phenolic compounds in their simple and also more polymerised forms, colour measurements provide a good indication of the status of phenolic structures in red wines (Kennedy *et al*., 2006). A significant change in colour was observed during the red wine ageing process, with bluish colourations being turned into more brownish hues (Morata, 2019). Interestingly, the yellow and blue tonalities in our study showed maximum values after the barrel-ageing process, with a subsequent slight decrease observed after bottle ageing (Table 1). Similar results were found for the red colouration; however, no significant differences were found between the initial and final sampling stage. These results are in agreement with a micro-oxygenation study, in which the three colour components increased after a 12-month ageing period (Baiano *et al*., 2016). On the other hand, the total colour intensity increased significantly when wines were exposed to barrel-ageing conditions, whereas a significant decrease was observed after bottle ageing (Table 1). The exposure to oxygen, in combination with the interactions between the wine and wood phenolic compounds, may explain the observed results (Versari *et al*., 2013; Gombau *et al*., 2016). Contradictory results were found in the literature, with studies reporting either an increase or a decrease in colour density during ageing in bottles of variable duration (McRae *et al*., 2015; Baiano *et al*., 2016; Gambuti *et al*., 2017; Petrozziello *et al*., 2018). However, other studies have reported increases and then decreases in colour intensity in red wines matured in barrels (Revilla & López, 2005; Laqui-Estaña *et al*., 2018). Noticeably, and despite the decreased levels of total phenolics observed over time, the new phenolic combinations seem to give rise to phenolic structures with enhanced colouration activity (Du Toit *et al*., 2006). In addition, a significant increase in the hue was also observed during the ageing period, with a greater increase occurring during barrel ageing compared to bottle ageing (Table 2). This is consistent with what has been reported in the literature. It could be explained not only by oxidation reactions, but also due to formation of some of the polymeric pigments, characterised by increased yellow-orange tonalities (pyranoanthocyanins, xanthylium or chalcone forms) (He *et al*., 2012b; Waterhouse *et al*., 2016).

CONCLUSIONS

Stability was observed for the total phenolic content of the wines over the ageing period evaluated. The decrease in phenolics found was mainly due to a decrease in the anthocyanin content. An intense polymeric pigment formation seems to have occurred, affecting the colour properties of the wines. Barrel ageing promotes the intensity of the wine colour, with a subsequent decrease after bottle ageing. Formation of polymeric pigmented structures with enhanced colour properties, but with chemical and oxidative degradation of anthocyanins, is expected to take place during ageing. In addition, cleavage and re-arrangement processes might be the cause of the decrease in colour intensity attributes during bottle ageing. The phenolic chemistry occurring during ageing thus seems to be based on a complex equilibrium involving additional pigment formation and the breakdown and re-arrangement of existing chemical structures. This study shows the importance of considering the ageing regime (barrel or bottle) to better understand the phenolic reactions taking place during wine ageing. However, further studies need to be conducted to fully understand the nature of the reactions, as well as the phenomena taking place in red wines during ageing.

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Alteration Index Three Facilitates Interpretation of β-Glucosidase, Acid-phosphatase and Urease Activities in Soils Irrigated with Diluted Winery Wastewater

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Soil enzyme activity data from a lysimeter pot trial involving four dissimilar soils irrigated over two simulated seasons (SS) with winery wastewater (WW) and municipal water (MW), were converted to numerical scores using the alteration index three (AI3). Unlike the activities of individual enzymes which are substrate specific and do not reflect the overall status of the soil microbiome, AI3 enabled differences in treatment-induced alteration states between combinations of soil and water to be quantified and statistically assessed. The more negative AI3 scores corresponded to a more altered state in the WW than the MW treatments, after SS3 than after SS4 and in the 0-10 cm compared to the 10-20 cm soil depth interval. AI3 is therefore a potentially useful adjunct to soil enzyme activity assays in monitoring and management of biological activity in vineyard soils. These findings, however, require verification under commercial vineyard conditions.

INTRODUCTION

Wineries produce substantial volumes of wastewater, so re-using this wastewater to irrigate vineyards could hold many potential benefits for the wine industry (Conradie *et al*., 2014; Howell & Myburgh, 2018). However, there are legal requirements in terms of the General Authorisations published in the Government Notice Nr. 399 (26 March 2004) in terms of section 39 of the National Water Act (1998) that govern the use of winery wastewater for irrigation purposes. Untreated winery wastewater may not be discharged into the environment but has to be treated prior to discharge.

The quantity and composition of winery wastewater (WW) vary, depending on the processes taking place in the winery, most of which are seasonal (Howell & Myburgh, 2018). Changes in water quality may affect soil chemistry and physical parameters such as hydraulic conductivity, ponding and gas exchange, all of which are likely to affect soil microbiology, enzyme activity and organic matter decomposition. Both water quality and soil enzyme activities should, therefore, be monitored frequently, for which a fast and reliable method of analysis, and easy data interpretation is essential.

Soil enzyme activities, notably that of β-glucosidase, phosphatase and urease (carbon (C), phosphorus (P) & nitrogen (N) cycling, respectively), are rapid and sensitive indicators of soil health and soil ecosystem sustainability (Pascual *et al*., 2000; Moebius-Clune *et al*., 2016; Adetunji *et al*., 2017), and are commonly used in biological assays (Adetunji *et al*., 2017). However, because soil enzymes are substrate specific, their individual activities are not indicative of overall biological activity (Adetunji *et al*., 2017). Neither do the activities of all soil enzymes react in the same way when soil or irrigation water parameters change (Mulidzi & Wooldridge, 2016). Consequently, the activities of single, or even of multiple enzymes are not readily interpreted. Interpretation of soil enzyme activities may nevertheless be facilitated by converting the enzyme activities to indexes (Puglisi *et al*., 2006).

Alteration index three (AI3), which was developed and validated by Puglisi *et al.* (2006), is a data reduction process that combines and balances the activities of β-glucosidase,

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phosphatase and urease into scores reflecting the degree of positive or negative change (alteration). This alteration may have been brought about in the soil microbiome by natural (e.g. erosion (Garcia & Hernández, 1997)) or anthropogenic processes (e.g. pollution (Leirós *et al*., 1999; Trasar-Cepeda *et al*., 2000)) and soil organic ameliorants (Meyer *et al.*, 2014)). In an apple orchard where factorial combinations of organic and conventional treatments had been applied, Meyer *et al*. (2014) reported that AI3 was able to differentiate between treatments, to correlate with soil organic matter (SOM) content, and to correlate with yield and yield efficiency, thereby linking AI3 with orchard soil surface management practices, and with tree performance.

Unlike soil parameters such as pH and P content, AI3 scores do not have ideal, or target values. They range from negative to positive (Puglisi *et al.,* 2006) and are commonly used to compare treated (altered) with untreated but otherwise similar soils (controls). After reviewing published data notably that of Caravaca *et al*. (2002), Puglisi *et al*. (2006) concluded that AI3 scores were more negative where control soils were generally characterised by higher total organic carbon contents (TOC) than negatively altered (treated) soils. Ghosh *et al.* (2020) also showed that AI3 scores become increasingly more negative with increasing soil organic content. For example, they showed that, in the 0-15 cm soil layers of soybean-wheat, AI3 tracked soil organic carbon (SOC) levels of 0.62, 0.68, 0.82, 0.90, 0.89, 1.04 with scores of, respectively, -28.5, -29.7, -30.8, -33.0, -35.2, -36.9. On the contrary, AI3 scores could also become progressively less negative, or even positive, as the degree of alteration increased, and *vice versa*. To this effect, Leirós *et al*. (1999) showed that, 28 days after application, AI3 tracked copper application rates of 0, 1 000, 2 000, 5 000 and 10 000 mg/kg with scores of, respectively, -257, -176, -111, -54 and 12. Alteration index three may also be used as an indicator of rehabilitation, as where Hinojosa *et al*. (2004) observed AI3 scores of -3.1, -24.4 and -36.6, in mining effluent, for polluted, rehabilitated and non-polluted catchments, respectively. According to Puglisi *et al*. (2006), the discriminating power of AI3 was appreciable ($p > 0.005$).

From the foregoing, AI3 appears to provide a simpler and more formally derived indication of the extent of soil alteration than can be obtained by interpreting the activities of individual enzymes. To test this supposition AI3 scores were calculated from, and compared with, enzyme activities in differently textured soils that had been irrigated with diluted (WW) and municipal water (MW).

MATERIALS AND METHODS

The research of which this article is an extension, has been described by Mulidzi and Wooldridge (2016), and Mulidzi *et al*. (2016). In summary, composite samples (homogenised after collection) of four pedogenetically different soils were collected from 0-30 cm soil intervals at four actual vineyard locations in the Western Cape (Table 1), packed into pots (200 mm lengths of 150 mm PVC pipe on perforated bases, compressed to a bulk density of 1 400 $kg.m^{-3}$) and drip irrigated to saturation whenever the gravimetric soil water potential decreased by 85%, using either MW, or WW that had been diluted (Myburgh *et al.*, 2015) with MW to a chemical oxygen demand (COD) of 3000 mg/L. Alluvial vineyard soil (Longlands form) from Rawsonville (Rv), an aeolian veld soil (Garies form) from Lutzville (Lv), as well as shale (St.s) and granite (St.g) derived soils (Oakleaf and Cartref forms, respectively) from Stellenbosch were used in this experiment (Soil Classification Work Group, 1991). Initial pH, COD and P levels in the MW were 7.4, 27.9 and 1.1 mg/L, respectively. Corresponding values for the WW were 5.4, 3210 and 4.7 mg/L, respectively. Difficulties were experienced in restoring field capacity in some of the soils, ponding and run-off of water being observed in some cases, and lack of drainage after irrigation in others (Mulidzi *et al.*, 2016).

After six irrigation events (one simulated season (SS)), a pot was removed from each water x soil treatment for analysis. Four such simulated seasons were applied, but the samples from SS1 and SS2 were set aside on the grounds that equilibrium had probably not been reached during these seasons. After SS3 and SS4, the soil from the 0-10 cm and 10-20 cm depth intervals from each pot were analysed to determine the activities of β-glucosidase, acid phosphatase and urease by colourimetry (Tabatabai & Bremner, 1969;

TABLE 1
Characteristics of soils used in the lysimeter trial (adapted from Mulidzi *et al.* (2016)).

Parameter	Lutzville (Lv)	Rawsonville (Rv)	Stellenbosch shale (St.s)	Stellenbosch granite (St.g)
Co-ordinates	-31.559 ˚S 18.353 ˚E	-33.694 ˚S 19.323 ˚E	-33.912 ˚S 18.871 ˚E	-33.917 ˚S 18.865 ˚E
Clay	0.4	3.3	20	13
Silt	1	1	13	17
Fine sand	69	60	50	33
Medium sand	26	29	5	3
Coarse sand	2	8	12	35
Soil textural class	Fine sand	Fine sand	Fine sandy loam	Coarse sandy loam
Kaolinite [a]	w, m	w, m	m	s, d

[a]X-ray diffraction peak intensity (t, trace; w, weak; m, moderate; s, subdominant, d, dominant). Wooldridge, 1988.

Eivazi & Tabatabai, 1988; Kandeler & Gerber, 1988). Total organic carbon (TOC) levels were determined by the Walkley and Black method (1934) in the original soil samples and after SS3 and SS4 (Table 2).

The enzyme activity data were converted to AI3 scores (Table 3) using the relationship of Puglisi *et al*. (2006):

AI3 = (7.87 x β-glucosidase) - (8.22 x phosphatase) – (0.49 x urease) Eq. 1.

where enzyme activities were expressed in micromoles of, respectively, p-nitrophenyl-β-D-glucoside and p-nitrophenylphosphate per gram of soil per hour, and micrograms of urea per gram of soil per hour. The loadings (7.87, 8.22 and 0.49) used in this relationship were derived by Puglisi *et al.* (2006) using a dimension reducing technique resembling principle component analysis (CDA) and the canonical discriminant analysis procedure (PROC CANDISC and PROC STEPDISC (SAS Institute, 1985)).

Each of the soil (4) x water (2) treatments was replicated in four blocks in a fully randomized split-plot design with soil and water as main treatments and simulated season and depth interval as sub-plot factors. The data were tested for normality by the method of Shapiro & Wilk (1965) and found to be acceptably normally distributed and subjected to analysis of variance (ANOVA) using SAS version 9.2 (SAS Institute, 2008). Student's t-test and least significant difference values (LSD) were calculated at the 5% probability level to facilitate comparison between treatment means (Ott, 1998). Means within data sets that differed at the 5% probability level were considered significantly different.

RESULTS AND DISCUSSION

The enzyme activity, soil and water data used in this article are derived from the research of Mulidzi *et al.* (2016) and Mulidzi and Wooldridge (2016). In the present article these data are discussed in the context of AI3.

Effects of soils on AI3

Mulidzi and Wooldridge (2016) reported that acid phosphatase activities responded to the soil treatments differently than urease and β-glucosidase, implying that interpretation of activities of disparate groups of enzymes will only be successful if variation in enzyme activity is taken into account, as in the AI3 relationship.

Averaged over all treatments, AI3 scores became increasingly negative, indicating a progressive change in soil alteration, in the sequence: Lv < Rv < St.s ≤ St.g, *i.e.* with increasing clay, silt and TOC content (Tables 2 & 3). Figure 1 supports this link between AI3, clay, silt and TOC; components that are usually associated with soil quality.

TABLE 2
Total organic carbon (TOC) contents of experimental soils, namely, Lutzville sand (Lv), Rawsonville sand (Rv), Stellenbosch shale (St.s) and Stellenbosch granite (St.g) before irrigation and after simulated seasons (SS) three and four (Mulidzi & Wooldridge, 2016).

Season	TOC (%)									
	Lv		Rv		St.s		St.g		Mean	
	Mean	SD[a]	Mean	SD	Mean	SD	Mean	SD		SD
Initial[b]	0.20	-	0.80	-	1.50	-	1.30	-	-	-
SS 3	0.19e[c]	0.06	0.82c	0.04	1.22a	0.22	1.28a	0.12	0.88a	0.46
SS 4	0.06f	0.02	0.61d	0.08	0.86c	0.09	1.02b	0.21	0.64b	0.38
p-value (time)									(<0.0001)	
p-value (soil x time)					(0.079)					
Decr.[d]	68.0%		25.6%		29.5%		20.3%		27.3%	

[a] Standard deviation. [b] Descriptive statistics of the means due to once off observation. [c] Values in the same data set, that are followed by the same letter, do not differ significantly ($p \leq 0.05$). [d] Decrease in TOC from SS 3 to SS 4 (%).

TABLE 3
Effect of soil over simulated seasons three and four, water source and combined sample depth intervals (0-20 cm) on alteration index three (AI3) of the four soils, namely, Lutzville sand (Lv), Rawsonville sand (Rv), Stellenbosch shale (St.s) and Stellenbosch granite (St.g).

Treatment	AI3*			
	Lv	Rv	St.s	St.g
Soil mean	-3,52c[a]	-10.31b	-19.18a	-20.59a

* Alteration decreases as AI3 scores become increasingly negative. [a] Values in the same data set, followed by the same letter, do not differ significantly ($p \leq 0.05$).

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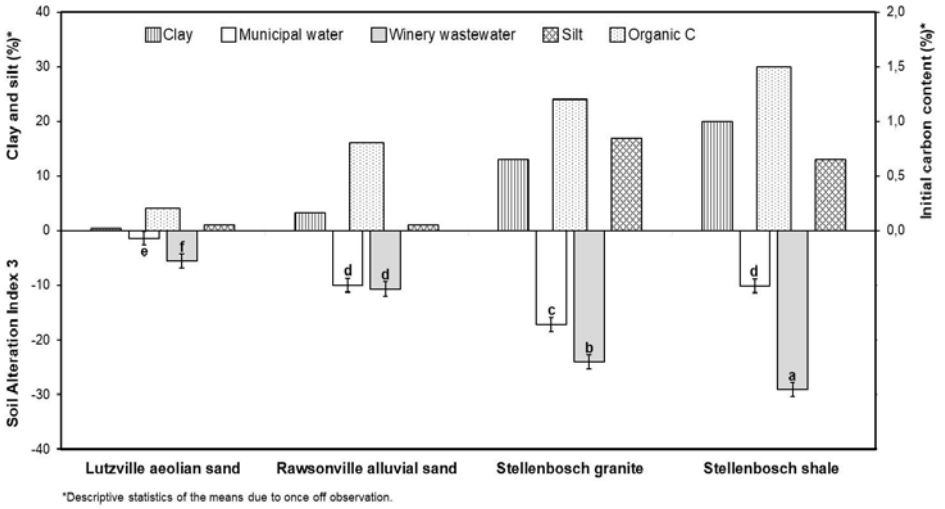

FIGURE 1

Soil alteration response, % clay, silt and organic carbon contents, following irrigation with diluted winery wastewater over four simulated seasons. Bar values represented by the same letters do not differ significantly ($p \leq 0.05$).

This indicated that AI3 was able to distinguish between soil quality, with Lv and Rv (*i.e.* sandy soils) being low fertility soils, and St.s and St.g (*i.e.* fine and coarse sandy loam soils) of better quality, associated with their higher TOC, silt and clay contents (Tables 2 & 3). A large portion of enzymes is protected by physio-chemically adsorption to soil clay minerals where they are immobilized and stabilized (Tabatabai, 1994), thereby influencing their activity (Dick & Tabatabai, 1992). Enzymes also naturally bind to the humus fraction in organic matter through various mechanisms (Ladd & Butler, 1975). Since organic matter also bind to clay minerals, it increases the surface area and number of active sites compared to silt and sand (Frankenberger & Johanson, 1982), thereby enhancing enzyme adsorption. Thus, soils with a higher clay and organic matter content tend to have higher soil enzyme activities than sandy soils (Dick *et al*., 1996) because they have a greater ability to store organic matter than sandy soils (Gispert *et al*., 2013). As derived from Mulidzi & Wooldridge (2016) and confirmed by AI3, activities of the three enzymes were higher in St.s and St.g soils, inferring that these soils function better than the low fertility sandy soils. It stresses the importance of knowing the clay and organic matter content of a soil that is to be altered because this determines its ability to protect microbial secreted enzymes responsible for decomposing organic matter and thus, the release of plant nutrients.

The AI3 scores did not differ between St.s and St.g despite differences in clay (Table 1), silt and organic carbon content, although the activities of the three individual enzymes differed significantly (Mulidzi & Wooldridge, 2016). This variation in the individual enzyme activity trends between these soils was strongly regulated by the difference in the type of clay mineral content (Olagoke *et al.* 2019) in each soil or by the combination of clay, silt and TOC. Mulidzi and Wooldridge (2016) reported that the respective activity trends of β-glucosidase, acid phosphatase and urease in St.s followed the exact opposite trend in St.g, thereby influencing the outcome of the AI3 scores. Since the weighting applied to acid phosphatase (8.22) and to β-glucosidase (7.87) in the AI3 equation is higher than that of urease (0.49), small changes in acid phosphatase and β-glucosidase activities have a greater effect on AI3 than a change of similar magnitude in urease. Therefore, when applied, the AI3 can quantify the balance between the activities of these three enzymes.

The AI3 showed that the degree of alteration in the St.s and St.g after WW irrigation, would be similar in magnitude because both soil types hold similar enzymatic activity potential compared to Lv and Rv soils.

Water source

Average (all soils) AI3 scores were more negative in the WW than the MW soils (Table 4), implying a greater degree of alteration in the former. The differences in AI3 ranged from 7.6% in the high-P alluvial vineyard soil (Rv) to 286% in the aeolian soil (Lv), with an average change for the four soils of 77.5%.

An explanation for the more negative AI3 scores from the WW compared with the MW supply is that soil enzyme activity increased significantly after inputs of WW, suggesting that WW may contain substrates that induced the activities of β-glucosidase, acid phosphatase and urease. Previous studies have shown that WW contained easily decomposable organic compounds such as urethane and cellubiose (Fidaleo *et al.*, 2006; Villena *et al*., 2007) found in grape musts and yeasts that are substrates for urease and β-glucosidase activity, respectively. Winery wastewater also contains traces of phosphorous in the form of inorganic and organic compounds, of which the latter may act as substrate for acid phosphatase activity.

The Lv soils had the highest AI3 WW to AI3 MW ratio (3.9:1), and by implication, was altered the most, i.e. was most responsive to WW irrigation, followed by the St.s (2.9:1) and the St.g (1.4:1), whereas Rv remained relatively unaltered (1.1:1). The intensity of these alterations was nevertheless dependent on soil aggregate stability (Mulidzi & Myburgh, 2014; Mulidzi *et al*., 2015), as well as a decline

TABLE 4
Effect of winery wastewater (WW) and municipal water (MW) over soil, simulated seasons (SS) three and four and combined sample depth intervals (0- 20 cm) on alteration index three (AI3).

Treatment	AI3*				
	Lv	Rv	St.s	St.g	Mean
MW	-1.45f[a]	-9.93d	-10.05d	-17.13c	-9.64b
WW	-5.59e	-10.68d	-29.13a	-24.06b	-17.11a
Difference	286%	7.6%	190%	40.5%	77.5%

* Alteration decreases as AI3 scores become increasingly negative. [a] Values in the same data set, followed by the same letter, do not differ significantly ($p \leq 0.05$).

in the initial TOC (Table 2).

According to Six and Jastrow (2002) organic matter has a shorter mean residence time in sandy soils (macroaggregates), as was the case with Lv which showed a phenomenal decline of about 70% of the initial TOC. Thus, soil with low clay and organic carbon contents will have the quickest turnover of organic matter after multiple WW inputs. The high clay dispersion reported by Mulidzi & Myburgh (2014) and Mulidzi (2015), together with an excessive decline of 43% in initial TOC after WW inputs caused the alteration in the St.s. Although the St.g showed signs of structural damage (Mulidzi & Myburgh, 2014; Mulidzi, 2015) it still retained around 80% of the initial TOC after 24 WW inputs. The Rv soil was well-aerated and well-drained during WW inputs (Mulidzi *et al*., 2016) and retained 80% of its initial TOC, hence its relatively unaltered state.

This study indicated that enzyme activity induced by WW inputs will play an important role in the decomposition of organic matter which will vary across soil textural range.

Simulated season

Average AI3 scores were less negative after SS4 than after SS3 (Table 5). Changes in AI3 between simulated seasons did not appear to be linked to texture (Table 1), but rather to differences in the availability of metabolizable substrate after SS3 sampling date. In support of this assumption the mean TOC levels (across both water treatments) in the SS4 soil samples were 27.3% lower than in SS3 soils and their initial pre-trial state (Table 2); most likely due to leaching of TOC and of enzymes after the initial accumulation or build-up to SS3.

A further explanation for the less negative AI3 scores at SS4, is that repeated additions of MW and WW had also affected the pH of soils. The pH of the MW decreased from 7.4 to 5.6, while that of the diluted WW increased from 5.4 to 7.1, which will in turn affect the optimum range of the three enzyme activities and thus the AI3 scores (data not shown).

Sample depth

AI3 could accurately reflect gradients in mineralizable substrates across a transect of two soil layers with the top layers being consistently higher than the subsoil. For each of the soils, as well as the all-soils average, the AI3 scores were more negative in the 0-10 cm than the 10-20 cm soil depth interval (Table 6), which supports work reported by Mulidzi & Wooldridge (2016). Percentage-wise, the difference between the two depths in each soil ranged in sequence: 61% (Lv) > 58% (St.s) > 33% (Rv) > 16% St.g. That AI3 was more negative (by 39.4% on average) in the 0-10 than the 10-20 cm intervals may have been at least partially due to differences in soil oxygen content and a shortage of metabolizable substrate energy (Jackson *et al*., 2019).

Treatment interactions

From the season x water interaction (Table 7) it is evident that alteration levels were signified by strongly negative average AI3 scores in the SS3 x WW treatment combination, in which alteration increased in intensity in the soil sequence: Lv ≥ Rv > St.g > St.s. Alteration levels were signified with the least negative mean AI3 scores in the SS4 x MW combination, notably in the fine, sandy Lv soil. Evidently, under the prevailing trial conditions, irrigating soils with WW improved AI3 scores to a greater extent than irrigation with MW. This supports Kumar *et al.* (2006) who reported that irrigation with WW does not adversely impact microbial activity and, in their research, promoted it.

Implications

As shown in Table 3, AI3 was able to quantify, compare and contrast alteration states, apparently with a reasonable, though as yet unquantified, level of sensitivity. AI3 not only differentiated between soils, water sources, simulated seasons and sample depths but also, using the scores allocated to each combination of the foregoing factors, facilitated direct comparison between combinations of these factors. AI3 scores not only reflect changes in enzyme activity due to natural changes in the environment, such as erosion, or to management practices, as in the present case where WW was substituted with for MW, but also to stages of recovery from such events or practices (Puglisi *et al*., 2006).

Although AI3 facilitates interpretation of soil enzyme activities, it does not identify the cause of any observed alteration. In terms of the soil health concept, which entails managing soils so that they remain fit for their purpose and able to support crops into the future (Moebius-Clune *et al.*, 2016), monitoring of soil physical, chemical and biochemical parameters must therefore be an ongoing process (Howell & Myburgh, 2018).

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TABLE 5
Effect of simulated seasons (SS) three and four over soil, water source and combined sample depth intervals (0-20 cm) on alteration index three (AI3).

Treatment	AI3*				
	Lv	Rv	St.s	St.g	Mean
SS3	-4.89de[a]	-11.57bc	-23.07a	-25.93a	-16.22a
SS4	-2.15e	-9.05cd	-15.61b	-15.27b	-10.52b

* Alteration decreases as AI3 scores become increasingly negative. [a] Values in the same data set, followed by the same letter, do not differ significantly ($p \leq 0.05$).

TABLE 6
Effect of two sample depth intervals (0-10 and 10-20 cm) over soil, water source and simulated seasons (SS) three and four on alteration index three (AI3).

Sample depth interval	AI3*				
	Lv	Rv	St.s	St.g	Mean
0-10	-5.05f[a]	-12.34d	-26.55a	-22.34b	-16.57a
10-20	-1.98g	-8.27e	-11.13d	-18.85c	-10.04b

*Alteration decreases as AI3 scores become increasingly negative. [a] Values in the same data set, followed by the same letter, do not differ significantly ($p \leq 0.05$).

TABLE 7
Effect of treatment interaction over soil, water source (winery wastewater (WW) and municipal water (MW)), simulated seasons (SS) three and four and combined sample depth intervals (0-20 cm) on alteration index three (AI3).

Treatment combination	Alteration Index (AI3)*				Mean
	Lutzville (Lv)	Rawsonville (Rv)	Stellenbosch		
			Shale (St.s)	Granite (St.g)	
SS3 x MW	-1.76f[a]	-11.53d	-10.79d	-22.44c	-11.63b
SS4 x MW	-1.14f	-8.33de	-9.32d	-11.83c	-7.65c
SS3 x WW	-8.02de	-11.61d	-37.81a	-29.42b	-21.01a
SS4 x WW	-3.16ef	-9.76d	-21.89c	-18.70c	-13.38b

* Alteration decreases as AI3 scores become increasingly negative. [a] Values in the same data set, followed by the same letter, do not differ significantly ($p \leq 0.05$).

CONCLUSIONS

AI3 generates numerical scores from the activities of urease, phosphatase and β-glucosidase. These scores enable differences in treatment-induced alteration states between applied treatment combinations to be quantified and compared. Uncertainties that arise where the activities of enzymes are interpreted individually are eliminated, although the activities of urease, phosphatase and β-glucosidase must still be determined. The AI3 index is therefore an aid or adjunct to interpretation rather than an alternative to enzyme analysis. In this role it has potential for use in the monitoring and management of enzymatic activity in vineyard soils. The results obtained from this lysimeter trial must be verified by field testing under vineyard field conditions over a range of soil and water combinations, ideally in combination with different soil surface management practices.

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Is There a Link Between Coffee Aroma and the Level of Furanmethanethiol (FMT) in Pinotage Wines?

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Over the years, Pinotage has found its way into the South African and international market. Producers have used the flavour potential of this "original" South African grape to produce different wine styles, one of them being the so-called "coffee-style Pinotage". The current study aims to explain the impact of furanmethanethiol (FMT) on the characteristic coffee aroma of these coffee-style wines. Chemical and sensory evaluation, as well as data mining of the technical information available, was performed. Not all wines marketed as "coffee Pinotage" showed a high "coffee" rating. However, the results showed a good correlation between the aroma perception and FMT concentrations ($R^2 = 0.81$). However, RV coefficients were low when comparing the coffee rating with the information provided on both the front and the back label, which shows that, in some cases, the use of the "coffee Pinotage" term was rather part of the marketing strategy.

INTRODUCTION

Innovation and product development are key to the success of any industry. Any changes introduced during processing can alter the physicochemical properties of a product and consequently influence its sensory space. In winemaking, different vineyard management techniques, as well as the use of different winemaking techniques, can modulate the aroma (Ruiz *et al*., 2019), taste and mouthfeel of a wine (Smith *et al*., 2015). Although the global wine industry has always been associated with tradition, it continuously seeks to explore new markets, as well as to implement new technologies to ensure better control of the winemaking process.

Wine aroma is one of the main drivers of the identity of a wine. The smell of a wine is the result of series of synergistic and masking interactions between aroma compounds and the non-volatile compounds in the wine matrix (Ferreira *et al*., 2015; Garrido-Bañuelos *et al*., 2020; McKay & Buica, 2020). These molecules are like the different characters in a book: individually they can be associated with specific features, but when interacting they can build a story. In short, the aroma of a wine can help us to detect not only the wine faults (Chatonnet *et al*., 2004; Mayr *et al*., 2015), but also to identify the grape cultivar, region, country or even some of the winemaking techniques employed in the process (Ferreira, 2010). Some of these compounds are strongly associated with certain wines, such as rotundone and the black pepper smell in Australian Shiraz (Siebert *et al*., 2008), and the presence of certain lactones in dessert wines (Stamatopoulos *et al*., 2015). Some of these compounds can be considered quality drivers for wines (Brand *et al*., 2020).

Similarly, the coffee aroma in wines has been associated with specific molecules, such as 2-furanmethanethiol (FMT) in Bordeaux wines (Tominaga *et al*., 2000). Despite certain furan derivatives being converted into alcohols with coffee-like notes by the yeast, this aroma is generally linked to the wine ageing in oak barrels and the size and level of toasting of the wood pieces (Fourie, 2005; Fernández de Simón *et al*., 2010).

The understanding of the odorant impact of FMT is of special interest for the South African wine industry. Pinotage is the "original" South African grape of excellence, but achieving an understanding of its flavour potential and acceptance in specific markets is an ongoing pursuit (Vannevel, 2015). Pinotage can be produced in different styles, and the so-called "coffee style Pinotage" is well known. Traditional Pinotage-style wine displays notes of 'chocolate box', 'banana', 'fruity', 'tobacco' and 'toasty', whereas the coffee-style wines have notes of 'chocolate box', 'banana', 'smoky', 'burnt rubber' and 'roast coffee bean' (Marais & Jolly, 2004; Naudé & Rohwer, 2013). The volatile fingerprint of Pinotage wines has not been investigated widely (Weldegergis *et al*., 2011), but the combination of furan and 2-furanmethanol and its role in the perception of the coffee

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aroma have been demonstrated by comparing traditional Pinotage to coffee-style Pinotage (Naudé & Rohwer, 2013). FMT and 2-furanmethanol have a similar chemical structure, where an S atom in the former (a thiol) replaces the O atom in the latter (an alcohol). Both compounds are found in roasted coffee and exhibit its particular aroma (pubchem.ncbi.nlm.nih.gov). The presence of FMT was not found in Pinotage wines by Naudé and Rohwer (2013), but a more recent study has shown that the levels of this thiol in Pinotage wines can range between 0.9 and 186 ng/L (Mafata *et al*., 2018).

The present study investigated the correlation between the levels of FMT and the perception of the "coffee" aroma in some South African Pinotage wines from both the traditional and coffee styles. In addition, the information available on the labels and in technical notes was evaluated to see how accurate it is in relation to this wine style.

MATERIALS AND METHODS

Wines and sensory evaluation

The study took place early in 2018. A total of thirteen wines, all commercially available, were selected for the study (Table 1). Nine out of the thirteen wines were marketed as "coffee Pinotage" by using images and descriptors on the front and back labels related to coffee, mocha and/or chocolate. The following Pinotage wines were from the 2015 vintage: PT1, PT2, PT10 and PT 11; the rest of the selected wines were from 2016 vintage. All wines had an alcohol content of 13.5% to 14% according to the labels.

All wines were evaluated in duplicate by a total of 15 trained panellists from the analytical panel of the Department of Viticulture and Oenology of Stellenbosch University. The wines were presented in a randomised order, introducing two blind duplicates (PT1-R and PT2-R) according to a Williams Latin square design. The evaluation was performed under room-controlled conditions. The coffee aroma of the wines was rated on an unstructured linear scale (from 0, corresponding with no coffee aroma, to 100, corresponding with high coffee aroma). The experimental design and data capturing were done with Compusense cloud software (Compusense Inc, Guelph, ON, Canada).

Thiol analysis

Thiol analysis was performed for all the wines according to the method of Mafata *et al*. (2018). The main interest of this work was to assess the relationship between FMT and coffee aroma, but the levels of 3MH, 3MHA and 4MMP were also quantified. Sample preparation was done by derivatising the thiol moiety (-SH) of 3MH, 3MHA, 4MMP and FMT with 4,4'-dithiodipyridine (DTDP). Sample purification was based on a solid-phase extraction on an SPE-ENVI-C18 cartridge (Supelco, Bellefonte, USA), followed by analysis by UPC^2-MS/MS (Waters Corporation, Milford, USA) on a BEH C18 column (Waters Corporation). Detailed information regarding the derivatisation, sample preparation and chromatographic conditions are as described in Mafata *et al*. (2018).

Label information

Graphic or text references to coffee were obtained from the front and back labels of the selected wines. If the labels did not include aroma profiles, descriptors were extracted from the technical notes provided on the producers' sites.

Statistical analysis

The sensory results were analysed with a two-way ANOVA (including the judge as a random factor and the wine as fixed factor) and post-hoc Tukey's test with STATISTICA 13 (Palo Alto, CA, USA). The results obtained on thiol content were analysed with a one-way ANOVA. Multifactorial analysis (MFA) was performed between the text data based on the frequency of citation of the different descriptors found on the wines labels. Text data was separated into three categories. First, front label, for which a data matrix was made on a binary base. A value of 1 was given to the wines that at least made reference to 'coffee/mocha/chocolate' on the front label, whereas a value of 0 was assigned to wine with no mention of these attributes. Back label data were split into two categories: coffee-related attributes (coffee, mocha and chocolate) and other descriptors (rest of attributes displayed on the back label of all selected wines). Other descriptors found on the wine labels were 'berry', 'fruity', 'fruitcake', 'raspberry', 'cranberries', 'spicy', 'plum', 'cinnamon', 'cherry', 'mulberry', 'prune', 'oak', 'Turkish delight', 'nuts', 'rooibos' and 'honeybush'. A data matrix for the sensory results was included as intensity values. The RV coefficient obtained from MFA was used to compare the configuration of the distribution of the wines according to the different variables.

TABLE 1
Wines used for the study

Wine codes	Vintage	Marketed as "coffee Pinotage"
PT1	2015	Yes
PT1-R	2015	Yes
PT2	2015	No
PT2-R	2015	No
PT3	2016	No
PT4	2016	Yes
PT5	2016	Yes
PT6	2016	Yes
PT7	2016	Yes
PT8	2016	No
PT9	2016	Yes
PT10	2015	No
PT11	2015	Yes
PT12	2016	Yes
PT13	2016	Yes

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RESULTS AND DISCUSSION

Rating results

The rating task was considered of medium difficulty by the judges. Fig. 1 illustrates the results of the one-way ANOVA on the rating results. The repeatability of the two blind duplicates was excellent (PT1 vs PT1-R and PT2 vs PT2-R, Fig. 1). The rating of the coffee aroma of the wines was therefore found to be a discriminant variable between the products. The ANOVA analysis showed that the PT9 wine was perceived as having the highest coffee aroma intensity on the nose (average 70.03 out of 100), followed by PT4 and PT6. However, the coffee aroma in PT4 and PT6 were not significantly higher than in PT5 (Fig. 1). Despite ten (nine plus blind duplicate) of the total wines being marketed as "coffee" Pinotage, the rest of the wines showed relatively lower and not statistically different intensities in the coffee aroma. Also, no significant differences were found between the Pinotage wines not marketed as "coffee" Pinotage (PT2, PT3, PT8 and PT10) and some of those that were.

Chemistry results and correlation with sensory results

The concentrations of the four thiols are given in Table 2. The level of FMT covered a wide range, from 6 ng/L to 138 ng/L; three wines stood out through their very high level of this compound, namely PT9, PT4 and PT6 (133 ng/L to 138 ng/L FMT), while most of the wines were in the range of 20 ng/L to 40 ng/L, and none were in the mid-range. Considering the concentration ranges of the other thiols, the wines were not very different from each other. All thiols were present above their odour thresholds, of 60, 4.2, 0.8 and 0.4 ng/L for 3MH, 3MHA, 4MMP and FMT, respectively (Mafata *et al*., 2018).

A good correlation was found between the level of FMT and the rating of the wines (R^2 = 0.81). This finding was indicative of a good correlation between the thiol concentration and the sensory perception – better than previously found for other single thiols in complex matrices (Garrido-Bañuelos *et al*., 2020). This possibly was due to the particular aroma imparted by this thiol to the wine, or to the Pinotage matrix, or a combination of both. Fig. 2 illustrates the relationship between the concentration of FMT and the intensity of the coffee aroma perceived in the wines. It can be observed that the three wines that showed the highest intensity of coffee aroma had the highest levels of FMT. It can also be noted that some wines with a lower FMT concentration were perceived to have a more intense coffee aroma than some with higher FMT concentrations, such as PT5 compared to PT13. In fact, PT5 (marketed as coffee style) had a lower level of FMT than some non-coffee Pinotage wines, but a higher perceived intensity of the aroma, indicating that the interaction of FMT with other compounds could have had an influence on the perception.

The next step was to explore the possible role of the other measured thiols (3-MH, 3-MHA and 4-MMP) in the perception of the coffee aroma. A recent study has shown that the interaction between 3-MH and 4-MMP in dearomatised red wine matrices results in "herbaceous, buttery and coffee aromas", with high levels of 3MH (Garrido-Bañuelos *et al*., 2020), but the interaction with FMT was not explored. In the current study, 3-MH was found to be the major thiol in all wines, with the exception of PT4. A good correlation was not found between the levels of 3-MH and the coffee smell (R^2 = 0.04). However, a good negative correlation was found between the percentage of total thiols represented by 3-MH and the rating of the coffee smell (R^2 = 0.85). In other words, the lower the percentage of 3-MH, the higher the perception of coffee aroma. Two of the wines with the highest

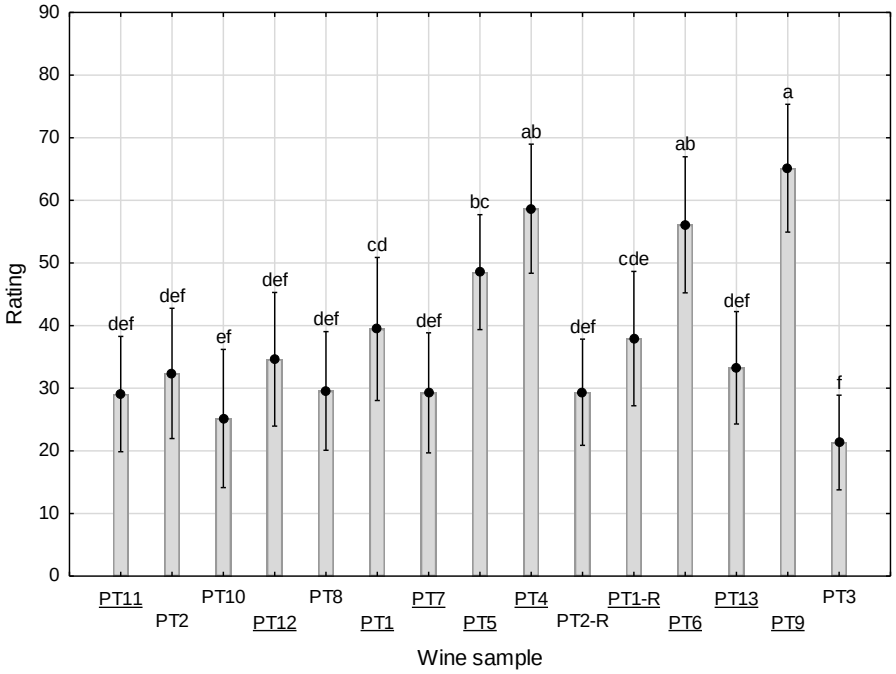

FIGURE 1

Results of the coffee aroma rating. Letters represent significant differences. Underlined codes correspond to samples of wines marketed as coffee/mocha/chocolate Pinotage.

TABLE 2
Thiol content (ng/L) of the Pinotage wines included in the experiment. Underlined codes correspond to samples of wines marketed as coffee/mocha/chocolate Pinotage.

Wine code	3MHA	4MMP	4FMT	3MH
PT1	5.98	1.88	44.06	126.66
PT2	5.91	2.38	29.82	155.06
PT3	7.79	1.65	9.56	172.11
PT4	5.77	1.1	133.36	108.79
PT5	5.20	1.9	26.24	98.92
PT6	9.79	2.37	138.00	163.42
PT7	9.03	1.75	39.27	150.82
PT8	7.12	1.96	23.62	178.06
PT9	6.82	1.26	136.58	161.54
PT10	4.86	1.31	6.03	149.25
PT11	5.87	1.07	5.92	82.15
PT12	5.54	1.20	25.96	119.30
PT13	4.62	1.38	56.15	151.52

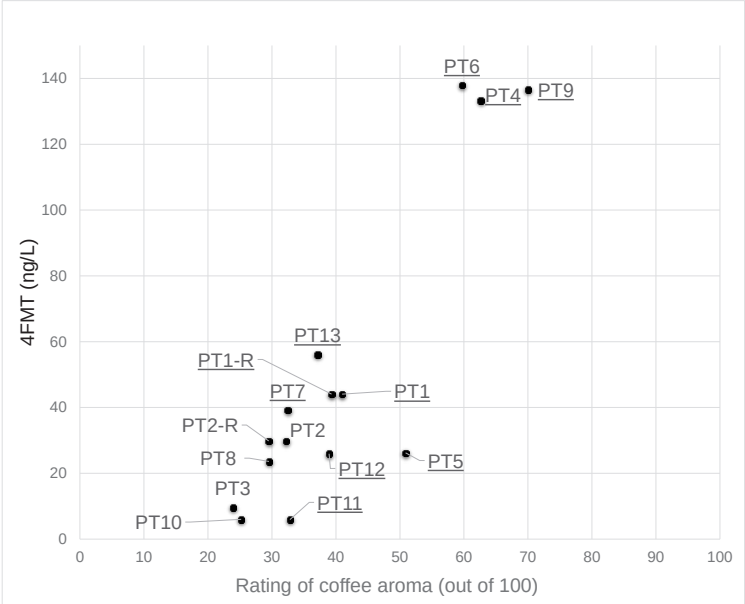

FIGURE 2
Correlation between FMT concentrations (ng/L) and rating of coffee aroma. Underlined codes correspond to samples of wines marketed as coffee/mocha/chocolate Pinotage.

concentration of 3-MH (PT8 and PT3) were perceived to have the lowest intensity of coffee smell. However, wines such as PT4 and PT9, with a high level of 3-MH, were perceived to have a high intensity of coffee aroma; this could simply be attributed to their levels of FMT. In the previously cited work (Garrido-Bañuelos *et al.*, 2020), the authors did not find any particular attribute associated with the presence of 3-MH in red wines. On the other hand, FMT has a prominent coffee smell and, as shown in this work, the high levels of FMT correlated with a high perception of coffee aroma.

Correlation with label descriptors

An MFA was performed to establish the relationships between the configuration of the wines according to the information offered on the front and back labels and the rating of the coffee aroma perceived by the panel. The explained variance

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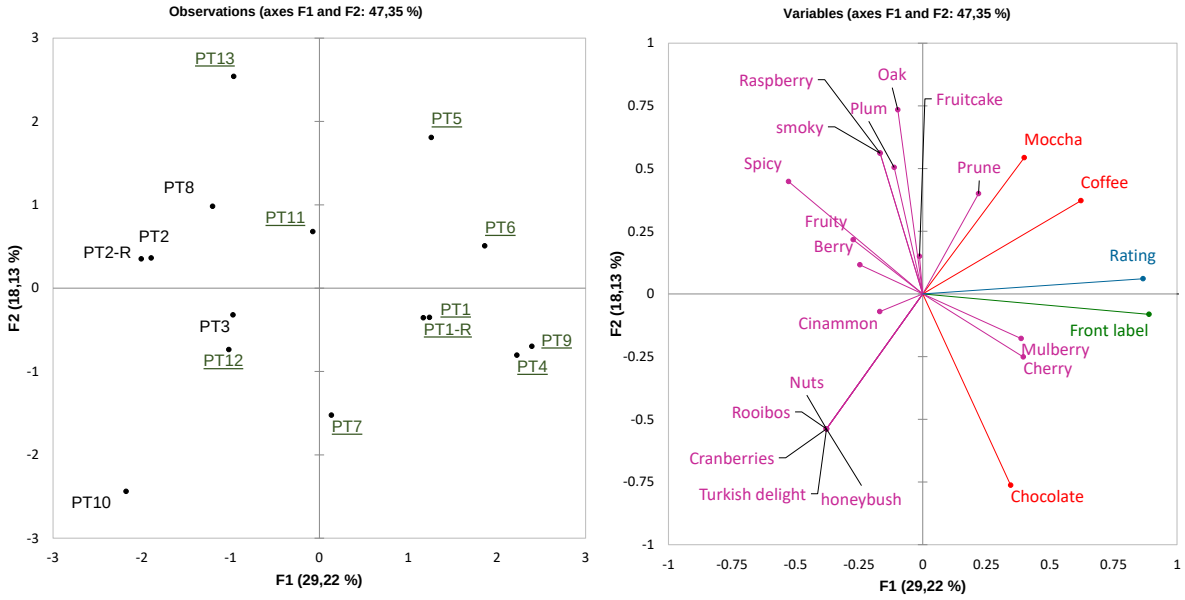

FIGURE 3

MFA (scores on the left, loadings on the right) representing the relationship between the descriptors from the technical information and the rating of the coffee aroma perceived by the analytical panellists. Underlined codes correspond to samples of wines marketed as coffee/mocha/chocolate Pinotage.

TABLE 3

Pair-wise RV coefficient values between the blocks constituting the matrix subjected to MFA and between the blocks and the multiblock matrix.

	MFA DATA BLOCKS				
RV COEFFICIENT	Front label	Coffee-related descriptors	Other descriptors	Rating	MFA
Front label	1.000	0.251	0.175	0.417	0.619
Coffee-related descriptors	0.251	1.000	0.290	0.196	0.670
Other descriptors	0.175	0.290	1.000	0.170	0.727
Rating	0.417	0.196	0.170	1.000	0.595
MFA	0.619	0.670	0.727	0.595	1.000

of the MFA map for the first two factors was 47.35%. On the score plot in Fig. 3, the wines not marketed as coffee style can be observed on the left side of the horizontal axis, opposite to most of the coffee-style samples. This also corresponds (on the loadings plot) to the configuration of the rating and the usage of coffee-related descriptors on the front and back labels, where 'coffee', 'mocha' and 'chocolate' were considered as sensory attributes linked to the coffee style. When considering the data blocks, the highest RV coefficients (Table 3) were found between the coffee-related descriptors on the front label and the coffee rating (0.42); however, this is a low RV value. The RV coefficient of the data block of coffee-related attributes on the back label vs the coffee rating from the sensory evaluation was even lower (0.20). This could be explained by the presence of other attributes to describe the wines on the back label; the similarity in the configuration from the MFA (including all blocks) and the block containing the "other descriptors" was the highest (RV 0.727).

CONCLUSIONS

The study has shown a clear relationship between the levels of FMT and the perception of coffee aroma in South African coffee-style Pinotage wines. However, not all the wines marketed in this way are either perceived to have a coffee aroma or are chemically characterised by higher levels of FMT. This shows that some of the wines marketed as coffee-style Pinotage would be perceived as such; however, in some cases, it appears to be more of a marketing strategy.

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