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# Periodic Determination of Physical and Physiological Responses to High Temperature Stress in cv. Narince Grapes

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**Stress is a condition that affects or even inhibits growth, development and metabolism in plants. The type of stress, duration of application and severity also affect plants significantly. In this study, the weekly responses of Narince, a wine grape variety, to high temperature stress (HTS) in *in vitro* conditions were followed and stress was applied in two different periods, for 12 and 18 hours at 40°C. Some growth development (viability, fresh shoot weight, dry shoot weight, number of leaves, shoot length), physiological (ion flow and relative water content (RWC)) and biochemical (malondialdehyde (MDA)) analyses were done, with weekly follow-ups after the applications. In addition, the damage was graded visually. At the end of the application of both stresses, the percentage viability rate started to decrease. This rate decreased more slowly during HTS for 12 hours, while a faster decrease was observed in HTS for 18 hours. The number of healthy plants to be evaluated could not be reached after the fourth week. Growth and development parameters – fresh shoot weight (g), dry shoot weight (g) and shoot length (mm) – decreased after both stress applications, and these values were found to be statistically significant. After 12 hours of HTS application, the highest MDA value was determined at 0.274 nmol in the sixth week, while the lowest MDA value was determined in the first week, at 0.137 nmol. After 18 hours of HTS application, the highest MDA value was determined in the fourth week, at 0.263 nmol, while the lowest MDA value of 0.103 nmol was determined in the first week.**

## INTRODUCTION

Most species belonging to the genus *Vitis* are easily grown in many areas of the world where there are favourable climatic conditions. *Vitis vinifera* L., which is one of these species, is grown in many countries as table, dried and wine grapes due to its high adaptability to temperate climatic regions (Keller, 2015).

Successful viticulture depends on the complementary relationship between vine genetics and climatic factors (Keller 2015; Fraga *et al.*, 2016). This has come about because individual species carrying the genes for tolerance to cold and/or hot climates have adapted to climates suitable for their genetic characteristics. However, the anomalies that occur in the climate from time to time affect the vines negatively (Köse & Güleriyüz, 2009; Fraga *et al.*, 2016).

Plants do not always have the optimum conditions necessary for their development, and since they do not possess a movement mechanism, such as moving away from the environment where they are located, the lack or excess of these conditions causes stress. Stress is examined in two ways: abiotic (drought, high/low temperature, salinity, etc.) and biotic (viruses, bacteria, etc.) stress (Levitt, 1980).

The temperature conditions of the environment where the plant is located affect plant growth and development

significantly (Cramer, 2010). High temperature affects plant growth, metabolic activities and production, and is defined as the increase in temperature above the critical level for a certain period of time and is one of the most important abiotic stresses (Wahid, 2007; Hasaruzzaman *et al.*, 2012).

The optimum daily temperature for photosynthesis, yield and fruit ripening in vine development is 30°C. It has been stated that heat stress can occur with an increase in temperature of 5°C in relation to optimum growth conditions in general (Keller, 2010). The rate of adaptation to high temperatures is high in vines. However, this adaptation is not unlimited, and it has been stated that the optimum leaf temperature for photosynthesis can increase up to 33°C (Schultz, 2000).

At high temperatures, cell damage and even death occur. The reason for this can be attributed to the destructive collapse of cellular organisation (Wahid *et al.*, 2007). Many physical properties can be investigated as indicators of temperature damage. Some of the indicators of gas exchange in photosynthesis are net photosynthesis rate, stomatal conductivity, thermostability in membranes, ion flux and thiobarbituric acid-reactive substance content, and chlorophyll content (Xu *et al.*, 2000; Rosyara *et al.*, 2010).

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The response of the vine to high temperature stress is one of the most important issues in vine biology and is important for the sustainability of viticulture. In this study, it was aimed to observe the growth-development, physiological and biochemical changes of Narince, one of the grape varieties with a high wine value, under *in vitro* conditions with high temperature stress by monitoring these changes weekly.

## MATERIALS AND METHODS

### Material

The experiment was carried out by planting micro-cuttings obtained by maintaining the buds of the Narince grape variety. The explants were obtained from the Narince vineyard of the Agricultural Research and Application Farm of Tokat Gaziosmanpaşa University using the method of continuing the dormant cuttings. Cuttings were taken when dormant in February and maintained in the plant growth chamber.

### Method

#### *In vitro* propagation

*In vitro* clonal propagation of the cultivar used in the study was carried out by the axillary bud culture method in accordance with Sivritepe (1995). The aim of the *in vitro* clonal propagation process is to propagate a sufficient number for the experiment in shoot information. The shoots, which developed five to six buds in the plant-growing room, were taken from the end and made into one-bud shoots (micro-cutting) and brought to the tissue culture laboratory for surface sterilisation in autoclaved jars containing pure water. The micro-cuttings brought to the laboratory were washed under running water for 20 minutes, with one or two drops of Tween 20 being added at intervals of 10 minutes. The micro-cuttings, which were kept in 70% ethyl alcohol for 15 seconds, were then moved to a sterile cabinet and rinsed with pure water. Here, the surface sterilisation process was continued, and the micro-cuttings were kept in 0.5% sodium hypochlorite solution twice for 10 minutes, then passed through distilled water three times and kept in the third distilled water, which concluded the process (Sivritepe, 1995). MS nutrient medium (Murashige and Skoog, 1962; MS; M-5519i Sigma Chemical Co.), which is preferred for plant tissue cultures, was used as the basic nutrient medium. To this nutrient medium was added 0.5 mg/l BAP (6-benzylaminopurin; B3408, Sigma), 3% sucrose and 0.7% Bacto agar. The pH of the nutrient medium was adjusted to 5.8 without adding agar (Sivritepe, 1995). Regarding the shoots, 15 ml of nutrient medium was put into 105 cc jars and autoclaved at 121°C under 1.06 bar pressure for 20 minutes. One explant was planted in each jar. Stress treatments were started three weeks after planting.

#### Applications of high temperature stress

Heat stress was gradually increased to 40°C, which was the application temperature, for the explants in the plant growth cabinet, and the cycle was adjusted to 16 hours light and eight hours dark photoperiod at 25°C. The explants were exposed to high temperature stress at 40°C for 12 and 18 h.

After the stress treatments, the plants were brought back to the plant growth room of the tissue culture at 25°C, with a 16-hour light and eight-hour dark photoperiod. The

parameters examined were taken at 7-day intervals for 6 weeks, and data collection started on the day the stress applications ended. Growth-development and physiological parameters were recorded, and the samples for biochemical analysis were stored at -20°C until the date of analysis.

## Observations and analyses

### Growth development parameters

The percentage viability – the number of explants that survived after the high-temperature application – was determined. Shoot fresh weights (g) were obtained by weighing using precision scales sensitive to  $\pm 0.001$  g. shoot dry weights (g) were subsequently determined using a precision scale with  $\pm 0.001$  g sensitivity after drying in an oven at 65°C for 72 h. The shoots length were measured using a digital calliper. The number of leaves/shoots (pieces) was the total number of leaves on each shoot (Dalsou & Short, 1987).

### Physiological parameters

In terms of colour characteristics, the explants were removed from the nutrient medium after the applications and leaf colour was measured using a Minolta portable chromameter (Minolta, Model CR-400).

Explant water content was determined using the formula for relative water content (RWC) (%):  $[(FW-DW) / (TW-DW) \times 100]$  (Yamasaki & Dillenburg, 1999). Using the aforementioned formula, fresh weights (FW) and turgor weights (TW) were determined after keeping them in distilled water for six hours, and dry weights (DW) were determined after 24 h of storage at 80°C.

The ion flux (%) was determined using the method of Ozden *et al.* (2009) using leaf samples divided into equal parts. These samples of 0.3 g were placed in 25 mm  $\times$  150 mm glass tubes, to which 15 ml of distilled water was added. The prepared samples were shaken at 100 rpm for 24 hours in a shaker. After incubation, the electrical conductivity (EC1) of the solution was measured using an EC meter. Then the same samples were autoclaved at 115°C for 10 minutes. After the samples were kept at room temperature for 24 hours, the electrical conductivity (EC2) value of the solution was measured again. The ion flux in the leaves was calculated as  $EC1/EC2 \times 100$  and expressed as %.

### Determination of temperature damage

The degree of damage to the explants (1 to 4) was determined according to Topcu Altıncı (2016). The plant shoots and leaves that were not damaged were graded as zero degrees; those with yellowing and drying up to 25% of the leaves and shoots were rated as one degree; those with yellowing and drying in 50% of the leaves and shoots of the plants were rated as two degrees; those with yellowing and drying in 75% of the leaves and shoots of the plants were rated as three degrees; and those with yellowing and drying in 100% of the leaves and shoots of the plants (dead plants) were rated as four degrees.

### Biochemical analysis

Lipid peroxidation (MDA) was estimated by measuring the concentration of thiobarbituric acid reactive substances

(TBARS) using the thiobarbituric acid method described by Heath and Packer (1968). A total of 0.3 g of tissue was homogenised in 3 mL of 0.1 % (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10 000 x g for 10 min and 3 mL of 20 % TCA containing 0.5 % (w/v) 2-thiobarbituric acid (TBA) was added to 1 mL of supernatant. The mixture was heated at 95°C for 30 min and the reaction was stopped by quickly placing it in an ice bath. The cooled mixture was centrifuged at 10 000 x g for 10 min, and the absorbance of the supernatant was read at 532 and 600 nm. After subtracting the non-specific absorbance at 600 nm, the TBARS concentration was determined by its extinction coefficient of 155 mM<sup>-1</sup> cm<sup>-1</sup>.

### Statistical analysis

The experiment was carried out according to the randomised plot design. In the high-temperature stress applications of the trial, three replications were found at each application dose and 40 explants were used in each replication. The data were analysed with ANOVA by using SAS Version 9.1 software (SAS Institute Inc., Cary, NC, USA). Duncan's multiple-range test was used to separate treatment means when ANOVA showed significant differences among the means. The level of significance was set at 5%.

### RESULTS AND DISCUSSION

Statistical evaluation of the weekly data obtained after 12 and 18 hours of HTS applications at 40°C in the Narince cultivar grown in vitro are given in the tables below. After 12 hours of HTS, a six-week evaluation was made, and in the 18-hour HTS application, it was terminated after four weeks due to an insufficient number of live plants.

After the applications, viable explant counts were made in 10 of the explants removed from the plant growth chamber (Table 1). The percentage vitality (%) values, which were evaluated on a weekly basis after 12 and 18 hours of HTS applications, were found to be statistically significant. The highest number of viable explants was detected after 12 hours of HTS stress, with 90% in the first week, and the

lowest in the sixth week, at 70%. The highest number of viable explants was detected after 18 hours of stress, with 80% in the first week, and the lowest was detected in the fourth week, at 40% (Table 1). A gradual decrease in vitality was encountered in the 12-hour HTS, while the 18-hour HTS experienced a higher and faster decline.

In terms of shoot length (mm), there was a statistically significant difference ( $p < 0.05$ ) over weeks in the result of HTS applied for 12 hours (Table 1), while there was no statistical difference in the application of HTS for 18 hours (Table 1). After 12 hours of HTS, the longest shoot length was measured at 36.316 mm in the first week, while the shortest shoot length was measured at 22.016 mm in the sixth week. In terms of the number of leaves (number), there was no statistically significant difference on a weekly basis as a result of HTS applied for 12 and 18 hours ( $p < 0.05$ ) (Table 1).

The explants, which were removed from the plant growth chamber every week, were removed from the medium in which they were found, and their shoot fresh weight (g) was weighed. According to these values, 12 and 18 hour HTS applications led to statistical differences ( $p < 0.05$ ) in shoot fresh weight (g) on a weekly basis. After 12 hours of HTS applications, the highest shoot fresh weight was measured with 0.990 mg in the second week, while the lowest fresh weight was measured at the sixth week with 0.354 mg. In the 18 hour HTS applications, the highest wet weight was measured with 0.506 grams in the wet shoot weight in the second week, and the lowest weight was determined with 0.351 mg in the fourth week (Table 1).

In terms of shoot dry weight (mg), there were statistical differences ( $p < 0.05$ ) on a weekly basis as a result of HTS applied for 12 hours (Table 1), while there was no statistical difference in relation to 18 hours of HTS application. According to Table 1, the highest shoot dry weight after 12 hours of HTS was measured at 0.358 g in the second week, while the shoot dry weight was measured at 0.042 mg in the sixth week.

TABLE 1

Some growth development parameters after 12 and 18 hours of HTS applied to the Narince variety at 40°C.

	App.	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
% Viability	<b>12 h</b>	A 100 a	A 100 a	A 90 b	A 90 b	80 c	70 d
	<b>18 h</b>	B 80 a	B 60 b	B 50 c	B 40 d	-	-
Shoot length (mm)	<b>12 h</b>	A 36.316a	A 34.790ab	A 34.943 ab	A 29.080 bc	28.290 c	22.016 d
	<b>18 h</b>	A 28.636 a	A 26.130 a	A 27.033 a	A 28.453 a	-	-
Number of leaves/ shoots (piece)	<b>12 h</b>	A 8.66 a	A 7.33 a	A 7.00 a	A 7.66 a	6.66 a	5.33 a
	<b>18 h</b>	B 5.00 a	A 5.66 a	A 7.00 a	A 5.66 a	-	-
Shoot fresh weight (g)	<b>12 h</b>	A 0.858 a	A 0.990 a	A 0.734 a	A 0.402 b	0.398 b	0.354 b
	<b>18 h</b>	B 0.501 a	B 0.506 a	B 0.434 a	B 0.351 b	-	-
Shoot dry weight (g)	<b>12 h</b>	A 0.358 a	A 0.387 a	A 0.126 b	A 0.060 c	0.052 c	0.042 c
	<b>18 h</b>	B 0.074 a	B 0.062 a	B 0.048 a	A 0.030 a	-	-

The different lowercase letters and capital letters indicate statistically significant differences between the treatments ( $p < 0.05$ )

In order for plants to survive under adverse environmental factors, the specific optimum environmental demands of that plant species must be met. Any increase or decrease in these optimum demands creates stress for the plant. In other words, stress is a condition that affects or inhibits metabolism, growth and development in plants (Levitt, 1980). Stress is generally one of the growth parameters of the plant; viability is measured based on productivity, growth (biomass accumulation) and primary extraction processes (CO<sub>2</sub> and mineral uptake) (Zeiger & Taiz, 2006). The decreases in the growth and development of plants because of stress are shown in Table 1. Viability (%) decreased in parallel with the duration of high temperature stress. The results show that the duration of the stress is as effective as its severity, and it also causes the plants' ability to regenerate to decrease. When damage from high temperatures exceeds the ability to regulate adversity, severe heat damage symptoms and even plant death may occur (Zha *et al.*, 2018; Xu *et al.*, 2020). Topcu Altıncı (2016) also reported decreases in fresh shoot and dry shoot weight, shoot length and leaf number in high temperature stress studies of six different wine grape varieties.

Table 2 shows the statistical evaluation of the physiological parameters of HTS applied for 12 and 18 hours at 40°C in the Narince cultivar grown in vitro. In terms of relative water content (%), there was no statistically significant difference when the two applications were evaluated on a weekly basis. However, as the weeks progressed, it was observed that this parameter decreased in both applications.

The RWC is an important factor in determining the effects of changing temperatures (Mazorra *et al.*, 2002). The RWC is accepted as an important measurement method to determine the water potential of the plant in terms of reflecting metabolic activity in the tissues (Bertamini *et al.*, 2006; Chylinski *et al.*, 2007). Xiao *et al.* (2017) determined that the high temperature stress applied to the saplings of the Hongti grape variety at 38°C and 40°C and on different days affected the proportional water content significantly and, as the high temperature levels increased, the RWC in the leaves decreased significantly. They also reported that the primary reason may be that HTS caused a reduction in hydraulic conductance, leading to a decrease in water absorption.

When the L\* value is 0, it means that the colour of the grain shell is black, that is, there is no reflection, while when the L\* value is 100, it means that the colour is white, that is, the reflection is complete. In the "a\*" value, negative values indicate green (-60: green), while positive values indicate red (+60: red). In Table 2, there is no statistical difference based on the weekly evaluation of 12 and 18 hours of HTS application for the values of L and a, and based on 12 hours of HTS application for the b value. The value "b\*" denotes the position between the colours yellow and blue, with negative values denoting blue, and positive values denoting yellow (-60: blue, +60: yellow). Only in the 18-hour HTS application of the b value did statistical differences ( $p < 0.05$ ) emerge on the basis of the number of weeks.

In relation to the ion flow rate (%), there were statistical differences ( $p < 0.05$ ) on a weekly basis when HTS was applied for 12 hours (Fig. 1), while there was no statistical difference in the application of HTS for 18 hours (Fig. 1). After 12 hours of HTS, the highest ion flow rate (61.38%) was determined in the sixth week, while the lowest rate was measured in the second week, at 24.42%. After 18 hours of HTS, the highest ion flow rate (50.33%) was measured in the fourth week, while the lowest rate was measured in the first week (32.11%).

In Fig. 2, the MDA values are given after 12 and 18 hours of HTS applied at 40°C. In terms of MDA (nmol), it is seen that there are statistical differences ( $p < 0.05$ ) on a weekly basis as a result of HTS applied for 12 and 18 hours (Fig. 2). After 12 hours of HTS application, the highest MDA value was determined in the sixth week, at 0.274 nmol, while the lowest MDA value was determined in the first week, at 0.137 nmol. After 18 hours of HTS application, the highest MDA value was determined at 0.263 nmol in the fourth week, while the lowest MDA value was determined at 0.103 in the first week.

Cell damage and even death can occur at high temperatures. The reason for this can be attributed to the destructive collapse of cellular organisation (Wahid *et al.*, 2007). Many physical properties can be investigated as indicators of temperature damage. Some of those are gas exchange indicators in photosynthesis, net photosynthesis rate, stomatal conductivity (Berry & Bjorkman, 1980;

TABLE 2  
Some physiological parameters after 12 and 18 hours of HTS applied to the Narince variety at 40°C.

	App.	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
*RWC (%)	<b>12 h</b>	A 92.41 a	A 89.60 a	A 86.76 a	A 74.48 a	73.63 a	64.50 a
	<b>18 h</b>	A 81.09 a	A 77.93 a	A 70.67 a	A 65.65 a	-	-
L *	<b>12 h</b>	A 37.72 a	A 34.03 a	A 34.03 a	A 39.40 a	35.90 a	38.96 a
	<b>18 h</b>	A 35.24 a	A 36.71 a	A 30.93 a	A 38.48 a	-	-
a*	<b>12 h</b>	A -10.52 a	A -11.18 a	A -11.83 a	A -11.81 a	-13.11 a	-12.57 a
	<b>18 h</b>	A -9.92 a	A -11.32 a	A -10.41 a	A -10.44 a	-	-
b*	<b>12 h</b>	A 18.06 a	A 14.68 a	A 15.82 a	A 17.93 a	15.12 a	18.13 a
	<b>18 h</b>	A 16.06 ab	A 17.80 a	A 12.55 b	A 16.49 ab	-	-

The different lowercase letters and capital letters indicate statically significant differences between the treatment ( $p < 0.05$ )

Stafne *et al.*, 2000, 2001; Herzog & Chai-Arree, 2012), thermostability in membranes, ion flux, thiobarbituric acid-reactive substance (TBARS) content and chlorophyll content (Rosyara *et al.*, 2010; Xu *et al.*, 2000).

The cell membrane is thought to be the primary physical site damaged by heat stress (Basra *et al.*, 1993). This damage arises on the leaf tissue, and high stress weakens the cell membrane. In this situation there is a tendency for ion flow out of the cell. For this reason, the measurement of ion flow is widely used to determine temperature damage (Wahid *et al.*, 2007).

MDA is a biochemical that tends to increase under many stress factors. Wang *et al.* (2004) and Topcu Altıncı (2016) have mentioned the increase in the amount of MDA under the conditions of high temperature stress to which they submitted grape varieties. MDA (lipid peroxidation) is an analysis method used in the biochemical evaluation of the stress factor in many stress studies. Lipid peroxidation can be viewed as the end product of essential cell membrane reactive damage to cellular mechanisms (Ali *et al.*, 2005; Liu *et al.*, 2006), and the MDA content is an important indicator of the extent of membrane lipid peroxidation. Most importantly, the accumulation of MDA can further damage the membrane and cells (Chen, 1989). These results show that high temperature stress causes significant increases in MDA and ion flow. In his study, Topcu Altıncı (2016) reported that the damage increased by showing the increase in MDA and ion flux values with the increase in heat stress. According to Xiao *et al.* (2017), high heat stress applied to

grapevine saplings causes an increase in MDA and relative electrical conductivity (REC). These authors state that the reason for this increase is that high temperature disrupts the dynamic equilibrium between the production and removal of free radicals, which increases membrane lipid oxidation. It has also been reported that the increase in MDA content and its association with membrane protein causes membrane disruption and loss of function, increased plasma membrane permeability and, as a result, electrolyte leakage.

After 12 and 18 hours of HTS application at 40°C, 10 explants were used to visually assess the damage. Subsequently, the values obtained were expressed as percentages. Figs 3 and 4 show the proportional expression of the visual evaluation of the damage after the HTS application. In addition, the visual form of the rating is given in Fig. 5. No damage was observed on the plants in the first three weeks after 12-hour HTS application. As the weeks progressed, damage began to occur, and in the fifth and sixth weeks, degree 4 (plant death) was observed. After 18 hours of HTS application, degrees 0 and 1 were not recorded for 4 weeks. Degree 4 was detected in plants starting from the second week, reaching 40% damage in the fourth week.

CONCLUSIONS

This study aimed to examine the weekly course of high temperature stress in the Narince grape variety. The greenhouse effect caused by the CO<sub>2</sub> released into the atmosphere since the industrial revolution has been observed to have effects of high temperature, which pose

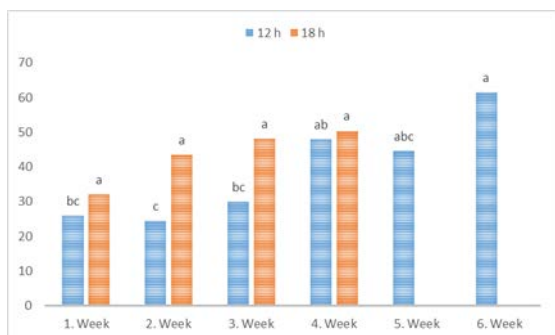


FIGURE 1  
Ion flow (%)



FIGURE 2  
MDA (nmol)

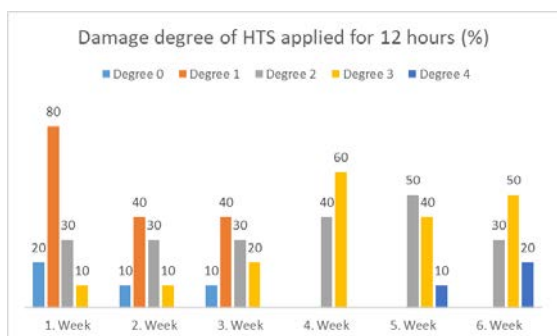


FIGURE 3  
Visual evaluation of damage after 12 hours of HTS treatment applied to the Narince variety at 40°C (%).

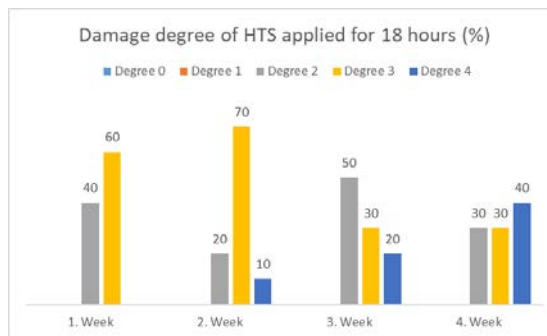


FIGURE 4  
Visual evaluation of damage after 18 hours of HTS treatment applied to the Narince variety at 40°C (%).



FIGURE 5

Photographs of the visual evaluation of damage after 12 and 18 hours of HTS treatment applied to the Narince variety at 40°C.

a threat to grape varieties. In this study, the morphological, physiological and biochemical effects of high temperature stress on plants were investigated. It was determined that the stress caused decreases in viability (%), shoot length (mm), and in shoot fresh and dry weight (g). No statistical differences were observed in the number of leaves, relative water content or changes in colour. While a gradual increase in ion flux (%) was observed in the treatment with 12 hours of HTS, no statistical difference was observed in the 18-hour HTS treatment. MDA content increased with stress to a statistically significant level. The results obtained showed that high temperature stress is not only recorded in climate change scenarios. The intensity of physical, physiological and biochemical damage to plants must be determined and the responses of plants should be analyzed. It is known that the type of stress, along with its duration and amount, are important in stress applications. In the light of these results, we need to develop studies that can offer solutions to the problems that high temperatures, which will be felt more intensely in the coming years, will create in plant production.

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# Long-term Effects of Irrigation with Treated Municipal Wastewater on Soil Chemical and Physical Responses in Commercial Vineyards in the Coastal Region of South Africa

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**The prolonged drought in the Western Cape province of South Africa in recent years has been particularly detrimental to the wine industry. Water restrictions imposed by the authorities, and the limited supply of fresh water that can be stored, have emphasised the need for alternative water sources for vineyard irrigation. Treated municipal wastewater has been used successfully as an alternative source of irrigation water in other countries. A long-term trial was conducted in commercial vineyards in the Coastal region of South Africa to assess the impact of treated municipal wastewater irrigation on vineyards. Cabernet Sauvignon and Sauvignon blanc grapevines were irrigated using treated municipal wastewater from the Potsdam wastewater treatment works for 11 years. Grapevines were either rainfed (RF), irrigated with treated municipal wastewater *via* a single dripper line (SLD), or received twice the volume of wastewater *via* a double dripper line (DLD). Irrigation using treated municipal wastewater increased soil  $\text{pH}_{(\text{KCl})}$ , the electrical conductivity of the saturated extract ( $\text{EC}_e$ ) and  $\text{Cl}$ . Substantial amounts of  $\text{Na}^+$  and  $\text{K}^+$  accumulated in the topsoil due to irrigation with treated municipal wastewater. These soil  $\text{K}^+$  increases could have a negative effect on wine colour stability should the levels of soil  $\text{K}^+$  be such that they are absorbed excessively by grapevines. The near-saturation hydraulic conductivity ( $K_{ns}$ ) at the surface of the soil could be related to the  $\text{EC}_e$  in the topsoil. The results represent specific in-field situations in three commercial vineyards under one set of climatic conditions.**

## INTRODUCTION

There have been frequent water shortages and below-average rainfall in the Western Cape of South Africa recently that have led to the worst drought in the province in recent memory. The drought was especially detrimental to the wine industry due to the carry-over effects of water constraints on grapevine growth and yield. Therefore, water scarcity is an increasingly important challenge for the viticultural sector in the region. These challenges have emphasised the need for alternative irrigation water sources. One such alternative could be treated municipal wastewater, which has been used as a source of irrigation water in many arid and semi-arid countries (Levy *et al.*, 2014). In South Africa, approximately 2 000 ha of vineyards in the Swartland region are being irrigated with treated municipal wastewater from the City of Cape Town's Potsdam wastewater treatment works

(WWTW) and the Malmesbury municipality (Myburgh, 2018). However, aside from the current study, there have been no other studies that have assessed the feasibility of using treated municipal wastewater for vineyard irrigation under South African conditions.

The irrigation of agricultural crops with municipal wastewater was recently reviewed by Hoogendijk *et al.* (2023a). In summary, there are possible benefits of using treated municipal wastewater for vineyard irrigation, as it can provide an extra source of water for irrigation that can improve grapevine growth and yield. Since domestic water sources often contain high amounts of macro-elements (Hoogendijk, 2019), nutrients such as nitrogen (N), phosphorous (P) and potassium ( $\text{K}^+$ ) can be recycled if applied *via* the irrigation water. Organic compounds present in the treated municipal wastewater may have

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positive effects on soil structural stability. However, the high levels of salts in treated municipal wastewater can affect the physical, chemical and biological properties of the soil. Sodium ( $\text{Na}^+$ ) and  $\text{K}^+$  are particularly detrimental in terms of soil structural stability and increasing soil salinity. The presence of large amounts of monovalent cations could also result in clay dispersion, which can subsequently clog soil pores and limit water movement into and through the soil. In addition, irrigation using  $\text{K}^+$ -rich wastewaters may lead to excessive  $\text{K}^+$  uptake by grapevines, which potentially can have a negative effect on wine quality (Laurenson *et al.*, 2012).

Although there is extensive literature available on the effect of irrigation with municipal wastewater on soil chemical properties (Hermon, 2011 and references therein; Hoogendijk *et al.* 2023a), there is very little information regarding the re-use of municipal wastewater for vineyard irrigation. In this regard, soil samples from vineyards in McLaren Vale, South Australia that were irrigated by means of drip irrigation with either mains water or municipal wastewater for four to 11 years indicated that irrigation with municipal wastewater increased soil  $\text{Na}^+$  and  $\text{Mg}^{2+}$ , but reduced soil Ca of a deep sand, clay loam and a hard-setting sandy loam soil (Laurenson, 2010). Increased soil salinity as a response to irrigation with municipal wastewater was reported for a vineyard in South Australia (McCarthy, 1981). In Spanish vineyards irrigated by means of drip irrigation, the use of treated municipal wastewater increased soil electrical conductivity of the saturated extract ( $\text{EC}_e$ ), N,  $\text{K}^+$ ,  $\text{Na}^+$ , magnesium ( $\text{Mg}^{2+}$ ) and manganese ( $\text{Mn}^{2+}$ ) compared to well water (De las Heras & Mañas, 2020). Treated municipal wastewater was used for vineyard irrigation in Australia and compared to freshwater irrigation where it was applied (Hermon, 2011). Pastures or areas adjacent to the vineyards and which were not irrigated were also sampled in a fence-line experimental design. The topsoil pH and EC were higher where treated municipal wastewater was used for irrigation, in comparison to irrigation with fresh water and in unirrigated pastures adjacent to the vineyards. The soil exchangeable sodium percentage (ESP) also increased, particularly in the soil layers up to 60 cm below the soil surface.

Taking the above-mentioned into consideration, the objective of this study was to assess the effects of long-term irrigation with treated municipal wastewater on soil chemical and physical properties in commercial vineyards in the Coastal region of the Western Cape.

## MATERIALS AND METHODS

### Site selection and vineyard characteristics

The field trial was carried out in commercial vineyards on a farm near the town of Philadelphia in the Western Cape, where three experimental sites were selected in different landscape positions. Details on site selection, vineyard characteristics and grapevine responses were reported previously (Howell *et al.*, 2022; Hoogendijk *et al.*, 2023b).

### Irrigation treatments and application

Details on the irrigation treatments and application were reported previously (Howell *et al.*, 2022; Hoogendijk *et al.*, 2023b). In summary, there were three different irrigation

treatments. The first treatment was rainfed (RF), which was considered to be a control treatment as no raw water was available for vineyard irrigation on the farm. The second treatment was drip irrigated with treated municipal wastewater *via* a single dripper line (SLD) in the grapevine row. The third treatment had a double dripper line (DLD), which supplied double the volume of wastewater in the grapevine row.

### Irrigation water origin and quality

An assessment of the water quality and nutrient load of the treated municipal wastewater applied in the study was reported by Howell *et al.* (2022).

### Soil chemical properties

Baseline soil samples were taken in 2006 before wastewater irrigation commenced (ARC, unpublished data). Following 11 years of irrigation with treated municipal wastewater, samples were taken at budbreak (September) of the 2017/2018 season. Soils were sampled in 30 cm increments to a depth of 90 cm in all plots, and up to 180 cm in all treatment plots where it was possible to sample deeper. Soil chemical analyses were carried out by a commercial laboratory. The  $\text{pH}_{(\text{KCl})}$  was determined in a suspension of 1 M potassium chloride (KCl) at 25°C. Soil electrical resistance of the saturated paste extract ( $R_s$ ) was determined according to methods presented by Jones (1999). Thereafter,  $R_s$  was converted to  $\text{EC}_e$  using the following equation:

$$\text{EC}_e = (0.25 \div R_s) \times 1\,000 \quad (\text{Eq. 1})$$

where  $\text{EC}_e$  is the electrical conductivity of the saturated paste extract (dS/m), 0.25 is the constant for the Bureau of Soils electrode cup (Richards, 1954),  $R_s$  is the electrical resistance of the saturated paste extract (ohm), and 1 000 is the conversion factor used to convert millimhos/cm to dS/m.

Basic cations ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$ ) were extracted with 1 M ammonium acetate at pH 7. The cation concentrations in the extracts were determined by inductively coupled plasma–optical emission spectrometry (ICP-OES) using a spectrometer (PerkinElmer Optima 7300 DV, Waltham, MA). The amounts of soluble cations were not determined, therefore the amount of exchangeable cations, which is the extractable minus the soluble amounts (Richards, 1954), could not be calculated. As a result, the cation exchange capacity (CEC) could not be calculated. The majority of South African laboratories solely determine extractable cations due to the laborious process of determining exchangeable cations and CEC (Conradie, 1994). Most laboratories calculate the sum of extractable cations to obtain an estimated CEC, which is referred to as the S-value (Howell, 2016). Subsequently, the ESP and exchangeable potassium percentage (EPP) of the soil could not be calculated. However, the extractable sodium percentage (ESP') was calculated using the following equation:

$$\text{ESP}' = (\text{Na}^+ \div \text{S}) \times 100 \quad (\text{Eq. 2})$$

where  $\text{Na}^+$  is the extractable sodium ( $\text{cmol}^{(+)}/\text{kg}$ ) and S is the S-value ( $\text{cmol}^{(+)}/\text{kg}$ ), *i.e.* the sum of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$  and  $\text{Na}^+$ . Similarly, the extractable potassium percentage (EPP') was calculated as follows:

$$EPP' = (K^+ \div S) \times 100 \quad (\text{Eq. 3})$$

where  $K^+$  is the extractable potassium ( $\text{cmol}^{(+)}/\text{kg}$ ) and  $S$  is the S-value ( $\text{cmol}^{(+)}/\text{kg}$ ), *i.e.* the sum of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$  and  $\text{Na}^+$ .

Bray II P and  $\text{K}^+$  were determined by extraction with 0.03 M ammonium fluoride ( $\text{NH}_4\text{F}$ ) in 0.01 M hydrochloric acid (HCl). The P and  $\text{K}^+$  concentrations in the extract were determined in the same manner as the basic cations. Soil organic carbon (SOC) was determined using methods described by Walkley and Black (1934). Soil chloride ( $\text{Cl}^-$ ) was not determined at the beginning of the larger study period. During the 2017/2018 season, soil  $\text{Cl}^-$  was determined volumetrically *via* titration of a 0.1 M potassium nitrate ( $\text{KNO}_3$ ) soil extract with 0.043 M silver nitrate ( $\text{AgNO}_3$ ), using potassium dichromate ( $\text{K}_2\text{CrO}_4$ ) as an indicator (Chapman & Pratt, 1961).

## Soil physical properties

### Soil texture

Soil textural characteristics were determined for each 30 cm soil increment except for the shoulder DLD plot, where samples deeper than 60 cm did not contain enough soil to perform the analyses. Particle size distribution was determined using the hydrometer method (Van der Watt, 1966).

### Near-saturation hydraulic conductivity

Mini disk infiltrometers (Decagon Devices, Pullman, Washington, USA) were used to measure the near-saturation hydraulic conductivity ( $K_{ns}$ ) of the soils in October 2017. Measurements were replicated five times in each treatment plot at each of the three landscape positions. Measurements were carried out on the grapevine row, where a thin layer of fine sand was added to the soil surface to ensure a level surface and adequate contact between the base of the infiltrometer and the soil surface (Köhne *et al.*, 2011). The treated municipal wastewater used for irrigation was also used for the  $K_{ns}$  measurements. The electrical conductivity of the irrigation water ( $\text{EC}_w$ ) and sodium adsorption ratio (SAR) of the irrigation water were 1.3 dS/m and 3.8, respectively. A suction head of 2 cm was maintained for each measurement.

Fixed time intervals between measurements were chosen to allow a decrease in water level of at least 1 mL to minimise reading errors. The  $K_{ns}$  values ( $\text{mm}/\text{h}$ ) were calculated using the following equation:

$$K_{ns} = \{[(V_i - V_f) \div 1000] \div 0.001521\} \times 60 \div \Delta t \quad (\text{Eq. 4})$$

where  $V_i$  is the initial volume reading (mL),  $V_f$  is the final volume reading (mL), 0.001521 is the area ( $\text{m}^2$ ) of the sintered stainless steel disk at the bottom of the infiltrometer, and  $\Delta t$  is the difference in time between measurements (min).

## Statistical analysis

Calculations of means and standard deviations (SD) were carried out using Microsoft Office Excel 365. Relationships between variables were calculated by means of linear regression at the 95% confidence level using Statgraphics® XV (StatPoint Technologies, Warrenton, Virginia, USA).

## RESULTS AND DISCUSSION

### Rainfall

The monthly rainfall for the 2017/2018 season measured from July 2017 to June 2018 in relation to the long-term mean (LTM) measured over the 12-year study period is shown in Fig. 1. The rainfall during the 2017/2018 season was below the LTM for most of the season and only exceeded the LTM during August 2017, as well as in April and May 2018 (Fig. 1). An average of 86 mm was measured during the summer months of September to March throughout the study period, whilst only 39 mm was measured during the summer of 2017/2018. The average winter rainfall from May to August was 160 mm. The LTM annual rainfall measured from July to June of each year was 253 mm, while only 118 mm was measured from July 2017 to June 2018.

### Soil texture

The soil texture at the shoulder site was relatively uniform and was predominantly clay to clay loam, with a clay content that ranged between 35% and 51%. The soils at this experimental site had high stone fractions that ranged between 15.3% and 45.8%. This was mainly due to the presence of the shale parent material, which was brought up to the soil surface when the soil was prepared before planting. The backslope

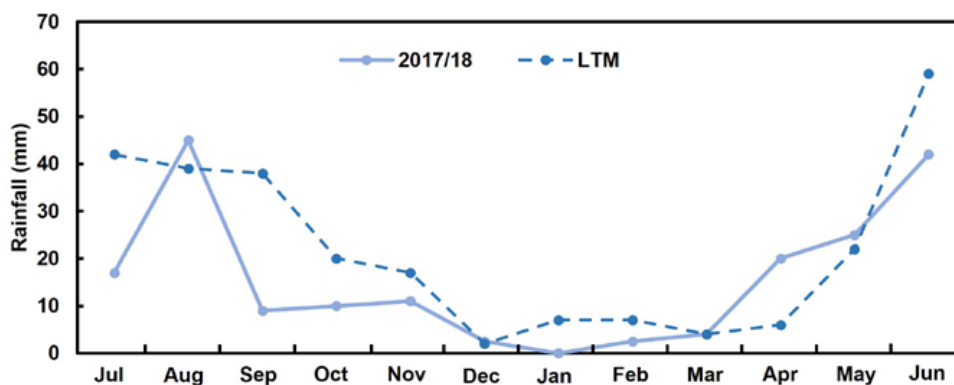


FIGURE 1

Monthly rainfall (mm) from July 2017 to June 2018 in relation to the long-term mean (LTM) measured over the 12-year study period on a farm near Philadelphia (ARC, unpublished data).

site had a sandy loam topsoil with a clay content of 15% to 19%, and a sandy clay loam to clay layer. The clay content ranged between 29% and 49% at 90 cm. The soil of the backslope did not contain any stones. The 0 cm to 30 cm layer of the footslope site ranged from sandy loam in the RF and SLD plots to sandy clay loam in the DLD plot. The clay content of the footslope DLD plot was between 17% and 25% in the 0 cm to 30 cm layer, and increased with depth to a maximum of 55% at 120 cm. The only presence of stones at the footslope site was in the 30 cm to 60 cm soil layer of the SLD plot. As expected, the soils differed substantially between the three landscape positions. However, soils were relatively uniform between treatment plots at the individual landscape positions.

**Soil chemical properties**

**pH<sub>(KCl)</sub>**

The pH<sub>(KCl)</sub> of the 0 cm to 30 cm soil layer increased in all of the landscape positions following 11 years of irrigation with treated municipal wastewater (Fig. 2A to 2C). At the shoulder site, the DLD plot had the highest topsoil pH at the end of the study period, followed by the SLD and RF plots (Fig. 2A), although the differences between the three treatments were relatively small. In contrast, the topsoil pH of the SLD and DLD plots on the backslope site was similar, whilst the RF was considerably lower (Fig. 2B). The topsoil pH of the backslope SLD plot increased by approximately 2.3 units compared to the baseline before irrigation commenced. Similar results were obtained at the footslope site (Fig. 2C). At this site, the topsoil pH of the RF treatment remained unchanged, but decreased with depth when compared to the baseline.

On average, the topsoil pH of the SLD and DLD plots increased by 1.3 units after 11 years of irrigation with treated municipal wastewater (Fig. 3). The increase in pH was most likely due to the pH of the irrigation water, which varied between 6.7 and 8.0 throughout the study period. The decarboxylation and hydrolysis of organic acids and bicarbonate anions present in the wastewater may also have contributed to the increased pH (Li *et al.*, 2008). The increased pH did not cause concern, as it remained near neutral and would therefore have had little effect on biological functioning (Schipper *et al.*, 1996). In addition, the pH of all of the treatment plots was within the recommended range to sustain optimal grapevine growth, viz. 5.0 to 7.5 (Saayman, 1981). Similar results have been reported by Sparling *et al.* (2006) and Schipper *et al.* (1996) where soils were irrigated with secondary and tertiary treated municipal wastewater, respectively. In contrast, Xu *et al.* (2010) reported a decrease in soil pH of *ca.* 1.1 units in a 150 cm soil profile following 20 years of irrigation with treated municipal wastewater.

**EC<sub>e</sub>**

The EC<sub>e</sub> of the 0 cm to 30 cm soil layer increased in all of the treatment plots at each of the landscape positions compared to the baseline values before irrigation commenced (Fig. 4A to 4C). The topsoil EC<sub>e</sub> of the shoulder DLD plot was nearly three times higher than the baseline value (Fig. 4A). In addition, it was the highest EC<sub>e</sub> measured across all three landscape positions. However, no clear trends could

be observed in the subsoil between the treatments of the shoulder site. On the backslope site, the SLD treatment had the highest topsoil EC<sub>e</sub>. However, the differences between the treatments and the baseline measurement were relatively small (Fig. 4B). An increase in EC<sub>e</sub> with soil depth was also evident at this site. The topsoil EC<sub>e</sub> of the footslope DLD plot increased from 0.17 dS/m before irrigation with treated municipal wastewater began to 0.56 dS/m following 11 years of irrigation (Fig. 4C).

The mean topsoil EC<sub>e</sub> increased with the amount of treated municipal wastewater applied (Fig. 5). However,

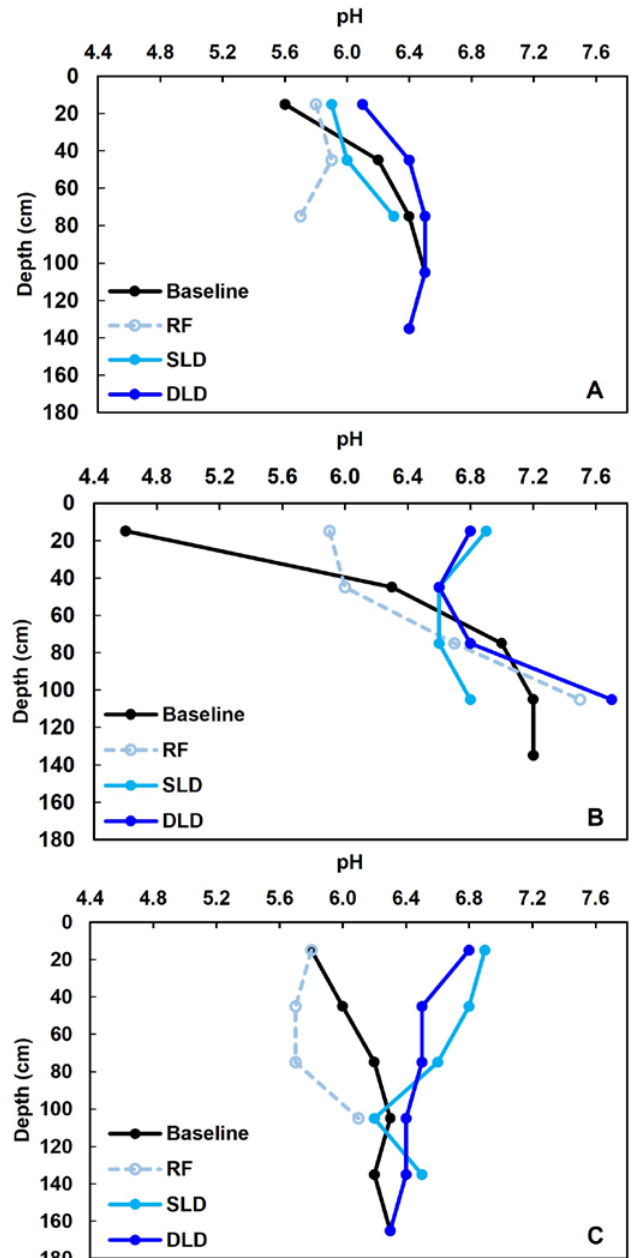


FIGURE 2

Effect of rainfed conditions (RF) and irrigation with treated municipal wastewater *via* single (SLD) and double dripper line (DLD) on the soil pH on (A) a shoulder, (B) a backslope and (C) a footslope after 11 years of wastewater irrigation compared to the baseline before irrigation commenced.

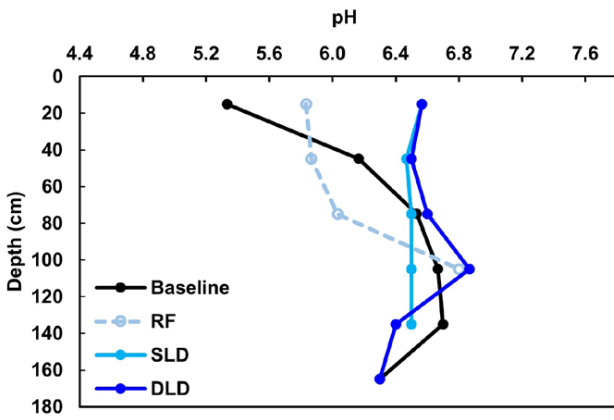


FIGURE 3

Effect of rainfed conditions (RF) and irrigation with treated municipal wastewater *via* single (SLD) and double dripper line (DLD) on the mean soil pH across the main experimental sites after 11 years of wastewater irrigation compared to the baseline before irrigation commenced.

there were no clear trends in the deeper soil layers that could be related to the different irrigation treatments compared to the baseline values. The increased  $EC_e$  of the topsoil indicates an accumulation of salts at the soil surface. The  $EC_w$  of the treated municipal wastewater ranged between 0.7 dS/m and 1.2 dS/m (Howell *et al.*, 2022), which could explain the increased  $EC_e$ . The accumulation of salts at the soil surface is most likely a result of high evapotranspiration during the irrigation season, which concentrated the salts in the upper parts of the root zone (Rhoades *et al.*, 1973). Similar results were reported for vineyard soils in Great Western, Australia that were irrigated with treated municipal wastewater for at least five years (Hermon, 2011). The accumulation of salts in the soil profile is of concern, as a progressive increase in soil salinity can result in deficiencies in grapevine nutrients (McCarthy, 1981; Paranychianakis *et al.*, 2006). However, the relatively small increase in  $EC_e$  after 11 years of wastewater irrigation in the current study suggests that winter rainfall might have leached some of the applied salts beyond the measured depth. In a laboratory study in which rainfall cycles were simulated, the EC of the drainage water was considerably higher than that of the input water (Laurenson, 2010), indicating a loss of salts from the soil during rainfall events. Therefore, regular rainfall events may help to alleviate high soil  $EC_e$  where municipal wastewater containing high levels of salts is used for irrigation.

**Bray II P**

Bray II P increased in the 0 cm to 30 cm soil layer at all of the experimental sites following 11 years of irrigation with treated municipal wastewater (Fig. 6A to 6C). The soil Bray II P content at the shoulder site was highest at the SLD, followed by the RF plot, whilst the Bray II P content of the DLD plot was approximately half that of the SLD (Fig. 6A). The SLD and RF plots at the shoulder site exceeded the norm of 30 mg/kg P recommended for grapevines in soils containing more than 15% clay (Conradie, 1994). The Bray

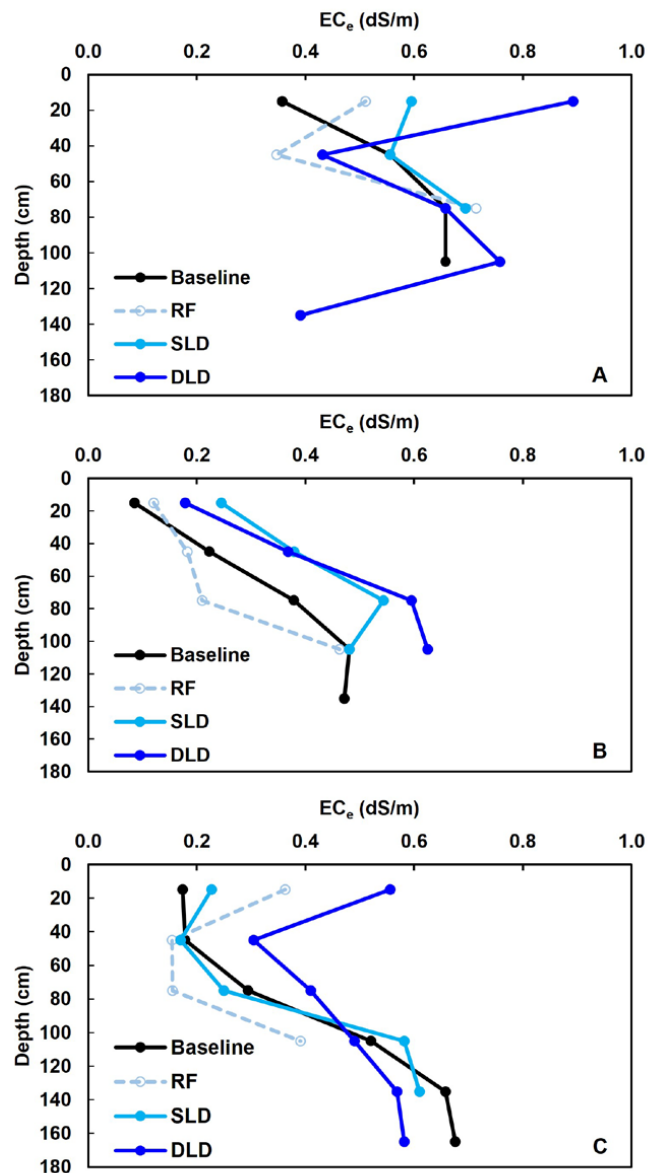


FIGURE 4

Effect of rainfed conditions (RF) and irrigation with treated municipal wastewater *via* single (SLD) and double dripper line (DLD) on the soil electrical conductivity ( $EC_e$ ) on (A) a shoulder, (B) a backslope and (C) a footslope after 11 years of wastewater irrigation compared to the baseline before irrigation commenced.

II P concentration in the 0 cm to 30 cm soil layer of the SLD and DLD plots at the backslope site was slightly less than the RF (Fig. 6B); however, all plots met the norm of 30 mg/kg. The RF plot at the footslope site had the highest Bray II P content in the 0 cm to 30 cm soil layer compared to the other treatments (Fig. 6C). The P content of the RF plot increased from 7 mg/kg measured before the study commenced to 46 mg/kg 11 years thereafter.

After 11 years of irrigation, the soil contained on average, 42.6 mg/kg, 39.3 mg/kg and 28.1 mg/kg Bray II P in the RF, SLD and DLD treatments, respectively, whilst the baseline value was 12.7 mg/kg. An accumulation of P in the topsoil was evident, as the concentration decreased sharply in the 30

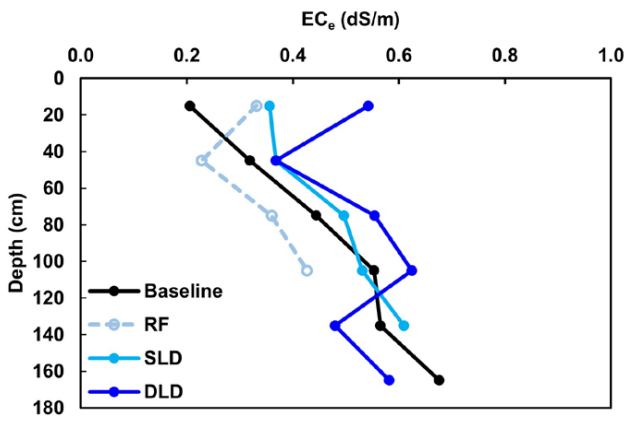


FIGURE 5

Effect of rainfed conditions (RF) and irrigation with treated municipal wastewater *via* single (SLD) and double dripper line (DLD) on the mean soil electrical conductivity ( $EC_e$ ) across the main experimental sites after 11 years of wastewater irrigation compared to the baseline before irrigation commenced.

cm to 60 cm soil layer in all of the treatments and remained at relatively constant levels in the 60 cm to 90 cm soil layer (Fig. 7). The 0 cm to 30 cm soil layers of the RF and SLD treatments consistently exceeded the norm of 30 mg/kg P for soils with a clay content of at least 15%. In contrast, the DLD treatments were on average only 2 mg/kg below the norm. The accumulation of P in the topsoil of the RF treatments could be explained by the application of P fertiliser by the grower and the low vigour that is expected of grapevines that are grown under dryland conditions, which would absorb very small amounts of P from the soil. In contrast, the high vigour that is expected from over-irrigated grapevines is reflected in the lower soil P content of the DLD plots, despite the application of additional P *via* treated municipal wastewater irrigation.

**Bray II K**

The Bray II  $K^+$  content of the 0 cm to 30 cm soil layer of the shoulder site increased in all of the treatment plots compared to the baseline values (Fig. 8A). The slightly increased  $K^+$  content of the RF plot on the shoulder site was due to the application of  $K^+$  fertiliser by the grower, whereas the high  $K^+$  content of the DLD plot may be the result of the low mobility of  $K^+$  in the soil and its retention by clay minerals (Pérez *et al.*, 2015), as well as an over-supply of  $K^+$  *via* treatment with treated municipal wastewater. On the backslope site, the  $K^+$  content of the 0 cm to 30 cm soil layer did not differ substantially between treatment plots, but increased in all of the plots compared to the baseline (Fig. 8B). The  $K^+$  content of the SLD and DLD plots was maintained in the deeper soil layers, whereas a gradual decrease up to 90 cm was observed in the RF plot. Bray II  $K^+$  levels in the 90 cm to 120 cm soil layer of the backslope site decreased in all of the treatment plots compared to the baseline. This was probably due to  $K^+$  uptake by grapevines from deeper soil layers, or the leaching of  $K^+$  beyond the measured depth. The  $K^+$  content

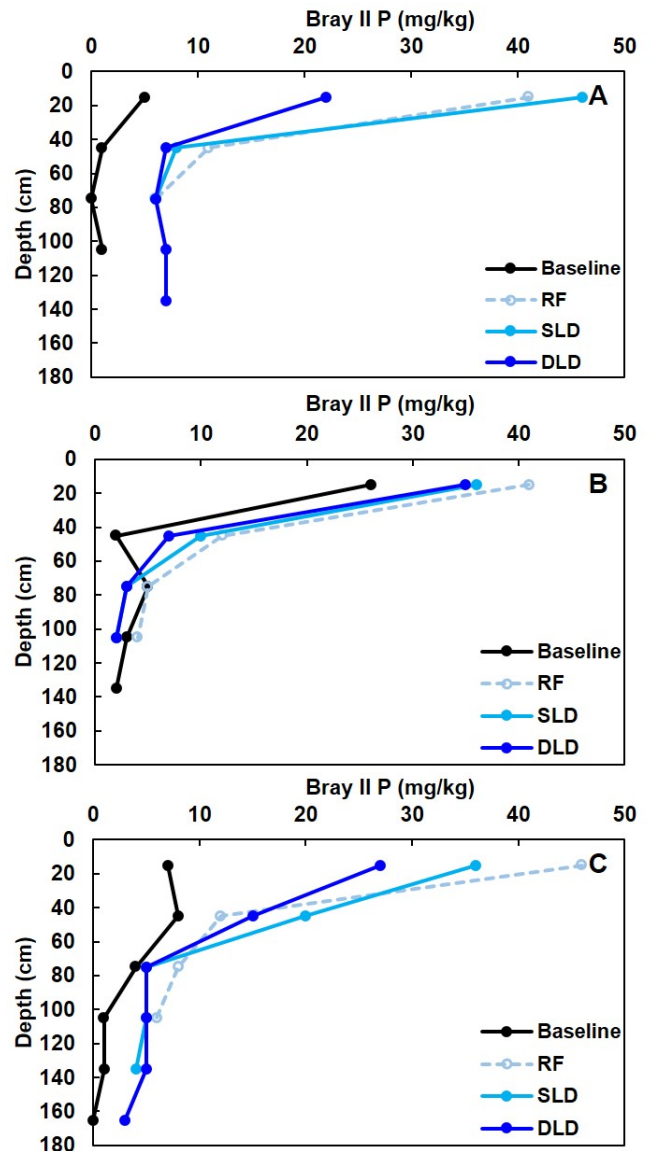


FIGURE 6

Effect of rainfed conditions (RF) and irrigation with treated municipal wastewater *via* single (SLD) and double dripper line (DLD) on the soil Bray II extractable phosphorous (P) content on (A) a shoulder, (B) a backslope and (C) a footslope after 11 years of wastewater irrigation compared to the baseline before irrigation commenced.

of the topsoil layer of the footslope increased under DLD compared to the baseline (Fig. 8C). Similar to the backslope site,  $K^+$  levels beyond 90 cm decreased below the baseline levels in all of the treatments.

On average, the Bray II  $K^+$  content of the 0 cm to 30 cm soil layer increased by 26 mg/kg, 42 mg/kg and 127 mg/kg for the RF, SLD and DLD treatments, respectively (Fig. 9). An accumulation of  $K^+$  in the topsoil due to municipal wastewater irrigation has previously been reported by Heidarpour *et al.* (2007), Kiziloglu *et al.* (2007) and Singh *et al.* (2012). The high  $K^+$  content under DLD is of concern, since an over-supply of  $K^+$  to grapevines may result in excessive  $K^+$  uptake. This could lead to musts with high pH and malate concentrations,

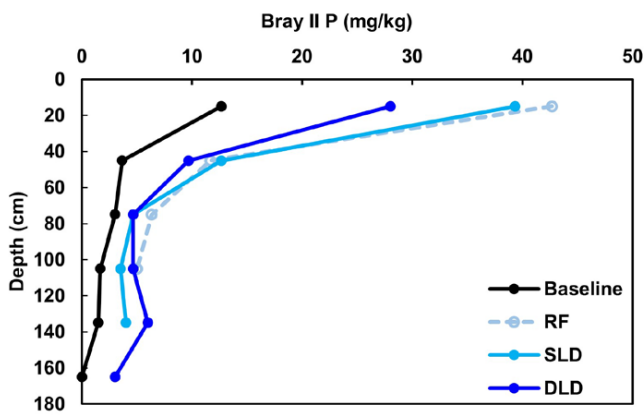


FIGURE 7

Effect of rainfed conditions (RF) and irrigation with treated municipal wastewater *via* single (SLD) and double dripper line (DLD) on the mean Bray II extractable phosphorous (P) content across the main experimental sites after 11 years of wastewater irrigation compared to the baseline before irrigation commenced.

as well as poor colour in red wines (Mpelasoka *et al.*, 2003). An accumulation of  $K^+$  in the soil can also have deleterious effects on soil structure (Laurenson *et al.*, 2012), and affect soil  $K$  and IR negatively due to its effects on clay dispersal (Arienzo *et al.*, 2009). However, clay dispersion is highly dependent on the electrolyte concentration of the infiltrating water (Shainberg *et al.*, 1981). Therefore, the high salinity that is often associated with wastewater might mitigate the negative effects of  $K^+$  on aggregate stability and  $K$  (Arienzo *et al.*, 2009). However, as long as the  $EC_w$  of the treated municipal wastewater remains above the critical coagulation value, no soil dispersion is expected to occur (Abedi-Koupai *et al.*, 2006).

**Extractable  $Ca^{2+}$  and  $Mg^{2+}$**

Extractable soil  $Ca^{2+}$  did not show any consistent trends in the various landscape positions that could be related to the different irrigation treatments (data not shown). The mean soil  $Ca^{2+}$  levels of the 0 cm to 30 cm soil layer increased only slightly for all of the treatments when compared to the baseline (data not shown). The lack of substantial response could be explained by the small amounts of  $Ca^{2+}$  applied *via* the treated municipal wastewater, the uptake of  $Ca^{2+}$  by grapevines, and the leaching from the soil profile through deep percolation. Similar results were presented by Duan *et al.* (2010) for sandy clay loam soil after one year of irrigation with secondary treated municipal wastewater. Irrigating golf course fairways on a clay loam topsoil with treated municipal wastewater for four to five years also did not affect soil  $Ca^{2+}$  (Qian & Mecham, 2005).

Similar to  $Ca^{2+}$ , extractable soil  $Mg^{2+}$  levels did not show any consistent trends at the various landscape positions that could be related to the different irrigation treatments (data not shown). The mean soil  $Mg^{2+}$  concentrations of the 0 cm to 30 cm soil layer in the RF and SLD treatments decreased when compared to the baseline values, whilst the DLD

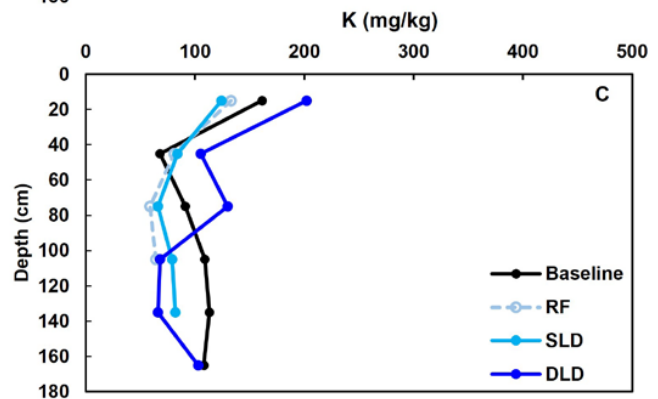
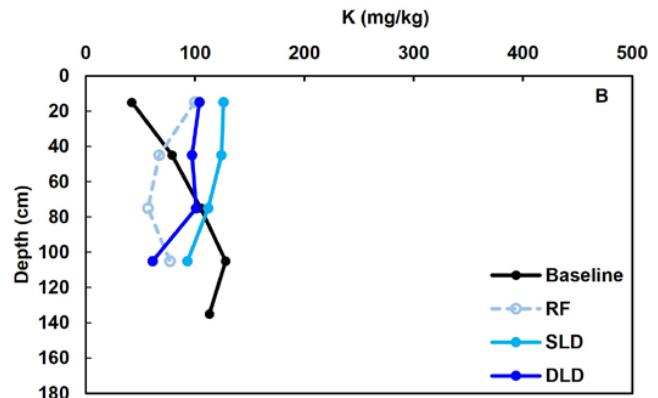
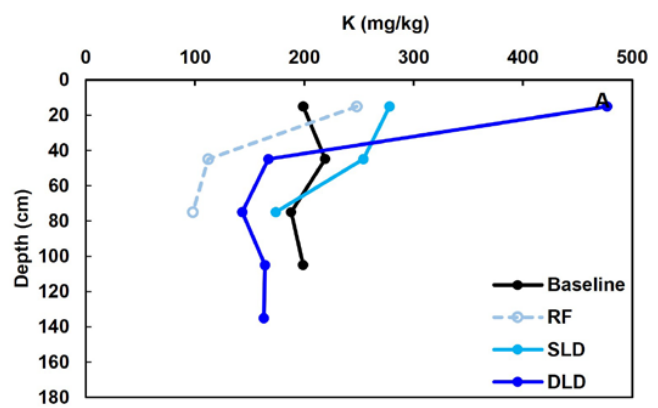


FIGURE 8

Effect of rainfed conditions (RF) and irrigation with treated municipal wastewater *via* single (SLD) and double dripper line (DLD) on the soil Bray II extractable potassium (K) content on (A) a shoulder, (B) a backslope and (C) a footslope after 11 years of wastewater irrigation compared to the baseline before irrigation commenced.

remained relatively unchanged after 11 years of irrigation using treated municipal wastewater (data not shown). This is probably due to small amounts of  $Mg^{2+}$  supplied to the soil and the uptake of  $Mg^{2+}$  by grapevines, which depleted soil  $Mg^{2+}$  levels over the long term. Nielsen *et al.* (1991) attributed the reduction in soil  $Mg^{2+}$  following five years of municipal wastewater irrigation to mass exchange by  $Na^+$  and  $K^+$ .

**EPP'**

Soil extractable  $K^+$  followed similar trends as observed for Bray II  $K^+$  (data not shown), therefore they will not be

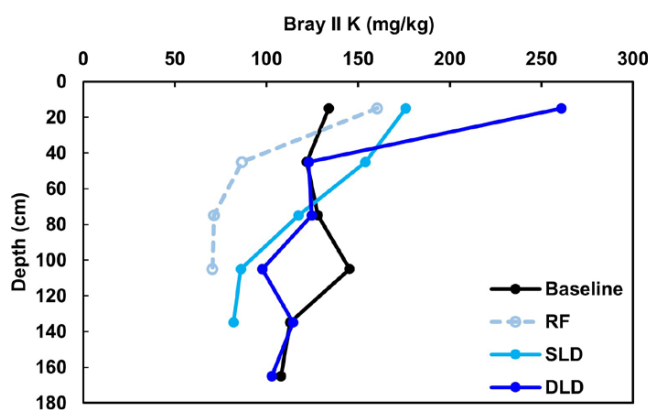


FIGURE 9

Effect of rainfed conditions (RF) and irrigation with treated municipal wastewater *via* single (SLD) and double dripper line (DLD) on the mean Bray II extractable potassium (K) content across the main experimental sites after 11 years of wastewater irrigation compared to the baseline before irrigation commenced.

discussed. Since exchangeable  $K^+$  was not determined, the  $EPP'$  was calculated rather than the  $EPP$ . Conradie (1994) recommended a ratio of 3% to 4% for exchangeable  $K^+$  to other cations. The baseline  $EPP'$  in the 0 cm to 30 cm soil layer of the shoulder site already exceeded this norm, and a further increase in all of the treatments was observed throughout the study period (Fig. 10A). These results were expected, as the topsoil of this particular site had a high clay content, which could retain a high amount of extractable  $K^+$ . Furthermore, the  $EPP'$  of the DLD plot was ca. 2% higher than the SLD plot and 3% higher than the RF plot, indicating a steady increase in  $EPP'$  due to irrigation with treated municipal wastewater. The  $EPP'$  of the 0 cm to 30 cm soil layer of the backslope site increased in all the treatment plots compared to the baseline, with the RF plot having the highest  $EPP'$ , followed by the SLD and DLD plots (Fig. 10B). The accumulation of extractable  $K^+$  on the RF plot could be explained by low vigour and grape production, which are associated with grapevines grown under dryland conditions, and the subsequent lower uptake of  $K^+$  from the soil.

The topsoil  $EPP'$  of the backslope DLD plot remained similar to the baseline, and the SLD plot only increased by 0.8% following 11 years of wastewater irrigation. The lack of response to wastewater irrigation in terms of  $EPP'$  at this site might be explained by the uptake of  $K^+$  by grapevines and the leaching of excess  $K^+$  from the soil profile, as the clay content of this site is considerably lower than that of the shoulder site (data not shown). At the footslope site, the baseline  $EPP'$  of the 0 cm to 30 cm soil layer was 10% and increased by 1% and 2% on the SLD and DLD plots, respectively, while the RF plot remained unchanged (Fig. 10C). The higher  $EPP'$  was expected on the DLD plot of this site due to the larger volume of K-containing wastewater applied to this site. The  $EPP'$  decreased with depth in all of the treatment plots, but increased in relation to the baseline in the 30 cm to 60 cm soil layer and reached levels below the baseline at a depth of

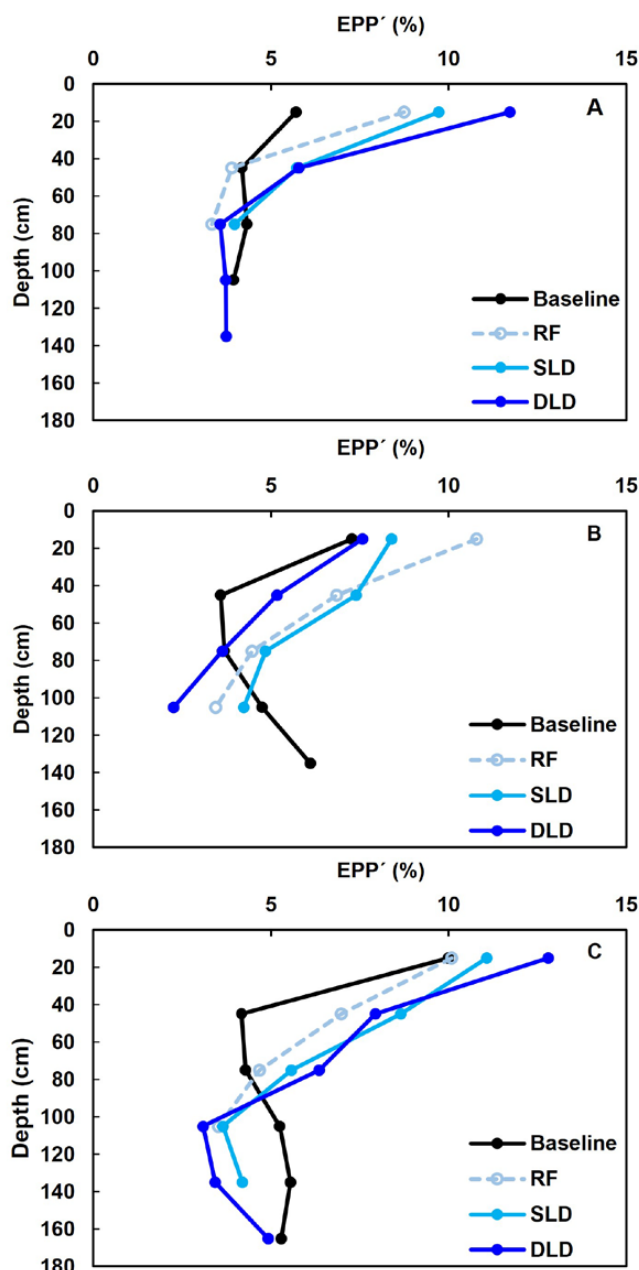


FIGURE 10

Effect of rainfed conditions (RF) and irrigation with treated municipal wastewater *via* single (SLD) and double dripper line (DLD) on the soil extractable potassium percentage ( $EPP'$ ) on (A) a shoulder, (B) a backslope and (C) a footslope after 11 years of wastewater irrigation compared to the baseline before irrigation commenced.

90 cm. The higher  $EPP'$  in the 30 cm to 60 cm layer could be explained by the higher clay content of this layer. The decrease in deeper layers may be due to a combination of grapevine  $K^+$  uptake and  $K^+$  leaching from the soil profile.

The mean  $EPP'$  for the baseline exceeded the recommended norm by far and increased substantially in all of the treatments throughout the study period (Fig. 11). There was little difference between the topsoil  $EPP'$  of the RF and SLD treatments, but the DLD was 2% higher than

in the other treatments. The high extractable  $K^+$  at all of the experimental sites is concerning, as it could lead to greater  $K^+$  uptake by the grapevines, which ultimately may result in unstable wines with a high pH (Gawel *et al.*, 2000; Mpelasoka *et al.*, 2003). In addition, high amounts of exchangeable  $K^+$  have been associated with reduced  $K$  (Quirk & Schofield, 1955; Chen *et al.*, 1983).

### ESP'

Soil extractable  $Na^+$  followed similar trends as those observed for ESP' (data not shown), therefore only ESP' is discussed. The ESP' of the 0 cm to 30 cm soil layer of the shoulder site decreased in the RF plot, but levels similar to the baseline were maintained in the SLD and DLD plots (Fig. 12A). An increase in ESP' was observed with depth in all of the treatment plots. However, the values were similar between treatment plots and lower than the baseline. An increase in ESP' with depth in three Israeli soils irrigated with treated municipal wastewater with SAR similar to the present study was attributed to an increase in clay content at deeper soil layers (Levy *et al.*, 2014). The reason for the high baseline ESP' values in the subsoil layers of the shoulder site could be explained by the increased weathering of clay minerals due to soil preparation. Irrigation with treated municipal wastewater increased the ESP' throughout the soil profile in the backslope site (Fig. 12B). However, little difference in  $Na^+$  accumulation could be seen between the SLD and DLD plots. The values measured in the RF plot of the backslope site at the end of the study period remained comparable to the baseline. Similar results were observed when olive orchards were irrigated with treated municipal wastewater and compared to a rainfed control treatment (Ayoub *et al.*, 2016).

The ESP' of the footslope site followed a similar trend to that of the backslope site (Fig. 12C), with the RF plot remaining largely unaffected, and the SLD and DLD plots increasing substantially following 11 years of irrigation with treated municipal wastewater. However, the SLD plot on the footslope site had greater ESP' values in the subsoil compared to the DLD plot, indicating more accumulation of salts in the subsoil under SLD. The increase in ESP' in the backslope and footslope sites is particularly concerning, as the SAR of the irrigation water largely remained below 5, which is the critical limit for wastewater used for irrigation (Department of Water Affairs [DWA], 2013). Similarly, Levy *et al.* (2014) reported ESP levels in sandy clay subsoils ( $\geq 15\%$  clay) reaching between 6% and 16% following ten years of irrigation with treated municipal wastewater with an SAR of 3 to 5.

The accumulation of  $Na^+$  was attributed to a possible lack of chemical equilibrium between the SAR of the treated wastewater, the SAR of the soil solution and the ESP of the subsoil layers. The replacement of exchangeable  $Na^+$  (applied *via* wastewater irrigation) in the topsoil by  $Ca^{2+}$  originating from the dissolution of calcium carbonate during the rainy season, and the subsequent leaching of  $Na^+$  to deeper soil layers where it is re-adsorbed to the soil exchange complex, was proposed as a possible mechanism for the  $Na^+$  accumulation (Levy *et al.*, 2014). Results presented by Myburgh (2018) indicated that  $Na^+$  accumulation in the

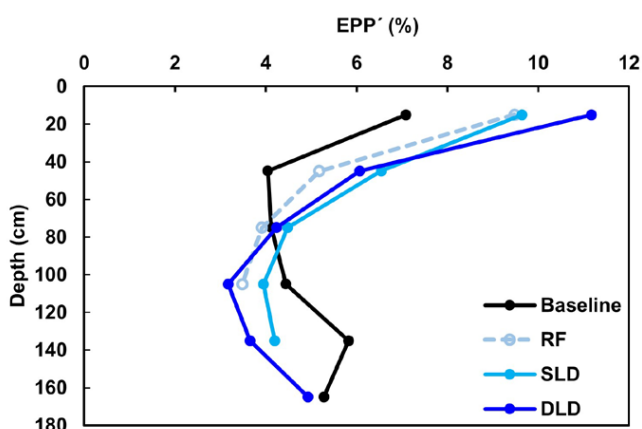


FIGURE 11

Effect of rainfed conditions (RF) and irrigation with treated municipal wastewater *via* single (SLD) and double dripper line (DLD) on the mean soil extractable potassium (EPP') across the main experimental sites after 11 years of wastewater irrigation compared to the baseline before irrigation commenced.

soil profiles of these sites was highly dependent on winter rainfall, implying that salts will accumulate in the soil during winters with low rainfall and leach to deeper layers following higher rainfall. The winter rainfall preceding the collection of the soil samples in 2017 was 138 mm (data not shown), which was lower than the LTM of 160 mm, and therefore could have contributed to the accumulation of  $Na^+$  in the wastewater-irrigated sites.

On average, the topsoil ESP' increased by 1% and 3% for the SLD and DLD treatments, respectively, whereas the RF treatment was 3% lower compared to the baseline (Fig. 13). A steady increase in ESP' with depth was evident in all of the treatments. This is probably due to the high  $Na^+$  levels present at the beginning of the study period, as the subsoil ESP' decreased in relation to the baseline, except for the SLD at 60 cm and 150 cm, as well as the DLD at 150 cm soil depth. Deeper than 60 cm, the mean ESP' of the SLD plots was higher than the DLD. This could be explained by the larger volumes of irrigation water applied to the latter plots, which facilitated the leaching of more  $Na^+$  from the soil profile. The importance of leaching salts from the soil profile has been highlighted previously (Hussain, 1981). Rengasamy and Olsson (1993) predicted that  $Na^+$  would accumulate in soil if the SAR of the applied water is greater than 3 and the leaching fraction is less than 50%. The high ESP' observed in the subsoil of the SLD and DLD treatments remains a concern, as it may reduce the movement of water through the soil profile. Lower macroporosity due to the accumulation of  $Na^+$  and  $K^+$  in the soil may affect the drainage capacity of soils, which in turn limits water percolation and, ultimately, the leaching of salts (Prior *et al.*, 1992; Halliwell *et al.*, 2001).

In soil with a permeable A horizon overlying a moderately draining B horizon, irrigation with treated municipal wastewater caused a reduction in  $K$  in the soil due to the increase of exchangeable  $Na^+$  in the B horizon,

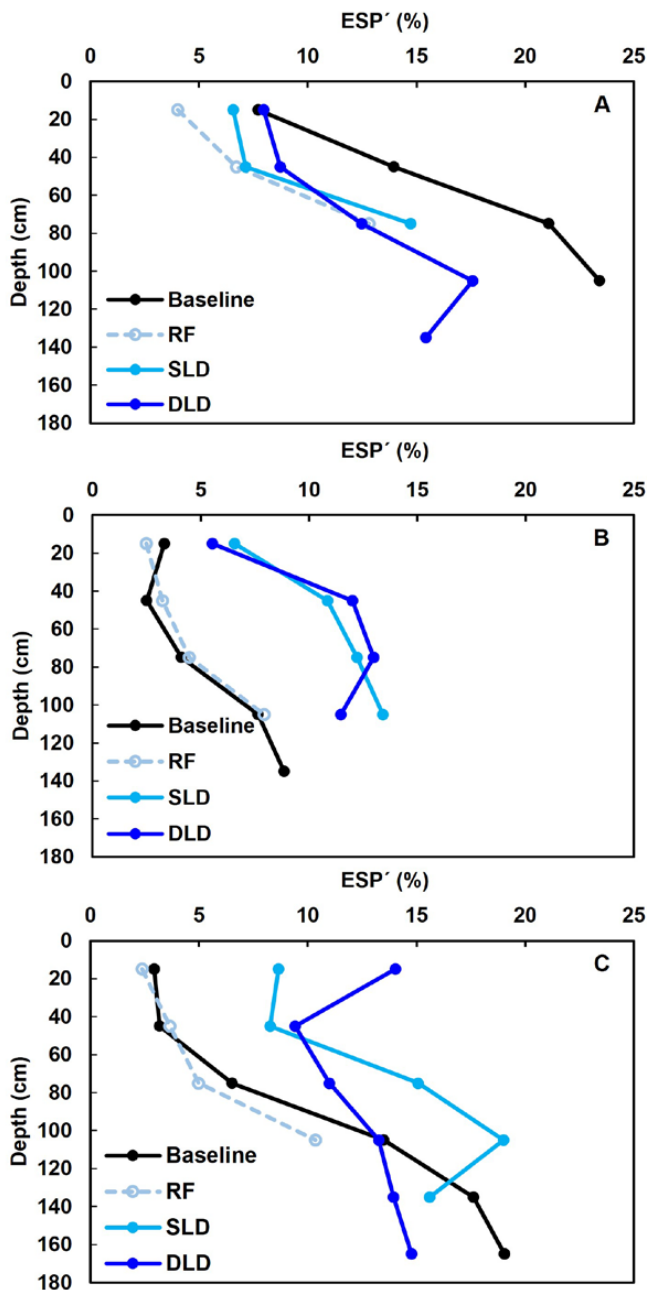


FIGURE 12

Effect of rainfed conditions (RF) and irrigation with treated municipal wastewater *via* single (SLD) and double dripper line (DLD) on the soil extractable sodium percentage (ESP') of (A) a shoulder, (B) a backslope and (C) a footslope after 11 years of wastewater irrigation compared to the baseline before irrigation commenced.

which reduced the leaching of salts and led to increased soil salinity in the A horizon (Stevens *et al.*, 2003). Soil permeability problems will also occur if a solution with very low electrolyte concentration, such as rainwater, percolates through the soil (Du Plessis & Shainberg, 1985). Therefore, the application of large volumes of water with higher salinity, as is the case with the DLD treatments, might help to mitigate the accumulation of  $\text{Na}^+$  at the soil surface and prevent reductions in  $K$  and IR.

### $\text{Cl}^-$

The soil  $\text{Cl}^-$  content was not determined at the beginning of the study period; therefore, no baseline value is available. Soil  $\text{Cl}^-$  concentrations in the 0 cm to 30 cm layer increased with the amount of irrigation water applied to all the treatment plots (Fig. 14A to 14C). The topsoil  $\text{Cl}^-$  content of the shoulder site was 17 mg/kg, 35 mg/kg and 79 mg/kg for the RF, SLD and DLD plots, respectively (Fig. 14A). Soil  $\text{Cl}^-$  levels decreased sharply in the 30 cm to 60 cm layer of this landscape position and values were comparable between the three treatment plots. In the 60 cm to 90 cm soil layer, the  $\text{Cl}^-$  concentration of all the treatments increased again, suggesting that the subsoil has inherently high  $\text{Cl}^-$  levels. Similar results were observed in the backslope site, although the  $\text{Cl}^-$  content increased rather than decreased in the 30 cm to 60 cm soil layer (Fig. 14B). In the footslope site, the soil  $\text{Cl}^-$  content of the SLD and DLD plots were consistently higher than the RF plot and reached similar concentrations to those observed in the shoulder and backslope sites (Fig. 14C).

The mean  $\text{Cl}^-$  content of the topsoil increased with the amount of treated municipal wastewater applied (Fig. 15). The high  $\text{Cl}^-$  levels were to be expected, as the wastewater was disinfected by a chlorination treatment at the WWTW, resulting in a mean  $\text{Cl}^-$  content of 160 mg/L over the 12-year study period (Howell *et al.*, 2022). An accumulation of  $\text{Cl}^-$  seems evident at a depth of 90 cm, but cannot necessarily be ascribed to the irrigation water, as high  $\text{Cl}^-$  levels were observed in the subsoil of the RF treatments as well.

### SOC

With the exception of the SLD plot on the footslope site, the SOC content of the 0 cm to 30 cm soil layer increased in all of the treatment plots in all of the landscape positions compared to the baseline values (data not shown). The accumulation of SOC could not be ascribed to the application of treated municipal wastewater, since the RF plots had the highest SOC content in each of the landscape positions. On average, the SOC content was 0.6% in the RF treatment, followed by 0.5% in the DLD and 0.4% in the SLD treatment (data not shown). An increase in SOC was also observed in the subsoil at the end of the study period, but no clear trend with regard to the different treatments could be seen. It should be noted that the chemical oxygen demand (COD) of the treated municipal wastewater was very low and would therefore not have made a significant contribution towards the SOC content (Howell, ARC, unpublished data). The increased SOC content could be explained by the accumulation of organic matter due to the annual establishment of a cover crop. The accumulation of grapevine plant material debris over the course of the 11-year study period also contributed to higher SOC levels. The greater accumulation of SOC in the RF treatments could be explained by a lack of water that would be needed to facilitate the decomposition of organic matter. Herpin *et al.* (2007) reported significant reductions in soil organic matter (SOM) due to the stimulation of soil microbial activity where soils were irrigated with secondary treated municipal wastewater. In contrast, an increase in total carbon was reported for the 0 cm to 10 cm layer of soils irrigated with treated municipal wastewater for eight and 10 years when compared to a rainfed control (Xu *et al.*, 2010).

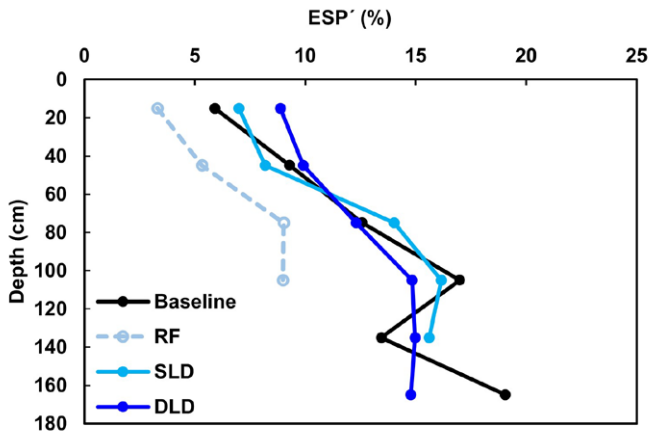


FIGURE 13

Effect of rainfed conditions (RF) and irrigation with treated municipal wastewater *via* single (SLD) and double dripper line (DLD) on the mean soil extractable sodium (ESP') across the main experimental sites after 11 years of wastewater irrigation compared to the baseline before irrigation commenced.

**Soil physical properties**

*Near-saturation hydraulic conductivity*

No clear trend was observed that could explain the effect of irrigation with treated municipal wastewater on the  $K_{ns}$  at the shoulder site (Fig. 16A). However, the results were similar to what was reported by Walker and Lin (2008) for summit landscape positions irrigated with treated municipal wastewater for over 40 years. Similarly, there was little difference between the treatments at the footslope in terms of  $K_{ns}$ , despite the slightly lower  $K_{ns}$  measured in the DLD plot (Fig. 16C). These results were comparable to reports by Sparling *et al.* (2006) and Vogeler (2009), who observed no significant difference in  $K_{ns}$  between wastewater-irrigated and non-irrigated soils. In contrast, in the backslope site,  $K_{ns}$  decreased with an increase in the amount of treated municipal wastewater applied (Fig. 16B). The  $K_{ns}$  of the backslope site was 103 mm/h, 66 mm/h and 38 mm/h for the RF, SLD and DLD plots, respectively. Bedbabis *et al.* (2014) reported a significant decrease in the IR of sandy soil (5.5% clay) following four years of irrigation with treated municipal wastewater. The decrease was also significant in relation to a rainfed control treatment and one irrigated with well water (Bedbabis *et al.*, 2014). In contrast, Lado and Ben-Hur (2010) reported improved saturated hydraulic conductivity ( $K_s$ ) in sandy soil (12% clay) irrigated with secondary-treated municipal wastewater for more than 12 years.

A strong correlation was found between the  $EC_e$  of the 0 cm to 30 cm topsoil and the  $K_{ns}$  (Fig. 17). The relationship between  $EC_e$  and  $K_{ns}$  could be described best using a reciprocal-Y logarithmic-X model, where  $K_{ns}$  decreased significantly with an increase in  $EC_e$  up to an  $EC_e$  of 0.4 dS/m whereafter it was expected to plateau. In contrast, Andrews *et al.* (2016) found no correlation between the  $K_s$  and  $EC_e$  of loam soils irrigated with treated municipal wastewater for over 50 years.

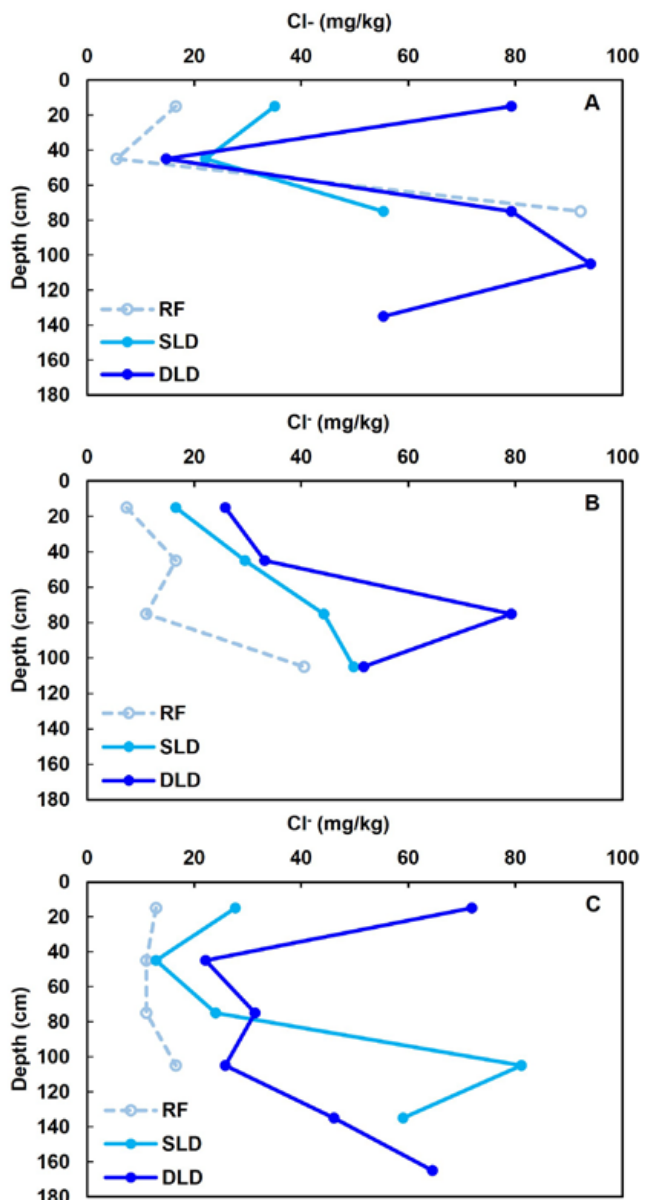


FIGURE 14

Effect of rainfed conditions (RF) and irrigation with treated municipal wastewater *via* single (SLD) and double dripper line (DLD) on the soil chloride content (Cl) of (A) a shoulder, (B) a backslope and (C) a footslope after 11 years of irrigation with treated municipal wastewater.

**CONCLUSIONS**

Irrigation using treated municipal wastewater increased soil pH and  $EC_e$ . There also was an accumulation of Cl<sup>-</sup> in the topsoil, which was likely due to the process of disinfection with chlorine at the wastewater treatment works. Substantial amounts of Na<sup>+</sup> and K<sup>+</sup> accumulated in the topsoil due to wastewater irrigation. Such soil K<sup>+</sup> increases could have a negative effect on the stability of wine colour should it be taken up by the grapevine in sufficient quantities, particularly if the levels of soil K<sup>+</sup> are such that it is absorbed excessively by the grapevines. In general, soil ESP' increased as a result of irrigation with treated municipal wastewater. The increase

was more prominent in the subsoil layers, possibly due to the seasonal leaching of salts by rainfall. Furthermore, the application of more water in the DLD treatment plots might also have contributed to more Na<sup>+</sup> being leached from the profile compared to the SLD plots. The  $K_{ns}$  at the surface of the soil could be related to the  $EC_e$  in the topsoil. Taking this into consideration, it may be beneficial in the future to quantify the formation of surface crusts that could form under wastewater irrigation. In addition, economically viable practices should be developed to alleviate such surface crusts. It should be noted that the results of this study represent specific in-field situations in three commercial vineyards under one set of climatic conditions. Future research should focus on the use of treated municipal wastewater for irrigation of vineyards or other crops on different soil types in different climatic regions.

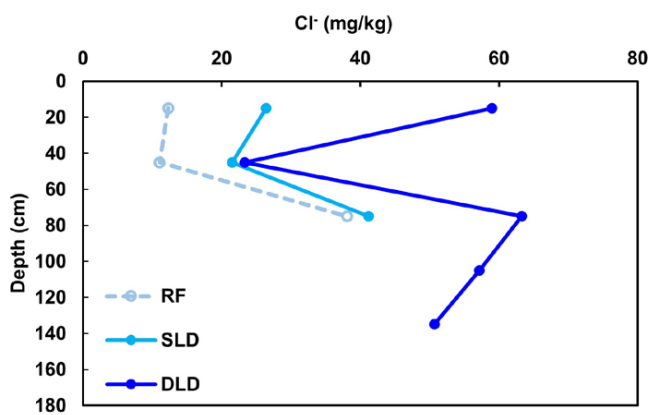


FIGURE 15

Effect of rainfed conditions (RF) and irrigation with treated municipal wastewater *via* single (SLD) and double dripper line (DLD) on the mean soil chloride (Cl) content across the main experimental sites after 11 years of irrigation with treated municipal wastewater.

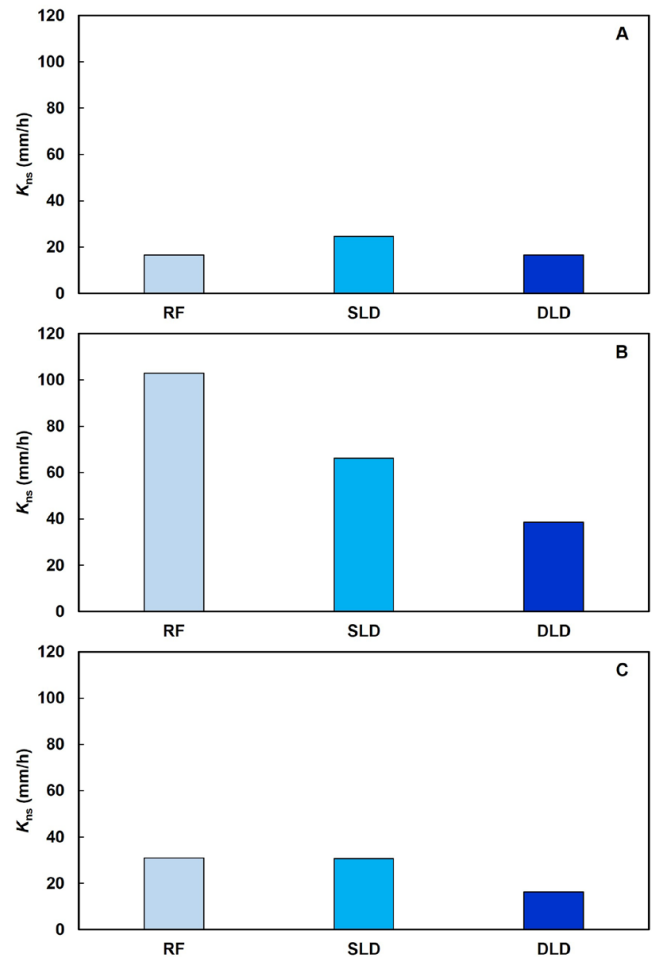


FIGURE 16

Effect of rainfed conditions (RF) and irrigation with treated municipal wastewater *via* single (SLD) and double dripper line (DLD) on the near-saturation hydraulic conductivity ( $K_{ns}$ ) (suction = 2 cm) of (A) a shoulder, (B) a backslope and (C) a footslope during the 2017/2018 season.

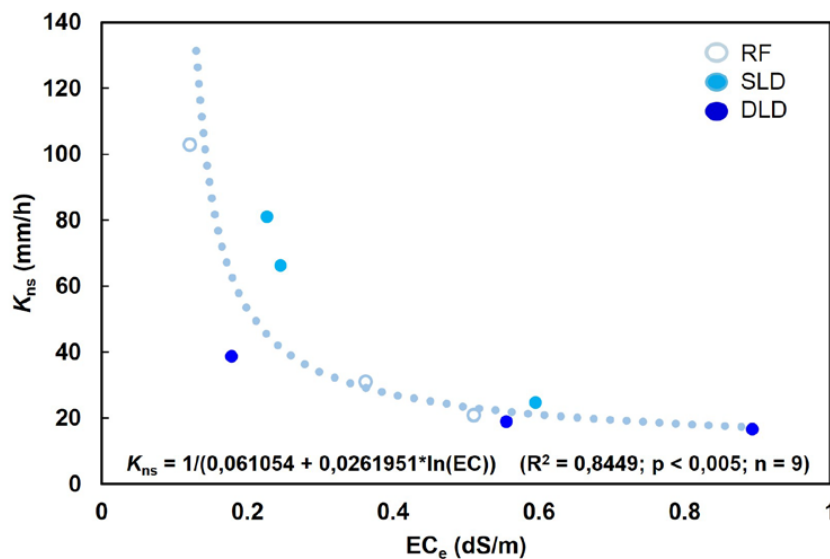


FIGURE 17

Effect of electrical conductivity ( $EC_e$ ) of the 0 cm to 30 cm topsoil layer on near-saturation hydraulic conductivity ( $K_{ns}$ ) during the 2017/2018 season.

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# Ripening Trends of *Vitis vinifera* L. cv Sugrasixteen (SABLE SEEDLESS®) Table Grapes at Different Crop Loads and Bunch Sizes

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**Crop load and bunch size manipulation are two prominent viticultural practices used in the production of *Vitis vinifera* L. cv. Sugrasixteen table grapes. However, their respective effects on berry ripening are unknown and were investigated in this study. A split-plot vineyard block design with three different crop loads (low, medium, and high) as the main treatments and two bunch size treatments (small and large) as the subplot factors were used. Weekly berry samples from veraison to harvest from each treatment were analysed for mass, total soluble solids (TSS), titratable acidity (TA), and pH. Data mining established the ripening trends and explored the possibility of estimating optimal harvest dates using  $EC_{50}$  values. Results showed that berries of large bunches had higher average mass than small bunches during ripening. Increased crop loads resulted in berries with lower mass, TSS and TSS:TA ratio, and delayed berry ripening. Conversely, berries from the low crop load increased in mass and accumulated TSS faster during ripening than higher crop loads, reaching  $EC_{50}$  two to four days earlier with accumulation starting to slow down at higher values.  $EC_{50}$  could be used to estimate harvest dates for low to medium crop loads, but not for high crop loads. This study provides the first in-depth analysis of SABLE SEEDLESS® ripening trends concerning crop load and bunch size management. The results have implications for the grape's overall eating quality. The statistical prediction of optimal harvest dates for lower crop loads could have practical benefits for the industry.**

## INTRODUCTION

Sugrasixteen (*Vitis vinifera* L.), also known as SABLE SEEDLESS®, is considered internationally one of the most important black seedless cultivars with its unique characteristic flavour (Maoz *et al.*, 2019). The berry size, total soluble solids (TSS), and skin colour of the South African SABLE SEEDLESS® for the export market are regulated by the Agricultural Product Standards Act of 1990 (Act No. 119, section 4(3)(a)(ii)). However, no set standard for TSS to titratable acidity (TA) ratio for SABLE SEEDLESS® has been published as for several other table grape cultivars intended for export. TSS:TA ratio plays a vital role in consumer preference (Jayasena & Cameron, 2008) and is a key characteristic determining the flavour of table grapes (Maoz *et al.*, 2016). The need arose to scientifically validate

the cultivar-specific recommendations regarding TSS and expand the profile of SABLE SEEDLESS® to include TSS:TA ratio. Additionally, the influence of the latter on the unique flavour of SABLE SEEDLESS® warrants further studies.

Furthermore, the timing when table grapes reach commercial ripeness is a critical consideration for producers and exporting companies when selecting final market destinations, which significantly impacts the economic value of a crop. The double sigmoidal growth curve of grape berries has been extensively discussed (Coombe, 1976; Ollat, 2002), indicating that the grape berry goes through a rapid development phase followed by a lag phase and finally, the ripening phase up to harvest. The ability to predict table grape berry ripening from veraison (onset of

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ripening) to optimal harvest date has been under research. Recent studies endeavoured to predict table grape berry maturity parameters non-destructively (Pigani *et al.*, 2018; Daniels *et al.*, 2019; Ferrara *et al.*, 2022) but estimating the rate of ripening, and optimal harvest date, for table grapes, has received little attention and is still questionable. Novel data mining techniques can be performed to determine ripening trends and possibly predict optimal harvest dates. In agriculture, the term  $EC_{50}$  is used as a statistical estimate to predict pesticides' effective concentration of achieving 50% of maximal control (Liang *et al.*, 2014; Rozman *et al.*, 2010). This statistical equation could be introduced to the table grape berry ripening trend to predict when the berry will reach 50% of optimal maturity.

Numerous scientific studies have focused on the aspects that influence the timing and evolution of chemical parameters during table grape berry ripening and therefore, the final berry quality (Peppi *et al.*, 2006; Jayasena and Cameron, 2008; Singh *et al.*, 2017; Yin *et al.*, 2022). Table grape vineyard management practices are intentionally performed to change final berry quality, for instance crop load manipulation are performed by removing entire bunches from the vine between pre-bloom and pea-size berry, depending on cultivar, to contribute to increased berry mass, total soluble solids, berry colour, and reduces post-harvest decay (Dokoozlian & Hirschfeld, 1995; Benavente *et al.*, 2014; Singh *et al.*, 2017; Söyler *et al.*, 2020). Improved berry set and increased berry size are obtained by girdling (Söyler *et al.*, 2020; Tyagi *et al.*, 2020), plant hormone applications (Avenant & Avenant, 2006), as well as bunch size shortening or thinning according to the required berries per bunch by cutting the berries from the distal part of the bunches to a specific bunch length (mm) or number of berries per bunch (Benavente *et al.*, 2014; Yin *et al.*, 2022). General viticultural management practices for SABLE SEEDLESS® include crop load manipulation and bunch shortening. However, the effect thereof on the grape berry ripening tempo and flavour development has been under-researched.

The key aspects that influence the economic value of SABLE SEEDLESS® are the berry ripening tempo, influencing harvest date, and if berries reach the required maturity indices coupled with the unique flavour at harvest. Several factors influence berry ripening and final quality. Still, in this study, the focus was on two predominant viticultural practices, namely crop load manipulation and bunch size manipulation (bunch shortening) and the effect thereof on SABLE SEEDLESS®'s ripening tempo. The commercial standard crop load of medium (6000 cartons/ha), as well as the extreme low (3000 cartons/ha) and high (9000 cartons/ha) crop loads, in combination with small and large bunches were investigated. Weekly sampling was done from veraison to harvest to determine maturity indices and ripening trends over time. Further data mining was performed on the ripening trends obtained for berry mass and TSS to determine the rate of accumulation, thereby statistically estimating when berries reach 50% of potential total accumulation ( $EC_{50}$ ), and when accumulation starts to slow down and plateau.

## MATERIALS AND METHODS

### Experimental vineyard layout

This study was performed over two consecutive seasons (2020/2021 and 2021/2022) in a commercial Sugrasixteen (*Vitis vinifera* L.) vineyard with a record of successfully attaining the required export market parameters for SABLE SEEDLESS®. The vines were grafted onto Ramsey rootstock and planted in 2015 at 3 m x 2 m spacing in De Doorns, Western Cape, South Africa. The grapevines were trained on a gable trellising system. Vineyard management practices for the cultivar and region were applied as recommended by the South African Table Grape Industry (SATI) (Van der Merwe, 2020). The experimental layout was a randomised block design containing 24 experimental units, four vines per unit, replicated in eight blocks. The treatment design was a split-plot design with the main treatments consisting of three different crop loads, and the subplot factors were the two bunch size treatments. The main treatments were performed on each vine of the experimental unit by selecting an even number of small and large bunches per vine and removing the rest to the desired crop load.

SATI recommends that optimal taste and quality of SABLE SEEDLESS® is achieved when working on a crop load factor of six bunches per square meter (Van Der Merwe, 2020). As a result, the optimal crop load is 6000 cartons/ha (cartons represent 4.5 kg commercially packed bunches for the export market), obtained with the recommended bunch size of 600 to 700 g/bunch with 110 berries/bunch and a berry mass of 5.5 to 6.3 g/berry (Van Der Merwe, 2020). However, producers strive to achieve a minimum of 5.0 g/berry (Sandhills, 2020), 0.5 g/berry lower compared to the 5.5 g/berry as recommended by Van Der Merwe (2020). The three crop load treatments were therefore set out as medium (6000 cartons/ha) with the extremes of low (3000 cartons/ha), and high (9000 cartons/ha) with cartons representing the commercial standard 4.5 kg packed carton. Bunch sizes consisted of small bunches (80 berries/bunch) and large bunches (120 berries/bunch). As recommended industry practice for the cultivar (Van der Merwe, 2020) both crop load and bunch size manipulation were performed at pea-size berry size (5 – 7 mm diameter) for both seasons. Crop load manipulation was performed on each vine by removing bunches to meet the required crop loads (20 bunches per vine for low crop load; 40 bunches per vine for medium crop load and 60 bunches per vine for high crop load) and shortening bunches by using a scissor and cutting the distal berries from a bunch to the specific number of berries required.

### Differences in bunch sizes between seasons

Initially, it was planned to have small and large bunches on the same vine for both seasons. In the first season, a sufficient number of bunches per vine could be trimmed to ensure an equal number of small and large bunches. However, in the second season, only small bunches occurred naturally throughout the trial site, and no sub-treatments of small and large bunches could be applied. The reason for different bunch sizes between seasons could be ascribed to a seasonal effect, but no further investigation was performed in this study.

### Grape sampling

From 5% veraison, the onset of ripening, five berries per bunch were randomly selected and cut from bunches, with pedicle attached, to obtain a total of 130 berries (65 berries from small bunches and 65 berries from large bunches) from the four vines per replicate. Grape berry samples were collected weekly from veraison until one week after the commercial harvest date (six weeks – 35 days after veraison) when all the crop loads had reached the minimum TSS of 17°Brix as set out by Act 1990 for SABLE SEEDLESS®.

### Fresh berry analyses

A sample consisting of 50 berries from each bunch size for each block replicate (n = 8) was used to determine the average berry mass. Berry samples were pulped, and total soluble solids (TSS) were measured as °Brix with a handheld digital refractometer (PAL-1, ATAGO, Tokyo, Japan). Titratable acidity (TA) and pH were measured by titrating 50 mL juice to end-point 7.0 using sodium hydroxide (Fenoll *et al.*, 2009; Moaz *et al.*, 2016) with a Metrohm, 785 DMP Titrimo instrument (CH-9101 Herisau, Switzerland). TSS:TA ratios were calculated.

### Yield and actual cartons/ha as measured at harvest

The actual number of 4.5 kg cartons packed for each treatment was determined at harvest to validate the experimental layout. All the bunches on each vine per replicate were removed and separated according to sub-treatment categories. Hereafter, the bunches from each sub-treatment were counted, weighed, and packed according to commercial standards in 4.5 kg cartons. The number of cartons was counted and converted to the number of cartons/ha for each sub-treatment.

### Ripening trend analysis

Exponential (least square) growth curves (Figure 1) were fitted to investigate how berry mass and TSS increased over time for the different treatments. Goodness-of-fit were

indicated by confidence intervals of the parameter estimates. The ripening trends were calculated using the following formula:

$$y = Y_0 + (\text{plateau} - Y_0) \left( 1 - e^{-\frac{\log(2) \times \text{DAV}}{EC_{50}}} \right)$$

The following three parameters were estimated from the formula, namely:  $Y_0$  - berry mass or TSS at the start of sampling; plateau - where berry mass or TSS accumulation starts to slow down; and  $EC_{50}$  - the number of days for the berry mass and TSS to reach 50% of the mass or TSS from  $Y_0$ . DAV denotes the number of days after veraison. Growth curves were fitted via R Statistical Software *drc* package version 3.0-1 (Ritz *et al.*, 2015).

### Statistical analysis

A mixed model ANOVA was conducted with plots as random effects and crop load, bunch size and days after veraison (with all interactions) as fixed effects. Normality was checked by inspecting normal probability plots and was judged to be acceptable. Fisher's least significant difference (LSD) was used for post hoc testing. Analysis was done using the R Statistical Software *ImerTest* package version 3.1-3 (Kuznetsova *et al.*, 2017). Statistica graphics facilities were used to make graphs from the results produced in R.

## RESULTS AND DISCUSSION

### Comparison of predicted to actual cartons/ha

The exact number of cartons/ha was not obtained as initially intended (Figure 2) since prediction calculations were based on industry standard bunch size (small) and crop load (medium). However, significant statistical differences between extreme crop loads were found, and therefore the treatments will be assigned as low, medium, and high crop load treatments. In the first season, the small bunch size treatments resulted in similar cartons/ha as intended, but the large bunch size treatments almost doubled the intended cartons/ha.

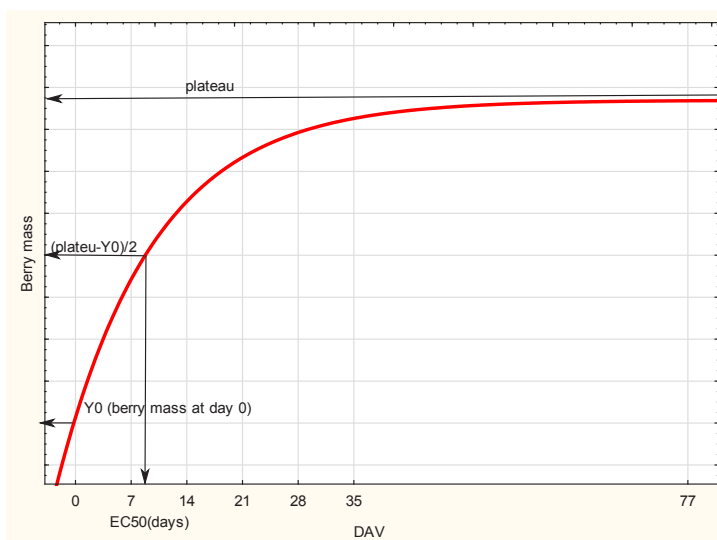


FIGURE 1

Statistical model graph displaying the exponential growth and representing the three parameters estimated namely  $Y_0$  (onset of sampling),  $EC_{50}$  (50% from  $Y_0$  to plateau) and where the ripening trend starts to slow down (plateau).

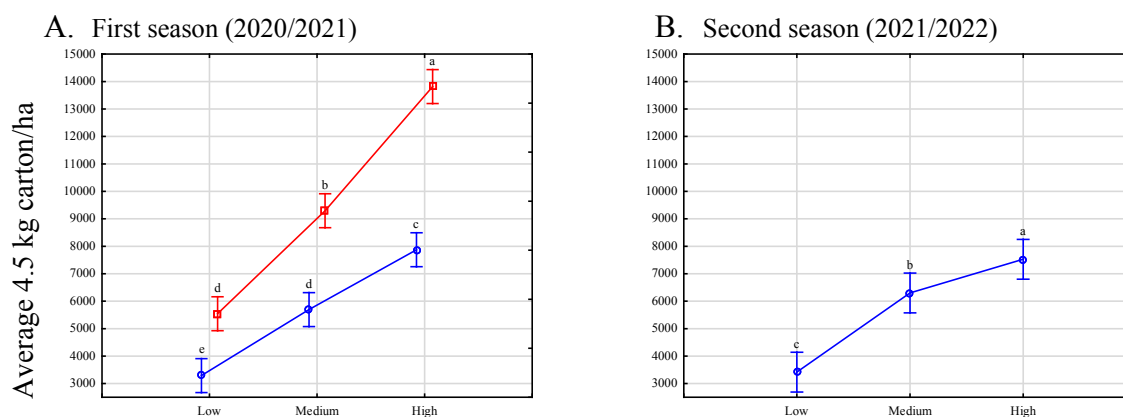


FIGURE 2

Actual 4.5 kg cartons/ha as intended for low (3000 cartons/ha), medium (6000 cartons/ha) and high crop loads (9000 cartons/ha) and bunch sizes (small and large) at harvest (35 days after veraison) for SABLE SEEDLESS® table grapes in A. First season (2020/2021) and B. Second season (2021/2022). Vertical bars represent 5% confidence intervals ( $p < 0.05$ ).

## Ripening and maturity indices

### Grape Berry mass

During the first season, crop load and bunch size significantly impacted the berry mass during ripening (Table 1). From the start of sampling to harvest, 0 to 35 days after veraison (DAV), statistical differences in berry mass between crop loads and between bunch sizes were found. Further data mining was done to determine the trendlines of berry mass increase (rate of accumulation) (Figure 3a) from the start of sampling, represented by  $Y_0$  (Figure 3b) until increase in berry mass stopped (Figure 3d). The trendline between  $Y_0$  and when berry mass stopped increasing was used to calculate the number of days it took for berries to reach 50% of the maximal mass ( $EC_{50}$ ) (Figure 3c). At the start of sampling, 0 DAV, the initial mass ( $Y_0$ ) of the berries from the low crop load was significantly higher compared to the medium and high crop load treatments (both bunch sizes) (Figure 3b). This indicated that berry growth during the development phase (before veraison) is influenced when the crop load decreased from medium to high (40 to 60 bunches/vine) to low crop load (20 bunches/vine) at 5 mm berry size. In contrast, Dokoozlian & Hirschfeld (1995) did not observe any differences in berry mass at the start of ripening when the crop load of Flame Seedless was reduced from 45 to 30 bunches/vine at either berry set or two and four weeks after berry set. This contrast could be ascribed to either the different cultivar, trellising system and climatic conditions compared to present study.

Furthermore, in both seasons of this study, the separation in trendlines of the different crop loads between 0 to 14 DAV indicates that berries for low crop load rapidly increase in berry mass between 0 and 7 DAV, followed by berries from medium crop load increasing between 7 to 14 DAV with berries from high crop load showing the slowest growth trend (Figure 3a). Low crop load resulted in berries with the highest mass throughout ripening compared to medium and high crop loads. The findings correspond to previous research indicating that crop load reduction leads to higher berry mass (Dokoozlian & Hirschfeld, 1995; Fallahi *et al.*, 2017; Singh *et al.*, 2017; Söyler *et al.*, 2020). The higher

berry mass in season two compared to season one at  $Y_0$  could indicate that a uniform bunch size across the vine results in less competition for resources between berries. As a result, nutrients can be located evenly throughout the bunches on the vine.

Opposed to the crop load effect, bunch size manipulation did not significantly influence berry mass during initial berry development up to veraison since no significant differences in berry mass were found within the crop load treatments at  $Y_0$ . Nevertheless, berries from large bunches tend to have higher berry mass compared to berries from small bunches across the three crop load treatments at  $Y_0$ . However, bunch size manipulation significantly affected berry mass during berry ripening since berries from larger bunches had significantly higher berry mass throughout ripening, and reached a higher berry mass compared to small bunches when increase started to slow down. A similar trend was found in a study by Fallahi *et al.* (2017), indicating that bunch shortening results in higher berry mass. However, in contrast, Benavente *et al.* (2014) found that small bunches (80 berries/bunch) had higher berry mass compared to large bunches (160 berries per bunch) of Thompson Seedless grapes. They did not report at what crop growth stage crop load and bunch size manipulation were performed. Furthermore, Yin *et al.*, (2022) found that bunch shortening at pea-size berry size (5-7 mm) did not affect berry mass at harvest of two cultivars (Baoguang and Cuiguang) produced under protected cultivation in China.

The rate of accumulation trendlines was used to predict at what berry mass the increase will slow down, and these values were compared to the actual values obtained from sampling. In both seasons, berries from lower crop load reached the producer-recommended 5.0 g/berry mass by 21 DAV and medium crop load at 28 DAV, while the predicted mass (between 5.5 to 6.1 g/berry) for both crop loads was reached at 35 DAV. Berries from the high crop load reached the recommended berry mass by 35 DAV but had not yet reached the predicted mass when increase will start to slow down (5.1 to 5.9 g/berry) in this study. This indicates that the berries from high crop loads tend to grow slower and reach the potential final mass later than the lower crop loads.

TABLE 1  
Berry mass (g/berry) throughout ripening, 0 to 35 days after veraison (DAV), for SABLE SEEDLESS® table grapes in first (2020/2021) and second (2021/2022) season.

Season	DAV	Low crop load			Medium crop load			High crop load			P-value	Crop load x Bunch size
		Small bunches	Large bunches	Small bunches	Large bunches	Small bunches	Large bunches	Small bunches	Large bunches	Crop load		
<b>First season (2020 / 2021)</b>	<b>0</b>	2,33 ± 0,28 ab	2,45 ± 0,27 a	2,01 ± 0,12 c	2,17 ± 0,19 bc	1,97 ± 0,17 c	2,18 ± 0,18 bc	<.0001	<b>0.0001</b>	<b>0.5551</b>		
	<b>7</b>	3,78 ± 0,35 a	3,65 ± 0,25 ab	3,43 ± 0,29 bc	3,22 ± 0,19 c	3,23 ± 0,18 c	3,18 ± 0,04 c	<.0001	<b>0.0089</b>	<b>0.3501</b>		
	<b>14</b>	4,43 ± 0,37 ab	4,59 ± 0,26 a	3,99 ± 0,24 c	4,27 ± 0,21 b	3,64 ± 0,16 d	3,80 ± 0,15 cd	<.0001	<b>&lt;.0001</b>	<b>0.3818</b>		
	<b>21</b>	5,13 ± 0,26 a	5,18 ± 0,32 a	4,63 ± 0,24 bc	4,85 ± 0,25 b	4,28 ± 0,15 d	4,43 ± 0,14 cd	<.0001	<b>0.0008</b>	<b>0.1739</b>		
	<b>28</b>	5,52 ± 0,29 a	5,41 ± 0,38 a	5,10 ± 0,18 b	5,07 ± 0,32 b	4,59 ± 0,21 c	4,72 ± 0,22 c	<.0001	<b>0.8993</b>	<b>0.1274</b>		
<b>35</b>	5,56 ± 0,22 b	5,90 ± 0,18 a	5,51 ± 0,38 b	5,56 ± 0,25 b	5,00 ± 0,29 c	5,43 ± 0,33 b	<.0001	<b>0.0006</b>	<b>0.0781</b>			
<b>Second season (2021 / 2022)</b>	<b>0</b>	2,65 ± 0,23 a		2,50 ± 0,18 a		2,39 ± 0,24 a		<b>0.0851</b>				
	<b>7</b>	4,07 ± 0,14 a		3,72 ± 0,25 b		3,49 ± 0,22 b		<b>0.0001</b>				
	<b>14</b>	4,76 ± 0,26 a		4,55 ± 0,24 a		4,22 ± 0,20 b		<b>0.0007</b>				
	<b>21</b>	5,39 ± 0,20 a		4,82 ± 0,29 b		4,55 ± 0,14 c		<b>&lt;.0001</b>				
	<b>28</b>	5,52 ± 0,27 a		5,24 ± 0,39 b		4,84 ± 0,25 c		<b>0.0009</b>				
<b>35</b>	5,79 ± 0,17 a		5,31 ± 0,49 b		5,15 ± 0,22 b		<b>0.0043</b>					

Each value represents the mean of eight replicates ± standard deviation. Different letters in the same row indicate significant differences at 5% significance according to the Least Significant Differences (LSD) post-hoc test.

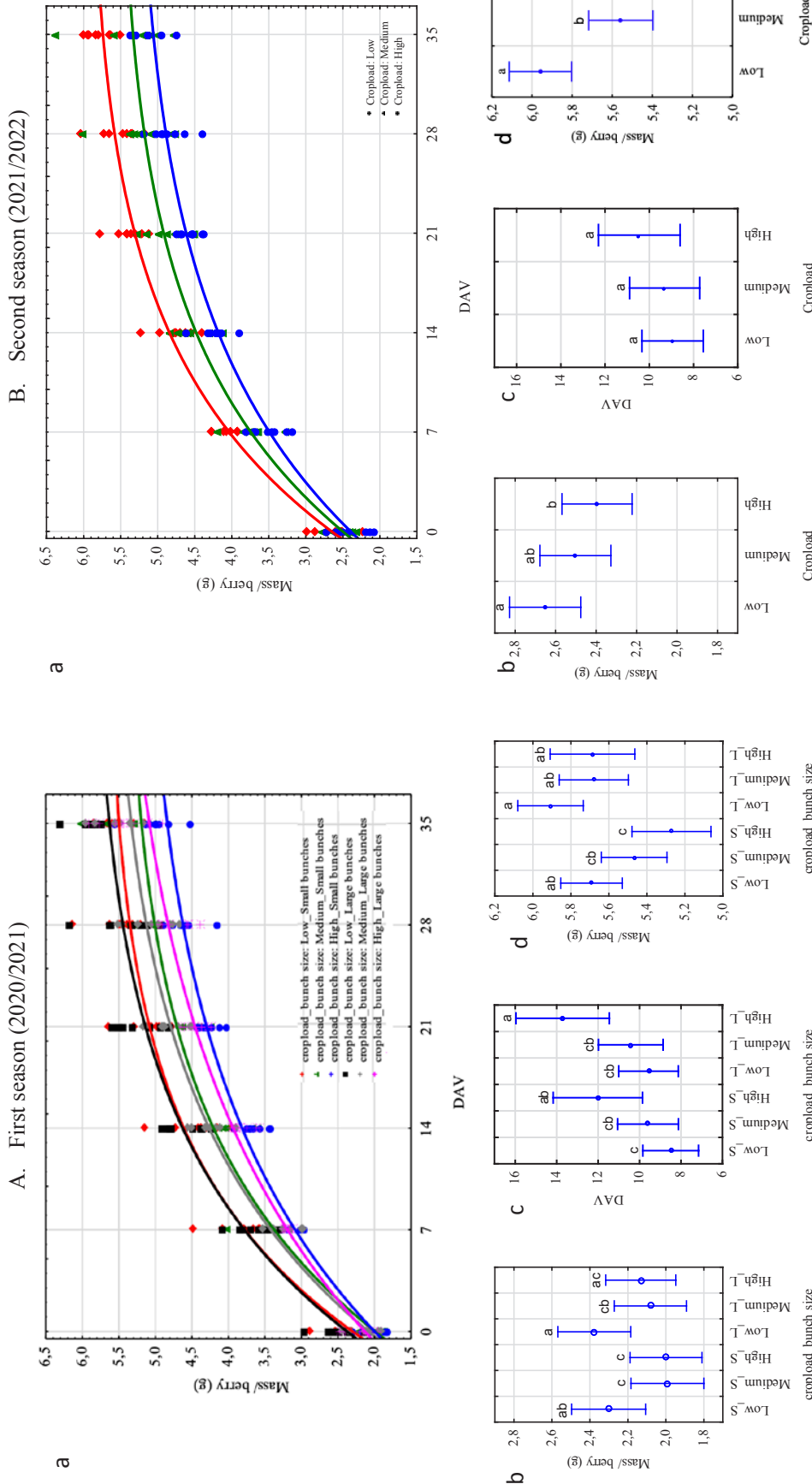


FIGURE 3

a - Growth curve as measured by mass per berry (g) for SABLE SEEDLESS® berry from veraison until harvest (0 to 35 DAV) for both seasons ( $R^2=0.97$  for both seasons). b - Berry mass (g) at start of sampling ( $Y_0$ ) at 0 days after veraison; c - Days after veraison when berries reached  $EC_{50}$  (50% of potential final mass); d - Berry mass (g) when increase starts to slow down. Vertical bars represent 5% confidence intervals ( $p \leq 0.05$ ). S represents small bunches; L represents large bunches.

Consequently, since berries from low crop load reached a higher berry mass before the increase started to slow down, it can be concluded that the larger the berry at the start of veraison ( $Y_0$ ), the greater the potential of rapidly reaching higher berry mass before reduction in berry mass increase is observed.

From the  $Y_0$  and plateau values, the  $EC_{50}$  was statistically calculated to predict the number of days after veraison (DAV) berries will reach 50% of the potential final berry mass (Figure 3c). In the first season, significant differences in  $EC_{50}$  were found between crop load treatments, with berries from the lowest crop load reaching  $EC_{50}$  earlier than higher crop loads. A similar trend was observed in the second season; however, differences were not significant. Bunch size also tends to influence the rate at which berry mass increases since berries from small bunches tend to reach  $EC_{50}$  earlier compared to berries from large bunches across the three crop loads. However, the differences were not significant within a crop load treatment.

In both seasons, low crop load reached  $EC_{50}$  between 7 to 11 DAV, one day earlier than the medium crop load (8 to 12 DAV). However, in the first season the high crop load reached  $EC_{50}$  between 12 to 16 DAV, 5 days later, compared to the low crop load. In the second season, the berries from high crop load reached  $EC_{50}$  earlier at 8.5 to 12.3 DAV, only one day later compared to low and medium crop loads. In both seasons, the  $EC_{50}$  graphs display greater variability in berry mass for the high crop load with a 95% confidence level line extending over 4 days compared to 3 days for low and medium crop loads.

Furthermore, it was found that the number of days predicted by  $EC_{50}$  could be used to determine when berries will reach the recommended 5.0 g/berry by multiplying the predicted  $EC_{50}$  value by two. The calculated predicted values were compared to actual values obtained during sampling (Table 1). For both low and medium crop loads, the recommended berry mass was reached within the predicted period. However, the recommended berry mass for berries from high crop load was reached one to seven days later than the predicted period. The trends found for both seasons indicate that predictions are more accurate with lower crop loads than with higher crop loads. This could be due to the variability in berries for higher crop loads.

### **Total soluble solids (TSS)**

In both seasons, the increase in TSS accumulation trends showed similar trends to berry mass (Figure 4). In the first season berries from low crop load with small bunches had significantly higher TSS at  $Y_0$  than berries of high crop load with large bunches (Figure 4a). In the second season, no significant differences in the TSS levels were found at  $Y_0$  (Figure 4d), which agrees with the study done by Dokoozlian & Hirschfeld (1995), who also found no difference in TSS in Flame Seedless berries between different crop load treatments at start of ripening. However, the TSS values were higher at  $Y_0$  for all the crop loads (7.0 to 8.7 °Brix) in the second season compared to the first season (5.5 to 8.0 °Brix), indicating that during the second season, berries could have started to accumulate sugars a few days earlier.

In the first season, the berries from small bunches tend

to have a more rapid accumulation rate compared to berries from large bunches (Figure 4a). However, bunch size had no continued significant effect on TSS accumulation during ripening (except at 7 and 21 DAV) (Table 2). Contradictory results were found in the study by Yin *et al.* (2022), where significant differences were found in TSS between the different size bunches for two table grape cultivars, Baoguang and Cuiguang. This could be because of the final number of berries per bunch in Yin *et al.*'s (2022) study, namely 30 to 60 berries per bunch, whereas the current study had 80 to 120 berries per bunch. This could indicate that the removal of more berries may potentially have a greater impact on TSS accumulation of SABLE SEEDLESS®.

Like  $Y_0$  in the first season, significant differences were found when berries reached  $EC_{50}$  from low crop load with small bunches compared to high crop load with large bunches. Berries from small bunches reached  $EC_{50}$  one to two days earlier compared to the large bunches. Similar to the  $EC_{50}$  values recorded for berry mass, low crop loads reached  $EC_{50}$  at 8 to 13 DAV (both bunch sizes), one to two days earlier than medium crop load (9 to 14 DAV for both bunch sizes), and two to three days earlier than high crop loads (10 to 16 DAV for both bunch sizes). In the second season, only small bunches were produced, and the berries took 1-2 days longer to reach the  $EC_{50}$  of the previous seasons' small bunches. Berries on small bunches from the second season reached  $EC_{50}$  in similar time periods compared to the berries from large bunches for the respective crop loads compared to the previous season. The delay in reaching  $EC_{50}$  in the second season for berries from small bunches could be ascribed to the mass difference between berries since the berries weighed on average 0.21 g/berry more compared to the first season. The increases in mass (and therefore berry size) could have influenced TSS in berries.

Significant differences in TSS were found between crop loads when reduction in the accumulation curves were observed (Figure 4c). TSS accumulation started to slow down between 19.6 and 21 °Brix for berries from the low crop load, between 18.7 and 20.5 °Brix for medium crop load, and between 17.6 and 19.8 °Brix for berries from high crop load (both bunch sizes) (Figure 4). In the second season, the TSS accumulation slowed down 2.0 °Brix increments higher compared to the first season for the corresponding crop loads (Figure 4c). In the first season, berries from the three crop loads reached the predicted TSS at 35 DAV, but in the second season, the berries from medium and high crop loads had not yet reached the predicted TSS.

In both seasons, significant differences in TSS were found between crop load treatments during ripening from 7 until 35 DAV, with berries from the low crop load, with higher berry mass, reaching higher TSS compared to medium and high crop load berries (Table 2). This corresponds to previous studies also indicating that berries from lower crop loads resulted in higher TSS at harvest (Sun *et al.*, 2012; Fallahi *et al.*, 2017; Singh *et al.*, 2017; Söyler *et al.*, 2020).

The predicted harvest date for low and medium crop load using  $EC_{50}$  were compared to the actual TSS measured. The actual TSS °Brix values measured during sampling were used to confirm if the harvest date can be predicted by using the equation as described in the berry mass section

TABLE 2  
Total Soluble Solids (TSS) (°Brix) throughout ripening, 0 to 35 days after veraison (DAV), for SABLE SEEDLESS® table grapes in first (2020/2021) and second (2021/2022) season.

Season	DAV	Low crop load			Medium crop load			High crop load			P-value	
		Small bunches	Large bunches	Small bunches	Large bunches	Small bunches	Large bunches	Small bunches	Large bunches	Crop load	Bunch size	Crop load x Bunch size
First season (2020 / 2021)	0	7.39 ± 0.85 a	7.25 ± 0.58 a	6.91 ± 0.58 ab	6.69 ± 0.84 b	7.03 ± 0.70 ab	6.70 ± 0.65 b	0.0155	0.1278	0.8698		
	7	12.51 ± 1.32 a	11.19 ± 1.00 b	11.73 ± 1.39 ab	9.99 ± 0.82 c	10.71 ± 1.71 bc	8.88 ± 0.63 d	<.0001	<.0001	0.7750		
	14	14.96 ± 0.42 a	14.49 ± 0.52 b	13.96 ± 0.33 c	13.98 ± 0.30 c	13.43 ± 0.75 d	13.34 ± 0.53 d	<.0001	0.1344	0.0220		
	21	17.10 ± 0.93 a	16.28 ± 1.35 b	15.31 ± 0.41 c	14.96 ± 0.68 cd	14.55 ± 0.33 d	14.39 ± 0.33 d	<.0001	0.0316	0.3720		
	28	18.68 ± 0.57 a	18.54 ± 1.74 a	17.13 ± 1.11 b	17.31 ± 1.59 b	15.06 ± 0.44 c	15.44 ± 0.85 c	<.0001	0.1175	0.9220		
	35	20.30 ± 1.31 a	19.84 ± 1.67 ab	19.36 ± 1.46 ab	18.91 ± 0.78 bc	18.39 ± 1.66 bc	18.18 ± 0.84 c	0.0015	0.0636	0.4992		
Second season (2021 / 2022)	0	7.93 ± 0.68		7.66 ± 0.46		7.86 ± 0.53		0.6296				
	7	13.70 ± 0.69 a		12.22 ± 1.12 b		11.35 ± 1.14 b		0.0005				
	14	14.83 ± 0.55 a		14.42 ± 0.44 ab		14.00 ± 0.19 b		0.0029				
	21	17.98 ± 0.64 a		16.19 ± 0.83 b		15.14 ± 0.71 c		<.0001				
	28	19.46 ± 0.83 a		18.59 ± 1.07 b		17.44 ± 1.06 c		0.0021				
	35	21.53 ± 0.78 a		20.29 ± 0.68 b		19.66 ± 0.47 c		<.0001				

Each value represents the mean of eight replicates ± standard deviation. Different letters in the same row indicate significant differences at 5% significance according to the Least Significant Differences (LSD) post-hoc test.

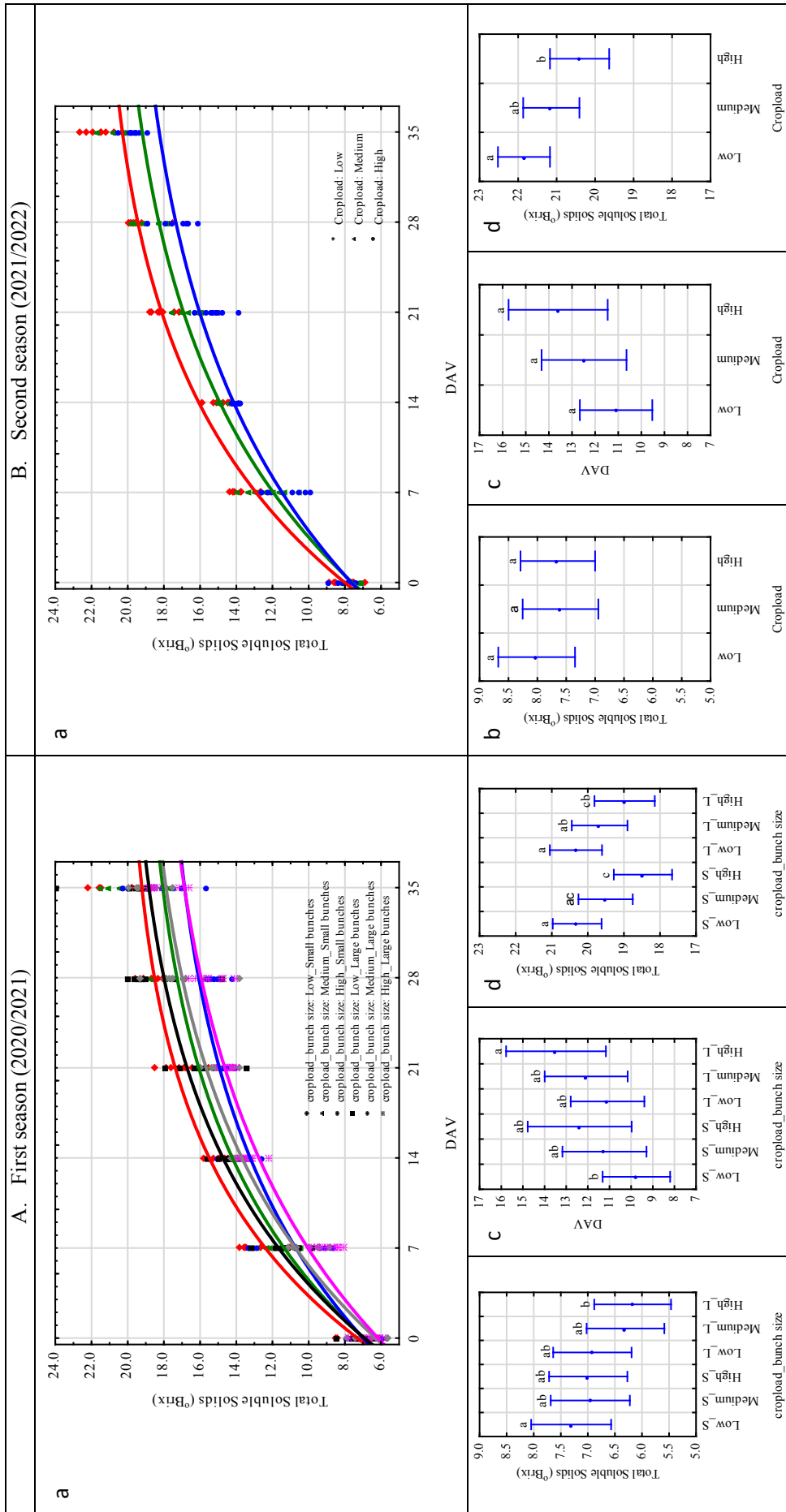


FIGURE 4

a -Accumulation of Total soluble solids (TSS) over time for SABLE SEEDLESS® berry from veraison until harvest (0 to 35 DAV) for both seasons (R<sup>2</sup> =0.97 for both seasons); b – TSS at the start of sampling at 0 DAV (Y<sub>0</sub>); c – DAV when berries reached EC<sub>50</sub> (50% of potential final TSS); d – TSS when accumulation starts to slow down . Vertical bars represent 5% confidence intervals (p<0.05). L represents small bunches; M represents medium bunches; H represents large bunches.

by multiplying the  $EC_{50}$  value by two. The predicted harvest dates for low crop load ranged between 20 to 22 DAV, and actual TSS met required standards (above 17 °Brix) between 21 to 28 DAV. Similar to medium crop load, berries reached the required TSS between 21 and 28 DAV compared to the prediction of 22 to 24 DAV. The high crop load reached the required TSS between 28 and 35 DAV, which was later compared to the predicted 25 to 27 DAV. Similar to the predictions for berry mass, the predictions of TSS by using the equation of  $EC_{50}$  multiplied by two did not correspond to actual measurements for the higher crop load. This difference could be ascribed to the variability in TSS of berries from high crop loads.

In both seasons, the berries from the lower crop load reached the required TSS (17 °Brix) a week earlier than the medium crop load and two weeks earlier than the high crop load. Previous studies also indicated that the differences in crop load influenced ripening trends with berries from vines with fewer bunches ripening earlier compared to vines with more bunches (Dokoozlian & Hirschfeld, 1995; Singh *et al.*, 2017; Söyler *et al.*, 2020). Even though, in this study, the required mass and TSS parameters were reached in one-week intervals between crop loads, the question arises whether

the prominent muscat flavour of SABLE SEEDLESS® has developed within the same time frame.

**Titrateable acidity (TA) and pH**

Similar trends for TA and pH were found between the different crop loads in both seasons. TA declined rapidly from 27 g/L at 0 DAV to below 4.5 g/L at 35 DAV (Figure 5). No significant differences in TA were found between the crop loads from 21 to 35 DAV. Crop load did not have any significant impact on pH during the ripening period, as well. Previous studies also found that crop load manipulation did not affect TA or pH (Dokoozlian & Hirschfeld, 1995; Sun *et al.*, 2012; Söyler *et al.*, 2020).

Bunch size (in the first season) had no significant effect on TA and pH, and therefore bunch size data is not presented in Figure 5. Similarly, Yin *et al.* (2022) found that a change in bunch size, early on in berry development stage, did not impact TA but altered the pH of berries at harvest. The change over time in TA and pH could be due to a dilution effect as berry size increase, malic acid is used or converted from malic acid to glucose and degradation of acids (Ramos & Romero, 2017).

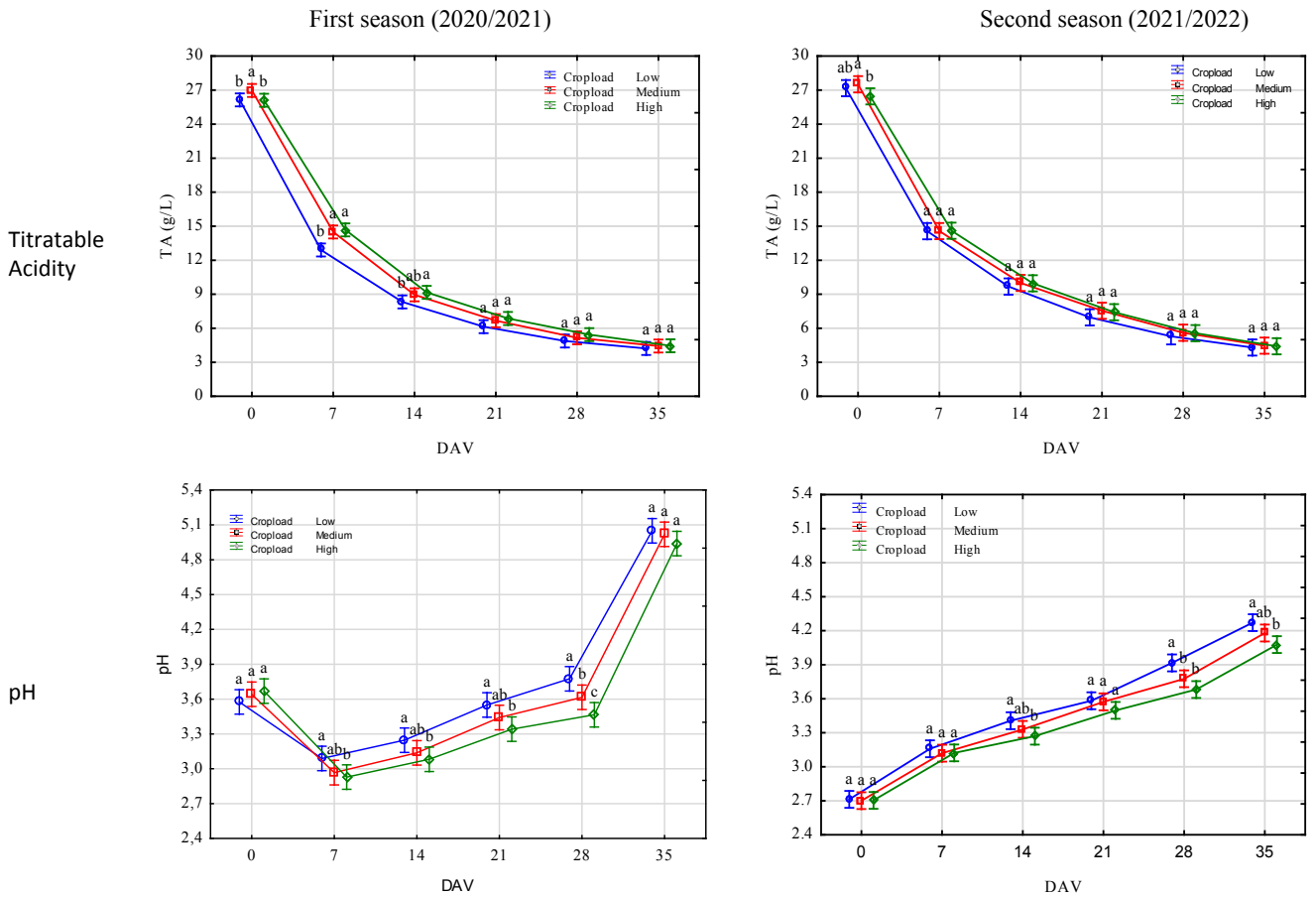


FIGURE 5

Titrateable acidity (TA) and pH of berries from low, medium and high crop loads from veraison until harvest (0 to 35 DAV) for SABLE SEEDLESS® table grapes in first (2020/2021) and second (2021/2022) season. Vertical bars represent 5% confidence intervals (p<0.05).

TABLE 3  
TSS: TA ratio throughout ripening, 0 to 35 days after veraison (DAV), for SABLE SEEDLESS® table grapes in first (2020/2021) and second (2021/2022) season.

TSS:TA ratio ± SD		P-value				
Season	DAV	Low crop load	Medium crop load	High crop load	Crop load	Crop load x Bunch size
First season (2020/ 2021)	0	2,82 ± 0,41 a	2,55 ± 0,43 b	2,66 ± 0,43 ab	0,0865	0,9956
	7	9,93 ± 4,20 a	7,65 ± 1,70 b	6,76 ± 1,50 b	<.0001	0,8953
	14	17,79 ± 1,61 a	15,72 ± 1,49 b	14,69 ± 1,44 c	<.0001	0,0135
	21	27,47 ± 3,81 a	22,78 ± 1,66 b	21,12 ± 0,98 c	<.0001	0,5275
	28	38,24 ± 3,42 a	33,53 ± 3,61 b	28,08 ± 1,92 c	<.0001	0,6113
	35	47,67 ± 4,58 a	43,26 ± 3,25 b	41,22 ± 4,09 c	<.0001	0,7515
Second season (2021/ 2022)	0	2,96 ± 0,54	2,80 ± 0,31	3,01 ± 0,50	0,6360	
	7	9,47 ± 1,04	8,45 ± 1,28	7,85 ± 1,26	0,0411	
	14	15,42 ± 1,64	14,55 ± 1,63	14,09 ± 0,92	0,1978	
	21	25,98 ± 2,52 b	21,76 ± 3,30 a	20,51 ± 1,88 a	0,0012	
	28	36,82 ± 1,67 b	33,32 ± 3,59 a	31,47 ± 3,41 a	0,0064	
	35	50,14 ± 3,60 b	45,55 ± 3,42 a	44,65 ± 3,10 a	0,0100	

Each value represents the mean of eight replicates ± standard deviation. Different letters in the same column indicate significant differences at 5% significance according to the Least Significant Differences (LSD) post-hoc test.

**TSS: TA ratio**

In the first season significant differences in TSS: TA ratio were found from 7 DAV between crop load treatments (Table 3). TSS had the greatest impact on the ratios. Even though similar trends were observed in the second season, the TSS: TA ratios for each respective crop load were higher. The lowest crop loads tend to be associated with a rapid increase in TSS, lower TA, and, therefore, higher TSS:TA ratio during ripening and at harvest, compared to the medium and higher crop loads. Contrary to previous findings (Yin *et al.*, 2022), in this study bunch size manipulation had no effect on TSS:TA ratio for both seasons. In both seasons the TSS:TA ratio ranged between 28 and 38 for the three crop loads at 28 DAV and increased to above 40 at 35 DAV. Under Egyptian conditions SABLE SEEDLESS® is harvested when TSS:TA ratios reach 40 to 45 (Mohamed & Khairey, 2017) which corresponds to the TSS:TA values found in the current study at 35 DAV. Jayasena & Cameron (2008) found the TSS:TA ratio to be a better indicator of consumer demand since the demand for Crimson Seedless table grapes increased by 54% once the TSS: TA ratio increased from 20 to 40. Therefore, further studies could be conducted to determine if the desirable muscat flavour of SABLE SEEDLESS® is correlated to the TSS:TA ratio and at what ratio the flavour becomes most prominent. The TSS:TA ratio parameter could be an important criterion to help determine the optimal timing to harvest SABLE SEEDLESS®.

**CONCLUSIONS**

Crop load and bunch size manipulation altered the ripening trends of SABLE SEEDLESS®, as well as the timing when berries reached the maturity indices required for the export market. Further data mining also proved beneficial since optimal harvest dates for low to medium crop loads could be predicted. This could influence harvest maturity and, thereby, the final quality and the sensory characteristics of SABLE SEEDLESS® berry. It is still not known how these parameters (reported in this study) relate to the unique Muscat flavour of SABLE SEEDLESS®.

Bunch size manipulation did not enhance the final berry TSS or TSS:TA ratio, and smaller bunches resulted in smaller berries (lower berry mass). Therefore, further research is required to establish if this viticultural management practice is needed for SABLE SEEDLESS®. No combination effect of crop load and bunch size was found for berry mass, indicating that crop load have the greatest impact on SABLE SEEDLESS® berry development. Grapes produced at a lower crop load tend to reach the required quality parameters earlier than the higher crop loads and could be distributed to the market earlier. The potential economic impact on the value per carton entering the market a day to week earlier should be further explored. Even though crop load tends to influence SABLE SEEDLESS® ripening trends, causing berries to reach the required maturity indices earlier, it is still to be ascertained whether the optimum flavour developed within a shorter period and if it is correlated to TSS and TSS: TA ratio.

The prediction models obtained in this study indicated that berry development and ripening, regarding berry mass and TSS, can be calculated using the  $Y_0$ ,  $EC_{50}$  and plateau

(when accumulation starts to slow down) values obtained from the rate of ripening curve for low to medium crop loads. The ripening trends can assist table grape producers in employing precision agriculture practices and farming sustainably since it will enable producers to plan and produce table grapes at specified berry mass (size) and TSS for the specific export market the grapes are destined to. The use of the ripening trends to predict  $EC_{50}$  and when berries will be harvest-ready, is a novel estimation technique in the table grape industry. Scientific-based predictions will allow producers to estimate when berries will reach the required maturity indices and, thereby, commercial ripeness. Additional research is required to establish the relationship between berry TSS, TSS:TA ratio and the unique desirable Muscat flavour of SABLE SEEDLESS®.

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# The Influence of Environmental Conditions and Vineyard Management Practices on the Composition and Content of Carotenoids in Grapevines (*Vitis vinifera* L.). A Review

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**Carotenoids are plant pigments that are important for photosynthesis and photo-protection. In recent years there has been great interest in the role of carotenoids in grapevines. The reason for this interest is that carotenoids are substrates for the synthesis of C13-norisoprenoids, which have a major influence on wine aroma. To optimise the concentration of C13-norisoprenoids in grapes and wine, the environmental conditions and vineyard management practices that may affect carotenoid metabolism in grapes need to be better understood. This paper reviews recent studies on vineyard management practices in terms of carotenoid accumulation, discusses grapevine plasticity, and provides new perspectives for understanding grapevine physiology in response to environmental conditions that affect carotenoid synthesis.**

## INTRODUCTION

Plant pigments are compounds perceptible to humans that can detect reflected or transmitted light with wavelengths between 380 and 730 nm. The most important classes of plant pigments are chlorophylls, anthocyanins and carotenoids. They impart orange/red, purple/blue, yellow and green colour to plants by acting as a visible signal to attract insects, birds and other animals for pollination and seed dispersal (Nzekoue *et al.*, 2022; Sun *et al.*, 2022). They also protect plants against UV and light damage (Matus *et al.*, 2016).

Today, the grape and wine industry is facing new challenges related to climate change. Air temperature, solar radiation, water availability in the soil and CO<sub>2</sub> concentration in the atmosphere are among the most important abiotic factors influenced by global warming, affecting vine development and grape ripening (Rienth *et al.*, 2021). The continuous depletion of the ozone layer over the last 50 years has led to an increase in UV radiation (Mackerness, 2000), forcing changes in plant physiology and development. Climate changes affect the vegetative growth and reproductive development of grapevines and affect the yield and quality of grapes (Van Leeuwen & Darriet, 2016). High and sudden exposure of bunches to sunlight can lead to photo-oxidative damage and sunburn, especially if the ambient temperature is too high (Torres *et al.*, 2021). Sunburn can affect the composition of the grapes, which can lead to significant economic losses due to a reduced yield and quality of the grapes (Yan *et al.*, 2020).

The grape berry has developed adaptations to mitigate the effects of excessive solar radiation, including

the accumulation of secondary metabolites (Gambetta *et al.*, 2022). Carotenoids are lipid-soluble plant pigments synthesised in the chloroplasts and chromoplasts of fruits and vegetables, where they play an active role in protecting plants from increased light intensity (Sun *et al.*, 2022). The composition and content of carotenoids in grapes are regulated by environmental conditions, phenological stage and variety characteristics (Oliveira *et al.*, 2004; Joubert *et al.*, 2016; Young *et al.*, 2016). Lower concentrations of carotenoids may be associated with shaded fruit, either through direct actions such as canopy management or indirectly through increased fertilisation or irrigation, resulting in a more vigorous canopy (Oliveira *et al.*, 2003; Bindon *et al.*, 2007; Gutiérrez-Gamboa *et al.*, 2018). The vine can react to increased solar radiation by changing the composition of the berries (Asproudi *et al.*, 2020) and increasing the concentration of carotenoids (Bureau *et al.*, 1998; Razungles *et al.*, 1996). Stress caused by water scarcity can also lead to an increase in carotenoids, as plants release excess energy from photosynthesis via the xanthophyll cycle in the form of heat (Hirschberg, 2001).

Since carotenoids are substrates for C13-norisoprenoid synthesis (Young *et al.*, 2012), higher carotenoid concentrations, and thus higher substrate availability, can lead to higher concentrations of C13-norisoprenoids in sun-exposed grapes (Mendes-Pinto, 2009). C13-norisoprenoids are secondary metabolites in grapes that have a positive influence on the complexity and quality of wine aroma. Many different C13-norisoprenoids have been identified in grapes and wine, but only a few of them affect aroma.

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These include  $\beta$ -damascenone,  $\beta$ -ionone, TDN, TPB and vitispirane (Yuan & Qian, 2016), which impart fruity and floral notes to wines. Sun exposure, higher temperatures and mild water stress are associated with an improvement in grape quality, and result in grapes with higher levels of sugars, polyphenolic and volatile compounds, and lower total acidity (Diago *et al.*, 2012). This has a positive effect on the sensory characteristics of the wine, as fruit and floral aromas are emphasised while vegetal and green aromas are reduced (Mendes-Pinto, 2009; Young *et al.*, 2016).

The altered microclimate due to climate change leads to stress responses, acclimatisation and altered quality-related metabolites in grape berries (Rienth *et al.*, 2021). High air temperatures can lead to changes in aroma compounds, leading to the conclusion that, with a further increase in air temperature due to climate change, the perception of overripe fruit notes will increase, as is now the case in hot years (Pons *et al.*, 2017). This may affect the organoleptic properties of wine (Van Leeuwen & Darriet *et al.*, 2016). Therefore, the traditional practices of viticulture and winemaking, as well as the typicality of the assortment, sensory characteristics and style of wines from certain wine-growing regions, are increasingly being questioned. For this reason, viticulture must adapt to the new climatic conditions by adopting new cultivation techniques, resistant varieties and ampelotechnical practices, but also finding new regions for the production of certain styles of wine (Fraga *et al.*, 2012).

Understanding vine physiology in response to environmental conditions may allow the exploration of berry metabolic plasticity in relation to desired wine styles

and could help grape growers make appropriate vine-manipulation decisions to produce the desired aromatic wine profile. The present review aims to demonstrate the influence of environmental conditions and vineyard management practices on the composition and content of carotenoids in grapevines, which can serve as a basis for grape growers and oenologists to synthesise aroma compounds in wine (C13-norisoprenoids).

## STRUCTURE AND SYNTHESIS OF CAROTENOIDS

### The structure of carotenoids

Carotenoids are isoprenoid compounds that belong to the family of terpenes with 40 carbon atoms (tetraterpenes). More than 700 carotenoids have been identified in plant tissues. Most carotenoids consist of eight isoprene units with a 40-carbon skeleton. Their general structure usually consists of a polyene chain with nine conjugated double bonds and an end group at both ends of the polyene chain. The carbon-carbon double bonds can be in the *cis* or *trans* isomer configuration, depending on the arrangement of the substituents. Based on their structure there are two classes of carotenoids, namely carotenes and xanthophylls (oxygenated carotenes). The structure and synthesis of carotenoids has been studied in detail by Tanaka *et al.* (2008) (see Fig. 1).

### The synthesis of carotenoids

Carotenoids are synthesised from isopentenyl pyrophosphate (IPP), which is derived from glyceraldehyde-3-phosphate and pyruvate via the methyl erythritol-4-phosphate (MEP) pathway in the plastids (Tanaka *et al.*, 2008). The condensation of four IPPs produces geranylgeranyl

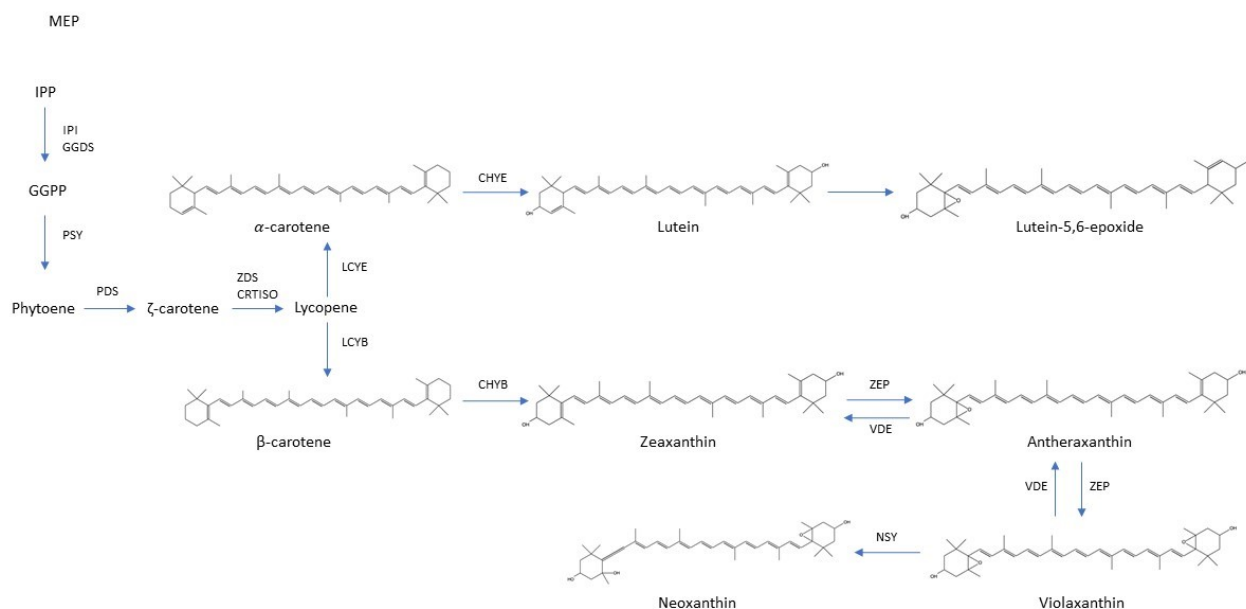


FIGURE 1

Synthesis and structure of different grape carotenoids: MEP – methyl erythritol-4-phosphate pathway; IPP – isopentenyl pyrophosphate; IPI – isopentenyl pyrophosphate isomerase; GGDS – geranylgeranyl diphosphate synthase; GGPP – geranylgeranylpyrophosphate; PSY – phytoene synthase; PDS – phytoene desaturase; ZDS –  $\zeta$ -carotene desaturase; CRTISO – carotenoid isomerase; LCYE – lycopene  $\epsilon$ -cyclase; LCYB – lycopene  $\beta$ -cyclase; ZEP – zeaxanthin epoxidase; VDE – violaxanthin de-epoxidase; NSY – neoxanthin synthase.

pyrophosphate (GGPP), and two GGPPs form phytoene, the first C40 carotenoid. Two enzymes, phytoene desaturase (PDS) and  $\zeta$ -carotene desaturase (ZDS), are responsible for the formation of phytofluene,  $\zeta$ -carotene, neurosporene and lycopene by adding conjugated double bonds. An increase in the number of conjugated double bonds leads to the formation of red lycopene, resulting in the formation of carotenoids with one  $\beta$ - and one  $\epsilon$ -ring ( $\alpha$ -carotene and its derivatives) or two  $\beta$ -rings ( $\beta$ -carotene and its derivatives).  $\alpha$ - and  $\beta$ -carotenes are further modified by hydroxylation or epoxidation, resulting in a variety of compounds (Tanaka *et al.*, 2008). The most important xanthophylls include neoxanthin, lutein, and flavoxanthin. The epoxidation of zeaxanthin catalysed by zeaxanthin epoxidase (ZEP) produces violaxanthin, which can be converted to neoxanthin by neoxanthin synthase (NSY) (Keller, 2010). The formation of violaxanthin from the conversion of  $\beta$ -carotene by activation of the xanthophyll cycle is described in Baumes *et al.* (2002).

The most abundant carotenoids in grapes are  $\beta$ -carotene and lutein (in the order of mg/kg), which account for 60% to 85% of the total carotenoid concentration in grape berries, while neochrome, neoxanthin, violaxanthin, luteoxanthin, flavoxanthin, lutein-5,6-epoxide, zeaxanthin and *cis*-isomers of lutein and  $\beta$ -carotene are present in smaller amounts (in the order of  $\mu$ g/kg) (Guedes de Pinho *et al.*, 2001; Mendes-Pinto, 2009; Fariña *et al.*, 2010; Kwasiewski *et al.*, 2010). Carotenoids are generally concentrated in the skin of the grapes (65%), while the rest is concentrated in the pulp (Guedes de Pinho *et al.*, 2001). The abundance of  $\beta$ -carotene and the xanthophylls neoxanthin, neochrome a, flavoxanthin and lutein is well documented in grape berries before véraison, while their concentrations decrease before or at véraison (Razungles *et al.*, 1996, 1998; Oliveira *et al.*, 2003, 2004; Bindon *et al.*, 2007; Yuan & Qian, 2016), which is due to the increased activity of carotenoid cleavage dioxygenase (CCD). The gene responsible for this, VvCCD1, was identified in research by Mathieu *et al.* (2005) and is induced in the early stages of grape berry development. Three other xanthophylls, namely violaxanthin, luteoxanthin and lutein-5,6-epoxide, appear in grapes after véraison, when the sugar concentration reaches about 160 g/L (Razungles *et al.*, 1996), although Yuan and Qian (2016) showed that neochrome b and violaxanthin may be synthesised from the pea-size stage until véraison, and then degraded after véraison. Carotenoids lutein,  $\beta$ -carotene and xanthophylls were also identified in grapevine leaves (in order of mg/kg). They are also synthesised during the first stage of berry growth and slowly decrease during ripening (Filimon *et al.*, 2016; Nzekoue *et al.*, 2022).

#### THE ROLE OF CAROTENOIDS IN PLANTS AND IN HUMAN HEALTH

Carotenoids are secondary metabolites that are responsible for various biological functions. They play an important role as plant pigments, giving flowers and fruits a yellow to red colour (Osorio, 2019). Carotenoids are essential for the production of the plant phytohormones abscisic acid (ABA) and strigolactone (Cazzonelli, 2011).

Carotenoids are natural plant pigments that play an important role in photosynthesis and photoprotection. They

accumulate in photosynthetic tissue as part of the photosystem II to protect tissues from light damage (Sun *et al.*, 2022). When too much oxygen is produced during photosynthesis in response to increased light intensity, it becomes toxic due to the formation of free radicals, so carotenoids, especially lutein and  $\beta$ -carotene, act as singlet oxygen scavengers (Hirschberg, 2001). They are also involved in dissipating excess light energy and converting it into heat by converting violaxanthin to zeaxanthin via antheraxanthin in the so-called xanthophyll cycle, a process that is controlled by increased light intensity. When the light intensity decreases, zeaxanthin is converted back into violaxanthin (Hirschberg, 2001). For this reason, the concentration of zeaxanthin and antheraxanthin in grapevine leaves increases in the morning and decreases in the evening (Keller, 2010).

Carotenoids have a positive effect on human health as they have antioxidant and anti-cancer properties (Pezzuto, 2008).  $\alpha$ - and  $\beta$ -carotene are important sources of vitamin A in the human diet, which is important for the eyes and the immune system. Carotenoids are widely used as food colorants and in the pharmaceutical industry (Tanaka *et al.*, 2008).

#### DEVELOPMENT OF C13-NORISOPRENOIDS FROM CAROTENOIDS

Carotenoids are unstable compounds due to their double-bonded conjugated structures. Therefore, their degradation in the berries during grape ripening seems to be due to a process of bioconversion of these compounds. This process produces different compounds, such as C13-norisoprenoids, which are responsible for the typical aromas of some grape varieties. These mechanisms are enzymatic or non-enzymatic reactions (Baumes *et al.*, 2002; Winterhalter *et al.*, 2002). The non-enzymatic reactions are stimulated by environmental conditions: light, temperature and oxygen. The enzymatic reaction is catalysed by carotenoid cleavage dioxygenase (CCD) (Mathieu *et al.*, 2005), followed by its degradation, either directly or via glycosylated intermediates (aroma precursors) (Baumes *et al.*, 2002). The aroma compounds are then released during fermentation and wine ageing by enzymatic and acid hydrolysis (Skouroumounis & Sefton, 2000).

Many different C13-norisoprenoids have been identified in grapes and wine, but only some of them affect aroma. These include  $\beta$ -damascenone,  $\beta$ -ionone, TDN, TPB and vitispirane.  $\beta$ -damascenone is responsible for fruity and floral notes in wine, while  $\beta$ -ionone gives a violet aroma to red wine (Darriet *et al.*, 2012). TDN has a detrimental effect on aroma due to its kerosene-like odour, while vitispirane imparts camphor/eucalyptus aromas to wine (Eggers *et al.*, 2006).

The correlation between carotenoids and C13-norisoprenoids in grape berries is compound-dependent, suggesting a dependence on enzyme activity and specificity (Yuan & Qian, 2016). The formation of TDN from lutein under acidic conditions has been illustrated in research by Marais (1992), while neoxanthin may be an important precursor for the formation of  $\beta$ -damascenone (Mendes-Pinto, 2009). In a study by Mathieu *et al.* (2005), the possible formation of  $\beta$ -ionone from zeaxanthin was investigated.

The researchers Guedes de Pinho *et al.* (2001) and Mendes-Pinto *et al.* (2005) even found carotenoids in Port wines from the Douro Valley. These possibly were extracted from the berry skin by ethanol added during alcoholic fermentation, but their concentration was lower than in the grapes. While the wines had lower levels of lutein and  $\beta$ -carotene, they were richer in oxygenated xanthophylls such as neoxanthin, violaxanthin and luteoxanthin.

#### DIFFERENCES BETWEEN GRAPEVINE VARIETIES IN CAROTENOID COMPOSITION AND CONTENT

The concentration of carotenoids in grapes and leaves can vary depending on environmental conditions, phenological stage and variety (Oliveira *et al.*, 2004; Filimon *et al.*, 2016; Joubert *et al.*, 2016; Young *et al.*, 2016). Although research shows that lutein and  $\beta$ -carotene are the most abundant carotenoids in grapes, their ratio depends on the grape variety and wine region. Higher levels of  $\beta$ -carotene compared to lutein were found in Tempranillo and Garnacha, with  $\beta$ -carotene ranging from 77% in Tempranillo to 81% in Garnacha, and lutein ranging from 17% in Garnacha to 21% in Tempranillo. Zeaxanthin was the least abundant carotenoid, varying between 1.3% (in Tempranillo) and 2.5% (in Garnacha), in the Rioja wine region in northern Spain (Gutiérrez-Gamboa *et al.*, 2018). Similar results were obtained by Crupi *et al.* (2010), who found that the concentrations of  $\beta$ -carotene in Chardonnay, Merlot, Primitivo and Negroamaro grown in the Italian region of Apulia were two to four times higher than those of lutein. Yuan and Qian (2016) discovered that the major carotenoids in Pinot noir grapes were lutein and  $\beta$ -carotene, with the lutein content almost twice that of  $\beta$ -carotene, while neochrome a, neochrome b, neoxanthin, violaxanthin and flavoxanthin were found in smaller amounts. In Nebbiolo (Asproudi *et al.*, 2020) and in Petit Verdot and Cabernet franc (Hickey *et al.*, 2018), the concentration of the two carotenoids was very similar. The concentration in the leaves was similar to that in the grapes. The levels of lutein ranged from 32 mg/g to 66 mg/g and those of  $\beta$ -carotene from 19 mg/g to 49 mg/g of dry weight (Castagna *et al.*, 2017).

In the study by Asproudi *et al.* (2020), the concentration of lutein and  $\beta$ -carotene varied between 3.5 and 9.7 mg/kg in the berries of Nebbiolo grapes. Oliveira *et al.* (2004) studied the concentrations in eight varieties from the Douro Valley, with concentrations of lutein and  $\beta$ -carotene ranging between 300  $\mu$ g/kg and 800  $\mu$ g/kg in the berries and of neoxanthin, violaxanthin and luteoxanthin ranging between 10  $\mu$ g/kg and 60  $\mu$ g/kg in the berries. Similarly, Yuan and Qian (2016) showed concentrations of lutein and  $\beta$ -carotene from 100  $\mu$ g/kg to 600  $\mu$ g/kg in the berries and of neoxanthin, violaxanthin and flavoxanthin from 10 to 60  $\mu$ g/kg in the berries of Pinot noir grapes.

#### THE INFLUENCE OF ENVIRONMENTAL CONDITIONS ON THE COMPOSITION AND CONTENT OF CAROTENOID IN GRAPEVINES

Grape varieties respond to changes in the environment and viticultural practices by altering the composition of the berries, which means they can produce different types and styles of wine (Young *et al.*, 2016). This ability of a single

genotype to produce alternative phenotypes as a result of environmental changes is referred to as phenotypic plasticity (Bradshaw, 1965; Arnold *et al.*, 2019). The concentration of carotenoids in grapes and leaves is related to the physiological processes of the vine, which depend on environmental conditions such as temperature, solar and UV radiation, soil properties, vineyard management, phenological stage and variety (Razungles *et al.*, 1996; Oliveira *et al.*, 2004; Kwasniewski *et al.*, 2010; Berli *et al.*, 2013; Castagna *et al.*, 2017; Gutiérrez-Gamboa *et al.*, 2018; Hickey *et al.*, 2018; Asproudi *et al.*, 2020; Doupis *et al.*, 2020; Rienth *et al.*, 2021). To better understand plasticity in relation to carotenoid synthesis, it is important to study grape varieties under different environmental conditions and at different stages of berry development.

#### Sunlight and temperature

The influence of sunlight on grape berry metabolism is complex. Solar radiation can modulate the development of the vine and influence metabolic processes in the berries. Direct sunlight can cause stress due to dehydration and increased temperature (González-Barreiro *et al.*, 2015), but it can also have a positive effect by causing a greater accumulation of sugars, anthocyanins and other polyphenolic compounds, as well as photosynthetic pigments (Fariña *et al.*, 2010; Song *et al.*, 2015).

Carotenoids are photosynthetic pigments whose synthesis in grapes continues from the early stages of berry development until véraison (Oliveira *et al.*, 2003). Sunlight exposure increases the concentration of carotenoids until véraison, and then affects their degradation (Razungles *et al.*, 1996; Bureau *et al.*, 1998). Solar radiation stimulates the photosynthetic activity of plants and consequently affects the biosynthesis of carotenoids, especially the activity of phytoene synthase, an enzyme responsible for the first step of the carotenoid biosynthetic pathway (Hirschberg, 2001), while the degradation of carotenoids in the final stage of berry development may be due to the VvCCD1 gene, which encodes the activity of the enzyme carotenoid cleavage dioxygenase (CCD) (Mathieu *et al.*, 2005).

Since carotenoids are substrates for C13-norisoprenoid synthesis (Young *et al.*, 2012), higher carotenoid concentrations, and thus higher substrate availability, may result in higher concentrations of C13-norisoprenoids in sun-exposed grapes (Mendes-Pinto, 2009). Some studies have shown that grapes exposed to sunlight may have a higher concentration of C13-norisoprenoids (Razungles *et al.*, 1998), while others showed no effect of sun exposure on C13-norisoprenoid concentration (Kwasniewski *et al.*, 2010; Song *et al.*, 2015). This difference can be explained by the effect of sunlight on the synthesis of the individual compounds in grapes, as different reactions can take place in each specific period during ripening, depending on the variety and microclimatic conditions (Asproudi *et al.*, 2020). In warm climates, grapes may have higher carotenoid concentrations due to higher solar radiation (Fariña *et al.*, 2010). However, the effects of temperature and light intensity on grape composition are complex, and the effects can vary. Other researchers have shown that higher temperatures (> 30°C) and higher solar radiation can promote the degradation

of berry carotenoids post-véraison (Oliveira *et al.*, 2004; Hickey *et al.*, 2018; Asproudi *et al.*, 2020). In the research by Gashu *et al.* (2022), the degradation of carotenoids was associated with increased temperature and solar radiation, and the relationship was dependent on the variety.

Under conditions of increased solar radiation, berries respond by adapting their metabolism to the specific stage of berry development, ensuring normal berry growth and development (Young *et al.*, 2016). In other words, the adaptation is reflected in the fact that the typical ripening process of the grapes is not affected, such as the physical parameters (berry weight and diameter) and the concentrations of primary metabolites (sugars and organic acids), but the perceived stress is managed by the synthesis of secondary metabolites predominant in the specific stage of development (Joubert *et al.*, 2016). In the pre-véraison stage of berry development, the secondary metabolites carotenoids are upregulated in response to increased light exposure, protecting photosynthetic membranes and ensuring the maintenance of photosynthesis, even under conditions of severe stress (De Oliveira *et al.*, 2019). After véraison, the increased light exposure leads to the synthesis of monoterpenes and phenols (Song *et al.*, 2015; Young *et al.*, 2016; Wang *et al.*, 2020). Plants use solar radiation for photosynthesis and energy production, but also as a source of information about the environment (Joubert *et al.*, 2016).

Increased sunlight intensity appears to affect the ratio of epoxyxanthophylls to non-epoxyxanthophylls by influencing the conversion of the epoxyxanthophyll violaxanthin to the de-epoxidised xanthophyll zeaxanthin as part of the xanthophyll cycle when excess light energy is dissipated (Hirschberg, 2001; Düring & Davtyan, 2002). In the study by Young *et al.* (2016), the content of zeaxanthin and antheraxanthin increased when grapes were exposed to sunlight by leaf removal, resulting in a larger xanthophyll pool (zeaxanthin, antheraxanthin and violaxanthin), and consequently in an increase in the de-epoxidation state of the xanthophylls (see Fig. 2). On the other hand, lutein epoxide concentrations were significantly lower in the exposed berries, with de-epoxidation of lutein epoxide to lutein

occurring after light exposure (Young *et al.*, 2012). Lutein had a higher degradation rate during grape ripening and a lower concentration of  $\beta$ -carotene at harvest under higher solar radiation and temperature conditions (Asproudi *et al.*, 2020).

### UV radiation

Ultraviolet (UV) radiation, including UV-A (315 nm to 400 nm) and UV-B (280 nm to 315 nm), represents a very small part of the solar spectrum, but has a very large biological effect. The amount of UV radiation changes depending on altitude, latitude, season, time of day and cloud cover (Matus, 2016). However, due to the damage to and thinning of the ozone layer, UV radiation increases and becomes more harmful, leading to forced changes in the life cycle of plants (Mackerness, 2000).

UV-B radiation is generally considered harmful to plants because it affects DNA and chlorophyll degradation, reduces photosynthesis and growth, and causes oxidative stress (Mackerness, 2000). However, most of the studies on it were conducted under unrealistic conditions, with excessively high doses of UV-B radiation (Del Castillo *et al.*, 2020). Instead, grapevine varieties are relatively well adapted to UV exposure under field conditions and generally show acclimatisation, as ecologically relevant doses of UV-B radiation act as environmental modulators and regulate gene expression, metabolism and growth (Downey *et al.*, 2003; Berli *et al.*, 2010; Hideg *et al.*, 2013; Joubert *et al.*, 2016). This new concept opens the possibility to explore different canopy management practices through UV manipulation. One of the most important measures to influence the UV exposure of grapes is leaf removal (Anić *et al.*, 2021). By improving microclimatic conditions through increasing light transmission and temperature in the fruit zone (Feng *et al.*, 2015; Young *et al.*, 2016), basal leaf removal can activate defence responses by increasing the concentration of stress-related defence molecules – polyphenols, monoterpenes and carotenoids (Joubert *et al.*, 2016; Hickey *et al.*, 2018) – which are associated with wine quality traits such as aroma, astringency, colour and stability (Downey *et al.*, 2003;

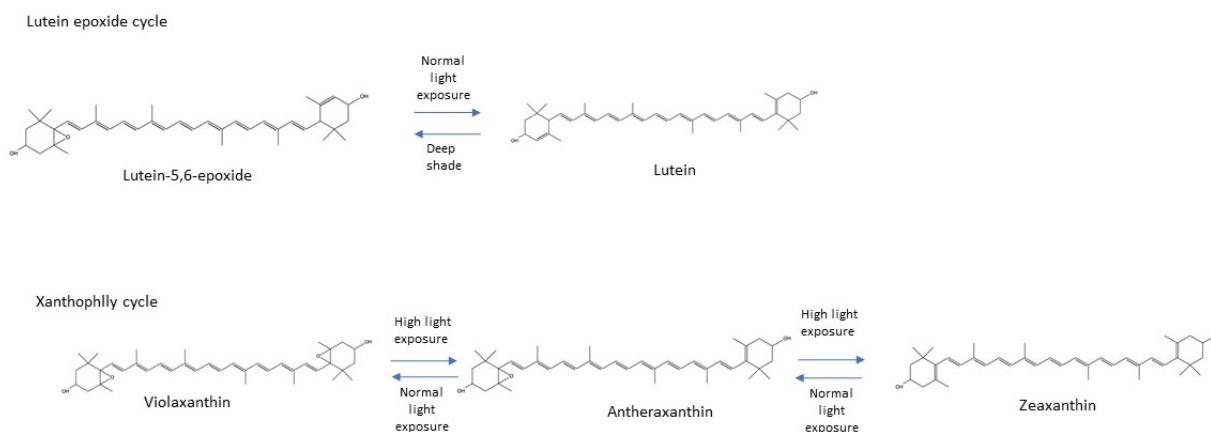


FIGURE 2  
Xanthophyll and lutein epoxide cycles.

González-Barreiro *et al.*, 2015; Wimalasari *et al.*, 2024).

The biosynthesis of secondary metabolites, especially polyphenols such as flavonols, is stimulated by UV radiation (Gregan *et al.*, 2012). Polyphenols have the function of absorbing and shielding UV-B radiation (Berli *et al.*, 2010), as it has been reported that grape exposure to UV radiation can increase the expression of genes involved in the regulation of flavonol accumulation via the phenylpropanoid metabolic pathway (Carbonell-Bejerano *et al.*, 2014; Martínez-Lüscher *et al.*, 2014). Monoterpenes are involved in the protection of berry tissues against UV-B radiation and other biotic and abiotic stresses (Joubert *et al.* 2016), and the exposure of grapes to sunlight promotes the expression of monoterpene metabolic genes in the berries (Friedel *et al.*, 2016).

Carotenoids are an important part of the grape UV-response machinery as they act as pigments for light harvesting (Matus *et al.*, 2016). Joubert *et al.* (2016) showed that UV-B can increase the accumulation of carotenoids and xanthophylls in Sauvignon blanc berries. The xanthophylls zeaxanthin and lutein responded most strongly to UV-B radiation in the early stages of berry development. In the absence of UV-B, berries require less zeaxanthin in the exposed environment (leaf removal treatment), and less lutein epoxide in shaded environments (Joubert *et al.*, 2016). Thus, the absence of UV-B could make grapes more susceptible to damage, as they are less acclimatised than grapes exposed to UV-B radiation. The latter grapes normally release higher concentrations of photoprotective xanthophylls and flavonols. Similar results were obtained in grapevine leaves, where the pigments of the xanthophyll cycle (violaxanthin, antheraxanthin and zeaxanthin) increased with an increased sum of UV radiation (Castagna *et al.*, 2016) (see Fig. 3).

### Topography

Topographic characteristics of the vineyard, such as elevation, slope and exposure of the terrain, as well as the orientation of the rows in the vineyard, the vigour of the

vineyard and different viticultural practices, can lead to great variability in microclimatic conditions, especially the quality and intensity of sunlight (Alem *et al.*, 2019). This can have an effect on carotenoid concentrations due to the direct effects on temperature, humidity, and other environmental factors that affect grape ripening (Castagna *et al.*, 2017). It is known that, in the northern hemisphere, slopes with south and southwest exposure of vineyards receive more sunlight. Higher temperatures in the less-vigorous and south-facing vineyards resulted in lower levels of carotenoids, especially lutein and  $\beta$ -carotene, both during ripening and at harvest (Asproudi *et al.*, 2020), while grapes grown on vines with higher vegetative height appear to have higher carotenoid content (Oliveira *et al.*, 2004) because the grapes are better protected from sunlight. In the Douro region of northern Portugal, high-altitude terraces with lower temperatures and higher humidity during the ripening period appear to produce grapes with higher carotenoid concentrations (Oliveira *et al.*, 2004).

### THE INFLUENCE OF VINEYARD MANAGEMENT PRACTICES ON THE COMPOSITION AND CONTENT OF CAROTENOIDS IN GRAPEVINES

#### Canopy management

Apart from environmental conditions, canopy management practices can also influence grape and wine quality by altering the vine microclimate (Hernandez-Orte *et al.*, 2015). Viticultural practices are known to affect grape berry secondary metabolites and regulate vine vigour, water and nutrient supply, and to increase the sunlight exposure of the grapes (Keller, 2010). Defoliation involves the removal of leaves around the cluster to improve air circulation and increase solar radiation and temperature inside the cluster (Alatzas *et al.*, 2023). In the study by Kwasniewski *et al.* (2010), leaf removal in the fruit zone 33 days after berry set was found to increase zeaxanthin concentrations in Riesling berries by 52 days after berry set, but not at harvest. On the

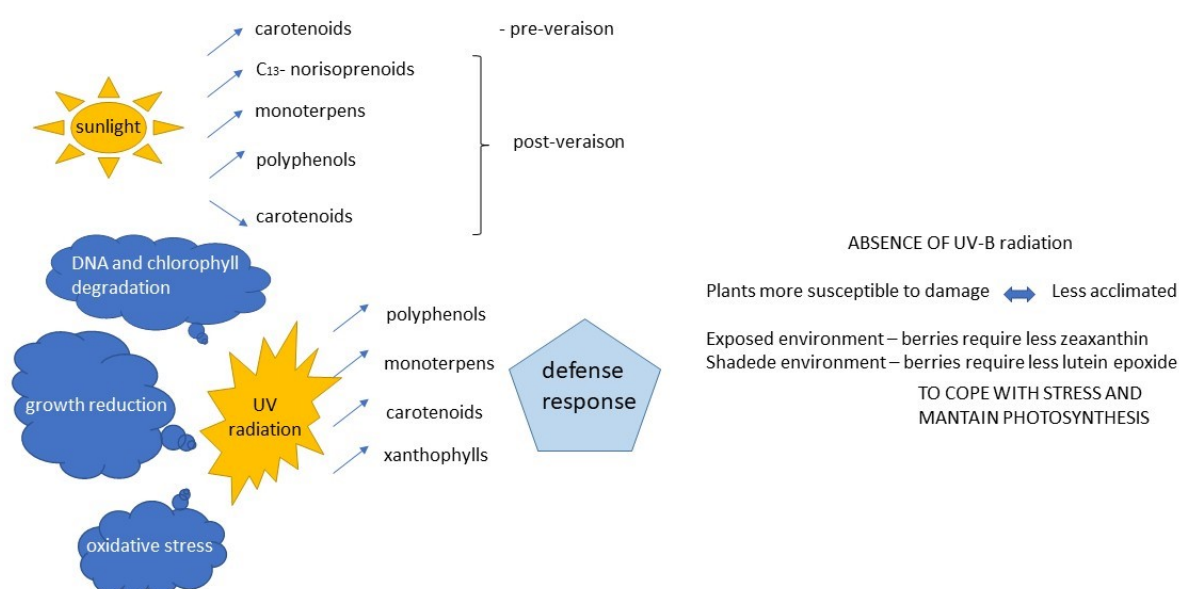


FIGURE 3

Synthesis of secondary metabolites in grapevines effected by sunlight and UV radiation.

other hand, Hickey *et al.* (2018) showed that leaf removal increased zeaxanthin concentration the most compared to all analysed carotenoids. Changes in microclimatic conditions as a result of leaf removal affect berry secondary metabolites (specific xanthophylls, monoterpenes and norisoprenoids) through transcriptional changes that influence enzyme activity and biochemical reactions during berry development and grape ripening, showing metabolic plasticity at different stages of development (Joubert *et al.*, 2016).

#### Irrigation management and tillage treatments

While direct practices such as leaf removal and shoot thinning alter the vine microclimate by modifying the crop level, canopy exposure and canopy density, indirect viticultural practices such as irrigation (Oliveira *et al.*, 2003; Bindon *et al.*, 2007; Jiao *et al.*, 2023) and fertilisation (Gutiérrez-Gamboa *et al.*, 2018) alter vine growth and canopy density by regulating the availability of nutrients and water, thereby influencing carotenoid synthesis in the grapes. The water balance of grapevines depends on soil texture, rainfall, root depth and evapotranspiration (Bois *et al.*, 2020). Depending on the phenological stage, a change in the water balance of the vine can affect vegetative growth, yield, canopy microclimate and the physiology of the berries (Baeza *et al.*, 2019; Chacón-Vozmediano *et al.*, 2020). Excessive water promotes more vegetative growth and reduces yield quality, while water deficit reduces photosynthesis, shoot growth, leaf area and berry size, and long-term water stress negatively affects vine metabolism and impedes ripening (Van Leeuwen & Darriet, 2016). However, moderate water stress can improve grape and wine quality by positively affecting the accumulation of polyphenolic compounds such as tannins and anthocyanins, as well as volatile compounds (González-Barreiro *et al.*, 2015; Van Leeuwen & Darriet, 2016). The water deficit reduces vegetative growth, which alters the vine microclimate and increases the amount of sunlight reaching the grapes, which is associated with higher grape quality (Baeza *et al.*, 2019). The study by Oliveira *et al.* (2003) showed that the irrigation treatment (30% of evapotranspiration (ET(0))) seems to contribute to lower carotenoid content in grapes, but only when the vines are planted in a soil with lower water-retention capacity compared to the non-irrigated treatment. Some studies have shown that partial root drying (PRD) could increase sunlight penetration in the fruit zone, possibly affecting the composition of carotenoids and C13-norisoprenoids (Bindon *et al.*, 2007). Under water stress, plants release excess energy from photosynthesis via the xanthophyll cycle in the form of heat (Hirschberg, 2001). This could lead to an increase in carotenoids, and consequently C13-norisoprenoids (Bindon *et al.*, 2007). A water deficit could also increase carotenoid cleavage dioxygenase (CCD) (Deluc *et al.*, 2009). Soil properties can also influence the rate of carotenoid degradation during grape ripening, with greater carotenoid degradation observed in vines in mulched soils than in conventional vineyards (Fariña *et al.*, 2010).

#### CONCLUSION

The grape-growing industry is highly dependent on the ability of grapevines to tolerate and adapt to the consequences of climate change. Among the most important consequences of climate change are high temperatures during grape ripening, a shift in phenological phases, changes in sugar and organic acid concentrations in grapes, and a change in the aroma profile of grapes, which affect grape and wine quality (De Orduña, 2010). Climate change could help lead to the development of appropriate adaptation strategies, which should be planned by the grape growers according to local environmental conditions. A major challenge for winegrowers and winemakers is therefore how to adapt to climatic changes while preserving the specificity and typicality of the sensory characteristics of a given region (Rienth *et al.*, 2021). This review contributes to knowledge of the influence of environmental conditions and vineyard management practices on the composition and content of carotenoids in grapevines in order to adapt canopy management strategies to maintain grape quality in warmer climates and produce the desired aromatic wine profile.

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# Diversity of *Xanthomonas citri* pv. *viticola* Populations on Grapevines from Different Locations in India

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The bacterial leaf spot (BLS) disease caused by *Xanthomonas citri* pv. *viticola* (*Xcv*) is a menace to the production of grapes all over India. The maximum incidence of BLS has been reported in the Maharashtra, Karnataka, and Andhra Pradesh states of India. The symptoms are characterised by water-soaked, angular spots which later become necrotic on leaves. The present study was conducted to investigate the variability of 23 *Xcv* strains isolated from infected samples collected from Maharashtra, Karnataka, and Tamil Nadu in India. Samples were collected on the basis of the characteristic symptoms. The diversity of all the isolates was assessed phenotypically and genetically. Phenotypic characters included the morphology of colonies, pathogenicity, hypersensitivity, and biochemical tests. Genetic characterisation was assessed using 16S rRNA sequencing. The results exhibited diversity with respect to various phenotypic aspects, like colony size, colour and opacity, the methyl red reduction test, enzyme production, pathogenicity, and a hypersensitivity reaction. The 16S rRNA sequencing showed a distribution of pathogens into two main clusters, which were further divided into nine sub-clusters. The salient findings on *Xcv* diversity in India will be useful to identify and characterise resistant germplasms against the disease. The non-uniform variability obtained among the isolates suggests that geographical location, climatic factors and the varieties were the driving forces for the evolution of the phytopathogens.

## INTRODUCTION

The grape industry is one of the major fruit industries in India, and the total output of grape production reached up to 3 489 000 metric ton from an average area of 162 000 ha in the 2021/2022 production year (Anonymous, 2022). The major grape-growing states are Maharashtra and Karnataka, accounting for about 95% of India's total grape production. Other table grape-growing states include Tamil Nadu, Telangana, Andhra Pradesh, Mizoram, Punjab, Madhya Pradesh, Jammu and Kashmir, Nagaland, Haryana and Rajasthan (Anonymous, 2022). India is one of the biggest grape exporters in the world, with reported earnings of 313.69 million USD in 2022/2023 (Anonymous, 2022). However, grape-related diseases like powdery mildew, downy mildew, anthracnose, bacterial leaf spot and rust have affected the growth of the grape industry and caused huge economic losses. According to a report, India loses 8.23%, i.e. about 223 000 tons, of its grapes every year (Agricultural and Processed Food Products Export Development Authority [APEDA], 2021).

Bacterial leaf spot (BLS) is a disease of grapevine that occurs worldwide, especially during the months of August to September in warm and humid climates (Jones *et al.*, 2014). *Xanthomonas citri* pv. *viticola* was reported to cause BLS of grapevine in Maharashtra, characterised by small, brown, angular, water-soaked lesions on the leaves, stems and fruit, leading to defoliation and direct fruit damage (Kamble *et al.*, 2019). Severe infection may cause substantial damage to crops, with significant yield losses (Pernezny *et al.*, 2003).

The causal bacteria are disseminated via various routes, like contaminated soil, leaves, berries, irrigation water and infected plant debris, which act as potential sources of inoculum (Gitaitis & Walcott, 2007). Initially, bacteria grow epiphytically and then enter the host through either stomata, hydathodes or wounds, spreading systemically to colonise the mesophyll parenchyma (Ryan *et al.*, 2011). The distribution and prevalence of BLS-causing *Xanthomonas* species in India is relatively unknown. BLS was first reported in India by Nayudu (1972), but its causal agent was mentioned as *Pseudomonas viticola*. Later, Dye (1978) redefined its taxonomic position and named it *X. campestris*

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pv. *viticola*. Chand and Kishun (1990) also reported the epidemic occurrence of the disease in India and its extensive influence on yield loss. In 2018, Da Gama *et al.* suggested reclassification of *X. campestris* pv. *viticola* as *X. citri* pv. *viticola*.

BLS is primarily controlled by resistant lines, antibiotic treatment, and copper sprays (Roach *et al.*, 2018). Due to reliance on the limited range of chemicals, resistance against copper and antibiotics was reported in the *Xanthomonas* population (Martin *et al.*, 2004; Griffin *et al.*, 2017). Hence, resistant lines, cultural interventions and biological control could be integrated for the successful management of the disease. The evolution of new *Xanthomonas* spp. and pathogenic strains over time has hindered the development and deployment of host resistance to manage bacterial spot disease in grape crops (Timilsina *et al.*, 2016). Variation and variability are natural phenomena in plant pathogenic bacteria, and their identification is always a challenge. 16S rDNA sequencing is one of the most rapid and accurate methods for identifying disease-causing bacteria and understanding their epidemiology (Clarridge *et al.*, 2004; Faniyan *et al.*, 2023). The rRNA genes, such as 16S, 23S and 5S, are highly conserved at the genus and species levels, and thus are believed to be useful tools for grouping bacteria at the taxonomic level.

Although outbreaks of BLS have occurred in most commercial vine-growing regions of India, scanty information is available regarding the genetic diversity and distribution of the causal *Xanthomonas* species. The development of effective management approaches, particularly the selection of resistant plant material, relies upon the accurate identification of the pathogens and a thorough understanding of pathogen diversity and pathogenicity. This study describes the phenotypic and genetic variability of pathogenic *Xanthomonas* spp. associated with BLS in India.

## MATERIALS AND METHODS

### Sample collection

Infected leaf samples were collected from various grapevine varieties in 23 different locations across Maharashtra, Karnataka and Tamil Nadu (Table 1), according to the symptoms shown in Fig. 1. The leaves were collected in labelled polythene bags and stored at 4°C prior to use.

### Isolation of *Xanthomonas*

The collected leaf samples were washed thoroughly under tap water and blot dried. Each leaf was segmented into pieces, 1 cm to 2 cm long, using a sterile scalpel. The pieces were dipped in sodium hypochlorite (1:100) (NaOCl) solution, followed by incubation for 30 sec, and subsequently treated



a) Infected leaves - red circle indicates symptoms



b) Healthy leaf

FIGURE 1

Symptomatology of bacterial leafspot in greenhouse trials

TABLE 1  
Samples collected from different locations

Sr no.	State	District	Village		Strains	Variety	
			Name	Position			
				Latitude (N)			Longitude (E)
1	Maharashtra	Nashik	Bhuvan	20.300	73.490	<i>Xcv</i> 1	Clone 2A
2	Maharashtra	Nashik	Sompur	20.590	74.198	<i>Xcv</i> 2	Crimson Seedless
3	Maharashtra	Nashik	Thengoda	20.590	74.198	<i>Xcv</i> 3	SSN
4	Maharashtra	Nashik	Thengoda	20.590	74.198	<i>Xcv</i> 4	Manikchaman
5	Maharashtra	Nashik	Palkhed	20.370	73.769	<i>Xcv</i> 5	Clone 2A
6	Maharashtra	Nashik	Verkheda	20.234	73.893	<i>Xcv</i> 6	Thompson Seedless
7	Maharashtra	Nashik	Verkheda	20.234	73.893	<i>Xcv</i> 7	Nanasaheb Purple
8	Maharashtra	Nashik	Satana	20.597	74.200	<i>Xcv</i> 8	Thompson Seedless
9	Maharashtra	Nashik	Karsul	20.370	73.769	<i>Xcv</i> 9	Thompson Seedless
10	Maharashtra	Nashik	Sarole	18.083	75.850	<i>Xcv</i> 10	Clone 2A
11	Maharashtra	Sangli	Belanki	16.817	74.642	<i>Xcv</i> 11	Sonaka
12	Maharashtra	Sangli	Walwa	16.494	74.230	<i>Xcv</i> 12	Sarita
13	Maharashtra	Sangli	Kuchi	17.021	74.863	<i>Xcv</i> 13	Manikchaman
14	Maharashtra	Sangli	Karoli	17.047	74.872	<i>Xcv</i> 14	Clone 2A
15	Maharashtra	Pune	Narayangaon	19.162	73.914	<i>Xcv</i> 15	Dhanaka
16	Maharashtra	Pune	Narayangaon	19.162	73.914	<i>Xcv</i> 16	Manikchaman
17	Maharashtra	Solapur	Mohal	17.809	75.637	<i>Xcv</i> 17	Red Globe
18	Maharashtra	Solapur	Pandharpur	17.673	75.294	<i>Xcv</i> 18	Thompson Seedless
19	Karnataka	Vijayapura	Vijayapura	16.827	75.725	<i>Xcv</i> 19	Crimson Seedless
20	Karnataka	Vijayapura	Vijayapura	16.827	75.725	<i>Xcv</i> 20	Thompson Seedless
21	Tamil Nadu	Theni	Theni	10.011	77.478	<i>Xcv</i> 21	Manikchaman
22	Tamil Nadu	Theni	Theni	10.011	77.478	<i>Xcv</i> 22	Crimson Seedless
23	Tamil Nadu	Theni	Theni	10.011	77.478	<i>Xcv</i> 23	Thompson Seedless

with a 70% ethanol solution for another 30 sec. Finally, the leaf segments were washed twice with sterile distilled water, blot dried and placed on a sterile nutrient agar (NA) plate to be incubated at 37°C for 24 h. After 24 h, the observed colonies were selected and characterised morphologically (Costa *et al.*, 2012). The selected colonies were sub-cultured and purified, following the method of Shah (2021) with minor modifications. Macroscopic features of the isolated bacterial colonies were assessed for the following criteria: colour, elevation, margin, opacity, consistency and surface of the colony. Microscopic features were determined through gram staining (Bartholomew & Mittwer, 1952).

#### Biochemical characterisation of purified bacterial isolates

The isolates were subjected to several biochemical tests – the potassium hydroxide (KOH) test, sugar utilisation test, catalase test, urease test, gelatine liquefaction test, indole production test, hydrogen cyanide (HCN) production test,

ammonia production, starch hydrolysis test, lipase production test, methylene red reduction, nitrate reduction, oxidase test, citrate reduction, hydrogen sulphide (H<sub>2</sub>S) production, cellulase production and protease production (Schaad *et al.*, 2001; Vashist *et al.*, 2013).

#### Abiotic stress-tolerance assay

##### Estimation of salt tolerance

Fresh bacterial culture was inoculated in test tubes containing nutrient broth with different concentrations of sodium chloride (NaCl), viz. 2%, 4%, 6% and 8% (Ullah *et al.*, 2018). Growth was observed after 24 h of incubation at 37°C, after which salt tolerance could be evaluated.

##### Estimation of temperature tolerance

Fresh bacterial culture was inoculated in test tubes containing nutrient broth. Growth was observed after 24 h of incubation at different temperatures, viz. 0°C, 4°C, 26°C and 30°C,

after which temperature tolerance could be evaluated (Ullah *et al.*, 2018).

### Virulence test

For the assessment of the virulence of *Xcv* on a grape leaf, an inoculum suspension was prepared in sterile water. Pinprick and injection infiltration methods were used for inoculation. In the pin prick method, paper pins were used to bruise the leaves, and the inoculum ( $10^6$  cfu/ml) of *Xcv* was applied. In the injection infiltration method, leaves of young seedlings were inoculated with 1 ml of inoculum using a hypodermic syringe. Control leaves (C) were inoculated with sterilised distilled water (Mazi *et al.*, 2015).

### Effect of different cultural conditions on the virulence of *Xcv*

#### Effect of age on the bacterium

Twenty-four hours of growth of the bacterial isolates on NA slants was used as a stock culture, and sub-culturing was done at one-day intervals in order to get five-, four-, three-, two- and one-day-old bacterial growth (Klement, 1963). Optical density of the bacterial suspension of each age level was adjusted to 0.5 and inoculated at the lower surface of the leaf using the syringe infiltration method. Symptoms were observed after 24 h of inoculation.

#### Effect of temperature

To study the effect of incubation temperature on virulence, each bacterial isolate was plated separately on NA and the plates were incubated at 25°C, 30°C and 35°C respectively. When the colonies emerged, one colony from each temperature treatment was sub-cultured, and the bacterial growth was suspended in sterile distilled water, centrifuged, and the optical density (OD) was adjusted to 0.5 (Klement, 1963). Isolates were inoculated at the lower surface of the leaf and symptoms were observed after 24 h.

#### Effect of pH

Nutrient agar plates were prepared with varying levels of pH, from 5.0 to 8.0 at intervals of 0.5. Fifty ml of the broth was dispensed in each of the 250 ml Erlenmeyer flasks and autoclaved at 121°C and 15 psi for 20 min. A loopful of 24 h growth of each isolate was suspended in 10 ml of sterile water. The flasks were then inoculated with 0.1 ml of the bacterial suspension prepared from the respective pH levels and incubated (Klement, 1963). Isolates were inoculated at the lower surface of the leaf and observed for lesions after 24 h of inoculation.

### Exopolysaccharide (EPS) production

Cultures were grown in basal medium prepared by mixing dipotassium phosphate ( $K_2HPO_4$ , 0.12%), potassium dihydrogen phosphate ( $KH_2PO_4$ , 0.08%), magnesium sulfate heptahydrate ( $MgSO_4 \cdot 7H_2O$ , 0.02 %) and ammonium nitrate ( $NH_4NO_3$ , 0.5%) on a rotary shaker for seven days at 28°C to 30°C (Nayudu, 1972). Bacterial cells were removed by centrifugation at 15 000 rpm for 15 min. N-cetylpyridinium chloride (2.0 g per litre) was added to the supernatant for the precipitation of polysaccharide, which was quickened by the addition of one to two pellets of KOH. The brownish-

white granular precipitate was removed by centrifugation and dissolved in 10% NaCl, and the polysaccharide were reprecipitated with two volumes of ethanol. This process was repeated thrice. The EPS produced was finally washed with ethanol, and dried before taking the final weight (Chowdhury *et al.*, 1980).

### Molecular identification

A single pure colony of the bacteria was inoculated in 100 ml of sterile nutrient broth (NB) and cultured on a rotatory incubator shaker at 120 rpm for 24 h at  $28 \pm 1^\circ C$ . The genomic deoxyribonucleic acid (DNA) was extracted using the HiMedia Bacterial Genomic Kit according to the manufacturer's instructions, and quantified with a Nanophotometer®NP120 (IMPLEN ver.2008). The universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify the 16S rDNA gene. Polymerase chain reaction amplifications were performed in 50 µl reaction mixture including 2U Taq polymerase (Bangalore Genei, Bangalore, India), 5 µl of 10X Taq buffer, dNTP at 200 µM, 10 pmol of each primer (IDT, USA) and 25 ng of DNA template. Initial denaturation was carried out at 94°C for four minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, primer extension at 72°C for 1.5 minutes, and final extension at 72°C for 10 minutes. The sequencing of the resultant polymerase chain reaction (PCR) amplicons was outsourced (GeneMatrix, Pune, India). Using BioEdit Sequence Alignment Editor, the sequences were aligned to obtain consensus sequences, which were then compared to three sequences of *Xcv* species available at the National Centre for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) using a BLAST search. MEGA 6.0 software was used to create the phylogenetic tree.

## RESULTS

### Isolation

Twenty-three isolates were obtained from the samples collected from various locations across India (Table 1). These isolates differed in morphological appearance, viz. colour, shape, size, margin, elevation, consistency and opacity.

### Morphological characterisation of *Xcv* isolates

It was observed that all the *Xcv* colonies had the characteristic features, namely round and white in colour, convex, smooth and butyrous in texture. The size of all the isolates ranged from 1 mm to 3 mm in diameter. Eight isolates from the Palkhed (*Xcv*5), Karsul (*Xcv*9), Sarole (*Xcv*10), Belanki (*Xcv*11), Walwa (*Xcv*12), Kuchi (*Xcv*13), Narayangaon (*Xcv*16) and Mohal (*Xcv*17) locations were opaque in nature, while the rest were translucent (see Fig. 2 and Table 2).

### Biochemical characterisation of *Xcv* isolates

All the isolates responded positively to the loop test by forming a thread when lifted gently. The loop formation provides confirmation of the Gram-negative bacteria to which all the isolates conformed. The isolates marked as Gram-negative from the loop test were further tested using Gram staining. Similar results were obtained twice, as all isolates retained a pinkish colour, thus confirming that they



FIGURE 2  
Morphological characterisation of 23 isolated *Xcv* from different locations

were Gram-negative.

All the isolates tested negative for H<sub>2</sub>S production, giving no black discoloration on lead acetate paper strips. It was observed that all the isolates had negative results for oxidase, urease and nitrate-reduction reactions, but were positive for the catalase test. All the isolates were able to ferment the tested sugars, viz. glucose, galactose, fructose, sucrose, dextrose and lactose. Isolates were neither able to utilise citrate as a sole source of carbon, nor did they show positive indole production. Isolates from eight locations, viz. Palkhed (*Xcv*5), Sarole (*Xcv*10), Walwa (*Xcv*12), Kuchi (*Xcv*13), Karoli (*Xcv*14), Pandharpur (*Xcv*18), Vijayapura (*Xcv*20) and Theni (*Xcv*21), were able to reduce methyl red reagent. It was observed that none of the isolates from Pune district were positive for the methyl red reduction test (Table 3a).

It was noted that eight isolates, from the Bhuvana (*Xcv*1), Satana (*Xcv*8), Walwa (*Xcv*12), Kuchi (*Xcv*13), Vijayapura (*Xcv*19 and *Xcv*20) and Theni (*Xcv*21 and *Xcv*21) regions, were unable to produce protease, lipase or gelatinase enzymes. Protease enzyme was produced by the isolates from Bhuvana (*Xcv*2), Verkheda (*Xcv*6), Balanki (*Xcv*11), Karoli (*Xcv*14), Narayangaon (*Xcv*16), Mohal (*Xcv*17), Pandharpur (*Xcv*18) and Theni (*Xcv*22), whereas lipase was secreted by isolates from Verkheda (*Xcv*6), Sarole (*Xcv*10), Pandharpur (*Xcv*18) and Theni (*Xcv*22). Ten isolates – from Sompur (*Xcv*2), Thengoda (*Xcv*4), Palkhed (*Xcv*5), Verkheda (*Xcv*7), Karsul (*Xcv*9), Sarole (*Xcv*10), Belanki (*Xcv*11), Narayangaon (*Xcv*15), Pandharpur (*Xcv*18) and Theni (*Xcv*22) – were able to hydrolyse gelatine. Amylase

and cellulase enzymes were produced by all the isolates from the different locations (Table 3b).

#### Abiotic stresses

None of the isolates were able to tolerate temperature stress. The maximum growth of *Xcv* took place at 26°C, and no growth was observed at 0°C, 4°C and -30°C. Tolerance to different salt concentrations were tested, and all the isolates were able to tolerate salt concentration of up to 4%, as no growth was observed in broth with a higher salt concentration (Table 4).

#### Virulence test

The infected grape leaves reacted positively to all isolates within 24 h to 72 h. Mild chlorosis to brown necrosis was observed around the injection point (Fig. 2). As per virulence reaction, the pathogen was categorised as strongly virulent, moderately virulent and mildly virulent. Isolates from eight locations, viz. Verkheda (*Xcv*7), Satana (*Xcv*8), Sarole (*Xcv*10), Walwa (*Xcv*12), Narayangaon (*Xcv*15, *Xcv*16), Vijayapura (*Xcv*20) and Theni (*Xcv*23), were considered to be highly virulent, as they manifested symptoms within 24 h of inoculation, whereas 12 isolates, from Bhuvana (*Xcv*1), Thengoda (*Xcv*3, *Xcv*4), Palkhed (*Xcv*5), Karsul (*Xcv*9), Belanki (*Xcv*11), Kuchi (*Xcv*13), Karoli (*Xcv*14), Mohal (*Xcv*17), Pandharpur (*Xcv*18) and Theni (*Xcv*21 and *Xcv*22), were regarded as moderately virulent, as the symptoms were seen within 48 h. After 72 h of inoculation, symptoms appeared in three of the isolates from Sompur (*Xcv*2), Verkheda (*Xcv*6) and Vijayapura (*Xcv*19) locations,

TABLE 2  
Morphological characterisation of purified cultures of *Xcv*

Sr no.	Strain	Shape	Size * (mm)	Colour	Consistency	Elevation	Texture	Opacity	Gram reaction
1	<i>Xcv1</i>	C	1.4	W	M	CX	S	T	N
2	<i>Xcv2</i>	C	2.1	W	M	CX	S	T	N
3	<i>Xcv3</i>	C	3.0	W	M	CX	S	T	N
4	<i>Xcv4</i>	C	2.1	W	M	CX	S	T	N
5	<i>Xcv5</i>	C	2.9	W	M	CX	S	O	N
6	<i>Xcv6</i>	C	1.1	W	M	CX	S	T	N
7	<i>Xcv7</i>	C	1.6	W	M	CX	S	T	N
8	<i>Xcv8</i>	C	2.8	W	M	CX	S	T	N
9	<i>Xcv9</i>	C	2.2	W	M	CX	S	O	N
10	<i>Xcv10</i>	C	3.1	W	M	CX	S	O	N
11	<i>Xcv11</i>	C	2.9	W	M	CX	S	O	N
12	<i>Xcv12</i>	C	2.4	W	M	CX	S	O	N
13	<i>Xcv13</i>	C	2.1	W	M	CX	S	O	N
14	<i>Xcv14</i>	C	1.9	W	M	CX	S	T	N
15	<i>Xcv15</i>	C	2.0	W	M	CX	S	T	N
16	<i>Xcv16</i>	C	2.4	W	M	CX	S	O	N
17	<i>Xcv17</i>	C	0.9	W	M	CX	S	O	N
18	<i>Xcv18</i>	C	1.5	W	M	CX	S	T	N
19	<i>Xcv19</i>	C	1.9	W	M	CX	S	T	N
20	<i>Xcv20</i>	C	2.3	W	M	CX	S	T	N
21	<i>Xcv21</i>	C	0.9	W	M	CX	S	T	N
22	<i>Xcv22</i>	C	1.7	W	M	CX	S	T	N
23	<i>Xcv23</i>	C	1.9	W	M	CX	S	T	N

\*Average of 50 colonies were observed

Key: C - circular; W - white; M - mucoid; CX - convex; S - smooth; N - negative; T - translucent; O - opaque

and these can be categorised as slightly virulent. It is clear from the data that Nashik and Sangli had the highest number of highly virulent strains, whereas strains from Pune, Theni and Solapur were of moderate virulence. No symptoms were recorded in the controls inoculated with distilled water (Table 5).

Two inoculation methods, viz. pin prick and syringe infiltration, were used, and they gave positive responses on the host plants. However, the syringe infiltration method gave more prominent symptoms compared to the pin prick method. The syringe infiltration method therefore was used for the further studies.

#### Effect of different cultural conditions on the virulence of *Xcv*

##### Effect of age of the bacterium

The virulence of the bacterial isolates causing BLS was influenced by the age and origin of the bacterial strains. Isolates from Sompur (*Xcv2*), Thengoda (*Xcv3*), Palkhed

(*Xcv5*), Verkheda (*Xcv7*), Satana (*Xcv8*), Walwa (*Xcv12*), Karoli (*Xcv14*), Narayangaon (*Xcv15*, *Xcv16*), Mohal (*Xcv17*), Vijayapura (*Xcv20*) and Theni (*Xcv22*, *Xcv23*) manifested the highest virulence in three- and four-day-old cultures, whereas isolates from Bhuvana (*Xcv1*), Thengoda (*Xcv4*), Verkheda (*Xcv6*), Karsul (*Xcv9*), Sarole (*Xcv10*), Belanki (*Xcv11*), Kuchi (*Xcv13*), Pandharpur (*Xcv18*), Vijayapura (*Xcv19*) and Theni (*Xcv21*) showed the highest virulence in two- and three-day-old cultures. Three-day-old cultures of all isolates manifested the maximum virulence compared to the other age levels. Five- and one-day-old cultures were the least virulent. The interaction between the bacterial isolates and their age in relation to virulence was significant.

##### Effect of temperature

Incubation temperature also influenced the virulence of the bacterial isolates. The pathogen was more virulent when it was incubated at 30°C. However, the virulence of the

TABLE 3a  
Biochemical characterisation of purified sample of Xcv

Sr no.	Strain	Sugar fermentation						Oxidase	Catalase	Indole production	Methyl red reduction	Nitrate utilisation	Citrate utilisation
		Glucose	Galactose	Fructose	Lactose	Sucrose	Dextrose						
1	Xcv1	+	+	+	+	+	+	+	-	+	-	-	-
2	Xcv2	+	+	+	+	+	+	+	-	+	-	-	-
3	Xcv3	+	+	+	+	+	+	+	-	+	-	-	-
4	Xcv4	+	+	+	+	+	+	+	-	+	-	-	-
5	Xcv5	+	+	+	+	+	+	+	-	+	+	-	-
6	Xcv6	+	+	+	+	+	+	+	-	+	-	-	-
7	Xcv7	+	+	+	+	+	+	+	-	+	-	-	-
8	Xcv8	+	+	+	+	+	+	+	-	+	-	-	-
9	Xcv9	+	+	+	+	+	+	+	-	+	-	-	-
10	Xcv10	+	+	+	+	+	+	+	-	+	+	-	-
11	Xcv11	+	+	+	+	+	+	+	-	+	-	-	-
12	Xcv12	+	+	+	+	+	+	+	-	+	+	-	-
13	Xcv13	+	+	+	+	+	+	+	-	+	+	-	-
14	Xcv14	+	+	+	+	+	+	+	-	+	+	-	-
15	Xcv15	+	+	+	+	+	+	+	-	+	-	-	-
16	Xcv16	+	+	+	+	+	+	+	-	+	-	-	-
17	Xcv17	+	+	+	+	+	+	+	-	+	-	-	-
18	Xcv18	+	+	+	+	+	+	+	-	+	+	-	-
19	Xcv19	+	+	+	+	+	+	+	-	+	-	-	-
20	Xcv20	+	+	+	+	+	+	+	-	+	+	-	-
21	Xcv21	+	+	+	+	+	+	+	-	+	+	-	-
22	Xcv22	+	+	+	+	+	+	+	-	+	-	-	-
23	Xcv23	+	+	+	+	+	+	+	-	+	-	-	-

Key: + = positive, - = negative

TABLE 3b  
Biochemical characterisation of purified sample of *Xcv*

Sr no.	Strains	Gelatine hydrolysis	H <sub>2</sub> S production	Urease test	KOH hydrolysis	Starch hydrolysis	Casein hydrolysis	Lipase production	Cellulose production
1	<i>Xcv1</i>	-	-	-	+	+	-	-	+
2	<i>Xcv2</i>	+	-	-	+	+	-	-	+
3	<i>Xcv3</i>	-	-	-	+	+	+	-	+
4	<i>Xcv4</i>	+	-	-	+	+	-	-	+
5	<i>Xcv5</i>	+	-	-	+	+	-	-	+
6	<i>Xcv6</i>	-	-	-	+	+	+	+	+
7	<i>Xcv7</i>	+	-	-	+	+	-	-	+
8	<i>Xcv8</i>	-	-	-	+	+	-	-	+
9	<i>Xcv9</i>	+	-	-	+	+	-	-	+
10	<i>Xcv10</i>	+	-	-	+	+	-	+	+
11	<i>Xcv11</i>	+	-	-	+	+	+	-	+
12	<i>Xcv12</i>	-	-	-	+	+	-	-	+
13	<i>Xcv13</i>	-	-	-	+	+	-	-	+
14	<i>Xcv14</i>	-	-	-	+	+	+	-	+
15	<i>Xcv15</i>	+	-	-	+	+	-	-	+
16	<i>Xcv16</i>	-	-	-	+	+	+	-	+
17	<i>Xcv17</i>	-	-	-	+	+	+	-	+
18	<i>Xcv18</i>	+	-	-	+	+	+	+	+
19	<i>Xcv19</i>	-	-	-	+	+	-	-	+
20	<i>Xcv20</i>	-	-	-	+	+	-	-	+
21	<i>Xcv21</i>	-	-	-	+	+	-	-	+
22	<i>Xcv22</i>	+	-	-	+	+	+	+	+
23	<i>Xcv23</i>	-	-	-	+	+	-	-	+

Key: + = positive; - = negative

pathogen was reduced when incubated at 25°C and 35°C, although these did not show a significant difference.

#### **Effect of pH**

The pH levels, ranging from 5.0 to 8.0, influenced the virulence of the causal bacterial isolates. All the isolates were significantly virulent at all the tested pH levels, with no direct relationship manifested between the pH levels and the virulence of the bacterial isolates.

#### **Exopolysaccharide production**

All the strains were able to produce a significant amount of exopolysaccharides (EPS), ranging from 0.3 mg/ml to 2.2 mg/ml (Table 5). Highly virulent strains of *Xcv* were able to produce larger amounts of EPS compared to the others. Isolates *Xcv7* (1.8 mg/ml), *Xcv8* (2.0 mg/ml), *Xcv10* (1.9 mg/ml), *Xcv12* (2.2 mg/ml), *Xcv15* (1.9 mg/ml), *Xcv16* (2.1 mg/ml), *Xcv20* (1.8 mg/ml) and *Xcv23* (2.1 mg/ml), from Verkheda, Satana, Sarole, Walwa, Narayangaon, Vijayapura

and Theni respectively, produced the maximum amount of EPS, ranging from 1.8 mg/ml to 2.2 mg/ml. Isolates from Bhuvana (*Xcv1*), Thengoda (*Xcv3*, *Xcv4*), Palkhed (*Xcv5*), Karsul (*Xcv9*), Belanki (*Xcv11*), Kuchi (*Xcv13*), Karoli (*Xcv14*), Mohal (*Xcv17*), Pandharpur (*Xcv18*) and Theni (*Xcv21* and *Xcv22*) produced moderate amounts of EPS, ranging from 0.8 mg/ml to 1.3 mg/ml. The smallest amounts of EPS were produced by *Xcv2* (0.5 mg/ml), *Xcv6* (0.3 mg/ml) and *Xcv19* (0.4 mg/ml) from Sompur, Verkheda and Vijayapura respectively (Table 5).

#### **Molecular characterisation and cluster analysis**

Polymerase chain reaction products produced a single band of approximately 1 500 base pairs (bp). 16S rDNA sequencing of these PCR-amplified products showed diversity in the *Xanthomonas* community. All the isolates had more than 97% sequence similarity with the *Xanthomonas citri* pv. *viticola* strain in a BLAST search. Sequences from seven *Xanthomonas* isolates were obtained from NCBI and

TABLE 4  
Stress tolerance, i.e. temperature and pH of purified sample of Xcv

Sr no.	Strains	Growth at different temperatures (°C)					Growth at different salt concentration (%)						
		0	4	26	-30	0	0.5	1	2	3	4	5	7
1	Xcv1	-	-	+	-	+	+	+	+	+	+	+	-
2	Xcv2	-	-	+	-	+	+	+	+	+	+	+	-
3	Xcv3	-	-	+	-	+	+	+	+	+	+	+	-
4	Xcv4	-	-	+	-	+	+	+	+	+	+	+	-
5	Xcv5	-	-	+	-	+	+	+	+	+	+	+	-
6	Xcv6	-	-	+	-	+	+	+	+	+	+	+	-
7	Xcv7	-	-	+	-	+	+	+	+	+	+	+	-
8	Xcv8	-	-	+	-	+	+	+	+	+	+	+	-
9	Xcv9	-	-	+	-	+	+	+	+	+	+	+	-
10	Xcv10	-	-	+	-	+	+	+	+	+	+	+	-
11	Xcv11	-	-	+	-	+	+	+	+	+	+	+	-
12	Xcv12	-	-	+	-	+	+	+	+	+	+	+	-
13	Xcv13	-	-	+	-	+	+	+	+	+	+	+	-
14	Xcv14	-	-	+	-	+	+	+	+	+	+	+	-
15	Xcv15	-	-	+	-	+	+	+	+	+	+	+	-
16	Xcv16	-	-	+	-	+	+	+	+	+	+	+	-
17	Xcv17	-	-	+	-	+	+	+	+	+	+	+	-
18	Xcv18	-	-	+	-	+	+	+	+	+	+	+	-
19	Xcv19	-	-	+	-	+	+	+	+	+	+	+	-
20	Xcv20	-	-	+	-	+	+	+	+	+	+	+	-
21	Xcv21	-	-	+	-	+	+	+	+	+	+	+	-
22	Xcv22	-	-	+	-	+	+	+	+	+	+	+	-
23	Xcv23	-	-	+	-	+	+	+	+	+	+	+	-

Key: + = positive, - = negative

TABLE 5  
Virulence and exopolysaccharide (mg/ml) produced by purified sample of *Xcv*

Strains	Virulence	Exopolysaccharide (mg/ml)
<i>Xcv1</i>	++	0.9
<i>Xcv2</i>	+	0.5
<i>Xcv3</i>	++	1.1
<i>Xcv4</i>	++	1.0
<i>Xcv5</i>	++	0.8
<i>Xcv6</i>	+	0.3
<i>Xcv7</i>	+++	1.8
<i>Xcv8</i>	+++	2.0
<i>Xcv9</i>	++	1.1
<i>Xcv10</i>	+++	1.9
<i>Xcv11</i>	++	1.2
<i>Xcv12</i>	+++	2.2
<i>Xcv13</i>	++	0.9
<i>Xcv14</i>	++	1.0
<i>Xcv15</i>	+++	1.9
<i>Xcv16</i>	+++	2.1
<i>Xcv17</i>	++	1.3
<i>Xcv18</i>	++	1.0
<i>Xcv19</i>	+	0.4
<i>Xcv20</i>	+++	1.8
<i>Xcv21</i>	++	0.9
<i>Xcv22</i>	++	1.3
<i>Xcv23</i>	+++	2.1
C	-	-

Key: + = slightly virulent; ++ = moderately virulent; +++ = highly virulent; - = negative

subjected to multiple alignments. A dendrogram depicting the estimated phylogenetic relationships was constructed by the neighbour-joining clustering method (Fig. 3). This dendrogram was based on comparisons of all the available 16S rDNA sequence data for the genus *Xanthomonas citri* pv. *viticola* species. The sequence obtained was submitted to GeneBank and an accession number was procured (Table 6). The cluster analysis also clearly discriminated *Xanthomonas citri* pv. *viticola* causing bacterial leafspot of grapes from other *Xanthomonas* species infecting different hosts (Fig. 3).

The dendrogram constructed from the pooled data had two main clusters (Fig. 3) – cluster I and cluster II. A clear distinction could be observed among the isolated *Xcv* and other *Xanthomonas* strains, as all the *Xcv* were grouped under cluster I, whereas cluster II consisted of *Xanthomonas* strains, viz. *Xanthomonas albilineans* (MH491194.1), *Xanthomonas sacchari* (KY486222.1), *Xanthomonas campestris*

(MH256551.1) and *Xanthomonas translucens* pv. *graminis* (AY855873.1). Cluster I was further divided into two sub-clusters. Isolates from Karoli (*Xcv14*), Pandharpur (*Xcv18*) and Vijayapur (*Xcv19*) were found to be closely related to each other. Isolates from the Walwa (*Xcv12*), Narayangaon (*Xcv15* and *Xcv16*) and Mohal (*Xcv17*) regions were grouped in one sub-cluster, whereas isolates from Theni (*Xcv21* and *Xcv23*) fell into another. All three *Xcv* isolates retrieved from the NCBI were closely related to the remaining isolates, as all were grouped in the same sub-cluster.

## DISCUSSION

The pathogenic and genetic diversity of the *Xcv* strains associated with BLS disease in India were analysed through pathogenicity, standard bacteriological tests and 16S rRNA sequence analysis. The results suggest that the *Xcv* populations investigated in the present study were composed of pathologically and genetically diverse strains.

The present study supports the previous findings relating to the occurrence of bacterial leaf spot in India and the characterisation of the causal bacterium, *Xcv* (Kamble *et al.*, 2019), and confirms that the disease is widespread in the grape-growing regions of India. Symptoms of the BLS, e.g. water-soaked, angular small spots on the lower surface of the leaves, were observed in all the visited fields. Similar symptoms of BLS have been described by Araujo and Robbs (2000) and Nascimento and Mariano (2004). Necrosis, yellow spots and cankerous lesions on the leaves and shoots are characteristic symptoms caused by any pathogen belonging to the genus *Xanthomonas* (Bradbury, 1970). The symptoms observed on the grapevines were homologous with the previous studies (Bradbury, 1970; Kamble *et al.*, 2019).

Bacteria of the genus *Xanthomonas* are straight, Gram-negative rods, typically with yellow pigmentation and a polar flagellum, strictly aerobic chemoorganotrophs, and mostly phytopathogenic. As observed, the colony morphology of *Xcv* revealed white-coloured colonies. In contrast to the present finding, a yellow-coloured colony of bacterial leaf spot pathogen of grapes was reported by Jambenal *et al.* (2011), which hints at phenotypic diversity. Hence, yellow colonies produce the pigment Xanthomonadin (Goel *et al.*, 2001), while white or albino colonies do not produce this pigment due to acquired mutations in the Xanthomonadin biosynthesis gene cluster, such as frameshift mutation, deletion and insertion, cause them to lose their pigmentation (Midha & Patil, 2014). Hence there is phenotypic diversity. Similar findings have been made in the case of other albino strains, *Xanthomonas campestris* pv. *mangiferaeindicae* and *Xanthomonas axonopodis* pv. *ricini* (Gama *et al.*, 2011). The size and shape of colonies were small to medium, convex and mucoid. The staining reaction was observed under microscope, and the bacteria were found to be Gram negative. The bacterial colonies identified were 1 mm to 3 mm in diameter, and Gram-negative cells were observed under the microscope. These observations regarding colony morphology in the present study correlate with reports by Breed *et al.* (1989) and Kamble *et al.* (2019). The results support those of a previous study (Arshiya *et al.*, 2014) in which morphological, biochemical and pathogenicity tests

were performed to identify and characterise the strains of *Xanthomonas* causing bacterial disease. The biochemical test results analysed in the present study confirm the pathogen.

In the present study, variations were observed with respect to the morphological and biochemical characterisation. All *Xcv* were able to utilise carbon in all the tested forms. This finding is in contrast with previous results reported by Young and Triggs (1994), who found that pathovars of *P. syringae* pv. *maculicola* were heterogeneous in their carbon source utilisation. Giri *et al.* (2011) reported that, amongst 16 strains of *Xanthomonas axonopodis* pv. *punicae*, only five could hydrolyse the starch and almost all the strains produced acid from different carbon sources. They further added that 13 strains had produced H<sub>2</sub>S gas. The results reflect the biochemical variation exhibited by the strains of *X. axonopodis* pv. *punicae* from different geographical locations (Tables 3a and 3b). Several researchers have reported variations in biochemical tests within the different pathovars (Wiebe *et al.*, 1993; Clerc *et al.* 1998; Zhao *et al.*, 2008).

Exopolysaccharide production is an important virulence determinant in numerous plant pathogenic bacteria, including *Xanthomonas* spp., *Erwinia amylovora* and *Pseudomonas syringae*, during infection by pathogens (Kemp *et al.*, 2004). The present study shows that all the pathogenic *Xcv* were able to produce a significant amount of EPS. It was also

seen that EPS production was directly proportional to the virulence, which supports the previous findings of Guo *et al.* (2015) and Nguyen *et al.* (2016). These authors reported that *X. oryzae* pv. *oryzae* and *X. citri* subsp. *citri* employed multiple virulence factors to promote their pathogenicity on rice and citrus respectively, including EPS production. Exopolysaccharide production helps the pathogen to grow and spread in plants by protecting the organism against toxic compounds like superoxide radicals, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and high or low pH produced by the hosts in defence reactions (Yu *et al.*, 2016; Bae *et al.*, 2018).

Here, we present a systematic approach to generating and validating diversity among species/supraspecies levels using the 16S rRNA gene, which was also proved by Escapa *et al.* (2020). Previous studies on membrane protein analysis, fatty acid profiling and 16s rRNA analysis showed a high similarity among several strains or pathovars of *Xanthomonas* (Ndongo *et al.*, 2018). Comparisons of sequence variation in the 16S rRNA region among 23 strains of *Xcv* from different locations showed more than 97% similarity. In addition, partial 16S rRNA sequences were identical among the strains of *Xcv* studied, and the current findings support the study of Kamble *et al.* (2019). There is limited information describing the genetic diversity of *Xcv*, compared to the closely related pathovars of *Xcc*.

There was a correlation between the groups and the

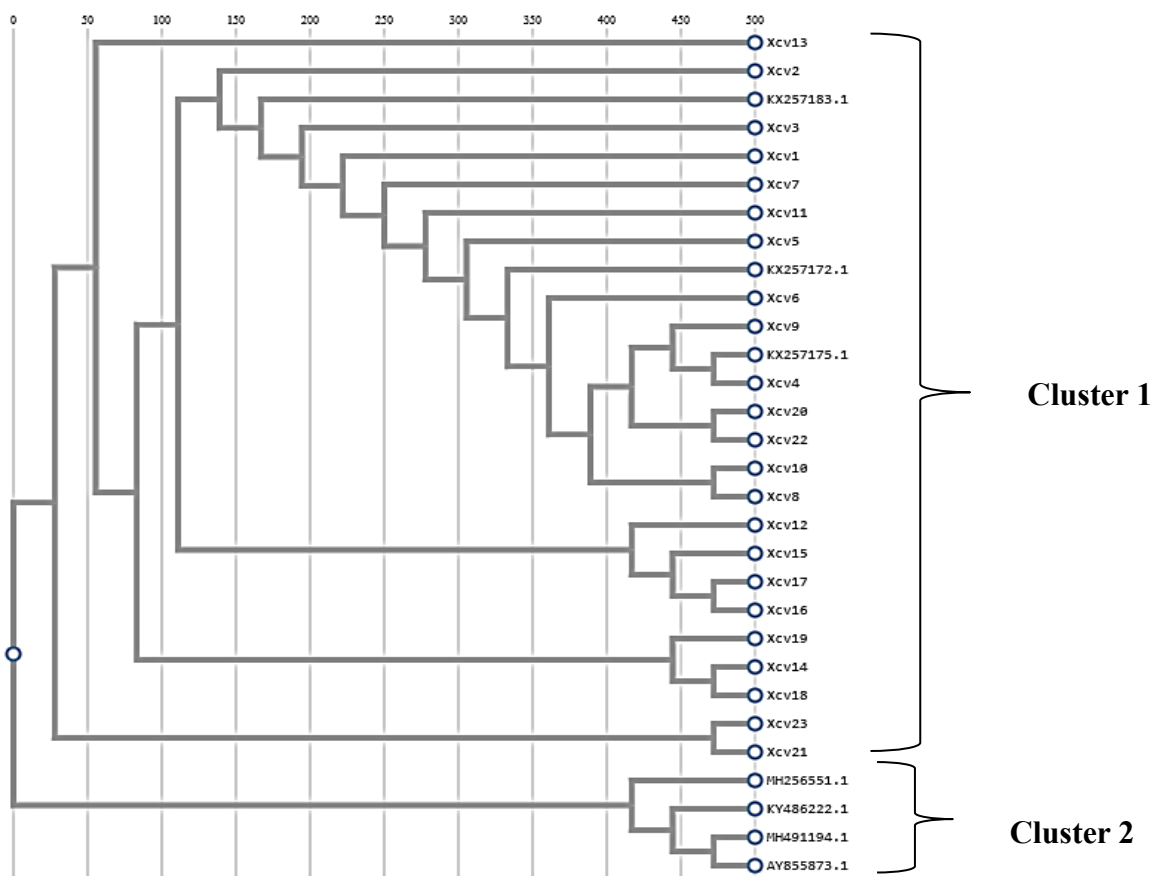


FIGURE 3  
Phylogenetic tree of *Xcv* inferred by analysis of 16S rDNA sequences

TABLE 6  
Accession number of isolated *Xcv* submitted to the NCBI

Strain	Accession number
<i>Xcv1</i>	OR485223
<i>Xcv2</i>	OR485224
<i>Xcv3</i>	OR485225
<i>Xcv4</i>	OR485226
<i>Xcv5</i>	OR485227
<i>Xcv6</i>	OR485228
<i>Xcv7</i>	OR485229
<i>Xcv8</i>	OR485230
<i>Xcv9</i>	OR485231
<i>Xcv10</i>	OR485232
<i>Xcv11</i>	OR485233
<i>Xcv12</i>	OR485234
<i>Xcv13</i>	OR485235
<i>Xcv14</i>	OR485236
<i>Xcv15</i>	OR485237
<i>Xcv16</i>	OR485238
<i>Xcv17</i>	OR485239
<i>Xcv18</i>	OR485240
<i>Xcv19</i>	OR485241
<i>Xcv20</i>	OR485242
<i>Xcv21</i>	OR485243
<i>Xcv22</i>	OR485244
<i>Xcv23</i>	OR485245

geographical origin of the strains, e.g. from Maharashtra, Karnataka and Tamil Nadu (Fig. 3). Alberts *et al.* (2002) state that it is plausible that the two groups may signify distinct, recent introductions of the pathogen, even though the origin of the distribution is not clear. Previous reports about *Xanthomonas* pv. *manihotis* have shown that there is divergence among the strains that were recently introduced to Africa (Verdier *et al.*, 1993). Linkages between groups, indicated by rep-PCR, and geographical origin have been reported for *Xanthomonas* strains pathogenic to other plants. Scortichini *et al.* (2001) reported the existence of genetic diversity in a worldwide collection of *X. axonopodis* pv. *juglandis* isolated from walnuts, and showed that the genetics of the pathogen was unique to each walnut cultivation area. Massomo *et al.* (2003) observed variation among *X. campestris* pv. *campestris* strains isolated from *Brassica* sp. collected in fields in Tanzania, and linked fingerprint patterns to specific geographical areas. They noted that adaptability might be the basis for this phenomenon. Likewise, Mkandawire *et al.* (2004) revealed that *X. campestris* pv. *phaseoli* strains isolated from the

common bean of Africa comprised three genotypes – two unique to East Africa and the other associated with strains collected from the New World. Selection for a specialised niche can affect genome organisation and the distribution of repetitive sequences in the bacterium genome, resulting in fingerprints unique to a specific pathovar or strain (Louws *et al.*, 1994). Yap *et al.* (2004) reported genetic diversity in *E. carotovora* subsp. *carotovora* strains isolated from the same region in the same season from the same host species. It would be interesting to study the *Xanthomonas citri* pv. *viticola* adaptation when different areas or climatic factors are involved.

The genetic diversity of *Xcv* was found to be based on the different geographical locations in India. The reasons for significant differences are yet to be investigated. However, several factors, such as the migration of individuals, sampling strategies and lumping of strains from different locations, could be possible mechanisms for such variations. Although *Xcv* populations appeared to be highly diverse, one possible explanation for the current observations might be horizontal gene transfer (HGT) and recombination, which probably occurred within the vines (Filip & Skuza, 2021; Ghaly *et al.*, 2024). It has been reported that HGT is the predominant force leading to similarities between the genomes of *X. citri* pv. *fuscans* and *X. phaseoli* pv. *phaseoli*. HGT was the main reason for the evolution of *Xanthomonas* strains with the common bean as host (Chen *et al.*, 2018). Further studies on *Xanthomonas citri* pv. *viticola* are necessary to clarify the transfer mechanism. Timilsina *et al.* (2019) say that the core genes reveal the extent, source and mechanisms of recombination events that shaped the current population and genomic structure of *X. perforans* in Florida.

## CONCLUSION

This study provides new insight into the phenotypic and genotypic diversity and evolution of *Xanthomonas citri* pv. *viticola* strains – the causal agent of bacterial leaf spot in grapevines – from Maharashtra, Karnataka and Tamil Nadu in India. Although the sample size was limited, the results indicate diversity in the population structure. Future studies are necessary to identify the factors responsible for the resistance in *Xcv*. Finally, this work provides an excellent basis for further exploration of the specific interaction between the *Xcv* strain and grapevines that will help to implement various preventive strategies. Furthermore, it can help to minimise the losses due to BLS through the development of a PCR-based diagnostic method for early detection of the pathogen.

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# GUIDE TO AUTHORS

## GENERAL INFORMATION

The South African Journal of Enology and Viticulture (SAJEV) publishes full-length original Research Papers, Research Notes and Review Papers on all subjects related to enology and viticulture including table grapes and raisins. The SAJEV does not accept articles published in, or submitted to, other journals.

**MANUSCRIPT:** Authorship of papers in the SAJEV is not limited to members of the South African Society for Enology and Viticulture (SASEV). The Editor, in conjunction with members of the Editorial Board, will determine the acceptability of papers. All full-length manuscripts, including research notes, have to be original research, neither simultaneously under consideration for submission nor previously published elsewhere. As from March 2019 supplementary files are no longer accepted and will also not be published on-line. All figures and tables have to be included in the main document. Only include the most relevant figures and tables.

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**REVIEWING:** Each manuscript is reviewed by at least two reviewers. Authors are encouraged to list three to five reviewers in their letter to the Editor when they submit their manuscript. Changes proposed by the reviewers will be forwarded to the author(s), but the Editor reserves the right to edit any manuscript for style. **Revised manuscripts must be uploaded directly onto the SAJEV submission platform (<http://www.journals.ac.za/index.php/sajev>) within two weeks (14 days).** Authors may apply directly to the Editor by e-mail ([LMTD@sun.ac.za](mailto:LMTD@sun.ac.za)) for extension. Authors need to highlight all changes made in the revised document and answer queries received from the reviewers in a separate letter to the Editor. The rebuttal letter must be uploaded together with the revised manuscript onto the SAJEV submission platform (<http://www.journals.ac.za/index.php/sajev>). Page proofs will be sent as a PDF file to the corresponding author. Queries or comments are highlighted on the page proofs, which need to be answered and e-mailed directly to the Editor ([LMTD@sun.ac.za](mailto:LMTD@sun.ac.za)) **within 48h**. Authors are not allowed to make changes to the text of the page proofs. All changes required must be made by inserting a note using the "new comment" option in Word. Failure to do so may delay the publication process. Once all corrections have been made on the page proofs to the satisfaction of the Editor, and the page fees have been paid, the manuscript receives a DOI (digital object identifier) number. At this stage of the publication process a DOI number, volume number, year of publication, and page numbers will appear on the manuscript. As from 2024, manuscripts will only be available online in electronic format and can be downloaded (free of charge) from either the SASEV website (<http://www.sasev.org>) or directly from the SAJEV submission platform (<http://www.journals.ac.za/index.php/sajev>). Papers accepted during December the previous year and January to May will be included in the first edition (May/June) and papers accepted from June towards the end of the year in the second edition (Nov/Dec).

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## MANUSCRIPT REQUIREMENTS AND SUBMISSION

All manuscripts must be written in English and grammatically edited to accepted standards of English style and usage before sub-mission. Spelling should be that of the Concise Oxford Dictionary, Oxford: Clarendon Press.

All manuscripts must be submitted in Word format online at <http://www.journals.ac.za/index.php/sajev> as from the 1st of June 2016. After registration, you will receive a user ID and password. The submission process is user-friendly, with exact instructions to lead you through the process. The Guide to Authors is also available on the same website (<http://www.journals.ac.za/index.php/sajev>).

The format must be in **Microsoft Word** (for PC not MAC). Pages must have the following layout: Font 12, Times New Roman, page size set at A4 (297 x 210 mm), 2.5 cm margins on all sides, double spaced lines. Paragraph formatting should be "Normal" and Text level formatting should be "Default Paragraph Font" throughout the whole document. All pages must be numbered. Lines must be numbered consecutively. Please consult a recent issue of the SAJEV for conventions and layout. Manuscripts with incorrect style will be returned to the corresponding author.

**Manuscripts should have a TITLE and, on separate lines, with one line spacing in-between, the following (in this order):**

- Initial(s) and Surnames of the author(s). Initials in capitals. Separate the authors with a comma. Clearly indicate the corresponding author with an \* and make sure the e-mail address of the corresponding author is listed
- The name of the organisation where the research was conducted, as well as the current postal and e-mail address(es) of the author(s). If more than one author and more than one address, number the address with (1), (2), etc. in front of the address and with a superscript (without brackets) above each author. Please use a recently published paper as an example.
- Submitted for publication: (date left open for insertion later)
- Accepted for publication: (date left open for insertion later)
- Key words (5 – 10): carefully selected for accurate electronic referencing (separated with commas)
- Running title: In italics, with the first letter of each word in capital. This is used as a condensed heading at the top of each page. Do NOT insert the running title as header or footnote into the text.

## GUIDE TO AUTHORS

The body of the manuscript should include the following sections, set off with headings in capital letters: ABSTRACT, INTRODUCTION, MATERIALS AND METHODS, RESULTS AND DISCUSSION (combined or as separate sections), CONCLUSIONS, LITERATURE CITED.

■ Sub-headings of the first order should be indicated at the beginning of a paragraph and printed in bold. The text starts on the following line as the sub-heading.

Second-order headings should be arranged likewise, but printed in italics and bold. Third-order headings should be arranged likewise, printed in italics only.

■ Tables should be on numbered pages following the Literature Cited section, followed by the legends for figures on a separate, numbered page. This is followed by the figures, each on a separate page. In the literature cited section the names of all authors must be presented in full.

### ABSTRACT

The abstract should be a short (less than 250 words), factual and informative summary of significant data collected.

### INTRODUCTION

The introduction should include a short, but appropriate, outline of selected literature bearing directly on the subject of the paper. The general problem involved, as well as reasons for the investigation, should be outlined. A detailed and extensive review of the literature is normally inappropriate.

### MATERIALS AND METHODS

These should be described briefly, but in sufficient detail, to allow repetition of the work. Variables and/or conditions which may affect the results should be specified. A reference is sufficient for a previously described method.

### RESULTS AND DISCUSSION OR RESULTS, DISCUSSION

The main results should be stated in the text, with reference to tables, diagrams or illustrations, where the supporting evidence is to be found. Although it is not necessary to describe the contents of tables in the text, the principal results should be critically discussed in logical order. Attention should be drawn to the implications of the results and to agreement or disagreement with previous work.

### CONCLUSIONS

This should not be a summary of results, but should focus on the implications of results and indications for possible applications. This section should not contain reference to figures, tables or any literature.

### LITERATURE CITED

References must be arranged alphabetically by author's surname. In text references must be listed chronologically. The sequence of reference must be as follows: author's surname, initials (the same for second and other authors, where applicable), year, title of paper (with only the first word capitalised; proper nouns excepted), name of periodical (abbreviated in the style of the Periodical Title Abbreviations, vol 1, By Abbreviation and vol 2, By Title 5th Edition, Gale Research Detroit, Michigan, 1986), volume, issue number (where necessary), pages. If the issue number is applicable, it appears after the volume number in parenthesis.

### ■ Examples of a journal paper citation:

Holmes, J.W., 1966. Influence of bulk density of the soil on neutron moisture meter calibration. *Soil Sci.* 102, 335-360.

Stelter, K.O., Luurer, G., Thomm, M. & Neuner, A., 1987. Isolation of extremely thermophile sulfate reducers: Evidence for a novel branch of archaeobacteria. *Science* 236, 822-824.

### ■ Example of a book citation:

Thring, M.W., 1975 (2nd ed). *Air Pollution*. Butterworths, London.

### ■ Example of an article quoted from a book:

Faith, W.T., Neubeck, C.E. & Reese, E.T., 1971. Production and application of enzymes. In: Ghose, T.K. & Fiechter, A. (eds). *Advances in biochemical engineering*, vol 1. Springer-Verlag, Berlin. pp. 77-111.

### ■ Example of a citation from unpublished data:

(P. Cilliers, personal communication, 1985).

### ■ Example of a proceedings citation:

Strauss, C.R., Wilson, B. & Williams, P.J., 1986. Flavour of non-muscat varieties. In: Lee, T. (ed). *Proc. 6th Aust. Wine Ind. Tech. Conf.*, July 1986, Adelaide, Australia. pp. 117 - 120.

### ■ Example of a thesis citation:

Du Plessis, L. de W., 1959. The study of the microorganisms associated with the flavours and ripening berries of a number of grape varieties (in Afrikaans). Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

### TABLES

Tables should be typed double-spaced on separate pages and numbered consecutively using Arabic numerals. They should also bear a short, yet adequately descriptive, caption and include enough information so that each table is interpretable without reference to other tables, figures or text. The layout of a table should be such that the data are presented clearly with brief sub-headings. Non-standard abbreviations must be explained in footnotes. When referring to a table in the text, it should be indicated as Table, followed by the number of the table. Please consult the latest edition of SAJEV for the correct style.

### FIGURES

Figures should be in **JPEG format (at least 600 dpi)** and not exceeding 297 x 210 mm. **Figures of low resolution and poor quality will not be accepted and will delay the evaluation of papers.** The figures, including lettering and detail, should be drawn so as to permit reduction to 84 mm (single column) or 175 mm (double column) width and still retain clarity. Each figure should be numbered at the bottom of the page and submitted as a separate file. Descriptive legends must be typed, double-spaced, on a separate sheet using Arabic numerals.

Legends should describe the contents so that each figure is understandable when considered apart from the text. When referring to a figure in the text, it should be indicated as Fig. or Figs followed by the number of the figure. Please consult the latest edition of SAJEV for the correct style.

The preferable positions of the tables in the text must be indicated as follows:

...text ...  
/ insert table 1 /  
...text ...

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...text ...  
/ insert figure 1 /  
...text ...

Tables and figures should be numbered according to the order in which they are referred to in the text.

# GUIDE TO AUTHORS

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Photographs submitted should be high quality, preferably as JPEG files. When necessary, the magnification should be indicated, e.g. x240. Photographs are expensive to print and should, therefore, be kept to a minimum and, if more than one, grouped together. Printing of full colour photographs will only be considered on rare occasions and these will be for the account of the author(s). **Poor quality will not be accepted and will delay publication.**

All figures and photographs must be referred to as figures and must be submitted in separate files. Only metric (S.I.) units may be used on figures.

## NUMERALS

Spell out all numbers or fractions which begin a sentence. Write out numerals one through nine, except with units of measure. If simple fractions are used they must be written out and hyphenated (e.g. three-quarters). It is preferable to use decimals instead of fractions. Between numerals the preposition "to" must be used instead of a hyphen (e.g. 15°C to 18°C). When reporting time, the 24-hour system with four digits must be used; the first two for hours followed by a colon and the last two digits for minutes (e.g. 09:00 for nine o'clock a.m., 21:30 for half past nine p.m.). Dates must be reported as year, month and then day of the month (e.g. 1992-12-14).

## UNITS

Wine and juice volumes should be reported as litres (L). The use of the capital is recommended to prevent confusion with the number one (1). Grape mass should be reported as grams (g), kilograms (kg) or metric tonnes (t). Temperatures should be reported as degrees Celsius with-out a space between the numerals and the unit (e.g. 15.8°C). All other numerals and units should be provided with a space (e.g. 15 mm, 5 mg/L, 2.5 M). Land surface area must be expressed as hectares (ha).

## ABBREVIATIONS AND SYMBOLS

For convenience certain chemical names may be abbreviated as long as the first usage of a certain abbreviation is defined in parentheses. Well known abbreviations, such as HPLC, DNA, etc., as well as chemical symbols may be used without definition.

The following abbreviations and symbols are accepted:

acetyl	Ac
adenosine diphosphate	ADP
adenosine triphosphate	ATP
ampere	A
and others	<i>et al.</i>
atmosphere	Atm
Degree Balling	°B
Degree Brix	°Brix
calorie	cal
Degree Celsius	°C
Coenzyme A	CoA
cosine	cos
cubic centimetre	cm <sup>3</sup>
cultivar	cv.
days post anthesis	DPA
deoxyribonucleic acid	DNA
experimental	exp
figure	Fig.
for example	e.g.
gas chromatography	GC
gram	g
gravity	g
hectare	ha
hectolitre	hL
Hertz	Hz
high performance liquid chromatography	HPLC
hour	h
hydrogen ion concentration (negative log)	pH
infra-red	IR
Joule	J
kilocalorie	kcal
kilogram	kg
kilometre	km
kilopascal	kPa
litre	L
light intensity	µE/m <sup>2</sup> /s
maximum	max.
Megapascal	MPa
Megalitre	ML
metre	m
micro-equivalent	µeq
microgram	µg
microlitre	µL
micrometer (micron)	µm
micromole	µmol
milli-equivalent	meq
milligram	mg
millilitre	mL
millimetre	mm
millivolt	mV
minimum	min.
minute	min
molar (concentration)	M
mole	mol
nanometre	nm
Newton	N
non-significant	ns
number	No.
page	p
pages	pp
Pascal	Pa
percent	%
probability	p
revolutions per minute	rpm
ribonucleic acid	RNA
second	sec
significant at 5 % level	*
significant at 1 % level	**
sine	sin
species	sp. / spp.
standard deviation	SD
sub species	subsp.
tangent	tan
that is	i.e.
tonne	t
variety	var
versus	vs
volt	V
volume	vol
Watt	W
year	yr



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- Where available, URLs for the references have been provided.
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