Preliminary results on the application of insect-based chitosan in a Mediterranean vineyard

Vitale Nuzzo[1], *Antonio* Carlomagno[1*],*Carmen* Scieuzo[2,3], *Patrizia* Falabella[2,3], and *Giuseppe* Montanaro[1]

[1] Università degli Studi della Basilicata - Dipartimento di Scienze Agrarie, Forestali, Alimentari e dell'Ambiente, Via dell'Ateneo Lucano 10, 85100 Potenza, Italy
[2] Università degli Studi della Basilicata – Dipartimento di Scienze di Base e Applicate, Via dell'Ateneo Lucano, 10, 85100 Potenza, Italy
[3]Spinoff XFlies s.r.l, Università degli Studi della Basilicata, Via dell'Ateneo Lucano 10, 85100 Potenza, Italy

Abstract. This study evaluated the effects of irrigation management and chitosan application in a field grown grapevine (*Vitis vinifera* L., cv 'Primitivo' N.) on physiological and productive responses. Factors were compared following a factorial experimental design ($2x2^n$). Irrigation management (factor A) significantly affected stomatal conductance (*gs*), with irrigated (I) vines exhibiting approximately 30% higher *gs* than drought-stressed (D) vines and significantly reduced cluster fresh mass (- 18% g in D), yield (- 52% g in D), berry (- 15% g in D) and skin (- 9% mg in D) fresh mass. Chitosan application (factor B) did not significantly affect *gs*, yield, or berry ripening parameters. The interactions of factors showed a reduction of *gs* but only in D vines. The study showed that water restrictions could impact on *g*s and yield components without compromising berry quality. The preliminary outcomes about chitosan spray on grapevines at veraison, suggest that it could act as a short-term anti-transpirant without affecting vine yield and berry quality. Further in-field research is needed to assess chitosan's role in mitigating water stress under severe water restriction restriction.

1 INTRODUCTION

Chitosan $(C_6H_{11}O_4N)n$, is a biocompatible and biodegradable polysaccharide obtained by the deacetylation of chitin, one of the most abundant polysaccharide in nature, being a component of fungi, diatoms, brown and green algae, crustaceans, mollusca, coelenterata and arthropods [1, 2]. Currently, crustaceans shell waste is the main source of raw material of chitin and then of commercial chitosan [3]. However, the global demand for chitin and chitosan is growing fast, with a compounded annual growth rate of 14.8% [4]. Hence, new sources of chitin and chitosan need to be explored. In line with this, insects are an alternative source of chitin and chitosan that might be more sustainable with respect to crustaceans [5]. Among other insect species, larvae of *Hermetia illucens* L. (the black soldier fly) contains up to 13% chitin and can be easily bred on a wide range of organic waste [6, 7].
Chitin can be extracted (demineralization and deproteinization processes) from different biomasses of *H. illucens* (larvae, pupal exuviae and dead adults) fed on various organic by-products. Thereafter chitin can be transformed into chitosan through a deacetylation process [7, 8].
Grapevine (*Vitis vinifera* L.) is a native species of the Mediterranean, Black Sea and the Caspian Sea basins and is extensively cultivated worldwide [9].
Mediterranean vineyards are increasingly facing drought and salinity stress triggering specific research including new strategies and tools to overcome these limitations.
For example, in the last half-century vineyards irrigation management has been the focus of interest of researchers and technicians to overcome drought and to control grape yield and quality [10, 11]. Moreover, change in amount, frequency and intensity of natural precipitation has globally reduced irrigation water resources [12]. This has led to study new tools to reduce plant transpiration, using foliar application of particle films, or to assess and enhance plant tolerance to salinity using imaging methods and chemical priming [13-15].
Chitosan has been proposed in a wide range of applications, serving in agriculture as fertilizer, fungicide, bactericide, virucide, natural rhizo-bacteria growth promoter (see [16]) and literature therein). Chitosan might also mitigate the adverse effect of drought, salinity and heat stress [17, 18]. Particularly, it was reported that chitosan foliar application helps to cope with drought stress enhancing the production of antioxidant enzymes [19], root growth [20], and WUE by inducing stomatal closure [21].
However, the effect of chitosan in field grown vineyard has been poorly explored.
In this background, the present experimental work aimed to preliminarily evaluate the effectiveness of commercial and insect-based chitosan application in mitigating drought induced stress in field grown vineyard.

* Corresponding author: antonio.carlomagno@unibas.it

2 Materials and Methods

2.1 Experimental vineyard

Trials were carried-out during 2023 growing season in a commercial vineyard, located in Nova Siri (N 40.140761311 E 16.616179014) at about 1 km from the Ionian Sea. The vineyard was established in 2017 with Primitivo N. cultivar grafted onto Couderc157-11 (*V. berlandieri*, cv Las Sorres x *V. riparia* cv Gloire). Vines were planted at a distance of 2.5 x 1.1 m, drip irrigated, monolateral Guyot pruned and trained at vertical shoots positioning (VSP). Rows were E-W oriented. Vapour pressure deficit (VPD) was obtained by means of an eddy covariance tower (LI-7500 open path, LI-COR Biosciences, Lincoln, USA), located in a vineyard close to the experimental plots.
Vineyard irrigation, mineral nutrition and defence were managed according to local practices.

2.2 Chitosan sources

Commercial chitosan was purchased from Merck Kga A (Darmstadt, Hesse, Germany), the molecular weight was in the range 150 – 190 kDa, and the deacetylation degree was 97%.
Insect-based chitosan was produced from *H. illucens* pupal exuvie by the spinoff XFlies s.r.l. at UniBAS and has a molecular weight in the range 160 - 180 kDa, and deacetylation degree was 85%.
Chitosan (2.5 g) was dissolved in distilled water (50 mL) containing 0.1% acetic acid. An aqueous solution containing 0.1% of acetic acid was also prepared.
These solutions were further diluted in tap water to achieve 0.15% chitosan concentration and 0.01% acetic acid concentration. Tap water was and used as control.

2.3 Experimental Design

Two main factors were evaluated: irrigation management and chitosan application as well as their interactions (Table 1). The irrigation management has two levels: full irrigation, 'I', and drought , 'D'.
In the I treatment an irrigation volume of about 255 m^3/ha was supplied weekly. . The onset of drought condition (D) was achieved stopping irrigation on 5 consecutive rows the 15th of June (pea size stage, BBCH 75). At veraison (BBCH 83, July 19th) irrigation was suspended also in I vines.
From drought imposition to harvest (47 days after) any relevant rain occurred (data not shown).
The chitosan application has four levels: vines treated with insect chitosan (CI), vines treated with commercial chitosan (CC), vines sprayed with the solvent acetic acid (AA) and untreated vines sprayed only with tap water (CTRL).
All chitosan solutions were applied once at veraison, 34 days after irrigation cut-off. Products were sprayed early in the morning using a backpack sprayer until the whole foliage and bunches were completely wet.

The two factors were arranged as a split-plot design with three replications each. Irrigation management was the whole plot, and chitosan application were the split plot. Whole plots consisted of three consecutive rows with a total of 180 vines; the split plots (10 vines per plot) were randomly selected within each row (Figure 1). A total of 240 vines was used for data collection (2 irrigation management x 4 chitosan treatments x 3 replications x 10 vines).

Table 1. Factorial experimental design to compare the two factors (irrigation management and chitosan applications) and their interactions.

Factors		**Irrigation management**	
		Irrigated (I)	Drought (D)
Chitosan application	Chitosan from Insect (CI)	I-CI	D-CI
	Commercial Chitosan (CC)	I-CC	D-CC
	Acid acetic (AA)	I-AA	D-AA
	Control (CTRL)	I-CTRL	D-CTRL

I		I		I		I	
I-CI		**I-CC**		**I-AA**		**I-CTRL**	
I-CTRL		**I-CI**		**I-CC**		**I-SS**	
I-AA		**I-CC**		**I-CTRL**		**I-CI**	
I		I		I		I	
D		D		D		D	
D-CI		**D-CC**		**D-AA**		**D-CTRL**	
D-AA		**D-CI**		**D-CTRL**		**D-CC**	
D-CC		**D-CTRL**		**D-CI**		**D-AA**	
D		D		D		D	

Figure 1. Experimental layout irrigated (I) and drought (D) plots. Treatments were distributed in the three central rows and sprayed with chitosan from insect (CI), commercial chitosan (CC), acetic acid (AA), tap water (CTRL).

2.4 Leaf functionality

Leaf functionality was assessed measuring the stomatal conductance (*gs*) to water vapour, light-adapted quantum yield of photosystem II (ΦPSII), and electronic transport rate (ETR). Measurements were done around midday (11:30 -13:30) on clear days at 34 day after irrigation withhold and a few hours after chitosan applications. Sampling of leaf functionality data was repeated at 42, and 47 days after irrigation withhold (DAIW).

Measurements were carried out on three randomly chosen vines for each chitosan application level and two fully developed, healthy and sun exposed main leaves per vine, were randomly chosen.
Measurements were carried out using the portable porometer/fluorometer LI-600 (LI-COR, Lincoln, NE, USA) . Instrument was set in auto mode with *gs* stability limit of 0.001 mol m^{-2} s^{-1}, slope limit (F) of 1 s and period of 2 s. Flow rate was set at 150 µmol s^{-1}, light pulse flash was set at 10000 µmol m^{-2} s^{-1}, flash duration of 800 ms, leaf absorbance of 0.8 and fraction absorbance of PSII of 0.5.

2.5 Yield, and berries quality traits

At harvest (September 7th, total solids soluble (TSS) > 20°Brix), the number of clusters and yield per vine were determined in each plot. For each vine, the cluster weight was calculated from the yield to the number of clusters ratio.
Samples of about 100 berries per plot were collected for analytical determinations. Berries were randomly picked from the clusters collected from the same vines, immediately enclosed in a plastic bag, stored in a portable refrigerator, and transported to the laboratory, where they were counted, weighed, and squeezed through two layers of cheesecloth. Aliquots of the expressed juice were immediately analyzed for total soluble solids (°Brix) with a digital refractometer. titratable acidity (TA) was measured by titration to a pH (HI1048; Hanna Instruments, Woonsocket, RI) endpoint of 7 with 0.1 N NaOH (expressed as g tartaric acid equiv. per L of juice).

2.6 Statistical analysis

A two-way ANOVA (Sigmaplot® 12.3 software (Systat Software, Inc.) followed by Tukey's pairwise *post-hoc* tests for means separation was employed for data analysis. Before the ANOVA, data were checked for normality (Shapiro-Wilk test) and homogeneity of variance (Levené's test). In case of failure of these tests, the non-parametric Kruskal-Wallis test, followed by Dunn's *post-hoc* for means separations, was used.

3 Results and Discussions

In this trial, the differences in irrigation management, established at pea size stage (BBCH 75), have significantly affected leaf functionality, yield and grape quality. While the chitosan treated leaves have shown a significant reduction of the stomatal conductance only on D vines on the same day of the treatment (34 DAIW).

3.1 Effects of irrigation management

Irrigation management significantly increased leaf *gs* in I compared to that in D treatments. Indeed, I vines exhibited approx. 30% higher *gs* compared to D at 34 (June 15th) and at 47 DAIW and about 15% at 42 DAIW (figure 2).

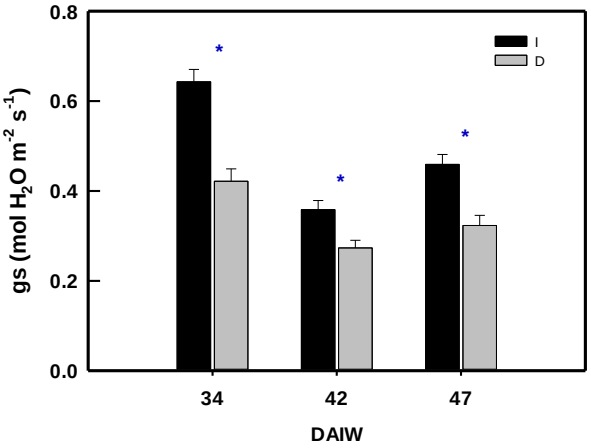

Figure 2. Mean leaf stomatal conductance (*gs*) measured in irrigated (I), and drought stressed (D) vines. DAIW: days after irrigation withhold. Comparing I and D at the same time, * indicates statistically significant differences (*t*- Student test, $p < 0.05$). Each bar on the top of column represents the standard error of the mean.

The low *gs* in D reflects the drought-induced stomatal closure to limit water loss as a physiological adaptation to cope with water deficit conditions [22]. Although we did not report soil moisture data, the different irrigation management conceivably influenced the soil moisture. Generally, *gs* responds to soil water content, and vapour pressure deficit (VPD) [11, 22]. In this study, both I and D vines were exposed at the same VPD that ranged from a maximum of 3.84 (2:00 pm) kPa at 34 DAIW to a minimum of 0.04 kPa (4:00 am) at 47 DAIW.
The *gs* ranged from a maximum of 0.643 mol H_2O m^{-2} s^{-1} in I to a minimum of 0.273 mol H_2O m^{-2} s^{-1} in D independently of chitosan factor.

Although there was a significant difference in *gs* between the two irrigation management, based on *gs* values both I and D likely were in a mild water stress phase [23]. In line with this, both ΦPSII (from 0.35 ± 0.01 to 0.44 ± 0.01) and ETR (from 241.75 ± 7.99 to 272.71 ± 8.50 µmol m^{-2} s^{-1}) measured for I and D vines, did not show significant differences.
Yield per vine, and fresh mass of cluster, berry, and skin were significantly influenced by the irrigation management. Indeed, I vines exhibited higher values than D vines (Table 2). Although both I and D vines experienced mild water stress (as previously discussed), the water limitations imposed from fruit set to harvest directly impacted berry growth. This period, characterized by a rapid cell division, is known to be the most sensitive to water stress [22].
Skin weight was significantly affected by water restrictions in this experiment (Table 2). However, the skin-to-berry mass ratio remained consistent, accounting for 6.6% in I and 7.0% in D. This contrasts with findings in [24], and the differing results may be attributable to variations in experimental conditions.

No significant differences were observed in TSS (21.0 ± 0.26 and 21.9 ± 0.45 °Brix in I and D, respectively) or TA (5.89 ± 0.23 and 5.28 ± 0.29 g tartaric ac eq. L^{-1} in I and D, respectively) between I and D berries. This outcome confirms that water deficits during early berry development often have a more pronounced effect on berry size rather than on sugar accumulation or acidity [25]. Both I and D vines were harvested on the same date (7th September 2024), with no significant differences observed in TSS accumulation. This suggests that water limitations imposed between fruit set and veraison did not influence the progression of berry ripening [24].
Considering the highest number of the cluster per vine in I than D vines, results suggest that water availability between fruit-set and harvest is crucial for ensuring berry growth and ripening under high crop load conditions [26].

Table 2. Yield, clusters fresh mass, and carpometric (berry and skin fresh mass) features in irrigated (I), and drought stressed (D) vines measured at harvest. Different letters indicate statistically significant differences within the same row (*t*-Student test, $p < 0.05$).

Parameters	**Water availability**	
	I	DS
Yield (g $vine^{-1}$)	1208.04 a	583.91 b
Cluster fresh mass (g $cluster^{-1}$)	164.32 a	134.43 b
Berry fresh mass (g $berry^{-1}$)	2.90 a	2.47 b
Skin fresh mass (mg $berry^{-1}$)	192.49 a	174.58 b

3.2 Effects of Chitosan treatments

In our experimental conditions, commercial and insect-based chitosan had no significant effects on the traits investigated.
During the monitored days, the *gs* varied from a maximum of 0.607 ± 0.035 (AA) to a minimum of 0.262 ± 0.029 (CI) mol H_2O m^{-2} s^{-1}. This contrasts with findings in pepper [21] and grapevine [27], where a chitosan-induced stomatal closure was reported. These discrepancies suggest that the effects of chitosan may vary depending on species, environmental conditions or its concentration.
Chitosan application did not exert an impact on berry (from 2.42 ± 0.19 to 2.79 ± 0.11 g) and cluster (from 135.28 ± 13.65 to 154.91 ± 14.84 g) fresh mass, as well as on vine yield (from 817.78 ± 79.27 to 1042 ± 160.08 g), in agreement with other results [28].
Oenological berry traits such as TSS (*i.e.* alcohol potential) and TA were not affected by chitosan application in this experiment, in line with findings of other authors [29]. TSS across all treatments ranged from about 20.9 (CI) to 21.7 °Brix (CTRL), whereas TA across all treatments ranged from 5.11 (CTRL) to 5.94 g tartaric ac. eq. L^{-1}.

3.3 Interaction irrigation management x chitosan applications

The interaction between the two main factors (water management and chitosan) led to significant differences only in D vines and soon after its application (Fig. 3). In addition, the source of chitosan (commercial or insect) induced a similar impact on *gs*. at 34 DAIW. This significant interaction suggests once again that chitosan application in droughted vines may initially reduce *gs*, potentially triggering ABA synthesis [30] which is known to be a chemical signal involved in stomatal regulation [31]. Hence, chitosan treated vines would adapt to drought via increasing stomatal closure and in turn save water.
In line with this, chitosan forms a semi-permeable film around sprayed surfaces [27] probably acting as a physical barrier impacting on *gs*. Indeed, [21] argued that chitosan may act as an anti-transpirant. However, no significant differences in factor interactions were observed at 42 and 47 DAIW. Overall, *gs* values ranged from 0.209 ± 0.028 to 0.397 ± 0.048 mol H_2O $m^{-2}s^{-1}$ at 42 DAIW and from 0.244 ± 0.023 to 0.545 ± 0.031 mol H_2O $m^{-2}s^{-1}$ at 47 DAIW. This transient effect of chitosan application on leaf *gs* might be associated to the chitosan mode of action [17] or to its persistency. However, this remain to be specifically examined.

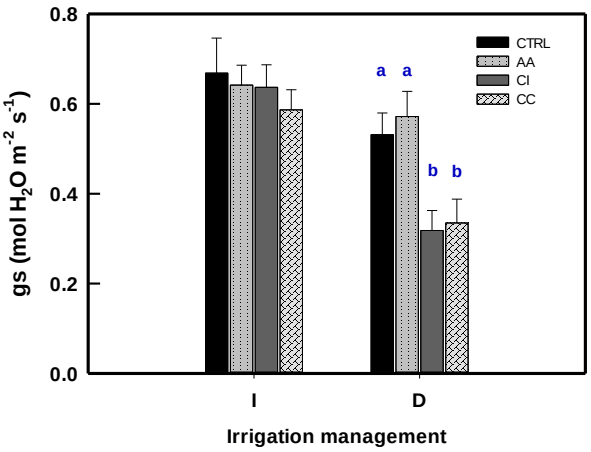

Figure 3. Leaf stomatal conductance (*gs*) measured at 34 DAIW in grapevines subjected to water (I: irrigated; D: drought stress) and chitosan treatments (CTRL: no treatment, AA: acetic acid, CI: insect chitosan, CC: commercial chitosan) soon after chitosan application (34 DAIW). Within each factor (water or chitosan), different letters indicate statistically significant differences (Student's t-test, $p < 0.05$). Absence of letters denotes non-significant differences. Each bar on the top of column represents the standard error of the mean

4 Conclusions

Chitosan application had a short-term significant effect on *gs* reduction with no impact on yield and berry quality. This suggests chitosan might be a valuable water-saving tool under drought.
The relatively mild water stress imposed in this study as inferred by the *gs* level likely masked the effectiveness of chitosan. Hence, future research should explore chitosan's potential under varying water stress levels to

better understanding its role in mitigating drought induced leaf function limitations in grapevines.

This study was carried out within the Agritech National Research Center and received funding from the European Union Next-GenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) – MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4 – D.D. 1032 17/06/2022, CN00000022). This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them.

The authors thank the Battifarano Farm for hosting the experiment.

Declaration: During the preparation of this work the authors used Grammarly in order to improve the readability and English language. After using this tool, the authors reviewed and edited the content as needed and takes full responsibility for the content of the published article.

1. M. Rinaudo, Prog. Polym. Sci., **31**, 603–632 (2006).
2. R. Sharif, M. Mujtaba, M. U. Rahman, A. Shalmani, H. Ahmad, T. Anwar, D. Tianchan, X. Wang, Molecules **28**, 872 (2018).
3. H. Amiri, M. Aghbashlo, M. Sharma, J. Gaffey, L. Manning, S. M. M. Basri, J. F. Kennedy, V. K. Gupta, M. Tabatabaei, Nature Food **3**, 822–828 (2022).
4. G.M. Oyatogun, T.A. Esan, E.I. Akpan, S.O. Adeosun, A.P.I. Popoola, B.I. Imasogie, W.O. Soboyejo, A.A. Afonja, S.A. Ibitoye, V.D. Abere, A.O. Oyatogun, K.M. Oluwasegun, I.E. Akinwole, K.J. Akinluwade, *Handbook of Chitin and Chitosan, vol. 1: Preparation and Properties* (Elsevier, 2020).
5. T. Hahn, E. Tafi, A. Paul, R. Salvia, P. Falabella, S. Zibeka, J. Chem. Technol. Biotechnol., **95**, 2775–2795 (2020).
6. C. Scieuzo, A. Franco, R. Salvia, M. Triunfo, N.F. Addeo, S. Vozzo, G. Piccolo, F. Bovera, A. Ritieni, A.D. Francia, A. Laginestra, E. Schmitt, P. Falabella, Insect Sci., **30**, (2022).
7. M. Triunfo, E. Tafi, A. Guarnieri, R. Salvia, C. Scieuzo, T. Hahn, S. Zibek, A. Gagliardini, L. Panariello, M.B. Coltelli, A. De Bonis, P. Falabella, Sci. Rep., **12**, 6613 (2022).
8. M. Triunfo, A. Guarnieri, D. Ianniciello, M.B. Coltelli, R. Salvia, C. Scieuzo, A. De Bonis, P. Falabella, Intern. J. Biol. Macromol., **271**, 132669 (2024).
9. International Organisation of Vine and Wine. State of the world vine and wine sector in 2023, **20** (2024).
10. P. Steduto, T.C Hsiao, E. Feres, D. Raes, *Crop yield response to water* (FAO, Rome, 2012).
11. H. Medrano, M. Tomás, S. Martorell, J.-M. Escalona, A. Pou, S. Fuentes, Agron. Sust. Dev., **35**, 499–517 (2015).
12. IPCC, *Climate change 2022: impacts, adaptation and vulnerability. Contribution of working group II to the sixth assessment report of the intergovernmental panel on climate change* (Cambridge University Press, 2022).
13. G. Montanaro, N. Briglia, L. Lopez, D. Amato, F. Panara, A. Petrozza, F. Cellini, V. Nuzzo, J. Plant Interac., **17**, 789-800 (2022).
14. D. Amato, G. Montanaro, S. Summerer, N. Briglia, F. Attia, E. Challet, V. Nuzzo, OENO One, **54**, 1007-1020 (2020).
15. G. Montanaro, N. Briglia, A. Petrozza, A. Carlomagno, L. Rustioni, F. Cellini, V. Nuzzo, Oeno-One, **58**, 1-7757 (2024).
16. R.K. Singh, E. Ruiz-May, V.D. Rajput, T. Minkina, R.L. Gómez-Peraza, K.K. Verma, M.S. Shekhawat, C. Pinto, V. Falco, F.R. Quiroz-Figueroa, Agriculture, **12**, 1369 (2022).
17. A. Hidangmayum, P. Dwivedi,, D. Katiyar, A. Hemantaranjan, Physiol. Mol. Biol. Plants, **25**, 313-326 (2019).
18. N.A. Shinde, P.G. Kawar, S.G. Dalvi, Plant Nano Biology, **7**, 100059 (2024).
19. H.L. Guo, Y.G. Du, X.F. Bai, X.M. Zhao, Chin J Mar Drugs, **1**, 11–12 (2003).
20. D. Zeng, X. Luo, Open J. Soil Sci., **2**, 282–288, (2012).
21. M. Bittelli, M. Flury, G.S. Campbell, E.J. Nichols, Agric. For. Meteorol. **107**, 167–175 (2001).
22. M. M. Chaves, O. Zarrouk, R. Francisco, J. M. Costa, T. Santos, A. P. Regalado, M. L. Rodrigues, C. M. Lopes, Ann. Bot., **105**, 661–676, (2010).
23. J. Cifre, J. Bota, J.M. Escalona, H. Medrano, J. Flexas, Agric. Ecosys. Environ., **106**, 159-170 (2005).
24. M.A. Matthews, M.M. Anderson, Am. J. Enol. Vitic., **39**, 313-320 (1988).
25. G. Roby, J.F. Harbertson, D.O. Adams, M.A. Matthews, Aust. J. Grape Wine Res., **10**, 100-107, (2004).
26. D.S. Intrigliolo, J.R. Castel, Irrig. Sci., **29**, 443-454 (2011).
27. G. Romanazzi, V. Mancini, E. Feliziani, A. Servili, S. Endeshaw, D Neri, Plant Dis., **100**, 739-748 (2016).
28. T. Garde-Cerden, V. Mancini, M. Carrasco-Quiroz, A Servili, G. Gutierrez-Gamboa, R. Foglia, E.P. Perez-Ãlvarez, G. Romanazzi, J. Agric. Food Chem., **65**, 7379–7386 (2017).
29. P. Tessarin, F. Chinnici, S Donnini, E. Liquori, C. Riponi, A.D. Rombolà, Food Chem., **210**, 512–519 (2016).
30. M. Iriti, F Faoro, Plant Physi. Biochem., **46**, 1106-1111 (2008).
31. C. Lovisolo, W. Hartung, A. Schubert, Funct. Plant Biol., **29**, 1349-1356 (2002).