MOLECULAR AND BIOCHEMICAL INSIGHT UPON CHITOSAN APPLICATION ON *VITIS VINIFERA* L. CV. TOURIGA FRANCA AND TINTO CÃO

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Introduction

Chitosan have gained the attention of the researchers globally for its use in agriculture pertaining to its multiple favorable properties, and its abundance in the environment which in turn makes it a cost-effective natural substance for a sustainable agriculture (Rinaudo, 2006; Malerba M and Cerana R., 2015). Chitosan is being extracted from chitin through a deacetylation process. Allan and Hadwiger (1979) were the first to report a fungal cell wall material; which potentially induced the plant inherent immune responses towards pathogen infestation and hence, trailed the attention towards this material for its future use in agriculture. Since then, it has been tested in various horticultural crops to meet diverse range of objectives (Sharif R. et al., 2018). Over the years, chitosan has been tested positively in agriculture for better post-harvest management and augmented agronomic traits. Moreover, it has shown improved defense response, higher growth and physiological activities, and better abiotic stress management in plants. In addition, it can be used as a biofertilizer and as a protectant in various crops including grapevines (Sharif R. et al., 2018; Hidangmayum, A. et al., 2018 and Iriti M. et al., 2011).

Despite of having numerous advantages, there are only a few reports on the effects of chitosan on the phenolic compounds elicitation in the grapevines at the molecular and biochemical level. Moreover, the mode of action of chitosan is still poorly understood. Previous recent reports on the use of chitosan from our group observed a higher accumulation of anthocyanins and secondary metabolite compounds by the modulation of the gene expression during veraison in grapevines (Singh et al., 2020). Our another report on chitosan application suggested an improved antioxidant potential, by modulating genes of reactive oxygen species (ROS) pathways and secondary metabolites syntheses in the different components of grapevine (Singh et al., 2019). An increased antimicrobial property against pathogenic bacteria has also been observed post-chitosan application in plants (Silva et al., 2020).

Present study investigates the application of chitosan in grapevine in the field condition and its subsequent effects on the secondary metabolites syntheses and accumulation at the molecular level. The findings suggested that chitosan has a stimulatory effect, in tissue-specific manner, on the key genes for secondary metabolites pathways. Thus, its use exhibits a qualitative improvement in grape berry quality as well as for the plant health. Overall, the authors would like to recommend chitosan as a basic material for the development of newer plant protection agents, especially in grapevine.

Materials and methods

Vineyards containing *Vitis vinifera* L. 'Touriga Franca' and *Vitis vinifera* L. 'Tinto Cão' (red varieties) at Quinta de Nossa Senhora de Loures vineyard (41º19'N, 7º44'W, 500 m above mean sea level, lower Corgo sub-region of the Douro Demarcated Region of northern Portugal) were considered for the present study. The grapevine plants (420A rootstock) consisted peculiar Guyot system, spaced 2.3×0.9 m with 15% of gravel, loamy, morainic soil. Phenotypically similar three lines with 12 vines each were marked in each cultivar for application of chitosan while similar three lines each were maintained as control. Application of crop protectants, weed control, and shoot guiding were kept similar for all the lines.

Chitosan (molecular weight of 76kDa and deacetylation degree of 85%) was dissolved for a concentration of 0.01% (w/v) in 0.01 M aqueous acetic acid. The plant leaves and grape bunches were sprayed with chitosan solution by using a manual spray lance. Total two applications were performed; first at the beginning of veraison at the onset of berry ripening, and second, at the completion of veraison with all the berries turned red-color. The meteorological conditions from the beginning of veraison till the complete maturation and sample harvesting were recorded (average temperature, humidity and wind velocity of 23.4 ºC, 57.4% and 6.5 km/h, respectively). Berries, leaves, cluster stems, and shoots were collected at the final maturation, and the berries were dissected into the skin and seeds. The same tissues from all control vines were also collected. The samples were frozen immediately in liquid nitrogen and stored at -80°C for further processing.

Crude total RNA was extracted from all the frozen tissues by using the CTAB (cetyl trimethyl ammonium bromide) method (Gasic et al., 2004) and DNase treatment was performed to eliminate any possible DNA contamination. Quality was checked by running samples on 1.0% denaturing agarose gels, followed by quantification with a ND-1000 Spectrophotometer at wavelengths of 230 and 280 nm, and 1 µg of DNase-treated RNA was used for cDNA synthesis, using the First Strand cDNA Synthesis Kit. Specific internal primers (Table 1) were designed by using Primer3 online tool (http://primer3.ut.ee/) and a quantitative real-time PCR (qRT-PCR) was carried out to record the relative expression of six selected genes of secondary metabolite pathway. Each reaction contained 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis Biodyne, Tartu, Estonia), 200 nM of each primer, 1 µL 10x diluted cDNA sample, and nuclease-free water to a final volume of 20 µl in PicoReal-Time Thermal Cycler (PRO 96-1500-

512, Thermo Scientific, Ratastie, Finland). The PCR program was: 95°C for 12 min (initial denaturation), followed by 40 cycles of heating at 95°C for 15 s (denaturation), 52 °C for 20 s (annealing) and 72°C for 20 s (extension). PicoReal 2.0 software was used to analyze the data, and relative expression for each gene was calculated as RQ values (relative quantification by using PicoReal 2.0 tool), using actin and GADPH as the internal reference genes.

Gene		**Primer sequence**	Tem
PAL	Phenylalanine amonia-lyase	F 5′ CCTACTGTTCAGAGCTCCAG 3′ R 5′ GCCACTAGGTATGTGGTAGACA 3′	55°C
CHS	Chalcone synthase	F 5′ CACTCTTCGAACTCGTCTCT 3′ R 5′ CCACCAAGCTCTTCTCTATG 3′	55°C
F3H	Flavanone3-hydroxylase	F5′CAGTGCAAGACTGGCGCGAGATCGTA3′ R 5′ TAGCCTCAGACAACACCTCCAGCAACT 3′	52°C
4CL	4-coumarate:CoA ligase	F 5′ TGCAGGGCCTAACTCACTCT 3′ R 5′ GCAGTCGCCTTAGGTAGCAC 3′	55°C
CAM	caffeic acid O-methyltransferase	F 5′ CTCCACTGGTCCTCTGCTTC 3′ R 5′ AGCCTGCTTCGAAAGTACCA 3′	55°C
STS	stilbene synthase	F 5′ TGCTTTTGTGATTTTGTTAGAGG 3′ R 5′ CCCTTCCCCGATTGAGAGTA 3′	55°C
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	F: 5'CACGGTCAGTGGAAGCATCAT 3′ R 5′ CCTTGTCAGTGAACACACCAG 3′	55°C
ACT	Actin	F 5′ GTGCCTGCCATGTATGTTGCC 3′ R 5′ GCAAGGTCAAGACGAAGGATA 3′	55°C

Table 1. *Primer sequences and annealing temperature for the genes of secondary metabolite pathway analyzed in this study.*

Results and discussion

There are only a few reports on the molecular mechanism regarding the mode of action of chitosan. The present study aimed to unveil this phenomenon, by investigating six genes key genes for secondary metabolite synthesis, i.e., PAL (phenylalanine ammonia lyase), CHS (Chalcone synthase), F3H (flavanone 3-hydroxylase), 4CL (4-coumarate:CoA ligase), CAM (caffeic acid O-methyltransferase) and STS (stilbene synthase) through qRT-PCR using all the tissues (leaf, berry skin, seeds, cluster stem and shoots) of both varieties (figure 1).

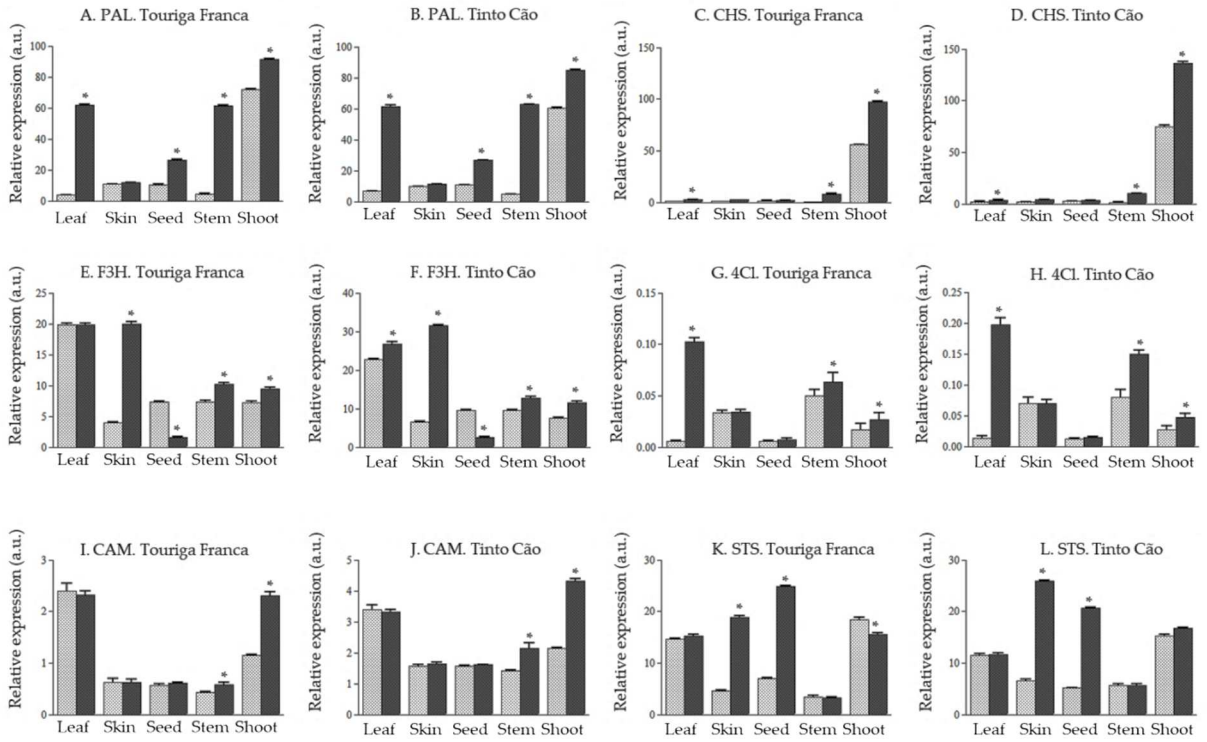

Figure 1. *Effect of chitosan on PAL (phenylalanine ammonia lyase), CHS (Chalcone synthase), F3H (flavanone 3-hydroxylase), 4CL (4-coumarate:CoA ligase), CAM (caffeic acid O-methyltransferase) and STS (stilbene synthase)genes in the tissues of grapevine. Tissues were collected at complete maturation of the berries. White column = control samples; black column = chitosan-treated samples.*

Phenylpropanoid pathway is a key factor for the production of the secondary metabolites, and PAL catalyzes the first step of this pathway (Arakawa 1988). Present study recorded the increased expression of PAL gene in leaf, seeds, cluster stems and shoot tissues in both the varieties upon chitosan treatment (figure 1A and 1B). Fruit color development was positively associated with PAL upregulation in previous studies (Boss et al., 1996; Kobayashi et al., 2002). PAL expression was reported to be induced during veraison until maturation of berries followed by a decrease in its transcript level (Zhao et al., 2016; Chen et al., 2006). Present study demonstrated increased expression in all the tissues except berry skins in maturation; although our recent previous study reported higher expression of PAL in leaf and berry skin during veraison in a grapevine variety Tinto cão (Singh et al., 2020).

Chalcone synthase is a key player in flavonoid biosynthesis in grapevines and the gene is reported to get upregulated from the veraison until the maturation of berries (Boss et al., 1996; Kobayashi et al., 2002). Leaf, clusters stems and shoot tissues showed higher expression of this gene in both varieties in chitosan-treated plants (figure 1C and 1D). This suggested an improved synthesis of flavonoids upon treatment. Flavanone 3-hydroxylase (F3H) was observed to be differentially expressed during berry development. It gets upregulated until maturation (Waters et al., 2005). Berry skins, cluster stems and shoots demonstrated high expression of F3H gene in the grapevine varieties Touriga Franca, while Tinto Cão exhibited

a higher expression in berry skins, cluster stems, shoots and leaf tissue as well in the treated plants during maturation. Our previous report recorded very high expression of F3H gene during veraison in leaf and berry skin tissues of Tinto cão upon chitosan treatment (Singh et al., 2020).

The 4-Coumarate:CoA ligases (4CLs) belongs to phenylpropanoid-derived compound and metabolisms (Yuan Y., 2014). Cluster stems, leaves, and shoot tissues showed an increased expression of this gene in both varieties upon treatment and suggested a positive induction in the grapevine. CAM gene also belongs to the flavonoid synthesis in grapevines (Giordano et al., 2016); especially during stress conditions. The present study recorded a higher expression of CAM gene in the stems and shoots of both varieties upon treatment, hinting an involvement for the improved plant health. Stilbene synthase is another key gene for stilbene/resveratrol synthesis in grapevines (Chialva et al., 2018). Present study recorded increased transcript accumulation in the berry skins, seeds, and shoots tissues in both the varieties upon chitosan application, and thus suggested a higher level of stilbene/resveratrol in the berries.

Total phenolics, total anthocyanins and total tannins were also analyzed and reported in the different tissues of both varieties in chitosan-treated grapevines in our previous report (Singh et al., 2019) and presented in an adapted form in the Table 2.

Variety/Tissue	Total phenolics		Total anthocyanins		Total tannins	
	control	chitosan	control	chitosan	control	chitosan
Touriga Franca						
berry seeds	94.67±2.26 c	93.65±5.10 c	0.00±0.00 a	0.00±0.00 a	95.35±5.32 c	125.98±4.54 b
berry skins	112.70±4.23 c	122.80±1.92 ab	26.02±1.02 a	23.28±0.55 bc	47.41±3.02 d	57.11±0.76 c
cluster stems	88.90±5.55 b	114.44±5.78 a	8.26±0.02 b	17.66±0.01 a	31.63±3.11 a	20.52±2.28 b
leaves	55.65±1.09 e	78.71±1.48 b	0.39±0.01 b	0.53±0.01 a	17.06±1.15 d	32.47±3.37 c
shoots	37.23±2.36 a	34.09±0.64 a	1.55±0.11 a	1.12±0.02 a	17.26±1.62 b	37.02±0.66 a
Tinto Cão						
berry seeds	146.32±4.25 e	144.05±1.23 e	0.00±0.00 a	0.00±0.00 a	106.81±1.23 c	113.64±4.25 c
berry skins	129.95±4.17 c	163.36±9.57 a	28.74±0.97 b	35.16±1.16 a	32.03±4.17 c	47.55±9.57 a
cluster stems	158.61±5.28 b	208.97±8.37 a	2.25±0.01 b	2.68±0.02 a	102.66±5.28 b	140.65±8.37 a
leaves	204.76±8.89 c	201.16±3.16 cd	0.78±0.03 b	0.77±0.03 b	89.43±8.89 a	78.72±3.16 b
shoots	49.59±2.44 a	52.65±1.51 a	0.14±0.02 a	0.15±0.01 a	30.75±2.44 b	37.97±1.51 a

Table 2 (adopted from Singh et al., 2019). *The total phenolic content (TPC; µg epicatechin equivalents per mg dry weight), total anthocyanin content (TAC; µg malvidin-3-O-glucoside equivalents per mg dry weight) and total tannin content (TTC; µg epicatechin equivalents per mg dry weight) in grapevine tissues in chitosan treated plants and control plants at berry maturation. All data were obtained from three biological replicates; means ± standard deviations within a row followed by different letters are statistically different (P < 0.05; ANOVA Turkey's test).*

Total phenolic content was recorded higher in berry skins, cluster stems, and leaves of the variety Touriga Franca in chitosan-treated grapevines, while Tinto Cão recorded higher phenolics in the berry skins and stem tissues. Cluster stems and leaves of Touriga Franca

variety showed higher total anthocyanins content, while a decrease was observed in the berry skins. Whereas, Tinto Cão berry skins showed a higher total anthocyanin content in the treated plants. Total tannins were high in seeds, berry skins, leaves and shoot tissues in Touriga Franca, while all the tissues except leaves in Tinto Cão showed higher total tannins upon chitosan treatment.

Elicitor molecules are known to induce secondary metabolite syntheses and accumulation in plants upon application (Rudolf 2005). Chitosan have been tested for its elicitation potential in the present study, as well as in our recent reports, and the results suggested a differential induction of the metabolites in the different tissues of grapevines upon application. The correlation was established between metabolite syntheses and the expression of the responsible key genes of the concerned pathways. The present study may provide a better understanding of the mode of action of chitosan at the molecular level. Moreover, in consideration to all the observations, chitosan have shown a positive effect on the quality of grape berries, and thus may assist the plant health as a bio-protectant.

Conclusions

In conclusion, chitosan have shown the potential towards an increased syntheses and accumulation of secondary metabolites in the grape berries by modulating key gene's expression. Our previous reports showed a higher antioxidant potential in the different tissues of grapevines upon chitosan application and a correlation was established with higher secondary metabolites. Moreover, increased antimicrobial properties were also noticed in the different grape components in the chitosan-treated grapevines in our recent reports. Keeping all the findings together, chitosan may be considered as a promising material for fruit quality improvement and for an overall development of more sustainable viticulture practices.

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References

Allan, C.R.; Hadwiger, L.A. The fungicidal effect of chitosan on fungi of varying cell wall composition. Exp. Mycol., 1979, 3, 285–287.

Arakawa, O. Photoregulation of anthocyanin synthesis in apple fruit under UV-B and red light. Plant Cell Physiol. 1988, 29, 1385–1389.

Boss, P.K.; Davies, C.; Robinson, S.P. Analysis of the expression of anthocyanin pathway genes in developing Vitis vinifera L. cv Shiraz grape berries and the implications for pathway regulation. Plant Physiol., 1996, 111, 1059–1066.

Chen, J.Y.; Wen, P.F.; Kong, W.F.; Pan, Q.H.; Wan, S.B.; Huang, W.D. Changes and subcellular localizations of the enzymes involved in phenylpropanoid metabolism during grape berry development. Plant Physiol., 2006, 163, 115–127.

Chialva, C.; Muñoz, C.; Miccono, M.; Eichler, E.; Calderón, L.; Prieto, H.; Lijavetzky, D. Differential Expression Patterns Within the Grapevine Stilbene Synthase Gene Family Revealed Through Their Regulatory Regions. Plant Mol. Biol. Rep., 2018, 36, 225–238.

Gasic, K.; Hernandez, A.; Korban, S.S. RNA extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction. Plant Mol. Bio. Rep. 2004, *22(4),* 437–438.

Giordano, D.; Provenzano, S.; Ferrandino, A.; Vitali, M.; Pagliarani, C.; Roman, F.; Cardinale, F.; Castellarin, S.D.; Schubert. A. Characterization of a multifunctional caffeoyl-CoA O-methyltransferase activated in grape berries upon drought stress. Plant Physiol. Biochem., 2016, 101, 23-32.

Hidangmayum, A.; Dwivedi, P.; Katiyar, D.; Hemantranjan, A. Application of chitosan on plant responses with special reference to abiotic stress. Physiol Mol Biol Plants, 2018, 25 (2), 313–326.
Iriti, M.; Vitalini, S.; Tommaso, G.; Amico, S.; Borgo, M.; Faoro, F. New chitosan formulation prevents grapevine powdery mildew infection and improves polyphenol content and free radical scavenging activity of grape and wine. Aust. J. Grape Wine R., 2011, 17, 263–269.

Kobayashi, S.; Ishimaru, M.; Hiraoka, K.; Honda, C. Myb-related genes of the Kyoho grape (Vitis labruscana) regulate anthocyanin biosynthesis. Planta, 2002, 215, 924–933.

Malerba, M.; Cerana, R. Reactive oxygen and nitrogen species indefense/stress responses activated by chitosan in sycamore cultured cells. Int. J. Mol. Sci. 2015, 16, 3019–3034.

Rinaudo, M. Chitin and chitosan: properties and application. Prog Polym Sci. 2006, 31(7), 603–632.

Rudolf, J.R.; Resurreccion, A.V.A. Elicitation of Resveratrol in Peanut Kernels by Application of Abiotic Stresses. J Agricul Food Chem., 2005, 53, 10186–10192.

Sharif, R.; Mujtaba, M.; Rahman, M.U.; Shalmani, A.; Ahmad, H.; Anwar, T.; Tianchan, D.; Wang, X. The Multifunctional Role of Chitosan in Horticultural Crops; A Review. Molecules. 2018, 23(4), 872.

Silva, V.; Singh, R.K.; Gomes, N.; Soares, B.G.; Silva, A.; Falco, V.; Capita, R.; Alonso-Calleja, C.; Pereira, J.E.; Amaral, J.S.; Igrejas, G.; Poeta, P. Comparative Insight upon Chitosan Solution and Chitosan Nanoparticles Application on the Phenolic Content, Antioxidant and Antimicrobial Activities of Individual Grape Components of Sousão Variety. Antioxidants**,** 2020, *9*, 178.

Singh, R.K.; Martins, V.; Soares, B.; Castro, I.; Falco, V. Chitosan application in vineyards (*Vitis vinifera* L. cv. Tinto Cão) induces accumulation of anthocyanins and other phenolics in berries mediated by modifications in the transcription of secondary metabolism genes. Int. J. Mol. Sci., 2020, 21, 306.

Singh, R.K.; Soares, B.; Goufo, P.; Castro, I.; Cosme, F.; Pinto-Sintra, A.L.; Inês, A.; Oliveira, A.A.; Falco, V. Chitosan Upregulates the Genes of the ROS Pathway and Enhances the Antioxidant Potential of Grape (*Vitis vinifera* L. 'Touriga Franca' and 'Tinto Cão') Tissues. Antioxidants, 2019, *8*, 525.

Waters, D.L.; Holton, T.A.; Ablett, E.M.; Lee, L.S.; Henry, R.J. cDNA microarray analysis of development grape (Vitis vinifera cv Shiraz) berry skin. Funct Integr Genomics*,* 2005, 5, 40–58.

Yuan, Y.; Yu, S.; Yu, J.; Zhan, Z.; Li, M.; Liu, G.; Wang, X.; Huang, L. Predicting the function of 4-coumarate:CoA ligase (LJ4CL1) in Lonicera japonica. Int. J. Mol. Sci., 2014, 15(2), 2386-2399.

Zhao, Q.; He, F.; Reeves, M.J.; Pan, Q.H.; Duan, C.Q.; Wang, J. Expression of structural genes related to anthocyanin biosynthesis of *Vitis amurensis*. J. For. Res., 2016, 27, 647-657.

Abstract

Chitosan is a biopolymer and correspond to the second most abundant material on earth after cellulose. Hence, its large scale use for applications in agriculture is economical viable. The diverse beneficial properties of chitosan make it an attractive material to use in sustainable agriculture. The present study aimed to evaluate the elicitation potential of chitosan on grapevines by assessing its effects on the molecular and biochemical level. Acidic chitosan solution (0.01% w/v) was applied in Vitis vinifera L. (cv. Touriga Franca and Tinto cão) vineyards. To study the effect in tissue-specific manners, the samples (leaf, berry skins, seeds, cluster stems and shoots) were collected at the maturation of grape berries. The expression of genes for Phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), 4-coumarate CoA ligase (4CL), caffeic acid O-methyltransferase (CAM), and stilbene synthase (STS) was increased in different tissues upon chitosan application, and the total phenolics, total anthocyanins, and the total tannins were relatively higher in different tissues. The findings of the present study suggest considering chitosan as a potential elicitor in grapevines, and chitosan-based formulations may lead to the development of newer practices for sustainable viticulture.

Keywords

Chitosan; elicitor; secondary metabolites; gene expression; sustainable viticulture.