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Effect of sodium nitroprusside (SNP) on physiological and biological responses of olive (*Olea europaea* cv. Conservolia) under water stress

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Abstract

Water deficit is the major environmental stress limiting agriculture worldwide by inducing physiological, morphological and biochemical changes in plants. This study was carried out to investigate the protective role of exogenous sodium nitroprusside (SNP) on alleviation of water stress in olive (cv. Conservolia). Our results showed that water deficit (D_2 treatment) significantly decrease g_s and RWC while increase peroxidase and polyphenol oxidase activity and proline content compared with D_1 treatment. However, peroxidase and polyphenol oxidase activity, RWC and proline content were improved by the application of SNP (the forth levels of SNP). Also, SNP treated plants showed the lower g_s. Hence, these findings suggested that SNP treatment could be used as a simple, practical and inexpensive method for modulating the negative effects of water deficit in olive.

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Introduction

Water deficit has been shown to influence various physiological and biochemical processes in plants. Plants can avoid water stress by maximizing water uptake or minimizing water loss (Kozlowski and Pallardy, 2002). A common effect of water stress is to cause oxidative damage. Water stress induces an oxidative stress because of the inhibition of the photosynthetic activity due to imbalance between the light capture and its utilization (Foyer and Noctor, 2000). Under water stress, changes in the photochemistry of the chloroplasts lead to generate reactive oxygen species (ROS). The toxicity of ROS is normally regulated by both non-enzymatic and enzymatic antioxidant systems in the plant cells (Aganchich *et al*., 2009). Flavonoids, carotenoids and tocopherols are from non-enzymatic antioxidant groups and enzymatic antioxidans include catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), peroxidase (POX) and polyphenol oxidase (PPO). These antioxidants can scavenge ROS and suppress oxidative stress process (Liu *et al*., 2011).

The maintenance of plant water potential during water deficit is essential for continued growth and can be achieved by osmotic adjustment mechanisms resulting from the accumulation of compatible solutes such as Proline in the cytoplasm (Moghaieb *et al*., 2004). Proline has a protective action which prevents membrane damage and protein denaturation during water stress (Ain-Lhout *et al*., 2001). It can act as an electron acceptor, avoiding damage of photosystems due to their photoinhibition by reactive e oxygen species (Sofo *et al*., 2004). In terms of water relations, the mechanisms controlling water loss through stomata seem to be an efficient process to maintain leaf turgor under water deficit. This includes stomatal responses to soil water potential (Cordeiro *et al*., 2009). Indeed, stomatal control is the major physiological factor in the optimization of water use in drought conditions (Moriana *et al*., 2002).

However, the olive tree is known to be relatively tolerant to water deficit (Aganchich *et al*., 2007). In spite of this character, water deficit leads to decrease growth and developmental processes in olive (Sofo, 2011). Thus, there is need for a simple, inexpensive and practical method to protect plants from the effects of water stress. Chemical products such as cycocel, brasinosteroids and paclobutrazol have previously been found to increase the resistance of various plants to water stress (Marshall *et al*., 1991). Nitric oxide (NO) is one of the molecules which have received much attention during the last decade from plant researchers (Hasanuzzaman *et al*., 2012). It is a short life bioactive molecule and first described as a toxic compound, but now recognized as an important signal and effective molecule involving in many key physiological processes including pathogen defense reaction, abiotic stresses tolerance, growth and development of plants (Pagnussat *et al*., 2002). Indeed, NO is an important signal molecule modulating plant responses to stress (Hasanuzzaman *et al*., 2012).. For example, Garcia-Mata and Lamattina (2001) reported that NO could induce the stomatal closure and enhance the adaptive plant responses against water stress by reducing water loss in plant cells which leads to increase relative water content (RWC) parameter. Also, NO acts as a secondary messenger in plant cells (Neill *et al*., 2002). In this regard, studies have recently provided genetic evidence that NO induces gene expression (Neill *et al*., 2008). Sheokand *et al*., (2010) mentioned that NO enhances the antioxidant capacity of cells by increasing the activities of antioxidant enzymes such as peroxidase (POX), superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT) and ascorbate peroxidase (APX). Exogenous application of SNP enhanced the enzymatic antioxidant activity and relative water content (RWC) in leaves of wheat seedling exposed to water stress (Tan *et al*., 2008). Also, Fan *et al*., (2012) found that the application of SNP, as a NO donor, increase proline content in cucumber seedling by improving its biosynthesis.

In this study, we exposed Olive plant to water deficit and investigated whether application of different levels of SNP could induce tolerance to water deficit

in Olive, and whether the induced tolerance to water deficit is associated with the enhancement of antioxidant activity and water relations and/or increase osmotic adjustment.

Materials and methods

Plant materials and treatments

One-year-old olive trees (*Olea europaea cv. Conservolia*) were obtained from Fadak Olive Orchard in Ghom city, Iran. The experiment was carried out in a greenhouse at agricultural and natural resources faculty of Moghan, Parsabad, Iran (39.65 °N, 47.92 °E) in 2013. Olive plants were transplanted in 30 L plastic pots containing freely drained light soil with a pH of 7.8, a field capacity of 25%. All trees were well watered for 2 months before the beginning of experiment. After the 2 months acclimation period, sodium nitroprusside (SNP) as a NO donor (N1= 0, N2= 0.1, N3= 0.5 and N4= 1 mM) was sprayed on the trees and the same time, two water regimes (D1= 100% FC and D2= 40% FC) were exposed for a period of one month (from early July till August). During the experiment, the soil water content was monitored by weighing with an electronic balance. Samples were collected for assays at the 30th days after treatment application.

Enzymatic assays

Each sample taken from newly developed shoots. The leaf samples were immediately frozen in liquid nitrogen and then stored at -80 °C until used. The frozen leaves were weighed (1.5 g of fresh mass), and ground in an ice-cold mortar and pestle with 0.1 M potassium phosphate buffer (pH 7.0) containing 5% (w/v) PVP. The homogenates were centrifuged at 4 °C for 10 min at 13000 rpm in a cooling centrifuge. The supernatants were then used to determine protein concentration and enzyme activity. Total soluble protein concentrations of the enzyme extracts were determined using BSA as a standard (Bradford, 1976). Peroxidase (POX) activity was assayed as described by Bacon *et al.* (1997) as follows: the assay mixture of POX contained 1 ml of potassium phosphate buffer (0.1 M), 500 µl of guaiacol (0.01 M) and 200 µl of enzyme extract. The reaction was started by adding 300 µl of H_2O_2 (0.03%). Then, the absorbency of the solutions at 470 nm was measured, for 3 min, using a spectrophotometer. Polyphenol oxidase (PPO) activity was assayed according Taneijia and Sachar (1974). The reaction mixture comprised 200 µl of enzyme extract, 500 µl of catechol (1 M) and 1300 µl of potassium phosphate buffer (0.1 M). The reaction was mixed and the absorbance was measured, for 3 min, at 430 nm.

Stomatal conductance (g_s)

Leaf stomatal conductance (g_s) was measured at midday with a portable porometer (Delta-T Device, Cambridge, UK). The device was calibrated before use on every occasion using the supplied calibration plate. During the water regime period, five fully expanded leaves from the middle part of branches of each tree were selected and marked and then the same selected leaves were used for stomatal conductance measurement (Aganchich *et al.*, 2007).

Relative water content (RWC)

Relative water content (RWC) was determined using two leaves per plant; they were detached in a similar position (third leaf from top). After cutting, the petiole was immediately immersed in distilled water inside a pre-weighed glass tube, which was sealed. The tubes were then taken to the laboratory and weighed; the increased weight of the tubes was used to determine leaf fresh weight (FW). After 48 h in dim light, the leaves were again weighed to obtain turgid weight (TW). Dry weight (DW) was then determined after over-drying at 80°C for 48 h and RWC was calculated as (Aganchich *et al.*, 2007): RWC= 100× (FW-DW)/(TW-DW).

Proline content

The free proline content was determined according to Claussen, (2005). Fresh leaf samples (0.5 g) were ground in 10 ml of sulfosalicilyc acid (3%) using a mortar and pestle. After filtering, 2 ml of the extract was placed in a test tube, and 2 ml each of glacial acetic acid and ninhydrin were added. The reaction mixture was boiled in a water bath at 100°C for 1 h.The reaction was terminated on ice, mixed with 4

ml of toluene. The chromophores containing toluene was separated from the hydrated phase. The absorbance was spectrophotometrically determined at 520 nm. The proline concentration was calculated based on a standard curve and was expressed as µmol proline g^{-1}FW.

Experimental design and statistical analysis

The experiment was conducted in a factorial design, completely randomized with four replications. In this study, water regimes and the different concentrations of SNP were the first and second factors, respectively. The data were analyzed using GLM producer SAS 9.1 version software package and the means were separated by Duncan test.

Results

Peroxidase and Polyphenol oxidase activity

Peroxidase (POX) and polyphenol oxidase (PPO) activity increased significantly during exposure water deficit (D_1) compared to well watered regime (D_1) treatment (Table 1). Also, SNP treatment led to increase these parameters. The minimum POX and PPO activity were obtained by N_4 and N_3 treatments, respectively. However, there was not significant difference between N_4 and N_3 treatments in PPO activity (Fig. 1).

Table 1. The effect of water regimes (D_1= 100% FC, D_2= 40% FC) on POX and PPO activity, Stomatal conductance (g_s), Relative water content (RWC) and proline.

Treatment					
POX	PPO	g_s	RWC	Proline	
(U mg^{-1} protein)	(U mg^{-1} protein)	(mmol $m^{-2}s^{-1}$)	(%)	(µmol g^{-1} fw)	
D_1	0.6444^b	0.0661^b	102.1^a	95.8^a	116.4^b
D_2	1.358^a	0.0909^a	51.7^b	65.2^b	152.5^a

Means followed by non-similar letters are significantly different at P≤ 0.01 according to Duncan test.

Stomatal conductance (g_s) and relative water content (RWC)

Water deficit (D_2) declined stomatal conductance and RWC compared to well watered regime (D_1) after 30 days of treatment (Table 1). Conversely, stomatal conductance was reduced with application of SNP whereas RWC was enhanced with this treatment. The minimum stomatal conductance and the maximum RWC were associated to the forth level of SNP (N_4) (Fig. 2).

Proline content

The rate of proline accumulation was significantly higher in D_2 treatment than D_1 treatment (Table 1). Also, SNP treatment led to increase this parameter. The highest proline content was obtained from N_4 treatment after 30 days of treatments. Indeed, proline content improved with increasing levels of SNP (Fig. 3) .

Discussion

Peroxidase and Polyphenol oxidase activity

Exposure of plants to water stress generally results in increased reactive oxygen species and accumulation of free radicals, which could damage cell membranes and cause the build-up of lipid peroxides (Aganchich *et al.*, 2007). ROS are efficiently eliminated by non-enzymatic and enzymatic antioxidants (Foyer and Noctor, 2000). Our results on the evolution of POX and PPO activities conform to previous findings in olive plants under water stress by Sofo *et. al.*, (2004). Plants subjected to water stress undergo increased exposure to ROS which, in turn, damage cellular structures and macromolecules. Plant cells are normally protected against such effects by a complex antioxidant system (Guerfel *et al.*, 2009). Therefore, the increase in ROS concentration likely causes the up-regulation of some antioxidant enzymes during water stress (Sofo *et al.*, 2005). Thus our results indicate that water deficit (D_2) treatment had possibly induced oxidative stress which resulted in up-regulated activities of POX and PPO under water stress. This can be an important protection mechanism of the olive plant against an excessive

increase of ROS under water stress (Aganchich *et al.*, 2007). POX is known to play a key role in the scavenging of ROS by regulating the levels of the peroxide hydrogen (H_2O_2) produced in plant cells (Liu *et al.*, 2011). Also, the observed increase in POX activity could possibly reflect the changed mechanical proprieties of the cell wall, which in turn, could be correlated with drought adaptation (Aganchich *et al.*, 2007). This author suggested that a high level of POX activity may cause growth limitation in olive plants under drought, which could be triggered by increased levels of lignifications and oxidation, and a consequent inactivation of auxin. Our results showed that water deficit (D_2) increase PPO activity. In this regard, Sofo *et al.*, (2004) reported an increase in PPO activity under water stress. PPO activity was previously reported to regulate the redox state of phenolic compounds, which are very abundant in olive leaves (Ryan *et al.*, 2002). It has been reported that phenolic compounds act as an antioxidant under water stress (Weidner *et al.*, 2009).

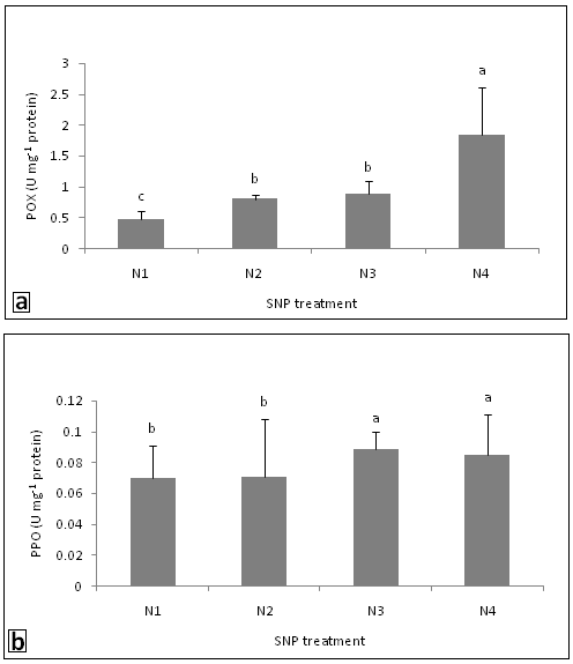

Fig. 1. Effects of SNP levels (N_1= 0, N_2= 0.1, N_3= 0.5, N_4= 1 mM) on POX (a) and PPO (b). Data are means of four replications. Mean values with different letters are significantly different by the Duncan test (P≤0.01). Vertical bars indicate standard deviation.

Also, in this research SNP treatment increased POX and PPO activity. In this regard, Hasanuzzaman *et al.*, (2012) found that SNP treatment improves the activity of antioxidant enzymes under abiotic stress. Indeed, nitric oxide acts as a signal molecule which induces gene expression attributed to antioxidant enzymes (Neill *et al.*, 2008). Kopyra and Gwozdz (2003) reported that SNP decreases the rate of ROS production by inducing ROS-scavengig enzymes activities of catalase, peroxidase, ascorbate peroxidase and polyphenol oxidase.

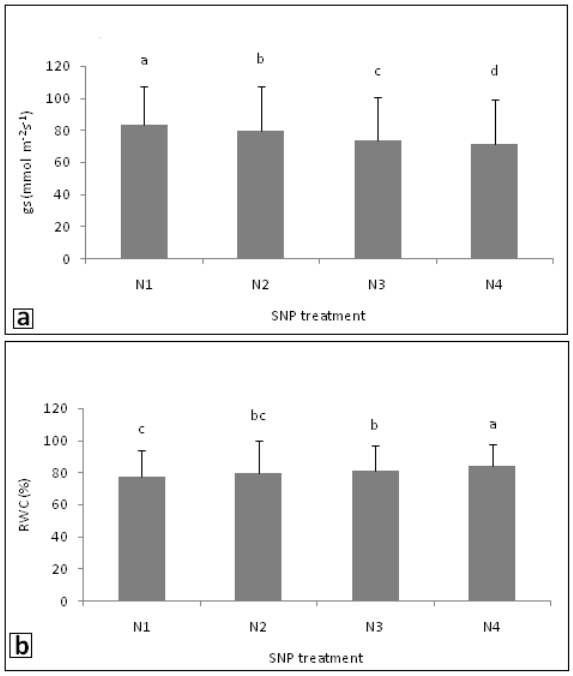

Fig. 2. Effects of SNP levels (N_1= 0, N_2= 0.1, N_3= 0.5, N_4= 1 mM) on g_s (a) and RWC (b). Data are means of four replications. Mean values with different letters are significantly different by the Duncan test (P≤0.01). Vertical bars indicate standard deviation.

Stomatal conductance (g_s) and relative water content (RWC)

Stomatal conductance (g_s) and relative water content (RWC) parameters significantly reduced under water deficit (D_2). Change in stomatal situation is the first symptom of water stress (Miyashita *et al.*, 2005). This process is a mechanism to avoid water deficit by preventing water loss (Rouhi *et al.*, 2007). Ordog *et al.*, (2013) mentioned that ABA has an important role in regulation of stomatal behavior under water deficit conditions. The stomatal closure was induced by accumulating ABA in guard cells of leaves potato under water stress (Tekalign and Hammes, 2005). In our study, the induction of stomatal closure was

observed with SNP application. No-induced stomatal closure has been reported by Ordog *et al.*, (2013). Hence, NO-induced Stomatal closure is probably associated with ABA biosynthesis under water stress.

RWC is an important parameter for evaluating plant water status (Aganchich *et al.*, 2007). In this research, RWC reduced with water deficit (D_2) treatment. The lower RWC observed under water stress could be explained by higher leaf dehydration at midday (Aganchich *et al.*, 2007).The similar results were obtained in Olive under water deficit by Ahmed *et al.*, (2007). However, the reduction of RWC improved with SNP treatment. Leaf Water maintenance is one of the main strategy for water deficit tolerance. NO induces ABA biosynthesis under water stress (Siddiqui *et al.*, 2010). In general, NO-induced ABA biosynthesis plays a key role in plant water maintenance under water deficit by inducing stomatal closure (Xing *et al.*, 2004). However, this researcher mentioned that the other mechanism can also control leaf water status. The regulation of genes correlated to osmoprotective proteins by NO maintained relative water content in wheat under water stress (Boyarshinov and Asafova, 2011).

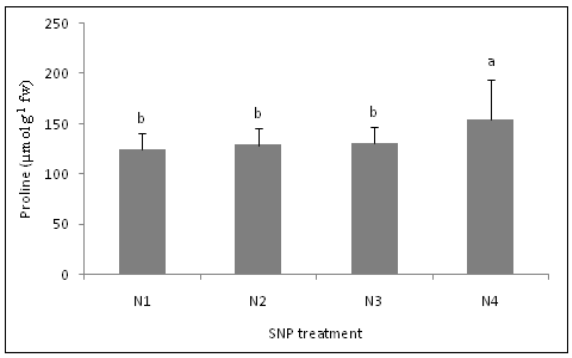

Fig. 3. Effects of SNP levels (N_1= 0, N_2= 0.1, N_3= 0.5, N_4= 1 mM) on proline. Data are means of four replications. Mean values with different letters are significantly different by the Duncan test (P≤0.01). Vertical bars indicate standard deviation.

Proline content

Osmotic adjustment is the most important mechanism that improves water uptake from soil and maintains cell turgor under water deficit (Cordeiro *et al.*, 2009). This process is induced by compatible solutes (such as Proline, soluble sugars, glycinebetaine and organic acids) in plants (Liu *et al.*, 2011). In the present study, Proline content increased with water deficit treatment (D_2). In many plants, Proline as a multifunctional amino acid accumulates in response to abiotic stress (Szabados and Savoure, 2009). It has been reported that proline as an osmolyte has suitable compatibility with enzymes and cell macromolecules (Zhang *et al.*, 2012). It protects protein structures and cell membranes under water stress (Sofo *et al.*, 2004). In order to osmotic adjustment, proline can also act as a hydroxyl radical scavenger (Claussen, 2005). Hence, the increase of proline content improves the plant tolerance in water shortage conditions (Claussen, 2005). In this regard, our results showed that proline content is increased with SNP treatment. Accumulation of proline could be associated with the improvement of its synthesis (Hong *et al.*, 2000). P5CS (delta-pyrroline-5-carboxylate synthetase) is the key enzyme of proline biosynthesis that this enzyme activity is increased with SNP treatment (Lei *et al.*, 2007). Fan *et al.*, (2012) found that the application of SNP increases proline content in cucumber seedling by improving P5CS activity. In our research, the increase of proline content is presumably associated with SNP induced P5CS activity.

Abbreviations

g_s, stomatal conductance; RWC, relative water content; POX, Peroxidase; PPO, Polyphenol oxidase; ROS; Reactive oxygen species; SNP, Sodium nitroprusside.

Conclusions

This study indicated that water deficit reduces olive plant efficiency by the influence on physiological and biochemical processes. However, the exogenous application of SNP induced water stress tolerance in olive (*Olea europaea* cv. *Conservolia*). Also, our results showed that the used concentration of SNP is the most important factor. In this research, N_3 treatment had positive effect on polyphenol oxidase (PPO) activity while stomatal conductance (g_s), relative water content (RWC), proline and peroxidase (POX) activity were improved by N_4 treatment. Therefore, the suitable concentration of SNP could be

used for reducing the negative effects of water deficit in Olive.

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