The effects of salt and drought stress on phenolic accumulation in greenhouse-grown *Hypericum pruinatum*

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Abstract

Hypericum pruinatum is a medicinal herb containing several bioactive compounds with important pharmacological activity. In this study, we investigated the effects of the salt (0.03 - control, 1, 2.5, 4 and 8 dS m^{-1} of $MgSO_4$, $CaCl_2$ and NaCl salts) and drought stress (80, 100 and 120% of required water) on the content of phenolic compounds, namely chlorogenic acid, rutin, hyperoside, isoquercetine, quercitrine and quercetine in greenhouse grown plantlets. In general, the salt stress especially in elevating doses increased the levels of all of the compounds analysed, whereas drought stress did not cause a significant chance in chemical content of the plantlets. The present results indicated that abiotic stress factors, particularly salinity, have a marked influence on the content of phenolic constituents in *H. pruinatum* and it is a salt tolerant species. The results also indicated that phenolic compounds play a significant physiological role in salinity tolerance.

Introduction

Among the different environmental constraints, salt and drought stress are considered as important abiotic factors limiting plant growth and productivity especially in arid and semi-arid regions (Zhu, 2001). The detrimental effects of salinity on plant physiology are though to be associated with specific ion effect (salt stress), low water potential in soil solution (drought stress), nutritional imbalance, redirection of energy from growth to extracting pure water from the saline water and to produce defensive chemicals or a combination of these different factors (Munns and Tester, 2008). Results from recent studies in the past few years, however, have pointed out that sensitivity to salt stress is associated mainly with oxidative stress, which is also caused by drought stress and due to the disturbance in balance between the rates of production and elimination of reactive oxygen species (ROS) (Turkan and Demiral, 2009; Tounekti *et al.,* 2011).

Plants have evolved different defence systems to avoid the oxidative damage caused by salt and drought stress including overproduction of antioxidant metabolites which stopping the propagation of oxidative chain reactions. In this case, polyphenolic compounds such as phenolic acids, flavonoids, proanthocyanidins and anthocyanins play an important role in reducing the detrimental effects of salinity (Hichem *et al.,* 2009). The proven antioxidant activity of phenolics allows them to act as ROS scavenging agents. As a result, their synthesis is generally triggered in response to biotic/abiotic stresses and especially under salt stress conditions (Souza and Devaraj, 2010). Except for their role in reducing the detrimental effects of salinity and drought, phenolic compounds exhibit wide range of physiological properties such as antiallergic, antiatherogenic, anti-inflammatory, antimicrobial, antithrombotic ones (Balasundram *et al.,* 2006). They have also defensive roles in protecting plants from biotic attacks by pathogens and herbivores (Conceicao *et al.,* 2006).

The genus *Hypericum* L. comprises more than 450 species divided in 36 sections with worldwide distribution in warm temperate, subtropical and mountainous tropical regions (Robson, 2001). Herbs belonging to this genus are very important in pharmacology, particularly *Hypericum perforatum* L. which has been studied deeply for the biologically activity of its extracts and isolated active components (Shelton, 2009). The pharmacological activities of *Hypericum perforatum* extracts namely, antidepressive and antiviral activities are mainly attributed to their flavonoid, hypericin and hyperforin contents (Avato, 2005). Turkey is an important center for *Hypericum* genus where it is represented by 89 species of which 43 are endemic (Bingol *et al.,* 2011). *Hypericum pruinatum* Boiss. and Bal., a perennial herbaceous plant which grows naturally in igneous slopes at high altitudes is one of the species of Turkish *Hypericum*. In previous studies, *H. pruinatum* were reported to have great pharmaceutical potential, with its well-documented contents of hypericin (Cirak *et al.,* 2006), hyperforin (Smelcerovic *et al.,* 2008), organic acids and flavonoids (Cirak *et al.,* 2007). Because of the similarities in the

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chemical compositions of *H. perforatum* and *H. pruinatum*, the latter species may eventually become a domesticated plant and serve as an alternative to *H. perforatum* for medicinal use.

Agronomic studies on all *Hypericum* species are rather low and the genus is quite understudied. Efforts undertaken to describe the effect of abiotic stress on chemical constituents of *Hypericum* plants are also very scarce and limited to those on *Hypericum perforatum* (Gray *et al.,* 2003; Zobayed *et al.,* 2007) and *Hypericum brasiliense* (Abreu and Mazzafera, 2005). In the previous studies, it was found that the drought stress greatly influenced the phenolic contents of both species of *Hypericum*. To the best of our knowledge, no study has been conducted concerning the changes of phenolic contents in *H. pruinatum* in response to abiotic stress. We investigate here for the first time the phenolic content of this species under salt and drought stress.

Materials and methods

Plant material and culture conditions

H. pruinatum plantlets were established from 5 months old seeds collected on approximately 30 plants representing the wild population from the Gümüş district of Amasya Province, Turkey (41° 04′ N; 36° 01′ E; 890 m a.s.l.). Plant samples were identified by Dr. Hasan Korkmaz, Department of Biology, University of Ondokuz Mayis, Samsun, Turkey. Voucher specimen was deposited in the herbarium of Ondokuz Mayis University Agricultural Faculty (OMUZF # 107). Seeds were germinated in a float system, commonly used for seedling production of broad-leaves tobacco Burley and Flue-Cured-Virginia under a 16 h light:8 h dark cycle. Newly emerged seedlings were transferred to pots, 30 cm in diameter (7L), filled with the mixture of commercial torf Tray Substrate and soil (35% sand, 45% silt and 20% clay) in 1:3 rate (w/w). The pots were watered daily until they reached full flowering at which chemical accumulation was reported to be highest for *H. pruinatum* (Cirak *et al.,* 2014b, 2015). Main chemical and physical properties of the peat are shown in Table 1. After maturation, the three-month-old plantlets were moved to greenhouse conditions (16/8 h light/darkness, 25°C temperature, 75% relative humidity, and 400 µmol m^{-2} s^{-1} PAR).

Abiotic stress experiments

City water supply (ECi: 0.03 dS m^{-1}) was used as irrigation during experiment. The salts $MgSO_4$, $CaCl_2$ and NaCl at doses of 0.03 (control), 1, 2.5, 4 and 8 dS m^{-1} were used for salt stress experiment. Sodium adsorption ratio (SAR) was kept about 1 in all salinity levels so that only effect of salinity could be evaluated without any negative effect of sodium accordingly the below equation. In order to determine the amount of chemical materials added to water a computer programme based on Visual Basic was used (Yurtseven and Baran, 2000):

$$SAR = \frac{Na^{+}}{\sqrt{\frac{Ca^{++} + Mg^{++}}{2}}}$$

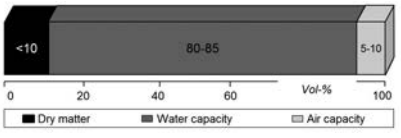
The doses were selected from the previous publications (Yurtseven and Baran, 2000; Ozturk *et al.,* 2004) and applied in three levels of water availability. Firstly control pots were watered with city water supply fully and left to leak. Then the amount of irrigation water not leaked but hold by pots was determined after leaks as the required water (RW). Thus, a total of 3 water amounts 80, 100 and 120% of RW, respectively were used when half of the pot water holding capacity was over for drought stress experiment. The salt doses with different levels of water availability were applied 13 times in accordance with the allowed depletion. The experimental design was a factorial experiment in completely randomised plots with 3 replications, each consisting of 5 pots (Cirak *et al.,* 2005, 2014a). Plantlets were harvested at the end of 39th day of experiment when severe wilting was observed in pots treated with higher salt doses. After dried at room temperature, the harvested aerial parts were assayed for phenolic contents by HPLC.

Preparation of plant extracts and high-performance liquid chromatography analysis

Air-dried plant material was mechanically ground with a laboratory mill to obtain a homogenous drug powder. Samples of about 0.5 g (weighed with 0.0001 g precision) were extracted in 50 mL of 100% methanol by ultrasonication at 40ºC for 30 min in a Sonorex Super model RK 225H ultrasonic bath. The prepared extracts were filtered through a membrane filter with pore size of 0.22 µm (Carl Roth GmbH, Karlsruhe, Germany) and kept in a refrigerator (+4°C) until analysis no longer than 3 hours.

A Shimadzu Prominence LC-20A (Shimadzu Europa GmbH, Duisburg, Germany) chromatographic system equipped with two LC-20AD model pumps, a SIL-20AC auto-injector, a thermostat CTO-20AC and a SPD-M20A detector was used for HPLC analysis. Separation of all compounds was carried out using an YMC Pack Pro-C18 (YMC Europe GmbH, Dinslaken, Germany) column (150 mm × 4 mm i.d.; 3 µm particle sizes) with 10 mm guard-precolumn. The mobile phase consists of solvent A [water containing 0.1% trifluoroacetic acid (TFA)] and solvent B (acetonitrile containing 0.1% TFA). The following binary gradient elution program was used: 0-1 min (B 5→5%), 1-14 min (B 5→20%), 14-20 min (B 20→80%), 20-30 min (B 80→100%), 30-39 min (B 100→100%), 39-39.5 min (B 100→5%), 39.5 - 45 min (B 5–5%). The mobile phase was delivered with a flow rate of 1.0 mL min^{-1}; volume of extract injected was 10 µL (Cirak *et al.,* 2016).

Table 1. Main chemical and physical properties and average amount of added nutrients for the commercial peat tray substrate.

Chemical data	Average amount of added nutrients	Physical properties
PH range (H_2O): 5.5-6.0	Nitrogen (mg N/l): 210	
Fertiliser (g/L): 1.5	Phosphorus (mg P_2O_5/l): 240	
Black sphagnum peat: 30%	Potassium (mg K_2O/l): 270	
White sphagnum peat: 70%	Magnesium (mg Mg/l): 100	

Detection was performed at 210-790 nm wavelength range with a constant column temperature at 40°C. The eluted compounds were identified on the basis of their retention time by comparison with retention time of reference standards and also confirmed with UV spectra's of reference standards in the wavelength range from 210 to 790 nm.

The quantification of detected compounds was achieved by using external standard method at the maximal absorption on the UV spectra of corresponding compounds: chlorogenic acid - 325 nm, rutin - 353 nm, hyperoside - 353 nm, isoquercetine - 353 nm, quercitrine - 347 nm and quercetine - 368 nm wavelengths. A six-point calibration curves were obtained with pure standards dissolved in MeOH in the concentration range of 0.2-110 µg mL^{-1}. All calibration cures showed good linear regression (r^2>0.999) within the test range. All solvents and standards of reference substances were of HPLC grade and purchased from Roth Chemical Company (Karlsruhe, Germany). Results were expressed as mg/g DW content of each tested compound which was calculated by multiplication of concentration and the plant mass (Paulsen and Selmar, 2016).

Data analysis

The data for chlorogenic acid, rutin, hyperoside, isoquercetine, quercitrine and quercetine contents of plant materials were objected to ANOVA. Differences among treatments were tested Duncan Multiple Range Test (P<0.01).

Results and discussion

Salt treatments resulted in wilting during time course of experiment and plant mortality was observed in several pots treated with 8 dS $m^{-1}$of salt as a result of severe wilting (Figure 1). The treatments, especially in increasing doses, also resulted in significantly higher phenolic production (P<0.01) and their positive effect on phenolic accumulation was more evident in decreasing water availability. Salt treated plantlets produced significantly higher content of chlorogenic acid, rutin, hyperoside, isoquercetine, quercitrine and quercetine in the presence of 80% of RW when compared to the plantlets treated with the same doses of salt but watered with 100 and 120% of RW. Quercetine content was the highest in plantlets treated with 8 dS m^{-1} of salt and watered with 80% of RW (2.08 mg/g DW) and 100% of RW (1.94 mg/g DW) while moderate concentrations of salt treatments produced the highest contents of the other tested phenolic compounds. At the level of 80% RW, it is clear that both factors tested affected the chemical content of plants. It can be concluded that this water application further increased the salinity effect. In case of 100% RW application, drought stress did not occur but only salt stress was observed. The effect of salinity at 120% RW was minimal because of leaching.

The treatment of 4 dS m^{-1} of salt plus 80% of RW culminated in the highest content of chlorogenic acid (8.36 mg/g DW), rutin (2.99 mg/g DW) and quercitrine (1.34 mg/g DW) accumulations, followed by 2.5 dS m^{-1}plus 80% of RW treatment (8.31, 2.79 and 1.21 mg/g DW chlorogenic acid, rutin and quercitrine, respectively). Similarly, plantlets treated with 2.5 and 4 dS m^{-1} of salt and watered with 80% of RW accumulated the highest content of hyperoside (10.11 and 10.05 mg/g DW, respectively) and isoquercetine (13.12 and 12.62 mg/g DW, respectively). On the contrary, drought stress by oneself caused no morphological change in appearance and significant change in the chemical contents. Control plantlets not salt treated but only watered with 80, 100 and 120% of RW produced similar content of chlorogenic acid, rutin, hyperoside, isoquercetine, quercitrine and quercetine (Figure 2).

Drought stress is one of the most important environmental stresses regulating plant growth and development and altering the main physiological and biochemical functions of plants. Drought stress is known to trigger the plant defense systems resulting in the enhanced production of secondary metabolites. In previous studies, the content of phenolic compounds namely, rutin, quercetin and total soluble phenols increased significantly in response to water and temperature stress in *H. brasiliense* (Abreu and Mazzafera, 2005) *H. perforatum* plants subjected to a brief drought stress showed an increase of quercetin and rutin (Gray *et al.,*

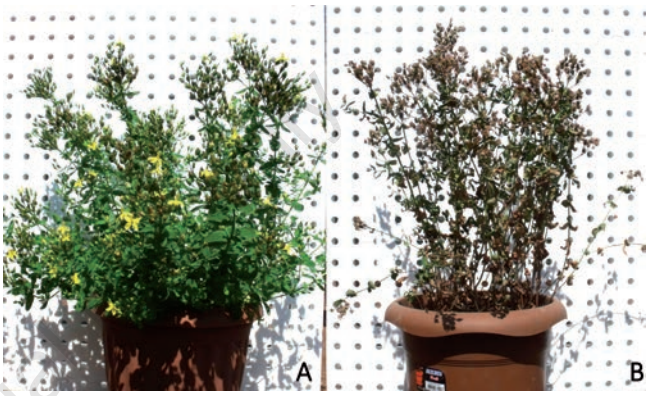


Figure 1. A view of control (watered with 100% of required water, A) and 8 dS/m salt treated (also watered with 80% of required water, B) *Hypericum pruinatum* plantlets at the end of the experiment.

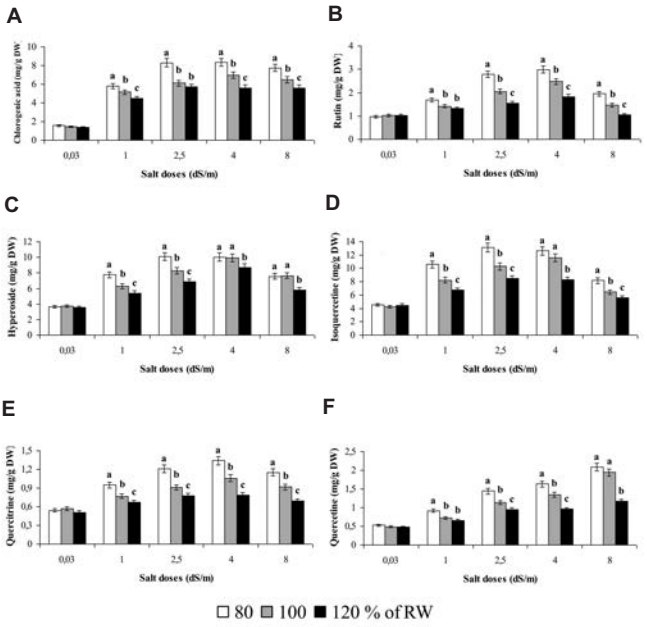


Figure 2. Chlorogenic acid (A), rutin (B), hyperoside (C), isoquercetine (D), quercitrine (E) and quercetine (F) contents of *Hypericum pruinatum* plantlets treated with different salt doses and water amounts (values with different small letters – a, b, c – within columns for each dose of salt differ significantly at the level of P<0.01).

2003). Similarly, hyperforin concentration of the same species increased significantly and was nearly double after 12 days of the drought stress treatment (Zobayed *et al.,* 2007). On the contrary, drought stress by oneself did not change the chemical content of *H. pruinatum* in the present study. This phenomenon was probably related to the stress intensity because we used only 80, 100 and 120% of RW for drought stress experiment which were not sufficient to create the oxidative stress resulting in enhanced secondary metabolite production. During the onset and development of salt stress, the all major physiological processes are affected within a plant. Under saline conditions, plants have produced excess amount of ROS as a result of oxidative stress and if they are poorly protected, these reactive molecules may damage macromolecules such as DNA, proteins and membrane lipids resulting in cell death. The degree of oxidative cellular damage is controlled by the capacity for protection against oxidative agents (Chinnusamy *et al.,* 2005; Plaza *et al.,* 2009). The ROS scavenging ability of plants mainly depends on the antioxidant defense system including nonenzymatic components (Cuin and Shabala, 2008). Nonenzymatic components of the antioxidant defense system consist of various secondary metabolites, such as hydrophilic phenolics and flavonols, organic acids, lipophilic carotenoids, and water-soluble ascorbate and the enhancement of the phenolics metabolism is considered one of the responses to abiotic stresses (Close and McArthur, 2002). Phenolics are well-known antioxidant compounds having powerful radical scavenging ability and the distinct increase in phenolic content of plant tissues under salinity is thought to be involved in the prevention of stress-induced oxidative damage (Bourgou *et al.,* 2010). It has been shown that salt stress induced disturbances in the secondary metabolic pathways, leading to an increase in phenolic compounds (Ksouri *et al.,* 2007). In the present study, accordingly, chlorogenic acid, rutin, hyperoside, isoquercetine, quercitrine and quercetine contents of *H. pruinatum* plantlets increased significantly with elevating doses of salt treatment. The observed increase in phenolic content in the present study was possibly due to the antioxidative mechanisms in response to oxidative stress induced by salt stress conditions because the phenolic enhancing effect of salinity was more distinct in limited water availability. However it should be noted that The ROS produced in the course of drought and salt stress, are detoxified by several antioxidants from the photosynthetic electron transport chain within the chloroplasts (Tounekti *et al.,* 2011), but the compounds tested in the present study are located in the vacuole. Nevertheless, they indeed contribute to avoid ROS, since their biosynthesis significantly reduce the stress related over-reduced status which leads to the generation of ROS as reported by Kleinwächter and Selmar (2015) and Selmar and Kleinwächter (2013). Increase in phenolic content in different tissues under increasing salinity has also been reported in a number of plant species. Ahmed *et al.* (2009) reported that major phenolic compounds namely, tyrosol, hydroxytyrosol, the vanillic, caffeic, syringic, p-coumaric, ferulic acids and total phenol concentrations in virgin olive oil increased under saline water irrigation. Plaza *et al.* (2009) reported two fold higher accumulation of total flavonoid and phenols in *Cordyline fruticosa* leaves in response to increasing salinity gradient in irrigation water and concluded that high flavonoids, phenols and sugars contents in this species are more useful indicators for salt resistance. Similarly, the total phenolic content in leaves was significantly increased by moderate salt treatment with a maximum at 50 µM NaCl in *Cynara cardunculus* (Hanen *et al.,* 2008). Ksouri *et al.* (2007) reported that salt-challenged halophyte *Cakile maritima* produced significanlty higher amount of polyphenol in its leaves. Souza and Devaraj (2010) reported that the total phenol content of the salt-stressed leaves exhibited a concentration-dependent increase from 100-400 µM NaCl during the first 24 h of exposure in hyacinth bean. In the medicinal herb *Echinacea angustifolia*, the salinity of the nutrient solution significantly enhanced the root contents of chlorogenic acid, cynarin and cichoric acid in hydroponically grown planlets (Montanari *et al.,* 2008). However, our data are not in accordance with those of several previous publications. After treated with different levels of saline irrigation water (0.39, 1.56, 3.13, 4.69, 6.25, 7.81 and 9.38 dS m^{-1}) consisting of NaCl, $CaCl_2$ and $MgCl_2$ salts, total flavonoids and carotenoids content were significantly reduced in *Calendula officinalis* (Khalid and da Silva, 2010). Total phenolic contents of 5- and 7-day old plantlets treated with 10 and 50 µM of NaCl were significantly decreased in radish sprouts (Yuan *et al.,* 2010). Similarly, total phenolic content decreased by 30, 54, and 61% in response to 20, 40, and 60 µM NaCl treatments in *Nigella sativa* (Bourgou *et al.,* 2010). The differential accumulation of phenolic fractions under different salt treatments in the plant species with different salinity resistance suggests that salt resistant species and varieties may employ phenolic compounds, especially phenolic acids and flavonoids differentially to adapt to contrasting salinities (Mahmoudi *et al.,* 2010). This phenomenon was confirmed by Salah *et al.,* (2011) who reported that The salt-tolerant *Medicago ciliaris* line TNC 1.8 was more efficient at managing salt-induced oxidative damage in leaves and in roots than the salt-sensitive line TNC 11.9, by preserving higher phenolic compound levels in both organs. In sugarcane and maize, similarly, salt tolerant varieties were found to accumulate significantly higher amount of soluble phenolics, anthocyanins, flavones and several polyphenols under salt stress conditions while content of the same metabolites decreased in response to salt treatment in case of salt sensitive varieties of both species (Wahid and Ghazanfar, 2006). Based on the results those authors pointed out the enhanced phenolic production in plant tissues under salt stress conditions as good indicator for salt resistance. The decrease in phenolic content of the aforasaid plant species in response to salt treatments could probably be due to their sensitivity to saline.

Conclusions

In conclusion, our results show that abiotic stress factors, particularly salinity, have a marked influence on the content of phenolic constituents in *H. pruinatum*. The increase in the contents of the compounds analysed indicates this medicinal herb as a salt tolerant species and agrees with data in the literature reporting that this is probably a response to the generation of ROS. These results also support the idea that phenolic compounds play a significant physiological role in salinity tolerance. Further studies are needed for broad spectrum understanding the roles of those metabolites in various plant species under saline conditions.

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