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EFFECTS OF FOLIAR OF THE APPLICATION CHITOSAN AND REDUCED IRRIGATION ON ESSENTIAL OIL YIELD, TOTAL PHENOL CONTENT AND ANTIOXIDANT ACTIVITY OF EXTRACTS FROM GREEN AND PURPLE BASIL

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ABSTRACT

Phenolic compounds are naturally occurring substances in plants. Basil (*Ocimum basilicum* L.) belongs to the family Lamiaceae, is a good source of phenolic compounds and natural antioxidants. Elicitors can activate specific genes involved in secondary metabolite biosynthesis. Chitosan, as elicitor, is a natural biopolymer modified from chitin, which is the main structural component of squid pens, cell walls of some fungi and shrimp and crab shells. On the other hand, water deficit stress is one of the most abiotic stress, which effects on the levels of secondary metabolites. To evaluate the effect of chitosan and different irrigation regimes on essential oil yield, total phenol content and antioxidant activity of extracts from green and purple basil (*Ocimum basilicum* L.), an experiment was conducted at Shahrekord, southwestern Iran. Treatments comprised control, 0.0, 0.2, and 0.4 $g \cdot L^{-1}$ chitosan applied to plants under normal irrigation, slight and mild drought stress conditions. Results indicated that the different levels of chitosan and irrigation had significant effects on the essential oil yield, total phenol content, and the antioxidant activity of the extracts. Foliar-applied chitosan in particular 0.4 $g \cdot L^{-1}$ increased total phenolic content in the basil as compared to untreated plants. In conclusion, it is suggested that the foliar application of chitosan as an elicitor could be a promising material used to increase biological activity and pro-health functional value of basil plants.

Key words: chitosan, *Ocimum basilicum*, water deficit stress, total phenolic compounds

INTRODUCTION

Sweet basil (*Ocimum basilicum* L.) is one of the most frequently used pharmacological materials. Basil is cultivated worldwide: in particular because it is a rich source of natural compounds, such as monoterpenes, sesquiterpenes, phenylpropanoids, anthocyanins, and phenolic acids [Hussain et al. 2008]. Phenolics in vegetables and herbs are the major bioactive compounds known for health benefits especially due to their antioxidant properties. The antioxidant activity of phenolic compounds is mainly

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caused by their redox properties, which permit them to act as reducing agents, hydrogen donors and singlet oxygen quenchers [Hakkim et al. 2007].

Several thousand such compounds have been identified in various plant species [Steyn et al. 2002, Winkel-Shirley 2002]. Many of which are thought to play roles, such as antibacterial, antiviral, anticancer agents and scavengers of most types of oxidizing molecules [Solecka and Kacperska 2003]. Phenolic compounds are ubiquitous in all plant organs and are an integral part of the human diet. Interest in food phenolics has recently increased greatly, because of the antioxidant and free radical scavenging abilities, associated with some phenolic compounds and their potential effects on human health [Bravo 1998].

The main phenolics reported in basil are phenolic acid (e.g. rosmarinic, lithospermic, vanillic, p-coumaric, hydroxybenzoic, syringic, ferulic, protocatechuic, caffeic and gentisic) and flavonol–glycosides [Jungmin and Carolyn 2009]. Therefore, basil extracts are used in the cosmetic and pharmaceutical products. On the other hand, the extract and essential oil from the aerial parts of basil have been used extensively in the food industry as a flavoring agent [Simon et al. 1999, Ghasemi Pirbalouti et al. 2013a,b, Ghasemi Pirbalouti 2014]. Overall, basil has also shown antialergic, anticancer, antimicrobial, antiseptic, antispasmodic, antifungal, antiviral, anti-inflammatory, analgesic, immune–stimulating, and sedative and antioxidant activities due to its polyphenols and aromatic compounds [Taie et al. 2010]. Recently scientists have carried out extensive research to increasing polyphenols concentration in plants to further enhance their overall nutritional and pharmaceutical value [Taie et al. 2010].

Elicitation is a technique that stimulates plants to accumulate secondary metabolites. It has been reported that various elicitors interact with plant membrane receptors and generate signal compounds that induce expression of genes encoding enzymes of secondary metabolites biosynthesis and can also have an indirect effect on phenolics accumulation in plants [Fang et al. 1999, Saniewski et al. 2006]. Chitin and chitosan are naturally-occurring compounds that have potential in the agriculture, medicine, pharmaceutical and food industries [Polish Chitim Society 2006, El Hadrami et al. 2010]. Fragments from chitin and chitosan are known to have eliciting activities leading to a variety of defense responses in host plants in response to microbial infections, including the accumulation of phytoalexins, pathogen-related (PR) proteins and proteinase inhibitors, lignin synthesis, and callose formation [El Hadrami et al. 2010]. Based on these and other proprieties that help strengthen host plant defenses, interest has been growing in using them in agricultural systems to reduce the negative impact of diseases on yield and quality of crops [El Hadrami et al. 2010]. Chitosan is considered to be potent elicitor of secondary metabolite accumulation in plants. Both chitin and chitosan have demonstrated antiviral, antibacterial, and antifungal properties, and have been explored for many agricultural uses [El Hadrami et al. 2010]. Chitosan is an exogenous biotic elicitor that is derived from the fungal cell wall [Montesano et al. 2003], it has been studied for their effects on phenylpropanoid metabolic enzymes [Chakraborty et al. 2009], and secondary metabolite production [Wiktorowska et al. 2010].

On the other hand, under abiotic stresses such as drought stress plants it can induce a defense response and increase secondary metabolite levels [Ali et al. 2006]. Moisture deficit has a significant influence on growth and metabolic activities of plant species. Secondary metabolites of plants can be altered by environmental factors specially water deficit and improper temperature on many aspects of plant metabolism. A lot of investigators [Ghasemi Pirbalouti et al. 2014] showed the effect of water stress on secondary metabolites such as essential oils in different plant species.

To the best of our knowledge there has been no previous report regarding the combined effects of the foliar application of chitosan and environment stress on essential oil yield, total phenol content and antioxidant activity of extracts from green and purple basil (*Ocimum basilicum* L.). Therefore, the present work was aimed towards studying the effect of chitosan and deficit irrigation on essential oil yield, phenolic compounds and antioxidant activity levels in green and purple basil.

MATERIALS AND METHODS

Experimental design and treatments. Pot experiments were conducted at the Research Field, Islamic Azad University of Shahrekord (latitude 32°20'N; longitude 50°51'E; altitude 2070 m a.s.l.), southwestern Iran, May to August 2014. Seeds of two Iranian landraces of basil were sown in plastic pots with a diameter of 20 cm and a depth of 25 cm. The climate of the area of study is classified as cold, semiarid and semi humid with temperate summer by Emberger's climatology method and very cold winter by Karimi's climatology method [IRIMO 2012]. The pots were filled with clay loam with a pH of 7.23, containing 0.8% organic matter comprised of 0.01% total N, 11.20 $mg \cdot kg^{-1}$ available phosphorus, 694 $mg \cdot kg^{-1}$ available potassium, and a saline value measured at E.C.: 1.35 $dS \cdot m^{-1}$. The soil moisture for all pots was kept at 100% field capacity until 35 days after sowing.

Experimental treatments were arranged as 3 × 4 × 2 factorial. Each treatment included three replicates, producing a total of 72 experimental units (pots). Factor A included three irrigation regimes, viz., I_1 (unstressed or control), I_2 (irrigation in 70% field capacity when 30% of maximum total available soil water was depleted in the upper 30 cm of the soil profile), and I_3 (irrigation in 40% field capacity when 60% of maximum total available soil water was depleted in the upper 30 cm of the soil profile). Factor B comprised three chitosan treatments (control, 0.0, 0.2 and 0.4 $g \cdot L^{-1}$) sprayed thrice at 10–12 leaves, before flowering, and two weeks later. Chitosan was dissolved in acetic acid 5%, diluted in distilled water with various concentrations. These solutions were sprayed at dew point (approximately 100 ml per plant) with a hand sprayer (untreated control plants were sprayed with an equivalent volume of distilled water). Factor C comprised two Iranian landraces, including green and purple basil. Field water capacity or *field capacity* (FC) is defined "the amount of water held in soil after the excess gravitational water has drained away and after the rate of downward movement of water has materially decreased". FC is the upper limit of the *available soil water* (AW) reservoir, from which water can be released but not necessarily absorbed by plants, until the *permanent wilting point* (PWP) is reached [Vanderlinden and Giraldez 2014].

Essential oil isolation. The inflorescences of the plants were harvested from each pot at the early flowering stage. Dried plant material (50 g) was powdered and subjected to hydrodistillation for three hours using a Clevenger–type apparatus [British Pharmacopoeia 1988]. The essential oils were dried with anhydrous sodium sulphate and kept in amber vials at 4°C.

Extract preparation. The aerial parts of the plants were shade dried. The aerial parts powder of the plant (50 g) was extracted with ethanol 70% by maceration method and filtered and then were dried at 35°C under rotary vacuum (Model Zirbus 302 w, Italy). The extract samples were stored in universal bottles and refrigerated at 4°C prior to use.

Determination of total phenolic content (TPC). The total amount of phenolic compounds in each extract was determined using the Folin–Ciocalteu method following procedure of Singleton and Rossi (1965) with some modifications. Briefly, 0.5 ml of the sample were mixed with 2.5 ml of Folin––Ciocalteu's phenol reagent and kept for 5 min at 37°C. Then 2 ml of saturated Na_2CO_3 (7.5%) was added, and the mixture was brought to 10 ml with the addition of deionized–distilled water. The mixture was maintained at room temperature in the dark for 120 min and then the absorbance of the mixture was measured at 765 nm against a reagent blank using a UV–Vis spectrophotometer (Perkin–Elmer Lambda, US). Gallic acid equivalent (GAE) was used as the reference standard and results were expressed as milligrams (mg) of Gallic acid equivalents per gram of each extract on dry basis (mg $GAE \cdot g^{-1}$).

Antioxidant test. The antioxidant capacity of the extracts was evaluated by the method of Hung et al. [2005]. The extracts at different concentrations (16 to 500 $\mu g \cdot ml^{-1}$) were mixed with the same volume of 0.2 mM methanol solution of DPPH. The disappearance of DPPH by extracts after 30 min of incubation at room temperature was determined spectrophotometrically at 515 nm. Methanol was used to zero spectrophotometer. The absorbance of the DPPH radical without antioxidant, i.e. the control was

measured daily using a Perkin–Elmer Lambda UV/VIS spectrophotometer at 515 nm against a blank, i.e. without DPPH. All tests were run in triplicate and an average was used. Decreasing of DPPH solution absorbance indicates an increase of DPPH radical scavenging activity. The percentage inhibition calculated according to the equation and then IC_{50} calculated graphically.

$$\% \text{ inhibition} = \left[\frac{AC(0) - AA(T)}{AC(0)}\right] \cdot 100$$

where $A_{C(0)}$ is the absorbance of the control at t = 0 min and $A_{A(t)}$ is the absorbance of the antioxidant at t = 30 min.

Statistical analysis. Simple and interaction effects of experimental factors were derived from two––way analysis of variance (ANOVA) based on the GLM procedure of the SAS statistical package (SAS/STAT® v.9.2. SAS Institute Inc., Cary, NC). The assumptions of variance analysis were tested by ensuring that the residuals were random and homogenous, with a normal distribution about a zero mean. The significance of differences among treatment means was tested using Duncan's multiple range test (DMRT) at $p \leq 0.05$.

RESULTS AND DISCUSSION

Essential oil. The color of oil extracted from the aerial parts of basil in all treatments was yellow. Results indicated that there was a significant difference between the irrigation regimes for essential oil yield. The highest oil yield was obtained from mild drought stress treatment (I_3) with 0.82 ml·100 g^{-1} dry material (tab. 1). Similarly, Simon et al. [1992] reported that water stress increased essential oil accu-

Table 1. Effect of the foliar application of chitosan, irrigation regime, landraces on some characteristics of basil

Treatments		TPC (mg GAE·g^{-1} extract)	IC_{50} (mg·g^{-1})	Essential oil yield (ml·100 g^{-1})
Irrigation				
I1 (control)		15.6c	1.80a	0.183c
I2 (60% FC)		19.3b	1.48b	0.488b
I3 (30% FC)		23.7a	1.28c	0.822a
Chitosan				
C1(control)		18.9c	1.58b	0.412d
C2 (acetic acid)		18.9d	1.58a	0.417c
C3 (0.2·g·L^{-1})		19.7b	1.48c	0.49b
C4 (0.4·g·L^{-1})		20.7a	1.45d	0.671a
Landraces				
S1(green)		0.1896b	1.511.9b	0.710a
S2(purple)		0.2021a	1534.7a	0.285b
ANOVA	Irrigation (I)		**	**
	Chitosan (C)		**	**
	Landraces (L)		**	**
	I × C		**	**
	I × L		**	**
	C × L		**	**
	I × C × L		**	**

** High significant at $p < 0.01$

Table 2. The effects of irrigation × chitosan, irrigation × landrace, and chitosan × landrace on some characteristicsof the landraces of basil

Treatments	TPC (mg GAE·g^{-1} extract)	IC_{50} (mg·g^{-1})	Essential oil yield (ml·100 g^{-1})
Irrigation × Chitosan			
I1×C1	15.4j	1.84b	0.138i
I1×C2	15.2k	1.85a	0.138i
I1×C3	15.7i	1.76c	0.133i
I1×C4	16.2h	1.76c	0.325h
I2×C1	18.7g	1.56e	0.388g
I2×C2	18.7g	1.57d	0.400f
I2×C3	19.6f	1.44f	0.513e
I2×C4	20.6e	1.38g	0.65d
I3×C1	22.9c	1.34h	0.712c
I3×C2	22.7d	1.33i	0.712c
I3×C3	23.8b	1.25j	0.825b
I3×C4	25.4a	1.20k	1.03a
Irrigation × Landrace			
I1×S1	0.1471f	1823.6a	0.225d
I1×S2	0.1658e	1782.9b	0.142e
I2×S1	0.1836d	1493.3c	0.75b
I2×S2	0.2043c	1478.4d	0.225d
I3×S1	0.2381a	1218.8f	1.15a
I3×S2	0.2361b	1343.0e	0.488c
Chitosan × Landrace			
C1×S1	0.1823e	1542.9c	0.617c
C1×S2	0.1971c	1612.5a	0.208g
C2×S1	0.1902d	1486.2d	0.617c
C2×S2	0. 2038b	1479.6e	0.217f
C3×S1	0.2042b	1460.0f	0.7b
C3×S2	0.2107a	1434.9g	0.281e
C4×S1	0.1816e	1558.4b	0.908a
C4×S2	0.1965c	1611.9a	0.433d

Means with common letters are not significant ($p \leq 0.05$), according to Duncan's multiple range test

Table 3. The effects of irrigation × chitosan × landrace on some characteristics of the landraces of basil

Treatments	TPC (mg GAE·g^{-1} extract)	IC_{50} (mg·g^{-1})	Essential oil yield (ml·100 g^{-1})
I1×C1×S1	0.1454r	1842.2b	0.175k
I1×C1×S2	0.1622o	1829.8c	0.1o
I1×C2×S1	0.1445r	1795.8d	0.175k
I1×C2×S2	0.1698n	1723.2e	0.1o
I1×C3×S1	0.1539q	1799.6d	0.15l
I1×C3×S2	0.1708n	1726.4e	0.117n
I1×C4×S1	0.1444r	1856.7a	0.4h
I1×C4×S2	0.1604p	1852.0a	0.25i
I2×C1×S1	0.1762m	1539.0h	0.65f
I2×C1×S2	0.1974j	1575.0f	0.125m
I2×C2×S1	0.1864l	1456.0i	0.65f
I2×C2×S2	0.2049i	1421.3k	0.15l
I2×C3×S1	0.1951k	1408.9l	0.8e
I2×C3×S2	0.2165h	1351.8m	0.225j
I2×C4×S1	0.1766m	1569.1fg	0.9d
I2×C4×S2	0.1983j	1565.3g	0.4h
I3×C1×S1	0.2253f	1247.5o	1.02c
I3×C1×S2	0.2318e	1432.8j	0.4h
I3×C2×S1	0.2398c	1206.9q	1.025c
I3×C2×S2	0.2367d	1294.3n	0.4h
I3×C3×S1	0.2633a	1171.4r	1.15b
I3×C3×S2	0.2449b	1226.5p	0.5g
I3×C4×S1	0.2239g	1249.5o	1.425a
I3×C4×S2	0.2309e	1418.4k	0.65f

Means with common letters are not significant ($p \leq 0.05$), according to Duncan's multiple range test

mulation via higher density of oil glands. Opposing results, however, indicated that optimum irrigation either resulted in higher essential oil accumulation [Figueiredo et al. 2008] or had no effect on essential oil content [Khazaie et al. 2008]. Different results in water deficit effects on the essential oil yield could be related to stress level, species as well as environmental conditions. Chitosan also increased the essential oil yield of basil (tab. 1). Our results indicated that chitosan at 0.4 $g \cdot L^{-1}$ produced maximum oil yield. Improvement in the essential oil yield by the foliar application of chitosan might be due to the increase in cycle growth, nutrients uptake or changes in leaf oil gland population and monoterpenes biosynthesis [Ghasemi Pirbalouti et al. 2014]. However, the effect of foliar application of chitosan on the essential oil yield of basil was influenced by irrigation regime (tab. 1). The highest essential oil yield (1.03 $ml \cdot 100\ g^{-1}$) was achieved by foliar spray of 0.4 $g \cdot L^{-1}$ chitosan under reduced irrigation (I3). The lowest essential oil yield (0.133 $ml \cdot 100\ g^{-1}$) was obtained by the foliar spray of 0.2 $g \cdot L^{-1}$ chitosan under normal irrigation (I1). The interaction effects of irrigation regime × landraces, irrigation regime × landraces and chitosan × landraces had significant influences on the essential oil yield (tab. 2). Moreover, the interaction effect of irrigation regime × chitosan × landraces had a significant influence on the essential oil yield (tab. 3).

Total phenolic content. The conducted studies aimed at determining the influence of irrigation regimes and chitosan on the content of phenolic compound in green and purple basil. Results of statistical analysis indicated significant differences in the total phenolic content of the extracts of basil under different treatments (tab. 1). Results of this study showed that the extract from the two landrace of basil had higher total phenolic content under reduced irrigation than normal irrigation (tab. 1). In addition, Manukyan [2011] reported that drought stress affected positively polyphenolic content in lemon balm (*Melissa officinalis* L.). In general many authors demonstrated that the production of phenols in plant tissues raises under abiotic stress conditions [Janas et al. 2002, Wrobel et al., 2005, Weidner et al. 2009]. In addition, the results indicated that different levels of chitosan significantly affected on the total phenolic content of the extracts (tab. 1). Cai et al. [2011] have been reported that treatment with chitosan efficiently enhanced biosynthesis of phenolic compounds in *Vitis vinifera*. Similarly, Kim et al. [2003] reported that total amount of the phenolic and terpenic compounds in sweet basil increased after the chitosan treatment.

Synthesis of phenolic compounds is recognized initiated very quickly after elicitation. Overall, elicitors such as chitosan is considered to be plant signaling molecules that are involved in some signal transduction systems, and induce gene expression levels of enzymes of the secondary metabolic pathway such as PAL (phenylalanine ammonia lyase), an enzyme involved in the synthesis of phenolics through phenylpropanoid pathway and in consequence increase the amount of phenolic compounds [Yao and Tian 2005, Reymond and Farmer 1998, Ding et al. 2002]. The initial step of phenylpropanoid synthesis is mediated by phenylalanine ammonia–lyase (PAL) enzyme. An increase in PAL activity could often be considered as a marker of plant reaction to elicitors. Plants phenolics present in herbs, because of their potential antioxidant, antimutagenic and antitumor activities, have been received considerable attention [Lupez-Velez et al. 2003]. Phenolic compounds, due to their antioxidant activities and free radical scavenging abilities, are widely distributed in plants [Li et al. 2007], which have gained much attention and potentially have beneficial implications for human health [Govindarajan et al. 2007]. Therefore, phenolic compounds are the major group contributing to the antioxidant activity of vegetables, fruit, cereals and other plant–based materials. The antioxidant activity of phenolics is mainly due to their redox properties, which make them acting as reducing agents, hydrogen donors, and singlet oxygen quenchers [Chan et al. 2007].

In this experimental, the interaction effect of irrigation regime × chitosan had a significant effect on total phenolic content. The maximum total phenolic content was obtained by foliar spray of chitosan (0.4 $g \cdot L^{-1}$) under reduced irrigation or mild stress drought (tab. 2). The interaction effect of irrigation

regime × landraces and chitosan × landraces had a significant effect on total phenolic content (tab. 2). Moreover, the interaction effects of irrigation regime × chitosan × landraces had significant effect on the total phenolic content (tab. 3).

Antioxidant activity. The antioxidant activity of the green and purple basil extracts was determined by measuring their ability to remove free DPPH radicals present in a methanol solution. The DPPH is a stable free radical, which has been widely accepted as a tool for estimating the free radical scavenging activities of antioxidants [Hu et al. 2004]. The antioxidant activity in basil is attributed both to its extract and soluble phenolic fractions. The lower IC_{50} value indicates a stronger ability of the extract to act as a DPPH scavenger, while the higher IC_{50} value indicates a lower scavenging activity of the scavengers as more scavengers are required to achieve 50% scavenging reaction. Results of this study showed that the extract from basil had higher antioxidant activity under mild drought stress, which there is maximum amount of phenolic compounds (tab. 1).

Our results are in accordance with a previous report, which has shown that higher phenolic compound levels increase the antioxidant activity [Ali et al. 2006] and also showed a linear correlation between phenolics contents and antioxidant activity [Kim et al. 2006]. There is a positive correlation between phenols content and antioxidant properties in plants [Li et al. 2008]. The antioxidant activity in basil is attributed both to its extract and soluble phenolic fractions. The antioxidant activity of phenolic compounds in plants is mainly due to their redox properties and chemical structure, which can play an important role in neutralizing ROS, such as free radicals, singlet and triplet oxygen and peroxides [Zheng and Wang 2001]. Because of the high relative antioxidant activity basil, this plant can be a good source for pharmaceutical industries and a new sources of antioxidant phenolics in the diet.

Results of this study indicated that spray chitosan treatments had a significant impact ($p \leq 0.01$) on the antioxidant activity (tab. 1). The most antioxidant activity was exhibited by the extract from the plants under the foliar spray of 0.4 $g \cdot L^{-1}$ chitosan (tab. 1). Probably, chitosan could regulate the activities of antioxidant enzymes and increase plant tolerance to biotic and abiotic stresses [Ghasemi Pirbalouti et al. 2014]. Treatment with signaling molecules like chitosan may also, induce H_2O_2 production, which in turn may induce the synthesis or activation of various transcription factors and are associated with the induction of different antioxidant enzymes [Ahuja et al. 1984].

The interaction effects of irrigation regime × landraces and chitosan × landraces had significant influences on antioxidant activity (tab. 2). Moreover, the interaction effects of irrigation regime × chitosan × landraces had significant effect on antioxidant activity (tab. 3).

CONCLUSIONS

This study has demonstrated drought stress and different concentrations of the foliar spray of chitosan increased the phenolic compounds, and the antioxidant activity of the extracts and the essential oil yield in two Iranian landraces of basil. These results emphasized the importance of biotic and abiotic elicitors for enhancement of the phenolic compounds and the antioxidant activity of basil plant extracts, which might be alternative and effective means instead of genetic modification. Overall, with regard to the results of this study the extract of green and purple basil could be an important source of phenolic compounds with antioxidant capacity. Finally, it could be concluded that foliar application of chitosan and reduced irrigation, as a possible technique, can be used to increase the phenolic compounds and antioxidant activity in cultivation system of basil. This study provides the basis for further research on improving the quality and pro-health functional value of two landraces of basil using elicitors.

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