Biochemical response of *Ocimum basilicum* L. inoculated with *Rhizophagus fasciculatus* as a NaCl-stress mitigator

Respuesta bioquímica de *Ocimum basilicum* L. inoculado con *Rhizophagus fasciculatus* como mitigador del estrés por NaCl

Resposta bioquímica de *Ocimum basilicum* L. inoculado com *Rhizophagus fasciculatus* como mitigador de estresse por NaCl

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Abstract

Basil (*Ocimum basilicum* L.) is a medicinal and aromatic plant of commercial interest; it can be grown in salinized soils by applying a stress mitigator. The objective was to evaluate the biochemical response of two basil varieties inoculated with AMF *Rhizophagus fasciculatus* and appraise its usefulness as a NaCl-stress mitigator. A completely randomized design with a factorial arrangement, four replicates per treatment and four plants per replicate was used. Three factors were considered, (1) two basil varieties (Napoletano and Nufar); (2) three NaCl concentrations (0, 50 and 100 mM); and (3) *R. fasciculatus* inoculum absence or presence (0 and 10 g). The variables evaluated were a substrate chemical analysis; shoot (STP) and root (RTP) total protein content; shoot (SP) and root (RP) proline content; shoot (SGA) and root (RGA) glutathione peroxidase activity; spore count and colonization. The spore content was 50 to 70 spores per gram of inoculum. The STP and RTP were highest in both varieties in 0 mM with AMF and decreased in Napoletano in 100 mM. The SP and RP were highest in Nufar in 50 and 100 mM with AMF and lowest in Napoletano in 0 and 50 with AMF. The SGA and RGA were highest in Napoletano in 50 and 100 mM with AMF. The colonization was high; however, decreased as NaCl increased. These results suggest that inoculation with AMF has a positive effect to mitigate NaCl-stress and a biochemical benefit for basil plants.

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Resumen

La albahaca (*Ocimum basilicum* L.) es una planta medicinal y aromática; se puede cultivar en suelos salinizados aplicando un mitigador del estrés. El objetivo fue evaluar la respuesta bioquímica de dos variedades de albahaca inoculadas con HMA *Rhizophagus fasciculatus* y valorar su utilidad como mitigador del estrés por NaCl. Se utilizó un diseño completamente al azar con arreglo factorial con cuatro repeticiones por tratamiento y cuatro plantas por repetición. Se consideraron tres factores, (1) dos variedades de albahaca (Napoletano y Nufar); (2) tres concentraciones de NaCl (0, 50 y 100 mM) y (3) ausencia o presencia del inóculo *R. fasciculatus* (0 y 10 g). Las variables evaluadas fueron el análisis químico del sustrato; contenido total de proteínas en brotes (PTB) y raíces (PTR); prolina en brotes (PB) y raíces (PR); actividad del glutatión peroxidasa en brotes (GPB) y raíces (GPR) y colonización. El contenido de esporas fue de 50 a 70 esporas por gramo de inóculo. La PTB y PTR fueron mayores en ambas variedades en 0 mM con HMA y disminuyó en Napoletano en 100 mM. La PB y PR fueron mayores en Nufar en 50 y 100 mM con HMA y menores en Napoletano en 0 y 50 con HMA. La GPB y GPR fueron mayores en Napoletano en 50 y 100 mM con HMA. La colonización fue alta, pero disminuyó conforme aumentó NaCl. Estos resultados muestran que la inoculación con HMA tiene efecto positivo para mitigar el estrés por NaCl y un beneficio bioquímico para albahaca.

Palabras clave: Hongos micorrízicos arbusculares, albahaca, estrés abiótico, bioquímica.

Resumo

Manjericão (*Ocimum basilicum* L.) é uma planta medicinal e aromática de interesse comercial; puede cultivarse em solos salinizados aplicando um atenuador. O objetivo foi avaliar a resposta bioquímica de manjericão inoculada com FMA *Rhizophagus fasciculatus* e avaliar sua utilidade como mitigador de NaCl. Utilizou-se o delineamento inteiramente casualizado com arranjo fatorial, quatro repetições por tratamento e quatro plantas por repetição. Três fatores foram considerados, (1) duas variedades de manjericão (Napoletano e Nufar); (2) três concentrações de NaCl (0, 50 e 100 mM); e (3) ausência ou presença de inóculo (0 e 10 g). As variáveis avaliadas foram uma análise química do substrato; teor de proteína total da parte aérea (STP) e da raiz (RTP); conteúdo de prolina da parte aérea (SP) e da raiz (RP); atividade da glutationa peroxidase da parte aérea (SGA) e da raiz (RGA); contagem de esporos e colonização. O conteúdo de esporos foi de 50 a 70 esporos por grama de inóculo. O STP e RTP foram maiores em ambas as variedades em 0 mM com FMA e diminuíram em Napoletano em 100 mM. O SP e RP foram maiores em Nufar em 50 e 100 mM com FMA e menores em Napoletano em 0 e 50 com FMA. O SGA e RGA foram maiores em Napoletano em 50 e 100 mM com FMA. A colonização foi alta; no entanto, diminuiu à medida que o NaCl aumentou. Esses resultados sugerem que a inoculação com FMA tem um efeito positivo para mitigar o estresse por NaCl e um benefício bioquímico para manjericão.

Palabras-chave: Fungos micorrízicos arbusculares, manjericão, estresse abiótico, bioquímica.

Introduction

Soil salinization in agricultural production areas is increasing worldwide and implies challenges to researchers due to its foreseeable negative impact in the short, medium, and long term. High salinity promotes imbalance in plant metabolism and in its osmotic relationships with soil (Agüero-Fernández *et al*., 2018). The plants activate physiological mechanisms, such as osmotic adjustment to ensure ionic homeostasis in response to saline-stress. The increment of Na and Cl contents in soil produce a decrease in osmotic potential, and subsequently, in water potential, avoiding ionic toxicity and interference in the assimilation of important cations. At genetic level, this adjustment induces the osmotically active organic compound synthesis, such as glycine-betaine and proline and modifies protein metabolism (González *et al*., 2005). Proline, betaine, glycine betaine, various carbohydrates and osmotically active proteins are synthesized in plants and used as indicators to select tolerant genotypes to salinity-stress (Feitosa de Lacerda *et al*., 2001). Some studies have been demonstrated that, the arbuscular mycorrhizal fungus (AMF) colonize the roots as an environmental alternative to increases tolerance to salinity and improves crop productivity (Medina-García, 2016; Agüero-Fernández *et al*., 2018). Arbuscular mycorrhizal symbiosis is the effect of beneficial interaction among roots and AMF, so the fungus receives photosynthates from the plant and improves its ability to absorb nutrients and water, increasing its tolerance stress (Aggarwal *et al*., 2012). Mycorrhizal colonization produces physical, biochemical, and physiological changes in roots. These changes improve the plant conditions and contribute to alleviating stress (Medina-García, 2016).

Mexico has regions with great potential to produce aromatic species. One of them is Baja California Sur, with the highest production of certified organic basil (SIAP, 2021). Basil generates income and is an economic and technological diversification for many farmers. Basil added value derives from its medicinal and culinary properties (Juárez-Rosete *et al*., 2013) with therapeutic applications around the world (Masarovičová and Králóvá, 2007). Baja California Sur is a semiarid region which soil and water for agriculture tend to salinization (Mazón-Suástegui *et al*., 2018). The objective of the study was to evaluate the biochemical response of two basil varieties inoculated with AMF *R. fasciculatus* and appraise its usefulness as a NaCl-stress mitigator.

Materials and methods

Experimental area

The experiment was carried-out in the Biological Research Center of the Northwest, S.C. (CIBNOR) located in La Paz, Baja California Sur, Mexico at 24° 08' 10.03 LN and 110° 25 '35.31 LW, at 7 m.a.s.l. The experiment was done inside a greenhouse with roof covered with white anti-aphid model 55. 30 % shade mesh. Under this mesh, another black mesh model 20 with 35 % shade was placed for a total shading of 65 %. The site has a type Bw (h') hw (e) climate considered as semi-arid with xerophilous vegetation (García, 2004). The temperatures mean, maximum and minimum in the site were 29°, 30° and 25° C, respectively; the mean relative humidity was 67 %, dew point of 21°C, with a total precipitation of 14.6 mm and solar radiation of 293.3 Wm^2. The weather variables were logged with a weather station (Vantage Pro2® Davis Instruments, USA).

Basil plant material

The seeds of Napoletano and Nufar varieties with differential response to NaCl (Batista-Sánchez *et al*., 2017) were obtained from the Vis Seed Company® (Arcadia, CA, USA).

Seedling production

The seeds were disinfected by soaking for 5 min in calcium hypochlorite with 5 % active chlorine and successively washed with sterilized distilled water. Then, were sown in 50-cavity polystyrene trays with a commercial sterile medium-size expanded horticultural mineral perlite as substrate (Hortiperl, Termolita® S.A de C.V., Mexico). Irrigation was applied daily to achieve a uniform seedling emergence, which was attained at 7 days after sown.

Transplant and experimental design

Transplant was performed when seedlings had an average height of 15 cm, placing one in each pot of ~10 kg, using the commercial substrate mentioned. The experimental design was completely randomized with factorial arrangement being factor 1, two varieties (Napoletano and Nufar); factor 2, three NaCl (0, 50, and 100 mM) concentrations; and factor 3, presence or absence of the AMF *R. fasciculatus* (0 and 10 g of inoculum) with four replications per treatment and four plants per replication. The duration of the experiment was 100 days after transplant with four harvests of biomass and the evaluation of other variables. The biochemical variables were determined 50 days after transplant.

Inoculation, irrigation, and nutrition

The AMF *R. fasciculatus* belong to the CIBNOR no commercial collection. The seedlings were inoculated with AMF during transplant, using the dose (control and 10 g of the inoculum), depositing the inoculum at the bottom of each seedling according to Rivera *et al*. (2003). The 10 g of AMF is equivalent to 50-70 spores per gram of inoculum. Irrigation began with daily application using water with an electrical conductivity (EC) of 0.04 $dS.m^{-1}$ and a nutrient solution (Samperio, 1997) which was modified in P^{+} content, that is, P^{+} was not included in the nutrient solution. One week after transplant, the gradual application of NaCl began when the plants were established. The amount applied daily per irrigation was 500 mL, allowing that the applied solution drained through the holes of the pots to avoid NaCl accumulation in the substrate. The pH and EC readings were taken after preparation the saline solution and subsequently to the drained liquid to compare the values of pH and EC (prepared and drained). No changes were detected in the drained solution.

Chemical analysis of the substrate (mineral perlite)

The samples of the substrate used were taken and sieved with mesh No. 10 (2 mm). The EC and pH were determined with a soil solution ratio of 1:5 using a potentiometer (Hanna®, Model 211, USA) (Jackson, 1958). Electrical conductivity was measured with a conductivity meter (Hach®, Model Sension+, Loveland, CO, USA) (Jackson, 1976). Phosphorus that is soluble in water (P^{+5}, $mg.kg^{-1}$) was measured from the aqueous extract with a soil solution ratio of 1:5 using Multiskan Acent® (Labsystems Model, No. 354, Finland) (Jackson, 1976). Extractable potassium (K^{+}, $mg.kg^{-1}$) was determined with flame atomic absorption spectrophotometry (GBC®, Avanta model, Australia). The extractable Ca^{+2} and Mg^{+2} were measured by complexometric volumetric method by titration (titration with EDTA 0.01 N) (Cheng and Bray, 1951). The organic matter content was determined by the Walkley and Black method using mesh No. 35 (0.5 mm). Total nitrogen was determined by the Dumas method (Leco®, model FP-528, USA), using mesh No. 100 (0.150 mm).

Total protein content (shoots and roots)

The total protein content was measured by bicinchoninic acid or BCA method (Provenzano *et al*., 1985). Briefly, digestion of samples and aliquot of the homogenate was performed. The diluted samples were taken, placed at the bottom of a microplate and the reagent prepared from BCA was added. Then, the samples were incubated, and absorbance was determined (Termo, Multiskan spectrum, Vanta, Finland). Each sample was analyzed in triplicate.

Proline content (shoots and roots)

The proline content was determined agreeing to Bates *et al*. (1973). Briefly, samples of plant tissue were homogenized in sulfosalicylic acid and centrifuged. Then, supernatant sample was pipetted into borosilicate and ninhydrin reagent. Subsequently, samples were heated in a boiling water bath and then cooled. The toluene phase was separated by measuring absorbance (Thermo Helios Omega®, Vanta, Finland). Each sample was analyzed in triplicate.

Glutathione peroxidase (GPx) activity (shoots and roots)

The activity of the glutathione peroxidase enzyme was determined according to the Folhé and Günzler (1984) method. Briefly, the assay mixture was composed of phosphate buffer, EDTA, sodium azide, glutathione reductase, NADPH, deionized water, reduced glutathione, and H_2O_2. The absorbance was measured (Beckman Coulter DU 800, Beckman Coulter, Inc. Brea, CA, USA). One unit of GPx is defined as the amount of enzyme that oxidizes 1 µmol of NADPH per minute. Each sample was analyzed in triplicate.

Spore count and colonization

Arbuscular mycorrhizal fungi spores were recovered from inoculum by wet sieving followed by sucrose gradient centrifugation method (Daniels and Skipper, 1982). Spores were counted under ×35 magnification in a dissecting microscope and the density was expressed as the number of spore's g^{-1} in the dry inoculum. The colonization (%) was calculated agreeing to Abeer e*t al*. (2014) with the following formula:

$$\text{Colonization (\%)} = \frac{\text{Total number of AM positive segments}}{\text{Total number of segments studied}} \times 100$$

Statistical analysis

Analysis of variance and multiple comparisons of means were performed (Tukey's HSD test p=0.05). The colonization (%) was transformed by arcsine (Little and Hills, 1989; Steel and Torrie, 1995), to comply with the normality assumption. Statistical analyses were done with Statistica® v. 10.0 for Windows (StatSoft®, 2011).

Results and discussion

Chemical analysis of the substrate (mineral perlite)

The mineral perlite showed low fertility with a content of 12.20 $mg.kg^{-1}$ of Mg^{+2}; low exchangeable K^{+} (34.43 $mg.kg^{-1}$); low available P^{+} (12.95 $mg.kg^{-1}$); low N (0.059 %); 0 (zero) organic matter; 40.10 $mg.kg^{-1}$ of Ca^{+2}; a pH of 7.46 and low EC (0.15 $dS.m^{-1}$). According to Castellanos *et al*. (2000) the analysis of the substrate confirmed its suitability for development seedlings and the *R. fasciculatus* as inoculum.

Total protein content (shoots and roots)

The shoot total protein content (STP) showed significant differences (Table 1). Nufar showed highest STP in 0 mM with and without AMF followed by Napoletano without AMF both in 0 mM. Napoletano showed the lowest STP in 100 mM without AMF. Root

total protein (RTP) content did not show significant differences; however, the highest RTP was observed in both varieties at 0 mM with and without AMF. This response is related to the salt stress tolerance of Napoletano and Nufar, which previously showed to be tolerant to NaCl-stress (Batista-Sánchez *et al*., 2019). This tolerance is attributed to the osmoprotective compound synthesis, such as proteins and proline (Argentel *et al*., 2012). A prior study showed that the AMF inoculation increased total soluble proteins in basil showing the additive effect of the fungus action once it reaches the time to colonize the root and produce enough external mycelium (Terry-Alfonso and Leyva-Galán, 2006). The results suggest that basil synthesized new proteins in response to the NaCl-stress. Similar results reported Mollasadeghi *et al*. (2011) in wheat under water deficit.

Proline content (shoots and roots)

Shoot (SP) and root (RP) proline content showed significant differences (Table 1). Nufar in 50 and 100 mM with AMF showed highest SP. Napoletano showed lowest SP in 0 mM without AMF. The RP content was highest in Nufar in 100 and 50 mM NaCl with AMF. The RP content was lowest in Napoletano in 0 and 50 mM NaCl with AMF and 0 NaCl without AMF. Both varieties increased SP or RP as NaCl increased except SP in Nufar. Similar results reported Larrinaga-Arce (2014), who indicated that basil has enzymatic capacity to reduce the superoxide radical under 100 mM NaCl. Proline is a biochemical marker to evaluate plants under saline stress (Shamshiri and Fattahi, 2014). The increase in proline in basil inoculated with AMF under NaCl-stress is evidence that AMF act as mitigator of NaCl. The proline protects plants against salt-stress, acts as an enzymatic protector, pH stabilizer, cytosolic buffer, and cell balance (Chelli-Chaabouni *et al*., 2010; Verbruggen *et al*., 2013). A variety with higher accumulation of proline under saline-stress could be assumed to be more tolerant compared to another one with less proline accumulation, since the increase of metabolites act as compatible solutes (Munns and Tester, 2008).

Glutathione peroxidase (GPx) activity (shoots and roots)

The shoot (SGA) and root (RGA) glutathione peroxidase activity showed significant differences (Table 1). Napoletano showed highest SGA in 100 and 50 mM NaCl with AMF. Nufar showed lowest SGA in 50 and 100 mM with and without AMF. Napoletano showed highest RGA in 100 and 50 mM with AMF while the lowest RGA was showed by Nufar and Napoletano in 0 mM with and without AMF. In Nufar with or without AMF, RGA increased as NaCl increased but SGA with or without AMF showed the contrary. In Napoletano with or without AMF, SGA and RGA increased as NaCl increased. Similar results reported Abeer *et al*. (2015) when plants inoculated increased proline and glutathione peroxidase, which is attributed to the AMF effect on these osmoprotective compounds. The increase in GPx in the inoculated plants is attributed to the improvement of the proline synthesizing enzyme activity and reduction of their restricted incorporation during protein synthesis. Salinity generates reactive oxygen species, causing oxidative stress at cellular level. Superoxide and hydrogen peroxide cause oxidative damage through the hydroxyl radical, affecting lipids and proteins (Porcel *et al*., 2015). Proline and glutathione peroxidase benefit plants maintaining water input balance, mitigating stress-induced damage (Ahanger *et al*., 2014). This study showed that AMF caused an increase in proline, glutathione, and protein in the inoculated plants, which is attributed to the effect of this endophyte in NaCl-stress. The increase of SGA and RGA activity in 100 mM with AMF is evidence that basil under NaCl-stress activate the osmoprotective compound synthesis to counteract damage caused by NaCl-stress.

Spore count and colonization

The AMF spore content ween to 50 to 70 spores g^{-1} of inoculum. The mycorrhizal colonization (MC) showed significant differences (Table 1). Both varieties showed the highest MC in 0 mM with AMF. The MC tends to decrease as NaCl increased. The uninoculated plants did not colonize, which indicates that no native strains colonized the culture medium. The number of spores in the AMF inoculum was considered adequate for mycorrhizal symbiosis according to Gloria *et al*. (2010). This result is related to the infectivity of the species, its ability to produce external hyphae, hypha speed to colonize the roots and its ability to maintain colonization levels in a competitive condition (Rivera *et al*., 2003). The mycorrhizal colonization shown by Nufar and Napoletano in 0 mM NaCl (Table 1), exceeded the reference value (45 %) reported in wheat (Al-Karaki *et al*., 2004). However, the mycorrhizal colonization of this study was lower than those reported (70.3 %) by Rojas-Martínez (2014) in *C. annuum* var. inoculated with *G. manihotis*. As expected, the colonization tends to decrease as NaCl increased. Similar effects were described by Wu *et al*. (2010) in citrus and Aroca *et al*. (2013) in lettuce seedlings, while Al-Karaki (2000) exposed *L. esculentum* to salinity with AMF and concluded that an increase of EC from 4.7 to 7.4 dS m^{-1} decreased the colonization of *F. mosseae*. A decrease in AMF colonization was observed in roots of tomatoes (Latef and Chaoxing, 2011) and *Jatropha curcas* L. (Kumar *et al*., 2010) subjected to saline-stress. Regardless of NaCl, the level of colonization by *R. fasciculatus* in basil is considered too high compared to other studies (Shekoofeh *et al*., 2012; Cartmill *et al*., 2013). The decrease in colonization despite the increase in NaCl does not interfere with the benefic effect of this endophyte on plant species development under NaCl-stress.

Conclusions

The substrate used is suitable to develop basil seedlings using *R. fasciculatus* as inoculum. The colonization was high; however, decreased as NaCl increased. The results showed a differential biochemical response to NaCl and the use of AMF between varieties (Napoletano and Nufar). Both varieties increased STP, RTP with AMF while SP and RP increased as NaCl increased except SP in Nufar. In Napoletano with AMF, SGA and RGA increased as NaCl increased. These results confirm that mycorrhization favors synthesis of osmoprotector compounds, increasing the biochemical response of basil plants to increase the capacity to facing NaCl-stress conditions.

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Table 1. Variety × NaCl × AMF interaction in response to the effect of *Rhizophagus fasciculatum* (AMF) as a NaCl stress mitigator on biochemical and fungal variables of *Ocimum basilicum* varieties subjected to NaCl stress.

Variety	NaCl (mM)	AMF (g)	STP ($mg.g^{-1}$)	RTP ($mg.g^{-1}$)	SP ($mg.g^{-1}$)	RP ($mg.g^{-1}$)	SGA (U mg protein)	RGA (U mg protein)	Colonization (%)
Napoletano	0	CM	22.59±0.13b	16.37±0.109a	0.24±0.00d	0.21±0.00i	0.96±0.00c	0.83±0.02e	64.50±0.58a
Napoletano	50	CM	9.56±0.03g	6.55±0.36a	0.35±0.00b	0.28±0.00de	1.45±0.03ab	2.23±0.00b	50.00±0.00c
Napoletano	100	CM	9.48±0.00g	6.13±0.01a	0.36±0.00b	0.30±0.00c	1.50±0.02a	3.19±0.01a	37.75±0.50e
Napoletano	0	SM	22.20±0.14c	16.17±0.06a	0.22±0.00e	0.21±0.00i	0.97±0.01c	0.74±0.19e	0.00±0.00f
Napoletano	50	SM	9.29±0.00gh	6.40±0.13a	0.29±0.00c	0.21±0.00i	1.43±0.03b	2.02±0.04c	0.00±0.00f
Napoletano	100	SM	9.14±0.00h	6.10±0.00a	0.30±0.01c	0.26±0.01fg	1.44±0.04b	2.10±0.04bc	0.00±0.00f
Nufar	0	CM	24.96±0.15a	16.48±0.06a	0.25±0.01d	0.26±0.00g	0.98±0.01c	0.83±0.01e	65.25±2.06a
Nufar	50	CM	19.44±0.11d	12.86±0.09a	0.46±0.01a	0.32±0.01b	0.44±0.01d	1.71±0.07d	58.50±0.58b
Nufar	100	CM	18.85±0.11e	12.60±0.27a	0.37±0.01b	0.44±0.00a	0.45±0.01d	1.74±0.02d	44.00±1.15d
Nufar	0	SM	22.59±0.13b	16.42±0.03a	0.25±0.00d	0.25±0.00h	0.96±0.00c	0.82±0.01e	0.00±0.00f
Nufar	50	SM	18.22±0.12f	12.55±0.31a	0.30±0.01c	0.27±0.00ef	0.44±0.02d	1.76±0.05d	0.00±0.00f
Nufar	100	SM	18.51±0.12f	12.15±0.09a	0.30±0.00c	0.29±0.00d	0.45±0.00d	1.75±0.02d	0.00±0.00f
Significance level			***	*ns*	***	***	**	***	***

NaCl= Sodium chloride (mM); AMF= Arbuscular mycorrhizal fungi (CM = with AMF, SM = without AMF) (g); STP= Shoot total protein content; RTP= Root total protein content; SP= Shoot proline content; RP=Root proline content; SGA= Shoot glutathione activity (U mg^{-1} of protein); RGA= Root glutathione activity. Average values ± standard deviation with different letters in the same column differ statistically (Tukey's HSD, $P = 0.05$). Significance level: ns= not significant; ** = $P \leq 0.01$; *** = $P \leq 0.001$.

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