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Effects of combined salt and flooding stresses on the growth and physiological behavior of alfalfa (*Medicago sativa* L.)

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The present work focused on evaluation of the physiological and metabolic behavior of alfalfa (*Medicago sativa* L., var. Siriver), when faced with two important ecosystems constraints, namely salinity and flooding-associated hypoxia. After germination, seedlings in symbiosis with *Sinorhizobium meliloti* were grown on different salt concentrations (0, 20 and 50 mM NaCl). At four weeks post-germination, plants were submitted to a five-day long flooding treatment and then analyzed for various physiological (growth, biomass) and biochemical (proteins, chlorophylls, ions, proline) parameters. Our results showed that when *M. sativa* was cultured on moderate (20 mM NaCl) or average (50 mM NaCl) salt stress, the classical mechanisms of stress response (ions and proline accumulation) are set up and the growth (FW, DW, growth of aerial parts) was moderately affected by salt. In a situation of average salt stress (50 mM NaCl), the combined salinity and short-term flooding led to an early alteration of the photosynthetic machinery and a modification of ions and proline contents in the leaves.

Key words: Chlorophyll, hypoxia, legume, NaCl, proline, salinity.

INTRODUCTION

Agricultural soils in North Africa are subject to numerous environmental constraints such as salinity, drought during the dry season, flooding or waterlogging during the rainy season, and pollutions caused by industrial and mining discharges (Zahran, 1999). Soil reduced fertility is often due to the presence of salt, and the use of salted water supplies for irrigation resulting in decreased crop productivity (Glenn et al., 1999). Depending on the agricultural region, limitation of water reserves and irrigation practices lead to periodic irrigation of the fields. Thus, either during the rainy seasons or between two irrigation turns, agricultural areas may be flooded or waterlogged for several days, even weeks, at the time of vegetative growth of crops. Both flooding and

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waterlogging result in hypoxia due to the rapid consumption of oxygen in the rhizosphere and a near 10^4 reduction in diffusion of oxygen in water relative to air (Armstrong, 1980).

Alfalfa, or lucerne (*Medicago sativa* L.), is a forage legume cultivated around the world, and among others in North Africa (Bouton, 2012; Zahran, 1999). *M. sativa* is considered to be relatively tolerant to salinity (Bruning and Rozema, 2013; Zahran, 1999) and drought (Bouton, 2012), but sensitive to flooding-induced hypoxia (Striker and Colmer, 2017). Several studies have analyzed its behavior under either saline stress or flooding-induced hypoxia (Li et al., 2010; Rogers et al., 2008, 2009, 2011; Smethurst et al., 2005), but the effects of the double saline and flooding constraints on its growth have not been investigated. In the present work, we initiated the analysis of young *M. sativa* (var. Siriver) plant response to moderate/average salinity combined to short-term flooding treatment. This was accomplished through the measurement of biometric and physiological parameters of the aerial part to evaluate the effects of the combined constraint on forage production, as well as concentrations of mineral ions (Na^+, K^+, Cl^-) and proline, chosen as markers of salt-stress response.

MATERIALS AND METHODS

Ionic analysis of irrigation waters of *M. sativa* cultures

To carry out this study, the salinity of irrigation water of two agricultural soils in northern Tunisia, where alfalfa (*M. sativa* var siriver) is cultivated, was analyzed. The first site of Ghezala Mateur (37° 05' 02'' North, 9° 32' 08'' East) is located in the heart of an area of 5,300 ha from the Northwest of the town of Mateur to the South of Lake Ichkeul, and it includes the plain of Mateur and the perimeter of Ghezala, which is fed from the dam of Joumine. The second site of Oum Hani, in the suburb of Menzel Bourguiba (37° 09' 13'' North, 9° 47' 09'' East) is located in agricultural land close to the lakes of Ichkeul and Bizerte. Electrical conductivity and salinity of irrigation waters during the winter of 2014 were measured as in Bahri (1993). The averaged electrical conductivity (one measurement) and nitrate and NaCl concentrations (3 measurements) of the irrigation water were as follows: 1) water of the Ghezala well: 0.86 dS/m, 0.21 ± 0.04 mM NO_3^-, 19 ± 5 mM NaCl; 2) water of the Oum Hani well: 4.65 dS/m, 0.92 ± 0.03 mM NO_3^-, 52 ± 6 mM NaCl.

Based on nitrate and NaCl contents measured in irrigation waters, we chose to test the behavior of *M. sativa* seedlings under the following salt conditions: 1) a control condition (Ctrl0), with 0.2 mM KNO_3 (a low concentration of nitrate which allows a minimum supply of nitrogen without affecting the symbiotic process) in the absence of NaCl, 2) a T20 treatment, with 0.2 mM KNO_3 and 20 mM NaCl, representing the irrigation water characteristics of the Ghezala well, which corresponds to a moderate salt stress, and 3) a T50 treatment, with 0.9 mM KNO_3 and 50 mM NaCl, representing the characteristics of the irrigation water of the Oum Hani well, which corresponds to an average salt stress.

Plant material

Seeds of *M. sativa* (var. Siriver) were sterilized with sodium hypochlorite (2% active chloride) for 20 min, thoroughly rinsed, and imbibed in sterile distilled water for 3 h. Then, germination was carried out at 22°C in Petri dishes, lined with filter papers moistened with distilled water. Germination, identifiable by the emergence of radicles, took place in the dark after 5 days. The germinated seeds were transplanted in plastic pots (8 cm deep) filled with B5 sand (0.6-1.6 mm diameter), provided with adequate drainage device and then grown in culture room at 25°C, with a 16 h/8 h (day/night) photoperiod, and 60/80% (day/night) humidity. Plants were watered every 3 days, once with water, once with a mineral solution containing the following basic macro- and micro-nutrients: 2.5 mM $MgSO_4$, 0.5 mM K_2SO_4, 2 mM KH_2PO, 1 mM $CaCl_2$, 0.05 mM Fe-EDTA, 16 µM H_3BO_3, 5 µM $MnSO_4$,$4H_2O$, 1 µM $ZnSO_4$,$7H_2O$, 1 µM $CuSO_4$,$5H_2O$, 0.7 µM $NaMoO_4$,$2H_2O$, and 0.5 µM $CoCl_2$,$6H_2O$. In addition to the basic nutrients, either water or mineral solution also contained either 0.2 mM KNO_3 (Ctrl0), 0.2 mM KNO_3 and 20 mM NaCl (T20), or 0.9 mM KNO_3 and 50 mM NaCl (T50). Plants were inoculated 7 days post-imbibition with *Ensifer meliloti* 2011 (previously named *Sinorhizobium meliloti* 2011), the predominant microbial symbiotic partner (Frendo et al., 2005).

At 28 days post-imbibition (three-leaf vegetative stage approximately), the plants were either maintained under normoxia (N), or subjected to flooding (H) for 5 days. Flooding treatment was done by submerging pots containing plants to 1 cm above substrate level in the mineral solution supplemented, or not, with NaCl and KNO_3 to maintain a medium close to that of the irrigation waters of Ghezala and Oum Hani. At 33 days post-imbibition, the shoots of the plants were harvested, and either measured for biometric parameters or quickly frozen in liquid nitrogen and stored at -80°C until further analysis.

Vegetative growth analysis

The growth and biomass production of shoots was assessed by measuring the following parameters: height, fresh weight (FW), dry weight (DW) and water content, expressed as WC (%) = 100 (FW – DW)/FW, and leaf area (LA), that was measured using the MESURIM free access software: (http://acces.ens-lyon.fr/acces/logiciels/mesurim/telechargement). The dry weight is measured after drying the fresh material for 48 h at 70°C.

Protein measurements

Shoot samples (50 to 150 mg) were ground in a mortar and pestle in a buffer containing 25 mM Tris-HCl, pH 7.0, 0.5 mM β-mercaptoethanol, and 0.3% (p/v) polyvinylpolypyrrolidone. Homogenates were centrifuged for 15 min at 13400 g, and the clarified extracts in the supernatant were recovered. Soluble proteins were quantified on clarified extracts (Bradfor, 1976), using bovine serum albumin as standard.

Determination of chlorophylls

The extraction and determination of the concentration of leaf chlorophyll *a*, chlorophyll *b* and total chlorophyll were performed as in Arnon (1949), and calculations were according to the following formula:

Chlorophyll *a* = 12.7 (A663) - 2.69 (A645),
Chlorophyll *b* = 22.9 (A645) - 4.68 (A663),
Total chlorophyll = 20.2 (A645) + 8.02 (A663).

Determination of proline

Proline extraction and measurement was carried out according to

the method of Bates et al. (1973), on alfalfa shoots previously dried in an oven at 60°C. The optical density was measured at 520 nm. The standard range was established based on pure proline.

Determination of mineral elements

Mineral elements were extracted using the technique of cold nitric extraction from dried plant material according to (Gauquelin et al., 1992). Potassium (K^+) and sodium (Na^+) were determined by flame emission spectrometry according to Morard and Gullo (1970). The determination of chloride (Cl^-) was done by coulometry (chloridometer Buchler-Cotlove) according to Morard and Gullo (1970).

Statistical analysis

Every parameter was analyzed in three independent experiments, and measurements were carried out on samples of five to nine plants per treatment. One-way ANOVA followed by a Tuckey test (http://www.statskingdom.com/index.html) was carried out to determine the effect of the treatments in the analyzed variables. Differences were considered to be significant at the threshold of $P < 0.05$.

RESULTS AND DISCUSSION

Vegetative growth analysis

The effects of saline and hypoxic constraints were first analyzed on the growth of *M. sativa* shoots (Figure 1). As compared to Ctrl 0 in normoxia, after 33 days of growth, the size of the shoots and the leaf area (LA) were not significantly affected by moderate salt treatment (T20), whereas they decreased by 33% and 65%, respectively, in the presence of 50 mM NaCl (T50) (Figure 1A and B). The fresh weight (FW) and dry weight (DW) masses of the shoots were not significantly modified by the salt treatment (Table 1), and the water contents (WC) remained constant (close to 80%) regardless of the NaCl content in the nutrient medium (Table 1). A similar pattern was maintained in shoot dry weight in response to salt stress in 3 month-old *M. sativa* (var sativa) plants subjected to varying concentrations of NaCl for 4 weeks (Rogers et al., 2008, 2009), which confirms that *M. sativa* is quite tolerant to salinity. The imposition of 5-day flooding stress did not result in a profound change in growth parameters (Figuure 1 and Table 1), which means that short-term hypoxia did not significantly affect the growth of *Medicago sativa* in the absence as well as in the presence of NaCl.

Considered together, these results show that the biomass production of *M. sativa* was not significantly affected by average salt stress, while the growth of aerial parts and LA already showed a moderate reduction. Such a pattern of maintaining biomass production, while aerial parts and LA are reduced, may be explained by an increase in leaf thickness and mass per area as already observed in *M. sativa* response to salinity (Smethurst et al., 2008). Interestingly, an increase in leaf mass area was also observed in *M. truncatula* response to hypoxia (El Msehli et al., 2016), and it may be thought that such response is reinforced in response to combined salinity and hypoxia stresses. Actually, the maintenance of water content revealed that *M. sativa* has a good ability to adjust its osmotic potential. The absence of additional deleterious effects on the growth parameter, when exposed to the combined treatments of salinity and flooding-induced hypoxia, indicated that, at least over a 5 day-long hypoxia period, *M. sativa* is able to cope with this double constraint. To go deeper into the physiological study of the effects of single and combined stress, we analyzed parameters related to shoot protein content and photosynthetic capacity on the one hand; and we measured the major ions accumulated under salt constraint (Na^+, K^+, Cl^-), as well as proline which is a salt stress response marker in plants (Tujeta, 2007), on the other hand.

Effect of salinity and hypoxia on protein and chlorophyll contents

The soluble protein content is a valuable indicator to evaluate the impact of stress on the overall functioning of cells and tissues. As shown in Table 1, shoot protein contents were not modified significantly by either saline treatment, or after 5 days of flooding, indicating that *M. sativa* tolerates the imposition of the combined constraint at least for short periods. Chlorophyll contents were also analyzed to evaluate the capacity of *M. sativa* shoots to maintain their photosynthesis under combined salinity and hypoxia. Table 1 shows that under normoxia chlorophyll contents (*a*, *b* and total) were unaffected by salinity treatments. Similarly, a 5-day long flooding treatment did not change significantly the global chlorophyll contents when compared to the other hypoxically treated plants. But, it should be noted that for plants combining both 50 mM NaCl and flooding treatments (T50, H), their chlorophyll contents were approximately 50% lower than that of 50 mM treated normoxic plants (T50, N). Decreased chlorophyll levels (Table 1), as well as reduced leaf area in plants treated with 50 mM NaCl (Figure 1B), indicate a decline in overall plant photosynthetic capacity. It has been reported that under salt stress, as well as under hypoxia, a close relationship exists between chlorophyll level and photosynthetic capacities (Netto et al., 2005; Striker and Colmer, 2017). Present data indicate, first, that the photosynthetic machinery is affected earlier than is the overall protein content, and second that each stress, considered separately, does not affect the photosynthetic machinery; as already observed in previous studies (El Msehli et al., 2016; Li et al., 2010). However, the combination of the two stresses clearly affects it and

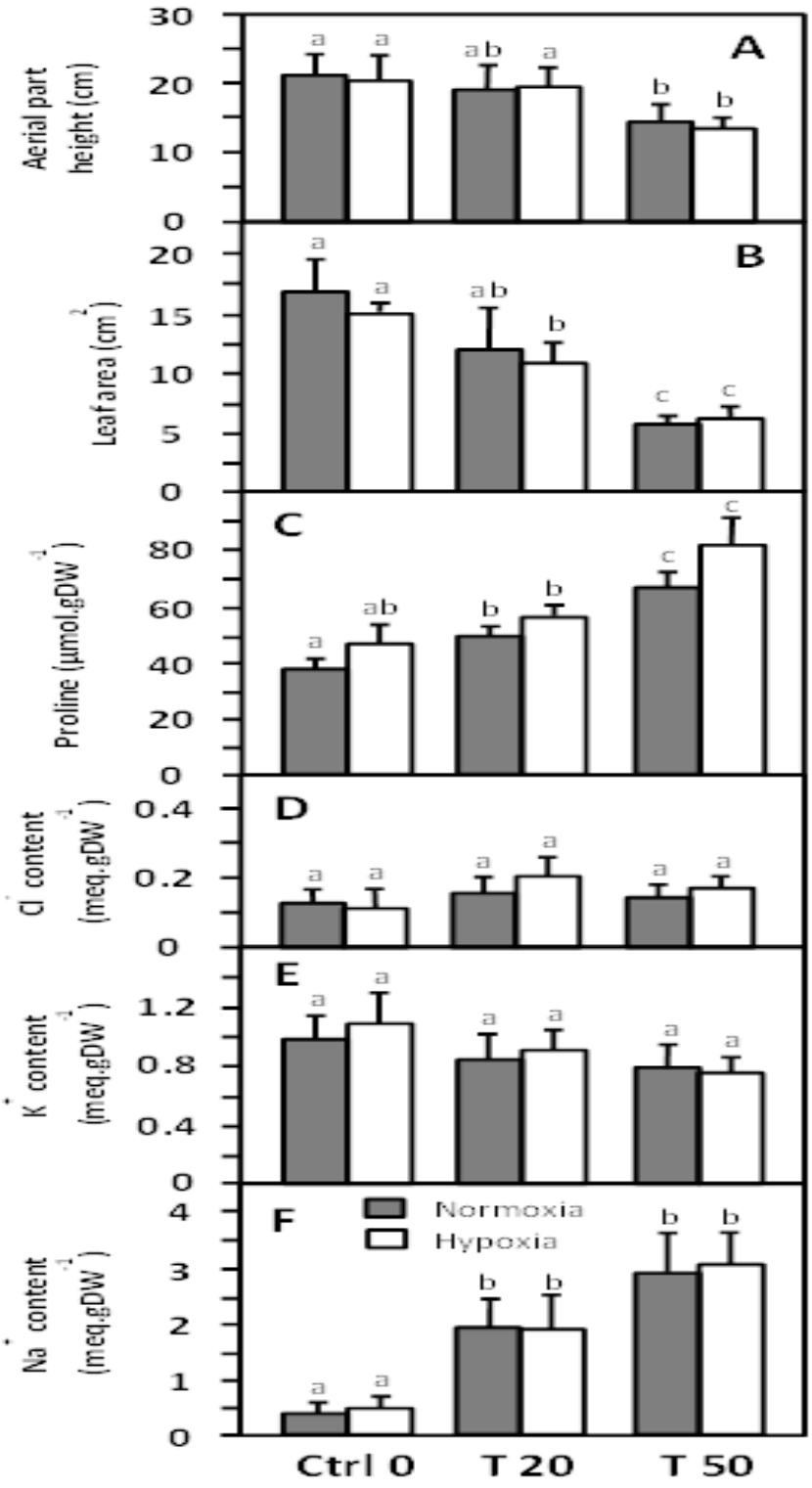

Figure 1. Effects of salinity and hypoxia on biometric parameters (height, leaf area), protein and metabolite contents (ions, proline) of *M. sativa* aerial parts. A, aerial part height; B, leaf area; C, proline content; D, Cl^- content; E, K^+ content; F, Na^+ content. Normoxic and hypoxic treatments were respectively figured as shaded and non-shaded bars. Treatments with 0, 20 and 50 Mm NaCl were respectively labeled Ctrl0, T20 and T50. The values are means ± SD of 3 independent experiments. Values followed by different letters are significantly different with $P<0.05$.

Table 1. Effects of salinity and hypoxia on dry and fresh weight, water content and chlorophylls of *M. sativa* plant shoots.

Variable	Treatment	NaCl		
		Ctrl0	T20	T50
Fresh weight (mg/plant)	N	288±79[a]	290±79[a]	255±68[a]
	H	274±84[a]	326±61[a]	325±89[a]
Dry weight (mg/plant)	N	52±15[a]	49±12[a]	44±14[a]
	H	51±18[a]	56±20[a]	51±16[a]
Water content (%)	N	82±3[a]	83±3[a]	83±7[a]
	H	81±3[a]	83±2[a]	84±2[a]
Protein content (mg/g FW)	N	10.5±1.8[a]	10.7±2.0[a]	9.7±1.3[a]
	H	9.6±1.5[a]	8.9±1.6[a]	8.8±2.1[a]
Chlorophyll a (mg/g FW)	N	0.36±0.06[a]	0.36±0.06[a]	0.41±0.10[a]
	H	0.30±0.09[ab]	0.24±0.08[ab]	0.23±0.05[b]
Chlorophyll b (mg/g FW)	N	0.29±0.09[a]	0.30±0.04[a]	0.45±0.14[a]
	H	0.22±0.07[ab]	0.28±0.07[a]	0.15±0.05[b]
Total Chlorophyll (mg/g FW)	N	0.65±0.15[ab]	0.65±0.09[a]	0.86±0.24[a]
	H	0.52±0.16[ab]	0.52±0.10[ab]	0.42±0.10[b]

Normoxic and hypoxic treatments were respectively labeled N and H. Treatments with 0, 20 and 50 mM NaCl were respectively labeled 0, 20 and 50. The values are means ± SD of 3 independent experiments. Values followed by different letters are significantly different with $P<0.05$.

impairs the capacity of *M. sativa* plants to maintain photosynthesis. In this situation, insufficient sugar production cannot cover the needs in maintaining the carbon skeleton for symbiotic nitrogen assimilation and plant growth, and compromises the plant capacity to cope with longer-lasting stress conditions.

Effect of salinity and hypoxia on mineral nutrition

The submission of *M. sativa* plants to increase salt stress in normoxia was accompanied by a significant increase in the Na^+ content in the leaves, from 0.43 meq.g^{-1} DW in Ctrl0 shoots to 2.0 and 2.95 meq. g^{-1} DW in T20 and T50 shoots, respectively (Figure 1F). The content of K^+, on the order of 1 meq. g^{-1} DW, did not change significantly as a function of salinity; while the Cl^- concentration remained close to 1.6-1.8 meq.vg^{-1} DW in T50 plants (Figure 1D and E). Such observations are comparable to the results obtained for other saline-treated varieties of *M. sativa* (Li et al., 2010; Rogers et al., 2008, 2009, 2011). However, in the present study, the Na^+/K^+ ratios measured in T20 and T50 plant shoots (2.3 and 3.7, respectively) are much higher than those measured in previous reports (Li et al., 2010; Rogers et al., 2009). This is likely due to the fact that in these reports *M. sativa* plants were first grown in the absence of NaCl for several weeks before being subjected to 2-4 weeks salt stress, whereas in our study plants were subjected to salinity from germination, as is the case in the field, and they have developed from the beginning a system of salt storage in the aerial parts. It also shows that *M. sativa* can store high amounts of NaCl in the leaves without significantly affecting plant growth. In comparison with normoxia treated plants, the application of flooding stress did not change the Na^+, K^+ and Cl^- contents in the shoots (Figure 1D, E and F), which means that ion transport and metabolism is not significantly altered by short-term flooding.

Effect of salinity and hypoxia on proline accumulation

As shown in Figure 1C, compared to normoxic treatment (Ctrl0), exposure to increasing concentrations of NaCl resulted in a significant and expected increase in leaf proline contents of 29 and 79%, in T20 and T50 shoots, respectively. Proline is a general marker of salt stress response in most plants (Mansour and Ali. 2017; Per et al., 2017), including legumes (Bruning and Rozema, 2013). Proline biosynthesis was expected to occur as a consequence of disturbance in cell homeostasis and to

reflect damage in response to salt stress (Khalil et al.,2016). Either an external supply (Ehsanpour and Fatahian, 2003), or an increased cellular level (Campanelli et al., 2013) in proline, promotes salt tolerance in *Medicago* species. Moreover, salt tolerance was found to be enhanced in *Medicago truncatula* plants transformed with a gene for proline synthesis (de la Pena et al., 2010). As compared with normoxia-treated plants, the application of hypoxia led to a slight increase (8-24%) in proline contents after 5 days of treatment. Although the difference was not significant between N-50 and H-50 plants, such an increase is of interest, because proline accumulation has recently been observed in *Phaseolus vulgaris* response to hypoxia (Velasco et al., 2019). It was suggested to serve as a source of energy, nitrogen or carbon to reinitiate growth after the hypoxia stress period (Aloni and Rosenshtein, 1982). However, further study would deserve investigation to confirm this observation and to analyze the potential role of proline in the combined salinity-hypoxia stress response.

Conclusion

M. sativa is a legume crop known to be tolerant to salinity (Bruning and Rozema, 2013; Zahran, 1999) and drought stress (Bouton, 2012), and sensitive to waterlogging and hypoxia (Rogers, 1974; Striker and Colmer, 2017). In northern Africa, *M. sativa* is often grown in areas where, during the rainy season or after irrigation turn, cultivated fields are subject to the double stress of salinity and flooding. Our results showed that when *M. sativa* is cultured on moderate (20 mM) and medium (50 mM) NaCl concentrations, the classical mechanisms of stress response (mineral ion accumulation, proline synthesis) are set up and the growth (FW, DW, growth of aerial parts) is only moderately affected by salt. At low NaCl concentration, short-term hypoxia did not significantly affect growth. But, in a situation of average salt stress (that is, 50 mM NaCl), the combined stresses seemed to lead, starting from 5 days of hypoxia, to an early alteration of the photosynthetic machinery and a modification of the compartmentalization of ions between roots and leaves. A study of the combined effects of salt and hypoxia during a longer period of hypoxia is needed to evaluate more deeply the impact of combined stresses on growth and yield of *M. sativa*. However, as a first step, it is recommended to control crop irrigation regimes to avoid too long period of flooding and the risk to significantly impact final yields.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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