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Original Article

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Effect of Seed Inoculation on Alfalfa Tolerance to Water Deficit Stress

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Abstract

Water deficit is one of the most important environmental stresses that adversely affect crop growth and production and mycorrhizal fungi and symbiotic bacteria have important role in resistance to drought stress. The effect of biofertilizers on alfalfa stress tolerance was studied at the greenhouse condition. Treatments comprised three water-deficit stresses (35%, 55% and 75% of field capacity) and four seeds inoculations (*Glomus mosseae*, *Sinorhizobium meliloti*, *G. mosseae* + *S. meliloti* and non-inoculated). Water-deficit stress decrease cell membrane stability (39%), total Chl (24.05%), carotenoid (35.55%), quantum yield (50.64%) and forage yield (28.20%), while increased the proline and soluble sugars content (68.55 and 46.53% respectively) and osmotic potential (45.84%). The inoculation of seeds increased the capability of the plants in counteracting the stress, so that the production of compatible solutes was increased and the photosynthetic indices, proline, osmotic potential, membrane stability and forage yield were improved by seed inoculation. Mycorrhiza improved photosynthetic indexes and proline, but bacteria had more efficacy on membrane stability and forage yield. However, double inoculation due to the synergistic effect of mycorrhiza and *Sinorhizobium*, had the greatest effect than Solitary inoculation. Our results suggest that biofertilized alfalfa plants were better adapted than non- biofertilized ones to cope with water deficit.

Keywords: drought, *Medicago sativa*, mycorrhizal fungi, resistance, symbiotic bacteria

Introduction

Water deficit changes the pathways of some metabolic processes. This stress ceases cell development and plant growth due to the low turgor potential (Sunka *et al.*, 2003). In addition, decrease in water uptake by roots is accompanied by decrease in cell turgor resulting in lower cell division and cell growth inhibition (Yordanov and Tsonev, 2003). Low water potential in plant tissues results in stomatal closure, reduces photosynthesis, affects respiration, destroys proteins and enzymes, results in production of toxic substances, hormonal disruption and increased level of different kinds of reactive oxygen species (Shimshi *et al.,* 1992) and injures the pigments and plastids (Follows and Boyer, 1996).

Plants may use of various strategies for dealing with stresses. Protecting cell membranes and osmotic adjustment during stress is known as an important method for improving stress tolerance. Osmotic adjustment can be controlled by converting polysaccharides (starch and fructan) and oligosaccharides (sucrose and glucose) to each other (Cui *et al,* 2004). One of the most important methods for mitigating the impact of stress is using biofertilizers. Biofertilizers are preservatives abounded with beneficial soil-borne organisms as their metabolite products which are colonized around roots or internal parts of the plant and stimulate the growth of the host plant in different ways (Singh and Kapoor, 1999). The benefits of growth-inducing bacteria to plants include stimulation of the plants growth by rhizosphere bacteria through fixing atmospheric nitrogen, increasing the availability of nutrients in rhizosphere, increasing root contact area, producing growth regulators and improving beneficial symbiosis with host plant at different growth stages (Chogan, 2003). Through improving the ability of host plant in absorbing nutrients, such as N and P in particular, from unavailable resources and augmenting the activity of soil microbial community, mycorrhiza and rhizobia increase the plant hormones like auxin and enhance the water-deficit resistance (Zahir *et al.,* 2000; Barea *et al.*, 2005). Given the fact that the application of chemical fertilizers is accompanied by the pollution of soil and environment, the application of biological sources for improving the tolerance and performance of the plants can be regarded as the useful economic, social and environmental aspects of these materials.

The contribution of *Rhizobium* and arbuscular mycorrhizal fungi (AM) to soil fertility, productivity and crop yield have been well documented (Bir *et al*., 2000). The success co-inoculation depends not only on the infectivity of microbes

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but also on the compatibility of the interactions between the participants (Puppi *et al*., 1994). Compatible combinations of nitrogen fixing *Rhizobium* bacteria and AM may result in enhanced effects on plant development in the various microsymbiont legume systems (Bir *et al*., 2000).

The application of bio-inoculants - Arbuscular Mycorrhizal Fungi (AMF) and *Rhizobium* - for improving of drought-tolerant plants is one of great importance because it minimizes the production costs and environmental hazards (Javaid, 2010). Therefore, the objective of this study was determining the effect of Arbuscular mycorrhizal fungi (AMF) and *Rhizobium* (R) inoculation; individually or in combination (AMF+R) on water deficit stress alleviation in alfalfa plants, in order to improve growth, photosynthetic indices, osmotic adjustment and forage yield.

Materials and Methods

Experimental design

This study was conducted as a factorial experiment based on completely randomized design (CRD) with three replications under controlled conditions in University of Mohaghegh Ardabili, in 2014. The water limitation imposed at 35%, 55% and 75% of field capacity (FC) and seeds inoculated by mycorrhiza (*Glomus mosseae*), rhizobium (*Sinorhizobium meliloti*), double inoculation (*S. meliloti* + *G. mosseae*) and non-inoculation (as control). Field capacity was determined by gravimetric method.

The seeds were prepared from Seed and Plant Improvement Institute of Karaj, Iran. Then, seeds were inoculated with mycorrhiza (*G. mosseae*) and rhizobium (*S. meliloti*) for which the seeds were treated with 10 g inoculators per 100 g seed and Arabic Gum solution was used for improving the viscosity of inoculators to the seeds. For double inoculation, five grams of each stimulator mixed with each other per 100 g seeds. Sinorhizobium was prepared from Institute of Mehr Asia biotechnology, and mycorrhizal inoculum from Turan Institute of Biotechnology.

Photosynthetic pigments

Fresh leaf tissue was used for the measurement of Chlorophyll. 200 mg of leaf tissue was gradually grinded using 80% acetone in order to extract Chlorophyll into the acetone solution. The final volume of the solution was then brought up to 20 ml using 80% acetone. The resulted solution was centrifuged at 400 rpm for 10 min and the optical absorption of the supernatant was then read at 470, 645 and 663 nm using a spectrophotometer. Chlorophyll and carotenoids contents were obtained according to Arnon (1967).

Chlorophyll *a*= $(19.3\times A_{663}-0.86\times A_{645})$ V/100W

Chlorophyll *b*= $(19.3\times A_{645}-3.6\times A_{663})$ V/100W

Carotenoid = $(1000a_{470}-1.82C_a-85.02C_b) / 198$

Quantum yield

The quantum yield was measured by the uppermost full expanded leaf using a fluorometer (Chlorophyll fluorometer; Optic Science-OS-30 USA). So, the plants were adapted to darkness for 20 minutes by using one special clamp. Then, the PS_{II} fluorescence was measured in 1000 (μM photon m^{-2} s^{-1}), and calculation was performed using following equations (Arnon, 1949):

ØPSII = (Fm - Fo) / Fm,

where:

ØPSII was quantum yield amount of photosystem II,

Fm was maximum fluorescence after a saturated light pulse on plants adapted to darkness and

Fo was the minimal fluorescence in the light adapted, which was determined by illumination with far-red light.

Proline

For the measurement of proline, 500mg of plant fresh tissue was crushed in 10 ml sulpho acetic acid solution to obtain a homogeneous mixture. The homogenate filtrated using Whatman filter paper no. 2 and 2 ml dimenhydrinate reagent and 2 ml glacial acetic acid were added. The extract was mixed and stirred on bain-marie at 100 °C for one hour and then 4 ml toluene was added and the extract was vortexed to form two separate phases. The supernatant absorbance was read at 520 nm by a spectrophotometer (Bates *et al*., 1973).

Soluble sugars assay

To measure soluble sugars, 500 mg of leaf tissue was first completely homogenized. Then 5 ml of 95% ethanol were added and vortexed in test tube for 30 seconds. The supernatant was then centrifuged at 3,500 rpm for 15 min and used to measure the soluble sugars according to the method proposed by Ndoumou *et al*. (1996). The absorbance was measured using a spectrophotometer at 625 nm.

Osmotic potential

The osmotic potential was determined based on the electrical conductivity (Janardhan and Krishnamoorthy, 1975). One gram of fresh leaf tissue was crushed in 25 ml distilled water. Electrical conductivity (EC) was measured at 25 °C. Osmotic potential was calculated using the following equations:

DF= M × 25/W

OP= EC (in 25 °C) × 0.36 × DF / 0.987,

where:

DF is the dilution factor,

M, tissue fresh weight,

W, gram water content in one gram fresh tissue and

OP is osmotic potential.

Cell membrane

The cell membrane stability was measured by Blum and Ebercon (1981) method. Ten expanded leaves used half for treating and the rest as control. The control samples were soaked in 30 ml of distilled water and the treatments in 30 ml of 30% PEG_{6000} solution. The samples were situated for 24 hours at 10 °C. All samples were washed three times by distilled water and were soaked in 30 ml of distilled water for other 24 hours at 10 °C. Cell membrane stability (CMS) called injury was estimated from the following equation:

$I = [1-(1-T_1/T_2)/(1-C_1/C_2)] \times 100$,

where:

T_1 and T_2 are the first and second (after autoclaving) measurement of the electro-conductivity of the solutions in which the treated samples were immersed, and

C_1 and C_2 are the related values for the controls.

Forage yield

To measure forage yield, the aboveground weights of plants were oven-dried at 75 °C for 48 hours.

Statistical analysis

Statistical analysis was performed using SAS software and mean comparisons were also performed using $LSD_{5\%}$. Regression analysis and set of equations were conducted using Minitab software.

Results and Discussion

Photosynthetic indices

It was found that water stress and seeds inoculation significantly influenced photosynthetic indices ($P < 0.05$). The water restriction reduced photosynthetic indices while seeds inoculation played an important role in mitigating the impacts of stress. Severe stress (35% FC) significantly decreased Chlorophyll *a*, Chlorophyll *b*, total Chlorophyll, carotenoid content and quantum yield by 23, 24, 24, 35 and 50%, respectively compared to non-stressed. Seed inoculation improved photosynthetic indices and the application of *G. mussea* produced better results in terms of all these indices than *S. meliloti*. However, the double inoculation treatment resulted in the highest amount of Chlorophyll *a*, Chlorophyll *b*, total Chlorophyll and carotenoid content. In addition, it was revealed that combined inoculation had less effect on quantum yield, so unlike other traits, the highest quantum yield was obtained by inoculation with *G. mussea* (Table 1).

Leaf Chlorophyll content is a key parameter in determining photosynthesis and dry matter production. Chlorophylls destroy by reactive oxygen species and hydrogen peroxidase (Navari-Izzo *et al.,* 1990; Ghosh *et al.,* 2004).

The results of this study also confirmed the altering of photosynthetic indices under stress, The mycorrhizal symbiosis in rhizosphere helps the plants uptake water and nutrients including P, Fe, and K and reducing Na under stress conditions (Chen *et al.,* 2003; Soliman *et al.,* 2014). The increased absorption of micro and macro-elements under double inoculation with mycorrhizal and bacteria can supply the N and Fe deficiency caused by stress and could increase the photosynthesis and restore the production of photosynthetic pigments (Soliman *et al.,* 2014). In addition, as root hydraulic conductivity and water uptake is increased by inoculation, the turgor of cells is re-established and subsequently, their growth increased and results in greater radial absorption and photosynthesis. The higher level of photosynthesis and the accumulation of carotenoids mitigates NADPH/$NADP^+$ ratio, prevents photo-inhibition and increase quantum yield (Conming and Zang, 2000).

There were a linear relationships between Chl *a*, Chl *b* and total Chl, a quadratic relationship between Chl *a*, carotenoid and quantum yield, and total Chlorophyll had the greatest role ($R^2 = 0.983$) in predicting Chl *a*. Furthermore, Chl *b* had *a* linear relationship with total Chl, carotenoid and quantum yield among which carotenoid had the greatest impact ($R^2 = 0.949$) in predicting it. The variations of Chlorophyll *b* and total Chlorophyll were proportional to those of Chl *a* so the increase in their quantities resulted in the increase in Chl *a*, whereas the increase in carotenoid and quantum yield at lower levels depended to the increase in Chl *a* (Table 2).

Given the fact that Chl *a* plays the main role in the absorption and transfer of electrons from photosystem II to reaction centre of photosynthesis, it is the key in determining photosynthetic capacity. Carotenoids can protect light harvesting systems of photosynthesis against radical oxygen species (ROS). Also, they use oxygen in xanthophyll cycle and protect Chlorophyll against photo-oxidation (Koyro, 2006). The linear relationship between carotenoids and quantum yield, also, confirms this argument (Table 2).

Compatible solutes

Water-deficit increased proline and soluble sugars, while osmotic potential and membrane stability decreased ($P < 0.05$). Severe stress (35% FC) enhanced proline by 2.20 fold and soluble sugars 87%, but osmotic potential and membrane stability decreased 85% and 40% respectively. In addition, it was observed that inoculation of seeds with stimulators affected osmotic adjustment ($P < 0.05$). The inoculation of the seeds increased proline and membrane stability moreover, decreased soluble sugars and osmotic potential. The inoculation of seeds with *G. mussea* resulted in better performance of compatible solutes than *S. meliloti*, so that it caused the highest proline and soluble sugars content. However, double inoculation of seeds

Table 1. Effect of seed inoculation on Photosynthetic indices (Chl *a*, *b*, total and carotenoid content, F_V/F_M) of alfalfa

Treatment		Chl *a* (mg g^{-1} FW)	Chl *b* (mg g^{-1} FW)	Total Chl (mg g^{-1} FW)	Carotenoid (mg g^{-1} FW)	F_V/F_M
Irrigation schedules	Seed inoculation					
75%	Control	1.55 ± 0.04	1.61 ± 0.01	3.16 ± 0.04	16.09 ± 0.2	0.77 ± 0.02
	G. *mussea*	2.03 ± 0.03	1.70 ± 0.02	3.73 ± 0.01	17.53 ± 0.4	0.80 ± 0.00
	S. *meliloti*	1.91 ± 0.06	1.67 ± 0.01	3.58 ± 0.05	16.41 ± 0.4	0.79 ± 0.02
	S+G	2.32 ± 0.02	1.85 ± 0.00	4.16 ± 0.03	19.04 ± 0.2	0.77 ± 0.00
55%	Control	1.22 ± 0.01	1.38 ± 0.01	2.60 ± 0.02	13.08 ± 0.8	0.70 ± 0.10
	G. *mussea*	1.63 ± 0.03	1.54 ± 0.01	3.17 ± 0.04	15.23 ± 0.5	0.74 ± 0.05
	S. *meliloti*	1.64 ± 0.03	1.47 ± 0.01	3.12 ± 0.02	14.80 ± 0.4	0.74 ± 0.06
	S+G	2.06 ± 0.05	1.65 ± 0.00	3.71 ± 0.05	16.24 ± 0.9	0.78 ± 0.01
35%	Control	1.18 ± 0.02	1.22 ± 0.01	2.40 ± 0.02	10.37 ± 0.5	0.38 ± 0.21
	G. *mussea*	1.40 ± 0.02	1.38 ± 0.01	2.78 ± 0.03	13.19 ± 0.4	0.78 ± 0.00
	S. *meliloti*	1.37 ± 0.03	1.31 ± 0.01	2.68 ± 0.04	11.98 ± 0.3	0.61 ± 0.24
	S+G	1.72 ± 0.04	1.47 ± 0.01	3.19 ± 0.04	14.33 ± 0.4	0.75 ± 0.00
$LSD_{0.05}$		0.062	0.018	0.063	0.89	0.16

Values represent the average of three replicates per treatment ± SE. Means within the same column followed by the same letter are not different at $LSD_{5\%}$.

Table 2. The regression equations between photosynthetic indices

Independent	Dependent	Regression equations	R^2	n
Chl *a*	Chl *b*	y = 1.076 + (1.084x)	0.856	36
	Total Chl.	y = -0.458 + (0.666x)	0.983	36
	Carotenoid	y = 1.321 - (0.0912 x) + (0.00751 x^2)	0.831	36
	F_V/F_M	y = 2.068 - (4.523 x) + (5.324 x^2)	0.338	36
Chl *b*	Total Chl.	y = 0.458 + (0.333 x)	0.935	36
	Carotenoid	y = 0.457 + (0.071 x)	0.949	36
	F_V/F_M	y = 1.008 + (0.715 x)	0.337	36
Total Chl.	Carotenoid	y = - 2.149 - (0.0715 x) + (0.0092 x^2)	0.898	36
	F_V/F_M	y = 1.809 + (1.926 x)	0.290	36
Carotenoid	F_V/F_M	y = 7.572 + (10.17 x)	0.369	36

Table 3. Effect of seed inoculation on compatible solutes, osmotic Potential and cell membrane stability of alfalfa

Treatment		Proline (µg g -1 FW)	Soluble Sugar (mg g -1 FW)	Osmotic Potential (bar)	Membrane stability (%)
Irrigation schedules	Seed inoculation				
75%	Control	1.50 ± 0.045	3.24 ± 0.036	-10.24 ± 0.65	53.00 ± 2.00
	G. *mussea*	3.23 ± 0.030	3.04 ± 0.038	-9.51 ± 0.61	63.33 ± 1.53
	S. *meliloti*	2.71 ± 0.031	3.03 ± 0.052	-9.50 ± 0.25	69.00 ± 2.00
	S+G	4.65 ± 0.035	2.95 ± 0.045	-10.22 ± 0.07	71.00 ± 1.00
55%	Control	2.24 ± 0.032	4.55 ± 0.045	-13.35 ± 0.56	46.33 ± 1.53
	G. *mussea*	4.33 ± 0.026	3.41 ± 0.061	-9.59 ± 0.16	58.67 ± 1.15
	S. *meliloti*	3.80 ± 0.035	3.27 ± 0.021	-12.59 ± 0.21	63.00 ± 2.00
	S + G	5.62 ± 0.021	2.98 ± 0.035	-12.44 ± 0.13	67.00 ± 2.00
35%	Control	4.77 ± 0.021	6.06 ± 0.051	-18.91 ± 0.35	32.33 ± 2.52
	G. *mussea*	5.63 ± 0.023	5.27 ± 0.021	-12.92 ± 0.06	39.33 ± 1.15
	S. *meliloti*	5.16 ± 0.050	5.03 ± 0.031	-15.48 ± 0.66	42.00 ± 1.73
	S+G	7.14 ± 0.040	4.74 ± 0.032	-16.29 ± 0.28	46.33 ± 1.53
$LSD_{0.05}$		0.056	0.068	0.676	2.918

Values represent the average of three replicates per treatment ± SE ($LSD_{5\%}$).

Table 4. The relationship between compatible solutes and cell membrane stability

Independent	Dependent	Regression equations	R^2	n
Proline	Soluble Sugar	y = 84.83 - (58.18x) + (13.36x^2) - (0.979 x^3)	0.163	36
	osmotic potential	y = 0.749 - (0.276x)	0.275	36
	membrane stability	y = 5.940 + (0.0314x)	0.064	36
Soluble Sugar	osmotic potential	y = 0.098 - (0.307x)	0.736	36
	membrane stability	y = 3.75 + (0.301 x) - (0.0095 x^2) + (0.000072 x^3)	0.947	36
Osmotic Potential	membrane stability	y = 35.52 + (0.822x) - (0.0060x^2)	0.685	36

with *G. mussea* and *S. meliloti* resulted in the highest proline amount and membrane stability under both stress and optimum conditions (Table 3).

Also, regression equations revealed that the variations of soluble sugar significantly related to the proline amount under stress conditions. In addition, the correlation of proline with osmotic potential was higher than that with membrane leakage rate. Nonetheless, osmotic potential had the highest effect (R^2 = 0.736) in predicting the soluble sugars (Table 4).

The results of the present study are in agreement with those reported by Dehqanzadeh *et al.* (2008). It seems that during stress, cell water potential goes down the threshold and the synthesis of proline is enhances with increasing in proteolysis enzymes activity to increase water uptake. It is shown that the increase in proline biosynthesis with the supply of α-glutarate metabolite by sugars is possible (Ghorbanli and Niakan, 2005). Proline accumulation in stress condition is a defensive mechanism (Koocheki *et al*., 2004). So, proline accumulation protects the plant by osmotic adjustment as well as by stabilizing many functional units like complex II of the electron transport system and removal of hydroxyl radicals (Mattioli *et al*., 2009). Proline reduces cytoplasmic pH and maintains the proper ratio of $NADP^+$ / NADPH in metabolism and increase different enzymes activities (Szabados and Savoure, 2009).

A solution stabilizes the water potential of vacuoles and reduces cell osmotic potential (Masour *et al*., 2005). Moreover, results showed the decrease in osmotic potential during stress. The decrease in osmotic potential via accumulation of minerals and other solutes in cells for maintenance of turgor potential to help the cell growth and development under stress condition. Stress causes the oxidation of cell membrane fatty acids and the destruction of membrane stability through producing toxic radicals and free oxygen, as well (Wang & Huang, 2004). Sugars can cause the osmotic adjustment, membrane stability and drying proteins besides it can prevent the condensation of phospholipids by replacing the water of lipid membrane (Irigoyen *et al.,* 1992). The results of regression also confirm the close relationship between membrane stability and soluble sugars, the application of growth stimulators improved the osmotic adjustment indices resulting in greater stress tolerance. Such results have been reported in other studies, as well (Kaschuk *et al.,* 2010).

Forage yield

Forage yield was influenced by water-deficit stress and the inoculation of seeds with growth stimulators ($P < 0.05$). Water-deficit stress reduced forage yield so that the severe stress (35% FC) resulted in 27% forage loss. The application of growth stimulators increased the yield. Double inoculation gave rise to the highest forage yield and there was no significant difference between the inoculation treatments with *G. mussea* or *S. meliloti* (Fig. 1).

Regression equations showed that among photosynthetic indices, the Chl *b* and carotenoid had the highest effect in predicting forage yield. The relationship of yield with carotenoid and Chl b was quadratic. Therefore, lower levels of Chl *b* and carotenoid had more effect than their higher levels. The relationship of Chl a and total Chl and quantum yield with forage yield were linear. Among osmotic adjustment indices, soluble sugar and membrane stability had the highest impact in predicting the yield. The variation of soluble sugar and yield was of cubic kind and the variation of yield and membrane stability was as quadratic. Therefore, the increase in soluble sugar resulted in the loss of forage yield, but the higher membrane stability increased alfalfa forage yield.

Results revealed that stress decreased osmotic and turgor potential besides, since the growth and development of cell relies upon turgor, the development and the size of the cells are reduced and result in the loss of leaf area, intercepted light and photosynthesis and also result in the loss of leaf and shoot dry matter (Nilsen & Orcutt, 1996; Desuloux *et al.,* 2000). The production of compatible solutes increases the plants expense on the consumption of N and C and on the other hand, the loss of photosynthesis during stress results in yield loss. Regression results confirmed the high impact of osmotic potential, Chl *b* and carotenoid in predicting forage yield. Additionally, it was observed that greater production of proline and soluble sugar resulted in forage yield loss (Fig. 2). The application of growth stimulators mitigated the adverse effects of the stress and increased the yield by improving photosynthetic indices as well as increasing osmotic adjustment. In addition, it has been shown that as a result of inoculation, the presence of fungal hyphae resulted in the uptake of water and nutrients by plant leads to higher yield of the plant (Baon *et al.,* 1994; Soliman *et al.,* 2014).

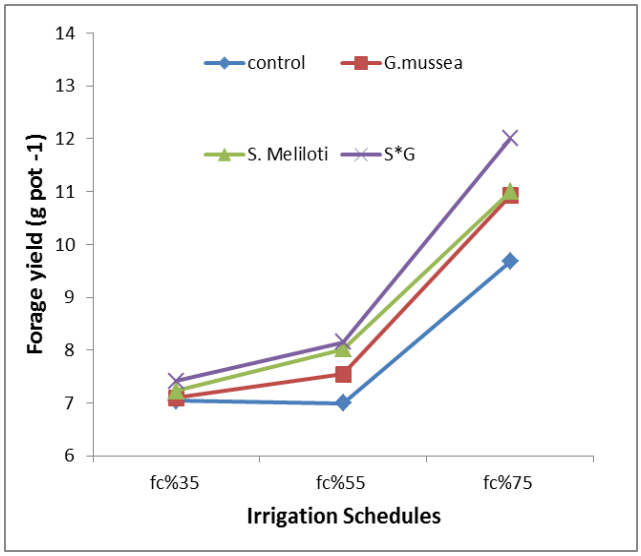

Fig. 1. Effect of seed inoculation on forage yield of alfalfa under water limitation

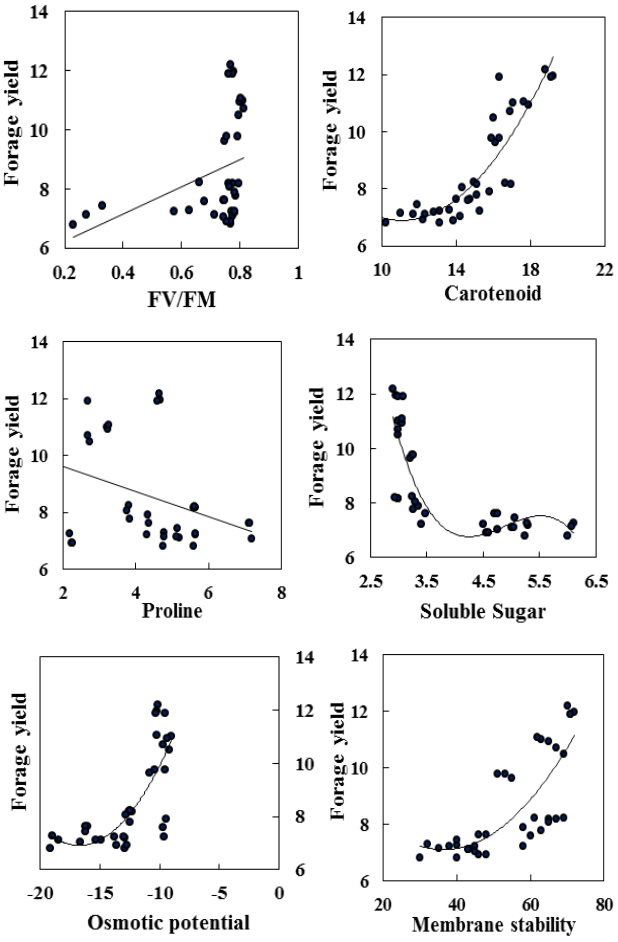

Fig. 2. Regression models forage yield changes by of alfalfa

Conclusions

It was found that water-deficit stress reduced osmotic potential, membrane stability and photosynthetic indices. The loss of osmotic potential and membrane stability as well as photosynthetic indices resulted in the loss of turgor potential and the required energy for the division and cell elongation and finally losses the forage yield. Under stress condition, plants increased their tolerance by producing adaptive metabolites (proline and soluble sugars). Also, it was observed that among the measured indices Chl *b*, carotenoid and soluble sugar had the highest effect in predicting forage yield. The inoculation of the seeds with growth stimulators improved the uptake of water by plant through forming hyphae in rhizosphere which decreased the effects of stress, improved photosynthetic indices, increased N and C sources of the plant for the production of compatible solutes and, improved the stress tolerance of the plant. The application of mycorrhiza plus rhizobia had the highest effect on measured traits owing to their synergistic relationship.

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