The effects of various levels of water deficit stress on forage yield and physiological characteristics of four cultivars of clover (*Trifolium* spp) under low input condition

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Abstract

This paper reports a study on the role of antioxidant enzymes and physiological characteristics of clover cultivars in their resistance against water deficit stress in the field conditions. Four clover cultivars namely, *Trifolium resupinatum* L., T. *incarnatum* L., *Trifolium pratense* L., and *T. alexandrinum* L. under three levels of irrigation including %35, %50, and %65 soil moisture discharge were studied as a split plot experiment based on completely randomized blocks design with three repetitions. Analysis of variance showed a significant effect of irrigation level on dry forage weight, leaf relative water content, and proline, catalase, peroxidase, chlorophyll b, and carotenoid contents. The effects of cultivars on dry forage weight, leaf relative water content, and proline, chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid contents of the plants were also significant ($P \leq 0.01$). Generally, with an increase in the percentage of soil moisture, the dry forage yield showed a significant decrease. Also, water deficit resulted in increased proline content and also increased activities of catalase, peroxidase, and superoxide dismutase antioxidants. On the other hand, no significant differences were observed between cultivars with respect to the level of antioxidant activities. In addition, the effects of soil moisture discharge on the activities of all antioxidants except for those of superoxide dismutase were significant. Moreover, increasing the percentage of moisture discharge led to the reduction in the relative water content of the leaves and chlorophyll and carotenoid contents of all four cultivars of clover under study. Finally, the study concludes with recommending *T. incarnatum* L. as the most suitable clover cultivar for the climatic condition of the study region and under low input management (low irrigation, minimum fertilizer application, no herbicide application, and no weeding).

Keywords: antioxidant enzymes, water deficit, forage, clover

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Introduction

Clover is a perennial forage plant belonging to the Papilionaceae family that has applications as fresh forage, pasture, silos, and for modifying soil components (Futsaether et al., 2009). Because of nitrogen stabilizing nodules on their rhizome, clovers play an important role in soil enrichment and fertility and their fast and dense growth coupled with the potential for multiple harvest makes it possible for them to override and destroy weeds. Therefore, these plants are considered as an important option for alternation with other crops (Vaseva et al., 2011).

Due to severe limitations in water resources in most regions of Iran, water deficit stress is introduced as the most important type of stress on crops. The majority of the provinces except Gilan and Mazandaran are classified as arid and semiarid lands with unreliable precipitation where average annual rainfall is less than evaporation. Iran generally belongs to the regions where the moisture needs of clover during its growth should be supplied through irrigation water. Since in many regions of the country the rainfall may not be sufficient and because the growth stages are generally simultaneous with hot and dry summer weather condition when the water needs of other crops are also high, it is very likely that irrigation periods are lengthened or 2-3 irrigation turns are postponed during the growth stage (Kouchaki et al., 1997).

When water is a limiting factor in production of a crop, suitable irrigation treatment is needed to achieve maximum yield for consumption of each unit of irrigation water volume. Low irrigation is one of the methods of improving Irrigation Water Use Efficiency (IWUE) (Bekele and Tilahun, 2007). The main aim of low irrigation is increasing IWUE in crops through reducing the water volume in irrigation or decreasing the irrigation turns. In recent years, the strategies for improving efficiency in agricultural practices have centered on improving the efficiency of using limited available irrigation water resources with the effect that low irrigation is becoming a quite possible option (Garg and Dadhich, 2014).

As a result of increase in non-reproducible energy costs and also their damaging biological effects, promotion of low input agriculture has grown considerably by government and non-government organizations in recent decades. The Since 1990, the United States has incorporated advising more low input and organic agricultural practices in its national programs with a view on the demands for these products in the market (Greene, 2013). Management methods are considered as an influential factor in the eventual efficiency of the farms. For example, introduction of groundcover plants and legumes in alternation with the main crops in the farm improves their yield and increases energy and nitrogen consumption efficiency. It must be noted that the introduction of fundamental changes in the main energy portals of the farm is time consuming. Also, replacement of the non-organic agricultural input has its own challenges and limitations. Therefore, increasing the efficiency of energy through low and high input management is a priority for many organizations that support the environment (Clerk et al., 2016).

Considering the increasing need of the society for healthy food and because of the limitations in water resources in the country and the high dependency of forage plants of the legume family to water, the capability of various cultivars of clover under different irrigation conditions is an important research issue. Moreover, livestock products are highly dependent upon forage plants of the legume family since legumes are an important resource for proteins in feeds. Therefore, taking into account the limitations in water resources, cutting the subsidies with a consequence of increased costs of agricultural inputs, and the increasing demands for healthy products, the need for research in this area is felt more than in the past.

Materials and Methods

This study was conducted in the research farm of Tarbiat Modares University of Tehran in a farm with an area of approximately 1000m^2 located in 35°, 74' N and 51°, 16'E. The farm was prepared for crop and in order to control the irrigation and level of drought stress, a drip irrigation system was implemented in the farm using special PVC and plastic pipes for drip irrigation and the relevant connection.

The experiment was carried out based on a split plot experiment with completely random blocks with three repetitions where the irrigation and clover cultivar treatments were considered for the main and periphery plots, respectively. The study treatments consisted of three levels of irrigation (irrigation after discharging %35, %50, and %65 available soil moisture) and four cultivars of clover including *T. resupinatum* L., T. *incarnatum L.*, *Trifolium pratense* L., and *T. alexandrinum* L. Seeds were obtained from Karaj Seed and Plant Improvement Institute. Nitrogen was supplied as urea $(NH_2)_2CO$ as a starter, 20 kg/ha for all plots. No other chemical or organic fertilizers were applied in the study. Time Domain Reflectometry method (TDR) was used for deciding on the irrigation time.

During sowing, the seeds of *T. resupinatum* L., T. *incarnatum L.*, *Trifolium pratense* L., and *T. alexandrinum* L. were inoculated with Rhizobium *legominosarum* (Bovary trifolii). Seeds were sown in late April 2015. Irrigation was done based on moisture discharge. Low irrigation started from 4-leaf stage to 6-leaf stage based on the soil moisture discharges under study and continued up to harvest stage. Each plot had four lines of m with a distance of 50 cm. The seeds sown per hectare were 20 kg for *T. resupinatum* L. and *Trifolium pratense* L., and25 kg for T. alexandrinum L. and T. incarnatum L. Twenty kg/ha nitrogen was used from the fertilizer tank as the starter. Soil profile of the study area is shown in Table 1.

In order to determine the qualitative yield of the forage, the plants were cropped from the two central lines of the plots disregarding those grown by 0.5 m of both ends which covered a total area of $2m^2$ at %20 flowering stage and the harvest was immediately weighed and the fresh forage yield was recorded in each plot per kilogram. A sample (1 kg) of the fresh harvest was then randomly selected, transported to the laboratory analysis, and dried at 75° C for 24 h in order to determine the dried weight. To determine dry forage yield, the total yield of different harvests was considered as the criterion. Farm management was implemented based on the low agricultural input. No herbicide was used and the weeds were controlled manually. Also, the RWC was measured using the method of Ritchie et al. (1990) based on the following equation:

$$RWC= [(FW - DW) / (SW - DW)] \times 100$$

Where FM is fresh leaf weight immediately after sampling, DW is the dry leaf weight after oven treatment, and SW is saturated leaf weight after immersion in distilled water.

Activities of catalase, peroxidase, and superoxide dismutase antioxidant enzymes were measured based on the methods reported by Dhindsa et al. (1981), Chance and Maehly (1995), and Laspina et al. (2005), respectively. Also, the proline content was obtained based on the method of Bates et al. (1973).

Chlorophyll and carotenoid assay

Lichtenthaler and Wellburn (1983) was used for chlorophyll and carotenoid assay. I this method, 0.25 g leaf tissue was homogenized in a porcelain mortar containing 5 mL acetone %80. The solution was then submitted to spectrophotometry and at 663, 646, and 470 nm to measure chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid contents based on the following equations (Lichtenthaler and Wellburn, 1983):

$$Chl\ a = (12.25\ A663 – 2.79\ A646),$$

$$Chl\ b = (21.21\ A646 – 5.1\ A663),$$

$$Chl\ t = Chl\ a + Chl\ b, \text{ and}$$

$$Car = (1000\ A470 – 1.8\ Chl\ a – 85.02\ Chl\ b)/198.$$

Table 1
Some physicochemical properties of the soil in depth of 60 cm

Ec ($dS.m^{-1}$)	pH	Clay%	Silt%	N%	N%	N%	P (ppm)	K (ppm)
1.2	7.67	10	18	72	0.12	1.267	36	209

Statistical analysis of the data and testing their normality were carried out using Microsoft Excel. Graphs were prepared using SAS 9.1 and the means were compared with Duncan multiple range test at P≤0.05.

Results

Dry forage yield

Results of ANOVA showed significant differences between cultivar, irrigation, and also interaction of cultivar-irrigation for dry forage yield (Table 2). Maximum dry forage yield (4411.7 kg/ha) was observed in *T. incarnatum* L. after %35 soil moisture discharge while minimum dry forage yield (406.6 kg/ha) was recorded in *T. pratense* L., after %65 soil moisture discharge. Increase in soil moisture discharge reduced yields in all clover cultivars; however, the reduction was not significant between %50 and %65 moisture discharge in *T. alexandrinum* L. (Table 3).

Relative water content

The effects of cultivars and irrigation levels on RWC were significant at P≤0.05 and P≤0.01, respectively while the interaction of cultivars and irrigation levels was not significant (Table 2). Maximum leaf RWC was recorded under %35 soil moisture discharge *T. incarnatum* L. Increase in the percentage of soil moisture discharge reduced leaf RWC in all clover cultivars under study; however, the reduction at %50 moisture discharge in *T. incarnatum* L. was not statistically significant compared to %65 moisture discharge. Also the minimum leaf RWC was observed in *T. pratense* L. under %65 moisture discharge (Table 3).

Proline content

Variations in the content of the proline were not similar in different clover cultivars and under increased drought treatment. Increase in soil moisture discharge from %35 to %65 led to a significant increasing trend in the proline content. Maximum proline content was recorded in *T. incarnatum* L. under %65 soil moisture discharge while this was not significantly different from the proline content under %50 discharge. On the other hand, minimum proline content was measured in *T. pratense* L. under %35 soil moisture discharge which showed no significant difference with cultivar *T. resupinatum* L. under %35 (Table 3).

Antioxidant enzymes activities

ANOVA showed that the effect of irrigation was statistically significant on the activities of catalase and peroxidase enzymes while it had no significant effect on the activities of superoxide dismutase. The effects of cultivars of clover on the activities of three enzymes were not significant; however, the interaction of cultivar and irrigation treatments were significant (Table 2).

T. resupinatum L. showed the highest catalase activities under %65 soil moisture discharge which was not significantly different from the %50 moisture discharge. Maximum peroxidase activity was also observed in *T. incarnatum* L. cultivar under %50 soil moisture discharge whereas the %65 moisture discharge in clover cultivar *T. incarnatum L*. had the highest activity of superoxide dismutase. Generally, increase in the soil moisture discharge led to increased antioxidant activities in all cultivars under study (Table 4).

Chlorophyll and carotenoid contents

Various levels of irrigation had significant effects on chlorophyll b and carotenoid contents while the effect of this treatment on chlorophyll a and total chlorophyll contents was not significant. Moreover, the effects of cultivars on chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid contents were significant at P≤0.01. Also, the interaction of the effects of cultivar-irrigation was significant on chlorophyll a and b contents (Table 5). The maximum chlorophyll a content (25.46 mg per fresh leaf weight) was observed under %35 moisture discharge treatment in *T. resupinatum* L. showing no significant difference from *T. incarnatum L*. under the same level of irrigation.

Table 2

Analysis of variance for irrigation and cultivars effects on yield and physiological traits of *Trifolium species*

S.O.V	Dry forage yield	RWC	Proline	CAT	POD	SOD
Replication	9685.7	49.4	72.3	506.3	11.8	2.3
Irrigation	222198.3**	983.5**	604.0**	51152.6**	250.2**	7.1ns
error a	3295.0	34.7	20.2	14.9	10.6	38.8
Species	120113.3**	155.1*	115.4*	16.1ns	53.1ns	9.1ns
I × S	30148.5**	25.9ns	14.8*	167.6*	47.0*	99.9**
error b	2431.2	21.9	15.0	0.1	0.2	2,14
CV(%)	29.11	12.76	11.37	13.49	13.43	6.28

ns,**, and *, indicate non-significant , significant at 1%, and significant at 5%., respectively.

Table 3

Comparison of mean dry forage yield, RWC, and proline content in clover species at different irrigation levels

Species	Irrigation regime	Dry forage yield (kg/ha)	RWC (%)	Proline (μg g^{-1} FW)
	35%	2678.8c	80.74c	0.1260cd
T. pratense	50%	1785.8e	70.25f	0.1292c
	65%	406.6h	61.28i	0.1296c
	35%	4020.3b	86.58b	0.1231d
T. resupinatum	50%	2140.5d	69.18f	0.1261cd
	65%	1455.9f	62.81h	0.1576a
	35%	4411.7a	88.50a	0.1269cd
T. incarnatum	50%	2152.7d	76.41d	0.1573a
	65%	741.8g	75.74d	0.1600a
	35%	2698.6c	80.52c	0.1283cd
T. alexandrinum	50%	1233.4fg	72.40e	0.1344b
	65%	777.0g	65.58g	0.1372b

Means with the same letter in each column have no significant difference (P<0.05).

The lowest chlorophyll a content was recorded in *Trifolium pratense* L. under %65 soil moisture discharge treatment. Increase in soil moisture discharge significantly decreased chlorophyll a content in all cultivars. Moreover, the chlorophyll a content in *T. resupinatum* L. and *T. incarnatum L.*, was higher than in *Trifolium pratense* L. and *T. alexandrinum* L. cultivars under all irrigation treatments and this dominance was not disturbed despite reduction under drought stress (Table 6).

T. incarnatum L. under %35 soil moisture discharge condition contained significantly higher chlorophyll b contents as compared with the other cultivars under all irrigation treatments. Because of higher level of the chlorophyll a compared with chlorophyll b, total chlorophyll showed a trend similar to that of chlorophyll a. The highest total chlorophyll content (35.83 mg per fresh leaf weight) was recorded in *T. incarnatum* L. cultivar under %35 soil moisture discharge treatment which was significantly different from other treatments. On the other hand, the lowest total chlorophyll content (13.42 mg per fresh leaf weight) was recorded in *Trifolium pratense* L. cultivar under %65 soil moisture discharge treatment. Generally, increased drought stress significantly decreased total chlorophyll content in all clover cultivars under the study.

Variations in leaf carotenoid contents were less than those of chlorophyll a, b, and total under drought stress so that with an increase in soil moisture discharge from %35 to %50 in *T. incarnatum L*. cultivar and from %50 to %65 in *Trifolium pratense* L. and *T. alexandrinum* L. cultivars, no significant decrease was observed in leaf carotenoid contents of the clovers under investigation (Table 6).

Discussion

Production of dry matter and the growth pattern are determined by the two factors of temperature and genotype (Smith, 1970). Many studies have confirmed the interaction of effects of abiotic, genetic, and agricultural factors on the variations in yields of clover cultivars (Pankiw et al., 1977; Taylor, 1985). According to the findings of the study, it could be concluded that forage yield under drought stress reduces due to many reasons including reduction in the growth period of the plants and the resulting reduction in the light they receive, reduction in photosynthesis due to chlorophyll damage, increase in antioxidant activities and consequently increase in the energy consumed by the plants to confront drought, and many other known and unknown factors. Water deficit induces different effects in cell metabolism and stimulates accumulation of chaperonins and dehydrins in cell tissue to protect it from stress conditions (Vaseva et al., 2011).

Relative water content reduced as a result of drought stress and this is also reported in *Rosmarinus officinalis* and *Triticum aestivum* under drought stress conditions (Grataniand Varone, 2004; Foulkes et al., 2007). Reduction in leaf RWC can occur as a result of decrease in percentage of the soil moisture and consequently decrease in leaf water potential due to increased drought stress. The reduction in the water content of plant tissues under drought stress can limit plant growth and incur physiological and metabolic changes in it (Mursalova et al., 2015). Sensitive cultivars produce more proline in comparison with resistant cultivars during drought stress and as a result, these differences in the proline content among various plants are because of their genetic differences and the different strategies they use against stress condition. Increased level of this amino acid under stress condition can be attributed to its probable role in osmotic regulation (Singh et al., 2000). In fact, the role of proline in resistance against drought is very complicated so that some researchers consider increased level of proline content under drought stress merely due to a damage incurred by the stress. In reality, accumulation of proline under drought stress is a general reaction that occurs as a result of proline synthesis in the tissue, inhibition of proline oxidation, and prevention of its participation in protein synthesis Pedrol et al., 2000). In a study on the effects of drought stress on antioxidant enzyme activities in clover, Antoline et al. (2010) reported that drought stress made a significant change in the activities of SOD, APX, and GR enzymes. Increase in catalase activities is also reported in alfalfa under water deficit conditions (Wang et al., 2008). The species with higher catalase activities were generally more resistant under water deficit condition and this suggests the role of catalase in the plants' resistance against oxidative stress resulting from drought (Table 4). Higher activity of peroxidase enzyme in plants depends on the mechanism of its operation in cells and their locus of production. Peroxidase not only plays a role in plants' resistance against drought through accumulation of peroxide hydrogen, but also it has a role in plant growth, suberification, cell connections, lignification, and detoxification of the toxic compounds all of which playing an important role in in increasing plant resistance against drought stress (Ahmad and Parasad, 2012). Increased activity of peroxidase in alfalfa can be attributed to its role in changing electrical conductance of roots for transferring water and nutrients (Li et al., 2010). Superoxide dismutase is a powerful antioxidant which destroys the superoxide radical and therefore, it is a primary defense layer against free oxygen radicals (Alscheret al., 2002). Research shows the increased activity of this enzyme in plant cells in response to various abiotic stresses such as drought (Badawi et al., 2003). There are also studies reporting the increased

Table 4
Comparison of mean antioxidant enzymes activity in clover species at different irrigation levels

Species	Irrigation regime	CAT (EU mg^{-1} protein min^{-1})	POD (EU mg^{-1} protein min^{-1})	SOD (EU mg^{-1} protein min^{-1})
	35%	30.37f	16.45i	6.96gh
T. pratense	50%	32.55f	20.62h	7.34fg
	65%	53.52c	35.83f	16.91c
	35%	35.43e	44.14e	8.45f
T. resupinatum	50%	80.21a	44.42e	12.13d
	65%	82.49a	47.83	24.34b
	35%	44.77d	35.07f	10.39e
T. incarnatum	50%	67.04b	25.52g	10.82de
	65%	68.38b	52.26c	26.36a
	35%	36.39e	23.05gh	5.44h
T. alexandrinum	50%	40.90de	70.85a	9.94e
	65%	44.22d	62.30b	11.99d

Means with the same letter in each column show no significant difference (P<0.05).

Table 5
Analysis of variance for irrigation and cultivars effects on physiological traits of Trifolium species

S.O.V	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Carotenoid
Replication	24.1	1.3	44.0	1361.0
Irrigation	41.7^{ns}	74.1^{**}	70.4^{ns}	132291.1^{**}
error a	83.4	7.9	124.7	163.9
Species	185.6^{**}	61.9^{**}	390.0^{**}	39786.6^{**}
I × S	16.3^{*}	25.1^{**}	112.7^{ns}	295.7^{ns}
error b	27.7	7.8	48.4	106.9
CV (%)	28.31	14.50	27.86	15.9

ns,**, and *, indicate non-significant, significant at %1, and significant %5, respectively.

Table 6
Comparison of mean chlorophyll and carotenoid content in clover species at different irrigation levels

Species	Irrigation	Chlorophyll a mg/g FW	Chlprpphyll b mg/g FW	Total chlorophiyll mg/g FW	Carotenoid mg/g FW
	35%	17.54e	6.19c	23.73e	5.84d
T. pratense	50%	14.02f	3.47f	17.49g	4.32f
	65%	10.75h	2.67h	13.42i	4.30f
	35%	25.46a	5.18d	30.64c	8.03a
T. resupinatum	50%	22.33b	4.36e	26.69d	6.27c
	65%	20.40d	4.16e	24.56d	5.01e
	35%	24.90a	10.93a	35.83a	8.11a
T. incarnatum	50%	21.60c	9.67b	31.27b	7.81a
	65%	20.27d	4.40e	24.67e	6.33bc
	35%	18.14e	6.24c	24.38e	6.67b
T. alexandrinum	50%	14.39f	5.95cd	20.34f	6.36bc
	65%	13.22g	3.33g	16.55h	6.38bc

Means with the same letter in each column show no significant difference (P<0.05).

antioxidant activities of the plants under drought stress in sunflower and alfalfa (Habibi et al., 2004; Antolin et al., 2010). In many plants, antioxidant mechanism is activated to reduce the damaging effects of free radicals resulted from the stress condition. Therefore, with an increase in the level of stress, so does the antioxidant activities of the plants. This approach shows the mechanism of plants' tolerance against stress. The more compounds effective in plants' antioxidant activities, the higher plants' capability to neutralize the harmful effects of free oxygen radicals in peroxiding lipids, proteins, and even nucleic acids and eventually the more tolerance against stress.

Chlorophyll content has always been used as a very useful criterion for evaluation of the plants' physiologic conditions. Studies have found variations in total chlorophyll content of different species under drought stress (Jiang and Haung, 2001). For example, Iannucci et al. (2002) found that leaf chlorophyll content in one-year-old clovers increased under moderate drought condition and with increase in stress level it significantly decreased. Reduced chlorophyll content under drought stress can be due to the stimulation of senescence even though the reaction of different plants would be different (Tize and Zeiger, 1991). Photosynthesis is influenced by drought stress in two ways: closing the stomata which results in CO2 deficit for chloroplast activities, and the direct effect of drought which is observed as chlorophyll damage. Reactive species of oxygen produced in plants during drought stress can cause damage in photosynthesis system and eventually decompose chlorophyll. Drought stress was reported to reduce carotenoid contents of wheat and clover (Akhkha et al., 2011; Lazaridou and Koutroubas, 2004) which confirms the findings of this study.

Conclusion

All biochemical characteristics in the study were influenced by irrigation, cultivar, and interaction of cultivar × irrigation. Soil moisture discharge increased proline contents and antioxidant enzyme activities in different cultivars of Trifolium which was dependent on the cultivar and the level of drought stress. On the other hand, with increasing soil moisture discharge, chlorophyll and carotenoid contents reduced. The findings showed that antioxidant enzymes play an effective role in the plant resistance against drought stress in clover cultivars and therefore have the potential for being utilized as biochemical indexes in clover cultivars. Comparison of the means in the characteristics under study revealed that in different soil moisture discharge treatments, cultivar *T. incarnatum L.* had the highest dry forage yield under low and moderate moisture discharge conditions whereas under high soil moisture discharge condition, *T. resupinatum* L., had the highest dry forage yield. Based on the findings of this study, regarding the limitations in water resources in the country and also consumption of less chemical inputs, determination of cultivars resistant to low water conditions is of great significance in conservation of the ecosystem.

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