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Research article

Physiological adaptations of lucerne under a limited irrigation system

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Abstract

A field study was conducted to identify the physiological tolerance mechanism of lucerne to limited irrigations. Lucerne needs irrigation once in 10 days. So, limited irrigation treatment was imposed once every 25 days. In the initial part of the experiment, accessions collected from the western part of India were screened for biomass potential under limited irrigation in an augmented RBD design. Accessions that performed well under these conditions were grown under temporal isolation, and seeds were collected. In the second part, a field experiment was conducted at SRRS, ICAR-IGFRI, Dharwad, from December 2018 to March 2020 to screen lucerne accessions under limited irrigation to identify the physiological and morphological characters that are required to impart tolerance in lucerne under the cut and carry limited irrigation system. An RBD design was adopted to screen 12 lucerne accessions under limited and full irrigated conditions. Accessions that maintained good persistence under limited irrigation also performed significantly (Acc38/2010, Acc23/2010, A2) regarding yield. Persistence during the juvenile phase depended on conductance, osmotic adjustment and antioxidant capacity. Accessions showed a constant increase in the number of branches after each harvest under limited irrigation was promising in yield performance. Specific-leaf weight (SLW), crude protein, conductance, increased number of branches, and persistence were significantly and positively correlated with yield.

Keywords: Antioxidants, Biomass, Juvenile phase, Limited irrigation, Persistence

Introduction

Lucerne (*Medicago sativa* L.), the queen of fodder crops, is a widely cultivated forage crop with significant economic value (Ghosh *et al*., 2022; Kumar *et al*., 2023). In India, lucerne occupies an area of over 1 million ha with a productivity of 60 to 130 tons per ha. Nutritionally, lucerne is known to have 65 to 72% digestible carbohydrates and about 12 to 24% protein; this quality is maintained nearly constant throughout the year (Ferreira *et al*., 2015). Lucerne also contains minerals like phosphorus, magnesium, calcium, and vitamins like A and D. Lucerne has lower digestible carbohydrates than other grasses. However, the total energy content is high, which helps farmers save on concentrates. Lucerne responds very well to nutrients and irrigation (Patan and Kamble, 2014). Moisture stress, a temporary deficiency in soil moisture in the crop root zone or a short-term reduction of soil moisture, causes a decrease in growth and development. Moisture stress reduces leaf size, stem extension, and root proliferation, disturbs plant water relations and reduces water-use efficiency and CO_2 assimilation by leaves. Lucerne is a perennial plant that can grow for over three years. If rainfall and irrigation are deficient, the plant suffers from moisture stress, which reduces plant population and yield. Indian Grassland and Fodder Research Institute (IGFRI) continually strives to identify abiotic and biotic tolerant lucerne varieties through pre-breeding and breeding initiatives. Germplasm accessions were collected from important lucerne growing areas like Rajasthan, Maharashtra, and Gujarat to assess its capacity to perform under limited irrigation conditions (Antony and Sridhar, 2014). Water stress also makes plants susceptible to biotic stress (SateeshKumar *et al*., 2022).
In India, lucerne is grown mainly for the cut-and-carry system. In contrast, genotypes suited for pasture may not be successful under the cut-and-carry system as the physiology of lucerne varies under the management system. Lucerne is perennial in growth habit, has relatively high water demand and is exposed to periodic harvesting (Singh *et al*., 2007). It is grown on irrigated

lands to realize its full fodder potential. Irrigation once in 10 days (recommended) is required under Indian conditions. With dwindling water resources, lucerne needs irrigation more frequently, which is becoming a significant constraint for its cultivation. This study aimed to understand the physiological responses to limited irrigation in lucerne, identify suitable varieties that could yield high biomass under limited irrigation systems, and dissect the tolerant characteristics of accessions under limited irrigation for a cut-and-carry system. Screening for limited irrigation was conducted under challenging situations in lucerne, *viz*., 1) Repeated harvesting schedule once in 25 days that affects root growth, 2) Juvenile stage of the crop that coincided with the non-rainy season that affects persistence.

Materials and Methods

Study area and plant materials: The experiment was conducted at Southern Regional Research Station, ICAR- IGFRI, Dharwad (15.4912° N, 74.9852° E, MSL of 720 m) from December 2018 to March 2020. The rainfall occurred during sowing in December 2018; there was no rain in December, January, February and March 2019. The maximum temperature fluctuated on an average of 34 to 37°C, and the minimum temperature was 18 to 21.5°C. The soil was clayey loam with a neutral pH. Two sets of plants were maintained in the field for all 12 lines. One set was irrigated once in 10 days (Full irrigation, FI) and the other once in 25 days (limited irrigation, LI). The different generations and phenotypes from the crosses between checks (RL-88, A2) and the Weevlchek (WC) were A2xWC (T-5), A2xWC (D-5), RL-88xWC (T-4), RL-88xWC (D-4), A2xWC (T-4) and A2xWC (D-4). The local checks used in this experiment were Anand-2 (A2) and RL-88. The four promising accessions were selected from (77 indigenous germplasm lines collected from Maharashtra and Rajasthan) a drought germplasm screening experiment conducted from November 2011 to May 2013, *viz*. RAJ 22, Acc. No. 45/2010, Acc. No. 23/2010 and Acc. No. 38/2010 (Antony and Sridhar, 2014) was included in this study. The experiment was laid out in RBD with three replications. During the study, average soil moisture (determined gravimetrically) was 40 to 42% and 4 to 6.3% (average) in full irrigated and limited irrigation conditions, respectively. Above-ground biomass was an indicator for selecting efficient genotypes under limited irrigation. Above-ground biomass under frequent harvesting was a cost-efficient and practical approach for selecting promising accessions with high biomass (Annicchiarico *et al*., 2015).

Morphological observations: The number of branches (second and third harvests) and number of plants (first and third harvests) was expressed as persistence percent of initial and final plant intensity per meter 2. The fresh and dry weight of the top portion of the plant was recorded and expressed as tonnes per hectare per cut at 25 to 30-day intervals.

Physiological observations: The membrane stability index (MSI) was calculated per the protocol of Heath and Packer (1968). The osmolytes in the sample were quantified using the vapor pressure osmometer (WESCOR, VAPRO model 5600). Osmotic adjustment (OA) was calculated as the difference between the osmotic potential at full turgor of control and stressed plants (Babu *et al*., 1999; Nounjan *et al.,* 2018). The phenol sulfuric acid method was used to estimate total sugars at the second harvest (Dubois *et al*., 1956). Photosynthesis and conductance were measured using IRGA LI6400XT and a fluorescence leaf chamber with an area of 2 cm^2. LI6400 had an open system. Fv/Fm, the maximum or optimum quantum yield, was measured in the dark-adapted leaves.

Biochemical and quality parameters: The catalase enzyme (EC) activity was estimated and calculated using the molar extinction coefficient of H_2O_2 (0.0436 mM cm^{-1}) and expressed as µmol H_2O_2 oxidized per minute per ml of the enzyme (Shukla *et al.,* 2017). Peroxidase (EC. 1. 11. 1.7) activity was determined spectrophotometrically by measuring the increase in absorption at 436 nm (Mahadevan and Sridhar, 1982). The activity of superoxide dismutase (SOD, EC.1.15.1.1) was assayed in leaf tissue of lucerne accessions from the measurement of inhibition of photochemical reduction of p-nitro blue tetrazolium chloride (NBT) spectrophotometrically at 560 nm following the method of Madamanchi *et al.* (1994). According to Sahoo *et al*. (2017), total glutathione was estimated. Crude protein content was calculated by multiplying the percent N of the sample by 6.25. It was expressed as percent values (Asija *et al*., 1956).

Statistical analysis: The data collected from the experiment were subjected to RBD statistical analysis in the WASP-2 (Web Agri Stat Package 2.0) statistical package hosted by Central Coastal Agricultural Research Institute CCARI, GOA (www.ccari.res.in). The significance levels used in the F and t-tests were $p = 0.05$. The least significant difference (LSD) values were calculated wherever the 'F' test was significant, denoted by Duncan's Multiple Range Test (DMRT).

Results and Discussion

Morphological characteristics: In this study, the selection of lucerne for water deficit conditions was conducted during the winter season when temperature, RH and rainfall were significantly lower than at other times of the year. The winter growth in lucerne allowed for use as a selection criterion for biomass under stress (Annicchirico, 2007). In India, lucerne sowing is initiated after the monsoon recedes, as lucerne is waterlogging intolerant, especially at the juvenile phase (Smethurst and Shabala, 2003). Only one life-saving irrigation once in 25 days was provided for the crop. Under the water deficit stress, there was a severe loss in the plant population. In lucerne, the number of plants surviving per unit area or persisting after each harvest indicated the biomass potential of the genotype. A lack of tolerance reduced the plant population under water deficit conditions after every harvest (Luo *et al*., 2020). Plant mortality eliminated less vigorous and less adapted accessions under water deficit conditions (Annicchiarico *et al*., 2015). Increased persistence provided a high long-term yield in lucerne under stress (Annicchiarico *et al*., 2015). Our study observed accessions with less than 100% persistence under water deficit conditions for four months after sowing. The accessions with high persistence ultimately produced very high biomass. In the germplasm screening experiment conducted during 2011-2013, it was observed that Maharastra accessions performed better and showed more persistence during the second year, while the Rajasthan accession population decreased in the second year with a reduced yield (Fig 1). The biomass accessions that performed better over the years originated in western regions of India, like Gujarat and Maharashtra, where monsoons (*kharif* and *rabi*) and summer showers are expected. Landraces of lucerne are adapted to the environmental conditions under which they evolved (Annicchiarico *et al*., 2015). The persistence percentage was highest in ACC 38/2010 and Anand-2 under limited irrigation conditions; genotypes from western India *viz*. Maharashtra and Gujarat. In our study, biomass had a significant positive correlation (0.59*) with persistence. A similar result was obtained in five lucerne accessions grown in drought-prone Hmadna regions of Italy (Annicchiarico *et al.,* 2013).

The yield of the top three performing accessions (Anand-2, ACC 45/2010 and ACC 23/2010) correlated significantly with the number of branches. There was a constant increase in the number of branches in these accessions after each harvest. The average number of branches was high in Anand 2 and crossed A2xWC (D-5) (Fig 2). The number of branches is a vital yield-determining factor in lucerne. A similar result was reported by Vinodkumar *et al.* (2023). Type I and type II branches in lucerne bear leaves and increase biomass yield. A variety having more branches or a tendency to increase the number of branches after every harvest ultimately produces more leaves and biomass yields under limited irrigation (Gosse *et al*.,1988; Baldissera *et al*., 2013). In this study, an increase in the number of branches had a significant positive correlation with fresh and dry biomass (r = 0.78**). The number of stems per plant also significantly correlated with yield under stress conditions in Italy (Annicchiarico *et al*., 2013).

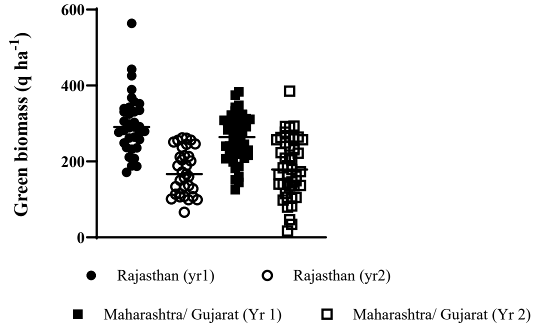

Fig 1. The difference in biomass in the indigenous collection from Rajasthan and Maharashtra/Gujarat during the first and second years under limited irrigation

Physiological parameters: Several physiological parameters also determine the tolerance to water deficit stress. SLW indicates the leaf weight per unit leaf area. In limited irrigation conditions, the highest SLW was recorded in ACC 45/2010 (3.95 mg cm^{-2}), followed by RL-88 and Raj 22/2007, whereas the least SLW (3.41 mg cm^{-2}) was in A2 × WC (T-5). The smaller the leaf area, the fewer the stomata and the more water use efficiency (WUE). This attribute was observed in the top three performers, where SLW significantly correlated with conductance (0.603*). The membrane stability index (MSI) is a vital physiological parameter that indicates the injury caused to cell membranes and the leakage of electrolytes from the cell. There was a significant difference in MSI among accessions compared to limited and full irrigated conditions (Fig 2). Among the accessions, ACC 23/2010 showed a maximum decrease in MSI under limited irrigation (47.58%) compared to fully

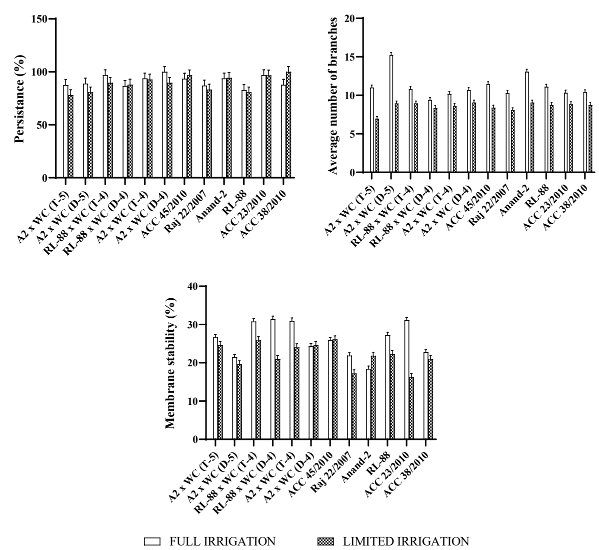

Fig 2. Persistence (%), average number of branches and membrane stability of lucerne accessions under full irrigation and limited irrigation. The vertical bars denote the standard error

irrigated conditions. The MDA content was also higher in limited irrigation. Indeed, some lipid peroxidation was unavoidable during water deficit stress (He *et al*., 2012). Still, the increase was less in the tolerant varieties.

The photosynthesis rate declined under limited irrigation conditions (Table 1). The chlorophyll stability index (CSI) in all accessions recorded higher values in limited irrigation. The CSI was significantly correlated to growth parameters like biomass, the number of branches and persistence. The increased chlorophyll stability might be the reason for the non-significant decrease in photosynthesis under limited irrigation.

A non-significant decrease in conductance among all the accessions under limited irrigation was observed except in Anand-2 and RL-88 (Table 1). The number of open stomata on the leaf surface was indicated by stomatal conductance. Conductance decides the WUE as stomata are the path for entry of CO_2 and water. Water extraction from soil layers below 2.5 meters occurs when the relative water content (RWC) and stomatal conductance drop below 17% and 0.3 cm per second in lucerne growing under pasture conditions. The RWC in the accessions varied between 35 and 50% under limited irrigation, and it was higher than the local check Anand 2. Several lines recorded conductance above 0.3 in this experiment, which yielded high biomass. Under limited irrigation, conductance was significantly correlated with biomass in lucerne accessions (r = 0.685*). Many accessions had less than a 25% decrease in biomass compared to Anand 2, and some genotypes had a decline of more than 50% (Table 2).

Conductance recorded a negative correlation with osmotic adjustment (OA). Osmotic adjustment indicates the capacity of genotypes to maintain tissue water potential by accumulating compatible solutes. The highest osmotic adjustment was recorded in Raj 22/2007 (2660.66 m moles kg^{-1}), followed by A2 ×WC (D-4) and ACC 38/2010. The Osmotic adjustment was significantly correlated with photosynthesis, indicating the requirement of osmotic adjustment to keep the photosynthetic machinery running under water deficit conditions. The relationship was significantly robust in the top three performers (Anand-2, ACC 45/2010 and ACC 23/2010; Table 2). Tolerance to water deficit stress in varieties from Mediterranean regions was attributed to OA (Slama *et al*., 2011). The capacity to adjust osmotically when the plant experiences water stress is an environmentally controlled inherited trait (Zhang *et al*., 1999). The osmotic adjustment was cultivar-specific and an energy-demanding physiological process (Munns *et al*., 2020). The energy needs to be shared between several physiological processes like growth and osmotic adjustment under limited irrigation. So genotypes with high biomass potential can only share the energy required for osmotic adjustment and grow, survive and persist under water deficit conditions. Soluble sugars also act as osmoticum. Soluble sugars are necessary for combating different abiotic stress (Sami *et al*., 2016). In our study, soluble sugars decreased under limited irrigation, and the decrease was smaller in tolerant accessions. There was an increase in soluble sugars in many genotypes compared to local check, Anand 2.

Table 1. Influence of moisture stress on photosynthetic parameters in lucerne accessions

Accessions	**Photosynthesis (μ moles $CO_2 m^{-2} s^{-1}$)**		**Conductance (moles of H_20 $m^{-2} s^{-1}$)**		***FV/Fm***		**Chlorophyll stability index**	
	FI	**LI**	**FI**	**LI**	**FI**	**LI**	**FI**	**LI**
A2xWC (T-5)	23.62	11.89	0.40	0.19	0.82	0.79^{c}	59.93^{d}	77.19^{ab}
A2xWC (D-5)	25.27	22.28	0.56	0.30	0.82	0.82^{ab}	61.62^{cd}	81.56^{a}
RL-88xWC (T-4)	25.94	10.16	0.47	0.19	0.82	0.82^{ab}	64.19^{bcd}	72.42^{bc}
RL-88xWC (D-4)	28.53	24.04	0.49	0.37	0.82	0.82^{ab}	69.00^{b}	67.71^{cd}
A2xWC (T-4)	21.66	18.45	0.33	0.31	0.82	0.83^{ab}	69.10^{b}	83.73^{a}
A2xWC (D-4)	26.44	21.25	0.51	0.36	0.82	0.82^{ab}	62.97^{bcd}	61.66^{d}
ACC 45/2010	25.86	16.10	0.49	0.27	0.82	0.81^{b}	69.60^{b}	79.31^{ab}
Raj 22/2007	25.77	14.28	0.37	0.19	0.82	0.83^{ab}	77.80^{a}	87.56^{a}
Anand-2	31.66	22.76	0.56	1.39	0.83	0.84^{a}	68.25^{bc}	72.87^{bc}
RL-88	23.45	21.76	0.33	0.37	0.81	0.82^{ab}	60.69^{d}	62.05^{d}
ACC 23/2010	25.29	21.62	0.51	0.31	0.83	0.83^{ab}	58.47^{d}	67.80^{cd}
ACC 38/2010	26.23	23.15	0.47	0.38	0.81	0.82^{b}	59.47^{d}	60.85^{d}
SEM	0.88	1.38	0.02	0.10	0.001	0.005	2.31	2.37

FI: Full irrigation; LI: Limited irrigation; Soil moisture level during the observation was 39.51% in FI and 3.85% in LI

Table 2. Percentage increase/decrease in physiological parameters in lucerne genotypes vs local check Anand-2

Genotypes	Fresh weight	SLW	RWC	Osmotic adjustment	Sugars	MDA
A2xWC (T-5)	-71.6	-8.57	19.52	-28.75	-21.05	-5.11
A2xWC (D-5)	-55.0	-8.57	10.85	-47.12	38.5	-13.12
RL-88xWC (T-4)	-56.8	-3.75-	18.38	-58.00	42.32	-3.31
RL-88xWC (D-4)	-62.9	-2.68	26.27	-21.89	-6.19	-9.94
A2xWC (T-4)	-55.8	-2.4	9.16	-23.33	-1.76	-3.31
A2xWC (D-4)	-28.6	-1.07	-12.90	32.57	5.87	-1.38
ACC 45/2010	-24.4	5.89	33.78	-30.14	20.34	-2.90
Raj 22/2007	-61.0	1.07	6.574	35.23	70.31	5.38
ACC 23/2010	-16.5	1.072	14.11	-36.21	63.12	1.65
ACC 38/2010	-26.1	1.072386	26.22	16.72	58.88	-3.31

Fluorescence or F_v/F_m is another crucial physiological parameter that helps lucerne to have a high water deficit tolerance. The genotype Anand-2 recorded the highest (0.84), and cross A2 × WC (T-5) recorded the lowest (0.79) fluorescence values under limited irrigation conditions (Table 1). The top three performing accessions had a significant correlation between fluorescence and biomass. Similar reports were observed in the drought-tolerant variety Longdong when subjected to drought (Zang *et al*., 1999). Under water-deficit stress conditions, electron transport activity and photosynthetic apparatus were less damaged in drought-tolerant lucerne varieties (Han *et al*., 2007). Among the physiological parameters, conductance, SLW and osmotic adjustment played an essential role in biomass production under limited irrigation.

Biochemical and quality parameters: Fresh biomass in lucerne is compromised if the accession does not have other mechanisms to combat the water deficit stress other than extending the roots. Extending the roots under limited irrigation under the cut-and-carry system decreases biomass and persistence, especially during the juvenile phase. So, tolerant mechanisms other than the extended root system are significant under limited irrigation.

The antioxidant enzyme system is another tolerance-imparting mechanism that differs in expression patterns under full and limited irrigation. Reduced glutathione detoxifies various electrophilic compounds by degenerating essential antioxidants such as vitamins E and C. Among all accessions, glutathione content was reduced in limited irrigation conditions compared to fully irrigated conditions (Fig 3). Catalase is an antioxidant enzyme that effectively removes the excess H_2O_2 and protects the membrane. There was a decrease in catalase enzyme activity in all accessions at limited irrigation compared to full irrigation conditions. In limited irrigation conditions, Raj 22/2007 showed the highest peroxidase enzyme activity and was at par with A2 × WC (D-5) and Anand-2. The lowest peroxidase enzyme activity was recorded in ACC 45/2010. In general, there was a decrease in peroxidase enzyme activity in limited irrigation conditions. SOD enzyme activity increased in limited irrigation conditions. Anand-2 (6.38 units mg^{-1} of protein) expressed the highest enzyme activity in limited irrigation conditions. The activity of these enzymes was high in the tolerant accessions, indicating the work of antioxidants to maintain cell function and resulting in high WUE (He *et al*., 2012).

Among the quality parameters, lucerne is well known for its crude protein content. Under full irrigation, lucerne accessions recorded 22 to 29% of crude protein. However, the water deficit caused by limited irrigation decreased the crude protein content in several accessions. ACC 45/2010 and A2 x WC (D-4) recorded significantly less decrease (less than 15% decrease) compared to its fully irrigated treatment. Crude protein was significantly correlated with biomass (Fig 4). Reduction in crude protein in lucerne due to water deficit stress was also observed by Liu *et al*. (2018). In lucerne, nitrogen fixation is the primary source of N, and lucerne fixes 10 to 30% higher nitrogen than other legumes (Gierus *et al*., 2012), resulting in a higher crude protein than other legumes. Thus, the degree of nitrogen fixation determines the availability of N for protein production. Nitrogen availability for protein production also depended on water availability in lucerne (Liu *et al*., 2018). The decrease in CP content under limited irrigation could be attributed to a combined stress response to water limitation and a reduction in N fixation.

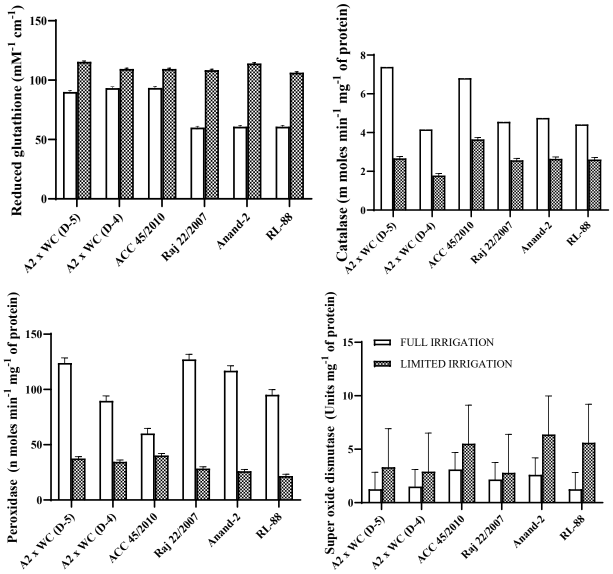

Fig 3. Influence of moisture stress on reduced glutathione, catalase, peroxidase and superoxide dismutase enzyme activity in *Medicago sativa* L. The vertical bars denote the standard error

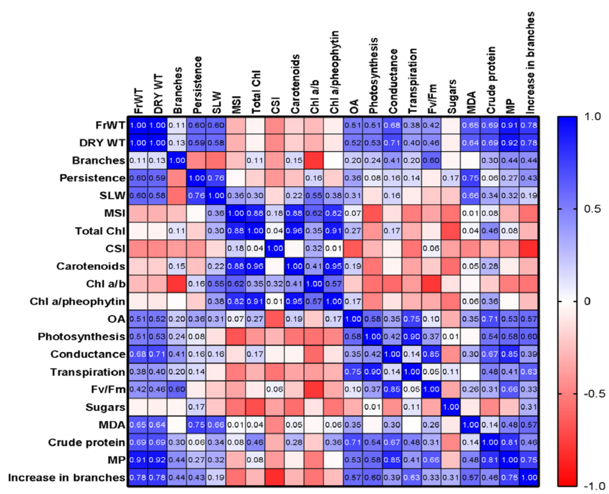

Fig 4. Heat map of Pearson's correlation coefficients of morphological, physiological and biochemical parameters calculated for lucerne accessions ranked in descending order according to biomass under limited irrigation; N = 8; at 5% p = 0.63 and 1% p = 0.76; SLW: Specific leaf weight; MSI: Membrane stability index; CSI: Chlorophyll stability index; OA: Osmotic adjustment; MDA: Malondialdehyde content; MP: Mean productivity

Conclusion

It was clear from this study that lucerne, being a perennial crop, exhibits varying growth characteristics related to genotype, environment and management practices. Under the irrigated cut and carry system, the accessions with a long root system establish and flourish well. However, persistence is the most crucial factor for higher yield, as a long root system is not beneficial under limited irrigation. Genotypes with a tolerance mechanism, especially during the juvenile phase, are necessary for limited irrigation systems. Accession Raj 22/2007, in spite of high SLW and OA, had very low biomass compared to the local check. This variety had a decreased antioxidant system, high lipid peroxidation and low conductance, leading to less photosynthesis and poor persistence. Tolerance mechanisms like osmotic adjustment, low conductance, SLW and increased fluorescence combined with a steady increase in a number of branches, leaves and biomass, along with the juvenile stage antioxidant system, are favorable characteristics for a genotype in a limited irrigation cut and carry management system. The accessions (Anand 2, Acc 45/2010 and Acc 23/20210) identified in this study could be used as parental material for developing more tolerant genotypes.

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