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Multitraits evaluation of Pakistani ecotypes of berseem clover (*Trifolium alexandrinum* L.) under full-irrigation and water restriction conditions

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Summary

Berseem clover (*Trifolium alexandrinum* L.) is an important forage crop in Pakistan and many ecotypes are grown across the country. Its yield is however frequently affected by insufficient irrigation due to unavailability of water. In the present study, twenty Pakistani ecotypes of berseem clover have been evaluated in lysimeters under full irrigation and water restriction conditions. In the full irrigation treatment soil humidity was maintained at field capacity, while in the water restriction treatment water was only supplied after severe wilting and to maintain humidity in the deep profile of the soil. Assessed traits included forage yield, calculated as the sum of the biomass harvested at 70 and 110 DA days after emergence, and morpho-physiological traits. Significant effects of water restriction were noted on yield, leaf gas exchange parameters, canopy temperature and osmotic adjustment. Most morpho-physiological traits had higher broad sense heritability than forage yield, both under full irrigation and water restriction conditions. Water restriction increased genetic and phenotypic variability and heritability of most traits under study. Under these conditions forage yield was positively associated to leaf temperature and recovery rate index and, under full irrigation, to net photosynthetic rate, canopy depression temperature and leaf area. The possible use of these traits as indirect selection criteria in berseem clover breeding programs is discussed. Some ecotypes with favorable traits such as high forage yield potential, good adaptation to water restriction and aptitude to multiple harvesting have also been identified.

Introduction

Trifolium alexandrinum L. (berseem clover or Egyptian clover) is one of the most important leguminous forages in the Mediterranean region and the Middle-East (Sardana and Narwal, 2000; El-Bably, 2002; Iannnucci, 2002; de Santis et al., 2004). It contributes to soil fertility and improves soil physical characteristics (Graves et al., 1996) and its forage is superior to grasses in protein and mineral contents (Laghari et al., 2000). Berseem clover originated in Syria and was introduced into Egypt in the 6th century (Hannaway and Larson, 2004), India in the 19th century and Pakistan, South Africa, the USA and Australia in the 20th century (Heuzé et al., 2014). It is grown in the semi-arid regions of the world under pure stand and crop mixture (Martinello and Iannucci, 1998; Vasilakoglou and Dhima, 2008). Limitation of water supply is a major production constraint for this crop (Iannucci et al., 2000; Lazaridou and Koutroubas, 2004).

Studies concerning the evaluation of tolerance to water restriction are scarce in berseem clover. Iannucci et al. (2000) reported a significant reduction in total dry weight, plant height and proline content due to water stress treatments. Lazaridou and Tsiridis (2004) noted a 75% decrease in biomass, leaf area and transpiration rate as a result of water restriction. Evaluation and selection of berseem clover germplasm in a specific environment have generally concerned small sets of ecotypes and have been based on a single or a reduced number of traits (Iannucci et al., 2000; Lazaridou and Koutroubas, 2004).

The present experiment compared the yield of a large set of berseem clover ecotypes originated from different regions of Pakistan under full irrigation and water restriction conditions. As in the semi-arid environment and under limited irrigation the berseem plant has often to rely on moisture stored in a deep profile of soil to maintain its growth, the water restriction treatment consisted in maintaining humidity only in the deep profile of the soil. The ability of the different ecotypes to utilize the stored soil moisture content and maintain its yield under these specific water restriction conditions was evaluated using a multiplicity of traits including canopy temperature (and its response to variation in air temperature), gas exchange parameters, osmotic potential, chlorophyll and carotenoid contents, water use efficiency, recovery rate index and wilting rate index, as well as calculating a drought resistance index on the basis of comparisons between the two treatments. The genetic variability and heritability of the assessed traits and their relation with yield were also examined, in order to identify potential criteria for the further selection of ecotypes under stored moisture stress.

Materials and methods

Plant material

Twenty Pakistani berseem clover ecotypes were provided by the National Agriculture Research Council and the Forage Research Institute of Faisalabad, Pakistan. Out of them, 17 originated from Punjab, one from Balochistan, one from Khyber Pakhtunkhwa and one from Singh Provinces (Tab. 1).

Experimental conditions

The experiments were conducted in lysimeters of 60 cm diameter and 40 cm depth. Field conditions are known to exploit full yield potential of the ecotypes but make difficult to ensure uniform moisture storage and avoid confounding factors such as soil heterogeneity or presence of multiple stress factors (Araus and Cairns, 2014). Conversely, the use of lysimeters facilitates better control in the application of uniform treatments (Masuka et al., 2012). Each lysimeter was filled with 50 kg of dry soil with an equal quantity of silt and loam. Soil fertility was increased by adding 5% of organic matter. The field capacity of the soil, measured using the gravimetric method, was 14% by weight. The seeds were inoculated before sowing with 5 µL of rhizobium bacterial suspension (*Rizobium leguminosarum* bv. *trifolii*). The experiment was conducted in a completely randomized design with three replications and two factors, i.e. ecotypes (20) and water regimes (2). The two contrasting water regimes consisted in a full irrigation and a water restriction regime. In the full irrigation regime, soil was flooded when the moisture level fell below the field capacity of the soil, while the water restriction regime was created by irrigating the crop when severe wilting (90%)

Tab. 1: List of ecotypes of berseem clover (*Trifolium alexandrium* L.) used in the study

Number	Ecotype	Origin	Source
1.	L-94	Punjab, Pakistan	Forage Research Institute, Faisalabad, Pakistan
2.	Anmol	Punjab, Pakistan	Forage Research Institute Faisalabad, Pakistan
3.	P-209	Punjab, Pakistan	Forage Research Institute Faisalabad, Pakistan
4.	BerseemQueta	Balochistan, Pakistan	National Agriculture Research Council, Pakistan
5.	Sandal Bad	Punjab, Pakistan	Forage Research Institute Faisalabad, Pakistan
6.	Agaiti	Punjab, Pakistan	Forage Research Institute Sargodha, Pakistan
7.	SB-12	Punjab, Pakistan	Forage Research Institute Faisalabad, Pakistan
8.	Berseem Peshawar	KPK, Pakistan	National Agriculture Research Council, Pakistan
9.	Berseem Tandojam	Sindh, Pakistan	National Agriculture Research Council, Pakistan
10.	Pachati	Punjab, Pakistan	Forage Research Institute Sargodha, Pakistan
11.	Pak Berseem	Punjab, Pakistan	National Agriculture Research Council, Pakistan
12.	Punjab Berseem	Punjab, Pakistan	National Agriculture Research Council, Pakistan
13.	Chenab	Punjab, Pakistan	National Agriculture Research Council, Pakistan
14.	Samarkand	Punjab, Pakistan	National Agriculture Research Council, Pakistan
15.	L-48	Punjab, Pakistan	National Agriculture Research Council, Pakistan
16.	SK-1	Punjab, Pakistan	National Agriculture Research Council, Pakistan
17.	Super Berseem	Punjab, Pakistan	Forage Research Institute Faisalabad, Pakistan
18.	P-22	Punjab, Pakistan	National Agriculture Research Council, Pakistan
19.	SB-10	Punjab, Pakistan	National Agriculture Research Council, Pakistan
20.	Sandal bar	Punjab, Pakistan	Forage Research Institute Faisalabad, Pakistan

was observed for each ecotype, through a 3 cm diameter pipe allowing storing the moisture in the lower profile of the soil (30-40 cm). The quantities of water supplied to each lysimeter were 16.5 L in the full-irrigation regime (11 irrigations) and 4.5 L in the water restriction regime (3 irrigations). The lysimeters were covered with a plastic tunnel to avoid chilling stress or damage due to frost during the night and maintain the optimum temperature for growth (25±3 °C) during the day. Relative humidity was around 40% and photon flux density was 600 µmol m^{-2} s^{-1} during the peak photosynthesis hour. Air temperature and humidity in the tunnel were registered at regular intervals using a digital thermometer and a hygrometer.

Thinning was carried out to keep the plant population close to 100 plants per lysimeter. During the entire crop growth cycle, weeds were removed manually and no herbicide or pesticide was applied. The different ecotypes were regularly evaluated for insect and disease, and no attacks were identified during the entire crop cycle.

Soil and plant trait measurements

A soil sampler with a coring cylinder of 3.8 × 10 cm was used to collect soil samples at various depths (10-40 cm) in each lysimeter. The samples were quickly transported to the laboratory without exposure to air. The soil sample was oven-dried at 60 °C (ED-115, Binder, Tuttlingen, Germany) to determine soil moisture content at constant weight.

All ecotypes were harvested at 70 and 110 DAE. For each harvest, fresh biomass (FB) was measured on a digital balance (GW 6202, Sartorius, Germany). Plants were then dried in a heating oven (ED-115, Binder, Tuttlingen, Germany) at 70 °C for 72 hours to determine dry biomass (DB). Forage yield was estimated as the sum of biomass of the two harvests (KRENZER et al., 1992). Leaf area (LA) was measured on well expanded leaves at 70 days after emergence (DAE), using a leaf area meter (CI-202, Camas, USA).

Canopy temperature was assessed at 60 DAE using a hand-held infrared thermometer (model IR-AHT, Chino Co., Tokyo, Japan) at a uniform height (1.5 m), angle (60°) and distance between the thermometer and the target. Measurements were made in windless conditions during the afternoon. Each measurement was repeated three times. The air temperature was subtracted from the canopy temperature to determine the canopy temperature depression (CTD).

Net photosynthesis rate (P_N), transpiration rate (T_r), leaf temperature and ambient air temperature were measured at 68 DAE on 10 days old leaves at the top of canopy around noon with a gas exchange apparatus (CI-340, Camas, USA). Each measurement was repeated three times. Leaf temperature depression (LTD) was measured by subtracting the temperature of ambient air around the leaf from the leaf temperature.

Chlorophyll contents were measured using the acetone extraction method. Collected samples (0.5 g of fresh leaf) were dissolved in 15 ml of acetone. The leaf extract was centrifuged at 8000 RPM for 5 minutes and absorbance was assessed at 663 nm, 645 nm and 470 nm using a UV-Vis spectrophotometer (UV 2600, Schemadzo, Japan). Chlorophyll *a* (Chl*a*), Chlorophyll *b* (Chl*b*) and carotenoids (Car) were calculated according to HISCOX and ISRAELSTAM (1979) as $Chla = 11.75A_{663} - 2.35A_{645}$, $Chlb = 18.61A_{645} - 3.96A_{663}$ and $Car = (1000A_{470} - 2.27\ Chla - 81.4\ Chlb)/227$ withA_{663}, A_{645} and A_{470} being the absorption values at 663, 645 and 470 nm, respectively.

Osmotic adjustment (OA) was determined for each ecotype by subtracting the osmotic potential under full irrigation from the osmotic potential under water restriction, after irrigation and recuperation of full turgor, as described by BABU et al. (1999). Osmotic potential was measured using a vapor pressure osmometer (VAPRO 5520, Wescor, Utah, USA). At 70 DAE and after irrigation leaf samples were collected in both treatments when 80% of plants recovered from wilting. The leaf samples were pressed in the Eppendorf tube to extract the cell sap which was centrifuged at 8000 RPM for five minutes to collect the supernatants. 10 µL of supernatants were used for the measurement of osmotic potential (in MPa) in both regimes.

The water used by the plant (WU) was estimated as the product of transpiration rate (T_r) by leaf area (LAZARIDOU and NOITSAKIS, 2003). Transpiration was asses on fully expanded leaves at the top of canopy with a hand-held photosynthesis system (CI-340 4845 NW, Camas, WA, USA) between 10:00 and 11:00 am. Plant water use efficiency (WUE) was calculated according to LAZARIDOU and KOUTROUBAS (2004) as the ratio of above-ground dry biomass to the water transpired by the plant. Drought resistance index was calculated as DRI = (Y_S/Y_N) / (M_S/M_N) where Y_S was the biomass of an ecotype under stress (water restriction) conditions, Y_N the biomass of this ecotype under non-stress (full irrigation) conditions, M_S the mean biomass yield of all ecotypes under stress and M_N the mean yield of all ecotypes under non-stress conditions. A recovery rate index was calculated for each ecotype as RRI = $[(R_1/T_1) + (R_2/T_2) + (R_3/T_3)]$ / R where R_1 was the number of plants which recovered from wilting the first day after restoring water in the lower profile of the lysimeter, R_2 the number of plants which recovered from wilting during the second day, R_3 the number of plants which recovered from wilting during the third day and R the final recovery after 3 days. T_1, T_2 and T_3 had values of 1, 2 and 3, respectively. A wilting rate index was also calculated for each ecotype as WRI = $(W_1/T_1+W_2/T_2+W_3/T_3)$ / % W where W_1 was the number of plants showing symptoms of wilting 10 days after restoring the soil moisture content, W_2 and W_3 being the number of plants that showed symptoms of wilting 2 and 3 days after the first measurement, respectively, and W the total number of wilted plants wilting after 3 days. T_1, T_2 and T_3 had values of 1, 2 and 3, respectively.

Statistical analyses

The data were subjected to the analysis of variance using the CROPSTAT 7.2 software. Genotypic and phenotypic coefficients of variation were estimated as GCV% = √Genotypic variance/overall average of the ecotypes × 100 and PCV% = √Phenotypic variance/overall average of the ecotypes × 100, respectively. PCV% and GCV% reflected the variation in the germplasm at phenotypic and genotypic levels. The phenotypic variation is the sum of genotypic variance (σ^2_g)+ environmental variance (σ^2_E). Genetic purity of each of the ecotypes (inbreeding coefficient = 1) was maintained for several generations by growing them in isolation. Therefore variation within ecotypes was considered environmental which was used to estimate overall σ^2_E. Variation between ecotypes was used to estimate the phenotypic variance (σ^2_p). Genotypic variance was estimated by subtracting the environmental variance from the phenotypic variance. Broad sense heritability (h^2) was calculated according to ALLARD (1960) as $h^2 = (\sigma^2_g/ \sigma^2_p) \times 100$.

A stepwise selection procedure based on the Akaike information criterion for multiple regression models (VENABLES and RIPLEY, 2002) was conducted to determine the traits associated with fresh and dry biomass. The plant trait values were used to describe multiple regression models for fresh forage yield (sum of first and second harvest) and dry forage yield (sum of first and second harvest), both measured in the water restriction treatment. Calculation of regressions and stepwise selection were carried out using the 'lm' and 'step' procedures of the R software (R 2013), respectively.

A genotype plus genotype by environment (GGE) analysis (YAN and KANG, 2003) was carried out to analyze the fresh and dry biomass in the first and second harvests for the two treatments. Both fresh and dry biomasses were arranged into a two-way genotype-by-combination of the harvest number and the water regime. A biplot analysis was carried out on traits showing positive influence on yield in order to select promising ecotypes. The traits were standardized before the analysis in accordance with different scales of the chosen variables. The biplot calculations were made using the 'scale' and 'svd' procedures of the R software (R 2013).

Results

As shown by the average pattern of water removal in different layers at various intervals (Tab. 2) moisture content was higher in upper layers in the full irrigation treatment, compared to the water restriction treatment. In this last one, moisture content was maintained higher in the lower layers than in the upper ones. Significant effects due to ecotypes, water regimes and interaction (ecotypes × water regimes) were noted for all traits, except carotene contents which showed insignificant differences due to water treatments (Tab. 3). Water restriction effects were particularly drastic on fresh forage yield (FFY), canopy temperature depression (CTD) and transpiration rate (T_r) which reduced 160, 79 and 61%. Water restriction increased the phenotypic and genotypic coefficients of variation for all traits except leaf area. High phenotypic and genotypic coefficients of variation were noted in both treatments except for leaf area. The broad sense heritability of FFY, leaf area (LA), chlorophyll b (Chl*b)* and carotene content (CAR) was less under water restriction conditions. Most physiological traits had a higher heritability than forage yield. Osmotic adjustment (OA) had higher phenotypic and genotypic coefficients of variation than drought resistance index (DRI), recovery rate index (RRI) and survival rate index (SRI) (Tab. 4). DRI and SRI had higher heritability than water use efficiency (WUE), OA and RRI.

Tab. 2: Moisture gradients in soil layers of lysimeters under two regimes at 60 days after emergence of plants.

Soil depth (cm)	Moisture content (%)					
	Full irrigation			Water restriction		
	24 h	48 h	72 h	24 h	48 h	72 h
10	17	14	10	5	5	4
20	14	10	8	7	6	6
30	10	8	7	10	9	7
40	5	6	7	15	12	10

The biplot analysis realized on biomass retained 88% of the variation for fresh biomass (Fig. 1) and 80% for dry biomass (Fig. 2). In both treatments, the evaluated ecotypes showed differential performance across the two harvests. Under full irrigation conditions, the highest fresh biomass was noted in 'SB-10' and 'L-94' for the first harvest and in 'Berseem Tandojam', 'Pachati' and 'Agaiti' for the second harvest. The highest dry biomass was noted in 'SB-10' and 'L-94' for the first harvest and in 'SB-10' and 'Sandal bar' for the second harvest. Under water restriction, the highest biomass was noted in 'SB-12', 'Berseem Queta' and 'P-22'.

The best fitted models for fresh biomass under water restriction and full irrigation conditions were respectively FB (fresh biomass) = 6.63 – 0.02 Chl*a* + 0.8 LTD – 0.17P_N + 3.18 DRI + 0.84 WUE + 0.03 RRI – 0.01 SRI (R^2 = 0.4) and FB = 15.07 + 0.06 LA + 2.09 LTD (R^2 = 0.1). The best fitted model explaining dry biomass under water restriction and full irrigation conditions were DB (dry biomass) = 1.560 + 0.26 LTD + 0.73 DRI + 0.21 WUE (R^2 = 0.32) and DB = 2.45 + 0.01 LA + 0.13 P_N – 1.02 E + 0.42 CTD (R^2 = 0.47), respectively.

The biplot analysis realized on the traits associated to yield under water restriction (ie, DRI, LTD, RRI and WUE) showed a negative association between WUE and LTD, and between DRI and RRI (Fig. 3). The ecotypes 'Anmol', 'Agaiti' and 'SB-12' had the highest WUE (and the lowest LTD values). Conversely, the ecotypes 'Super' and 'Chenab', followed by 'Samarkand', 'L-48', 'SK-1', 'P-

Tab. 3: Mean, analysis of variance, genotypic coefficient of variation (GCV%), phenotypic coefficient of variation (PCV%) and heritability (h^2) for fresh forage yield (FFY), dry forage yield (DFY), canopy temperature depression (CTD), leaf temperature depression (LTD), net photosynthesis rate (P_N) and transpiration rate (T_r) of berseemclover under full irrigation and water restriction conditions.

Plant traits	**Mean S**		**Mean sum of squares‡**			GCV%		PCV%		**h^2**	
	Full irrigation	Water restriction	**G**	**T**	**G × T**	Full irrigation	Water restriction	Full irrigation	Water restriction	Full irrigation	Water restriction
FFY† (g)	26.85	10.31	21.67**	4104.15**	11.73**	22.65	23.52	43.22	46.33	0.52	0.51
DMY† (g)	4.79	2.98	0.88*	49.27**	84.63**	25.21	27.87	47.41	51.58	0.53	0.54
CTD (°C)	2.80	1.56	0.52*	50.70**	0.34*	21.94	42.03	28.91	52.57	0.76	0.80
LTD (°C)	2.20	1.51	0.52*	16.65**	1.26**	18.64	41.05	23.3	49.51	0.80	0.83
LA (cm^2)	125.92	112.67	637.98**	4957.92**	381.39**	8.19	4.87	12.47	11.23	0.66	0.43
Chla (mg kg^{-1})	90.23	67.44	1637.60**	15258.72**	1510.28**	27.31	31.72	31.27	35.8	0.87	0.89
Chlb (mg kg-^{1})	17.41	26.92	112.56**	2582.44**	45.10*	23.02	27.19	28.02	35.31	0.82	0.77
Carotene	19.84	19.39	85.10**	19.14^{NS}	128.42**	30.44	31.94	43.44	46.38	0.70	0.69
P_N (µmolm^{-2} s^{-1})	8.01	5.61	70.78**	247.25**	17.95**	40.69	62.67	51.36	74.22	0.79	0.84
T_r (mmolm^{-2}s^{-1})	1.74	1.08	0.34*	12.93**	0.25*	14.90	28.07	21.43	34.64	0.70	0.81

† FFY and DMY were sum over two harvests
‡ G = variation due to ecotypes with df = 19; T = water treatment df = 1 and G×T = ecotypes × water regimes with df = 19
** P ≤ 0.01, *P ≤ 0.05

Tab. 4: Mean, analysis of variance, genotypic coefficient of variation (σ^2_g), phenotypic coefficient of variation (σ^2_p) and broad sense heritability (h^2) for osmotic adjustment (OA), drought resistance index (DRI), water use efficiency (WUE), recovery rate index (RRI) and survival rate index (SRI) under water restriction conditions.

Plant traits	Mean	ecotypes mean sum of square	σ^2_g	σ^2_p	h^2
OA	0.23	0.14**	71.34	85.31	0.81
DRI	1.08	0.32**	36.18	37.40	0.94
WUE	1.43	1.01**	39.39	42.93	0.84
RRI	44.75	1136.01**	48.35	60.43	0.78
SRI	83.58	5195.23**	50.38	52.41	0.92
**P ≤ 0.01)					

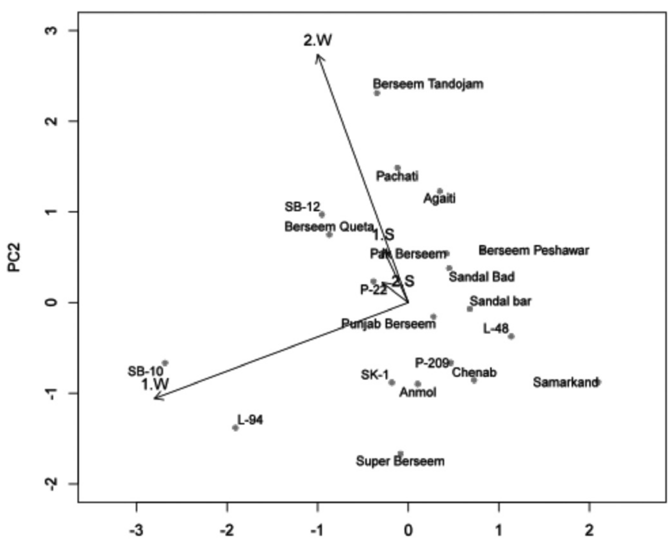

Fig. 1: GGE biplot of fresh biomass in the first (1) and second (2) cuts under water restriction (S) full irrigation (W) for twenty ecotypes of berseem clover (*Trifolium alexandrinum* L.).

22', 'SB-10', 'Sanadal bar', 'Pak berseem' and 'Punjab' had high LTD and low WUE values. The highest DRI and lowest RRI values were observed in 'Berseem Peshawar', 'Agati', 'Sandal Bad', 'Super', 'Pachati', 'L-48', 'Berseem Tandojam' and 'Sanadal bar'. The ecotypes 'L-94', 'SB-10', 'P-209', 'P-22', 'Anmol' and 'Punjab' had the highest RRI and lowest DRI values.

The biplot analysis realized on the traits associated to yield under full irrigation (ie, P_N, LA and CTD) showed a positive association between P_N and LA (Fig. 4). Those traits were poorly correlated to CTD. The ecotype 'SB-10' had high P_N and LA values. 'P-209', 'P-22', 'Sanadal Bar', 'Samarkand' and 'Pachati' showed high P_N values, while 'Berseem Peshawar', 'Berseem Queta', 'Sandal Bad', 'Berseem Tandojam', 'L-48', 'L-94', 'Anmol' and 'Punjab' had low $P_N$values. The ecotypes 'P-209', 'P-22', 'Sandal Bar', 'Samarkand', 'L-94' and 'SK-1' had the highest LA while 'Punjab', 'Berseem Peshawar', 'Agaiti', 'SB-12' and 'Chenab' had the lowest. 'Punjab', 'Chenab', 'Pak', 'Agaiti', 'SB-12' and 'Pachati' had high CTD values. Contrastingly, the ecotypes 'L-94', 'Sandal Bad', 'Anmol', 'Berseem Queta', 'Berseem Tandojam','P-209' and 'L-48' had low CTD values. P_N was not associated to CTD and LA. 'Pachati' and 'Pak Berseem' combined high CTD and $P_N$values, while 'P-209', 'P22', 'Sanadal Bar', 'Samarkand' and 'SK-1' had high LA and P_N values. The ecotypes 'Berseem Peshawar', 'Berseem Queta', 'Berseem Tandojam' and 'L-48' had lower CTD, P_N and LA values.

Discussion

After having used yield as an exclusive breeding objective, many breeders progressively replaced this empirical approach by indirect

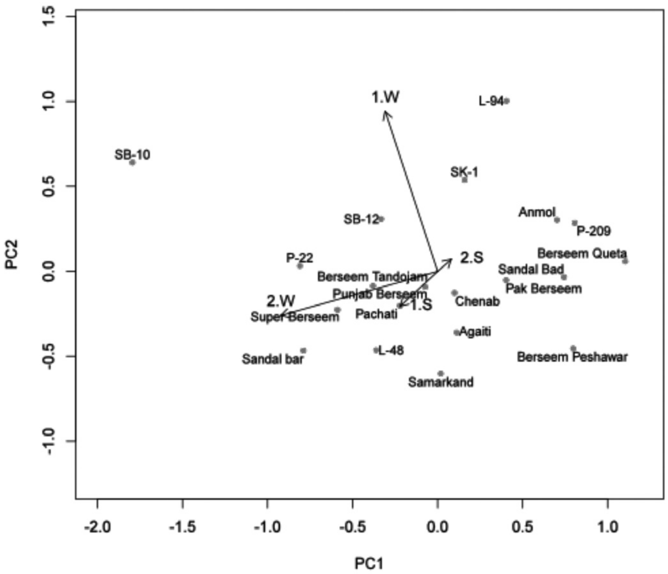

Fig. 2: GGE biplot of dry biomass in the first (1) or second (2) cut under water restriction (S) andfull irrigation (W) for twenty ecotypes of berseem clover (*Trifolium alexandrinum* L.).

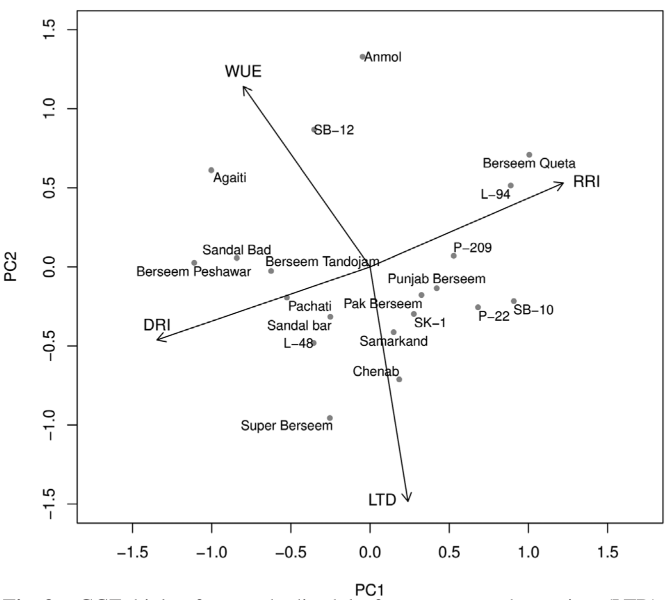

Fig. 3: GGE biplot for standardized leaf temperature depression (LTD), drought resistance index (DRI), recovery rate index (RRI) and water use efficiency (WUE) for twenty ecotypes of berseem clover (*Trifolium alexandrinum* L.) under water restriction. LTD, DRI, RRI, and WUE were averaged across replications for each combination of Genotype-by-Trait. The first principal component axis (PC1) retains 41 % and the second 31 % of sum of squares.

selection (JACKSON et al., 1996), based on the selection for 'secondary traits' or plant characteristics that provide additional information about how the plant performs under a given environment (LAFITTE et al., 2003). Any trait to be used as a surrogate of yield in the evaluation or selection process has to be genetically variable, highly heritable, genetically associated with yield, easy, inexpensive and fast to observe or measure, non-destructive and stable over the measurement period (EDMEADES et al., 1997). In the present study, all measured traits had high genotypic and phenotypic coefficients of variation. Moreover, water restriction increased the variability of various traits as previously observed by IANNUCCI et al. (2000), EL-BABLY (2002) and LAZARIDOU and KOUTROUBAS (2004). Physiological traits had higher broad sense heritability than yield. Finally, an association was noted between forage yield and LTD and RRI under water restriction conditions, suggesting the use of these traits as indirect selection criteria for yield under stress in berseem clover. Under full irrigated conditions, yield was positively associated to P_N, canopy temperature depression (CTD) and leaf area. Gas exchange measurements which provide simultaneous information about LTD and P_N appears provide useful prediction of forage yield. The use of leaf gas exchange traits has been proposed for the selection of sunflower (KALYAR et al., 2013). These measurements however require expensive equipment and are slow, stable over the measurement period. The use of the recovery index consequently appears as a good option for the prediction of forage yield under water restriction. Similarly, under full irrigated conditions, leaf area and canopy temperature depression could represent good candidates for indirect selection. Leaf area is strongly reduced by water stress in berseem clover (MARTINIELLO and TEXEIRA DA SILVA, 2011) and is closely associated to yield in these conditions (AHMED, 2006). CTD is a highly integrating trait resulting from the effects of several biochemical and morpho-physiological features acting at the root, stomata, leaf, and canopy levels. It is useful mainly in hot and dry environments, with high vapor pressure deficits (TUBEROSA, 2012). In wheat, significant genetic gains in yield have been reported in response to direct selection for CTD in these environments (BRENNAN et al., 2007).

The mean sum of square (MSS) due to ecotypes × water regimes was high when compared with MSS due to ecotypes for traits such as FFY and DMY (Tab. 3), suggesting differential performance of ecotypes over water regimes (DESMARAIS et al., 2013). This was confirmed by the magnitude of genetic variation (GCV%) which increased under water restriction, particularly for CTD, LTD and $P_{N,}$ indicating that this treatment successfully discriminated the ecotypes for their tolerance.

GGE biplot analysis of multiple harvests under water regimes showed that the biomass of the first cut is not always a good indicator of biomass of the second cut. Some ecotypes like 'SB-10' from Punjab, however, showed stable biomass across cuts. As the development of high-yielding and stable ecotypes across multiple harvests is a major breeding objective of forage breeding (CHAKROUN et al., 1990; KRENZER et al., 1992), these ecotypes should be recommended for cultivation.

The relationships observed between traits under water restriction conditions suggest that ecotypes with a higher LTD may recover well from drought stress. LTD is an important index of drought avoidance, transpiration and root growth under drought stress. It has been noted earlier that this trait positively correlated with biomass in various forage grass species (ACUNA et al., 2011; MEREWITZ et al., 2014). It also appears that simultaneous selection for LTD and DRI or for RRI and WUE might be difficult. However, simultaneous selection may be practiced to some extent for LTD and RRI or WUE and DRI. Under full irrigated conditions, simultaneous selection for P_N and CTD or P_N and LA may be possible for the selection of high-yielding ecotypes under irrigated conditions. Selection for low LA might lead to higher CTD. KALYAR et al. (2013) also showed that a higher temperature depression was negatively related with leaf area in irrigated sunflower and suggested that reduced leaf area could participate in leaf cooling.

A large variation has been reported in berseem clover for the adaptation to multiple cuts (GRAVES et al., 1996; PUTNAM et al., 1999; ROSS et al., 2003; RANJBAR, 2007) and the knowledge of productivity across cutting regimes is of primary importance in breeding programs (JUSKIW et al., 2000). Among the tested ecotypes, 'SB-10' (from Punjab) showed the highest productivity. This ecotype was also characterized a good aptitude for double harvesting in irrigated conditions. This aptitude that highly depends harvesting management

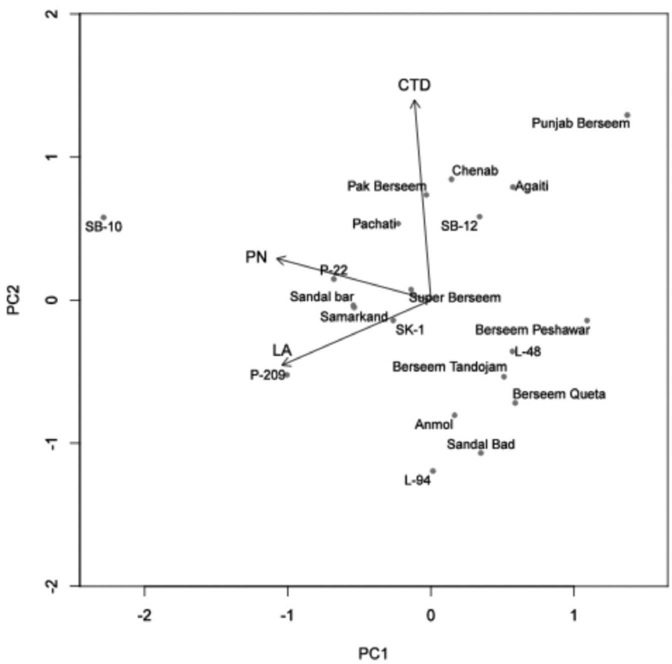

Fig. 4: GGE biplot for standardized net photosynthesis rate (P_N), leaf area (LA), and canopy temperature depression (CTD) under full irrigation for twenty ecotypes of berseem clover (*Trifolium alexandrinum* L.). The first principal component axis (PC1) retains 47% and the second 36% of sum of squares of averaged by replications trait-by-ecotypes combinations.

(Abdel-Gawad, 1993; Geweifel and Rammah 1990; Iannucci et al., 2000) should however be confirmed under in different dates of harvest. Under water restriction conditions, the highest forage yield was noted in 'SB-12' (Punjab), 'Berseem Queta' (Balochistan) and 'P-22' (Punjab). These ecotypes could be recommended to farmers according to their needs and demand and for further use as progenitors in breeding programs.

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