Acta Physiol Plant (2013) 35:3127–3136
DOI 10.1007/s11738-013-1345-3

ORIGINAL PAPER

Assessment of genetic diversity and morpho-physiological traits related to drought tolerance in *Stylosanthes scabra*

Deepti Nagaich · Kapil Kumar Tiwari · Nidhi Srivastva · Amaresh Chandra

Received: 11 June 2012 / Revised: 5 July 2013 / Accepted: 15 July 2013 / Published online: 2 August 2013

Abstract Among the many *Stylosanthes* species, *Stylosanthes scabra*, a range fodder legume, performs better under limited water condition. In the present investigation, thirty-four accessions of *S. scabra* were assessed under limited water condition, for various morpho-physiological characters associated with drought. In general, *S. scabra* exhibited better tolerance to drought, as evidenced by high leaf thickness and greater accumulation of proline, and malondialdehyde (MDA) in water stress condition. Transpiration efficiency (TE) was high, in both control and water stress conditions and positively correlated with root, shoot, and total dry matters, in both control and stress conditions (r^2 = ranged from 0.589 to 0.961 in control and from 0.351 to 0.985 in stress). Of these, 25 accessions were assessed for estimation of genetic diversity, employing random amplified polymorphic DNA (RAPD) markers. A total of 210 RAPD bands, obtained with 32 primers, revealed high polymorphic information content (0.49) and marker index (4.41). Dendrogram analysis indicated close proximity among the accessions of *S. scabra.* These accessions were clustered in high similarity range (84.01–98.36 %). Accession IG-366A separated from other clusters at 85.62 % similarity level. RAPD marker system revealed 13 accessions exhibiting >90 % genetic similarity while the other accessions exhibited similarity ranging from 68 to 90 %. A higher level of genetic similarity which was also evident from the similar levels of TE, biomass production, root/shoot ratio, MDA, proline contents and drought tolerance index, indicated a cause–effect relationship among them. Results also indicated that among the accessions, *S. scabra* rate-reducing resistance allo-tetraploid lines were better suited for hard and cracking soils, under complete rain-fed condition.

Keywords Drought · Malondialdehyde · Molecular marker · Proline · Rate-reducing resistance · Stylosanthes · Transpiration efficiency

Abbreviations

DTI	Drought tolerance index
MDA	Malondialdehyde
RAPD	Random amplified polymorphic DNA
RDM	Root dry matter
R/S ratio	Root/Shoot ratio
SLA	Specific leaf area
SDM	Shoot dry matter
TDM	Total dry matter
TE	Transpiration efficiency

Introduction

The genus *Stylosanthes* (Fabaceae) consists of more than 40 species (Kirkbride and de Kirkbride 1985). These

Communicated by B. Zheng.

D. Nagaich · K. K. Tiwari
Indian Grassland and Fodder Research Institute, Jhansi 284003, India

N. Srivastva
Banasthali University, Jaipur, India

A. Chandra (✉)
Indian Institute of Sugarcane Research, Lucknow 226002, India
e-mail: amaresh_chandra@rediffmail.com

Present Address:
A. Chandra
USDA-ARS, Sugarcane Research Laboratory, 5883 USDA Road, Houma, LA 70360, USA
e-mail: amaresh.chandra@ars.usda.gov

species are grouped into two subgeneric sections, sectt. *Styposanthes* and *Stylosanthes*, based on the presence of rudimentary secondary floral axis and two inner bracteoles in the former and no such axis and only one inner bracteole in the latter. Most species of *Stylosanthes* are diploid (2n = 20), but polyploid species (2n = 40 and 2n = 60) also exist. Five species namely *S. scabra*, *S. seabrana*, *S. hamata*, *S. guianensis*, and *S. viscosa* are predominantly used as fodder legumes in humid to semi-arid tropics of the country (Chandra et al. 2006; Ramesh et al. 1997). Due to their ability to restore soil fertility, improve soil physical properties and provide permanent vegetation cover, they are playing a vital role in the development of wastelands in India. However, the most important limitation w.r.t. this crop species is the narrow genetic variability and low availability of diverse accessions of drought-tolerant *S. scabra.*

DNA markers are considered the best tools for determining genetic relationships/diversity, as they are highly polymorphic and independent of environmental interactions, i.e. highly heritable. The choice of marker system(s) for genetic diversity studies is driven by several considerations. These include the availability of markers, marginal assay cost, size of experiment and preference between a high averages expected heterozygosity, and a high effective multiplex ratio (Powell et al. 1996). Among several markers, random amplified polymorphic DNA (RAPD) (Williams et al. 1990) data can be generated faster than others. It employs single short primer with an arbitrary sequence, to generate genome-specific 'finger print' of multiple amplification products. Polymorphism found between RAPD profiles can serve as a genetic marker (Williams et al. 1990). Owuor et al. (1999) studied *H. spontaneum*, using RAPDs to demonstrate strong association between specific loci and soil types, gene diversity and soil type, and also to depict the frequency with which rare alleles were observed in one soil type over another. Molecular markers have not only proved efficient in the analysis of genetic diversity in space and across eco-geographic gradients, but have also been successfully used to test the common assertion that scientific plant breeding has led to a narrowing in crop diversity over time (Donini et al. 2000).

Apart from genetical studies, morpho-physiological parameters have also been identified as important traits, which could be used in indirect measurement of transpiration efficiency (TE) in stylo (Thumma et al. 1998, 2001). Lines having low specific leaf area (SLA) (thicker leaves) are known to have higher TE, a suitable trait to identify genotypes, which grow better under dry environment, where water is a major environmental constraint for plants (Chandra and Bhatt 2008). Water use efficiency or TE (dry matter produced per unit of water transpired) has been extensively investigated as a trait associated with drought tolerance of plants. Studies have shown that a negative relationship exists between TE and SLA in *Stylosanthes scabra* (Thumma et al. 1998). Phenotypic and QTL data has indicated that SLA is more closely associated with biomass production than TE (Thumma et al. 2001). The accumulation of low molecular weight solutes like proline and other substances also provides better tolerance under low leaf water potential of this crop (Chandra et al. 2004). The reaction of plants to water stress differs significantly at various levels, depending upon intensity and duration of stress as well as plant species and its stage of growth (Chaves et al. 2002; Jaleel et al. 2008). It has been established that drought stress is a very important limiting factor at the initial phase of plant growth and establishment, affecting both elongation and expansion growth (Anjum et al. 2003; Bhatt and Srinivasa Rao 2005; Kusaka et al. 2005). Plant height was found to reduce up to 25 % in water-stressed citrus seedlings (Wu et al. 2008). Development of root system increases the water uptake and maintains requisite osmotic pressure through higher proline levels in *Phoenix dactylifera* (Djibril et al. 2005). An increased root growth due to water stress was reported in sunflower (Tahir et al. 2002) and *Catharanthus roseus* (Jaleel et al. 2008). Root dry weight decreased under mild and severe water stress, in *Populus* species (Wullschleger et al. 2005). An increase in root/shoot ratio under drought conditions was related to ABA content of roots and shoots (Sharp and LeNoble 2002; Manivannan et al. 2007). The root/shoot ratio differed significantly between the drought treatment levels. However, these differences could be caused by the loss of leaves, which was stronger in case of plants under dry conditions, than under moderate and wet conditions. Drought tolerance index based on biomass (DTI-BIO) showed significant variation in drought tolerance capacity among cultivars. DTI-BIO is negatively correlated to SLA and selection of cultivars using these two traits (low SLA and high DTI-BIO) has been found to be very useful, for improved performance of a crop under rain-fed condition (Nautiyal et al. 2002). Many plants cope with water stress and synthesize/accumulate compounds termed osmoprotectants (or compatible solutes) including polyols, sugars, amino acids, betaines, etc. (Bohnert and Jensen 1996). In many plants, in general, increased free proline and P5CS enzyme activity are observed, in response to a wide range of stresses, such as salinity (Delauney and Verma 1993), drought (Zhang et al. 1995; Chandra et al. 2004), extreme temperatures (Ruiz et al. 2002), heavy metal toxicity (Chen et al. 2001), and nutrient deficiency (Sanchez et al. 2002). However, the precise function of proline accumulation is still a controversial question (Hare et al. 1999). The imposition of both abiotic and biotic stresses causes over production of reactive oxygen species (ROS), which ultimately impose secondary oxidative stress in plant cells. The ROS, like H_2O_2, act as a signaling molecule/second messenger and eventually mediate the acquisition of tolerance to both biotic

and abiotic stresses. Accumulation of solutes under stress, not only decreases cell osmotic and water potential, but also allows maintenance of water absorption, cell membrane, and metabolic machinery under dehydration.

In the present paper, we report the genetical assessment of twenty-five accessions of *S. scabra*, from among thirty-four accessions used for testing morphological and biochemical parameters vis a vis Pearson's correlation coefficients' analysis among different traits, under control and water stress conditions. Thus, the identified and verified causal nature and established relationships between TE, total dry matter (TDM), and SLA will have larger impact on stylo research globally.

Materials and methods

Plant materials and growth conditions

Seeds of 34 accessions (IG-310, IG-331, IG-332, IG-335, IG-350, IG-351, IG-352, IG-357, IG-358, IG-361, IG-363, IG-365B, IG-365C, IG-366A, IG-366B, IG-369, IG-374, IG-376, IG-381, IG-390, IG-391, IG-393, IG-395, IG-36260, IG-93116, IG-94-100B, IG-408406, IG-RRR94-97, IG-RRR94-96A, IG-RRR94-96B, IG-RRR94-93, *S. scabra* cv Fitzroy, *S. scabra* cv Seca, and q10042) of *Stylosanthes scabra* were germinated in the month of June, at the research farm of Indian Grassland and Fodder Research Institute, Jhansi, India. The region is located at 78°555′ E, 25°526′ N, 242 masl, in the North-Central plains of India, having a typical semi-arid tropical climate, with about 850 mm average annual rainfall. During the crop growing season (June–November), the minimum and maximum temperatures were 15 °C and 42 °C, respectively, and the average bright sunshine was 9.9 h/day. At the time of seed sowing, a basal dose of nitrogen (20 kg/ha) and phosphorus (60 kg/ha) was applied in the soil. After 15 days of germination, three seedlings of each accession were transferred to pots (20 cm × 15 cm) containing 2 kg locally available red laterite soil having 11–15 % water holding capacity, with farm yard manure (FYM) (3:1) in each pot. After 2 weeks of establishment, each pot was thinned to one plant. Each accession had three replications arranged in three randomized treatments. In total, each accession was represented six times. After growing the plants for another 30 days, two treatments were imposed. In the control plants, water was given at field capacity (200 ml) while in stress-induced plants, water was given up to half of the field capacity. Both the control and stressed plants were watered every third/fourth day from the start of the experiment and on each watering day, pots were weighed manually, using electronic balance to calculate the amount of water loss. After 60 days of water treatment, plants were harvested (3 pots for each accession under each treatment) by carefully removing them from the pots and separating them into root and shoots. Both shoots and roots were oven-dried at 80 °C for 48 h.

Transpiration efficiency and drought stress parameters

Transpiration efficiency was calculated as total biomass produced per unit of water transpired (Thumma et al. 1998). At the time of the final harvest (60 days of stress treatment), leaf area of ten, youngest, fully trifoliate leaves, was measured, using the portable leaf area meter LI-3000A (LICOR, USA). They were oven-dried at 80 °C for 48 h, to calculate the SLA (leaf area/leaf dry weight). Water potential (ψ) was measured in the leaf using HR 33T Due Point Hygrometer, employing C-52 sample chamber (Wescor, USA), as described earlier (Chandra and Bhatt 2008). DTI-BIO was calculated by dividing the TDM of stress plants by that of the control plants.

Proline was extracted and its content determined, as described by Bates et al. (1973). Hundred milligrams of dry leaves were homogenized in 3 % (w/v) aqueous sulfosalicylic acid solution, centrifuged at 3,000 rpm for 20 min. The supernatant was treated with acid ninhydrin (2.5 g ninhydrin per 100 ml of a solution containing glacial acetic acid, distilled water, and 85 % ortho-phosphoric acid in the ratio 6:3:1) boiled for 1 h, and the reaction was terminated by keeping it in an ice bath for 10 min. The absorbance was then determined at 520 nm, using L-proline as standard, and proline content was expressed in μmoles gdw^{-1}. Malondialdehyde (MDA) was estimated in leaf by following the procedure devised by Heath and Packer (1968). The concentration of MDA was calculated from its extinction coefficient of 155 mM^{-1} cm^{-1} and its content was expressed in nanomoles gdw^{-1}.

Extraction of DNA

Genomic DNA was isolated from young and fresh leaves using buffer 'S' (100 mM Tris–HCl pH 8.0, 50 mM EDTA, 100 mM NaCl, and 2 % SDS), by following the procedure of Liu and Musial (1995). The quantity and quality of DNA was checked on 0.8 % agarose gel in 0.5× TBE buffer. It was diluted with TE, to a concentration of 5 ng/μl, for PCR analysis.

DNA amplification

Each PCR amplification was performed in a final volume of 20 μl reaction mixture, containing 67 mM Tris HCl (pH 8.0), 16.6 mM $(NH_4)_2SO_4$, 0.45 % (v/v) Triton X-100, 4 mg BSA, 3.5 mM $MgCl_2$, 150 μM of each of dATP, dCTP, dGTP, and dTTP, 7.5 pmol (15 ng) primer, 25 ng genomic DNA template, and 0.5 unit Taq polymerase (Bangalore Genei, India). Amplifications were performed on a DNA thermal cycler PTC-200 (MJ Research, USA),

with the cycling program consisting of 94 °C for 1 min, 37 °C for 1 min, and 72 °C for 2 min, for 40 cycles, followed by a 41st cycle at 37 °C for 1 min, and final extension at 72 °C for 10 min. The amplified products were kept at 4 °C, until loaded onto the gel. Amplified products were separated on 1.6 % agarose gel in 0.5× TBE buffer (pH 8.0) with ethidium bromide added for band visualization under UV light. Along with the unknown samples, 100 bp DNA ladder was also loaded, to know the size of the amplified products. Gels were analyzed using the gel documentation system (Alpha Imager 2200, Alpha Innotech Corp., USA). PCR reactions were repeated at least once, under strict control of the reaction conditions, to establish reproducibility of results.

Data analysis

Here, a locus was considered polymorphic, if the band was present in some accessions and absent in others, and monomorphic, if the band was present in all the accessions. RAPD marker input binary data matrix was developed by entering the data, assigning 1 to presence and 0 to absence of bands. Only reproducible and unambiguous RAPD fragments were used for analysis. The NTSYS program, version 2.0, was used to produce the similarity matrix (Simqual function). Jaccard similarity coefficient was used to estimate the genetic similarity. The resulting data was further processed with neighbor-joining algorithm, for clustering and generation of dendrograms (Saitou and Nei 1987). The MIDPOINT method of rooting was employed. The PIC value was calculated by employing the formula of Roldan-Ruize et al. (2000): $PIC_i = 2f_i\ (1 - f_i)$, where f_i is the frequency of the amplified allele (band present), and $(1 - f_i)$ is the frequency of the null allele (band absent) of marker i. Marker index (MI) was determined as the product of PIC and the number of polymorphic bands per assay unit (Powell et al. 1996).

The results were statistically evaluated for mean, standard deviation (SD), coefficient of variation (CV %) and correlation coefficient among the characteristics. Pearson correlation coefficient was used to assess the association between different traits. SPSS computer software was used to calculate significance levels and were marked as ($^{**}P < 0.05$) and ($^{*}P < 0.01$). The experimental design was a randomised complete treatment design, with two factors (water regimes) and three replicated plants, per factor and genotype. Two-way analysis of variance (ANOVA) analysis was also performed to test for significant difference.

Results and discussion

It is evident from the results presented in this paper that the metabolism in *Stylosanthes scabra* responds to changes in the availability of water. These changes can be considered as a morphological adaptation of the plants, to water stress, to reduce the evaporative surface area (De Herralde et al. 1998), and facilitate a lower consumption of water (Banon et al. 2004). TE was observed to be positively and significantly related to root dry matter (RDM), shoot dry matter (SDM), and TDM, in both control ($r = 0.589$–0.961) and stress ($r = 0.351$–0.985) treatments. Highest correlation ($r = 0.985$) was detected between TE and TDM, under drought stress condition. A significant negative relationship was also observed between TE and SLA, in control ($r = -0.473$) and stress ($r = -0.695$) treatments (Table 1).

Table 1 Pearson's correlation coefficients among various biochemical and physiological parameters

	Water deficit condition										
Parameters	Height	TFW	SFW	TDM	SDM	RDM	R/S ratio	TE	SLA	W.P.	MDA	Proline
Well watered condition												
Height	1	0.557*	0.498*	0.556*	0.582*	− 0.032	− 0.474*	0.526*	− 0.320	− 0.387	0.023	0.469*
TFW	0.563*	1	0.800*	0.815*	0.822*	0.182	− 0.272	0.864*	− 0.778*	− 0.676*	− 0.275	0.685*
SFW	0.587*	0.875*	1	0.967*	0.971*	0.278	− 0.386**	0.962*	− 0.639*	− 0.636*	− 0.259	0.615*
TDM	0.575*	0.851*	0.978*	1	0.984*	0.404**	− 0.394**	0.985*	− 0.650*	− 0.565*	− 0.280	0.578*
SDM	0.617*	0.883*	0.978*	0.980*	1	0.276	− 0.391**	0.980*	− 0.641*	− 0.592*	− 0.268	0.572*
RDM	0.168	0.397**	0.504*	0.593*	0.500*	1	0.073	0.351**	− 0.082	0.067	− 0.277	0.191
R/S ratio	− 0.268	− 0.020	− 0.026	0.062	− 0.017	0.716*	1	− 0.334	0.312	0.181	− 0.191	− 0.272
TE	0.589*	0.850*	0.943*	0.968*	0.961*	0.602*	0.081	1	− 0.695*	− 0.619*	− 0.294	0.593*
SLA	− 0.289	− 0.710*	− 0.571*	− 0.518*	− 0.553*	− 0.164	− 0.050	− 0.473*	1	0.496*	0.238	− 0.481*
Water potential	0.258	0.338**	0.291	0.266	0.299	0.024	− 0.133	0.184	− 0.151	1	0.113	− 0.338**
MDA	0.143	0.132	0.199	0.144	0.163	− 0.139	− 0.234	0.157	− 0.109	0.204	1	− 0.281
Proline	0.160	0.625*	0.511*	0.550*	0.524*	0.482*	0.263	0.566*	− 0.539*	0.213	0.308	1

* Significance at $p < 0.05$

** Significance at $p < 0.01$

Table 2 Maximum and minimum values of morphological and biochemical attributes as observed at maximum level of stress of 34 accessions of *Stylosanthes scabra*

	Maximum		Minimum		Mean		Stdev		CV %	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress
Height	56.67	51.67	31.17	30.84	45.78	42.85	6.22	5.76	14.00	13.96
TFW	30.57	19.79	13.06	6.89	29.52	22.14	4.52	3.16	20.23	24.11
SFW	26.56	17.29	5.56	4.76	21.37	13.15	4.79	3.04	26.73	27.61
TDM	13.66	9.21	3.96	3.03	11.10	7.14	2.14	1.54	21.91	25.16
SDM	11.49	7.37	3.01	2.08	9.08	5.86	1.93	1.37	24.96	27.72
RDM	2.88	1.76	0.76	0.70	2.05	1.19	0.48	0.30	26.35	25.63
R/S ratio	0.813	0.756	0.123	0.149	0.20	0.24	0.14	0.14	46.85	49.75
TE	3.74	4.91	1.24	1.19	2.97	3.82	0.62	0.88	24.23	28.71
SLA	164.13	188.09	113.34	101.08	121.63	123.91	14.71	20.47	11.13	14.84
Water potential	−1.11	−2.06	−2.47	−3.01	−2.00	−2.50	0.27	0.21	−13.44	−9.05
MDA	337.51	368.94	226.21	273.39	288.09	320.10	27.24	28.18	9.55	8.61
Proline	51.75	80.28	14.43	28.81	41.83	59.31	10.23	11.65	30.81	22.92

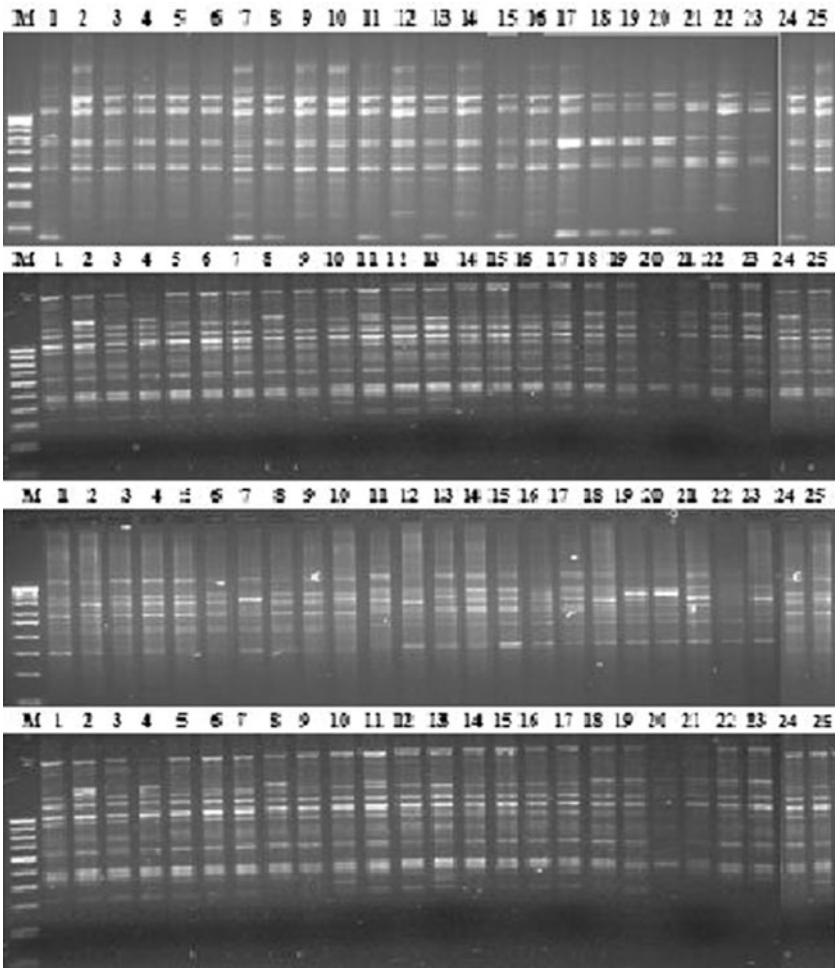

Fig. 1 RAPD profiles of a set of 25 *S. scabra* accessions with primer OPE-06, OPH-12, OPO-01 and OPH-1. M = 100 bp DNA ladder as molecular weight marker

Mean values for control treatment ranged between 1.24 and 5.11 g kg^{-1} for TE, 3.96 and 18.08 g for TDM, 3.01 and 16.69 g for SDM, 0.76 and 6.77 g for RDM, 0.111 and 0.477 for R/S ratio, 31.17 and 63.00 cm for plant height, −2.47 and −1.11 Mpa for water potential, 226.21 and 468.81 nmol gdw^{-1} for MDA, and 12.93 and 96.29 μmol

Table 3 Total no. of bands, no. of polymorphic bands, % of polymorphic bands, polymorphic information content and marker index of RAPD primers tested in *Stylosanthes scabra*

Primers	Total no. of bands	No. of polymorphic bands	% of polymorphic band	Polymorphic information content (PIC)	Marker index (MI)
AB-04	6	3	50.00	0.38	1.12
AB-14	7	3	42.86	0.41	1.23
AB-17	13	9	69.23	0.49	4.41
AD-02	8	4	50.00	0.44	1.76
P-12	5	0	Monomorphic	Monomorphic	Monomorphic
AH-05	10	3	30.00	0.49	1.47
N-19	4	2	50.00	0.14	0.28
B-05	8	2	25.00	0.49	0.98
B-11	2	0	Monomorphic	Monomorphic	Monomorphic
E-01	6	1	16.67	0.49	0.49
E-02	5	3	60.00	0.49	1.47
E-03	5	0	Monomorphic	Monomorphic	Monomorphic
E-05	5	1	20.00	0.26	0.26
E-06	7	1	14.29	0.49	0.49
E-07	9	0	Monomorphic	Monomorphic	Monomorphic
E-14	7	4	57.14	0.189	0.74
E-15	7	0	Monomorphic	Monomorphic	Monomorphic
U-05	4	2	50.00	0.42	0.84
H-12	7	5	71.43	0.48	2.4
Q-18	6	2	33.33	0.42	0.84
C-02	5	3	60.00	0.48	1.44
N-13	6	4	66.67	0.49	1.96
P-01	8	5	62.50	0.41	2.05
AF-11	5	3	60.00	0.34	1.02
N-09	10	3	30.00	0.43	1.29
N-15	8	4	50.00	0.49	1.87
A-20	7	3	42.86	0.41	1.23
AF-06	6	2	33.33	0.49	0.98
AA-14	6	2	33.33	0.26	0.52
D-18	4	0	Monomorphic	Monomorphic	Monomorphic
AD-12	7	3	42.86	0.38	1.14
AF-19	7	3	42.86	0.49	1.47

gdw^{-1} for proline. In case of stress treatment, TE ranged from 1.19 to 7.23 g kg^{-1}, SDM from 2.08 to 10.33 g, RDM from 0.70 to 1.84 g, TDM from 3.03 to 12.29 g, for R/S ratio from 0.025 to 1.65, SLA from 68.95 to 188.09 cm^2g^{-1}, plant height from 30.84 to 55 cm, water potential from −3.44 to −2.06 Mpa, MDA from 226.45 to 409.06 nmol gdw^{-1}, and proline content from 28.81 to 127.68 µmoles gdw^{-1} (Table 2).

Based on morphological variability and response to maximum drought condition, 25 accessions (IG-310, IG-331, IG-332, IG-335, IG-350, IG-351, IG-352, IG-357, IG-358, IG-363, IG-365B, IG-365C, IG-366A, IG-366B, IG-369, IG-374, IG-376, IG-381, IG-390, IG-393, IG-395, IG-RRR94-97, IG-RRR94-96A, IG-RRR94-93 and *S. scabra* cv Fitzroy) of the total 34 accessions, were selected and used for genetic assessment with 32 RAPD primers. This generated a total of 210 DNA fragments, of which 80 bands were polymorphic and 130 were monomorphic (Fig. 1). The maximum numbers of bands (13) were observed with primer OPAB-17 (Table 3). The percentage of polymorphism ranged from 14.29 to 71.23 % and polymorphic information content (PIC) ranged between 0.18 and 0.49. The marker index ranged from 0.26 to 4.14. Any trait which is used as an indirect selection criterion should have high genetic correlation with the traits. DNA-based markers such as RAPD, RFLP, AFLP and microsatellites provide opportunities to study genetic similarity. RAPD not only generated a high number of polymorphic bands, but sufficiently distributed to enable discrimination among accessions, by each primer. The cluster analysis (UPGMA), based on RAPD similarity matrix, was performed to generate

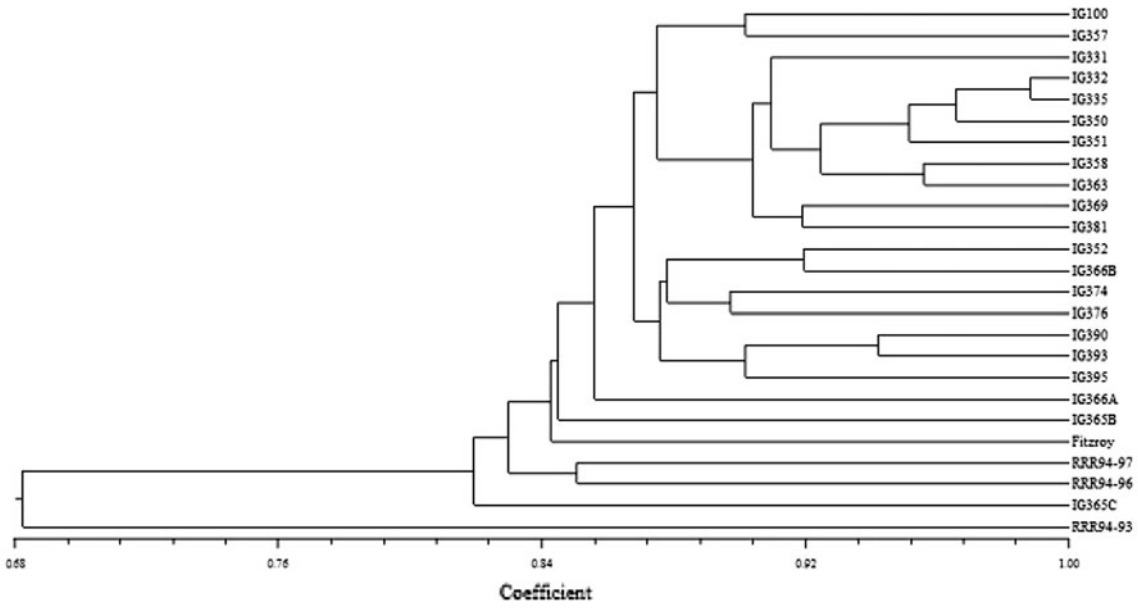

Fig. 2 Dendrogram based on RAPD markers generated with 25 accessions showing closeness among these accessions of *S. scabra* developed using UPGMA (Jaccard similarity coefficients) and SAHN clustering

dendrogram. The distribution of accessions, based on average cut off value, revealed a major cluster consisting of 18 accessions (Fig. 2). These accessions were clustered in high similarity range (84.01–98.36 %). Accession IG-366A was separated from this big cluster at 85.62 % similarity level. RAPD marker system revealed 13 accessions, possessing more than 90 % genetic similarity, while the rest exhibited similarities ranging from 68 to 90 %. Though these accessions were clustered in two different clusters, they were mostly centred around one part of the dendrogram, indicating some level of closeness among them.

Analysis of variance of the data indicated that variation due to different treatment was highly significant for all the traits, although the relative proportions of variance varied from one trait to another (Table 1). On the other hand, the plants growth in pots under water deficit had appreciable and rapid response in the relative growth rate. Also, comparison between the two watering treatments showed significant differences in results for SDM, TDM, SLA, TE (TDM produced per unit of water transpired; TE), water potential, MDA, proline, and DTI. All parameters were highly correlated with each other (Table 1). However, in several C_3 plant species like alfalfa, SLA was negatively correlated with biomass production (Nelson 1988; Wright et al. 1994). Although, the reason for this negative relationship is not established, it may be due to the fact that plants with low SLA (thicker leaves) will have more mesophyll cells per unit area or larger mesophyll cells, leading to higher rates of CO_2 assimilation (Thumma et al. 1998). Indeed, a negative relationship has been observed between SLA and leaf photosynthesis per unit area in many plant species (Pearce et al. 1969; Nelson 1988; Sheshshayee et al. 2003; Aniya and Herzog 2004). Additionally, mean SLA in stressed plants was found to be almost equal to control. This could be due to the reduction in leaf area expansion during stress treatment but it is not significant. Leaf growth is often more reduced than root growth as a result of water stress (Franco et al. 2006), indicating that shoots and roots respond differently to drought (Bacelar et al. 2007; Alvarez et al. 2009). This was confirmed in our experiment because the plants under water deficit showed a significant decrease in aerial dry matter accumulation, height and root/shoot ratio (Sanchez et al. 2002). The direct and strong relationship between TE and TDM (Table 1), as observed in the present study, indicated better photosynthetic capacity of the crop. The SLA was significantly correlated with biomass production. High transpiration not only leads to high photosynthetic rate, but also keeps the leaf surface cool, especially under hot conditions. Among the thirty-four accessions of *S. scabra*, q10042 accession had maximum biomass accumulation, expressed as TDM production, indicating highest TE in both sets of treatments. Even when TDM was subdivided into root and shoot, both these components showed positive and significant relationships with TE, in both, control and stress conditions. In general, root growth under drought had small negative effects on biomass. Thus, variation in root traits contributed very little toward reducing the drought stress. Effect of drought was more pronounced on biomass, as plant parts above the ground were more influenced than those below ground, hence causing an increase in the root/shoot ratio (Fig. 3a). Within

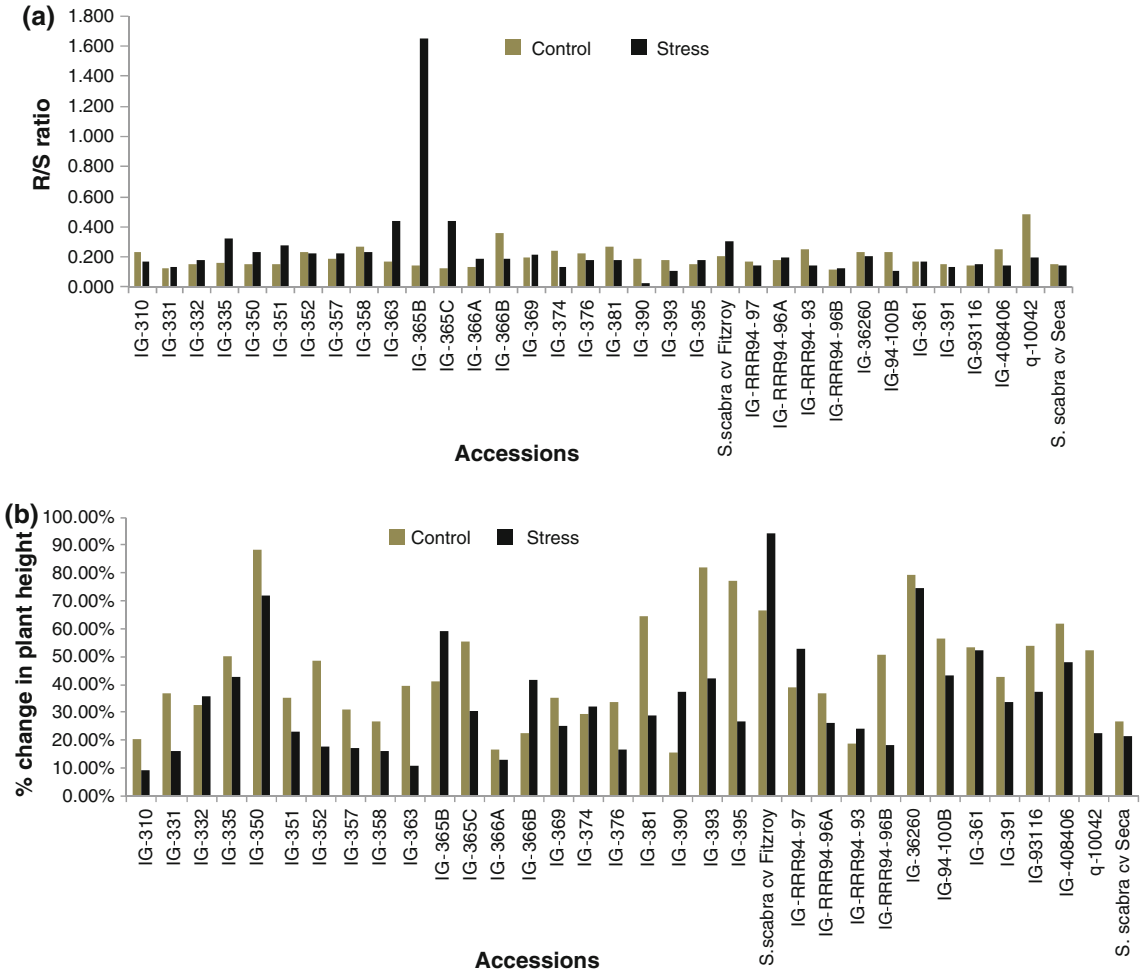

Fig. 3 Changes of root/shoot ratio (**a**) and plant height (**b**) after imposing water stress in *S. scabra* accessions (color figure online)

species, the proportion of biomass of roots, stems and leaves was the result of the effect of drought on total biomass (ontogenetic drift), rather than a direct response to the treatments. The root/shoot ratio increased when plants grown under drought condition showed a significant and positive correlation with RDM (0.716) under control while negatively correlated with TDM (−0.394) under stress condition. In moisture deficit condition, six accessions of *S. scabra* showed moderate increase in R/S ratio while 11 accessions, namely, IG-332, IG-335, IG-350, IG-351, IG-357, IG-363, IG-365B, IG-365C, IG-366A, Fitzroy, IG-RRR94-96, showed higher increase in this ratio (Fig. 3a). Plant height was significantly inhibited by imposing water stress (Fig. 3b). At the end of the experiment, the reduction was around 10–70 % compared to the control, except for four accessions, namely, IG-365B, IG-366B, IG-RRR96-97 and Fitzroy, which showed better drought tolerance. A similar pattern was observed in the relative growth rate, under both treatments. During the inhibition of water transport from root, osmotic regulation may have actively influenced water potential (ψ), as it was significantly correlated with total fresh weight (0.338) in control while negatively correlated with TDM and SDM under the water stress condition. Due to reduction in leaf water potential, accumulation of free amino acids starts, along with MDA. The level of MDA was negatively correlated with RDM, R/S ratio and water potential (Table 1). The level of proline under water deficit condition showed significant correlation with water potential (−0.338), total fresh weight (0.685), TDM (0.578), TE (0.593) and SLA (−0.481). Based on various physio-biochemical parameters, IG-335, IG-363, IG-365B, IG-365C, Fitzroy, IG-366B, IG-RRR94-97 and IG-36260 accessions were identified as better performing lines under drought.

In conclusion, the drought tolerance in *Stylosanthes* was found related to morphological and physiological adaptations, i.e. the ability to adjust osmotic potential to enhance rigidity and modify leaf gas exchange, to reduce water losses though transpiration. The reduction in aerial dry weight (leaf area, leaf number and height) together with increase in the R/S ratio and TE, could promote a more rapid establishment of these species. Different groups of *S.*

scabra might have evolved from single tetraploid plant, or more likely, from several tetraploid plants formed locally by independent hybridization events between their diploid progenitor species, i.e. *S. seabrana* and *S. viscosa* (Liu and Musial 1997). The present study provides information that could be exploited in screening large number of accessions, on the basis of established drought tolerance characters and molecular markers (Chandra et al. 2011). All of these are very valuable in pyramiding traits such as low SLA, WP, high TE, high biomass. The study also indicates that a cause–effect relationship may exist between SLA and biomass production.

Author contribution AC and DN designed the research, DN and KKT conducted the research, DN, AC, and NS analyzed the data and wrote the paper DN, and AC had primary responsibility for the final content. All authors have read and approved the final manuscript.

Acknowledgments Authors are thankful to Director and Head of the Division for providing the necessary facilities to carry out the work. The seed materials received under ACIAR stylo project through NBPGR are duly acknowledged.

References

Alvarez S, Navarro A, Banon S, Sanchez-Blanco MJ (2009) Regulated deficit irrigation in potted *Dianthus* plants: effects of severe and moderate water stress on growth and physiological responses. Sci Hortic 122:579–585

Aniya AO, Herzog H (2004) Water use efficiency, leaf area and leaf gas exchange of cowpeas under midseason drought. Eur J Agron 20:327–339

Anjum F, Yaseen M, Rasul E, Wahid A, Anjum S (2003) Water stress in barley (*Hordeum vulgare* L.). I. Effect on morphological characters. Pak J Agric Sci 40:43–44

Bacelar EA, Santos DL, Moutinho-Pereira JM, Lopes JL, Goncalves BC, Ferreira TC, Correira CM (2007) Physiological behaviour, oxidative damage and antioxidative protection of olive trees grown under different irrigation regimes. Plant Soil 292:1–12

Banon S, Fernandez JA, Franco JA, Torrecillas A, Alarcon JJ, Sanchez-Blanco MJ (2004) Effects of water stress and night temperature pre-conditioning on water relations and morphological and anatomical changes of *Lotus creticus* plants. Sci Hortic 101:333–342

Bates LS, Walbren RP, Teare TD (1973) Rapid determination of free proline water stress studies. Plant Soil 39:205–207

Bhatt RM, Srinivasa Rao NK (2005) Influence of pod load response of okra to water stress. Indian J Plant Physiol 10:54–59

Bohnert HJ, Jensen RG (1996) Strategies for engineering water stress tolerance in plants. Trends Biotech 14:89–97

Chandra A, Bhatt RK (2008) Carbon isotope discrimination function analysis and drought tolerance of stylo species grown under rain-fed environment. Acta Physiol Plant 30:63–69

Chandra A, Pathak PS, Bhatt RK, Dubey A (2004) Variation in drought tolerance of different *Stylosanthes* accessions. Biol Plant 48:457–460

Chandra A, Pathak PS, Bhatt RK (2006) Stylo research in India: prospects and challenges ahead. Curr Sci 90:915–921

Chandra A, Tiwari KK, Nagaich D, Dubey N, Kumar S, Roy AK (2011) Development and characterization of microsatellite markers from tropical forage *Stylosanthes* species and analysis of genetic variability and cross-species transferability. Genome 54:1016–1028

Chaves MM, Pereira JS, Maroco J, Rodriques ML, Ricardo CPP, Osorio ML, Carvatho I, Faria T, Pinheiro C (2002) How plants cope with water stress in the field photosynthesis and growth? Ann Bot 89:907–916

Chen C, Chen L, Lin C, Kao C (2001) Regulation of proline accumulation in detached rice leaves exposed to excess copper. Plant Sci 160:283–290

De Herralde F, Biel C, Save R, Morales MA, Torrecillas A, Alarcon JJ, Sanchez Blanco MJ (1998) Effect of water and salt stresses on the growth, gas exchange and water relations in *Argyranthemum coronopifolium* plants. Plant Sci 139:9–17

Delauney AJ, Verma DPS (1993) Proline biosynthesis and degradation in plants. Plant J 4:215–223

Djibril S, Mohamed OK, Diaga D, Diegane D, Abaye BF, Maurice S, Alain B (2005) Growth and development of date palm (*Phoenix dactylifera* L.) seedlings under drought and salinity stresses. Afr J Biotech 4:968–972

Donini P, Law JR, Koebner RMD, Koebner JC (2000) Temporal trends in the diversity of wheat. Theor Appl Genet 100:912–917

Franco JA, Martinez-sanchez JJ, Fernandez JA, Banon S (2006) Selection and nursery production of ornamental plants for landscaping and xero gardening in semi-arid environments. J Hortic Sci Biotech 81:3–17

Hare PD, Cress WA, Van Staden J (1999) Proline synthesis and degradation: a model system for elucidating stress related signal transduction. J Exp Bot 50:413–434

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acids peroxidation. Arch Biochem Biophys 125:189–198

Jaleel CA, Sankar B, Murali PV, Gomathinayagam M, Lakshmanan GMA, Panneerselvam R (2008) Water deficit stress effects on reactive oxygen metabolism in *Catharanthus roseus*: impacts on ajmalicine accumulation. Colloids Surf B Biointerfaces 62:105–111

Kirkbride JH, de Kirkbride CG (1985) Typification of *Stylosanthes* (Leguminosae) and its sections. Taxon 36:455–458

Kusaka M, Ohta M, Fujimura T (2005) Contribution of inorganic components to osmotic adjustment and leaf folding for drought tolerance in pearl millet. Physiol Plant 125:474–489

Liu CJ, Musial JM (1995) Restriction fragment length polymorphism detected by cDNA and genomic DNA clones in *Stylosanthes*. Theor Appl Genet 91:1210–1213

Liu CJ, Musial JM (1997) *Stylosanthes* sp. Aff. *Scabra*: a diploid putative progenitor of *Stylosanthes scabra*. Plant Syst Evol 208:99–105

Manivannan P, Jaleel CA, Sankar B, Kishorekumar A, Somasundaram R, AlaguLakshmanan GM, Panneerselvam R (2007) Growth, biochemical modifications and proline metabolism in *Helianthus annuus* L. as induced by drought stress. Colloids Surf B Biointerfaces 59:141–149

Nautiyal PC, Rachaputi NR, Joshi YC (2002) Moisture deficit induced changes in leaf water content leaf carbon exchange rate and biomass production in groundnut cultivars differing in specific leaf area. Field Crop Res 74:67–79

Nelson CJ (1988) Genetic association between photosynthetic characteristic and yield: review of evidence. Plant Physiol Biochem 26:543–554

Owuor ED, Fahima T, Beharav A, Korol A, Nevo E (1999) RAPD divergence caused by microsite natural selection. Genetica 105:177–192

Pearce RB, Carlson GE, Barnes DK, Hart RH, Hanson CH (1969) Specific leaf weight and photosynthesis in alfalfa. Crop Sci 9:423–426

Powell W, Machray GC, Provan J (1996) Polymorphism revealed by simple sequence repeats. Trends Plant Sci 1:215–222

Ramesh CR, Hazra CR, BhagMal, Sukanya DH, Ramamurthy V, Chakraborty S (1997) Status of Stylosanthes development in other countries III Stylosanthes development and utilization in India. Trop Grasslands 31:467–475

Roldan-Ruize I, Calsyn E, Gilliand TJ, Coll R, Vaneijk MJT, De Loose M (2000) Estimating genetic conformity between related ryegrass (*Lolium*) varieties. 2. AFLP characterization. Mol Breed 6:593–602

Ruiz JM, Sanchez E, Garcia P, Lopez-Lefebre LR, Rosa RM, Romero L (2002) Proline metabolism and NAD kinas activity in green bean plants subjected to cold-shock. Phytochemistry 59:473–478

Saitou N, Nei M (1987) The neighbour-joining method: a new method for reconstructing phylogenic tree. Mol Biol Evol 4:406–425

Sanchez E, Ruiz JM, Romero L (2002) Proline metabolism in response to nitrogen toxicity in fruit of French bean plants (*Phaseolus vulgaris* L. Cv. Strike). Sci Hortic 93:225–233

Sharp RE, LeNoble ME (2002) ABA, ethylene and the control of shoot and root growth under water stress. J Exp Bot 53:33–70

Sheshshayee MS, Bindumadhava H, Shankar AG, Prasad TG, Udayakumar M (2003) Breeding strategies to exploit water use efficiency for crop improvement. J Plant Biol 30:253–268

Tahir MHN, Imran M, Hussain MK (2002) Evaluation of sunflower (*Helianthus annuus* L.) inbred lines for drought tolerance. Indian J Agric Biol 3:398–400

Thumma BR, Naidu BP, Cameron DF, Bahnisch LM (1998) Transpiration efficiency and its relationship with carbon isotope discrimination under well watered and water stressed conditions in *Stylosanthes scabra*. Aust J Agric Res 49:1039–1045

Thumma BR, Naidu BP, Chandra A, Cameron DF, Bahnisch LM, Liu C (2001) Identification of causal relationships among traits related to drought resistance in *Stylosanthes scabra* using QTL analysis. J Exp Bot 52:203–214

Williams JGK, Kubehk AR, Livak KI, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535

Wright GC, Rao RCN, Farquhar GD (1994) Water use efficiency and carbon isotope discrimination in peanuts under water deficit conditions. Crop Sci 34:92–97

Wu QS, Xia R-X, Zou Y-N (2008) Improved soil structure and citrus growth after inoculation with three arbuscular mycorrhizal fungi under drought stress. Eur J Soil Biol 44:122–128

Wullschleger SD, Yin TM, DiFazio SP, Tschaplinski TJ, Gunter LE, Davis MF, Tuskan GA (2005) Phenotypic variation in growth and biomass distribution for two advanced-generation pedigrees of hybrid poplar. Canadian J Forestry Res 35:1779–1789

Zhang C, Lu Q, Verma DPS (1995) Removal of feedback inhibition of P5CS, a bio-functional enzyme catalyzing the first two steps of proline biosynthesis in plants. J Biol Chem 270:20491–20496