



Genetic and Biochemical Studies of *Thymelaea hirsuta* L. Growing Naturally at the North Western Coast of Egypt

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Authors' contributions

This work was carried out in collaboration between the two authors. The two authors MMAEM and MASEZ put the research points. Author MMAEM collected samples, performed biochemical analyses and wrote the first draft of the manuscript. Author MASEZ performed the ISSR and SRAP primers and phylogenetic relationship. Both authors read and approved the final manuscript.

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ABSTRACT

This study aims to elucidate the biochemical and genetic behaviors of *Thymelaea hirsuta* L. growing naturally at the following habitats; oletic sand dunes, slope of salt marshes, non-saline depression, road side, plateau, upstream of Wadi Halazeen, and wadi bed of Wadi Halazeen along the western Mediterranean coast of Egypt. Soil and plant samples were collected in May, 2017. Water content recorded the highest value in *Thymelaea* growing at slope of salt marshes. Total soluble carbohydrate attained the highest value in *Thymelaea* growing at up-stream of Wadi Halazeen while the highest values in non-soluble carbohydrate and proline were observed in plants growing at oletic sand dunes. Total carbohydrate recorded the highest value in *Thymelaea* growing at plateau. Total lipids and total phenols attained the highest content in *Thymelaea* growing at wadi bed. Genetically, ISSR & SRAP molecular markers were done. Seven ISSR and four SRAP primers produced 73.78% and 68.57% of polymorphism, respectively. PIC value, assay efficiency index, effective multiples ratio and marker index, were higher in SRAP than in ISSR.

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1. INTRODUCTION

Organic solutes metabolism is one of the highest significant biochemical characters affected by abiotic stress. Drought and salinity are the most common within abiotic stress. Plants response to drought stress by accumulating compatible solutes such as proline that can keep proteins stability to facilitate water absorption and to scavenge excess amounts of reactive oxygen species [1, 2]. The proline response to abiotic stress, while the proline contents also depend on plant species [3]. Also, soluble carbohydrates and free amino acids are often increased under water deprivation [4]. Drought also increased Phenolics which play significant role in plant protection [2].

Some techniques are commonly used in genetic diversity evaluation within plant species such as amplified fragment length (AFLP), random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), simple sequence repeat (SSR), [5], sequence-related amplified polymorphism (SRAP) [6]. ISSR is more preferable than RAPD due to use of longer primers, which allows the use of high annealing temperature performing to higher stringency. In addition to overcome most limitations such as high cost and low reproducibility [7, 8]. Moreover, this technique often reveals higher polymorphism per primer [5]. SRAP combines simplicity, reliability and facile sequencing of selected bands. Also, it targets coding sequences in the genome and results in a moderate number of co-dominant markers [6]. Actually, SRAP can give large numbers of information on the diversity of genetic resource [9]. Primarily, SRAP markers have been used for research addressing hypotheses in plant taxonomy, biogeography, systematics, ecology, horticultural and agronomic targets, developing quantitative trait loci in advanced hybrids and evaluating genetic diversity of large germplasm collections [10].

Thymelaea hirsuta is an evergreen shrub belonging to Thymelaeaceae family commonly known as "Methane". In Egypt, it is the only plant species present in the only genus of Thymelaeaceae [11]. The aerial part of *T. hirsuta* has been used in folk medicine for its antimelano-genesis [12], antioxidant and antitumor [13, 14] and hypoglycaemic and antidiabetic [15] properties. Recently, [16]

reported that *T. hirsuta* L has potential source for biodiesel production.

2. MATERIALS AND METHODS

2.1 Materials

Samples of fresh aerial parts of *Thymelaea hirsuta* L and accompanied soil were taken in May, 2017 from seven different habitats; oolitic sand dunes, slope of salt marshes, non-saline depression, road side, plateau, upstream of Wadi Halazeen, and wadi bed of Wadi Halazeen along the western Mediterranean coast of Egypt. The GPS reading was recorded as in Table 1.

Plant samples were washed with distilled water to be free from dust, then dried at 60°C up to constant weight and ground to fine powdered to estimate total soluble sugars, total carbohydrate, proline, total phenols and total lipids.

Sequence-related amplified polymorphism (SRAP) and inter-simple sequence repeat (ISSR) markers were used to determine the genetic diversity and genetic structure in *Thymelaea hirsuta* L. Fresh leaves from each site were put in liquid nitrogen and kept in freezer -20°C till DNA isolation. Genomic DNA was extracted from young leaves by the CTAB method according to Doyle and Doyle [17]. DNA concentration was measured with a microplate spectrophotometer. Extraction buffer consisted of 100 mM Tris-HCl, pH 8.0; 20 mM EDTA, pH 8.0; 1.4 M NaCl, 2% cetyl triethyl ammonium bromide (CTAB), 0.3% β mercapto ethanol and 20% PVP. Precipitation buffer consisted of 50 mM Tris HCl, pH 8.0; 10 mM EDTA, pH 8.0; 1% CTAB, and 0.15% β -mercaptoethanol. After washed twice with 70% ethanol, the total DNA was resuspended in 0.05 cm³ distilled water and quantified by UV-spectrophotometer.

The soil samples supporting plants were taken in three replicates at 0-30 cm depth, dried and powdered gently with wooden wallet and passed through 2 mm sieve. Mechanical analysis was performed using pipette method Kilmer and Alexander [18]. Electrical conductivity (EC) was estimated in soil water extract (1:1), pH and moisture content was estimated according to Rowell [19] and illustrated in Table 2.

Table 1. Geographical position for the studied locations

Sites	N	E	Altitude	Habitats
Site 1	31°22.460	27°11.009	16 m	Ooletic sand dunes
Site 2	31°26.312	26°54.636	-4 m	Slope of salt marshes
Site 3	31°31.621	26°17.986	11 m	Non-saline depression
Site 4	31°24.124	27°1.500	-1 m	Road side
Site 5	31°23.952	26°15.582	96 m	Plateau
Site 6	31°25.024	26°51.604	82 m	Upstream Wadi Halazeen
Site 7	31°25.160	26°51.650	64 m	Bed Wadi Halazeen

Table 2. Soil properties supporting *T. hirsuta*

Sites	Soil particle distribution				Chemical characteristics		
	Sand%	Silt%	Clay%	texture	Moisture%	pH	EC µmhos
Site 1	83.25	4.77	11.98	Loamy sand	2.4	8.4	320
Site 2	66.10	27.00	6.90	Sandy loam	8.85	8.1	1600
Site 3	77.07	20.01	2.92	Loamy sand	6.1	7.7	520
Site 4	80.87	12.33	6.80	Loamy sand	3.04	7.9	325
Site 5	79.30	18.08	2.62	Loamy sand	4.97	7.9	145
Site 6	76.27	13.18	10.55	Sandy loam	3.9	7.6	95
Site 7	71.02	20.75	8.23	Sandy loam	3.24	7.7	211

2.2 Methods

2.2.1 Biochemical parameters

Water content percentage of plant leaves was calculated as $TWC = 100 (Fw - Dw) / Fw$. Where TWC: total water contents, Fw: Fresh weight, Dw: dry weight. Total carbohydrate and soluble carbohydrates were extracted and estimated calorimetrically using the general phenol-sulphoric acid method according to Chaplin and Kennedy [20]. Total lipids was extracted with equal volume from ether and petroleum ether using Soxhlet apparatus and calculated as lipid content % = mass of lipid extracted (g)/sample weight (g) × 100. Free proline was determined by the sulfosalicylic acid-ninhydrin method as described by Bates et al. [21]. Total phenols were estimated using Folin-Denis reagent according to Shahidi and Nacz [22]. A known weight of plant powder was macerated in ethanol 80%. 1 ml of the extract and 0.5 ml of Folin reagent was mixed in a test tube, 1 ml of saturated Na_2CO_3 was added and mixed well then 3 ml of dist. water were added. After 1 hour, absorbance of blue color was read at 725 nm by spectrophotometer using catechol as a standard.

2.2.1.1 Statistical analysis

The experiment was designed in a randomized complete block design using three replicates. Analysis of variance (ANOVA) was conducted

according to Casella [23], using MSTAT-C software program [24]. For separating the treatment means, the LSD 0.05 was used.

2.2.2. Genetic

2.2.2.1. SRAP-PCR analysis

Among 10 pairs of SRAP primer combinations, four pairs with good repeatability and high polymorphism were determined to expand the genomic DNA of *Thymelaea hirsuta* from seven sites by SRAP-PCR. 20 µL is the optimum SRAP-PCR reaction system contains 2.0 µL 10 × PCR buffer, 3 U Taq DNA polymerase, 150 µmol/L dNTPs, 0.4 µmol/L each of SRAP forward and reverse primers and 40 ng genomic DNA as described by Yan et al. [25]. PCR reactions were occurred in (Thermocycler, Biometra, Desert Research Center, Egypt) under the following conditions: initial denaturation at 94°C for 5 min followed by five cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min and extension at 72°C for 1 min. Regarding the following 35 cycles, denaturation at 94°C for 1 min, annealing at 48°C for 1 min and extension at 72°C for 1 min; the last extension step at 72°C for 10 min. The amplification products were resolved on 2% agarose gels.

2.2.2.2. ISSR-PCR analysis

Genomic DNA for young leaves was extracted using the CTAB method as described by Doyle

and Doyle [17]. The genomic DNA of *Thymelaea hirsuta* L from seven sites expanded by ISSR-PCR. The optimum ISSR-PCR reaction system (20 µL) contains 2.0 µL 10 × PCR buffer, 1.5 U Taq DNA polymerase, 300 µmol/L dNTPs, 0.6 µmol/L ISSR primers and 80 ng genomic DNA. ISSR-PCR reactions were accomplished in (Thermocycler, Biometra, Desert Research Center, Egypt). PCR cycling conditions were: initial denaturation at 94°C for 4 min followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 50°C for 45 s and extension at 72°C for 2 min; a final extension step at 72°C for 8 min. The products of amplification were resolved on 2% agarose gels.

2.2.2.3. Data analysis

Each band was scored as present (1) or absent (0) and data were analyzed with the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc version 2.1) software package Rohlf [26]. A similarity matrix was constructed based on Dice's coefficient Dice [27] which considers only one to one matches between two taxa for similarity. The similarity matrix was performed to draw a dendrogram using the unweight pair group method arithmetic average (UPGMA) to determine genetic relationships among the germplasm studied. The representativeness of dendrogram was evaluated by estimating Cophenetic correlation for the dendrogram and comparing it with the similarity matrix, using Mantel's matrix correspondence test Mantel [28]. The result of this test is a Cophenetic correlation coefficient, r , indicating how well the dendrogram represents similarity data. Polymorphism information content (PIC) values were determined according to Smith et al. [29], using the algorithm for all primer combinations as follows:

$$PIC=1-\sum fi^2$$

where:

fi^2 —frequency of the i^{th} allele PIC provides an estimate of the discriminatory power of a locus by taking into account not only the number of alleles that are expressed but bands were scored manually as well. According to the weight of the DNA ladder (1 kb DNA Ladder Plus in ISSR but 100 bp DNA Ladder in SRAP), the same weight bands were marked as a line. The bands that were distinctly seen and repeatable on the electrophoresis map were marked as "1" and the disappearance of a band at the same site was marked as "0". A binary data matrix was

collected for each primer set. The total numbers of bands expanded by each primer in addition to polymorphic bands number were calculated. Most of the following analyses were achieved based on adjoined matrix data. The genetic diversity of different *Thymelaea hirsuta* L. populations was assessed by calculating Total number of effective alleles (N_e), Fraction of polymorphic loci (β), Assay efficiency index (A_i), Effective multiples ratio (E) and Marker Index (MI), Effective number of patterns/ assay unit (p), Multiplex ratio (MR) (L/T)(MR), PIC value (PIC).

3. RESULTS & DISCUSSION

3.1 Biochemical

Data in Table 3 showed that all investigated parameters in *Thymelaea* were significantly changed under different sites. Water content recorded the highest value in plants growing at site 2 (slope of salt marshes), followed by those at site 4, while the lowest value was recorded in those at site 3 (non-saline depression) as represented in Table 3. The highly increase in moisture content in *Thymelaea* growing at slope of salt marshes response to the increase in soil moisture content and EC. In this respect, [30] found that *Limoniastrum monopetalum* acquired the highest value of water content pledge of the high salinity in the salt marshes habitat.

Thymelaea growing at up-stream of Wadi Halazeen recorded the highest value of total soluble carbohydrate followed by those at wadi bed and the lowest value in those at site 2 (slope of salt marshes). Non-soluble carbohydrate recorded the highest value in those growing at site 1 and total carbohydrate recorded the highest value in those at site 5 and site 1, while those growing at site 3 recorded the lowest value in both as observed in Table 3.

Our data indicate that drought stress motivates soluble sugars accumulation in *T. hirsuta*. Such effects of drought stress on soluble carbohydrate accumulation were also reported by [2, 31]. It is suggested that soluble sugars play significant role in the stabilization of cell membranes and proteins and improve plant tolerance during drought stress. Besides supporting energy, sugars modulate expression of multiple genes as regulatory messengers [32]. However the reduction in soluble sugar at slope of salt marshes are in disagreement with [33] who found over increasing by 25% under 300 mM NaCl in *Haloxylon stocksii*. On the other hand, Peng J et

al.[34] found a reduction in soluble sugar by 9%-34% in cotton leaves under salinity stress. The higher values of non-soluble carbohydrate and total carbohydrate in *T. hirsuta* growing at coastal oolitic sand which is characterized by the lowest soil moisture content respond to drought stress. Similar results were obtained by Abd El-Maboud MM [35] who declared that accumulation of total carbohydrate, non-soluble carbohydrate and organic osmolytes like proline and glycinebetaine may support plants to face abiotic stresses via osmoregulation. Concerning total lipids, the highest value was recorded in *Thymelaea* growing at site 7 and the lowest one was recorded in those at site 3. Total lipids in *T. hirsuta* fluctuate between 6.25% and 7.17% with non-specific trend. There is no relation between total lipids and soil properties in the studied species. In general, plant lipids act as energy stores or as plant membrane components. In addition to lipid provide an impervious barrier on the plant surface represented in cuticle and epicuticular wax to restrict water loss to save protection against desiccation and pathogens [36]. Also, they are used as a compact energy source for seed germination [37]. In general, the high amount of total lipids in *T. hirsuta* reflects the adaptive response under harsh environmental conditions.

Proline content recorded the highest accumulation in those growing at site 1 and 4 while the lowest value in those at site 5. Regarding phenolics, the highest content was noticed in those at site 7 followed by those at site 5, site 1, site 6, site 2, site 3 and the lowest one at site 4.

Like lipids, proline and total phenols fluctuate in *T. hirsuta* among different sites without obvious trend. The highest proline accumulation in *T.*

hirsuta at coastal oolitic sand dunes combined with the lowest soil moisture content. Proline motivates cell division or cell death and stimulates specific gene expression, which may be important for plant recovery from stress [38]. Furthermore, it is a multifunctional molecule which is associated with respond to abiotic stress [39]. In the past, proline was used as stress indicator. Since few years proline was not related to water stress [4]. Phenolic compounds have many biological properties such as antimutagenic, antitumor, antibacterial properties [40] and antioxidant [41].

3.2. Molecular ISSR

Seven primers; 807, 814, 844B, 17899B, 17898A, HB 01, HB 04 were analyzed and presented 22, 16, 8, 10, 12, 17, 18 bands, respectively. Percent of polymorphism ranged from 25% (17898A) to 94% (HB 01) with 73.7% polymorphism as observed in Table 4 and illustrated in Fig 1.

These results are in line with those obtained by Yao H et al. [42], who detected the genetic diversity in 4 wild populations of *Glycyrriza uralensis* collected from Inner Mongolia, China using fourteen ISSR primers which produced 92.2% polymorphism. Verma KS et al. [43] found 13 primers of ISSR produced 166 amplification products, and out of which 99 were polymorphic, with an average of 7.6 polymorphic bands per primer. The number of bands amplified per primer varied between 9 (UBC-807, 802) and 16 (UBC-803, 812). Polymorphic percentage ranged from 45.4% to 73.3% with an average of 60.05% polymorphism. Three *Miscanthus* species were examined using 15 ISSR primers, produced 443 bands with 98% polymorphism [44].

Table 3. Biochemical determinations in *T. hirsuta*

Sites	Water content %	Soluble carbohydrate %	Non-soluble Carbohydrate %	Total carbohydrate %	Total lipids %	Proline mg 100g ⁻¹	Total phenols mg g ⁻¹
Site 1	47.62	8.87	32.1	40.97	6.81	261.73	27.67
Site 2	69.18	6.92	26.16	33.08	6.99	217.78	24.36
Site 3	41.56	9.8	22.13	31.93	6.25	192.37	23.66
Site 4	57.32	10.37	30.23	40.6	6.46	255.67	22.59
Site 5	44.38	9.93	31.45	41.39	6.67	174.56	28.31
Site 6	48.22	10.87	24.36	35.23	6.69	186.51	26.37
Site 7	50.07	10.66	25.54	36.2	7.17	184.8	32.22
LSD at 5%	0.95	0.16	0.44	1.04	0.36	10.74	0.52

Table 4. Results on ISSR primers used in *T. hirsuta* L

Primers	Sequence (5' to 3')	Bands	Polymorphic bands	Percentage Polymorphic bands (%)
807	(AGA) 4GT	22	18	82
814	(CT) 8TG	16	12	75
844B	(CT) 8 GC	8	6	75
17899B	(CA) 6 GG	10	9	90
17898A	(CA) 6 AC	12	3	25
HB 01	(CAA) 5	17	16	94
HB 04	(GACA) 4	18	12	67
Total		103	76	73.7

3.3 SRAP Marker

Data in Table 5 and Fig. 1 revealed that the ME1-EM1, ME1-EM2 and ME1-EM4 produced 16 bands for each primer except the ME1-EM3 produced 22 bands. The highest polymorphism was recorded with the primer ME1-EM1 (75%). The SRAP technique is a simple marker

detection method arised for preferable amplification of open reading frames based on two primers [6]. Aneja et al. [45] using 29 SRAP primer pairs observed a total of 121 polymorphic bands in *Vigna radiata* (mungbean), ranging from 1 to 12 bands per primer combination, with an average of 4.65 bands per primer set.

Table 5. Results on SRAP primers used in *T. hirsuta* L

Primers	Sequence(5' to 3')	Bands	Polymorphic bands	Percentage Polymorphic bands (%)
ME1-EM1	TGAGTCCAAACCGGATA GACTGCGTACGAATTAAT	16	12	75
ME1-EM2	TGAGTCCAAACCGGATA GACTGCGTACGAATTTGC	16	11	69
ME1-EM3	TGAGTCCAAACCGGATA GACTGCGTACGAATTGAC	22	16	73
ME1-EM4	TGAGTCCAAACCGGATA GACTGCGTACGAATTACG	16	9	56
Total		70	48	68.5

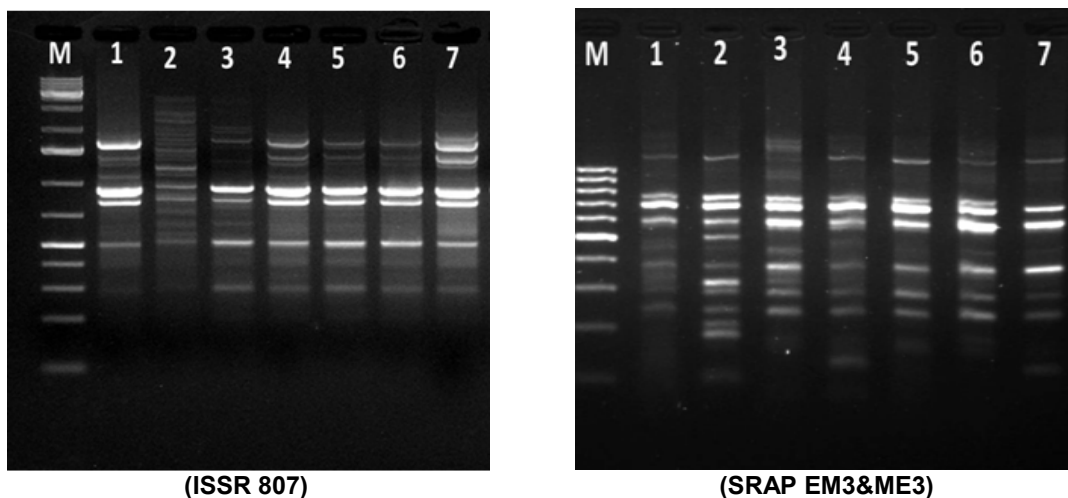


Fig. 1. ISSR and SRAP marker

A comparative scenario of the discriminating capacity of ISSR and SRAP markers are summarized in (Table 6) and (Fig. 1). On average, the two parameters, mean of polymorphism per assay unit, PIC value, assay efficiency index (A_i), effective multiples ratio (E) and marker index, were higher in the SRAP marker, highlights the advantage nature of these markers compared ISSR.

It is worth emphasizing that the comparison of efficiency between ISSR and SRAP markers has not been done in *Thymelaea hirsuta* L, till date. In this research, the highest assay efficiency index and marker index value for SRAP marker as result of greater effective multiples ratio component, proposing that SRAP has a higher discriminating capacity for quantifying the genetic diversity and can detect numerous polymorphic markers per reaction at the same time. Despite the differences in some of the diversity statistics, these results show that SRAP and ISSR markers can be used to evaluate the level of polymorphism in *Thymelaea hirsuta* L. [10] performed statistic to 151 studies on SRAP analysis. They found 65 (42.8%) of them produced more than 10 polymorphisms per locus

and an average number of polymorphisms was 11.8 per locus.

3.4 Genetic Diversity and Phylogenetic Relationship

The data from all ISSR and SRAP amplification amplicons were used in the similarity assessment. The genetic similarity matrix among all studied materials was shown (Table 7). Based on 173 alleles similarity matrix was calculated according to Dice's coefficient. The similarity values among the studied location ranged between 0.32 and 0.74. The highest similarity was between the site 2 & site 3 (0.74), site 1 & site 3 (0.69) and site 3 & site 4 (0.68), while the lowest similarity (0.32) was between, site 5 & site 6 for ISSR, and SRAP. Based on data obtain from ISSR and SRAP tree, a combined UPGMA tree was illustrated in (Fig 2). In this paper, a little modification in the positioning of some location was observed in the sub-clade tree formed, using different markers systems and the phylogenetic tree from SRAP data was most compatible with a combined tree. The phylogenetic analysis showed numerous well supported clades with great bootstrap values.

Table 6. Summary statistics of the information obtained with and discriminating capacity of ISSR and SRAP markers in *T. hirsuta*

No	marker efficiency		ISSR	SRAP
1	Number of assay units		7	4
2	Total number of bands	(L)	103	70
3	Polymorphic bands	(p)	76	48
4	Number of loci/assay unit	(nu)	14.7	17.5
5	Total number of effective alleles	(Ne)	242	156
6	Average number of polymorphic bands/assay unit	(np/U)	10.8	12
7	Polymorphic information content	(PIC)	0.95	0.96
8	Fraction of polymorphic loci	(β)	0.73	0.68
9	Assay efficiency index	(A _i)	34.6	39
10	Effective multiples ratio	(E)	10.8	12
11	Marker index = $H_{av} \times MR$	(MI)	10.3	11.6

Table 7. Similarity matrix result from ISSR and SRAP data

Case	Proximity Matrix					
	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
Site 1						
Site 2	0.59					
Site 3	0.69	0.74				
Site 4	0.45	0.66	0.68			
Site 5	0.44	0.63	0.67	0.41		
Site 6	0.44	0.67	0.63	0.41	0.32	
Site 7	0.41	0.58	0.62	0.46	0.35	0.37

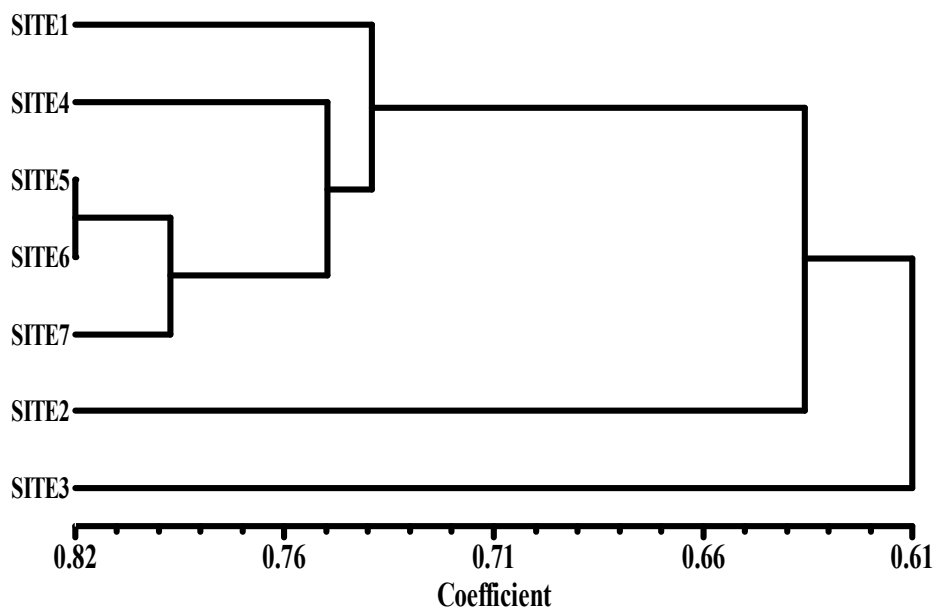


Fig. 2. Dendrogram of the seven studied sites for *T. hirsuta* using ISSR and SRAR markers

4. CONCLUSION

In conclusion, *Thymelaea hirsuta* L can be said to have high genetic diversity, that increased genetic diversity of plant populations leads to increased plant adaptability and resilience to different environmental conditions and even propagation, this is consistent with [46]. *Thymelaea hirsuta* is rich with total lipids and total phenols which enable the plant to adapt under harsh environmental conditions.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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