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Inter-population differences in active limestone tolerance of three ecologically distinct *Medicago tunetana* (Murb.) A.W. Hill accessions (Fabaceae) from Tunisia

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Abstract:
Background*: Medicago tunetana (Murb.) A.W. Hill is a perennial pastoral species endemic to Tunisia and Algeria of agronomic, ecological and economic interests.*
Materials and Methods*: This work aims to evaluate the effect of active limestone on physiological and morphological growth parameters of three M. tunetana accessions collected from different geographical regions from Western North Tunisia (Makthar, Thala and Sekiet Sidi Youssef) including leaf number, stem height, internodes length, leaflet length, and chlorophyll content. For this purpose, a pot assay was conducted following a randomized complete block design under greenhouse conditions. Plants were subjected to seven active limestone levels (0, 5, 10, 15, 20, 25 and 30%).*
Results*: The statistical analysis highlighted the high significant effect of active limestone treatment ($p<0.001$) on all the studied parameters. All measured parameters were negatively affected when compared to the control treatment and when the rate exceeded 25%. For active limestone percentage ranging between 5 and 20%, all the growth parameters were significantly affected. The highest leaf number per plant (87 leaves) was recorded at 20% limestone level.*
Conclusion: *In fact, the excess of active limestone in soil (above 25%) can generate the death of the principal stem. However, M. tunetana continues their cycle thanks to rhizomes. As a calcicole plant, this species can tolerate the moderate to excessive levels of active limestone in the soil as long as there is no hypoxia problem in soil.*
Key Word*: Medicago tunetana, endemic species, biodiversity, tolerance variability, growth parameters, rhizomes, calcicole plant.*

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I. Introduction

Tunisia has wide plant diversity with thirty-four endemic taxa [1], among which, seven taxa belong to the Fabaceae family [2]. This latter constitutes, a precious capital that should be preserved in order to perform its agronomic task with its aptitude for the symbiotic fixing of nitrogen, allowing an abundant production of plant proteins [3] and to environmental protection by avoiding erosion phenomena via wind action or rains' surface flow [4]. *Medicago tunetana* (Murb.) A.W. Hill is a perennial forage legume endemic to Tunisia and Algeria [5] and threatened to disappear because of overgrazing [6] and soil water erosion [7]. It presents an essential preoccupation in the region of Western North and Western Center of Tunisia. This region is characterized by difficult climatic factors (low winter temperatures) and extreme edaphic conditions (calcareous and basic soil) [8]. The calcium which exists in calcareous soils is the essential mineral element for plant growth [9]. In addition, it represents a way for the pH soil balance and it stimulates nodules formation at alfalfa plants [10]. The excess of this macro-nutrient presents an abiotic stress for certain plants because it slows down the assimilation of oligo-mineral elements such as iron, manganese and aluminum [11] leading to the appearance of deficiency symptoms. Generally, species of Fabaceae have a physiological mechanism of adaptation towards the excess of calcium in soil; in the situation of ferric chlorosis, Fabaceae plants reduce Fe^{3+} to Fe^{2+} by using the ferric-chelate reductase activity [12].

In order to evaluate the *M. tunetana* plants response under calcareous soil stress, a collection of *M. tunetana* was collected and several agronomical and physiological parameters were analyzed.

II. Material And Methods

Plant material and sites description

Three accessions of *M. tunetana* were collected throughout three years of prospecting (2011-2017) from three sites in mountain regions of Tunisian dorsal (Makthar, Thala and Sekiet Sidi Youssef). The prospected governorates, their geographical coordinates, their altitude and their bioclimatic zones are shown in the Table 1. In order to evaluate the edaphic characterization of *M. tunetana* sites, physico-chemical soil analyses were carried out and the characteristics of prospected stations soil are presented in the Table 2.

Table 1: The prospected governorates, their code, their bioclimatic zone, their altitude and their geographical coordinates for three *M. tunetana* accessions

Prospected sites	Code	Bioclimatic zone	Altitude (m)	Geographical coordinates	
				Latitude	Longitude
Makthar, Siliana	A1	Superior semi-arid at cool winter	1010	35.828826°	9.357835°
Thala, Kassrine	A2	Medium semi-arid at cool winter	1041	35.484876°	8.741761°
Sekiet Sidi Youssef, Kef	A3	Sub-humid at cool winter	905	36.24237°	8.36897°

Table 2: Physico-chimical soil characteristics of different prospected sites of *M. tunetana* accessions

Regions	%Clay	%Silt	%Sand	Total limestone (%)	Active limestone (%)	Salinity (g/l)	Soil pH	Organic material (%)	K (meq)
Makthar	36.35	29.4	33.29	34.4	11.73	0.31	8.32	8.4	0.297
Thala	28.85	26.35	42.74	53.19	22.625	0,66	8.01	2.41	0.137
Sekiet Sidi Youssef	25.15	41.55	38.43	44	18.51	0.5	7.92	6.59	0.052

Experimental site

The experiment was carried out in the experimental station of the Higher School of Agriculture of Mograne. The station is located at 156 m of altitude, 10.092049° of longitude and 36.428272° of latitude. It belongs to the Superior Semi-Arid climate zone. Five plants per accessions were transplanted for each level of active limestone in soil (0, 5, 10, 15, 20, 25 and 30%) in order to evaluate the tolerance of *M. tunetana* plants under calcareous soil stress.

Growth parameters

The measurements of the studied growth parameters (the stem height, the leaflet length, the leaf number and the internodes length) were realized once per week during six successive weeks.

The determination of chlorophyll a (Ch_a), chlorophyll b (Ch_b) and total chlorophylls (Ch_{a+b}) was analyzed using a spectrophotometer (JENWAY model 6300) in two different wave lengths:

λ= 645 nm and 663 nm according to Yentsch and Menzel [13] formulas cited above:

$Ch_a = 12.25\times A_{663} - 2.79\times A_{645}$

$Ch_b = 21.5\times A_{645} - 5.10 \times A_{663}$

$Ch_{a+b} = 7.15\times A_{663} + 18.71\times A_{645}$

Statistical analysis

The experimental dispositive is the factorial essay with three factors where the treatments are three different accessions of *M. tunetana*: A1, A2 and A3 and seven active limestone contents: 0, 5, 10, 15, 20, 25 and 30% and time of measurements: week 1, 2, 3, 4, 5 and 6. In order to facilitate the essay installation, we realized a split-plot dispositive where the active limestone contents are considerate as a large plot and genotypes are the secondary plot. The mathematical model of the statistical analysis is:

$$Y_{ijkl} = \mu + \rho_i + \alpha_j + \beta_k + (\alpha \beta)_{jk} + \gamma_l + (\alpha \gamma)_{jl} + (\beta \gamma)_{kl} + (\alpha \beta \gamma)_{jkl} + \sum_{ijk}$$

With: μ = mean; α_j = j^{th} accession effect j= 1, 2, 3; β_k = k^{th} active limestone content effect; k= 1,..,7; $\alpha\beta_{jk}$= interaction between j^{th} accession and k^{th} active limestone content; γ_l = l^{th} time of measurement effect; l=

1,....,6; $(\alpha\ \gamma)_{jl}$ = interaction between j^{th} accession and l^{th} time; $(\beta\ \gamma)_{kl}$ = interaction between k^{th} active limestone content and l^{th} time; $(\alpha\ \beta\ \gamma)_{jkl}$ = interaction between j^{th} accession, k^{th} active limestone content and l^{th} time ;ε_{ijk}= experimental error. The statistical analysis of this data was done using the statistical software SPSS statistics version 22.0 (IBM, Armonk, NY, USA). Data were statistically significant at $p<0.001$ for Tukey test.

III. Results

In order to study the impact of calcareous soil stress on *M. Tunetana* plant species, agronomical and physiological parameters of three different accessions were measured during six successive weeks. A three-way ANOVA was performed to assess the effects of the factors 'active limestone content', 'accessions', 'time' and their interactions, on all measured parameters (Table 3) whereas, the third factor 'time' had not showed a significant interaction with other factors. Therefore, for all results presentation, only the factors accessions and active limestone content and their interaction were presented and discussed. The four agronomical and three physiological traits displayed significant differences for the interaction 'species x active limestone content' effect. Differences between active limestone treatment were high significant for all agronomical parameters ($p<0.001$).

Table 3. Results of two-way ANOVA (F ratios) for the independent factors 'accessions' (A), 'active limestone content' (ALC) and the interaction 'accessions × active limestone content' (A x ALC).

Parameters	A	ALC	A x ALC
Leaf number	39.33 ***	41 ***	***
Stem length	13.80 ***	15.32 ***	***
Leaflet length	1.01 NS	1.03 ***	*
Internodes length	1.76 ***	1.79 ***	*
Chlorophyll a	18.57 ***	18.38***	***
Chlorophyll b	16.53***	16.80***	***
Total chlorophyll	35.11***	33.69***	***

*: significant at the 95% confidence level; ***: significant at the 99.9% confidence level; NS: not significant.

Effect of interaction between active limestone content and genotype on the leaf number of *M. tunetana*

The statistical analysis shows that the interaction between active limestone contents x genotype had been significantly affected ($p<0.001$) the leaf number of *M. tunetana* during growth monitoring period. Table 4. shows the average of leaf number per accession for the three accessions and the seven active limestone contents. The lowest leaf number per plant was recorded for the active limestone contents of 0% and 30 %. Thereby *M. tunetana* does not tolerate the acidic soils with a pH lower than 5. The accession A2 from Kasserine owned the highest number of leaves per plant. In fact, the content of 20% generates the highest leaf number per plant with an average of 87 leaves per plant (table 4.). The highest leaf number per plant for the accession A3 from Sekiet Sidi Youssef is obtained for 5% content with an average of 76 leaves per plant. This result confirms the genetic variability between the accessions of *M. tunetana*. Furthermore, environment and initial conditions during accessions growth may affect their development and their vegetative growth towards the seven active limestone rates.

Table 4. Effect of accessions and active limestone content interaction (A x ALC) on leaf number of three *M. tunetana* accessions collected respectively from Makthar, Thala and Sekiet Sidi Youssef. The shown values are means ±SE (n=5). Means with the same letters are not significantly different (ANOVA, Tukey test at $p<0.001$) nd: not determined.

Active limestone content ***	Accessions ***			Means
	A1	A2	A3	
0 %	7±0,95	6±0,79	12±1,32	9±1,81 [a]
5 %	42±4,68	80±5,46	76±6,34	66±11,83 [d]
10 %	29±3,14	74±8,98	nd	52±22,70 [bc]
15 %	29±1,56	25±4,06	60±6,49	38±11,01 [b]
20 %	46±4,64	87±6,62	28±2,04	53±17,42 [cd]
25 %	22±1,38	85±6,81	nd	53±31,33 [cd]
30 %	9±1,50	18±1,99	22±1,45	16±3,54 [a]

Means	26±5,58 [a]	53±13,40 [c]	39±12,10 [b]	-

***: significant at the 99.9% confidence level.

Effect of interaction between active limestone content and genotype on the stem height of *M. Tunetana* accessions

The statistical analysis shows that interaction between active limestone content x genotype had been affected the stem height of *M. tunetana* plant accessions during the experimental period at $p<0.001$. The different stem height values for the three accessions and the seven active limestone contents are showed in Table 5. In fact, the accession A2 from Kasserine showed the highest plants for 5% content with an average stem height of 20.06 cm followed by A1 from Siliana with an average of 19.54 cm for 15 % active limestone content. The stem of the accession A3 plants was increased during the trial period: it had reached 18.13 cm for 15% rates in the end of experience while it measured only 15 cm just after transplantation. This result explains the adaptation of Sekiet Sidi Youssef accession with active limestone excess in soil.

Table 5. Effect of accessions and active limestone content interaction (A x ALC) on stem height of three *M. tunetana* accessions collected respectively from Makthar, Thala and Sekiet Sidi Youssef. The shown values are means ±SE (n=5). Means with the same letters are not significantly different (ANOVA, Tukey test at $p<0.001$) nd: not determined.

Active limestone content ***	Accessions***			Means
	A1	A2	A3	
0%	14,01±0,53	10,68±0,91	13,41±0,69	12,70±1,02 [a]
5%	18,93±0,55	23,45±0,77	16,28±0,89	19,55±2,09 [d]
10%	15,93±1,07	14,41±0,55	nd	15,17±0,76 [bc]
15%	19,54±1,01	13,84±1,00	18,13±1,30	17,17±1,72 [cd]
20%	15,85±0,38	17,51±0,85	9,63±0,50	14,33±2,40 [b]
25%	14,75±0,75	18,67±0,30	nd	16,71±1,96 [bc]
30%	13,62±0,12	11,69±0,58	9,60±0,53	11,64±1,16 [a]
Means	16,09±0,88 [b]	15,75±1,68 [b]	9,58±2,74 [a]	-

***: significant at the 99.9% confidence level.

Effect of interaction between active limestone content and genotype on the internodes length of *M. tunetana* accessions

The statistical analysis shows that interaction between active limestone x genotype had been significantly affected the length of the internodes of *M. tunetana* plants during the period of growth monitoring at $p<0.001$. Table 6. shows the difference in the internodes length in function of genotypes and active limestone content for the three accessions and the seven active limestone levels. We recorded the longest internodes for all studied plants at 30% of active limestone reaching 2.67 cm for plants of A1 from Siliana which explain the morphological response of *M. tunetana* plant response under active limestone excess in soil.

Table 6. Effect of accessions and active limestone content interaction (A x ALC) on internodes length of three *M. tunetana* accessions collected respectively from Makthar, Thala and Sekiet Sidi Youssef. The shown values are means ±SE (n=5). Means with the same letters are not significantly different (ANOVA, Tukey test at $p<0.001$) nd: not determined.

Active limestone content ***	Accessions ***			Means
	A1	A2	A3	
0 %	2,17±0,13	1,69±0,09	1,16±0,06	1.67±0.29 [a]
5 %	1,94±0,12	1,97±0,19	1,56±0,09	1.82±0.13 [ab]
10 %	1,32±0,09	1,70±0,10	nd	1.51±0.19 [a]
15 %	2,12±0,15	1,83±0,10	1,47±0,14	1.81±0.19 [ab]
20 %	1,97±0,10	2,06±0,09	1,29±0,14	1.78±0.24 [b]
25 %	1,67±0,25	2,07±0,10	nd	1.87±0.20 [b]
30 %	2,67±0,12	2,05±0,18	1,53±0,11	2.08±0.33 [b]

Means	1,98±0,16 b	1,91±0,06 b	1,40±0,08 a	-

***: significant at the 99.9% confidence level.

Effect of interaction between active limestone content and genotype on the leaflet length of *M. tunetana* accessions

The statistical analysis shows that interaction between active limestone x genotype had been significantly affected (p<0.05) the leaflet length of *M. tunetana* plants during the experimental period. The different leaflet length values for the three accessions under seven active limestone contents are shown in table 7. In fact, A3 from Sekiet Sidi Youssef showed the biggest leaflet with an average of 1.32 of leaflet length for the content of 15% of active limestone in soil. For all studied accessions, more the active limestone content in soil was concentrated; more the leaflet size was decreased.

Table 7. Effect of interaction between active limestone content and genotype on the leaflet length of three accessions of *M. tunetana* collected respectively from Makthar, Thala and Sekiet Sidi Youssef. The shown values are means ±SE (n=5). Means with the same letters are not significantly different (ANOVA, Tukey test at p<0.001). nd: not determined

Active limestone content ***	Accessions			Means
	A1	A2	A3	
0%	1,12±0,25	0,75±0,04	0,77±0,04	0,88±0,12 ab
5%	1,26±0,04	1,26±0,06	1,02±0,05	1,18±0,08 c
10%	0,95±0,04	1,04±0,04	nd	1,00±0,04 bc
15%	1,03±0,05	1,06±0,06	1,32±0,34	1,13±0,09 c
20%	1,26±0,04	1,29±0,04	0,85±0,03	1,13±0,14 c
25%	1,22±0,04	1,24±0,03	nd	1,23±0,01 c
30%	0,67±0,08	0,70±0,06	0,59±0,03	0,66±0,03 a
Means	1,07±0,08	1,05±0,09	0,91±0,12	-

***: significant at the 99.9% confidence level.

Effect of interaction between active limestone contents and genotype on the chlorophyll content of *M. tunetana* accessions

The statistical analysis shows that the interaction between active limestone content and genotype of *M. tunetana* had significantly affected the chlorophyll contents of *M. tunetana* at p<0.001. Tables 8, 9, 10 show the variation of the chlorophyll concentrations (a, b and total) for the three accessions of *M. tunetana*. In fact, the lowest chlorophyll contents were shown in 0% and 30% active limestone content in soil. These results are explained by the increase of the plant photosynthetic activity. While the highest content of total chrophyll was observed for the content of 20% for the accession A2 from Kasserine with 58.06 ppm. For the other accessions A1 and A3, the highest values of total chlorophyll contents were showed at 5% of active limestone content (Table 10.).

Table 8. Results of the interaction between accessions and active limestone content (A x ALC) impact on chlorophyll (a) content of three *M. tunetana* accessions collected respectively from Makthar, Thala and Sekiet Sidi Youssef. The shown values are means on ppm (n=5). Means with the same letters are not significantly different (ANOVA, Tukey test at p<0.001) nd: not determined

Active limestone contents***	Accessions ***			Means
	A1	A2	A3	
0 %	16.04	15.09	19	16.71 c
5 %	12.98	19.91	22.42	18.44 d
10 %	15.18	17.22	nd	16.2 b
15 %	21.03	16.41	23.68	20.37 e
20 %	19.15	23.26	19.63	20.68 f
25 %	17.84	23.02	nd	20.43 e
30 %	15.49	15.04	17	15.84 a
Means	16.81 a	18.56 b	20.35 c	-

***: significant at the 99.9% confidence level.

Table 9. Results of the interaction between accessions and active limestone content (A x ALC) impact on chlorophyll (b) content of three *M. tunetana* accessions collected respectively from Makthar, Thala and Sekiet Sidi Youssef. The shown values are means on ppm (n=5). Means with the same letters are not significantly different (ANOVA, Tukey test at p<0.001) nd: not determined

Active limestone contents***	Accessions ***			Means
	A1	A2	A3	
0 %	12.24	10.5	12.3	11.68 [a]
5 %	25.9	12.39	17.94	18.74 [d]
10 %	17.47	10.03	nd	13.75 [b]
15 %	15.76	10.13	21.32	15.74 [c]
20 %	13.99	34.8	13.97	20.92 [e]
25 %	12.59	34.76	nd	23.67 [f]
30 %	11.85	13.51	13.91	13.09 [ab]
Means	15.69 [a]	18.02 [b]	15.89 [a]	-

***: significant at the 99.9% confidence level.

Table 10. Results of the interaction between accessions and active limestone content (A x ALC) impact on total chlorophyll content of three *M. tunetana* accessions collected respectively from Makthar, Thala and Sekiet Sidi Youssef. The shown values are means on ppm (n=5). Means with the same letters are not significantly different (ANOVA, Tukey test at p<0.001) nd: not determined

Active limestone contents***	Accessions ***			Means
	A1	A2	A3	
0 %	28.28	25.59	31.30	17.96 [a]
5 %	38.88	32.31	40.36	37.18 [e]
10 %	32.65	27.26	-	29.96 [c]
15 %	36.80	26.53	45	36.11 [d]
20 %	33.14	58.06	33.61	41.60 [f]
25 %	30.43	57.78	-	44.11 [g]
30 %	27.35	28.55	30.91	28.94 [b]
Means	32.50 [a]	36.58 [c]	36.24 [b]	-

***: significant at the 99.9% confidence level.

IV. Discussion

The *M. tunetana* plants were moderately resistant by a slight increase in leaf mean number at the beginning of the essay for the content of 30 % of soil active limestone. However, it showed that the average of leaf number decreased lately (Table 3.). The excess of the active limestone in the soil can promote the appearance of superficial crusts [14] which promotes the water stagnation known as the phenomena of hypoxia caused by a bad drained ground, poorly ventilated or compact can generate the hypoxia [15] which involves a stress for the plants and stimulates the synthesis of ethylene [16, 17, 18, 19, 20]. The ethylene steps in the stem elongation fall of the fruits and also the abscission of leaves and flowers [21, 18, 22, 23, 24, 25].The high pH content in the calcareous soil reduces iron solubility and causes iron chlorosis in plants [26, 27]. The lowest contents of chlorophyll a, b and total are shown in 30% of active limestone rate in soil for the three *M. tunetana* accessions plants (Table 8, 9, 10). According to [27], more than 8% of active limestone content in soil causes many physiological symptoms of Fe deficiency for *Medicago scutellata* such as the decrease of the chlorophyll content in leaves which is an interesting physiological indicator of the plant tolerance to abiotic stress [26].The fall of leaves of the three accessions of *M. tunetana* is bounded to the ethylene synthesis. Calcium excess and hypoxia has negative and positive effects on the ethylene synthesis) while acting on its production [28, 29, 30, 31, 32]. On the other hand, the low vegetative growth on the content of 0% can be explained by the nature of the acidophil soil. The perennial species of *Medicago*, specially *M. tunetana*, tend to appear in the calcareous or almost neutral soils [33].

The same for the parameters of the stem height and the internodes length, the content of 30% of active limestone content has the lowest values and this is for the three accessions of *M. tunetana* (Table 5. and 6.). We can explain these phenomena by the death of the principal stem on this content opposite the excess of soil active limestone; thus, the plants of *M. tunetana* continue their cycle thanks to the rhizomes. This rhizome gives birth to the air stems and adventitious roots from its buds and nodes [34]. On the physiological level, the nanism or the reduction of the internodes length supposes the nitrogen deficiency and this generates the absence of auxin which is responsible for the elongation. In addition, it is important to report that during the period of this essay, we have observed a yellowing marked by the old leaves. This yellowing is due to the nitrogen deficiency. The nitrogen nutrition is incontestably more difficult in the calcareous soil because of the exaggerated absorption of calcium which would cause an imbalance of the nutrition [35]. The perennial alfalfa fixes nitrogen thanks to a bacterium of the soil (*Rhizobium meliloti* Frank) that lives in symbiosis with plants [36] and which is a very sensitive bacterium to the acidic soil, the absence of the exchangeable calcium and also it is strictly aerobic that's why it cannot be encountered in the soil gorged with water. Consequently, the hypoxia, caused by the active limestone excess in soil, generates the nitrogen deficiency at the three accessions of *M. tunetana.* Sanaa [14] showed that the calcium excess prevented the assimilation of some biogenic salts such as Bore and Magnesium. Also, the excess of Ca^{2+} in the soil has a negative impact on the enzymatic activities and the iron acquisition genes [37, 38, 39, 40]. García et al., [15] indicated that hypoxia and calcium excess may induce iron chlorosis by limiting the expression of iron acquisition genes and also by negatively affects different steps of ethylene synthesis. Although the calcareous soil causes the ferric chlorosis, no young leaves yellowing was observed for the three accessions of *M. tunetana* during the essay. The absence of the iron deficiency symptoms is due essentially to the leguminous adaptation to the unfavourable soil conditions. Fabaceae plant absorbs iron through the excretion of ferric-chelate reductase which reduces Fe^{3+} (not assimilable by plants) to the Fe^{2+} (assimilable by plants)[41, 12]. In addition, the ethylene takes part in the regulation of several iron acquisition genes [42, 43, 44, 45, 46, 47, 48].

M. tunetana is a calcicole species [38], indeed it tolerates the excess of active limestone in soil whereas it has difficulties with the hypoxia of soil. *M. Sativa* and *M. tunetana,* don't tolerate the hydromorphy.

V. Conclusion

The conservation of the threatened and endemic phytogenetic resources is gaining broad interest. Then, it seems to be interesting to discover all Tunisian endemic species in order to preserve their disappearance and especially to have a clear idea on their agronomic and economic potentials. In order to evaluate this rare and endemic species, this work evaluates the response of *M. tunetana* plants under calcareous soil stress by studying agronomical and physiological parameters (leaf number, stem height, internodes length, leaflet length and the chlorophyll contents: a, b, total). In fact, the accession A2 from Kasserine showed the best adaptation to the active limestone appears on the content of 25% in terms of studied growth parameters followed by the accession A1 from Siliana which showed the best adaptation to the active limestone on the level 20%.However, the accession A3 from Kef has a weak aptitude to be adapted to active limestone since it presents its best growth to the active limestone level of 5% except the height of the stem which has its best growth to the level 15%. These results give an idea on genetic variability for the different accessions of *M. tunetana* that highlights the wealth of this biodiversity.

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