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Seed Germination and Early Seedling Growth Responses to Drought Stress in Annual *Medicago* L. and *Trifolium* L. Forages

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Abstract: Climate change in the Mediterranean-like regions of South Africa has resulted in increased rainfall variability, a delayed start to the wet winter season, and increased occurrences of out-of-season summer rainfall events. These changes in bioclimatic conditions are predicted to become more pronounced and, therefore, could result in poor seedling establishment and false breaks from the soil seed bank, followed by seedling desiccation in annual medic and clover pastures. This study therefore aimed to quantify and compare the responses of three annual *Medicago* species and five annual *Trifolium* species to drought stress imposed at the seed germination, seedling establishment, and early seedling growth stages. Three separate controlled trials were conducted. Firstly, the seeds were germinated in seed germination chambers at constant temperatures ranging from 5 to 30 °C, in 5 °C increments, with five osmotic treatments within each temperature, and germination was recorded daily. For the second experiment, seeds were planted at 100, 70, 50, and 30% of the soil's moisture-holding capacity without subsequent watering, and emergence was recorded daily. For the third experiment, one-month-old seedlings were subjected to two water-limitation periods (15 or 30 days), and their subsequent morphological responses were measured. The results from these experiments indicated that the species differed significantly in their responses to drought, and the best-performing species often differed when drought was imposed at different development stages. Five species—*M. polymorpha* L., *M. truncatula* Gaertn., *T. alexandrinum* L., *T. vesiculosum* Savi., and *T. subterraneum* L. ssp. *subterraneum*—were able to tolerate incidences of drought better than other species and, thus, should be prioritized for further research into the variation in drought tolerance between cultivars within these species.

Keywords: clovers; crop rotations; ley farming; medics; pastures

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1. Introduction

Global climate change is a major concern for current livestock production systems. Apart from the effects on livestock health, production, and water availability, global warming and the associated changes in the variability of bioclimatic conditions will also affect the production, availability, and quality of forage [1]. Mediterranean-type ecosystems are especially vulnerable to climate change, where an intensification of extreme climatic events is expected to result in increases in aridity [2,3]. In the Mediterranean-like region of South Africa, which primarily falls within the Western Cape Province, climate change has resulted in a decrease in winter rainfall and a slight shift to summer rainfall. Furthermore, increases in maximum winter temperatures, out-of-season rainfall events, variability in the periods between rainfall events, and a delay in the start of the winter rainfall season have already been reported, with these conditions predicted to be more pronounced under future climate change conditions [4,5]. Within the Mediterranean-like region of South Africa, the Swartland, Rûens, and Overberg areas of the Western Cape Province are major cropping zones. Here, a large diversity of pasture species are used in the mixed crop–livestock production region, in which approximately 24% of the available agricultural land is planted with legume pastures consisting primarily of annual *Medicago* (medics), *Trifolium* (clover), and the perennial forage legume species *Medicago sativa* (lucerne/alfalfa) [6,7]. In these areas, the forages are planted under dryland conditions in rotation with cash crops such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), canola (*Brassica napus*), and oats (*Avena sativa*) [6–8].

Annual forage legumes have been introduced into these rotation systems because of their positive impacts on livestock production, nutritional quality, nitrogen fixation abilities, and ability to increase soil fertility by maintaining soil organic matter and improving the soil's physical conditions [6,9–13]. These pasture–crop rotations have been shown to be highly effective, and the inclusion of the legume component results in various benefits, often leading to an improvement in overall farm productivity [8,14–21].

Further climate change within the area may, however, significantly affect the productivity of the pasture phase of these rotation systems by influencing the seed germination, seedling establishment, and growth of these annual medic and clover pastures [22–24]. This is especially true under dryland production systems, where the water needed for seed germination and subsequent seedling establishment and growth is limited and available only for short periods [24–27]. Under the predicted climate change scenarios for the winter rainfall region of South Africa, a major concern is that increased rainfall variability and the delayed start to the wet season (with accompanying increases in temperatures) will result in poor seedling establishment and survival after establishment. Further increases in the occurrence of out-of-season rainfall events and extended dry periods could also result in more false breaks from the soil seed bank, followed by seedling desiccation. This, in turn, could significantly reduce the soil seed bank, which is one of the key features that makes these pasture–crop rotation systems effective [15,28,29].

Due to the importance of these annual legume forages in pasture–crop rotation systems, it is necessary to obtain a greater understanding of how these species will respond to the predicted climatic changes. Particularly, a greater understanding of how changes in moisture availability and increases in temperatures during the germination and early establishment stages of the pastures could play a major role in the establishment of these forages and help stabilize pasture performance under future bioclimatic conditions.

The aim of this study was therefore to quantify and compare the responses of three annual *Medicago* and four annual *Trifolium* species to drought stress imposed at the seed germination, seedling establishment, and early seedling growth stages. It was hypothesized that (1) certain species evaluated would be better suited to germination and establishment under drought stress conditions, and (2) certain species evaluated would be better adapted to recover from drought. This will inform future breeding and selection initiatives to improve drought tolerance in these forages.

2. Materials and Methods

2.1. Seed Germination under Increasing Water Limitation and Temperatures

Seeds of annual *Medicago* (*M. polymorpha*, *M. truncatula*, and *M. littoralis* Loisel.) and *Trifolium* (*T. alexandrinum*, *T. michelianum* L., *T. subterraneum* ssp. *brachycalycinum* Katzn. & Morley, *T. subterraneum* ssp. *subterraneum*, and *T. vesiculosum*) forage legumes were obtained from local seed distributors. The initial germination potential of these seeds was determined by germinating four replicates of 100 seeds of each species in the dark in 9 cm Petri dishes on filter paper, in germination chambers set at a constant temperature of 20 °C. The seeds were watered as needed, and germination was recorded daily for 15 days. Seeds were regarded as germinated after the emergence of a radicle of approximately 0.5 cm. All species evaluated had an initial germination potential below 100%; therefore, all germination achieved in the subsequent seed germination experiments for all species was calculated as a percentage of the initial germination. This was calculated according to Equation (1):

$$\text{FGP } (\%) = (x_n/x_i) \times 100 \quad (1)$$

where FGP (%) is the final germination percentage expressed as a percentage of the initial germination potential of the species, X_n is the germination percentage obtained under the different experimental treatments, and X_i is the initial germination percentage.

Thereafter, four replicates of 100 seeds for each temperature and osmotic treatment combination within a species were placed in 9 cm Petri dishes on a layer of filter paper. Germination chambers were calibrated to constant temperatures of 5–30 °C in increments of 5 °C, under continuous dark conditions. Within each temperature treatment, five osmotic treatments (0 MPa, −0.1 MPa, −0.3 MPa, −0.5 MPa, and −0.7 MPa) were imposed on the seeds. The osmotic treatments were prepared using polyethylene glycol 6000 (PEG6000) in accordance with the methods of Michael and Kaufmann [30] at each of the temperatures evaluated. The osmotic solutions were stored in the germination chambers for each of the associated temperature treatments to maintain the desired osmotic potential of the solutions. Then, 5 mL of each osmotic solution was added to the Petri dishes, and distilled water was used as the 0 MPa or control treatment. After watering, the Petri dishes were sealed using parafilm to prevent excessive water loss. The filter paper and osmotic solutions were replaced every five days to keep the osmotic conditions within the Petri dishes relatively constant. Seed germination was recorded daily for 15 days, and germinated seeds were removed from the Petri dishes as required to minimize excessive uptake of the available water resources by germinated seeds. The day when the first germinated seed was recorded was regarded as the time taken for germination to commence.

2.2. Seedling Emergence under Moisture Stress

Seedling emergence in annual medic and clover species was evaluated under greenhouse conditions with natural light and an average temperature of 20 ± 3 °C. Prior to planting, four pots (15 cm wide and 17 cm deep) filled with a sandy loam soil (Table 1) were irrigated until water started draining from the bottom of the pots. Draining of excess water from the pots was allowed for 12 hours to reach field capacity, after which the gravimetric water content (θ_g g.g^{-1}) was determined. The soil moisture content of these pots was regarded as the field capacity (100%). Thereafter, the experimental pots were watered to soil moisture contents of approximately 100%, 70%, 50%, and 30% of capacity and expressed as a percentage of the initial field capacity. In each soil moisture treatment, four replicates of 25 pre-germinated seeds (radicle $\geq$ 0.3 cm) were planted at a depth of 1 cm and arranged randomly on the greenhouse benches. Pre-germinated seeds were used to ensure that all seeds planted were able to establish. Seedling emergence (i.e., two expanded cotyledons visible) and mortality were counted daily for 14 days after planting, along with the number of emerged seedlings and the number of seedlings that eventually died in each pot. The dates of the first and last seedlings' emergence were recorded, and at the end of the trial the rate of seedling mortality was calculated from the maximum seedling emergence per pot.

Table 1. Soil characteristics.

N (g/kg)	P (g/kg)	K (g/kg)	Ca (g/kg)	Mg (g/kg)	Na (g/kg)	pH (Water)	Sand (%)	Silt (%)	Clay (%)
1.42 ± 0.17	0.06 ± 0.01	2.35 ± 0.06	2.23 ± 0.20	0.59 ± 0.04	0.02 ± 0.001	7.73 ± 0.14	69.8 ± 4.45	10.0 ± 0.75	20.2 ± 1.90

2.3. Early Growth Responses to Moisture Stress in Medicago and Trifolium Seedlings

A greenhouse pot study was conducted to determine the phenotypic adjustments made in response to moisture stress by annual medic and clover seedlings. The experiment consisted of five replicates of two treatments; the amount of water (well-watered or water-limited) and the time of harvest (15 or 30 days after water limitation) were arranged randomly on the greenhouse benches. Before planting, the seeds were pre-germinated in Petri dishes on two layers of filter paper. After radicle emergence ($\geq$0.3 cm), five pre-germinated seeds were transplanted into 15 cm tall × 10 cm wide plastic planting bags filled with a sandy loam soil. After seedling emergence, the planting bags were thinned to one plant per bag. The bags were watered to capacity (until water started draining from the planting bags) once per week until 30 days after establishment, after which watering was withheld for the 30-day moisture-stressed plants. Watering continued up to 45 days for the 15-day moisture-stressed plants, after which water was withheld. Water limitation was staggered so that all plants could be harvested at the same time and the same age for comparative purposes. At each harvesting time, the seedling was removed. The roots were carefully washed with distilled water and then blotted dry, after which shoot and root length measurements were taken using a Grip GV9371 digital Vernier caliper. After length measurements, the seedlings were separated into roots and shoots and oven-dried (Drying oven 620, Scientific Engineering (Pty) Ltd., Roodepoort, South Africa) at 60 °C until a constant mass was achieved for the determination of dry mass using a digital scale (Digital Pocket Mini Scale, Tennessee - Laboratory Supplies, Louisville, TN, USA).

2.4. Statistical Analyses

All data were statistically analyzed using IBM SPSS Statistics for Windows Version 22.0 (IBM Corporation, Armonk, NY, USA). Where necessary, seed germination data were ARCSINE-transformed to obtain normality and back-transformed for final illustrations. One-way analysis of variance (ANOVA) with Fishers' LSD post hoc test was used to determine whether significant differences were obtained in seed germination between temperatures and osmotic treatments within each species, as well as in seedling emergence and early seedling growth measurements within each species. For comparisons of seed germination between species, within each temperature, all results were standardized by calculating the z-score using the equation $z = (x - \mu)/\delta$, where x is the raw value (germination percentage), μ is the population mean, and δ is the population's standard deviation. The z-scores obtained were used to determine differences in germination potential between different species at the same temperatures and within the same osmotic treatment using ANOVA in SPSS. For final illustration, all z-scores were back-transformed to raw percentages using the equation $x = (\delta \times z) + \mu$. Seedling vigor (maximum seedling emergence, days to first seedling emergence, and days to 100% seedling mortality) was correlated with soil moisture content and statistically analyzed using Pearson's correlation coefficients for each of the medic and clover species.

3. Results

3.1. Seed Germination

The results from the germination trial (Figure 1) indicated that annual medic and clover species differ in their germination responses to temperature and osmotic stress conditions, as well as to combinations of these stresses (Table 2). Species such as *T. vesiculosum*, *T. alexandrinum*, *M. polymorpha*, and *M. littoralis* were able to germinate optimally (>70%) at temperatures up to 30 °C, 30 °C, 25 °C, and 25 °C, respectively, under well-watered or slightly drought-stressed conditions (−0.1 MPa) (Figure 1). The results also showed that occasionally there were species that could germinate optimally (>70%) under moderately drought-stressed conditions (−0.3 MPa). Species that could achieve this included *M. polymorpha* (up to temperatures of 15 °C), *T. alexandrinum*, *M. truncatula*, and *M. littoralis* (up to temperatures of 20 °C), and *T. vesiculosum* (up to temperatures of 25 °C). Furthermore, *T. vesiculosum* was able to achieve a germination percentage of more than 80% at temperatures of 10 °C and 15 °C, even under severe water limitation (−0.5 MPa).

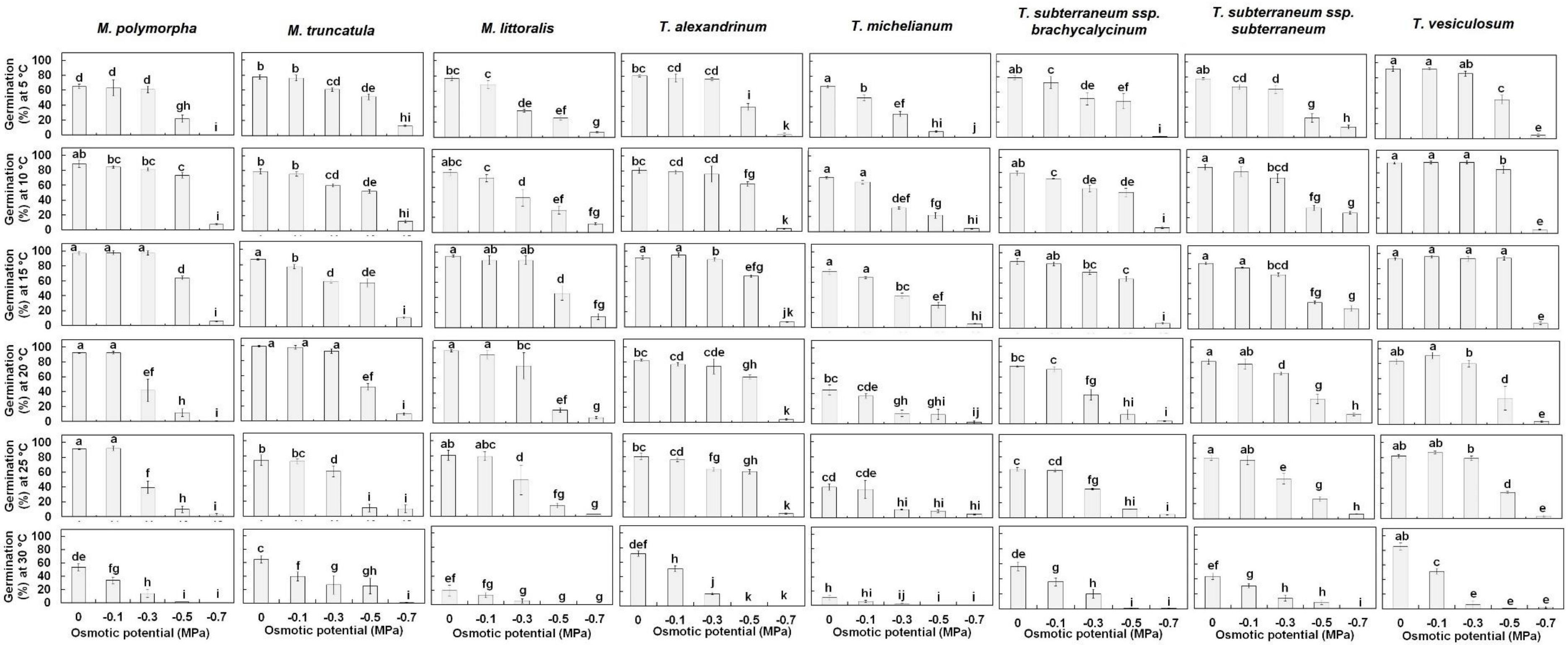

Figure 1. Seed germination (%) in three *Medicago* and five *Trifolium* species under different germination temperatures and osmotic treatments. Different letters indicate significant differences ($p < 0.05$) in seed germination between different osmotic and temperature treatments. Columns with error bars represents means ± SEMs. Comparisons are made within species.

Table 2. Differences in seed germination potential (mean % ± SEM) between three annual *Medicago* and five annual *Trifolium* species under different germination temperatures and osmotic treatments. Different letters indicate significant differences ($p < 0.05$) in germination between the different species. Comparisons were made within each osmotic treatment within a temperature.

Temperature Treatment	Osmotic Treatment	*T. alexandrinum*	*T. michelianum*	*T. subterraneum* ssp. *brachycalycinum*	*T. subterraneum* ssp. *subterraneum*	*T. vesiculosum*	*M. polymorpha*	*M. truncatula*	*M. littoralis*
5 °C	0 MPa	80 ± 1.2 ^{b}	67 ± 1.3 ^{c}	67 ± 2.1 ^{c}	65 ± 1.0 ^{cd}	92 ± 3.3 ^{a}	59 ± 2.7 ^{d}	67 ± 2.5 ^{c}	77± 2.7 ^{b}
	−0.1 MPa	78 ± 5.5 ^{b}	51 ± 3.8 ^{d}	62 ± 6.6 ^{cd}	56 ± 2.6 ^{cd}	92 ± 1.5 ^{a}	57 ± 9.7 ^{cd}	66 ± 3.2 ^{bcd}	69 ± 5.1 ^{bc}
	−0.3 MPa	76 ± 2.0 ^{a}	30 ± 3.6 ^{e}	43 ± 7.1 ^{cd}	53 ± 4.4 ^{bc}	86 ± 3.3 ^{a}	55 ± 4.1 ^{b}	52 ± 2.0 ^{bc}	35 ± 1.9 ^{de}
	−0.5 MPa	39 ± 4.4 ^{b}	7 ± 1.1 ^{d}	41 ± 8.6 ^{b}	22 ± 4.6 ^{c}	52 ± 5.9 ^{a}	20 ± 4.5 ^{cd}	44 ± 3.2 ^{b}	25 ± 2.3 ^{c}
	−0.7 MPa	3 ± 1.7 ^{c}	0 ± 0.0 ^{d}	1 ± 0.5 ^{d}	12 ± 2.1 ^{a}	5 ± 1.8 ^{bc}	0 ± 0.0 ^{d}	11 ± 0.9 ^{a}	7 ± 0.8 ^{b}
10 °C	0 MPa	81 ± 3.5 ^{b}	71 ± 1.8 ^{cd}	67 ± 2.5 ^{d}	72 ± 2.9 ^{bcd}	93 ± 1.2 ^{a}	80 ± 4.2 ^{bc}	68 ± 2.9 ^{d}	79 ± 4.0 ^{bc}
	−0.1 MPa	76 ± 2.8 ^{b}	65 ± 3.4 ^{de}	61 ± 0.5 ^{e}	67 ± 5.3 ^{cde}	94 ± 1.8 ^{a}	76 ± 1.6 ^{bc}	65 ± 2.4 ^{de}	72± 4.7 ^{bcd}
	−0.3 MPa	76 ± 10.7 ^{b}	31 ± 2.3 ^{d}	50 ± 3.8 ^{c}	60 ± 4.9 ^{bc}	94 ± 2.4 ^{a}	73 ± 2.0 ^{b}	52 ± 1.9 ^{c}	46 ± 10.9 ^{cd}
	−0.5 MPa	63 ± 3.0 ^{b}	21 ± 5.4 ^{d}	46 ± 4.6 ^{c}	28 ± 2.6 ^{d}	84 ± 4.9 ^{a}	66 ± 3.3 ^{b}	45 ± 1.9 ^{c}	29 ± 5.2 ^{d}
	−0.7 MPa	4 ± 0.6 ^{d}	4 ± 1.1 ^{d}	6 ± 1.1 ^{cd}	22 ± 1.4 ^{a}	5 ± 0.4 ^{cd}	7 ± 0.9 ^{c}	11 ± 1.5 ^{b}	11 ± 1.3 ^{b}
15 °C	0 MPa	92 ± 2.4 ^{ab}	74 ± 3.4 ^{cd}	75 ± 3.4 ^{cd}	72 ± 1.6 ^{d}	93 ± 1.7 ^{ab}	88 ± 1.7 ^{b}	75 ± 0.8 ^{c}	95 ± 1.3 ^{a}
	−0.1 MPa	95 ± 2.6 ^{a}	66 ± 1.4 ^{b}	73 ± 2.1 ^{b}	67 ± 0.6 ^{b}	96 ± 1.7 ^{a}	88 ± 2.6 ^{a}	67 ± 2.3 ^{b}	89 ± 6.0 ^{a}
	−0.3 MPa	89 ± 2.4 ^{a}	42 ± 3.4 ^{d}	63 ± 2.4 ^{b}	60 ± 1.7 ^{bc}	93 ± 3.3 ^{a}	88 ± 2.5 ^{a}	50 ± 1.8 ^{cd}	89 ± 5.8 ^{a}
	−0.5 MPa	67 ± 1.7 ^{b}	30 ± 3.6 ^{d}	56 ± 2.8 ^{b}	29 ± 1.7 ^{d}	94 ± 2.2 ^{a}	58 ± 2.4 ^{bc}	49 ± 4.3 ^{c}	45 ± 9.3 ^{c}
	−0.7 MPa	7 ± 0.5 ^{c}	5 ± 0.4 ^{c}	6 ± 0.9 ^{c}	23 ± 3.1 ^{a}	8 ± 1.7 ^{c}	5 ± 0.7 ^{c}	10 ± 0.8 ^{bc}	15 ± 4.1 ^{b}
20 °C	0 MPa	82 ± 1.4 ^{b}	45 ± 6.6 ^{d}	63 ± 1.1 ^{c}	68 ± 3.0 ^{c}	83 ± 3.9 ^{ab}	83 ± 0.7 ^{ab}	90 ± 3.6 ^{ab}	96 ± 1.5 ^{a}
	−0.1 MPa	77 ± 2.2 ^{b}	37 ± 3.0 ^{d}	60 ± 2.7 ^{c}	65 ± 5.7 ^{c}	91 ± 4.5 ^{a}	83 ± 1.6 ^{ab}	91 ± 4.5 ^{a}	91 ± 5.1 ^{a}
	−0.3 MPa	74 ± 10.2 ^{b}	14 ± 4.3 ^{d}	32 ± 5.8 ^{d}	55 ± 1.7 ^{c}	80 ± 4.5 ^{a}	50 ± 7.2 ^{c}	81 ± 3.2 ^{a}	76 ± 17.5 ^{b}
	−0.5 MPa	60 ± 2.9 ^{a}	12 ± 6.8 ^{d}	10 ± 5.3 ^{d}	27 ± 5.0 ^{bc}	35 ± 4.6 ^{bc}	11 ± 4.6 ^{d}	39 ± 3.9 ^{b}	17 ± 2.8 ^{c}
	−0.7 MPa	4 ± 1.2 ^{bc}	2 ± 1.7 ^{c}	3 ± 0.4 ^{c}	10 ± 1.7 ^{a}	4 ± 1.1 ^{bc}	1 ± 0.3 ^{c}	8 ± 1.6 ^{a}	7 ± 1.7 ^{ab}
25 °C	0 MPa	80 ± 4.3 ^{a}	40 ± 4.0 ^{d}	55 ± 2.1 ^{c}	67 ± 2.6 ^{bc}	82 ± 2.2 ^{a}	82 ± 0.8 ^{a}	64 ± 6.2 ^{c}	81 ±7.0 ^{a}
	−0.1 MPa	76 ± 2.6 ^{b}	37 ± 12.0 ^{e}	53 ± 1.8 ^{d}	64 ± 4.8 ^{cd}	87 ± 1.9 ^{a}	83 ± 2.9 ^{a}	63 ± 2.8 ^{cd}	79 ± 5.6 ^{b}
	−0.3 MPa	63 ± 2.0 ^{ab}	11 ± 0.4 ^{d}	32 ± 0.8 ^{c}	44 ± 5.7 ^{bc}	79 ± 3.0 ^{a}	36 ± 7.5 ^{c}	51 ± 6.2 ^{bc}	48 ± 19.5 ^{bc}
	−0.5 MPa	60 ± 2.8 ^{a}	8 ± 1.9 ^{d}	10 ± 0.3 ^{d}	22 ± 2.8 ^{c}	34 ± 1.5 ^{b}	9 ± 3.8 ^{d}	13 ± 4.7 ^{d}	14 ± 3.3 ^{cd}
	−0.7 MPa	5 ± 0.9 ^{a}	4 ± 0.8 ^{a}	4 ± 0.3 ^{a}	5 ± 0.6 ^{a}	3 ± 0.9 ^{a}	3 ± 1.1 ^{a}	9 ± 4.3 ^{a}	2 ± 0.0 ^{a}
30 °C	0 MPa	72 ± 3.6 ^{a}	11 ± 3.6 ^{d}	48 ± 4.9 ^{bc}	36 ± 3.4 ^{c}	85 ± 4.6 ^{a}	48 ± 4.9 ^{bc}	56 ± 4.8 ^{b}	20 ± 7.4 ^{d}
	−0.1 MPa	51 ± 4.2 ^{a}	5 ± 1.5 ^{c}	31 ± 4.4 ^{b}	26 ± 2.2 ^{b}	51 ± 3.8 ^{a}	31 ± 4.4 ^{b}	34 ± 5.8 ^{b}	13 ± 3.3 ^{c}
	−0.3 MPa	17 ± 1.3 ^{a}	1 ± 0.8 ^{a}	17 ± 4.9 ^{a}	12 ± 3.4 ^{a}	6 ± 5.4 ^{a}	17 ± 4.9 ^{a}	23 ± 11.9 ^{a}	4 ± 2.0 ^{a}
	−0.5 MPa	0 ± 0.0 ^{b}	0 ± 0.0 ^{b}	1 ± 0.5 ^{b}	7 ± 2.4 ^{b}	1 ± 0.3 ^{b}	1 ± 0.3 ^{b}	29 ± 9.5 ^{a}	0 ± 0.0 ^{b}
	−0.7 MPa	0 ± 0.0 ^{a}	0 ± 0.0 ^{a}	1 ± 0.5 ^{a}	0 ± 0.0 ^{a}	2 ± 1.2 ^{a}	0 ± 0.0 ^{a}	1 ± 0.5 ^{a}	0 ± 0.0 ^{a}

When comparing all species to one another (Table 2), it was found that *T. vesiculosum* had the highest or one of the highest germination percentages at each of the temperatures, under each of the osmotic stress conditions evaluated. However, when water became more limited (i.e., −0.3 MPa and −0.5 MPa), this was only true up to 25 °C and 15 °C, respectively. At −0.5 MPa water limitation, *T. alexandrinum* had the highest germination percentages at 20 °C and 25 °C, reaching a germination percentage of 60%, while at 30 °C, *M. truncatula* had the highest germination percentage (29%). At −0.7 MPa, *T. subterraneum* ssp. *subterraneum* had the highest germination percentages from 5 °C to 20 °C, although the germination percentages under these conditions were generally low (Table 2). The germination percentages of some of the medic species did not differ significantly from *T. vesiculosum* at 15 °C to 25 °C under osmotic conditions ranging from optimal to moderately water-limited (−0.3 MPa).

3.2. Seedling Emergence

The results from this trial indicated that soil moisture content at the time of planting, without subsequent watering, significantly influenced the maximum number of seedlings emerging, the time to initial seedling emergence, the number of days between the maximum seedling emergence and the initial record of seedling mortality, and the time to 100% seedling mortality (Table 3). The maximum seedling emergence for each of the species evaluated was positively and significantly correlated with the soil moisture content at the time of planting (Table 3). However, for *T. alexandrinum, T. vesiculosum, T. michelianum*, and *T. subterraneum ssp. Subterraneum*, the maximum number of seedlings emerging did not differ when planted at soil moisture contents of 100% and 70% of capacity (Table 4).

Table 3. Pearson's correlation coefficients between the different vigor indices and soil moisture contents at planting of three annual *Medicago* and five annual *Trifolium* species (* $p < 0.05$).

		Maximum Seedling Emergence	**Days to First Seedling Emergence**	**Days to 100% Seedling Mortality**
T. alexandrinum	Soil moisture at planting	0.807 *	−0.848 *	0.879 *
	Maximum seedling emergence		−0.945 *	0.888 *
	Days to first seedling emergence			−0.933 *
T. vesiculosum	Soil moisture at planting	0.875 *	−0.868 *	0.861 *
	Maximum seedling emergence		−0.955 *	0.888 *
	Days to first seedling emergence			0.952 *
T. michelianum	Soil moisture at planting	0.945 *	−0.836 *	0.764 *
	Maximum seedling emergence		−0.901 *	0.908 *
	Days to first seedling emergence			−0.787 *
T. subterraneum ssp. *brachycalycinum*	Soil moisture at planting	0.853 *	−0.658 *	0.849 *
	Maximum seedling emergence		−0.816 *	0.945 *
	Days to first seedling emergence			−0.841 *
T. subterraneum ssp. *subterraneum*	Soil moisture at planting	0.944 *	−0.792 *	0.762 *
	Maximum seedling emergence		−0.820 *	0.906 *
	Days to first seedling emergence			−0.785 *
M. polymorpha	Soil moisture at planting	0.763 *	−0.892 *	0.877 *
	Maximum seedling emergence		−0.910 *	0.930 *
	Days to first seedling emergence			−0.968 *
M. truncatula	Soil moisture at planting	0.813 *	−0.775 *	0.821 *
	Maximum seedling emergence		−0.844 *	0.905 *
	Days to first seedling emergence			−0.871 *
M. littoralis	Soil moisture at planting	0.946 *	−0.725 *	0.844 *
	Maximum seedling emergence		−0.781 *	0.798 *
	Days to first seedling emergence			−0.847 *

Table 4. Mean (± SEM) seedling emergence of three Medicago and five Trifolium species in relation to soil moisture content (% of soil water capacity) at planting. Significant differences ($p < 0.05$) in seedling emergence within a species at each planting time and moisture content at planting are indicated by different superscript letters.

Species	Soil Moisture	Seedling Emergence (%)													
		Days after Planting													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
T. alexandrinum	100%	20 ± 2^{d}	60 ± 0^{hi}	81 ± 3^{k}	97 ± 2^{l}	98 ± 1^{l}	98 ± 1^{l}	98 ± 1^{l}	98 ± 1^{l}	90 ± 3^{l}	57 ± 4^{hi}	24 ± 3^{de}	8 ± 2^{bc}	7 ± 3^{b}	0 ± 0^{a}
	70%	16 ± 3^{cd}	53 ± 2^{h}	65 ± 5^{ij}	96 ± 2^{l}	98 ± 1^{l}	98 ± 1^{l}	72 ± 3^{j}	53 ± 2^{h}	22 ± 1^{de}	9 ± 1^{bc}	5 ± 3^{b}	5 ± 3^{b}	1 ± 1^{a}	0 ± 0^{a}
	50%	0 ± 0^{a}	7 ± 1^{ab}	18 ± 1^{d}	36 ± 7^{fg}	54 ± 6^{h}	65 ± 6^{ij}	64 ± 12^{ij}	39 ± 4^{g}	29 ± 3^{ef}	6 ± 1^{b}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
	30%	0 ± 0^{a}	0 ± 0^{a}	1 ± 1^{a}	23 ± 10^{de}	17 ± 12^{cd}	1 ± 1^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
T. vesiculosum	100%	26 ± 4^{efg}	55 ± 2^{jk}	74 ± 3^{l}	88 ± 8^{mn}	95 ± 3^{n}	96 ± 3^{n}	96 ± 3^{n}	94 ± 5^{n}	79 ± 7^{lm}	50 ± 5^{j}	18 ± 1^{cde}	7 ± 2^{b}	5 ± 3^{b}	0 ± 0^{a}
	70%	26 ± 4^{efg}	36 ± 3^{hi}	40 ± 5^{i}	74 ± 7^{l}	92 ± 5^{n}	93 ± 6^{n}	52 ± 5^{j}	49 ± 5^{j}	18 ± 1^{cde}	9 ± 3^{b}	4 ± 3^{ab}	4 ± 3^{ab}	1 ± 1^{a}	0 ± 0^{a}
	50%	0 ± 0^{a}	8 ± 2^{b}	21 ± 4^{cde}	55 ± 7^{jk}	56 ± 7^{jk}	64 ± 6^{k}	58 ± 7^{jk}	34 ± 2^{ghi}	28 ± 7^{fgh}	12 ± 3^{bc}	1 ± 1^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
	30%	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	24 ± 4^{def}	16 ± 3^{cd}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
T. michelianum	100%	12 ± 2^{c}	24 ± 3^{d}	34 ± 4^{fg}	55 ± 5^{jk}	75 ± 2^{l}	75 ± 2^{l}	75 ± 2^{l}	75 ± 2^{l}	51 ± 6^{ijk}	32 ± 2^{rf}	12 ± 0^{c}	5 ± 3^{bc}	5 ± 3^{bc}	0 ± 0^{a}
	70%	8 ± 2^{bc}	21 ± 1^{d}	27 ± 5^{def}	59 ± 7^{k}	68 ± 8^{l}	76 ± 2^{l}	53 ± 7^{ijk}	51 ± 5^{ijk}	20 ± 2^{d}	9 ± 1^{bc}	4 ± 2^{ab}	4 ± 2^{ab}	1 ± 1^{a}	0 ± 0^{a}
	50%	0 ± 0^{a}	2 ± 1^{a}	6 ± 3^{bc}	41 ± 2^{gh}	47 ± 2^{hi}	56 ± 3^{jk}	49 ± 5^{ij}	26 ± 1^{de}	11 ± 2^{c}	6 ± 1^{bc}	1 ± 1^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
	30%	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	12 ± 0^{c}	10 ± 6^{bc}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
T. subterraneum ssp. *brachycalycinum*	100%	1 ± 1^{a}	11 ± 2^{bc}	43 ± 2^{g}	80 ± 2^{ij}	97 ± 2^{l}	97 ± 2^{l}	97 ± 2^{l}	93 ± 2^{kl}	83 ± 6^{j}	47 ± 4^{g}	15 ± 1^{cde}	9 ± 3^{bc}	4 ± 4^{ab}	0 ± 0^{a}
	70%	17 ± 3^{cde}	31 ± 7^{f}	49 ± 6^{g}	73 ± 11^{i}	84 ± 7^{jk}	85 ± 8^{jk}	72 ± 6^{i}	42 ± 1^{g}	24 ± 2^{ef}	17 ± 3^{cde}	12 ± 3^{cd}	5 ± 3^{ab}	1 ± 1^{a}	0 ± 0^{a}
	50%	0 ± 0^{a}	6 ± 2^{ab}	21 ± 6^{de}	44 ± 5^{g}	49 ± 7^{g}	60 ± 9^{h}	71 ± 6^{i}	42 ± 7^{g}	22 ± 1^{def}	11 ± 2^{bc}	1 ± 1^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
	30%	0 ± 0^{a}	0 ± 0^{a}	2 ± 1^{ab}	3 ± 1^{ab}	5 ± 2^{ab}	2 ± 2^{ab}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
T. subterraneum ssp. *subterraneum*	100%	8 ± 4^{bc}	16 ± 3^{d}	51 ± 5^{hi}	62 ± 7^{k}	93 ± 3^{l}	59 ± 2^{jk}	59 ± 2^{jk}	59 ± 2^{jk}	41 ± 1^{g}	28 ± 0^{f}	5 ± 1^{b}	3 ± 2^{ab}	2 ± 2^{a}	0 ± 0^{a}
	70%	5 ± 3^{b}	25 ± 5^{ef}	46 ± 5^{gh}	53 ± 6^{hij}	57 ± 6^{ijk}	60 ± 5^{jk}	47 ± 4^{gh}	32 ± 3^{f}	18 ± 2^{de}	6 ± 1^{bc}	6 ± 1^{bc}	3 ± 2^{ab}	1 ± 1^{a}	0 ± 0^{a}
	50%	0 ± 0^{a}	4 ± 2^{ab}	13 ± 4^{cd}	47 ± 1^{gh}	50 ± 1^{hi}	57 ± 2^{ijk}	57 ± 2^{ijk}	42 ± 1^{g}	32 ± 3^{f}	12 ± 2^{cd}	2 ± 1^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
	30%	0 ± 0^{a}	0 ± 0^{a}	3 ± 1^{ab}	5 ± 4^{ab}	5 ± 1^{ab}	3 ± 1^{ab}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
M. polymorpha	100%	10 ± 3^{bc}	22 ± 3^{def}	69 ± 3^{l}	80 ± 4^{m}	97 ± 2^{n}	97 ± 2^{n}	97 ± 2^{n}	97 ± 2^{n}	81 ± 8^{m}	44 ± 3^{h}	16 ± 4^{cd}	16 ± 4^{cd}	2 ± 1^{a}	0 ± 0^{a}
	70%	21 ± 5^{def}	52 ± 4^{i}	72 ± 10^{l}	92 ± 3^{n}	97 ± 2^{n}	97 ± 2^{n}	54 ± 2^{ij}	31 ± 3^{g}	18 ± 3^{de}	8 ± 2^{b}	4 ± 0^{ab}	3 ± 1^{a}	1 ± 1^{a}	0 ± 0^{a}
	50%	0 ± 0^{a}	9 ± 1^{bc}	31 ± 3^{g}	64 ± 2^{kl}	70 ± 1^{l}	87 ± 3^{mn}	87 ± 3^{mn}	60 ± 6^{jk}	28 ± 5^{fg}	10 ± 1^{bc}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
	30%	0 ± 0^{a}	0 ± 0^{a}	19 ± 1^{de}	25 ± 4^{efg}	25 ± 3^{efg}	19 ± 1^{de}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
M. truncatula	100%	4 ± 2^{a}	23 ± 4^{cd}	54 ± 3^{gh}	64 ± 4^{hi}	84 ± 5^{l}	86 ± 3^{l}	86 ± 3^{l}	83 ± 4^{kl}	60 ± 6^{ghi}	32 ± 2^{de}	6 ± 2^{ab}	6 ± 1^{ab}	1 ± 1^{a}	0 ± 0^{a}
	70%	15 ± 4^{bc}	40 ± 8^{ef}	50 ± 8^{fg}	69 ± 3^{ij}	74 ± 3^{jk}	76 ± 2^{jk}	50 ± 3^{fg}	33 ± 2^{de}	17 ± 1^{bc}	9 ± 1^{b}	2 ± 1^{a}	2 ± 1^{a}	1 ± 1^{a}	0 ± 0^{a}
	50%	0 ± 0^{a}	8 ± 2^{ab}	28 ± 3^{d}	56 ± 10^{gh}	63 ± 9^{hi}	73 ± 10^{jk}	73 ± 10^{jk}	59 ± 8^{gh}	40 ± 3^{ef}	17 ± 2^{bc}	2 ± 1^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
	30%	0 ± 0^{a}	0 ± 0^{a}	1 ± 1^{a}	8 ± 2^{ab}	9 ± 4^{ab}	1 ± 1^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
M. littoralis	100%	4 ± 3^{a}	14 ± 2^{bc}	34 ± 8^{f}	52 ± 5^{hi}	72 ± 4^{k}	75 ± 4^{k}	75 ± 4^{k}	75 ± 4^{k}	64 ± 4^{jk}	25 ± 5^{e}	8 ± 2^{bc}	8 ± 2^{bc}	2 ± 1^{a}	0 ± 0^{a}
	70%	10 ± 7^{bc}	19 ± 5^{de}	34 ± 1^{f}	45 ± 1^{gh}	58 ± 2^{ij}	45 ± 2^{gh}	26 ± 4^{e}	22 ± 1^{de}	11 ± 1^{bc}	6 ± 1^{ab}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
	50%	0 ± 0^{a}	4 ± 2^{ab}	16 ± 4^{cd}	16 ± 4^{cd}	19 ± 5^{de}	24 ± 6^{d}	24 ± 6^{d}	43 ± 2^{g}	23 ± 4^{de}	6 ± 3^{ab}	2 ± 1^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
	30%	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	18 ± 14^{cd}	8 ± 4^{bc}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}

Seedling establishment for all species was below 25% when planted at soil moisture contents below 50% of capacity (Table 4). All species that were planted when soil moisture content was high (i.e., 100%) were able to obtain a maximum establishment of greater than 75%. However, when planted at a soil moisture content that was 70% of capacity, *T. subterraneum* ssp. *subterraneum*, *T. subterraneum* ssp. *brachycalycinum*, and *M. littoralis* had significantly lower levels of seedling establishment, with only 60% and 58% of *T. subterraneum* ssp. *subterraneum* and *M. littoralis* seedlings established, respectively (Table 4). When the soil moisture content dropped to 50% of capacity, all species apart from *M. polymorpha* and *M. truncatula* had significantly fewer seedlings established, ranging from 43% to 74%, with *M. polymorpha* and *M. truncatula* still having a 93% and 77% successful seedling establishment, respectively (Tables 3 and 4). At the end of the trial, significant seedling mortality was observed, with only seedlings that were established at soil moisture contents of 100%, 70%, and 50% having seedlings that survived, while 100% mortality was observed in seedlings established at 30% of soil moisture capacity (Table 4).

3.3. Drought Stress Resistance in the Seedling Stage

The results from this trial indicated that a significant decrease in above- and below-ground plant biomass was observed under moisture stress conditions for all species evaluated (Figure 2). However, in certain species—such as *T. alexandrinum* and *T. subterraneum* ssp. *subterraneum*—the reduction in shoot biomass only occurred under severe moisture stress conditions (i.e., 30 days of water limitation), and these species were able to maintain their shoot biomass at well-watered levels under moderate moisture stress conditions (i.e., 15 days of water limitation). This suggests that these species are somewhat adapted to cope with short durations of moisture stress without compromising biomass production. Furthermore, even though root biomass was severely reduced in all species, most species were found to have longer roots under moisture stress conditions, suggesting that increased root growth for improved water harvesting is a major adaptation to drought tolerance in medics and clovers (Figure 2). Although only *T. alexandrinum* and *T. subterraneum* ssp. *subterraneum* were able to maintain biomass production under water-limited conditions, the fact that longer roots developed under water-limited conditions means that these species can access deeper water resources when the topsoil is starting to dry. However, in species such as *T. alexandrinum*, *T. michelianum*, and *T. vesiculosum*, this increased root growth was only seen during severe moisture stress conditions, while in species such as *M. polymorpha*, *M. truncatula*, and *T. subterraneum* ssp. *subterraneum* increased allocation to root growth was found even under moderate moisture stress conditions (Figure 2).

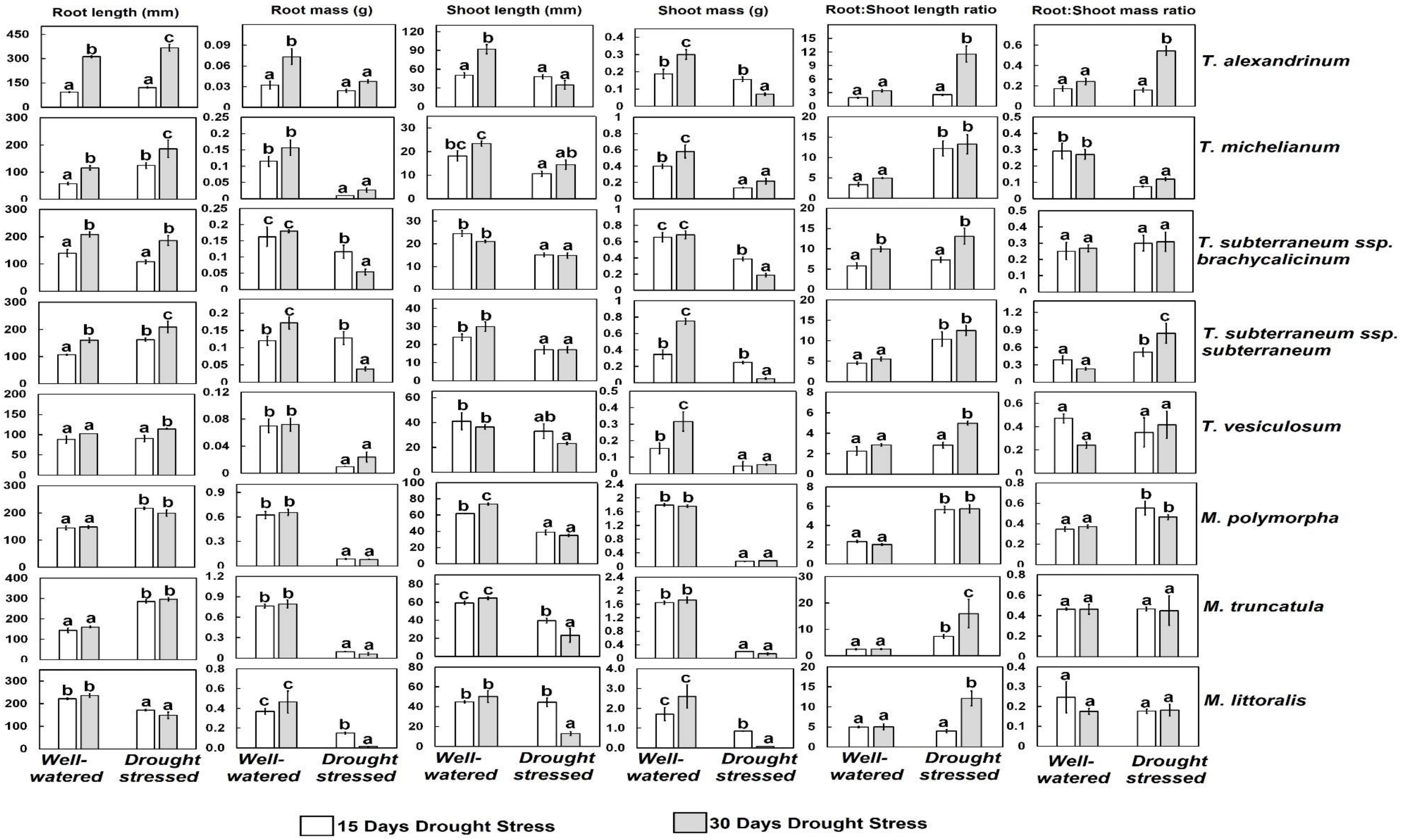

Figure 2. Morphological characteristics of annual medics and clovers subjected to 15 and 30 days of water limitation. Columns with error bars represent means ± SEMs. Comparisons are made within each species for each measured variable.

4. Discussion

The favorability of environmental conditions—especially temperature and moisture availability—is considered to be the most important variable influencing the success with which seeds germinate and seedlings establish [24]. The results from this study indicate that annual medic and clover species respond differently to drought stress. Generally, annual species escape droughts by completing their life cycle in the wet season, after which they survive as dormant seeds, which can remain in the soil until conditions become favorable for their establishment [24]. Under agronomic conditions, seeds are sown with their dormancy already broken down, and seeds will imbibe water and germinate even under suboptimal growing conditions. This is also true for seeds in the soil seed bank that have been scarified under livestock production systems. Thus, there is a need to understand how these species would respond to periods of unfavorable growing conditions. Furthermore, because medics and clovers in pasture–crop rotation systems should establish from the soil seed bank after the cropping phase, the ecology of seeds and seedlings under water-limited conditions is an important consideration for stabilizing this type of agronomic production system under future bioclimatic conditions.

The results from this study showed that all species evaluated had seeds that could germinate at high temperatures and under moderately water-limited conditions, since dormancy was already removed. The success and rate of germination at different germination temperatures and/or osmotic stress levels is usually related to the ecological and geographical conditions from where the seeds were collected or the agro-ecological conditions for which the species have developed. Thus, because these medic and clover species are primarily bred for use in Mediterranean-type agro-ecosystems, the optimal germination temperatures of 5–15 °C under well-watered conditions in this study were expected. However, it was clear that some of the species evaluated were able to tolerate higher germination temperatures. Although these species can germinate at higher temperatures, often these higher temperatures mean that moisture content will decrease rapidly prior to the seedlings having adequate time to develop deeper root systems to access water from deeper in the soil. This is especially true within semi-arid and arid environments, where the water needed for seed germination and subsequent establishment and growth of seedlings is limited and available only for short periods, and follow-up rains are spread over longer periods. This corresponds to the findings of the present study, as it was clear that some of the medics and clovers could germinate at higher germination temperatures, but this was only possible as long as water availability was not a limiting factor.

With follow-up rains expected to become more variable and the durations between rainfall events expected to become longer under future bioclimatic conditions [5], it is important to select species that can survive for extended periods of moisture stress until follow-up rains occur. In this study, *M. polymorpha* and *M. truncatula* were found to be better suited for establishment and survival under water-limited agro-ecological conditions. Similarly, *T. vesiculosum* was able to reach 26% seedling establishment when planted at a soil moisture content of only 30%, with *M. polymorpha* and *T. alexandrinum* achieving 22% and 14% seedling establishment under these moisture stress conditions, respectively. In areas with more variable rainfall early in the wet season, the results obtained from this study show that *M. polymorpha* and *M. truncatula* may be better options for planting, as more of their established seedlings may survive subsequent moisture-stressed conditions for longer periods until follow-up rains occur.

However, the results from these trials also have implications for seedlings' establishment from the soil seed bank. These annual forage legume species are rotated with cash crops [6–8], after which the pasture phase establishes from a soil seed bank. The results from the germination study showed that seeds of these species, if dormancy had been sufficiently broken down throughout the summer months and during the cropping phase of the rotation systems, could result in large amounts of seeds germinating and seedlings emerging under unfavorable growing conditions. Out-of-season rainfall, with distant follow-up rains, could result in significant false breaks from the seed bank, which could significantly influence subsequent pasture productivity. This is because at the time of optimal conditions for the pastures to establish from the soil seed bank, fewer seedlings will establish. One of the consequences of reduced pasture establishment is the intrusion of weeds. Generally, these weeds are more vigorous and are able to establish faster [31] and compete with the remaining legumes for resources such as water and light [24]. The reduction in the capture of incident sunlight, as a result of reduced growth and subsequent shading by weeds during the early stages of seedling growth, could result in a lower rate of branch development by the legumes. This, in turn, could result in reduced leaf formation and, ultimately, a reduced leaf area available to capture sunlight, resulting in reduced productivity [32]. The intrusion of broadleaf and grass weeds into legume pastures also significantly reduces the nutritional quality of the pastures [32,33], with van Heerden et al. [34] and Nichol and Edwards [35] indicating that pure legume pastures outyield grass-dominant pastures in terms of livestock production.

5. Conclusions

Based on our results, five species—*M. polymorpha*, *M. truncatula*, *T. alexandrinum*, *T. vesiculosum*, and *T. subterraneum* ssp. *Subterraneum*—can be provisionally prioritized for further research into variations in drought tolerance between cultivars within these species. Significant genetic resources exist within each of these species [28,29] (Nichols et al. 2007, 2010), which could lead to the identification of cultivars within species with beneficial traits for drought tolerance. These traits may not be found in a single existing cultivar, but could be bred through crossing different genotypes with beneficial traits into a new cultivar.

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