Drought tolerance and AFLP-based genetic diversity in purslane (*Portulaca oleracea* L.)[‡]

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Drought stress is an environmental factor that causes severe yield loss in agriculture. Identifying new genetic resources is key to improving crop drought tolerance. Purslane (*Portulaca spp*.) is a xerophyte that grows in extreme drought conditions worldwide. Here, we report genetic variation in drought tolerant phenotypes, and AFLP-based genetic diversity among purslane accessions collected from geographically diverse regions. Variations among different purslane accessions occurred at seed germination, seedling and adult stages with Tokombiya identified as the most drought tolerant accession at adult stage. Genetic diversity among purslane accessions was evaluated by AFLP fingerprinting. Using the average genetic similarity as cut-off value, four distinct groups were classified by UPGMA-cluster analysis, and Tokombiya was found to be distinct from all other purslane accessions. Together, these results suggest that significant genetic variations exist among purslane accessions and Tokombiya is a unique accession with strong drought tolerance. Further examination of this accession may benefit efforts to improve crop tolerance to drought stress.

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Introduction

Drought, heat, and their combination are environmental factors that pose a significant threat to plant life cycles [1, 2]. The response to water deficit caused by drought has been a major force in plant evolution [3]. In agricultural production, drought stress is the most prevalent abiotic constraint responsible for widespread and irreversible crop loss [4].

As moisture levels decrease, plants respond by inducing diverse physiological and molecular changes including altered gene expression, metabolism, and osmotic adjustment [5]. Different mechanisms and various genes involved in drought response signaling have been identified in model species [6-9], as well as in crop species [10-12]. While efforts to improve plant stress tolerance by genetic transformation have resulted in several important achievements [13-16], there are no reports to date on successful application of biotechnology to increase drought tolerance in agriculture. This is mainly because the genetic complexes that respond to drought stress are controlled by multigenes. In addition, genes identified in model species often have pleiotrophic effects; for example, the drought tolerance phenotypes controlled by Arabidopsis CBF1 and DREB1A genes are always coupled with a dwarfing phenotype that significantly limits their potential use in agriculture [17-19].

In the past decade, attention was also focused on searching for new genetic resources with unique features related to abiotic stress tolerance. Many wild landraces and desert-adapted species have been studied for their stress tolerance properties at ecological, physiological and molecular levels [20-26]. In a study on a wild watermelon that maintains leaf turgidity even under severe drought conditions, Yokota et al [23] found that the species survived drought stress by accumulating high concentrations of citrulline, glutamate and

arginine in the leaves. Brosche et al. [25] studied gene expression profiles of *Populus euphratica* growing in the Negev desert as compared with the sequenced *P. trichocarpa* genome. They concluded that the *P. euphratica* genome did not contain different genes *per se*. Instead, it expressed a different set of genes in response to abiotic stress indicating the importance of gene networks and regulatory elements in determination of plant abiotic stress tolerance.

Purslane, *Portulaca oleracea*, is a highly efficient drought resistant plant species that grows in hostile environmental conditions in many countries [27]. However, little is known about the mechanism(s) conferring drought tolerance. Further identification of genetic variations and elucidation of the mechanisms responsible for stress tolerance at a molecular level in this unique drought resistant species will facilitate the development of new strategies for sustainable crop improvement in the face of declining water resources and global climate change.

Materials and methods

Plant materials

Ten purslane accessions: POS, PO and Turkey (from Turkey), Keren and Tokombiya (from Eritrea), Golden E (from UK), Golden G and Golden T (from Netherlands), and wild accessions from Greece (Wild Greece), and Egypt (Egyptium) were evaluated for drought tolerance and genetic diversity with the soybean cultivar "Union" included as reference for drought tolerance. Experiments were conducted at the M. T. Carter Agriculture Research Center (Molecular Biology Lab) and in the Greenhouse at Randolph Farm of Virginia State University.

Polyethylene glycol (PEG) sensitivity during germination and seedling development

To test the effect of osmotic stress on seed germination, purslane accessions were germinated in Petri dishes on a layer of Whatman® filter paper saturated with 4 ml of 0, 5, 10, 15, 20, 30, and 40% (w/v) PEG. Petri dishes were maintained at room temperature and irradiated at ~120µmol/m^2 in a 14/10 (light/dark) photoperiod. Seed germination data was collected after 4 days.

Three day old seedlings were used to assay seedling sensitivity to PEG for all accessions except Tokombiya and Egyptium, when five day old seedlings were used. Seeds were surface sterilized in 50% (v/v) bleach and 0.1% (v/v) Triton X-100 for 8 minutes, rinsed with sterile distilled water, and germinated on solid MS media in conditions described above. The three or five day old seedlings were transferred to 50 mL liquid MS media containing 0, 5, 10, 15, 20, and 30% (w/v) PEG. Seedlings were grown for 10 days with gentle shaking (90 rpm) and irradiated as described above. At the end of the 10-day growth period, seedlings were rinsed and blot dried before total and shoot fresh weight were measured. Treatments were duplicated and contained five seedlings from each of the 11 accessions.

Evaluation of purslane adult response to drought

To evaluate drought response at adult stage, seeds from purslane accessions were grown in 7" pots filled with RediEarth Plug Mix under greenhouse conditions and natural light. After 14 days, all pots were watered to field capacity prior to the start of drought treatments. Drought was imposed on sets of seedlings from each accession for a 30- or 60-day period before pots were re-watered and seedlings left to recover for a period of 7 days. Survival was visually assessed by comparing pictures taken before re-watering and after the recovery period.

Effect of drought stress on root architecture

An experiment was set up to study root growth under well watered, and water-deficit conditions in drought sensitive Golden E and drought tolerant Tokombiya accessions. Seedlings from each accession were grown for two weeks before one half was subjected to

Table 1. Primer pair combinations used for AFLP analysis and number of DNA fragments with polymorphism.

Code	Primer Combination	No. of polymorphic bands
E01M08	E-AAA, M-CCT	75
E01M09	E-AAA, M-CGA	48
E03M08	E-AAG, M-CCT	36
E05M09	E-ACA, M-CGA	34
E08M09	E-ACT, M-CGA	19
E09M08	E-AGA, M-CCT	35
E10M09	E-AGC, M-CGA	27
E11M09	E-AGG, M-CGA	84
Total		358
Average		45

drought and the other to well watered conditions. After 30 days, roots were recovered by careful washing with water and dry weight measured after drying in a forced air oven for 5 days at 42^{o}C.

Amplified Fragment Length Polymorphism (AFLP) analysis of purslane accessions

The genetic composition of different purslane accessions was determined by AFLP analysis. Genomic DNA from each accession was extracted from fresh seedlings by using the CTAB method [28]. The analysis was carried out as originally described by Vos et al [29]. EcoRI and MseI were used to digest purslane DNA samples and 8 primer combinations between EcoRI-NNN and MseI-NNN (Table 1) were used for PCR amplification. AFLP products were separated using a CEQ 8000 Genetic Analyzer System (Beckman Coulter), and data matrix was used to calculate genetic similarities between samples. Cluster analysis was performed on the similarity matrix using the unweighted pair group method with arithmetic mean (UPGMA) algorithm [30] provided in the software package NTSYSpc (v2.11X). Average similarity score was used as cut-off value to classify the distinct groups.

Results

Effect of osmotic stress on seed germination in purslane

Except for Tokombiya and Turkey, purslane seed germination was not affected by PEG8000 at concentrations below 20% (Figure 1). At 30% PEG, there were significant differences in seed germination ranging from 0% for Tokombiya to 84.4% for POS. Among the different accessions, POS, Wild Greece, Keren, and Golden E recorded germination rates above 50% at 30% PEG, but germination in Turkey, Golden G, Golden T, PO and Tokombiya was strongly inhibited (Figure 1). At 40%, PEG completely inhibited germination of all purslane accessions (data not shown).

Effects of PEG on seedling development

In general, total seedling weight decreased with increasing PEG concentrations (Figure 2a), reaching 5-15% of weight of control seedlings (0% PEG in MS media) in all accessions in 30% PEG (data not shown). Seedlings grown in 15% PEG weighed less than half the weight of control seedlings (0% PEG in MS media) in all accessions except Keran (Figure 2a). This was mainly due to the dramatic decrease in shoot weight since all but Golden G fell to less than 25% of control shoot weight in 15% PEG. Furthermore, mild drought stress (5-10% PEG) caused an increase in root weight in Golden G, Golden E, Wild Greece, Golden T, POS, Turkey, and Keran (Figure 2b). Wild Greece demonstrated the greatest increase in root weight with roots from seedlings grown at 10% PEG contributing to over 80% of seedling

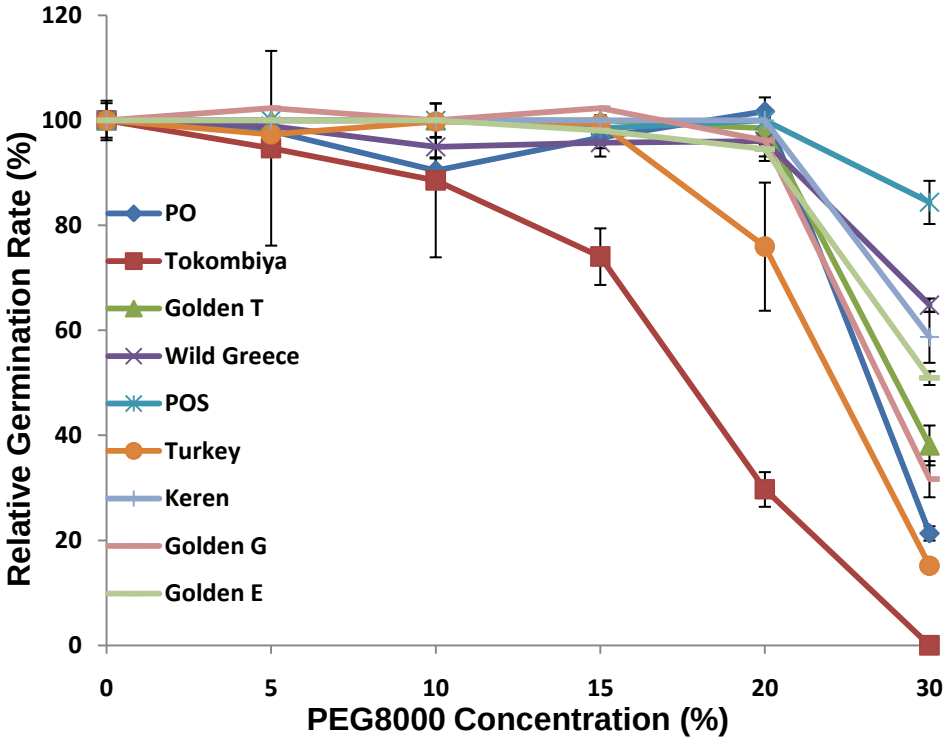


Figure 1. Effect of osmotic stress on seed germination of purslane accessions. Quantification of the relative percentage of germinated seedlings of 9 purslane accessions grown on various concentrations of PEG8000 (0, 5%, 10%, 15%, 20% and 30%). Approximately 40 seedlings per accession per plate were assayed, and duplicates per treatment were used. Error bars indicate standard deviation (SD).

weight. At 15% PEG, root fresh weight of Wild Greece and Keren was greater than roots of seedlings grown in 0% PEG. Interestingly, root weight fell to 30% of total seedling weight when media contained 30% PEG.

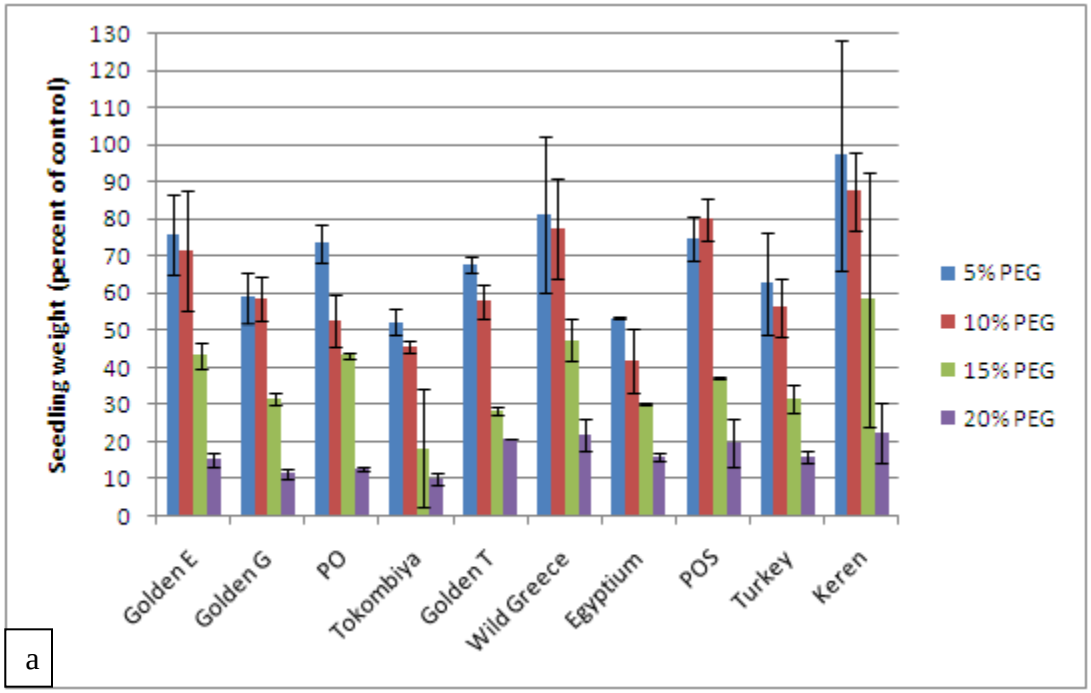
Response of purslane accessions to drought stress at adult stage

All soybean plants (controls) died after 9 days of drought but it was 20 days before purslane seedlings started to show symptoms of moisture stress (Figure 3a). All purslane accessions recovered from 30 days of drought but significant differences in drought tolerance were observed after 60 days. Tokombiya and Egyptium (100% recovery) emerged as the most drought tolerant, and Golden E (0% recovery) as the least tolerant accession after a 7-day recovery period following re-watering (Figure 3b and 3c).

Because of significant differences in drought tolerance among different accessions, we further compared root architecture under both normal and drought conditions between drought tolerant Tokombiya and drought sensitive Golden E. As shown in Figure 4, Tokombiya developed significantly more root branches than Golden E under both normal and drought conditions. Dry weight data showed that relative to Golden E, Tokombiya allocated up to 5-fold more dry matter to below ground growth under both normal and drought conditions.

Physical adaptation to drought in different purslane accessions

Drought tolerant Tokombiya and Egyptium were studied for growth related adaptation to drought stress. In Tokombiya, there was severe wilting and arrested growth but no leaf senescence after 20 days of drought. On the other hand, Egyptium plants responded to moisture stress by initiating flowering followed by leaf senescence after seed set. Upon re-watering, both accessions resumed growth with



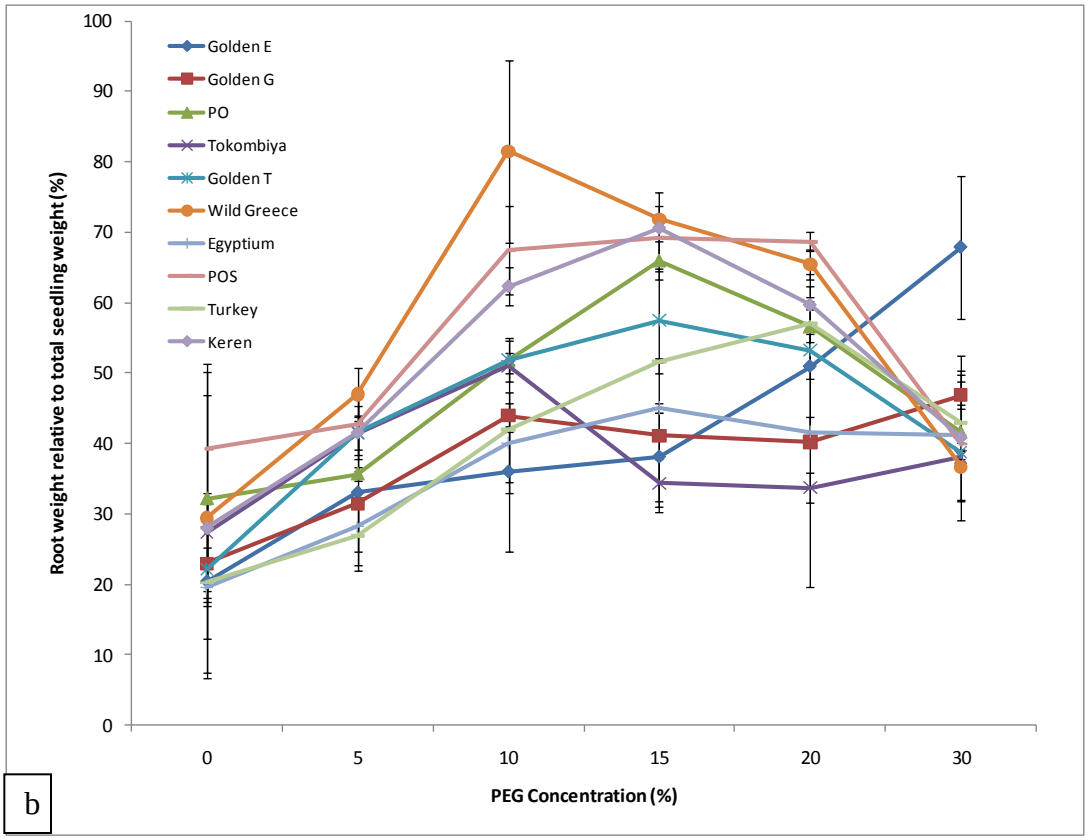


Figure 2. Effect of osmotic stress on developing purslane seedlings. Three or five day old seedlings were grown in liquid MS media containing various concentrations of PEG (0%, 5%, 10%, 15%, 20% and 30%). **a.** Total weight as expressed as percent of control (0% PEG) of seedlings grown for 10 days under osmotic stress. **b.** Contribution of root weight to total seedling weight. Five seedlings per accession per PEG treatment were measured, and duplicates per treatment were used. Error bars indicate standard error (SE, n=2).

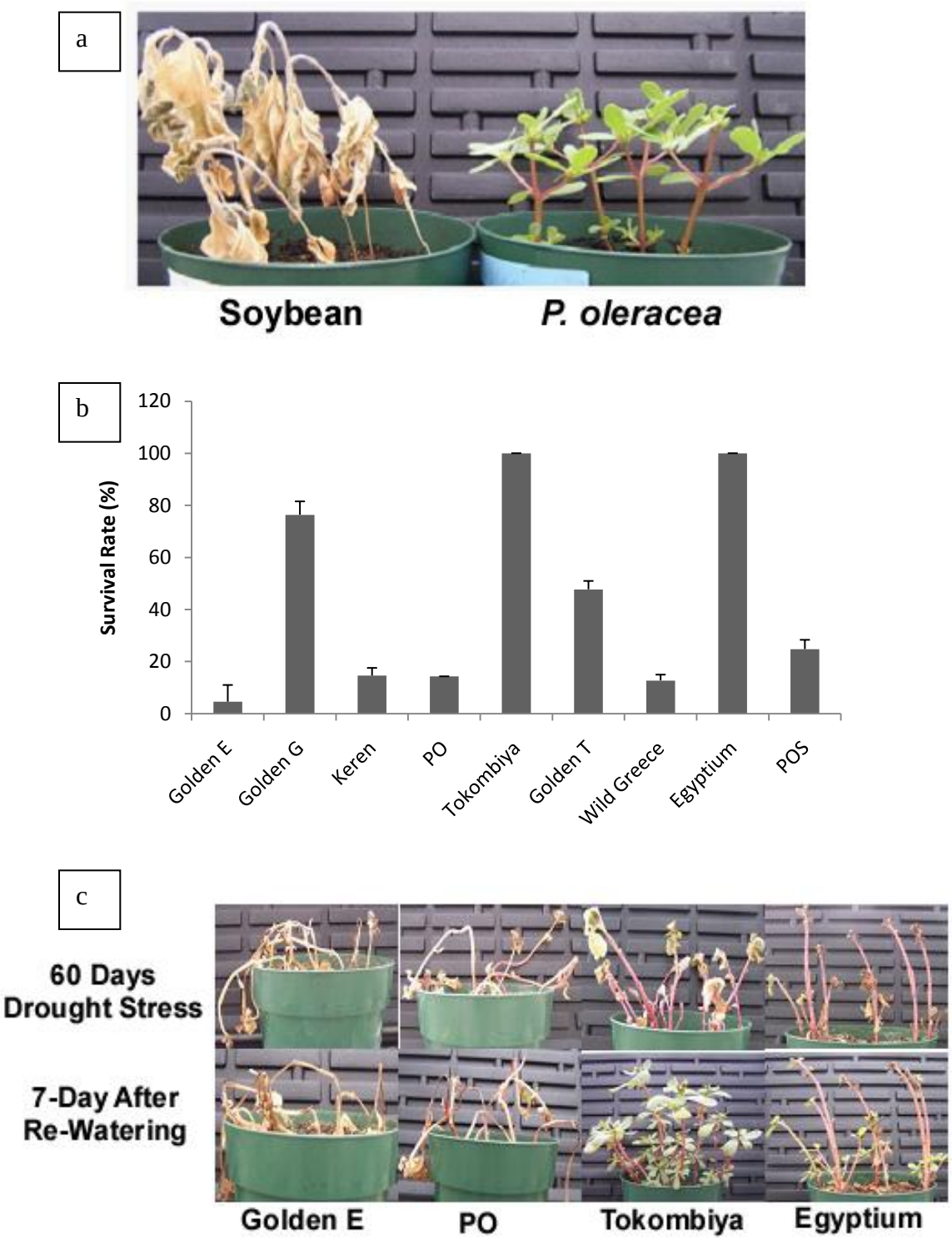


Figure 3. Seedling drought tolerance variations of purslane accessions. **a.** comparison of drought tolerant phenotypes between soybean (*G. Max*) and purslane (*P. oleracea*). Photo was taken after 10 days drought stress treatment. **b.** Survival rate of purslane accessions with 60-day drought stress followed by re-watering and recovering for 7 days. All experiments were biologically duplicated. Error bars indicate SD. **c.** Representatives of purslane accessions responding to drought treatment and recovering after re-watering.

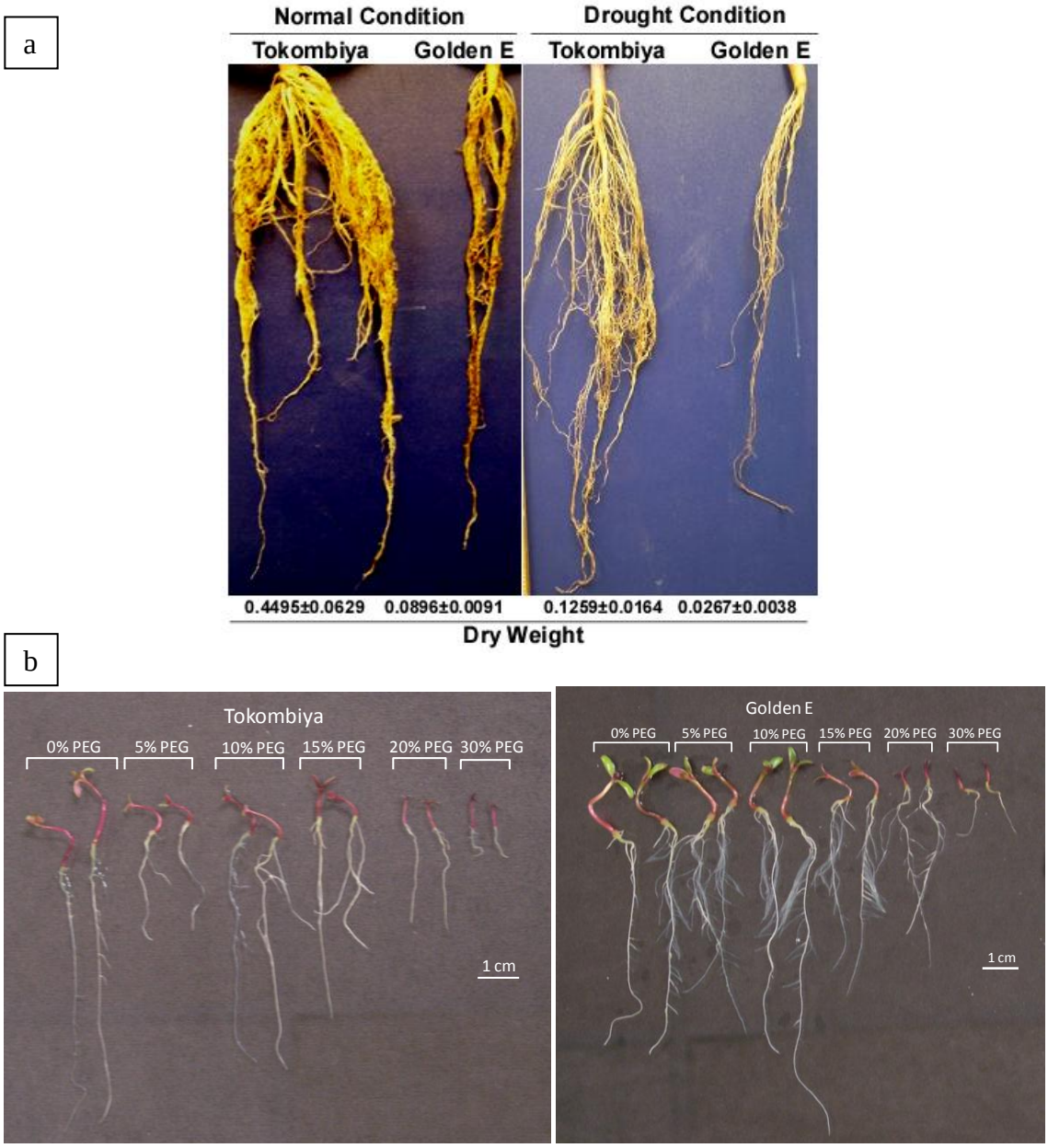


Figure 4. Root architecture of Tokombiya and Golden E purslane accessions. a. under both well-watered and drought conditions. Values below the figure are mean root dry mass±standard deviation. Drought tolerant accession Tokombiya develops more branch roots than that of Golden E, **a.** drought sensitive accession, under both well-watered and drought stressed conditions. **b.** after 10 days growth in various concentrations of PEG (0, 5%, 10%, 15%, 20%, 30%). Golden E develops more branched roots at earlier stages of seedling development than Tokombiya under osmotic stress.

the wilted leaves in Tokombiya showing remarkable recovery and resumption of physiological activity (Figure 5). These observations suggest that the mechanisms regulating drought resistance in Tokombiya and Egyptium are different. Egyptium escapes drought by maintaining vigor long enough to complete a shortened lifecycle while Tokombiya avoids drought by entering into a state of dormancy to limit moisture loss.

Genetic diversity among P. oleracea accessions

A high level of genetic diversity among the 10 *P. oleracea* accessions was revealed by AFLP

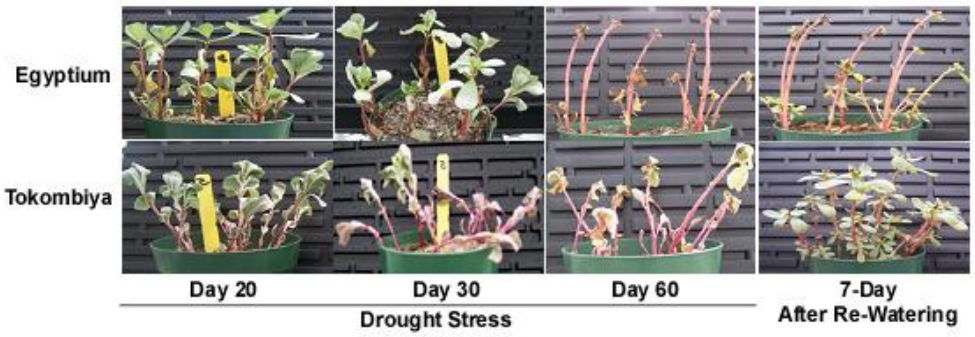


Figure 5. Drought response mechanisms in Egyptium and Tokombyia purslane accessions. Egyptium showed partially drought escape as evident by early flowering, but it kept survive after setting seeds; Tokombiya was wilted upon drought stress and arrested at the stage until water supplied.

fingerprinting (Figure 6). The average pairwise genetic similarity was 0.665 and ranged from 0.550 to 0.780. Using the average genetic similarity (0.665) as a cut-off value, accessions were clustered into 4 distinct groups. Most European accessions, together with Keren and PO, were clustered into the same group. Accessions POS and Turkey were grouped together and formed another group. Tokombiya and Egyptium were distinct from all others and from each other, and formed unique individual clusters.

Discussion

Purslane (*P. oleracea*) is a wild species that is highly adaptable to hostile environmental conditions [31]. It grows in many geographically different areas worldwide and is well adapted to extreme drought and heat, and to saline and nutrient-deficient conditions [27, 32]. In this paper, we report genetic variations in drought tolerance among *P. oleracea* accessions collected from geographically different regions worldwide. Drought tolerance was highly variable among *P. oleracea* accessions at seed germination, seedling development and adult stages, and although *P. oleracea*, is generally more drought tolerant than most crops, (Figure 3a) [27, 32], variations in this trait do exist among accessions from different regions with two African accessions, Tokombiya and Egypitum being the most drought tolerant. In our study, Tokombiya, collected from the west lowlands of Eritrea, a dry hot area with mean annual temperature above 30^{o}C, and about 200 mm of rainfall survived 60 days of drought.

We also examined variations in drought tolerance by evaluating the effect of osmotic stress on *P. oleracea* at germination. There was significant genetic variation in osmotic stress tolerance among accessions at germination stage, but similar trends were not observed at the seedling development stage. For example, even though Tokombiya adult plants were highly resistant to drought stress, seed germination and seedling development was almost completely inhibited by high osmotic stress (Figures 1 and 2). Taken together, these results suggest that distinct mechanisms or regulatory pathways control response to drought at germination and more developed seedling stages in *P. oleracea*.

When water deficit reaches a critical level, it is believed that roots will first perceive a dehydration stress signal [33]. Intrinsic and environmental response pathways may regulate root system architecture [34]. Furthermore, improvement of root architecture in *edt1* Arabidopsis mutants significantly enhanced drought tolerance [35]. To further elucidate mechanisms that regulate drought tolerance in *P. oleracea*, we compared root system architecture between drought tolerant

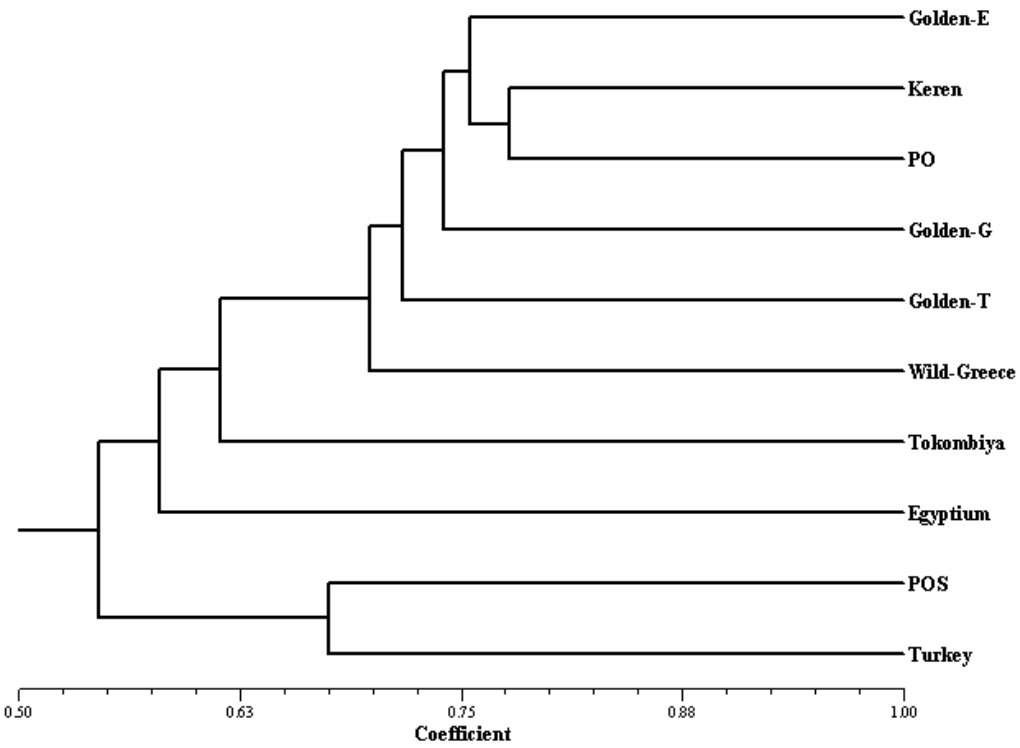


Figure 6. UPGMA dendrogram based on Dice coefficient of genetic similarity among 9 purslane accessions.

Tokombiya and drought sensitive Golden E and found that two-week old Tokombiya seedlings had better root architecture with a more extensive root system than Golden E. This difference in root development between two accessions was observed under both well-watered and drought conditions. Our results are consistent with previous studies [34, 35] that demonstrate root architecture as modulating plant response to drought. We made the opposite observation in three and five day old seedlings treated with PEG regarding root structure. Golden E roots were more branched and extensive than Tokombiya roots after 10 days of drought stress (Figure 4b). This suggests that the mechanism of drought tolerance in some accessions (i.e. Tokombiya) may require an extensive root system to be established prior to drought.

Stomatal density and movement affects transpirational water loss and is a key determinant of drought tolerance [36-38]. We compared stomatal density between soybean and *P. oleracea* accessions and found significantly less (about 3~4 fold) stomata across all *P. oleracea* accessions than that of soybean (data not shown). Surprisingly, stomata in *P. oleracea* are concentrated on the adaxial rather than abaxial side of leaves in contrast to other dicots like soybean and Arabidopsis. This unique property may be due to *P. oleracea* adaptation to dry, hot, and sandy environments where ground heat could lead to higher transpirational water loss on the abaxial side. Overall, lower stomatal density may account for increased drought resistance in *P. oleracea* but is not strongly linked to phenotypic variation in *P. oleracea* drought tolerance.

First reported by Vos et al [29], AFLP fingerprinting is widely used for genetic diversity studies [39-43]. We examined genetic composition in *P. oleracea* using AFLP and found a high level of genetic diversity among accessions (Figure 6). The significant genetic

differences may be partially due to differences in climate and environment, but are not directly linked to geographic location. For example, Keren collected from a region with moderate climate and abundant rainfall in the highlands of Eritrea differed significantly from Tokombiya that originated from the dry and hot lowlands of the same country. This suggests that localized conditions rather than geographic region accounts for such genetic diversity. Previous studies on Australian *P. oleracea* accessions revealed similar phenomena [44-47].

The *P. oleracea* accessions used in the current study differ in morphology (leaf size, stem color, plant height, and seed size), but genetic variations detected by AFLP among the accessions did not correspond to the wide range of morphological differences. This could be due to the effect of one or a few genes influencing plant stature, leaf size, stem color and seed size as observed by Dooner and Kermicle [48]. On the other hand, when considering drought resistance, Tokombiya and Egyptium were distinct from other accessions and from each other (Figure 6) indicating that plant environmental adaptation plays an important role in *P. oleracea* diversity.

P. oleracea has been identified as one of the richest vegetable source of omega-3 fatty acids [49, 50]. In addition, *P. oleracea* is rich in Vitamin A, Vitamin C, Calcium, Phosphorus and Iron [27, 51], and antioxidants, such as α-tocopherol [51] and melatonin [52]. Its nutritional characteristics suggest that it can offer better nourishment than the 20 major crops currently providing most of our food needs. Our study, together with previous discoveries [31, 32, 44, 45], demonstrate that *P. oleracea* is adapted to different environments and conditions. This provides a unique opportunity to produce high quality food for human consumption and/or reduce agricultural use of precious water resources in agriculturally marginal regions of the world. On the other hand, understanding the mechanisms that regulate abiotic stress tolerance in this species will help in the development of other crops with similar abiotic stress tolerance.

Acknowledgement

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