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Abstract

Cowpea [*Vigna unguiculate* (L.) Walp.] is not only a healthy, nutritious and versatile leguminous crop, it also has a relatively high adaptation to drought. Researches have shown that cowpea lines have a high tolerance to drought, and many of them can survive over 40 days under very hot and dry conditions. The cowpea (Southern pea) breeding program at the University of Arkansas (UA) has been active for over 50 years and has produced more than 1,000 advanced breeding lines. The purpose of this study is to evaluate the drought-tolerant ability in Arkansas cowpea lines and use the drought tolerant lines in cowpea production or as parents in cowpea breeding. A total of 36 UA breeding lines were used to screen drought tolerance at the seedling stage in this study. The experiment was conducted in greenhouse using randomized complete block design (RCBD) with two replicates, organized in a split-plot manner, where the drought treatment as the main plot and the cowpea genotypes as the sub-plot. Drought stress was applied for four weeks, and three drought tolerant related traits were collected and analyzed. Results showed that cowpea breading line: 17-81, 17-86, Early Scarlet, and AR Blackeye #1 were found to be drought tolerant.

Introduction

Cowpea [*Vigna unguiculate* (L.) Walp.], which is also called southern pea or black-eye pea in the United States is a versatile leguminous crop. Multiple parts of the cowpea plant are used for human consumption and livestock feeding (Diaga, 2011). People around the world consume dry and fresh seeds as the main product of the cowpea plant. The fresh leaves are consumed by people in Africa as an additional dish. In Asia and the Caribbean, the fresh green pods are also viewed as a delicious food source (Ehlers & Hall, 1997). Even the leaves and stems are also used as high-value hay for livestock in Africa (Timko & Singh, 2008). Because cowpea is a leguminous crop, it is also used as green manure in the southern part of the United States and Australia (Ehlers & Hall, 1997)

Among all leguminous crops, cowpea has a relatively high adaptation to drought (Hall & Patel, 1985). However, since the majority of cowpea is grown in the dry, sub-tropic area, drought is still one of the major abiotic constraints for cowpea production (Agbicodo et al., 2009). In some drier places in Niger, the average yield is nearly 20 times less than the average yield in the United States where the water is abundant (Agbicodo et al., 2009).

Research has showed that there is a huge difference among cowpea varieties in drought tolerance (Mai-Kodomi et al., 1999). Many cowpea breeding programs have created to breed drought tolerant cowpea varieties, and some cowpea cultivars were proved to be drought tolerant. For example, the cowpea cultivars "Machakos 66" and "Katumani 80" released by Kenya National program are drought resistance varieties (Ehlers & Hall, 1997). Early maturing varieties are also developed to avoid many late-stage biotic and abiotic constraints including drought (Ehlers & Hall, 1997). However, research showed that these varieties were vulnerable to mid-season drought (Thiaw et al., 1993).

Many studies show that cowpea has a strong ability to tolerant drought in the vegetative stage. However, cowpea drought tolerance at seedling stage has not gained much attention (Muchero et al., 2008). Since vegetative stage drought-tolerant cowpea has been extensively studied, it is important to explore more seedling-stage drought tolerant cowpea lines for future researchers to study more about the seedling stage drought-tolerant mechanism and genes in the genome of cowpea.

In a water stress test, although most of the cowpea seedlings were stunted, many of them survived for 43 days under very hot and dry conditions and recovered after they were irrigated (Hall, 2012). Various studies also found that the cultivars which showed seedling stage drought tolerance had a big chance to survive the whole life circle in the drought stress environment with a reasonable level of yield (Hall, 2012; Muchero et al., 2013; Singh et al., 1999). Due to cowpea's strong ability to tolerate drought, and its relatively small genome size (about 620 Mb) (Arumuganathan & Earle, 1991), cowpea can be an "ideal model to study the molecular mechanisms of drought tolerance in crops" (Agbicodo et al., 2009, p. 361).

The cowpea (Southern pea) breeding program at the University of Arkansas has been active for over 50 years and has produced more than 1,000 advanced breeding lines and released more than 12 varieties. The varieties have been utilized by the processing industry and widely grown by producers for fresh-market. The program has developed a diversity of cowpea types with a range of mature seed pattern and color including blackeye, pinkeye, and red holstein, and various seed coat colors including black, brown, green, cream, and yellow. However, the seedling stage drought tolerant ability of that group of cowpea lines is remaining unknown. The purpose of this study is to evaluate the drought-tolerant ability in Arkansas cowpea lines to use drought tolerance in cowpea breeding. The specific research objectives of this research are to evaluate drought

tolerance in cowpea lines at seedling stage under greenhouse condition.

Materials and Methods

Plant material

A total of 36 Arkansas cowpea lines were used in this study for drought tolerance evaluation. In addition, two previous reported drought tolerant lines PI293568 and PI349674, and one drought susceptible line, PI255774 (Ravelombola et al., 2018) were used as controls (Table 1).

Table 1. ***Cowpea Breeding Lines (39 genotypes) for Drought Tolerance Screening Experiment***

Cowpea Lines	Source	Cowpea Lines	Soruce
PI255774	Susceptible Control	01-1781	AR Breedinglines
09-741	AR Breedinglines	Ebony	AR Breedinglines
17-133	AR Breedinglines	17-118	AR Breedinglines
Epic	AR Breedinglines	09-211	AR Breedinglines
17-109	AR Breedinglines	16-181	AR Breedinglines
17-129	AR Breedinglines	07-303	AR Breedinglines
17-131	AR Breedinglines	17-127	AR Breedinglines
17-135	AR Breedinglines	17-40	AR Breedinglines
17-81	AR Breedinglines	17-61	AR Breedinglines
16-166	AR Breedinglines	09-393	AR Breedinglines
Early Scarlet	AR Breedinglines	09-714	AR Breedinglines
Empire	AR Breedinglines	17-102	AR Breedinglines
09-692	AR Breedinglines	17-107	AR Breedinglines
PI293568	Tolerant Control	17-111	AR Breedinglines
17-117	AR Breedinglines	17-114	AR Breedinglines
AR Blackeye #1	AR Breedinglines	17-124	AR Breedinglines
09-529	AR Breedinglines	17-128	AR Breedinglines
PI349674	Tolerant Control	17-137	AR Breedinglines
Early Acre	AR Breedinglines	17-86	AR Breedinglines
Early Set Select	AR Breedinglines		

Greenhouse evaluation for drought tolerance

Greenhouse evaluation was carried out in the greenhouse at the Arkansas Agricultural Research & Extension Center, Fayetteville, AR. During the experiment, the day/night temperatures in the greenhouse was maintained at 25°C/20°C. The screening method was similar to the 'Wooden Box' screening methodology established by Singh et al. (1999) and Verbree et al. (2015) with minor modifications (Ravelombola et al., 2018). All cowpea lines were planted in the Sterilite polypropylene boxes (Sterilite Corporation, Townsend, MA, dimensions 88.6 cm X 42.2-cm X 15.6 cm) that were filled with Sunshine® Mix #1 Natural & Organic (Agawan, MA) up to 10.5 cm high. 6 L of tap water was added into each box two days before planting to make sure that the potting mix at its field capacity stage when seeds were planted.

The experimental design was randomized complete block design (RCBD) with two replicates per genotype, organized in a split-plot manner, where the drought treatment as the main plot and the cowpea genotypes as the sub-plot. For each replicate, the 39 cowpea lines (36 tested lines plus three controls) were planted with a total of eight boxed where four boxes were used as control with watering without drought stress, and the other four boxes as the treatment group with drought stress (Fig. 1).

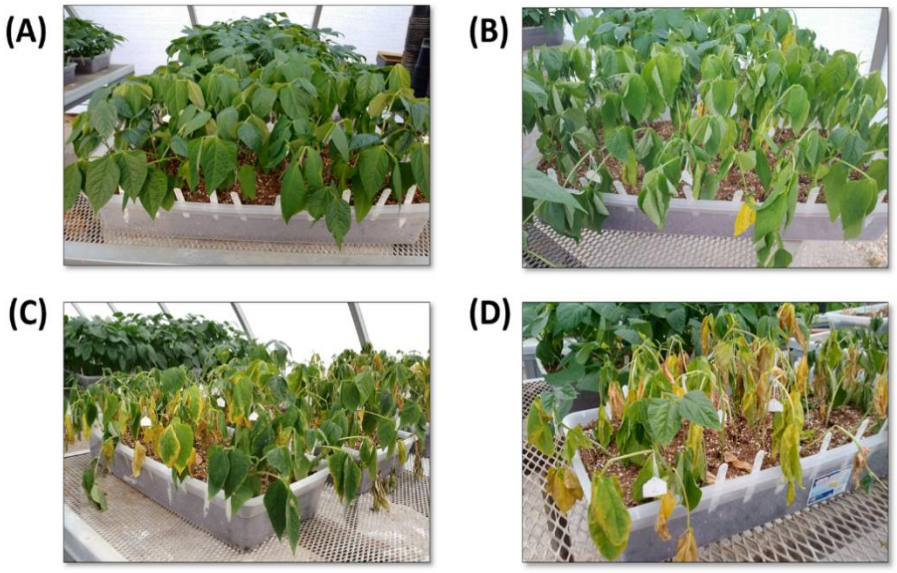

Figure.1. Greenhouse phenotyping experiments for cowpea drought tolerance: (A) drought stress was applied for 7 days, (B) for 14 days, (C) for 21 days, and (D) for 28 days

For each box, a total of ten 7.5 cm-spaced rows were designed across the box length. Each testing cowpea line was planted within each row. A total of 6 uniform and vigorous plants were kept at each row when the first trifoliate leaf began to expand. Fertilizers (Miracle-Gro, Detroit, MI) was applied one week after the emergence of cowpea seed from the medium. For each row in every box, 150 mL of tap water was irrigated every three days until the first trifoliate leaf was fully developed. Drought stress was imposed for the stress treatment by stopping water irrigation when the first trifoliate was completely expanded, and water stress lasted for four weeks until some cowpea genotypes were completely dead, indicating susceptibility to drought stress. The control (un-stress of drought) treatment was irrigated with 150 mL of tap water every three days continuously.

Measurements

Three drought-related traits: chlorophyll content in trifoliate and unifoliate leaves, overall plant greenness scores, and the plant main stem lodging scores were measured from the 39 cowpea lines.

Leaf chlorophyll content was measured using the SPAD-502 Plus Chlorophyll Meter (Spectrum Technologies, Inc., Plainfield, IL). For each plant, three types of leaves: unifoliate (U), first trifoliate (T), and new (second) trifoliate (NT) were measured separately.

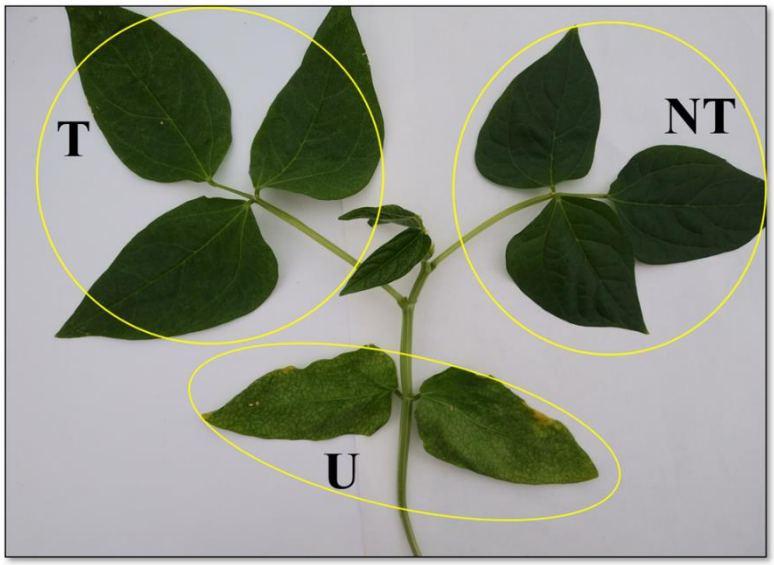

Figure 2. Different types of leaves measured for chlorophyll content. U= Unifoliate, T= Trifoliate, NT= New Trifoliate

Measurements were taken weekly after drought stress was imposed. For each leaflet of the unifoliate or trifoliate, the measurement was done three times to avoid edge effect, and the average of the three measurements was kept as the final chlorophyll content value. To evaluate the drought tolerance in each cowpea line, two other parameters: AD (Absolute Decrease), and II (Inflect Index) (González, 1996), were also estimated using the following formulas:

Absolute Decrease (AD) = CC-CS; Inflect Index (II) = 100* (CC-CS)/ (CC)

Where CC = chlorophyll content for control, CS= chlorophyll content for drought stress.

The overall plant's greenness scores were measured using a 1 to 5 scale (1= plant completely green, 2= new trifoliate completely green with chlorotic unifoliate and trifoliate, 3= new trifoliate is chlorotic, 4= severe signs of necrotic on all leaves with a green growing tip, and 5= dead plants.) (Fig. 3). Overall plant greenness was measured twice on per plant basis at the third and fourth week after first applying drought stress. The average score of the six plants from one row was recorded as the final greenness score for the specific cowpea line. In order to see how the greenness was changed over weeks, the greenness change value was calculated using the formula: Greenness Change = 100* (5 - Overall plant greenness score)/4. The plant main stem lodging scores were

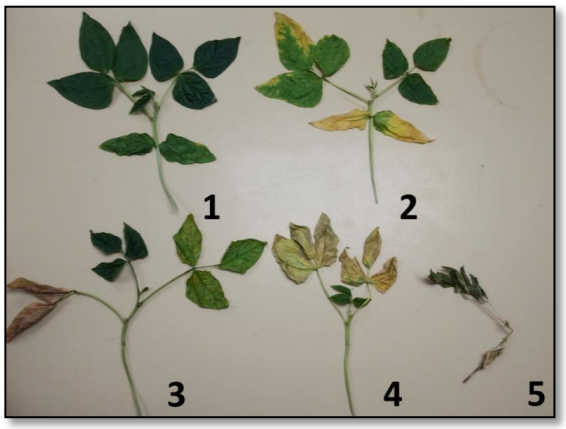

Figure 3. Overall-plant greenness assessed on a 1-5 scale: 1= green, 2= green new trifoliate with chlorotic unifoliate and first trifoliate, 3 = chlorotic new trifoliate with necrotic unifoliate and first trifoliate, 4 = severe signs of necrotic

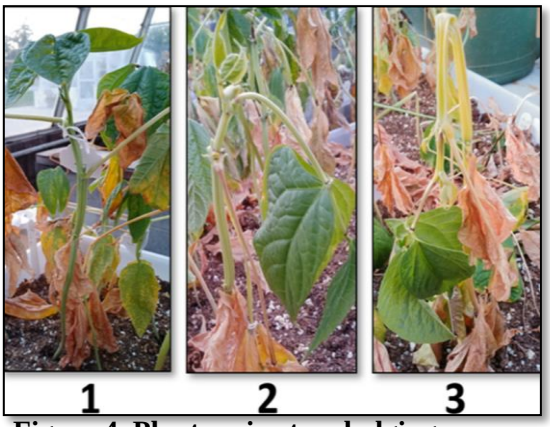

Figure 4. Plant main stem lodging score assessed on a 1-3 scale: 1= vigorous, green main stem, 2= mildly wilting, light green main stem, 3= completely lodged, yellow main stem

measured using a 1 to 3 scale (1= vigorous, green main stem, 2= mildly wilting and light green main stem, and 3= completely lodged and yellow main stem) (Fig. 4). Plant main stem lodging score was assessed on a per plant basis in the fourth week after the drought stress was initiated. The average score of the six plants from one-row records as the final main stem lodging score for that specific cowpea breeding line.

Phenotypic data analysis

The cowpea drought tolerant data were analyzed using analysis of variance (ANOVA) with the general linear models (GLM) procedure of JMP Genomics 9 (SAS Institute, Cary, NC). The distribution of the data was drawn using 'Distribution'; and the scatter plot matrix graphs for chlorophyll content were generated by JMP Genomics 9 (SAS Institute, Cary, NC). Overall greenness score and leaf fall percentage change by week charts were generated using the Excel chart drawing tool.

Results

Chlorophyll content in trifoliate and unifoliate leaves

The drought stress was imposed for four weeks (March 25th to April 22nd), and chlorophyll content difference started to show from the second week (April 1st to April 8th). Across the 39 cowpea lines, all three types of the leaf [unifoliate, first trifoliate and new (second) trifoliate] had a significant chlorophyll content drop during the four weeks of drought stress (Figure 5). Overall, unifoliate

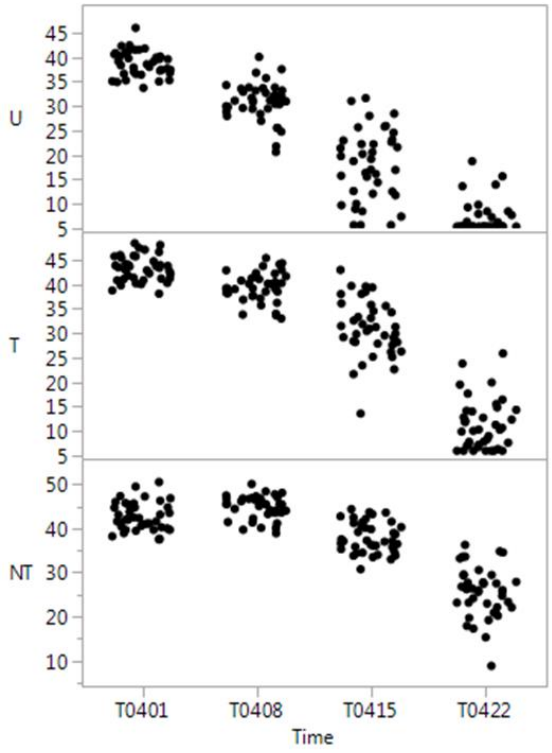

Figure 5. Scatter Plot Matrix represent the chlorophyll content change in U (unifoliate), T (trifoliate), NT (new trifoliate) in the period of 4 weeks.

and first trifoliate chlorophyll contents dropped more than new trifoliate over time; and few cowpea lines managed to remain their new trifoliate chlorophyll at the higher level. After four weeks of drought stress, the average unifoliate chlorophyll content dropped from 38.6 to 7.0; and the average first trifoliate chlorophyll content drops from 43.4 to 11.2; while the average new trifoliate chlorophyll content only drops from 40.7 to 25. The new trifoliate chlorophyll content in four weeks after drought stress varied from 36.3 to 8.8. Six cowpea line: 17-81 (34.8), PI293568 (34.6, drought tolerant control), AR Blackeye#1 (33.6), 07-303 (36.3), 17-124 (33.2) and 17-86 (33.6) have the highest new trifoliate chlorophyll content.

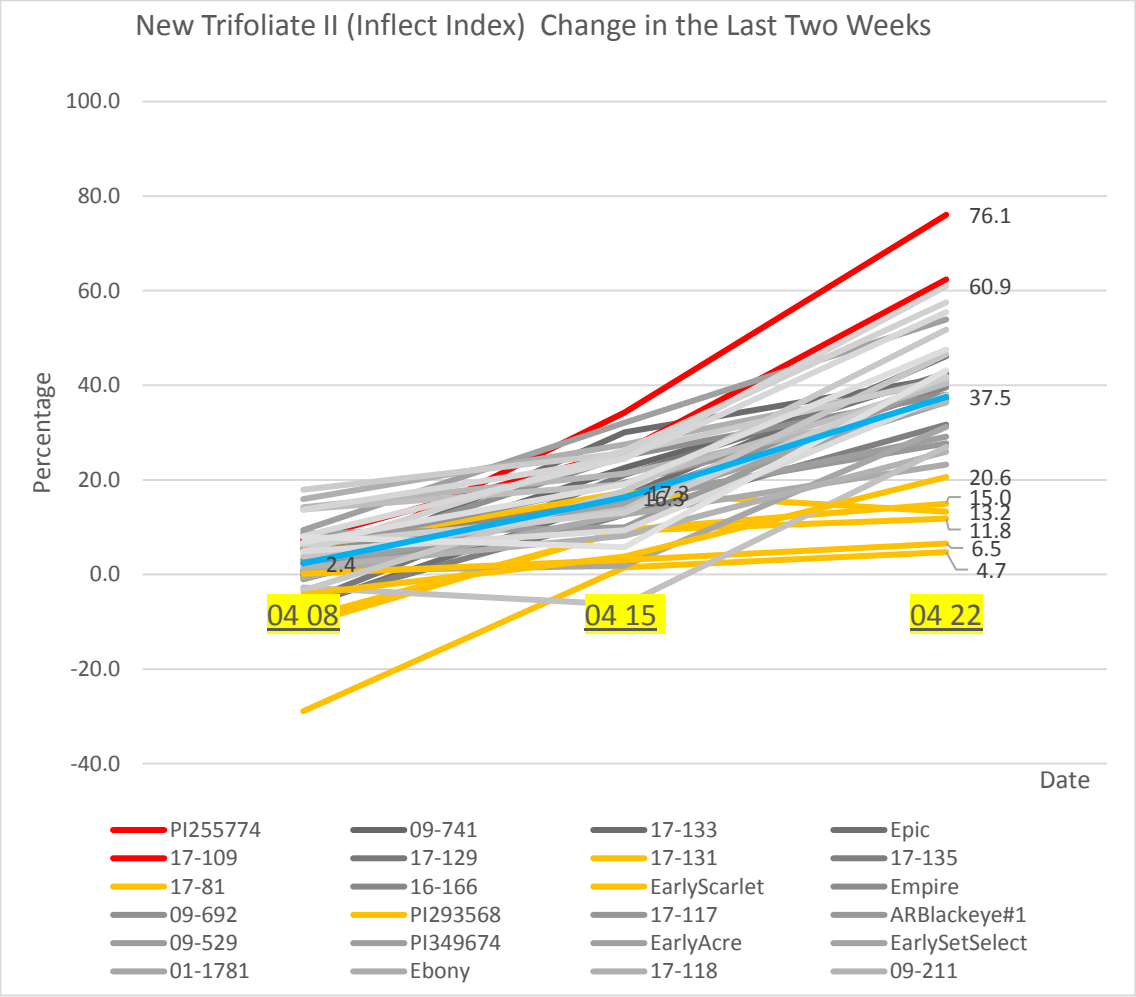

Figure 6. 2D line chart that represent the II (Inflect Index) change in NT (new trifoliate) over the last two weeks of drought stress from April 08th 2019 to April 22th 2019.

Inflect index (II) indicates how much the chlorophyll content of that cowpea plant leaf has changed (as percentage) in the drought stress condition compared to the same genotype, healthy cowpea plant in the control without drought stress but watering. The higher the II value indicates more chlorophyll content lost compare to the healthy plant, i.e. the higher the II value, the more susceptible the cowpea line is. The new trifoliate II value for most cowpea lines started to rise up significantly in two weeks after the drought stress was imposed (From April 8th) (Figure 6). At the end of the fourth week, the new trifoliate II value for each cowpea line varied from 76.1 to 4.7. Among the 39 cowpea lines, PI255774 (76.1) and 17-109 (60.9) had the highest II value, indicating that their inability to keep their chlorophyll content stable in the drought stress and showing that their susceptibility to drought stress condition. On the other hand, six cowpea lines: 17-131 (4.7), 07-303 (6.5), 17-81 (11.8), PI293568 (13.2), Early Scarlet (15.0) and 17-86 (20.6) had relatively low II value, indicating that the six lines were drought tolerant.

Overall plant greenness scores

Overall plant greenness scores were recorded at week three and week four after the drought stress treatment. For week three, the over plant greenness score varied from 1.42 to 3.67, with an average of 2.53 and an SD of 0.49. For week four, the plant greenness score varied from 2.08 to 4.42, with an average of 3.06 and an SD of 0.56 (Figure 7).

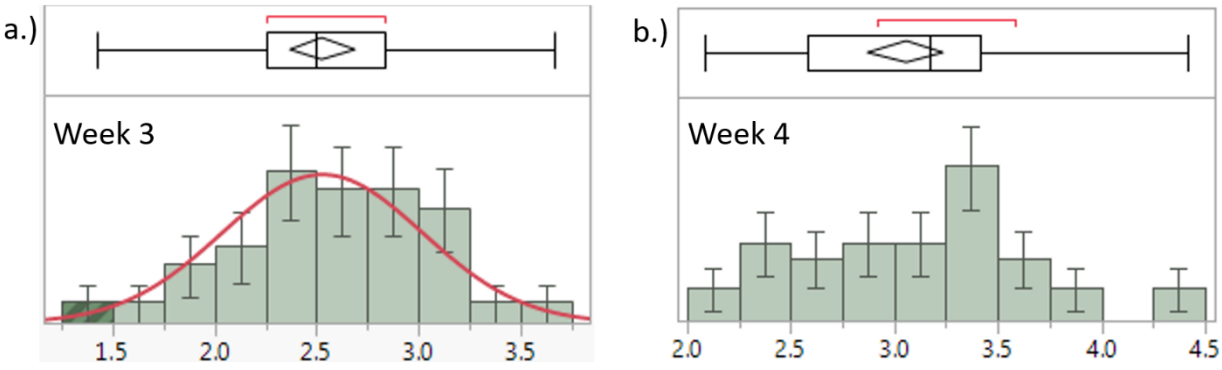

Figure 7. a) Distribution chart for overall plant greenness score at week 3 of the drought stress. b) Distribution chart for overall plant greenness score at week 4 of the drought stress.

The plant greenness change chart was generated using both weeks' data. The higher value (percentage) meant more greenness the plant had kept in the drought stress condition, suggesting the cowpea line was drought tolerance. The greenness change percentage varied from 14.58% to 72.92% with an average of 48.57%. Based on the percentage change, two cowpea line: PI255774 (14.58%) and 17-109 (18.75%) had the lowest greenness change percentage, indicating their susceptibility to drought stress. The six line: PI 349674 (72.92%), 17-86 (72.92%), PI 293568 (68.75), Early Scarlet (68.75), AR Blackeye #1 (66.67%) and 17-81 (66.67%) had the highest greenness change percentage, indicating that the six lines were drought tolerant.

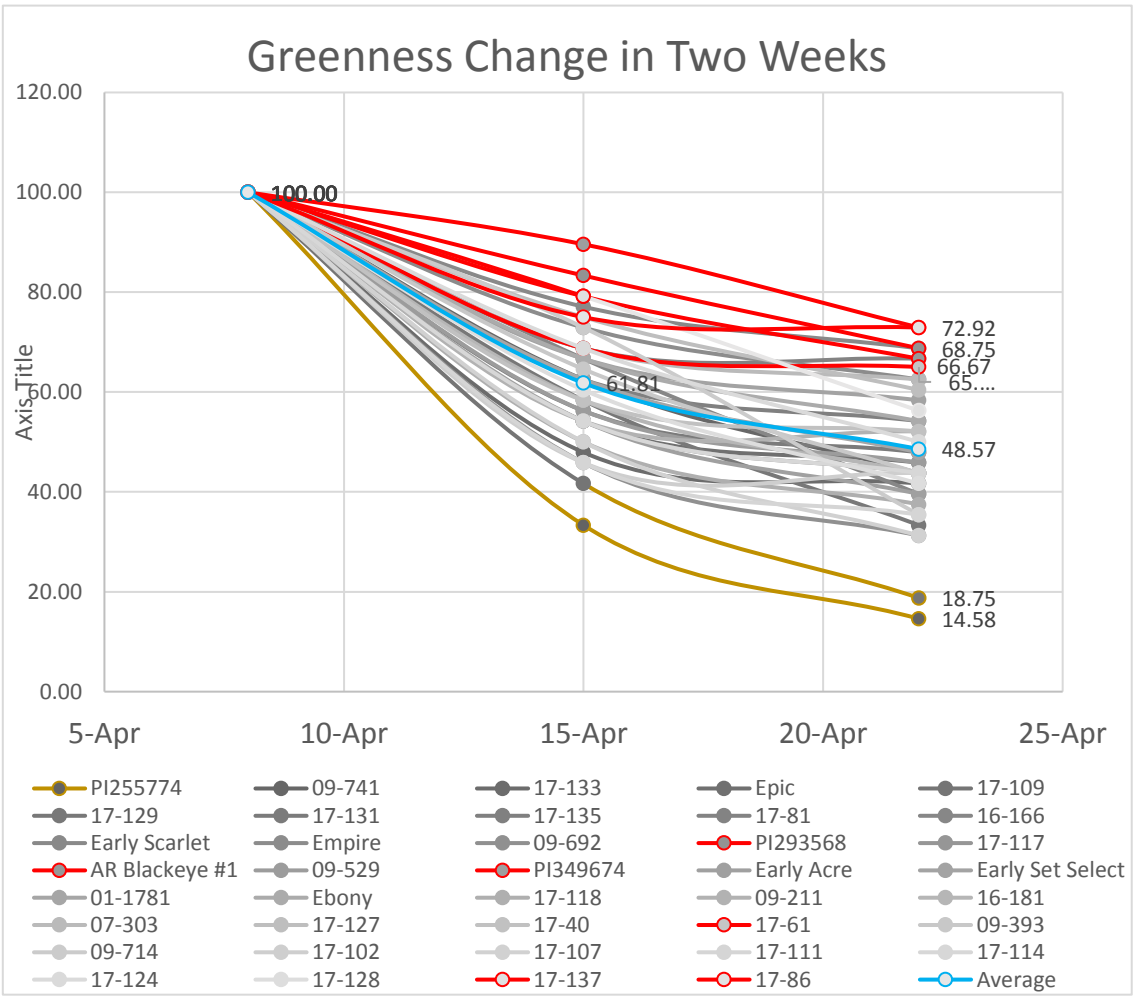

Figure 8. 2D line chart that represents the greenness score change over the last two weeks of drought stress from April 08th 2019 to April 22th 2019.

Plant main stem lodging scores

Plant main stem lodging scores were recorded on fourth week after the drought stress. The plant main stem lodging scores varied from 1.5 to 3, with an average of 2.19 and an SD of 0.41 (Figure 9). Among the 39 cowpea lines, three cowpea lines: 17-118, 16-181, and 17-127 had a stem lodging score of 3, indicating that the three lines were completely susceptible to drought stress. On the other hand, five cowpea lines: 17-81 (1.50), PI349674 (1.50), 17-61 (1.50), AR Blackeye #1 (1.67), and 17-86 (1.67) had a very low stem lodging score, indicating that the five lines were drought tolerant.

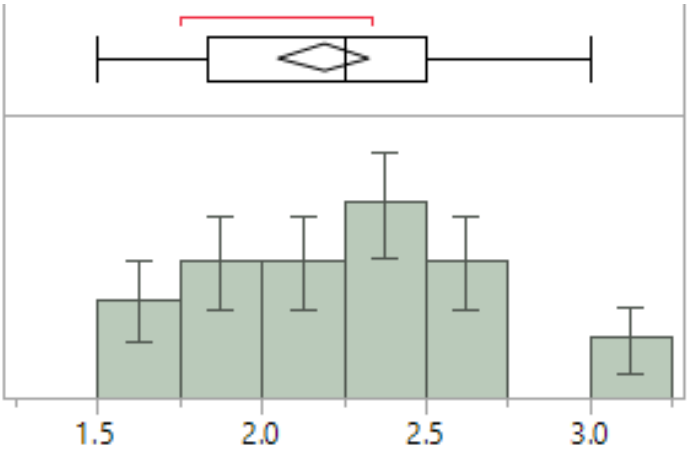

Figure 9. Distribution chart for plant main stem lodging score at week 4 of the drought stress.

Discussion

By analyzing the chlorophyll content data in three different types of the leaf over four-week drought stress, we found that both unifoliate and first trifoliate chlorophyll content dropped to a very low level (chlorophyll content lower than 10) at the end of the week four. The new trifoliate chlorophyll content also dropped significantly, but the magnitude was not as dramatic as the unifoliate and trifoliate. We also found that some cowpea lines were capable of keeping the new trifoliate chlorophyll content level, which suggested that new trifoliate chlorophyll content and its related parameters, inflect index, can be a good trait to measure for the drought-tolerant

assessment. Among the 39 cowpea lines, huge differences were also found in both overall plant greenness score and plant main stem lodging score, indicating that that both traits can contribute to the overall drought tolerance level for a cowpea line. However, different cowpea lines performed and scored differently based on the trait we measured. Because of the complexity of drought-tolerant trait, it is hard only to use one parameter to decide the overall drought tolerance of a certain line.

In this experiment, three parameters: Inflect index of new trifoliate, greenness change percentage for the overall plant greenness scale, and main stem lodging scale were used as the main parameters for evaluating drought tolerance. We assumed all the three traits had the same importance value for the overall drought tolerance and used them to rank each cowpea line for its drought tolerance summed the values of the three traits. In the end, an overall ranking score was given to each cowpea line, and the overall ranking score from the sum of the three ranking number drought tolerance. The cowpea lines with the low overall ranking score were considered to be overall drought tolerant, and the cowpea lines with higher overall ranking score were considered to be drought susceptible (Table 2).

The two previous reported drought tolerant lines PI293568 and PI349674, and one drought susceptible line, PI255774 (Ravelombola et al., 2018) were also ranked (Table 2). Cowpea line PI293568 was ranked as number 6, and PI349674 as number 3 in the overall drought tolerant ranking, indicating their drought tolerance, and PI255774 ranked number 39 as the most susceptible to drought tolerance in the study, which showed and confirmed our previous results (Ravelombola et al., 2018). The three cowpea genotypes showed stable tolerance or susceptibility across the three parameters and were used as controls in this study.

Based on the overall ranking, four cowpea lines: 17-81, 17-86, Early Scarlet, and AR Blackeye #1 were listed as overall rank 1, overall rank 2, overall rank 4, and overall rank 5, respectively, suggesting that they were considered to be drought tolerant (Table 2). The cowpea line, 17-109 was listed as overall rank 38 and it was considered to be a very susceptible line under drought stress.

Table 2. Overall ranking of drought tolerance for 39 cowpea breeding lines.

Cowpea_Lines	II	Greenness Change Score	Stem lodging Score	Overall Ranking Score
17-81	3	5	1	9
17-86	6	1	4	11
PI349674	11	1	1	13
EarlyScarlet	5	3	6	14
ARBlackeye#1	10	5	4	19
PI293568	4	3	13	20
17-131	1	18	6	25
17-135	13	13	6	32
17-40	9	15	10	34
07-303	2	8	27	37
Empire	17	8	13	38
EarlySetSelect	15	11	13	39
17-61	31	7	1	39
09-211	8	15	21	44
Ebony	7	13	35	55
17-124	18	17	21	56
01-1781	12	18	27	57
17-117	16	22	21	59
17-137	29	12	18	59
09-529	23	20	18	61
Epic	30	20	13	63
09-393	33	22	12	67
17-127	21	10	37	68
09-741	27	22	21	70
EarlyAcre	34	30	6	70
16-166	20	30	21	71
17-129	28	35	10	73
16-181	14	22	37	73
17-133	25	28	21	74
09-714	24	33	20	77
17-102	26	22	30	78
09-692	19	36	27	82
17-107	36	36	13	85
17-114	35	22	31	88
17-118	22	32	37	91
17-128	32	28	31	91
17-111	37	33	31	101
17-109	38	36	31	105
PI255774	39	39	35	113

Improvement and Future Direction

In the experiment of this study, we used only two replicates. It will be nice to add one more replication in order to reduce experiment error. For the four newly discovered drought tolerant lines, it will also good to confirm their drought tolerance ability further in next experiment.

In this study, we conducted drought tolerance in 39 cowpea lines. We plan to do more than 300 cowpea lines for both drought tolerance phenotyping and genotyping through DNA sequencing to have SNPs, and then conduct genome wide association study in order to identify QTLs/genes and SNP markers for drought tolerance and use them in cowpea breeding.

The drought cowpea lines from this study can be used as parents in cowpea breeding to breed new drought tolerance cultivars.

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