Evaluation of Salt Tolerance at Germination Stage in Cowpea [*Vigna unguiculata* (L.) Walp]

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Additional index words. *Vigna unguiculata*, breeding, germplasm, salinity, screening

Abstract. Cowpea is a leguminous and versatile crop which provides nutritional food for human consumption. However, salinity unfavorably reduces cowpea seed germination, thus significantly decreasing cowpea production. Little has been done for evaluating and developing salt-tolerant cowpea genotypes at germination stage. The objectives of this research were to evaluate the response of cowpea genotypes to salinity stress through seed germination rate and to select salt-tolerant cowpea genotypes. The seed germination rates under nonsalt condition and salinity stress (150 mM NaCl) were evaluated in 151 cowpea genotypes. Four parameters, absolute decrease (AD), the inhibition index (II), the relative salt tolerance (RST), and the salt tolerance index (STI) were used to measure salt tolerance in cowpea. The results showed that there were significant differences among the 151 cowpea genotypes for all parameters (P values <0.0001). The AD in germination rate was 5.8% to 94.2%; the II varied from 7.7% to 100%; the RST ranged from 0 to 0.92; and STI varied from 0 to 0.92. A high broad sense heritability (H^2) was observed for all four parameters. High correlation coefficients (r) were estimated among the four parameters. PI582422, 09–529, PI293584, and PI582570 were highly salt tolerant at germination stage. In addition, genotypes from the Caribbean and Southern Asia exhibited better tolerance to salinity, whereas those from Europe and North America were the most salt-susceptible.

Cowpea [*Vigna unguiculata* (L.) Walp] ($2n = 2x = 22$) is a legume of economic importance worldwide. It is widely grown in Central and West Africa with a production estimated at 5.4 million tons of dry seed (Olufajo, 2012) and also grown in Latin America, Southeast Asia, and in the United States (Muchero et al., 2009; Tan et al., 2012). Cowpea plays an essential role for food security and ecosystem services. Cowpea has high seed protein content and other nutritional components for human benefit. It also supplies nitrogen to soils (Duke, 1990). Mamiro et al., (2011) reported that cowpea dried seeds had a fat concentration ranging from 5.4% to 11.2% depending on cultivar. The calcium content ranged from 360 to 992.4 mg/kg. The concentration of zinc

varied between 31.5 and 35.6 mg/kg. The iron content ranged from 27.6 to 28.9 mg/kg. In addition, their results suggested that cowpea leaves were also rich in micronutrients. In the United States, cowpea, also commonly referred as southern pea, blackeye pea, crowder pea, lubia, niebe, coupe, or frijole is usually grown by small-scale farmers (less than 50 ha) mainly in Southern States, and there is a significant blackeye bean industry in California. It is a profitable crop for growers (Davis et al., 2012; Okiror et al., 2008).

Germination is a pivotal step for crop propagation. Studies reported that germination is highly influenced by a large number of environmental factors. Mistura et al., (2011) stated that salinity affected germination of legumes and plant vigor. Dutta and Bera (2014) found a noticeable decrease in seed germination, plant growth, and vigor indices for mung bean supplied with salt treatment. In addition, Zhang et al., (2013) stated that

low temperatures undermine cowpea germination. Their results indicated that germination rate decreased to 18% at 10 °C. Reddy and Reddy (2016) reported that excessive and incessant rainfall decreased the seed germination of soybean in India. Low germination unfavorably affects crop production. Germination ensures the propagation of plants to properly ensure food supplies for human consumption (Bewley, 2003). Moreover, seed quality is an important feature required by seed testing centers. To ensure a stable market, seed producer companies have to provide good quality seeds exhibiting high germination rate.

Saline soil is characterized by high concentration of cations (K^+ , Mg^{2+} , Ca^{2+} , and Na^+) and anions (NO_3^- , HCO_3^- , SO_4^{2-} , and Cl^-) (Wallender and Tanji, 2011). Sodium chloride is the most well-known salt causing damage in agriculture (Ayers and Westcot, 1985). In addition, Shannon (1997) reported that the concentration of salt in cultivated areas has been increasing because of inappropriate irrigation, fertilization, or other factors. Such increase is estimated at 1 to 60 t/ha annually. Läuchli and Lüttge (2002) pointed out that costs related to salinity issue were 12 billion US dollars per year. Rock weathering, seawater, rain, deforestation, air pollution, and contamination of river waters by chemical and domestic animals are substantial sources of salinity in agriculture (Omami and Hammes, 2006; Rengasamy et al., 2006).

However, little has been done regarding studies on seed germination in cowpea along with the factors that markedly affect germination rate. A study conducted by Zahedi et al., (2012) reported that salinity decreased germination rate in cowpea, and poor seed germination resulted in a significant reduction in yield. In addition, salinity had negative effects on plant growth, cell extension, cell division, and photosynthesis (Maas and Hoffman, 1977).

The evaluation of salt tolerance requires a controlled environment. Field screening is an available technique to screen plant genotypes for salinity. For this method, seeds are sown directly in soil with high salt concentration. However, because of the variability of salt content in the field and the considerable influence of other factors such as moisture content in soil, soil fertility, temperature, light, transpiration, and weather, the results might be highly biased and nonconclusive in a field setting (Parker et al., 1983; Pathan et al., 2007; Yang and Blanchard, 1993). Plant genotypes can be also hydroponically screened by using nutrient solution as a medium growth and adding salt solution after germination and establishment (An et al., 2001). However, such a method is expensive. Therefore, evaluating salt tolerance at germination stage would be of interest because doing so will add information to genotype response at different stages of plant growth and development.

Screening for salt-tolerant genotypes at germination stage is one of the most cost

Received for publication 9 June 2017. Accepted for publication 11 July 2017.

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effective ways to tackle salinity-related issues. In this view, some research has dealt particularly with the effects of salinity on germination rate and emergence (Badia and Meiri, 1994; Carter et al., 2006; Kent and Lauchli, 1985; Mauromicale and Licandro, 2002). Kan et al. (2015) evaluated the effects of salt stress on a panel of 191 soybean (*Glycine max* L.) genotypes and identified eight SNP markers highly associated with tolerance to salinity at germination. Concerning cowpea, Wests and Francois (1982) suggested that a salt concentration higher than 120 mM would reduce cowpea germination. Salinity engenders osmotic or toxicity effects, which result in low seed germination (Waisel, 1972). In addition, Taffouo et al. (2009) reported that high sodium chloride concentration significantly affected germination rate in cowpea. Ashebir et al. (2013) studied the effects of salinity at germination in cowpea. Their results revealed that there was a significant variability in response to salt stress among cowpea genotypes. They found that the genotypes ‘211557’ and ‘Asebot’ were salt tolerant at germination stage. The objectives of this study were to evaluate the responses of cowpea genotypes to salinity (NaCl) at germination stage, to screen salt-tolerant cowpea genotypes, and to select for the most accurate parameter for assessing cowpea salt tolerance at germination stage for a salt-tolerance breeding program.

Materials and Methods

Plant materials. A total of 151 cowpea genotypes consisting of 116 United States Department of Agriculture (USDA) Germplasm Resources Information Network (GRIN) germplasm accessions and 35 University of Arkansas lines were used in this study (Table 1). The 116 germplasm accessions were originally collected from 31 countries and classified into 12 regions depending on their origin (Caribbean, Eastern Africa, Southern Africa, Western Africa, Eastern Asia, Southeastern Asia, Southern Asia, Western Asia Europe, Latin America, and North America). All original seeds of the GRIN germplasm were obtained from USDA Plant Genetic Resources Conservation Unit at Griffin, GA, and were increased at the Research and Extension Station of University of Arkansas at Fayetteville, AR, in the summers 2014 and 2015.

Determination of optimal seed germination temperature and salt concentration. Two pilot experiments were conducted to determine the optimal temperature and salt (NaCl) concentration for cowpea seed germination. For the temperature experiment, the cowpea cultivar Early Scarlet was used to test seed germination rates under three thermogradient temperatures (25, 28, and 31 °C). The results showed that ‘Early Scarlet’ had the highest seed germination rate at 28 °C, which was the same optimum temperature for cowpea seed germination reported by Souza et al., (2004). In regard to salt concentration, five cowpea genotypes (‘Early

Scarlet’, ‘07-303’, ‘09-655’, ‘PI293584’, and ‘PI527561’) were tested for seed germination under six levels of concentrations (0, 50, 100, 150, 200, and 250 mM NaCl) to determine the optimal NaCl concentration for salt stress. The concentrations were obtained by dissolving at 2.92, 5.84, 8.77, 11.69, and 14.61 g of sodium chloride powder of Science Company®, Lakewood, CO, into 1 L of distilled water, respectively. The salt concentration where germination rate between cowpea genotypes was the most significantly different was chosen as the optimal salt concentration to evaluate salt tolerance at germination stage.

Germination conditions. The cowpea seeds used in this study were harvested from the field of University of Arkansas Research and Extension Center at Fayetteville, AR, during Summer 2015. Seeds having uniform size from each cowpea genotype were selected. To avoid any contamination, clean seeds were selected, and petri dishes used for germination were sterilized 60 s by washing with bleach 2% followed by 75% ethanol.

Forty seeds from each cowpea genotype were put on paper filter (Laboratory Nerd) (Avogadro’s Laboratory Supply, Inc, Shamong NJ 08088), which was previously placed in a petri dish of 9 cm in diameter. The treatment consisted of adding 14 mL of NaCl solution and deionized water for the control (0 mM NaCl) to each dish. After treatments were applied, the petri dishes were placed in an incubator New Brunswick Scientific Innova 4230® (Manassquan, NJ) at 28 °C for 48 h. Each genotype and salt treatment combination was placed on three different shelves in the incubator with three replicates, and each shelf was treated as a block. The experiment was run in multiple times because of space limitations. After each run, 75% ethanol solution was sprayed into the incubator to limit any microbial growth.

Measurements. The seed germination data were gathered in 48 h after placing the petri dishes in the incubator. The seed germination rate was recorded when the radicle reached one third of the seed length. The performance of the cowpea genotype under salinity stress was evaluated by computing the values of AD due to salinity, II (González, 1996), RST, and STI (Fernandez, 1992; Saad et al., 2014). These parameters were estimated using the following formulas:

$$\text{Absolute Decrease(AD)} = \text{GC} - \text{GS};$$

$$\text{Inhibition Index(II)} = 100 * (\text{GC} - \text{GS})/(\text{GC});$$

$$\text{Relative Salt Tolerance(RST)} = \text{GS}/\text{GC};$$

$$\text{Salt Tolerance Index(STI)} = (\text{GS} * \text{GC})/(\text{GC}_{\text{av}})^2,$$

where GC = seed germination rate without salt stress, GS = seed germination under salt stress, and GC_{av} = average of the seed germination rate of a cowpea genotype without salt stress.

Experimental design. Regarding the preliminary test related to the determination of the optimal salt (NaCl) concentration,

a two-factor factorial (genotype X salt) organized in a randomized complete block design (RCBD) with three blocks was adopted. The genotype consisted of ‘Early Scarlet’, ‘07-303’, ‘09-655’, ‘PI293584’, and ‘PI527561’, and the salt treatment levels were 0, 50, 100, 150, 200, and 250 mM. Three replications per genotype and salt treatment combination were used.

With respect to the assessment of salt tolerance among the 151 cowpea genotypes, the design was similar to that of the preliminary test using RCBD. However, the salt treatments were the optimal salt concentration from the preliminary test (150 mM NaCl) and the deionized water with 0 mM NaCl. Each salt-genotype combination was assigned to petri dishes, replicated three times, and each replication corresponded to each of the three shelves in the incubator and the shelf was used as a block.

Data analysis. The parameters used for the analysis resulted from pairing data on a genotype under salinity treatment and without salt stress. Therefore, the statistical model for the analysis was as described below.

In the calculations, Y_{ijk} = value of the parameters for the j th cowpea genotype on the i th shelf at the k th replication, for $i = 1, 2, 3$; $j = 1, \dots, 151$, and $k = 1, 2, 3$.

$$Y_{ijk} = \mu + S_i + G_j + \epsilon_{ijk}$$

where μ = constant (overall mean), S_i = effect of the i th shelf (random effect) on the variability of the response, G_j = effect of the j th genotype (fixed effect) on the mean response, and ϵ_{ijk} = experimental error associated with the ijk th observation. In this study, the effects of experiment runs would be assumed as negligible as the germination study was carried out in an incubator. However, the effect of shelves in the incubator should be taken into account because there could be within incubator temperature variability.

The analysis of variance (ANOVA) test was carried out using the general linear model (GLM) procedure of JMP Genomics 7 (SAS Institute, Cary, NC). The mean separation was performed using the Student’s t test at $\alpha = 0.05$. The descriptive statistics were generated using “Tabulate”; the correlations among the parameters were analyzed using “Multivariate Methods” by “Multivariate” function; and the distributions of the data were drawn using “Distribution” in JMP Genomics 7.

The broad sense heritability (H^2) was obtained from (Hosseini et al., 2012)

$$H^2 = (\sigma_G^2 / \sigma_P^2) \times 100 \\ = [\sigma_G^2 / (\sigma_G^2 + (\sigma_E^2 / r) + (\sigma_S^2 / s))] \times 100$$

where σ_G^2 = genotypic variance, σ_P^2 = phenotypic variance, σ_E^2 = variance associated with the experimental error, σ_S^2 = variance associated with the shelf, s = number of shelves, and r = number of replications per treatment.

Table 1. Cowpea name (accession number), seed color, origin, and germination rates without salt treatment and under salt stress, absolute decrease (AD), inhibition index (II), relative salt tolerance (RST), and salt tolerance index (STI).

Accession number	Seed color	Origin	Nonstress (%)	Stress (%)	AD_(%)	II_(%)	RST	STI
07-303	Red	AR, USA	82.50	60.83	21.67	25.83	0.74	0.73
09-105	Cream	AR, USA	89.17	46.67	42.50	47.59	0.52	0.52
09-1090	Pinkeye	AR, USA	85.00	35.00	50.00	58.85	0.41	0.41
09-181	Pinkeye	AR, USA	72.50	49.17	23.33	32.09	0.68	0.68
09-231	Pinkeye	AR, USA	68.50	42.50	26.00	37.65	0.62	0.62
09-239	Pinkeye	AR, USA	80.83	34.17	46.67	57.88	0.42	0.43
09-268	Cream	AR, USA	83.33	16.67	66.67	79.92	0.20	0.20
09-295	Pinkeye	AR, USA	64.17	35.83	28.33	44.12	0.56	0.56
09-307	Blackeye	AR, USA	96.67	46.67	50.00	51.71	0.48	0.48
09-316	Black Holstein	AR, USA	90.00	31.67	58.33	64.75	0.35	0.35
09-323	Pinkeye	AR, USA	86.67	50.00	36.67	42.05	0.58	0.58
09-393	Pinkeye	AR, USA	87.50	0.83	86.67	99.02	0.01	0.01
09-452	Black Holstein	AR, USA	88.33	11.67	76.67	86.76	0.13	0.13
09-455	Blackeye	AR, USA	77.50	57.50	20.00	25.89	0.74	0.75
09-470	Pinkeye	AR, USA	91.67	44.17	47.50	51.61	0.48	0.48
09-529	Blackeye	AR, USA	85.50	75.00	10.50	12.16	0.88	0.88
09-655	Pinkeye	AR, USA	92.50	28.33	64.17	69.01	0.31	0.30
09-686	Pinkeye	AR, USA	93.33	37.50	55.83	59.80	0.40	0.40
09-692	Pinkeye	AR, USA	92.50	39.17	53.33	57.33	0.43	0.42
09-697	Pinkeye	AR, USA	68.33	32.50	35.83	52.26	0.48	0.47
09-714	Pinkeye	AR, USA	80.83	40.00	40.83	50.47	0.50	0.50
09-741	Red Holstein	AR, USA	87.50	65.83	21.67	24.41	0.76	0.75
09-744	Cream	AR, USA	85.83	42.50	43.33	50.40	0.50	0.49
09-745	Red Holstein	AR, USA	89.17	6.67	82.50	92.49	0.08	0.07
09-749	Red Holstein	AR, USA	76.67	27.50	49.17	64.21	0.36	0.36
09-752	Black Holstein	AR, USA	81.67	4.17	77.50	94.88	0.05	0.05
Arkansas Blackeye #1	Blackeye	AR, USA	76.67	50.83	25.83	33.52	0.66	0.66
Early Acre	Cream	AR, USA	67.50	32.50	35.00	50.92	0.49	0.49
Early scarlet	Pinkeye	AR, USA	81.67	29.17	52.50	64.39	0.36	0.36
Ebony	Black	AR, USA	90.00	69.17	20.83	23.06	0.77	0.77
ENCORE	Pinkeye	AR, USA	71.67	30.00	41.67	58.10	0.42	0.42
Envoy	Red Holstein	AR, USA	99.17	30.00	69.17	69.72	0.30	0.30
Epic Select	Tan	AR, USA	81.67	10.83	70.83	86.92	0.13	0.13
EXCEL	Browneye	AR, USA	86.67	70.00	16.67	19.24	0.81	0.81
PI152195	Red	Paraguay	82.50	44.17	38.33	46.71	0.53	0.54
PI152196	Mixed (Cream Brown)	Paraguay	95.00	58.33	36.67	39.42	0.61	0.62
PI152197	Red	Paraguay	88.33	25.00	63.33	71.42	0.29	0.28
PI152199	Mixed (Purple Cream)	Paraguay	79.17	45.83	33.33	42.02	0.58	0.58
PI162924	Mixed (Cream Brown)	Paraguay	83.33	66.67	16.67	18.72	0.81	0.79
PI167284	Mixed (Cream Brown)	Turkey	79.17	32.50	46.67	59.52	0.40	0.42
PI175332	Tan	NA	77.50	31.67	45.83	58.33	0.42	0.41
PI180014	Tan	India	71.67	27.50	44.17	62.76	0.37	0.40
PI190191	Tan	Mexico	63.33	17.50	45.83	72.09	0.28	0.28
PI201498	Blackeye	Mexico	92.50	77.50	15.00	16.24	0.84	0.84
PI218123	Browneye	Pakistan	97.50	30.83	66.67	68.22	0.32	0.32
PI221730	Red Holstein	South_Africa	69.17	30.83	38.33	55.17	0.45	0.44
PI221731	Red Holstein	South_Africa	95.83	50.83	45.00	46.87	0.53	0.53
PI223023	Browneye	Iran	95.00	56.67	38.33	40.74	0.59	0.60
PI225922	Mixed (Brown Tan)	Zambia	60.83	46.67	14.17	20.25	0.80	0.75
PI229551	Browneye	Iran	90.83	70.83	20.00	21.52	0.78	0.78
PI229734	Blackeye	Iran	88.33	26.67	61.67	70.13	0.30	0.31
PI244571	Mixed (Brown Cream)	Guatemala	66.67	17.50	49.17	73.74	0.26	0.26
PI250416	Cream	Pakistan	88.33	22.50	65.83	73.98	0.26	0.25
PI250587	Dark brown	Madagascar	70.00	5.83	64.17	91.73	0.08	0.08
PI253428	Dark brown	Spain	97.50	38.33	59.17	60.67	0.39	0.39
PI255765	Blackeye	Nigeria	97.50	56.67	40.83	41.85	0.58	0.58
PI255815	Browneye	Nigeria	77.50	54.17	23.33	28.81	0.71	0.69
PI257463	Red Holstein	Nigeria	79.17	46.67	32.50	40.51	0.59	0.59
PI262179	Blackeye	Portugal	73.33	55.00	18.33	24.68	0.75	0.75
PI292897	Grey	Hungary	67.50	28.33	39.17	58.30	0.42	0.42
PI292898	Black	Hungary	86.67	71.67	15.00	17.09	0.83	0.83
PI292899	Tan	Hungary	66.67	11.67	55.00	83.70	0.16	0.19
PI292913	Tan	Hungary	62.50	8.33	54.17	86.71	0.13	0.13
PI293467	Mixed (Tan Brown)	USA	84.17	28.33	55.83	66.45	0.34	0.34
PI293469	Tan	NA	91.67	23.33	68.33	74.72	0.25	0.26
PI293476	Variable (Grey Brown)	USA	70.83	7.50	63.33	87.68	0.12	0.09
PI293514	Grey	USA	78.33	21.67	56.67	72.51	0.27	0.28
PI293545	Mixed (Brown Cream)	NA	93.33	65.00	28.33	30.37	0.70	0.70
PI293570	Mixed (Brown Tan)	NA	62.50	7.50	55.00	87.63	0.12	0.12
PI293582	Grey	NA	70.00	22.50	47.50	68.13	0.32	0.33
PI293584	Variable (Red Brown Cream)	NA	67.50	58.33	9.17	13.31	0.87	0.86
PI297561	Mixed (Brown Grey)	NA	61.67	36.67	25.00	38.75	0.61	0.60
PI339587	Red Holstein	South_Africa	70.00	53.33	16.67	23.46	0.77	0.76

(Continued on next page)

Table 1. (Continued) Cowpea name (accession number), seed color, origin, and germination rates without salt treatment and under salt stress, absolute decrease (AD), inhibition index (II), relative salt tolerance (RST), and salt tolerance index (STI).

Accession number	Seed color	Origin	Nonstress (%)	Stress (%)	AD_(%)	II_(%)	RST	STI
PI339591	Brown Holstein	South_Africa	65.00	51.67	13.33	18.60	0.81	0.79
PI339592	Brown	South_Africa	70.83	44.17	26.67	36.71	0.63	0.62
PI339594	Variable (Red Holstein Red Tan)	NA	71.67	51.67	20.00	23.91	0.76	0.71
PI339609	Tan	Tanzania	89.17	50.00	39.17	43.40	0.57	0.56
PI339610	Variable (Red Brown Cream)	Tanzania	60.83	38.33	22.50	36.71	0.63	0.63
PI339611	Tan	Tanzania	73.33	62.50	10.83	14.60	0.85	0.85
PI339613	Variable (Grey Tan Brown)	Tanzania	93.33	45.00	48.33	51.62	0.48	0.48
PI347639	Tan	NA	60.00	36.67	23.33	38.34	0.62	0.61
PI353045	Red Holstein	India	77.50	45.83	31.67	40.68	0.59	0.59
PI353062	Mixed (Cream Purple)	India	94.17	68.33	25.83	27.51	0.72	0.73
PI367921	Tan	NA	89.17	73.33	15.83	17.68	0.82	0.82
PI406290	Browneye	Mozambique	65.00	18.33	46.67	72.02	0.28	0.28
PI430687	Red	China	68.33	32.50	35.83	51.75	0.48	0.47
PI487518	Tan	Indonesia	98.33	44.17	54.17	55.26	0.45	0.45
PI527561	Tan	Burindi	96.67	58.33	38.33	39.64	0.60	0.60
PI578902	Red	China	78.33	22.50	55.83	69.99	0.30	0.28
PI578907	Black	China	87.50	44.17	43.33	49.38	0.51	0.51
PI578911	Tan	China	82.50	47.50	35.00	41.91	0.58	0.57
PI582340	Mixed (Brown Pink)	NA	69.17	26.67	42.50	61.38	0.39	0.39
PI582347	Tan	Myanmar	80.83	49.17	31.67	39.10	0.61	0.61
PI582352	Blackeye	Saudi_Arabia	88.33	49.17	39.17	43.35	0.57	0.55
PI582354	Blackeye	NA	69.17	40.83	28.33	39.97	0.60	0.58
PI582415	Tan	Mexico	89.17	52.50	36.67	41.13	0.59	0.59
PI582416	Variable (Red Brown Cream)	Mexico	92.50	25.00	67.50	72.94	0.27	0.27
PI582420	Variable (Red Brown)	NA	77.50	65.00	12.50	15.36	0.85	0.83
PI582421	Grey	NA	97.50	65.00	32.50	33.50	0.66	0.67
PI582422	Blackeye	NA	75.83	70.00	5.83	7.67	0.92	0.92
PI582425	Mixed (Tan Brown)	NA	82.50	40.83	41.67	50.44	0.50	0.49
PI582428	Blackeye	Trinidad and Tobago	64.72	49.17	15.56	22.14	0.78	0.76
PI582467	Black Holstein	NA	75.83	37.50	38.33	49.07	0.51	0.49
PI582474	Purple	Botswana	64.17	25.83	38.33	59.41	0.41	0.40
PI582522	Tan	Mexico	95.00	0.83	94.17	99.12	0.01	0.01
PI582531	Variable (Grey Brown Tan)	Ghana	65.83	42.50	23.33	31.66	0.68	0.62
PI582542	Browneye	Mexico	88.33	58.33	30.00	33.67	0.66	0.66
PI582551	Blackeye	Botswana	84.17	64.17	20.00	23.85	0.76	0.76
PI582570	Browneye	India	67.78	58.33	9.44	13.65	0.86	0.86
PI582573	Browneye	Kenya	67.50	53.33	14.17	20.60	0.79	0.79
PI582574	Tan	Kenya	75.00	28.33	46.67	61.01	0.39	0.38
PI582575	Black	Kenya	91.67	28.33	63.33	68.91	0.31	0.31
PI582578	Tan	Kenya	77.50	42.50	35.00	41.86	0.58	0.53
PI582579	Tan	Kenya	97.50	21.67	75.83	77.66	0.22	0.22
PI582665	Variable (Grey Brown Tan)	Botswana	89.17	1.67	87.50	98.20	0.02	0.02
PI582666	Tan	Botswana	83.33	46.67	36.67	44.14	0.56	0.56
PI582680	Browneye	Botswana	65.83	26.67	39.17	59.28	0.41	0.41
PI582696	Brown	Botswana	74.17	44.17	30.00	40.27	0.60	0.59
PI582703	Red Holstein	Botswana	69.17	27.50	41.67	60.29	0.40	0.40
PI582789	Tan	Kenya	95.83	36.67	59.17	61.62	0.38	0.38
PI582809	Tan	Botswana	61.67	23.33	38.33	62.28	0.38	0.38
PI582813	Mixed(Grey Cream Black eye)	Botswana	77.50	0.00	77.50	100.00	0.00	0.00
PI582815	Purple	Botswana	84.17	41.67	42.50	50.45	0.50	0.49
PI582818	Black Holstein	Botswana	90.00	20.00	70.00	77.74	0.22	0.22
PI582821	Mixed (Brown Tan)	Botswana	90.83	40.00	50.83	56.15	0.44	0.44
PI582852	Brown	Botswana	61.67	31.67	30.00	48.31	0.52	0.51
PI582863	Browneye	Botswana	87.50	59.17	28.33	32.17	0.68	0.68
PI582873	Tan	Botswana	67.50	7.50	60.00	88.53	0.11	0.11
PI582874	Tan	Botswana	85.00	37.50	47.50	55.91	0.44	0.45
PI582875	Tan	Botswana	70.83	11.67	59.17	83.54	0.16	0.17
PI582878	Green	NA	72.50	12.50	60.00	81.76	0.18	0.16
PI582894	Tan	Botswana	71.67	27.50	44.17	61.35	0.39	0.38
PI582932	Browneye	Malawi	70.00	57.50	12.50	17.56	0.82	0.82
PI583193	Variable (Gray Brown)	Senegal	94.17	15.83	78.33	83.11	0.17	0.17
PI583194	Variable (Tan Grey)	Senegal	99.17	9.17	90.00	90.83	0.09	0.09
PI583198	Tan	Senegal	96.67	57.50	39.17	40.82	0.59	0.60
PI583201	Browneye	Senegal	70.83	51.67	19.17	24.75	0.75	0.73
PI583202	Browneye	Senegal	89.17	56.67	32.50	36.56	0.63	0.64
PI583209	Tan	Nigeria	90.83	23.33	67.50	74.27	0.26	0.26
PI583244	Tan	Senegal	80.83	35.83	45.00	55.53	0.44	0.44
PI583248	Browneye	Senegal	77.78	63.33	14.44	18.13	0.82	0.81
PI583249	Browneye	Senegal	69.17	47.50	21.67	30.62	0.69	0.69
PI583274	Browneye	Senegal	88.33	62.50	25.83	28.92	0.71	0.71
PI583487	Tan	Senegal	62.50	39.17	23.33	33.91	0.66	0.61
PI583488	Brown	Australia	69.17	30.83	38.33	55.67	0.44	0.45
PI583550	Purple	Mali	93.33	42.50	50.83	54.27	0.46	0.45

(Continued on next page)

Table 1. (Continued) Cowpea name (accession number), seed color, origin, and germination rates without salt treatment and under salt stress, absolute decrease (AD), inhibition index (II), relative salt tolerance (RST), and salt tolerance index (STI).

Accession number	Seed color	Origin	Nonstress (%)	Stress (%)	AD_(%)	II_(%)	RST	STI
PI583551	Browneye	Mali	70.83	46.67	24.17	33.91	0.66	0.67
PI610520	Black	Italy	69.17	12.50	56.67	81.33	0.19	0.18
PI610533	Blackeye	NA	80.00	49.17	30.83	37.96	0.62	0.61
PI610604	Purple	Italy	65.83	23.33	42.50	64.19	0.36	0.37
PI666252	Tan	USA	90.00	26.67	63.33	70.33	0.30	0.30

Fig. 1. Seed germination rates under six salt (NaCl) concentrations (0, 50, 100, 150, 200, and 250 mM): (A) five cowpea genotypes, respectively, and (B) the pooled five cowpea genotypes.

Fig. 2. An example of the seed germination rate in two cowpea genotypes, '09-655' and 'PI293584' under six salt (NaCl) concentrations in the petri dishes (left) and the radicle length (right) in 48 h after NaCl treatment at 28 °C condition.

σ_G^2 , σ_E^2 , and σ_S^2 were obtained using the following formulas:

$$\sigma_G^2 = (\text{MSG} - \text{MSE})/r, \sigma_E^2 = \text{MSE},$$

$$\sigma_S^2 = (\text{MSS} - \text{MSE})/n$$

where MSG = mean square genotype, MSE = mean square error, MSS = mean square shelf, r = number of replications, and n = number of genotypes.

In addition, ANOVA of the effects of the origin (region) of the cowpea genotypes on

salt tolerance were analyzed using GLM of JMP Genomics. Before analysis, the genotypes without any information on their origin were discarded so that 132 genotypes were analyzed in this study.

The cluster analysis involved 151 individuals which were measured using six parameters: germination rates without salt treatment (nonstress) and under salt stress (stress), AD, II, RST, and STI. Ward's method was used as a clustering technique (Sahu, 2013). The

phylogenetic tree diagram was constructed using "Multivariate Methods" by "Cluster" in JMP Genomics 7 (SAS Institute, Cary, NC).

Results and Discussion

Optimal concentration for evaluation of cowpea salt tolerance at germination stage. From our preliminary experiments with six salt concentrations: 0, 50, 100, 150, 200, and 250 mM of NaCl, when the NaCl concentration increased, the seed germination rate decreased either pooled across cowpea genotypes or individually (Figs. 1 and 2; Tables 2 and 3). At the salinity level at 250 mM, the seeds had minimal germination rate which made it difficult to separate the tolerant from the susceptible genotypes (Tables 2 and 3). At 150 mM, seed germination differed the most among the cowpea genotypes ($F = 27.37$, P value = 0.0001) (Table 3). It was reported that increasing salt concentration reduced the germination rate of cowpea genotype in other studies (Thiam et al., 2013; Wests and Francois, 1982; Zahedi et al., 2012). Lobato et al. (2009) used 150 mM NaCl to conduct their study on the effects of salinity on cowpea germination. Therefore, the salt concentration of 150 mM NaCl would be a reasonable concentration to perform salt tolerance testing in cowpea and it was used in our study.

Germination at nonsalt stress and salt stress conditions. The average germination rate among the 151 cowpea genotypes under the nonsalt stress condition varied from 60.0% to 99.2%, with a mean of 80.2% and a standard deviation of 11.0%. Incubating seeds in 150 mM NaCl resulted in a drop of

germination to 0% to 77.5%, with an overall average of 38.7% and a standard deviation of 18.3%. These results suggested that salinity significantly reduced the germination rate in cowpea. For all parameters, there were no significant incubator shelf effects. The germination rate was significantly different among the cowpea genotypes under non-stress condition ($F = 7.37$, P value < 0.0001) (Table 4). ‘Envoy’ (99.2%), ‘PI583194’ (99.2%), ‘PI487518’ (98.3%), ‘PI582579’ (97.5%), ‘PI218123’ (97.5%), ‘PI253428’ (97.5%), ‘PI255765’ (97.5%), and ‘PI582421’ (97.5%) had the highest germination rates, and ‘PI225922’ (60.8%), ‘PI339610’ (60.8%), and ‘PI347639’ (60%) had the lowest germination rates. Under salinity condition, ‘PI201498’ performed well with a germination rate of 77.5%, indicating it was a salt-tolerant cowpea

accession at germination stage; however, the lowest germination rate was recorded for ‘PI252665’ (1.7%), ‘09-393’ (0.8%), ‘PI582522’ (0.8%), and ‘PI582813’ (0.0%), indicating that they were very susceptible to salt stress at the germination stage. The germination rate among the cowpea genotypes was significantly different under salt stress ($F = 16.62$, P value < 0.0001) (Table 4).

Absolute decrease and inhibition index. The AD indicated the decrease of the germination rate between the nonsalinity conditions and the salt treatment. In this study, salinity reduced the germination rate from 5.8% to 94.2%. The cowpea genotypes responded differently to salinity environment at germination stage in terms of AD ($F = 10.1$, P value < 0.0001) (Table 4). ‘PI582522’

(94.2%) exhibited the highest AD. ‘PI585422’ had the lowest AD, 5.8%. The higher the AD was, the more salt susceptible the genotype.

The II was a parameter which was widely used for studies related to plant stress (González, 1996). The II of the germination ranged from 7.7% to 100.0%, with an average of 51.2% and a standard deviation of 22.6%, indicating a large variability in responses to salinity among the cowpea genotypes. In addition, the inhibition of the germination because of salinity significantly differed among the cowpea genotypes ($F = 11.6$, P value < 0.0001) (Table 4). ‘PI582813’, ‘PI582522’, and ‘09-393’ had a very high II, over 99% under salt stress. These results suggested that these lines are highly salt sensitive at germination stage. ‘PI582422’, ‘09-529’, ‘PI293584’, ‘PI582570’, and

Table 2. ANOVA for pooled seed germination rates of five cowpea genotypes under six NaCl concentrations (0, 50, 100, 150, 200, and 250 mM).

Source	DF	Sum of squares	Mean square	F ratio	Prob > F
Shelf	2	2.534	1.267	0.5061	0.6175
Concentration	5	23,605.7	4,721.1472	1,885.53	<0.0001
Error	10	25.039	2.5		

DF = degree(s) of freedom.

Table 3. ANOVA for seed germination rates of five cowpea genotypes under six salt concentrations of NaCl.

NaCl concn (mM)	Source	DF	Sum of squares	Mean square	F ratio	Prob > F
0	Shelf	2	7.50	3.75	0.2553	0.7807
	Genotype	4	397.50	99.38	6.766	0.0111
	Error	8	117.50	14.69		
50	Shelf	2	22.50	11.25	0.4269	0.6666
	Genotype	4	354.17	88.54	3.3597	0.068
	Error	8	210.83	26.35		
100	Shelf	2	40.83	20.42	1.0262	0.4011
	Genotype	4	373.33	93.33	4.6911	0.0304
	Error	8	159.17	19.90		
150	Shelf	2	55.83	27.92	1.8741	0.215
	Genotype	4	1,630.83	407.71	27.3706	0.0001
	Error	8	119.17	14.90		
200	Shelf	2	3.70	1.85	0.2441	0.789
	Genotype	4	458.57	114.64	15.1259	0.0008
	Error	8	60.63	7.58		
250	Shelf	2	7.50	3.75	0.7059	0.522
	Genotype	4	137.50	34.38	6.4706	0.0126
	Error	8	42.50	5.31		

DF = degree(s) of freedom.

Table 4. ANOVA for six parameters related to seed germination based on cowpea genotypes.

Parameters*	Source	DF	Sum of squares	Mean square	F ratio	Prob > F	Estimated Mean square
Germination without salt stress (nonstress)	Shelf	2	214.528	107.264	2.163	0.1167	$\sigma_e^2 + n\sigma_s^2$
	Genotype	150	54,845.211	365.635	7.374	<0.0001	$\sigma_e^2 + r\sigma_g^2$
	Error	300	14,875.217	49.584			σ_e^2
Germination under 150 mM NaCl (stress)	Shelf	2	86.865	43.433	0.715	0.4899	$\sigma_e^2 + n\sigma_s^2$
	Genotype	150	151,389.901	1,009.266	16.621	<0.0001	$\sigma_e^2 + r\sigma_g^2$
	Error	300	18,217.301	60.724			σ_e^2
Absolute decrease (AD)	Shelf	2	55.025	27.513	0.245	0.7828	$\sigma_e^2 + n\sigma_s^2$
	Genotype	150	169,967.007	1,133.113	10.093	<0.0001	$\sigma_e^2 + r\sigma_g^2$
	Error	300	33,681.420	112.271			σ_e^2
Inhibition index (II)	Shelf	2	40.441	20.221	0.153	0.8581	$\sigma_e^2 + n\sigma_s^2$
	Genotype	150	229,783.382	1,531.889	11.597	<0.0001	$\sigma_e^2 + r\sigma_g^2$
	Error	300	39,629.810	132.099			σ_e^2
Relative salt tolerance (RST)	Shelf	2	0.004	0.002	0.139	0.8699	$\sigma_e^2 + n\sigma_s^2$
	Genotype	150	22.947	0.153	11.587	<0.0001	$\sigma_e^2 + r\sigma_g^2$
	Error	300	3.961	0.013			σ_e^2
Salt tolerance index (STI)	Shelf	2	0.058	0.029	2.560	0.079	$\sigma_e^2 + n\sigma_s^2$
	Genotype	150	22.465	0.150	13.164	<0.0001	$\sigma_e^2 + r\sigma_g^2$
	Error	300	3.413	0.011			σ_e^2

*Germination rate under non-salinity condition, salt stress, and different formulas used to assess genotype performed to salinity.

DF = degree(s) of freedom.

‘PI339611’ had the lowest II, which were 7.7%, 12.2%, 13.3%, 13.6%, and 14.6%, respectively. These accessions could be excellent sources for salt tolerance at germination stage. The lower the II was, the more likely the genotype withstood salt stress (González, 1996).

Relative salt tolerance and salt-tolerance index. The RST and STI were two parameters to measure cowpea salt tolerance. The higher the RST or STI parameter was, the more likely the genotype was salt tolerant (Fernandez, 1992; Saad et al., 2014).

The RST varied from 0 to 0.92, with a mean of 0.49 and a standard deviation of 0.23. RST was significantly different among the cowpea genotypes ($F = 11.99$, P value < 0.0001) (Table 4). ‘PI582422’, ‘09-529’, ‘PI293584’, and ‘PI582570’ had the highest RST scores, indicating that they were salt tolerant based on RST. ‘PI582813’, ‘09-393’, ‘PI582522’, and ‘PI582665’ exhibited the lowest RST scores, suggesting that they were salt-sensitive at germination stage.

The STI ranged from 0 to 0.92, with a mean of 0.48 and a standard deviation of

0.22. Significant differences were observed among the cowpea accessions in terms of STI ($F = 13.16$, P value < 0.0001) (Table 4). ‘PI582422’ (0.92), ‘09-529’ (0.87), ‘PI293584’ (0.86), and ‘PI582570’ (0.85) exhibited the highest STI, suggesting that these lines were highly tolerant to salinity at germination stage. The lowest STI was found in ‘PI582813’ (0), ‘09-393’ (0.01), ‘PI582522’ (0.01), and ‘PI582665’ (0.02) (Table 4), indicating that these cowpea genotypes are highly salt-sensitive.

The population dynamic of a crop is closely related to its seed germination (TeKrony and Egli, 1991). In this study, large variation of responses to salinity among the cowpea genotypes was observed for all parameters. As expected, there was a significant effect of salinity on the germination rate of the cowpea panel. Similar results have been found in other studies (Ashebir et al., 2013; Taffouo et al., 2009; Wests and Francois, 1982).

Analysis by geographical location. Significant differences were observed in seed germination rate without salt treatment and

the germination rate under salt stress, AD, II, RST, and STI among the 12 different regions of origin of the cowpea genotypes (P values < 0.0001) (Table 5). Genotypes from the Caribbean and Southern Asia showed better salt tolerance at the germination stage. The II was smaller than 40%, and both RST and STIs were greater than 0.6 on average. Cowpea genotypes from Europe and North America were more sensitive to salinity at the germination stage (Table 6). The results suggested that the origins of those genotypes markedly impacted their response to salinity, so the origin should be taken into a consideration when one selects cowpea genotypes for salt tolerance. Little has been done regarding the effect of geographical distribution on plant salt tolerance. However, geographical distribution proved to be a strong driving factor on plant adaptation to stress. Burke (1990) reported that crops grown in semiarid areas developed mechanisms which enabled them to overcome permanent exposure to high temperature. Those mechanisms involved both cellular adaptation and photosynthetic responses to heat stress, indicating

Table 5. ANOVA for six parameters related to seed germination based on the origin of cowpea genotypes.

Parameter ²	Source	DF	Sum of squares	Mean square	F ratio	Prob > F
Germination without salt stress (nonstress)	Region ³	11	6,038.717	548.974	3.9444	<0.0001
	Error	384	53,444.976	139.18		
	C. Total	395	59,483.693			
Germination under 150 mM NaCl (stress)	Region	11	9,729.556	884.505	2.4725	0.0053
	Error	384	137,371.517	357.738		
	C. Total	395	147,101.073			
Absolute decrease (AD)	Region	11	10,023.452	911.223	2.0521	0.0229
	Error	384	170,513.885	444.047		
	C. Total	395	180,537.337			
Inhibition index (II)	Region	11	14,775.172	1,343.197	2.3674	0.0077
	Error	384	217,873.973	567.38		
	C. Total	395	232,649.145			
Relative salt tolerance(RST)	Region	11	1.479	0.134	2.3737	0.0076
	Error	384	21.754	0.057		
	C. Total	395	23.233			
Salt tolerance index (STI)	Region	11	1.411	0.128	2.3359	0.0086
	Error	384	21.081	0.055		
	C. Total	395	22.491			

²Parameters indicate the germination rate under nonsalinity condition, salt stress, and different formulas used to assess genotype performed to salinity.

³Geographical areas where cowpea genotypes originated.

DF = degree(s) of freedom.

Table 6. Mean separation for salt tolerance parameters of different regions.

Regions ²	Relative salt tolerance index ³	Salt tolerance index ⁴	Absolute decrease (%) ⁵	Inhibition index (%) ⁶
Caribbean	0.80 A ³	0.79 A	15.70 D	19.91 D
Southern Asia	0.61 AB	0.61 AB	33.02 CD	39.57 CD
Southeastern Asia	0.53 ABCD	0.53 ABCD	42.92 ABC	47.18 ABC
Western Africa	0.57 BC	0.56 BC	37.52 BC	43.31 BC
Southern Africa	0.50 BCD	0.49 CD	38.97 BC	50.45 ABC
Eastern Africa	0.48 BCD	0.45 BCD	43.56 ABC	51.79 ABC
Latin America	0.48 BCD	0.47 CD	43.89 ABC	52.27 ABC
Eastern Asia	0.47 BCD	0.46 BCD	42.50 ABC	53.26 ABC
Oceania	0.44 BCD	0.46 CD	38.33 ABCD	55.66 ABC
Western Asia	0.46 CD	0.48 CD	47.81 AB	53.82 AB
North America	0.45 D	0.45 D	45.76 A	54.81 A
Europe	0.40 D	0.41 D	42.50 ABC	59.58 A

²Geographical areas where cowpea genotypes originated.

³Germination rate under salt stress condition divided by germination rate under nonsalt condition.

⁴Germination rate under salt stress condition times germination rate under nonsalt condition divided by square of germination rate under nonsalt condition.

⁵Germination rated under nonsalt condition subtracted by the germination under salt stress.

⁶Germination rated under nonsalt condition subtracted by the germination under salt stress, divided by germination rate under nonsalt condition, and multiplied by one hundred.

⁷Means followed by the same letter are not significantly different using the Student’s *t* test at alpha = 0.05.

Fig. 3. Distribution of seed germination rates among 151 cowpea genotypes: (A) without salt stress and (B) with salt stress.

Fig. 4. Distributions of seed germination rates among 151 cowpea genotypes in four parameters: (A) absolute decrease, (B) inhibition index, (C) relative salt tolerance, and (D) salt tolerance index.

Table 7. Correlation among six parameters, without salt stress (nonstress), with salt stress (stress), absolute decrease (AD), inhibition index (II), relative salt tolerance index (RST), and salt tolerance index (STI).

Correlation	Nonstress	Stress	AD ^z	II ^y	RST ^x	STI ^w
Nonstress	1	0.20	0.38	0.10	-0.10	-0.08
Stress	0.20	1	-0.83	-0.95	0.95	0.95
AD	0.38	-0.83	1	0.95	-0.95	-0.95
II	0.10	-0.95	0.95	1	-1	-1
RST	-0.10	0.95	-0.95	-1	1	1
STI	-0.08	0.95	-0.95	-1	1	1

^zAbsolute decrease.

^yInhibition index.

^xRelative salt tolerance.

^wSalt tolerance index.

‘PI582813’ and ‘09-393’ were salt tolerant. In addition, Stankowski et al. (2015) showed that ecology played a pivotal role in plant adaptation. Their study on *Mimulus aurantiacus* C., a flower plant, revealed that floral trait had evolved according to the local conditions, suggesting that geographical location had shaped the adaptation of some traits to a specific environment. With respect to salt tolerance in cowpea, further investigation is required to unravel the mechanisms

behind these significant differences in response to salinity among the genotypes from different countries.

Broad sense heritability. H^2 associated with the seed germination without salt stress was 68%. However, it was 83.9% under saline conditions. H^2 was also observed as 75.2% for the AD in germination rate due to salt stress. The results indicated a high H^2 for the parameters related to the II, RST, and STI, with H^2 equal to 77.9%, 78.2%, and

80.8%, respectively. Foolad and Jones (1992) found high heritability for tomato salt tolerance at seed germination stage. They estimated heritability to be 76.0% for tomato salt tolerance of an $F_{2:3}$ population derived from a cross between ‘PI174263’ and ‘UCT5’, which was close to those of salt tolerance in cowpea in this current investigation.

Correlation between parameters. The six parameters, without salt stress (nonstress), with salt stress (stress), AD, RST, and STI involved in this study showed near normal distributions (Figs. 3 and 4). The correlation coefficients among the six parameters were estimated (Table 7). There was a relatively low linear correlation between the germination rate under nonsalt stress condition and the other five parameters related to salinity stress, with $r = 0.20, 0.38, 0.10, -0.10$, and 0.08 , respectively (Table 7), suggesting that salt tolerance at germination stage had a weak association with the germination rate in normal conditions in cowpea. However, the seed germination rate under salt stress had a very high negative linear correlation with the AD ($r = -0.83$) and the II ($r = -0.95$), but a high positive linear correlation observed between the germination rate under salt treatment and the RST ($r = 0.95$), and the STI ($r = 0.95$). These results indicate that salt tolerance at germination stage is highly associated with germination rate under salt stress.

Cluster analysis. Five different groups were identified among the 151 cowpea genotypes involved in this study (Supplemental Fig. 1). The three cowpea accessions, ‘PI582422’, ‘PI293584’, and ‘PI582570’ having the highest STI were clustered together and located at the same cluster, and the four lowest STI genotypes, ‘PI582813’, ‘09-393’, ‘PI582522’, and ‘PI582665’ were clustered together and belonged to another cluster. These results indicated that the STI was reliable method to distinguish the high salt tolerant to high salt sensitive cowpea genotypes.

Conclusion

This study provides data on the responses of cowpea genotype to salinity, which could

be used to screen for salt tolerant parents for breeding purposes. To our knowledge, this is one of the first reports dealing with cowpea salt tolerance at germination stage which involved a large number of cowpea genotypes having a wide range of variability in terms of country of origin. The results suggest that 'PI582422', 'PI293584', 'PI582570', and '09-529' have strong salt tolerance at germination stage. In addition, the most salt-sensitive lines 'PI582813', '09-393', 'PI582522', and 'PI582665' could be used as parents for developing populations for QTL mapping for cowpea salt tolerance at germination stage. Findings from this current report will add information on cowpea genotype responses to salinity at germination stage, which definitely helps breeders to select potential lines for further variety improvement process.

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Supplemental Fig. 1. Phylogenetic tree diagram among 151 cowpea genotypes based on six salt tolerant parameters: germination rates without salt treatment (Non-stress) and under salt stress (Stress), absolute decrease (AD), inhibition index (II), relative salt tolerance (RST), and salt tolerance index (STI).