Development and evaluation of drought resistant mutant germ-plasm of *Vigna unguiculata*[#]

JA de Ronde* and MH Spreeth

Agricultural Research Council (ARC) – Vegetable and Ornamental Plant Institute (VOPI), Private Bag X293, Pretoria, South Africa

Abstract

The aim of this project was to select cowpea plants with improved levels of drought resistance without alteration to the colour of the testa or the growth form. Seed from M_2 to M_5 generations (M = mutant) were used in the study. The M_2 to M_4 seeds were planted and evaluated in wooden boxes in the greenhouse and in the field. It was demonstrated that it was possible to examine mutant lines at the seedling stage in wooden boxes. Mature plants were screened in a rain-out shelter and physiological traits for drought stress were identified among the lines tested. Roots of mature plants were also assessed and the variation observed could be correlated with drought tolerance. Six mutant cowpea lines were included in a physiological screening experiment that was conducted on greenhouse plants. The data demonstrated that the mutant line 217 performed very well in terms of relative water content, free proline concentration and yield. The yield performance of the mutant lines 447, MA_2 and 217 proved to be outstanding under well-watered conditions, whereas lines 447, 217 and 346 performed well under drought stress conditions.

Keywords: abiotic stress, cowpea, JIP test, mutation technology

Introduction

The most well-known Papilionaceae species with an African origin is *Vigna unguiculata* (L) walp (cowpea) (Schippers, 2002). The large amount of diversity and wild types found in the Niger River basin suggest that the species probably originated there. Cowpea is known under many different names, such as southern pea, black-eyed pea, crowder pea (English); frijol or judia (traditional English); akkerboon (Afrikaans); dinawa (Sotho, Tswana); munawa (Venda); caupies (Sp.); faseol (Gr.); faseolus (L.); fasulia (Arabic); augenbohne, lubia, niebe, coupe, pois a vaches (Cubero 1994; Davis et al., 2000; Duke, 1981; Wiersema, 1999). Cowpea has been used for many years as a source of protein, vitamins and minerals for human consumption, as well as for cattle fodder and green manure. Under favourable environmental conditions the protein content may be as high as 36% in some of the genotypes (Coertze and Venter, 1996). Subsistence farmers in countries such as Nigeria, Niger, Mali and Malawi in Africa and Myanmar in South East Asia are the main producers of cowpea (Fowler, 2000). Cowpea is produced for household purposes and as a cash crop. It is a multipurpose crop, since it is cultivated for leaf and seed yield (Schippers, 2002). In areas facing food insecurity, such as Africa, peasants or small-scale farmers have practised intercropping since old times. Typically, cereal crops such as maize (*Zea mays*), millet (*Pennisetum glaucum*) and sorghum (*Sorghum bicolor*) are dominant crop/plant species, whereas legume crops such as beans (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), groundnut (*Arachis hypogaea*), pigeonpea (*Cajanus cajan*) and soybean (*Glycine max*) are the associated plant species (Tsubo et al., 2003).

Young and tender leaves are prepared as a pot herb in a manner similar to that of spinach. Immature snapped pods are often mixed with other foods. Green cowpea seeds are boiled as a fresh vegetable and may be canned or frozen. Dry, mature seeds are suitable for boiling and canning.

The number of African countries that are classified as water stressed is higher than in any other region of the world and this number is likely to increase as a result of population growth, degradation of watersheds caused by changes in land-use practices, siltation of river basins as well as climate change (Watson et al., 2000). Although cowpea is regarded as drought tolerant, much variation in drought tolerance occurs within the genotype. The challenge in breeding for drought tolerance is to improve yield and productivity by investigating different breeding strategies, and to suggest possible ways of selection for drought conditions (Slabbert et al., 2004). Mutation technology is a way to create variation in the search of a desired trait such as drought tolerance.

The objective of this study was to improve the drought tolerance and yield of this neglected crop to such an extent that it could be used in marginal areas where rainfall is either scarce or unreliable. The selected line(s) would be expected to have the potential to expand the utilisation of the crop in these areas and, thus, be of economic importance for both commercial and subsistence farmers. Seeds of the selected cowpea line, IT93K129-4, were irradiated with gamma radiation, after which the plants were screened for increased drought tolerance and yield. Screening was performed using physiological screening methods as well as phenotypical observations in glasshouse and field trials (Spreeth et al., 2004).

Materials and methods

Plant material

Over the duration of this study various cowpea mutant lines were screened and compared with the control lines received from the

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* To whom all correspondence should be addressed.
☎ +2712 841 9775; fax: +12 808 1499/ +27 12 808 0844;
e-mail: kderonde@arc.agric.za

International Institute of Tropical Agriculture (IITA) in Nigeria. These lines included the control line IT96D-602 (drought tolerant), TVu7778 (susceptible to drought) and a parent line of the mutant IT93K129-4. The latter line was selected for its colour, growth form and yield. It has white to light beige coloured seeds, an upright growth form and good yield. Gamma irradiation was used to obtain a high frequency of gene mutation and chromosomal alterations. Various irradiation dosages between 0 and 300 Gy were applied in order to determine the optimal irradiation dosage of 180 Gy. M_1 (mutant generation 1) plants were cultivated in the field and seeds were harvested for the subsequent M_2 plantings. Each plant was allocated a number that was used for that specific plant and its offspring.

Drought screening of M_2 to M_4 mutants

Various cowpea mutant lines were screened in the field under rain-out shelters and greenhouses, and compared with the control lines received from IITA. A total of 17 000 cowpea seeds were irradiated, using a dosage of 180 Gy Gamma irradiation. M_1 seeds were planted in the field, after which 8 230 M_2 true to type plants, that had survived the irradiation process and yielded seed, were selected. A number of aberrations were observed, including leaf mutation and chlorophyll deficiencies. Some of the M_2 seeds were subsequently planted in wooden boxes in a greenhouse as well as in a rain-out shelter and 487 lines with higher levels of drought tolerance were selected (Table 1). Lines planted in the field were watered for the first 2 weeks to achieve good establishment, after which watering was terminated. During the stress period the plants experienced a severe drought stress due to low rainfall. Seeds were harvested from all plants that survived the stress treatment. Lines planted under rain-out shelters were watered until the plants had germinated and the first trifoliate leaves were fully extended. Subsequently, the plants were not watered for 2 months, at which stage watering resumed, since the leaves were severely wilted. All plants were left to produce seed and the yield of each line was determined.

The wooden box procedure of Singh et al. (1999) was used for screening of M_3 and M_4 mutant seedlings. Selected M_3 and M_4 lines were replanted in wooden boxes in dry land trials in the field and under rain-out shelters. Nine of the best performing lines were selected on the basis of yield in the field, 22 promising lines were identified in the rain-out shelter trials and 36 in the wooden box trials. Out of these the six most drought-tolerant cowpea lines were selected. These lines were evaluated in the greenhouse using physiological parameters**.**

Plants were allowed to germinate and grow for 2 weeks, whereafter water was withheld. Wilting and dying of plants were monitored and recorded until 75% of the plants had reached the permanent wilting stage. Plants were then re-watered and the survival as well as recovery of the plants was noted. By definition the permanent wilting point is the soil water content at which plants do not recover turgor overnight, but will recover if water is applied.

Selected mutant and control plants were planted in pots in a greenhouse and drought stressed at flowering stage, after which various screening methods were used to determine the levels of drought resistance. Measurements were taken every 2nd day for phenotypical observations, combined with biochemical and physiological screening in the laboratory as the stress intensified. Just before the plants reached the permanent wilting stage, they were re-watered and measurements taken 2 to 3 d later in order to determine the recovery potential of the plants.

Physiological evaluation of M_4 mutants

Chlorophyll-a fluorescence transients were measured using a plant efficiency analyser (PEA, Hansatech Ltd.). The fluorescence transients were induced using a red light wavelength of 600 W/m^2 intensity (excitation intensity) provided by 6 light-emitting diodes. Leaves were covered for 1 h, using leaf clips, whereafter measurements were taken using the PEA. During the first second of illumination the following data were recorded: F_m (maximal fluorescence intensity when all the reaction centres (RCs) are closed); F_O (fluorescence intensity at 50 µs when all RC's are open); F_J (fluorescence intensity at 100 µs, 300 µs and 2 ms); F_I (fluorescence intensity at 30 ms); the time taken for t_{Fmax} to reach F_M, and the area between the fluorescence transient and the level of $F_{M.}$ These fluorescence transients were quantified using the Biolizer program. These data were used to calculate the phenomenological and biophysical expressions. The JIP-test refers to the main steps for F_O-J-I-P (Strasser and Strasser, 1995).

The calorimetric method of Bates et al. (1973) was used to determine the proline concentrations of freeze dried leaves. Solution absorbance was determined with a multiscan reader at 520 nm. The proline concentration was then determined using a standard curve and the concentration expressed as µg proline/g dry mass.

Leaves were collected early in the morning to determine relative water content (RWC) according to the method described in De Ronde (2004). Leaf disks were cut and weighed immediately after harvest in order to obtain the fresh weight (W). The samples were re-hydrated by placing of the disks into small glass bottles and the addition of approximately 3 mℓ distilled water, the disks being floated at ambient temperature, approximately 20°C. After 4 h the leaf disks were blot-dried, using towelling paper and weighed to obtain the turgid weight (TW). The samples were then oven dried overnight at 70°C, cooled in a desiccator, and weighed to obtain the dry weight (DW). The RWC was calculated using the following equation:

$$RWC = [(W-DW)/(TW-DW)] \times 100$$

TABLE 1
Number of mutant cowpea plants screened from a number of generations in order to select the six most promising drought tolerant mutant lines

Total number of seeds irradiated	Total number progenies selected	Screened in wooden boxes	Screened in rain out-shelters and field	Subjected to physiological screening
17 000	M_2= 8 230	891	1 239	
	M_3=487	294	190	
	M_4=54	48		6

M = mutant generation

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TABLE 2 Ranking of eight cowpea lines according to the specific and phenomenological fluxes after 24 days without water										
Line	RC/CSo	ABS/CSo	TRo/CSo	ETo/CSo	DIo/CSo	ABS/RC	TRo/RC	ETo/RC	DIo/RC	Ranking
IT96D602	0.829	1.004	0.993	1.546	1.034	1.213	1.198	1.853	1.252	1
IT93K129-4	0.878	1.169	1.076	1.153	1.473	1.335	1.229	1.312	1.683	1
164	0.779	1.079	0.992	1.052	1.390	1.402	1.283	1.337	1.828	5
217	0.868	1.052	0.984	1.101	1.274	1.200	1.128	1.279	1.432	7
346	0.887	0.875	0.897	1.020	0.804	0.990	1.012	1.142	0.914	8
447	0.862	1.062	0.993	1.078	1.302	1.233	1.152	1.253	1.511	5
MA1	0.830	1.017	0.976	1.165	1.115	1.180	1.163	1.400	1.219	4
MA2	0.918	1.185	1.090	1.115	1.520	1.293	1.188	1.211	1.664	3

The root systems of the plants were evaluated using the root architecture box technique developed by Singh et al. (1999). Plants were grown in a flat box in which they were able to develop a two-dimensional root system in order to obtain an impression of root distribution. Once the plants had developed a good rooting system (at approximately 4 weeks), the box was opened, the sandy soil removed by washing and the root system examined. A nail board was used to keep the roots in their original position while the sand was being removed by washing.

Results and discussion

Two types of drought tolerance were observed: In the first type plants produced seed before reaching the permanent wilting stage. Although the plants did not recover after the stress period, viable seeds were produced, thus ensuring a next generation (drought evasion). The other type was to withstand the period of drought stress and being able to recover to such an extent so that the plants were able to produce seed after being re-watered.

Using the chlorophyll fluorescence technique, specific and phenomenological fluxes were compared (Table 2). Of all the selected cowpea lines, line MA_2 had the highest number of reaction centres (RCs) per cross-section (RS_o), as calculated using RC/CS_o. When a lower number of RCs are activated, the remaining RCs have to absorb, trap and transport much more energy in order to have the same output as the RCs of line MA_2. This is evident from the high absorption (ABS) per RC (as calculated using ABS/RC) registered by line 164. When the rate of absorption of photons by the antenna in the cross section (ABS/CS_o) and trapping of electrons (TR) (as calculated using TR/CS) were examined, it was found that line MA_2 also had the highest rate, while line 364 had the lowest rate. Line 364 lost the least energy (DI/CS), while MA_2 lost the most energy. The specific energy fluxes of each individual RC of most of the lines were higher than that of the control line IT96D-602 (Table 2).

Contrary to observations in a number of other crops (Bandurska, 2000), it is the more drought-tolerant cowpea plants that start to produce proline in the latter part of the stress period and at lower concentrations. The proline levels of all of the stressed plants increased over time. However, there were differences in the time of the onset of proline production as well as in the levels of proline production. Lines 217 and IT96D-602 produced the lowest levels, followed by MA_1, 164 and IT93K129-4 (Table 3). The highest proline production was in line 447, followed by 346 and MA_2. In line MA_2 this increase was already visible as from day 21 without water, while the levels in the other lines only increased as from day 24 without water. The ranking of the lines was not only determined by the levels of free proline at the height of the drought stress treatment, but also according to the time at which the proline levels started to increase.

TABLE 3 The ranking of eight cowpea lines according to free proline production after 24 days without water		
Cowpea line or mutant	Free proline concentration after 24 days without water (µg proline/g dry weight)	Ranking
IT96D-602	276.51	2
IT93K129-4	788.75	5
164	690.00	3
217	98.56	1
346	1053.86	6
447	1892.08	8
MA_1	585.97	3
MA_2	1106.76	8

After 12 d without water the plants started to react visibly to the drought stress condition. Some plants started to lose chlorophyll in their lower leaves, while others inclined their leaves away from the sun. The RWC of the stressed plants at this stage was between 80 and 90%. As the stress condition intensified the RWC dropped further and after 24 days without water the RWC of the mutant line 346 was as low as 65%, compared to the 75% of MA_1 and IT96D-602 (Table 4 – see next page).

The plants were ranked according to the number of seeds produced as well as mean seed mass (Table 5). The well watered lines yielding the highest number of seeds were lines 217 (250.8 g), IT93K129-4 (245.3 g), 447 (239.8 g) and 346 (221.3 g). Of all the stressed plants, line 346 produced the highest number of seeds (212.3 g), ranking 6^{th} highest overall, followed by lines 217 (205.8 g), 447 (199.3 g) and IT93K129-4 (188.8 g). Regarding mean seed mass, line IT93K129-4 produced the heaviest seed under well watered conditions (45.9 g), followed by line MA_2 (37.0 g), line 447 (36.9 g) and line 164 (33.1 g). Under drought conditions lines IT93K129-4 (36.4 g), 447 (30.4 g) and 164 (27.8 g) produced the heaviest seed. In terms of total yield, which is the most important parameter, the four highest yielding lines under well-watered conditions were IT93K129-4, 447, MA_2 and 217 (in decreasing order). Under stress conditions the four highest yielding lines were IT93K129-4, 447, 217 and 346 (in decreasing order). Most noteworthy is that lines IT93K129-4 and 447 ranked first and second under both well-watered and stress conditions. The ideal plant for subsistence farmers would be one that produces good yields under all conditions, such as lines IT93K129-4 and 447 (Table 5). In contrast line MA_2

TABLE 4
The percentage relative water content (RWC) of eight cowpea lines as determined at the height of the drought stress, as well as after rewatering

Cowpea line	24th day without water		Recovery after rewatering		Ranking
	Control	Stress	Control	Stress	
IT96D-602	87.95	74.15	89.44	85.43	2
IT93K129-4	92.26	69.51	91.45	87.57	5
164	89.18	72.12	91.73	86.70	2
217	92.45	73.60	92.82	89.37	2
346	91.08	65.27	90.79	88.00	7
447	90.52	70.62	90.02	89.47	5
MA_1	91.61	76.98	90.44	89.03	1
MA_2	90.14	70.90	92.27	87.22	7

TABLE 5
The yield of eight cowpea lines under drought stress and control conditions. The plants were ranked according to the mean number of seeds produced per plant, mean seed mass per plant as well as mean yield.

Cowpea line	Treatment	Mean number of seeds			Mean seed mass (g)			Mean yield		
		Number	Ranking		Mass (g)	Ranking		Mass (g)	Ranking	
			Overall	W = well watered S = stress		Overall	W = well watered S = stress		Overall	W = well watered S = stress
IT96D-602	Well watered	88.5	15	W8	17.3	15	W8	1531.05	15	W8
	Stress	108.8	14	S7	22.6	14	S7	2458.88	14	S7
IT93K129-4	Well watered	245.3	2	W2	45.9	1	W1	11259.27	1	W1
	Stress	188.8	9	S4	36.4	4	S1	6872.32	5	S1
164	Well watered	178.3	11	W7	33.1	5	W4	5901.73	7	W5
	Stress	146.8	13	S6	27.8	9	S3	4081.04	13	S6
217	Well watered	250.8	1	W1	29.5	8	W6	7398.6	4	W4
	Stress	205.8	7	S2	25.0	11	S5	5145	10	S3
346	Well watered	221.3	4	W4	24.5	12	W7	5421.85	9	W7
	Stress	212.3	6	S1	24.2	13	S6	5137.66	11	S4
447	Well watered	239.8	3	W3	36.9	3	W3	8848.62	2	W2
	Stress	199.3	8	S3	30.4	7	S2	6058.72	6	S2
MA_1	Well watered	181.0	10	W6	31.1	6	W5	5629.1	8	W6
	Stress	158.0	12	S5	27.7	10	S4	4376.6	12	S5
MA_2	Well watered	214.0	5	W5	37.0	2	W2	7918	3	W3
	Stress	80.5	16	S8	14.8	16	S8	1191.4	16	S8

performed well under well-watered conditions (ranked 3rd), but worst of all lines under stress conditions (ranked 16 overall and 8th under stressed conditions). In view of its drastic reduction in yield (85%) due to drought stress, compared with its yield under well-watered conditions, MA_2 can be classified as being extremely sensitive to drought stress. The so-called drought tolerant line IT96D-602 performed second worst under stress conditions (ranked 7th under stress conditions in terms of all three parameters) and performed even worse under well-watered conditions than under stress conditions (8th), its overall rankings for well-watered and stress conditions being 15 and 14 respectively (Table 5). Because it did not suffer any yield decrease due to drought stress, it is technically correct to classify IT96D-602 as extremely drought tolerant. Due to its very poor overall yield performance, such line can however never be recommended to farmers on the basis of its drought tolerance.

When the root architecture of drought tolerant (IT96D-602) and susceptible (TVu7778) lines were examined, it became clear that there were marked differences in the distribution of the roots, but not in total root length (Table 6). The drought tolerant IT96D-602 had much more roots in the subsoil (deeper than 25 cm) than in the topsoil. For the drought sensitive Tvu7778 the reverse was true. This difference is very clearly illustrated in Figure 1. The distribution of the roots of the mutant plants 217 and 164 was more similar to that of the drought tolerant control line, IT96D-602, than to that of the susceptible line (TVu7778) (Table 6). Although the root distribution patterns were the same, the mutant plants had bigger root systems than line IT96D-602, especially in the subsoil. The root system plays an important role in the uptake of soil water, and for cowpeas the principle of maximum uptake and minimum loss can be applied (Slabbert et al., 2004). A strong, deep root system that can utilize the plant-available water in subsoils efficiently to great depths is very important in this regard.

Conclusions

The aim of this study was to use mutation breeding as a tool to improve the drought tolerance of cowpea lines, having favourable traits, to such an extent so that these plants can survive and

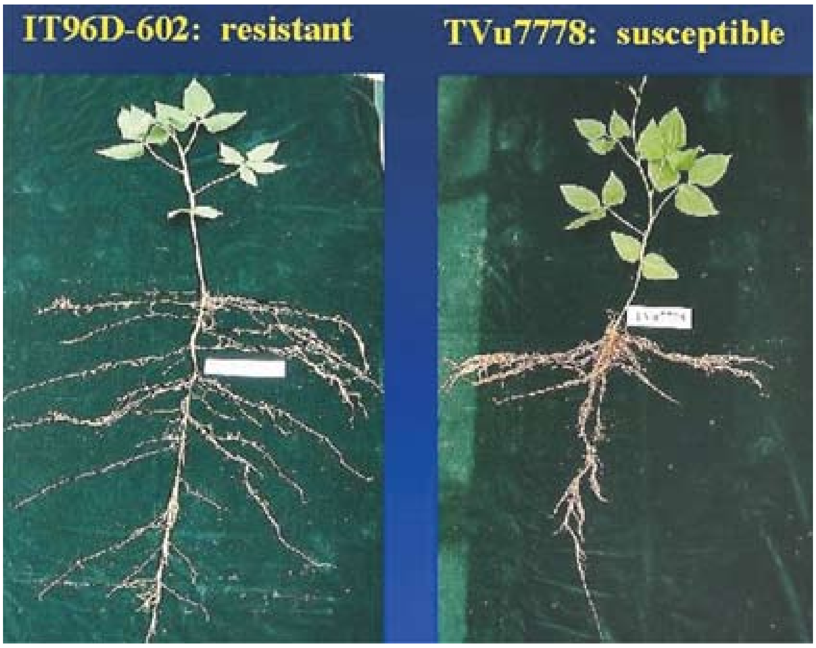

Figure 1
Root patterns of the cowpea drought tolerant control line IT96D-602 and the more susceptible line TVu7778

TABLE 6
Root lengths of four cowpea lines and mutants in the topsoil, subsoil (below 25 cm) and total soil profile

Line	Length		Total length
	Top 25 cm	Below 25 cm	
IT96D-602	452 mm	586 mm	1038 mm
TVu7778	557 mm	475 mm	1032 mm
Mutant no. 217	519 mm	676 mm	1195 mm
Mutant no. 164	520 mm	659 mm	1179 mm

produce good yields even under adverse environmental conditions. Results indicated that five mutant cowpea lines exhibited drought tolerance levels similar to or better than that of the parent line, in terms of percentage yield reduction under drought stress conditions. One mutant line (MA_2) was highly sensitive to drought stress. All the mutant lines gave lower yields than the parent line (Table 5). The yield performance of the mutant lines 447, MA_2 and 217 proved to compare fairly to moderately well with that of the parent line under well watered conditions, giving yields of 79%, 70% and 66% of that of the parent line respectively. Under drought stress conditions mutant lines 447, 217 and 346 compared best with the parent line, giving yields of 88%, 75% and 75% of that of the parent line respectively. Mutant line 217 also performed very well in terms of RWC and free proline concentration. Using mutation technology, we have, thus, succeeded in obtaining drought tolerant lines giving relatively good yields under drought stress conditions and having favourable traits. These have the potential to be tested for adaptation to local environmental conditions in rigorous statically designed experiments.

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