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Full Length Research paper

Screening cowpea [*Vigna unguiculata* (L.) Walp.] varieties by inducing water deficit and RAPD analyses

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The effects of water deficit induced by polyethylene glycol-6000 on some cowpea varieties, which belong to the national germplasm in Senegal are reported. Our results showed that, the length of the epicotyl was not affected by water deficit but the length of primary root was influenced only in Mouride variety. Water deficit influenced mostly the number of lateral roots. The 985 variety showed a great increase of its lateral root numbers and could be considered a drought tolerant variety. In contrast, the IT81D-1137 variety is very sensitive to water deficit because its lateral root number were reduced 3.8 fold compared to the control. These physiological studies were complemented by analyzing the genetic diversity of these varieties with random amplified polymorphic DNA (RAPD). The RAPD analysis suggested that the samples were also genetically diverse.

Key words: *Vigna unguiculata*, drought tolerance, PEG, RAPD.

INTRODUCTION

Cowpea, *Vigna unguiculata* (L.) Walp., is an essential crop in developing countries of Africa, Asia and Latin America where it is consumed as dry seeds, fresh green pods or leaves. Because of its high proteins, vitamins and minerals contain, cowpea plays an important role in human consumption and animal feeding (Singh et al., 1997; Nielsen et al., 1997). However, cowpea is relatively sensitive to soil water deficit. Depending on timing and the magnitude of the water deficit, cowpea responds by stomatal regulation of water loss, leaf area reduction, hastening or delaying its reproductive cycle or by developing a deep root system (Gwathmey and Hall, 1992).

Presently only a few studies have been reported in the selection of cowpea varieties on the basis of their tolerance to water stress. The first strategy developed to screen cowpea varieties according their tolerance to water stress was based on the assessment of shoot dry matter and leaf area under well-watered and drought treatments in field conditions. These studies allow to identify cowpea varieties showing tolerance to water stress (Hall and Grantz, 1981; Gwathmey and Hall, 1992). The second strategy was based on the intrinsic water-use efficiency, which should be associated with differences in the extent to which C_3 plants discriminate against ^{13}C (carbon isotope) compared with ^{12}C (Δ) during CO_2 fixation. Measurements of plant composition of stable isotopes ($^{13}C/^{12}C$) in field conditions suggest a correlation between Δ and genotype in cowpea (Hall et al., 1990; Hall et al., 1994).

These strategies developed in order to screen cowpea varieties tolerant to water stress are very difficult to apply in field conditions and they are time consuming. To overcome these problems, we developed an *in vitro* technique using polyethylene glycol (PEG) to induce water stress. PEG is described as a non-ionic water-soluble polymers, which is not excepted to penetrate intact plant tissues rapidly and is widely used to induce

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Abbreviations: PEG, Polyethylen glycol; RAPD, Random Amplified Polymorphic DNA; ANOVA, Analysis of variance.

Table 1. Effects of water deficit induced by polyethylene glycol on six cowpea varieties. The values followed by the same letter do not differ significantly for the same variety in different concentrations of PEG at P=0.05 (Table have to be read by line). Means±SE (n=24).

Varieties	Length of the primary root (cm)				Number of lateral roots			
	Control	6.9 g/l	13.75 g/l	27.5 g/l	Control	6.9 g/l	13.75 g/l	27.5 g/l
977	7.83 ± 0.67a	7.26 ± 0.57a	7.54 ± 0.60a	4.94 ± 0.85a	39.79 ± 4.76a	34.54 ± 2.35ab	0.83 ± 3.12ac	28.29 ± 4.47d
985	4.62 ± 0.73a	4.15 ± 0.77a	4.64 ± 0.70a	5.59 ± 0.68a	13.87 ± 3.50a	14.04 ± 3.49a	5.50 ± 3.21ab	20.21 ± 3.81b
58-74	5.19 ± 0.57a	4.47 ±.0.70a	6.93 ± 0.26a	5.77 ± 0.56a	15.25 ± 2.35a	16.12 ± 2.82ab	6.37 ± 1.78c	19.37 ± 2.59b
IT81D1137	8.72 ± 0.95a	5.48 ± 0.86a	6.06 ± 0.91a	4.01 ± 0.83a	43.33 ± 4.04a	23.29 ± 4.30b	1.25 ± 5.98c	11.37 ± 3.36d
ISRA 819	2.86 ± 0.57a	3.85 ± 0.63a	2.88 ± 0.56a	3.49 ± 0.57a	6.21 ± 1.99a	7.79 ± 2.03ab	.17 ± 1.61a	9.91 ± 2.28b
Mouride	6.28 ± 0.30a	5.08 ± 0.20ab	4.81 ± 0.23ab	4.14 ± 0.25b	22.17 ± 1.15a	16.37 ± 1.05b	7.75 ± 1.05b	17.21 ± 1.19b

water stress in higher plants (Nepomuceno et al., 1998). Our objective is to use the PEG technique to quickly screen cowpea varieties on the basis of their drought tolerance and assessment for DNA polymorphism.

MATERIALS AND METHODS

Plants *in vitro* culture

Cowpea seeds were sterilized in 1% mercuric chloride for 8 min and washed with sterile distilled water 3 times. They were sterilized a second time in sodium hypochloride at 8°C for 2 min and washed 3 times with sterile distilled water. After removing the teguments, the seeds were pre-germinated in a bottle containing 8 g/l of agar (Sigma) and incubated in a dark oven at 28°C. After 24 h of pre-germination, the seeds were transferred in test tubes containing different concentrations of PEG solidified with 8 g/l of agar and grown in a greenhouse with a photoperiod of 12h, 20 w/m2 at 34°C. After 4 days, the length of the epicotyl, the length of the primary root and the number of the lateral roots were measured.

Statistical analysis

Each treatment consisted of 24 test tubes with 1 plant per tube. Position of the tubes were randomized to minimize the positional effects in the grow chamber. Analysis of variance was conducted on the six varieties in water stressed and control treatments by using ANOVA.

Cowpea genotyping

DNA was extracted from the six cowpea varieties from senegalese national germplasm (Table1) according to Fulton et al. (1995). PCR amplifications were performed in a 0.2 ml tube containing 2 mM $MgCl_2$, 1 µM of each primer (operons A, B, F, N16 of Kit N, O15 of Kit O, Genosphere Biotechnologies and N4, N6, Oligo Express), 200 µM of each dNTPs (Promega), 1.5 U of Taq (Amersham Biosciences) and 25 ng of genomic DNA in a final volume of 25 µl. Sixty four primers were used in this study. DNA sequences were amplified using a PTC-100 thermocycler (MJ Research, Inc) with the following parameters: a predenaturing step of 3 min at 94°C followed by 45 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C and a final extension of 10 min at 72°C. After amplification, 5 µl of loading buffer was added to each sample and separated by electrophoresis at 120 V for 4 h on a 1.8% agarose gel (Sigma). After staining for 30 min with 1 mg/l of ethidium bromide, the gel was exposed to UV light and images were photographed using BIO-CaptMW (Vilber Lourmat, France) software.

Determination of glutamine synthetase activity

Glutamine synthetase (GS) was extracted from 1 g of lyophized leaves of stressed plant in a mortar containing Fontainebleau sand and 5 ml of buffer [25 mM Tris-HCl (pH 7,6), 1 mM $MgCl_2$,7H_2O, 14 mM ß-mercaptoethanol, 500 mM EDTA and 10 mg polyvinylpolypyrrolidone (PVP)] at 4°C. The extract was centrifuged at 15 000 rpm for 20 min at 4°C. In 200 µl of the supernatant collected in a sterile Eppendorf tube, was added 100 µl of mix solution [150 mM MgSO4,7H_2O, 470 mM glutamate, 50 mM NH_2OH.HCl, 30 mM EDTA], 100 µl of ATP 8 mM and 750 µl of buffer containing 25 mM Tris-HCl (7.6) and 1 mM $MgCl_2$.7H_2O. The samples were incubated at 30°C for 30 min. The reaction was stopped by adding 750 µl of stop solution [370mM $FeCl_3$, 200 mM TCA and 660 mM HCl] and centrifuged at 15 000 rpm at 4°C for 5 min and GS activity was measured spectrophotometrically (540 nm).

RESULTS

Drought tolerance assessment among cowpea varieties

The statistical analysis of the results showed that the PEG concentration did not affect the length of the epicotyl (data not shown) and the primary root of the varieties except Mouride, which had a significant reduction of the length of its primary root (Table 1). The reduction of the number of lateral roots was between 1.3 and 3.8 fold lower than the control for 977, IT81D-1137 and Mouride varieties. In contrast, the number of the lateral roots increases for the 985, 58-74 and ISRA 819 varieties (Table 1). Mouride and IT81D-1137 were sensitive to water deficit at 6.9 g/l of PEG. Varieties 977, 985, and ISRA819 were only sensitive to water deficit at 27.5 g/l of PEG. The effects of PEG on varieties 985 and IT81D-1137 is depicted in Figure 1. While the glutamine synthetase activity in IT81D-1137 and 985 with or without PEG is shown in Figure 2.

Table 2 showed a comparison between the varieties for each concentration of PEG. The significant difference

Table 2. Varietal effects of water deficit on the length of the primary root and the number of lateral roots.The values followed by the same letter do not differ significantly between species at P=0.05 (Table have to be read by column). Means ± SE (n=24).

Varieties	Length of the primary root (cm)				Number of lateral roots			
	Control	6.9 g/l	13.75 g/l	27.5 g/l	Control	6.9 g/l	13.75 g/l	27.5 g/l
977	7.83 ± 0.67bcd	7.26 ± 0.57b	7.54 ± 0.60a	4.94 ± 0.85ab	39.79 ± 4.76d	34.54 ± 2.35d	40.83 ± 3.12d	28.29 ± 4.47d
985	4.62 ± 0.73a	4.15 ± 0.77a	4.64 ± 0.70bd	5.59 ± 0.68ac	13.87 ± 3.50bc	14.04 ± 3.49ac	15.50 ± 3.21b	20.21 ± 3.81cd
58-74	5.19 ± 0.57a	4.47 ±.0.70a	6.93 ± 0.26a	5.77 ± 0.56a	15.25 ± 2.35ab	16.12 ± 2.82a	26.37 ± 1.78a	19.37 ± 2.59bc
IT81D1137	8.72 ± 0.95d	5.48 ± 0.86ac	6.06 ± 0.91ad	4.01 ± 0.83bcd	43.33 ± 4.04d	23.29 ± 4.30ab	31.25 ± 5.98a	11.37 ± 3.36ab
ISRA 819	2.86 ± 0.57e	3.85 ± 0.63a	2.88 ± 0.56c	3.49 ± 0.57bd	6.21 ± 1.99c	7.79 ± 2.03c	5.17 ± 1.61c	9.91 ± 2.28a
Mouride	6.28 ± 0.30ac	5.08 ± 0.20ac	4.81 ± 0.23bd	4.14 ± 0.25ad	22.17 ± 1.15a	16.37 ± 1.05a	17.75 ± 1.05b	17.21 ± 1.19ac

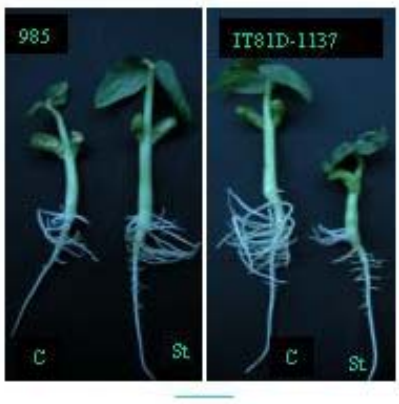

Figure 1. Effect of water stress induced by PEG on two cowpea varieties (985 and IT81D-1137) after 4 days. C: Controlled, non-stressed plants. St: Stressed plants with 27.5 g/l of PEG. Bar=2 cm.

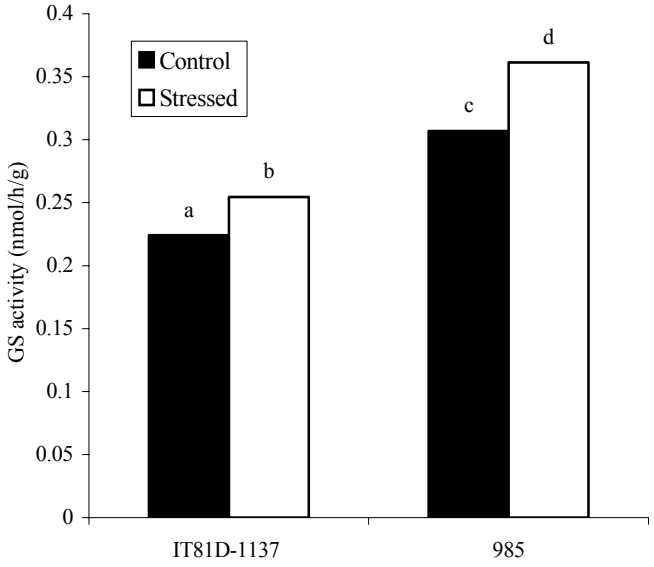

Figure 2. Effect of water deficit on glutamine synthetase activity. Means with the same letters are not statistically different at 5% level according Newman and Keuls test.

observed between plants in term of the length of the primary root and the number of the lateral roots resulted from the specific effects of the varieties. The results also indicated that the IT81D-1137 and 977 varieties produced more secondary roots in a medium without PEG but the number decreased considerably for IT81D-1137 when 6.9 g/l of PEG were added. There was no significant difference of the number of lateral roots for IT81D-1137, ISRA819 and Mouride at 27.5 g/l of PEG (Table 2).

Fingerprint patterns

Sixty four 10-mer primers were tested for their capacity to identify polymorphism between the six cowpea varieties. Two primers gave either no bands but one gave very complex banding patterns, these primers were not included in this study. Of the remaining, 17 (26.5%) were found informative. These primers generated a total of 682 scorable markers (bands) and 72 (11%) of them were polymorphics. The non polymorphics markers i.e. present in all the six varieties were not considered. Figure 3 illustrates the amplification pattern obtained with A12 and O15 primers.

DISCUSSION

To our knowledge, this is the first work aimed at selecting drought tolerant cowpea varieties by using PEG. Most previous studies have relied on carbon isotope method in field conditions (Hall et al., 1990; Hall et al., 1994). Such technique is time consuming and unable to estimate adequately the root system, which is one of the strategies developed by certain plant species to tolerate drought. The pattern of analysis developed in this study, which has also been used in many plant species, allows a quick selection of drought tolerant variety, and their differentiation by DNA polymorphism.

A wide range of concentrations of PEG-6000, 6.9 g/l to 82.5 g/l was initially tested. At 82.5 g/l of PEG, cowpea seeds could not germinate and this concentration was not

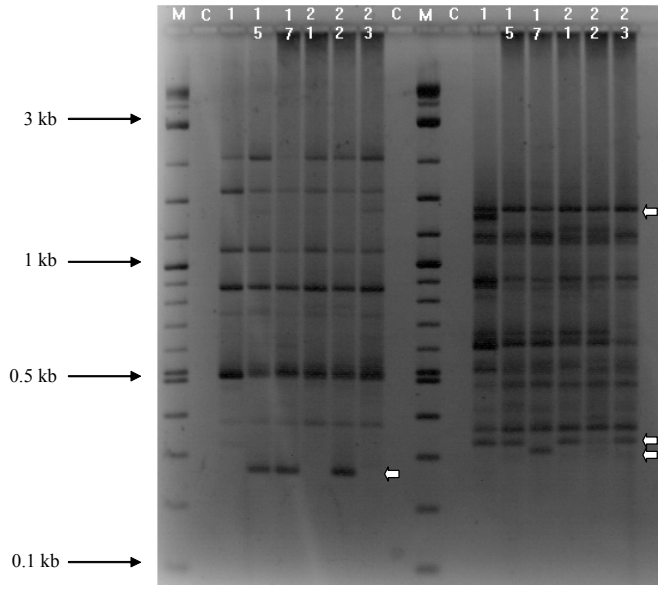

Figure 3. DNA banding pattern by A12 (left) and O15 (right) primers in six cowpea varieties. M: Molecular size markers (2-Log DNA Ladder), Biolabs, New England] C: Control, amplification without DNA; 1: 58-74; 15: Mouride; 17: IT81D-1137; 21: 977; 22: 985; 23: ISRA819.

included in subsequent experiments. Most of the varieties were sensitive to water deficit at 27.5 g/l of PEG and this concentration was considered as sufficient to induce stress for the plants. Our results suggest that cowpea drought tolerance can be quickly determined in early stage of development by using PEG. This allows for the assessment of the number of roots, which is one of the strategy developed by drought tolerant plants for exploring a wide range of soil surface. Similar method has been used in many cultivated plants like cotton in order to select the best tolerant varieties to water deficit (Nepomuceno et al., 1998).

To overcome the negative effect of PEG-6000 on plants chlorophyll content a short (4 days) application of PEG was considered. Phenotype observations showed that there was no difference between the control and stressed plants in term of color suggesting that PEG should not affect chlorophyll content during this period. This procedure was easier than the one developed by Plaut and Federman (1985) which recycles the nutrient solution containing PEG-6000 to avoid its negative effects (Ranjbafordoei et al., 2000).

Statistical analyses showed that the most interesting variety was 985, which increased the number of its lateral roots 1.4 fold compared to the control and could be considered as a tolerant variety to water deficit. The high GS activity observed in the 985 variety could be linked to the fact that the chloroplastic GS did have sufficient energy (ferredoxin, ATP) from photosynthesis because of the higher level of chlorophyll content (Kar and Feierabend, 1984). This result suggests that 985 variety should be evaluated by the farmers in the Sahelian regions where the shortage of the rainfall is a real problem. This variety offers a great advantage because it has big seeds (27 g for 100 seeds) and its productivity is around 1,992 kg per ha in wet conditions. However, IT81D-1137 variety was sensitive to water deficit because the number of its lateral roots decreased at 3.8 fold compared to the control when the nutrient medium contains 27.5 g/l of PEG (Table 1).

The low level of polymorphism (11%) observed in the RAPD analysis were similar to other results reported on cowpea (Li et al., 2001; Tosti and Negri, 2002; Fall et al., 2003; Diouf and Hilu, 2004). Our data showed that the drought tolerant cowpea variety 985, was genetically different to the sensitive variety IT81D-1137 suggesting possibility of DNA products controlling this phenomenon. The development of the root system in response to water deficit also suggests that the expression of certain genes controlling root formation is stimulated by drought conditions. Our technique will be also helpful for selecting a drought tolerant variety for the Sahelian regions where water availability is the main constraint limiting production as well as in the genetic identification of plant parents in breeding programs.

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