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Genetic diversity in cowpea [*Vigna unguiculata* (L.) Walp.] varieties determined by ARA and RAPD techniques

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Cowpea, *Vigna unguiculata* (L.) Walp. presents phenotypical variabilities and in order to study the genetic diversity of cultivated Senegalese varieties, two experimental approaches were used. First, a physiological characterization based on nitrogen fixation was used to assess cowpea breeding lines. Inoculation with two *Bradyrhizobium* strains (NGR234 and ISRA312), showed a difference in nitrogen fixation potential between the cowpea varieties. Diongoma is the highest nitrogen fixing variety, whereas Mouride is the lowest. The second approach employed genetic characterization based on DNA polymorphism to screen. Results suggest that random amplified polymorphic DNA (RAPD) technology can be used to reorganize the national germplasm in order to eliminate the putative duplicates, and to identify elite varieties.

Key words: *Vigna unguiculata,* nitrogen fixation, cowpea, molecular markers, RAPD.

INTRODUCTION

Cowpea, *Vigna unguiculata* (L.) Walp. is a tropical grain legume which plays an important nutritional role in developing countries of the tropics and subtropics, especially in sub-saharan Africa, Asia, Central and South America (Singh et al., 1997). Because of its high protein content (20-25%), cowpea has been referred to as “poor man’s meat.” Cowpea young leaves, pods and peas contain vitamins and minerals which have fuelled its usage for human consumption and animal feeding (Nielsen et al., 1997; Rachie, 1985). Despite its importance, the production of cowpea which is about 1000 kg/ha in Sub-saharian regions, does not meet the need of consumers (Ehlers and Hall, 1997). The low yield is the result of poor soil, particularly in nitrogen and the high cost of chemical fertilizers. However, cowpea establishes symbiotic association with *Bradyrhizobium* bacteria enabling it to fix atmospheric nitrogen. Nevertheless, breeders and agronomists have not given high priority to cowpea varieties with high nitrogen fixing potential in breeding program in order to improve production and maintain soil fertility. Attempts have, however, been made to screen for a cowpea variety with higher nitrogen fixing potential (Gueye and Ndiaye, unpublished).

The objective of our work is to identify cowpea varieties with high nitrogen fixing potential, to verify the pattern of variation within cowpea breeding lines in Senegal, and to evaluate the applicability of ARA and RAPD techniques in the screening of cowpea varieties. The highest nitrogen fixing varieties will be selected for subsequent breeding programs.

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Abbreviations: ARA, Acetylene Reduction Activity; RAPD, Random Amplified Polymorphic DNA; AFLP, Amplified Fragment Length Polymorphism.

MATERIALS AND METHODS

In vitro culture, plant nodulation and nitrogen fixation assessment

Cowpea seeds were sterilized with 70% alcohol for 15 min and washed 3 times with sterile distilled water. Following sterilization, the seeds were imbibed for 3 h with sterilized distilled water and germinated in Petri dish containing 8 g/l of Agar-Agar (Sigma) for 48 h in the dark at 30ºC. Cowpea were grown in a greenhouse with a photoperiod of 12 h, 20 w/m^2 at 28ºC in Leonard apparatus containing sterilized sand from Cambérène. The plants were watered daily with Jensen medium (Vincent, 1970). In order to assess their nitrogen fixation, the cowpea plants were inoculated with fresh cultures of *Bradyrhizobium* strains, NGR234 and ISRA312. NGR234 is a promiscuous strain because it nodulates many legumes, whereas ISRA312 is only effective on *V. unguiculata* (Léwin et al., 1987). After 5 weeks of growing, nodules were harvested, the aboveground parts of plant weighed, and nitrogen fixation assessed by ARA method according the protocol described by Hardy et al. (1973).

Molecular characterization of cowpea with RAPD

DNA was extracted from leaves of seven cowpea varieties (Table 1) from the Senegalese national germplasm 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, 200 μM of each dNTP, 1.5 U of lyophilized Taq (Amersham Pharmacia Biotech) and 25 ng of genomic DNA in a final volume of 25 μl. Forty four primers (Genosphere Biotechnologies) were used in this study. The reaction were placed in a PTC-100 thermocycler (MJ Research Inc.) programmed for predenaturating step of 3 min at 94°C followed by 45 cycles of 1 min 94°C, 1 min at 36°C, 2 min at 72°C and a final extension of 5 min at 72°C. Amplification products were electrophoresed on 1.8% agarose gel, stained for 30 min with 1 mg/l of ethidium bromide and photographed under UV light.

RESULTS

Physiological genotyping of cowpea

The effect of cowpea varieties on dry weight of aboveground parts and nodules, as well as the number of nodules are presented in Table 1. Ndiambour and Diongoma varieties have the highest dry weight of the aboveground parts, while Mouride had the lowest. The number of nodule per plant and their dry weight also differed between the cowpea varieties. Table 1 also showed that the varieties with low number of nodules have higher nodule dry weight. We also observed that nodulation by ISRA312 was more profuse than that of NGR234 (data not shown). There were also differences in nitrogen fixation between the cowpea varieties when inoculated with the two *Bradyrhizobium* strains (Table 2). Diongoma variety has the highest nitrogen fixation (with ISRA312), while Mouride has the lowest.

Table 1. The main effect of cowpea varieties on the dry weights of aboveground parts and nodules, and nodule numbers.

Varieties	Dry weight of aboveground parts (g)	Numbers of nodules	Dry weight of nodules (g)
Diongoma	1.092 a	27 a	0.0145 c
CB5	0.652 ab	27 a	0.0194 c
Melakh	0.866 ab	27 a	0.0141 c
Bambey21	0.696 ab	30 a	0.0198 c
Mougne	0.488 b	15 b	0.0559 b
Ndiambour	1.170 a	20 ab	0.0982 a
Mouride	0.487 b	14 b	0.0187 c

The values followed by the same letter do not differ significantly at P= 0.05

Molecular genotyping of cowpea varieties

Forty-four primers were screened with DNA samples from seven cowpea varieties. Of these, ten primers did not amplify the DNA; nine amplified but did not show any discrimination, while 25 gave polymorphic RAPD patterns. Eleven (A1, A13, A14, B6, B10, B11, B12, B15, F4, F13, F16) of the 25 polymorphic primers were selected because they gave very clear bands. In total, 331 bands (amplification products) were produced by the PCR reactions. Sixty-one of the bands were polymorphic (Figure 1).

Table 2. Effect of cowpea varieties (*V. unguiculata*) x *Bradyrhizobium* strain interaction on ARA.

Varieties	*Bradyrhizobium*	ARA/plante (μmol C_2H_4/h/plant)
Diongoma	NGR 234	11.550 bc
	ISRA 312	18.716 a
CB5	NGR 234	11.74 bc
	ISRA 312	13,97 ab
Melakh	NGR 234	10,79 bc
	ISRA 312	11,59 bc
Bambey21	NGR 234	10,89 bc
	ISRA 312	11,30 bc
Mougne	NGR 234	14,912 ab
	ISRA 312	10,80 bc
Ndiambour	NGR 234	5,67 cd
	ISRA 312	4,23 d
Mouride	NGR 234	1,60 d
	ISRA 312	1,85 d

The values followed by the same letter do not differ significantly at P= 0.05

DISCUSSION

Assessment of nitrogen fixation in cowpea has been conducted in some Nigerian and Senegalese varieties (Gueye, 1982). However, varieties which are mainly cultivated in Diourbel, Thiès, Louga and Saint-Louis (North West regions of Senegal) where the lands are poor, were not represented in this study. The main objective of our study is to identify a better cowpea variety-*Bradyrhizobium* strain symbiotic combination for improving the yield of the farmer and soil fertility. Our results confirmed that biological nitrogen fixation is dependent on both the plant and the nodulating bacteria, as have been reported in other studies (Gueye and Bordeleau, 1988; Kishinevsky and Zur, 1997). Successful inoculation of the cowpea varieties with ISRA312 and NGR234 indicates that cowpea could be nodulated by more than one *Bradyrhizobium* strain. In general, ISRA312 induced more nodules and fixed more nitrogen than NGR234. Two main varieties, Diongoma having a higher nitrogen fixation, and Mouride which is a much lower nitrogen fixing variety, were identified in this study.

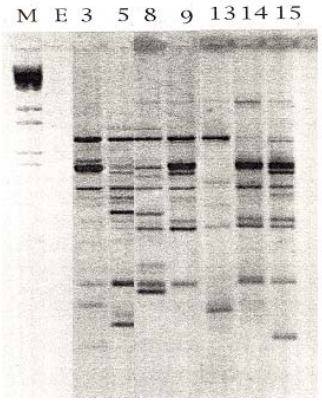

Figure 1. RAPD profiles of cowpea varieties generated with F4 primer.
M: Ladder, λ DNA digested by BstEII; E: Control (no DNA); 3: Diongoma; 5: CB5; 8: Melakh; 9: Bambey 21; 13: Mougne; 14: Ndiambour; 15: Mouride.

Variation in nitrogen fixation has also been reported in other legumes such as *Faidherbia albida* (Gueye et al., 1997; Sanginga et al., 1990). Other studies using ^{15}N in both semi-controlled and field conditions also indicate that Diongoma belongs to the group of higher nitrogen fixing cowpea varieties (Gueye M., unpublished). This difference in nitrogen fixation may be due to the genetic framework of the different varieties.

Physiological assessment is not sufficient for adequate characterization of plant varieties because nitrogen fixation can be influenced by many environment factors (Antolin et al., 1995). For these reasons, molecular markers offering more valid results were used in our studies in order to improve the physiological approach. Previous studies using alloenzymes, RAPD and microsatellites, indicate a low level of variation in cowpea (Li et al., 2001; Pasquet, 2000; Tosti and Negri, 2002). However, high level of polymorphism has been observed in lentil, soybean and cowpea using AFLP technique (Coulibaly et al., 2002; Maughan et al., 1996; Sharma et al., 1996). In addition, a high level of polymorphism was observed in *Phaseolus* using RAPD (Bai et al., 1998). In addition to physiological assessment, our data showed that some DNA fragments could be specific to the higher or lower nitrogen fixing varieties suggesting that some genes could govern the higher nitrogen fixation character in cowpea. Our findings also provide an alternative avenue for understanding the biological nitrogen fixation process and the genetic identification of parent plants in a breeding program.

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