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Full Length Research Paper

Determination of effective nodulation in early juvenile soybean plants for genetic and biotechnology studies

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Symbiotic fixation of atmospheric nitrogen (N_2) is a complex physiological process influenced by the interaction of genetic elements in the higher plant species and rhizobia. No standardized, efficient method is available to critically examine the effect of altering the genetic elements in either component by selection and/or genetic engineering. At planting, seeds of a tropical ('TGX-4E') and non-tropical ('Soma') soybean cultivar were inoculated individually in sand-filled Conetainers® in a greenhouse with each of two strains within two rhizobial types (*Bradyrhizobium japonicum* and cowpea). Six weeks after inoculation, each plant was classified into one of two categories; vigorous plant with dark green leaves indicating effective nodulation and N_2-fixation (+), and stunted plant with chlorotic yellow leaves indicating ineffective nodulation and no N_2-fixation (-). The results indicated that this non-destructive method could be used to identify major genetic differences in the soybean and inoculant. Therefore, this method could be used to rapidly identify genetic segregants resulting from selection in plant breeding programs and/or genetic engineering.

Key words: effective nodulation, rhizobia, tropical soybean type, symbiosis.

INTRODUCTION

The formation of effective (functional) nodules in soybean [*Glycine max* (L.) *Merr*.] when inoculated with compatible rhizobia leads to fixation of atmospheric nitrogen (N_2) making nitrogenous fertilization of the soybean unnecessary. Symbiotic N_2-fixation is a complex physiological process influenced by the interaction of genetic elements in the higher plant species and rhizobia.

Genetic variation for N_2-fixation ability has been reported involving both the soybean and rhizobium components of the symbiotic association (Hungria and Bohrer, 2000; Sanginga et al., 2000; Sinclair et al., 1991; Pulver et al., 1982). Currently, there is no reliable, simple and nondestructive method to identify N_2-fixation. Various methods, including acetylene reduction (Denison et al., 1983) and xylem ureide assay (McClure et al., 1980) that involve sophisticated and expensive equipment have been used for determining N_2-fixation. Destructive

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methods for the determination of shoot dry weight, which has a positive relationship to N_2-fixation ability (Neuhausen et al., 1988), have been reported.

This study reports a rapid, inexpensive and non-destructive method to screen soybean -rhizobium combinations which have improved N_2-fixation ability and have been generated by either plant breeding selection programs and/or genetic engineering.

MATERIALS AND METHODS

Genetic material

Pathogen-free seeds of two soybean cultivars ('Tropical *G. max* -4E' = TGX -4E and non-tropical 'Soma') were obtained from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria and the Crop Breeding Institute (CBI), Harare, Zimbabwe, respectively.

Two rhizobial strains of *Bradyrhizobium japonicum* (designated I and II) and cowpea type (designated III and IV), were used in the study. The *B. japonicum* strains were obtained from a commercial outlet in Gainesville, Florida and the cowpea-type strains were obtained from Dr. P. Singleton at NifTAL, Hawaii, USA. Each strain was maintained in the laboratory by sub-culturing periodically (every 60 days) on yeast-mannitol agar (YMA) growth medium described by Vincent (1970). The cultures were streaked directly on YMA plates (15.0 x 100.0 mm) using the method described by Somasegaran and Hoben (1994) and incubated at room temperature.

Inoculum was prepared by excising (under sterile conditions in the laboratory) two pieces of agar (20 x 20 mm) supporting rhizobial colonies from the subculturing dishes that were then placed in bottles containing 300 ml sterile distilled water. Two drops of the surfactant Tween® 80 (polyoxyethylene sorbitan monooleate) were added to each bottle before shaking vigorously to disperse the rhizobial cells. Each bottle was wrapped with aluminum foil to protect rhizobia from inactivation by UV light.

Planting procedure and evaluation of N_2-fixation

The planting procedure involved placing one seed in a hole (1 cm deep) in the center of a Conetainer® (Stuewe and Son, Inc., USA) measuring 4 cm in diameter x 20.5 cm in length filled with moist sterile washed sand. After adding inoculum (0.1 ml), each seed was immediately covered with the sand. The resulting plants were grown in the greenhouse with day/night temperatures of 28/20^{o}C without supplemental light in Gainesville, Florida, USA.

One week after emergence 0.1 g of microelement fertilizer (3.72% Fe, 9.28% Mg, 0.002% Mo and 2.32% Mn) was applied to each plant. Two weeks after emergence and at weekly intervals thereafter, 0.1 g of nitrogen-free (0-10-20) fertilizer was applied to each plant.

Six weeks after emergence, each plant was classified as either vigorous with dark green leaves indicating effective nodulation and N_2-fixation (+) or stunted with chlorotic yellow leaves indicating ineffective nodulation and no N_2-fixation (-). In each cultivar x strain combination, five plants (replications) were inoculated. One uninoculated (control) plant was included to assess effective nodulation.

Nodules from plants with green leaves and yellow leaves were harvested. Each nodule was washed and carefully rinsed with water before excision into halves in order to determine the color of the nodular tissue and relate it to the leaf color of the plant.

RESULTS AND DISCUSSION

After six weeks of growth under nitrogen-free conditions, all the uninoculated (control) plants were distinctly chlorotic, yellow and stunted. Rhizobial strain I induced effective nodules on both cultivars (Table 1). However, 'Soma' responded differentially to the strains. The cowpea-type rhizobial strain IV, was compatible with the cultivar TGX-4E but not with cultivar Soma (Table 1). This indicated that major cultivar and rhizobial differences were present and could be detected by this sensitive greenhouse conetainer method.

Table 1. The effectiveness of nodulation in early juvenile plants of two soybean cultivars grown in nitrogen-free medium inoculated individually with rhizobial strains. (+ = dark green leaves on normal, N_2-fixing plant; - = yellow leaves on chlorotic, nonfixing plant).

Cultivar	Rhizibial type			
	B. japonicum strain		Cowpea strain	
	I	II	III	IV
TGX-4E	+	+	+	+
Soma	+	+	-	-

Chlorotic plants with yellow leaves, were visually distinguishable from the vigorous plants with dark green leaves (Figure 1). Because the plants were grown in a nitrogen-free medium, the available nitrogen as indicated by the dark green leaves, was derived from the N_2-fixation process. Nitrogenous compounds resulting from N_2-fixation are exported from root nodules in the form of ureides (allantoin and allantoic acids) and translocated to the leaves where they are catabolized (Winkler et al. 1987) and used for the biosynthesis of chlorophyll and other proteins essential for photosynthesis.

All the inoculated plants had nodules regardless of cultivar or rhizobial strain. The morphology, size and exterior color of intact nodules were generally indistinguishable. However, the cross-sections of nodules indicated clear differences in the color of nodular tissue between effective and ineffective nodules (Figure 2). The ineffective nodules were white to light green inside while the effective nodules were characteristically pinkish-brown indicating differences in N_2-fixation capabilities between the two nodule types.

In summary, our results demonstrated a simple, inexpensive and reproducible method for identifying early juvenile soybean genotypes effective in N_2-fixation under nitrogen-free growth conditions. The method would be useful in plant breeding programs concerned with rapid screening of soybean for N_2-fixation effectiveness.

Figure 1. Differences in leaf color indicating differences in N_2-fixation effectiveness among early juvenile soybean plants grown in nitrogen-free medium inoculated with a cowpea type rhizobial strain IV.

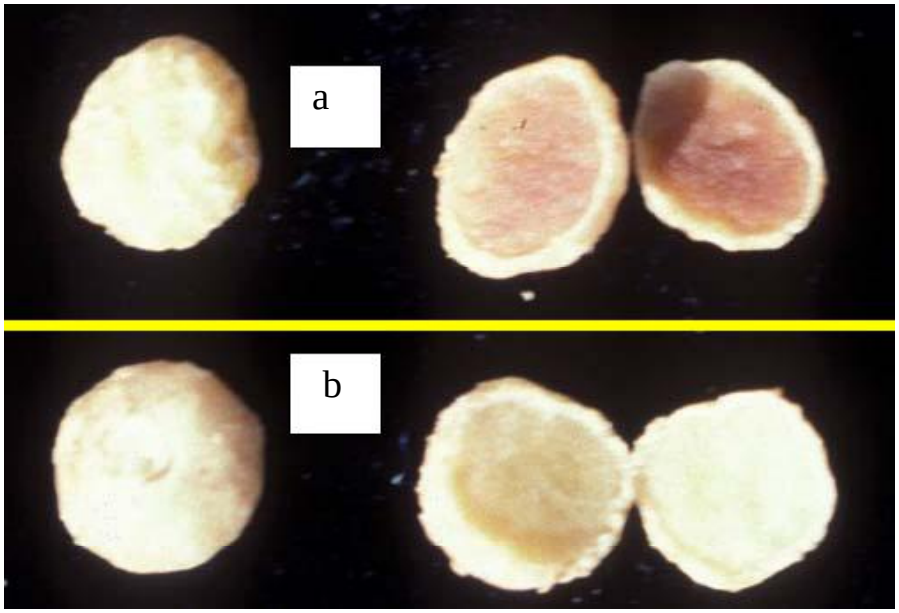

Figure 2. Intact and cross-section of root nodules from early juvenile soybean plants grown in a nitrogen-free medium and inoculated with a cowpea-type rhizobial strain IV. a = root nodules from plants with dark green leaves indicating effective nodulation and b = root nodules from plants with light yellow leaves indicating ineffective nodulation.

Moreover, the method is non-destructive and therefore allows for the development of advanced filial generations necessary for plant breeding, selection and genetic studies of variation in N_2-fixation in soybean and possibly other leguminous species. In addition, this method can also be useful in screening of transgenic plants as well as in biotechnology studies requiring identification of molecular markers using DNA extracted from early juvenile plant populations segregating for specific genetic traits such N_2-fixation effectiveness.

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