



## Polymer-entrapped rhizobium as an inoculant for legumes

G. JUNG, J. MUGNIER,

Rhône-Poulenc Industries, 182 Av. Aristide Briand, Antony, 92160, France;

H. G. DIEM and Y. R. DOMMERGUES

CNRS/ORSTOM BP 1386 Dakar, Sénégal

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**Summary** Field and cylinder experiments conducted in France and in Senegal showed that polyacrylamide, previously proposed as an entrapping gel for preparing *Rhizobium* inoculants, could be replaced by alginate (AER inoculant) or a mixture of xanthan and carob gum (XER inoculant). Semi-dried or dried AER and XER were used successfully provided that their storage time was less than 90 days. In soil inoculation trials, no marked differences were observed among semi-dried XER, dried AER, and dried XER. A number of seed inoculation experiments indicated that dried XER significantly outranked AER. Seeds preinoculated by up to 48 days with XER yielded plants which were comparable in nodulation and growth parameters to those derived from plant receiving peat inoculation at the time of planting.

### Introduction

The importance of legume crops to world food production, and compelling needs to exploit the nitrogen fixing potential of those crops have focused attention on technologies for the production of more effective legume inoculants<sup>2</sup>. Most legume inoculants have been prepared by adsorbing broth cultures of the selected rhizobia on a suitable carrier, usually peat but also on such adsorbents as talc, gypsum, clays, charcoal, lignite, cellulose powder, various powdered crop residues or soil compost mixtures<sup>4</sup>. In 1979, Dommergues *et al.*<sup>5</sup>, proposed to entrap rather than adsorb *Rhizobium* cells by incorporating the bacteria in a polymeric gel. The first experiments using polyacrylamide as the entrapping agent, showed that the resulting inoculant (PER) compared favorably with peat base inoculant, provided PER was maintained at a suitable moisture content. The survival of *Rhizobium japonicum* in moist PER was most satisfactory.

The present note reports the further development of polymer entrapped inoculants. In addition to polyacrylamide two other polymers were used, alginate and gum xanthan.

### Material and methods

*Strains of Rhizobium and soybean cv.*

We used three strains of *Rhizobium japonicum*: (1) USDA strain 138, (2) a streptomycin-resistant mutant of USDA strain 135<sup>17</sup> designated as strain 235 spl, (3) another streptomycin

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of USDA strain 135 designated as strain 135 sp2 this last strain being desiccation-sensitive. *R. japonicum* was grown in yeast-mannitol medium<sup>19</sup> on a rotary shaker (250 rpm) at 30°C.

Preliminary experiments indicated that the survival of entrapped *Rhizobium japonicum* was better for 6–7 day, than for younger cultures (4–5 days), hence 6-day old cultures were used throughout. The density of *Rhizobium* in the broth was  $1-3 \times 10^9$  per ml at 6 days.

Experiments that were carried out in Senegal used soybean (*Glycine max*) cv. 44A73, provided by CNRA (Centre National de la Recherche Agronomique) Bambey, Senegal. Seeds were surface sterilized by dipping them in a 0.1% (w/v) solution of HgCl<sub>2</sub> for 2 min followed by 5–8 rinses with sterile water.

Experiments that were carried out in France used soybean cultivar Amsoy provided by AMSOY, 12 Rue Georges V, 75 Paris, France. These seeds were not sterilized.

#### *Methods for preparing the inoculants and storage treatments\**

Three polymers were used: polyacrylamide, obtained by chemical synthesis<sup>5</sup>; alginate, extracted from algae<sup>13</sup>; and a complex resulting from the association of locust bean (carob) gum<sup>18</sup> and xanthan gum<sup>14</sup>.

*Polyacrylamide-entrapped Rhizobium (PER)* The *Rhizobium* cells were entrapped in polyacrylamide according to the method of Hicks and Updike<sup>11</sup> modified by Dommergues *et al.*<sup>5</sup>. The gel with entrapped *Rhizobium* cells (PER) was cut into 0.1–0.5 ml blocks and washed under running tap water overnight. The gel was stored as blocks in 0.2 M phosphate buffer, pH: 7.0 (*wet PER*), or crushed, spread out on a sheet of polyethylene, dried under forced air at a relative humidity of about 50% for 1–2 days at 25–28°C and then ground with a Wiley mill and stored as an airdried powder (*dried PER*) in the dark at 25–28°C. Dried PER was stored at 28°C in sealed 100 µm thick polyethylene bags. The water content of wet PER expressed on fresh weight basis was  $95 \pm 3\%$ , that of dried PER was  $10 \pm 2\%$ .

*Alginate-entrapped Rhizobium (AER)* *Rhizobium* cells were entrapped according to the principle previously used for the immobilization of cells and enzymes in calcium alginate gels<sup>6,9</sup>. Gelation was achieved either with CaCl<sub>2</sub><sup>8,12</sup> or with CaSO<sub>4</sub><sup>7</sup>.

*Gelation with CaCl<sub>2</sub>*. Two grams of commercial sodium alginate powder, Satialgine S 170 (société Bretonne de Produits Chimiques et Pharmaceutiques, 6 Impasse Latécoère Velizy, F: 78140), was poured into 100 ml of *Rhizobium* culture agitated with a magnetic stirrer until the powder was thoroughly mixed. The slurry thus obtained was passed drop-wise from a burette into about 300 ml of a 17% (w/v) CaCl<sub>2</sub> solution under magnetic stirring. The drops readily solidified as beads of 2.5–3.0 mm diameter. The beads were immediately washed under running tap water overnight, then carefully drained and placed in sealed 100 µm thick polyethylene bags (*wet AER beads*). The water content of AER beads expressed on a fresh weight basis was  $95 \pm 2\%$ .

*Gelation with CaSO<sub>4</sub>*. The slurry of sodium alginate (Satialgine S 170) and *Rhizobium* culture was prepared as indicated above, but in proportion of 1 g of sodium alginate to 80 ml of *Rhizobium* culture. Gelation was readily obtained by adding to the slurry 40 ml of a 0.6% (w/v) CaSO<sub>4</sub>·2H<sub>2</sub>O solution. The gel was crushed, then drained, spread on a sheet of polyethylene to form a 0.5 cm thick layer and air-dried as for PER. When it was dry, the gel was ground into powder (*dried AER*) and stored in sealed 100 µm thick polyethylene bags maintained at 28°C (Experiments 4, 5) or 4°C (Experiment 6). The water content of dried AER expressed on a fresh weight basis was 90%.

*Xanthan-entrapped Rhizobium (XER)* To obtain a gel with xanthan, it is necessary to associate this gum in solution with the β-1.4 linked galactomannans of carob gum with which it reacts

\* Patents 77 10254 (Y. Dommergues, Hoang Gia Diem and C. Divies, 1977); 7908597 (G. Jung, 1979); 7928956 (J. Mugnier, H. G. Diem; Y. Dommergues, 1979).

synergistically<sup>16</sup>. A 1.5% suspension of xanthan gum, a polysaccharide synthesized by *Xanthomonas campestris* (Rhodopol 23, Rhone-Poulenc Industries, 22, Avenue Montaigne, 75008 Paris, France), was prepared by agitating continuously for 20–30 min at 70–80°C and then decreasing the temperature to 40–45°C. A second suspension, 1.5% carob gum, ground seeds from *Ceratonia siliqua* (Etablissements Francois, Saint-Maur des Fossés, France) was obtained as above for xanthan.

The Rhizobium culture (50 ml) was added to 100 ml of the xanthan gum suspension, and 50 ml of culture was added to 100 ml of the carob gum suspension, both at 40–45°C. Both mixtures were then poured into a Waring blender, vigorously agitated for a min, and transferred into a beaker where gelation occurred rapidly. The product obtained was called XER gel. XER gel was either (1) stored in sterile distilled water or in saline (*wet XER*), (2) air-dried in the same way as indicated for PER (*dried XER*), or (3) mixed up with silica and only partially air-dried (*semi-dried XER*).

Semidried XER was obtained as follows. Forty g of natural or synthetic silica (such as silica 733, Rhone-Poulenc 25, Quai Paul-Doumer, 92408 Courbevoie Cédex, France) was added to 100 ml of crushed wet XER gel. The mixture was homogenized for 3–4 min at low speed (Küstner homogenizer, Küstner, 103 Av. Rouget de Lisle, Vitry sur Seine, F 94400) to obtain a product having the aspect of a wet powder. The wet powder could also be transformed into wet granules using an extruder (Alexanderwerk, Euraf, 4 place Constantin Pecqueur, Paris, F 75018).

Wet powder or wet granules were partially dried under forced air (relative humidity *ca.* 50%) for 7–8 h at 24–29°C. Drying was stopped when the water loss was 50–55% of the initial weight of the mixture. The water content of semidried XER expressed on a fresh weight basis was *ca.* 70%. Semidried XER was stored in 100 µm thick polyethylene bags at 29°C. The time elapsed between the preparation of inoculant and its application to soil or seeds is designated hereafter as the *age of the inoculant* (t).

#### *Assessment of the survival of Rhizobium entrapped in the polymers*

The number of colony-forming units in the different types of inoculants was determined after storage times of 0, 50, 100, 150 and 300 days at 28°C using standard agar plate counting techniques. Serial dilutions were prepared with suspensions obtained by grinding the inoculants as finely as possible in a Cheidolph-RGL-551 grinder for 2–3 min. Since the entrapping procedure was not carried out aseptically, contaminants occurred on the plates, especially at the lower dilutions. Fungal contaminants were avoided by the addition of cycloheximide (150 µg per ml).

#### *Soil and seed inoculation*

Since inoculation with wet inoculant had been studied previously<sup>5</sup>, we focussed our attention on the use of semidried and dried inoculants which generally appeared to be easy to handle in the field.

**Soil inoculation** In most experiments, soil was inoculated by manual application of the inoculant in the row. However, in experiment 2 the inoculant was applied mechanically (microgranulateur Nodet Gougis 77130, Montereau, France). Liquid inoculant, used as a reference treatment, was poured into the row with a watering can.

**Seed inoculation** The powdered inoculants (dried PER, AER, or XER; particle size: 150 µm) were applied to seeds previously moistened with 0.2% (w/v) solution of carboxy-methylcellulose (CMC) (1.6 ml of CMC solution for 150 g of soybean seeds) and stirred. Drying was completed by placing the seeds in an air-draft for 2–3 min. The weight of powdered inoculant adhering on each seed was *ca.* 6 mg (PER) or 3 mg (XER). Seeds coated with PER or XER contained 10<sup>5</sup>–10<sup>6</sup> cells of strain 138 per seed as freshly prepared. The seeds were used immediately after they had been inoculated or stored in 100 µm polyethylene bags punctured with 0.1 mm holes. The time T elapsed between inoculation of the seeds and seeding was designated as the *storage time* for inoculated seed.

Table 1. Main characteristics of the soils used in the experiments

	Guerina (South Senegal)	Bel Air ORSTROM Station (Dakar, Senegal)	Toulouse (South-West France)
Clay (%)	5.9	4.4	19.6
Silt (%)	6.6	0.9	13.2
Sand (%)	88.6	94.1	64.19
N total (%)	0.026	0.027	0.10
C total (%)	0.32	0.42	0.67
P total (ppm)	36	400	N.D.*
Available P (ppm)	8	239	360
pH (1:2 aqueous suspension)	5.7	7.5	8.1

\* Not determined

*Evaluation of the performance of the inoculants used for soil inoculation*

*Experiment 1 – Comparison of dried PER, dried XER and semi-dried XER* The experiment was carried out in the field at Guerina, South Senegal, in a sandy soil (Table 1) during the rainy season (July–October 1979). Soybean had never been grown in this soil. Since the soil was heavily infested with pathogenic nematodes, the entire experimental area was treated with nemagon (1.2 dibromo-3-chloropropane) at the rate of 50 l ha<sup>-1</sup> one month before sowing. A basal application of 30 kg P ha<sup>-1</sup> (as triple superphosphate) and 100 kg K ha<sup>-1</sup> (as KCl) was made prior to sowing. There were six treatments (Table 3); no inoculation (control); inoculation with semidried XER at the rate of 150 kg ha<sup>-1</sup>; inoculation with dried PER and with dried XER at the rate of 15 kg ha<sup>-1</sup>; inoculation with liquid culture which had been carried from the laboratory under refrigeration, at the rate of 150 l ha<sup>-1</sup>; application of urea, broadcast at sowing time (100 kg N ha<sup>-1</sup>) and at flowering (100 kg N ha<sup>-1</sup>). Usual precautions were taken to reduce risks of rhizobial contamination during the planting operation. One meter wide furrows were dug around all plots to minimize further rhizobial contaminations. The experimental design was four randomized blocks of five 8 × 8 m plots. Rainfall during the experiment was 892 mm (July 4 to October 15, 1979).

*Experiment 2 – Influence of the rate of application of semi-dried XER to soil* The experiment was carried out in the field at Deyme, 15 km from Toulouse in the Southwest of France in a sandy loam (Table 1) in 1980 (14 April–22 October). The soil, in which soybean had never been grown, received 500 kg ha<sup>-1</sup> of 0:25:25 fertilizer prior to seedbed preparation and 107 kg K ha<sup>-1</sup> (as K<sub>2</sub>SO<sub>4</sub>) after plowing. Semi-dried XER was placed in the row at the time of seeding using a pneumatic seeding machine (Pneumasem II, Nodet Gougis, 77130 Montereau, France). Rates are indicated in Table 4. Time *t* for semi-dried XER was 15–30 days, and the number of colony-forming units at that time was 5–11 × 10<sup>7</sup> per g of inoculant. Seed inoculation with the reference peat based inoculant (prepared by LIPHA, 115 Avenue Lacassagne 69, Lyon, France) was at the time of planting. The experimental design was 4 blocks of seven 9 × 15 m plots. Rainfall during the experiment was 290 mm; a supplement of 70 mm was added by irrigation.

*Experiment 3a. Influence of the age and rate of application of semi-dried XER to soil* The experiment was carried out at the ORSTOM research station of Dakar, Senegal, in March 1980, using Bel Air soil (Table 1) in which soybean had never been grown. Plants were grown in cylinders, a device already adopted by Legg and Sloger<sup>13</sup>. The 30 cm diameter 50 cm high PVC cylinders were driven into the soil of an open field and emptied of soil. Then a layer of 3–5 mm diameter stones was placed at the

bottom to facilitate drainage. Finally, the cylinders were filled up with carefully homogenized Bel Air soil. PK fertilization was the same as in experiment 1. There were eight cylinders with five plants each for each treatment. Treatments are indicated in Table 5. Two rates of XER inoculant, 15 and 30 kg ha<sup>-1</sup>, and two ages of inoculant (t), 20 and 90 days, were compared. The reference treatment was inoculation with liquid culture. A no-inoculation treatment was common to experiments 3a and 3b. Cylinders were irrigated throughout the experiment.

#### *Evaluation of seed inoculants*

*Experiment 3b. Effectiveness of dried XER on seeds as a function of storage time* This experiment was carried out in cylinders together with experiment 3a and the experimental material and design were similar. Seeds were coated with freshly prepared (t = 0) dried XER and stored for 20 days (T = 20), or 90 days (T = 90). Seeds freshly coated with peat based inoculant were used as the control treatment.

*Experiment 4. Influence of additives to AER* The soil and experimental design were the same as for experiments 3a and 3b, but with seven rather than eight cylinders. The experiment was carried out at the ORSTOM research Station of Dakar in October 1980. Treatments were 1 g of rock phosphate or 5 g of kaolinite added to the slurry obtained by mixing 100 ml of the Rhizobium culture with 2 g of sodium alginate.

*Experiment 5. Comparison of AER and XER for seed inoculation* This experiment was carried out in the field at Guerina, Senegal, i.e., at the same location as experiment 1, but one year later (July-October 1980). The treatments are indicated in Table 7. The main objective of the experiment was to compare dried AER and dried XER inoculants that were 80 days old (t = 80) and applied to the seeds immediately before sowing (T = 0). Soil inoculation with a liquid broth was used as the reference treatment since peat-inoculated control seeds failed to germinate due to damping off. Subsequent experiments not reported here confirmed the deleterious effect of peat based inoculant in other tropical soils, but only during the summer. The experimental design was four randomised blocs of five 8 × 8 m plots. The rainfall during the experiment was only 550 mm.

*Experiment 6. Influence of storage time on seeds inoculated with AER and XER* The experiment was carried out in the field at Toulouse in the vicinity of experiment 2 (April-October 1980) to compare the behavior of AER and XER inoculants under temperature soil conditions and to study the effect of storage time on inoculated seeds. Seed inoculation with peat based inoculant was used as the reference treatment. The experimental design was 4 blocks of six 9 × 15 m plots.

Nodulation and shoot weight were observed in all experiments. Grain yield expressed on weight and N content basis was determined only in the case of field experiments.

## **Results and discussion**

### *Survival of Rhizobium entrapped in the polymers*

Table 2 indicates that the survival of *R. japonicum* was excellent in PER and AER beads that were stored wet. In the case of wet PER, however, the number of colony-forming units counted was lower than in that of AER, probably because these units did not originate from single cells but from clumps that were not disrupted when crushing PER. PER and AER were never invaded by contaminants, probably because exogenous microorganisms could not readily colonize polyacrylamide nor penetrate into the AER beads. By contrast, wet

Table 2. Survival of *Rhizobium japonicum* entrapped in different polymers stored at 28°C

Type of inoculum	Strain of Rhizobium	Log <sub>10</sub> no. of Rhizobium per ml of broth before entrapping	Log <sub>10</sub> no. of colony-forming units per ml of broth entrapped in the polymer after storage (days)				
			0	50	100	150	300
		(1)	(2)	(3)	(4)	(5)	(6)
Wet PER	135 sp1	0.0-9.3	8.1	8.1	8.1	8.1	N.D.
Wet AER beads	135 sp1	9.0-9.3	9.3	9.3	9.3	9.3	N.D.
Wet XER	135 sp1	9.0-9.3	8.3	8.3	C	C	N.D.
Semidried XER	138	9.0-9.3	8.8-9.0	N.D.	8.4-8.6	8.5-8.7	8.5-8.7
Dried PER	138	9.0-9.3	6.9	5.4	3.9	1.5	N.D.
	135 sp1	9.0-9.3	7.1	N.D.	4.1	2.6	N.D.
	135 sp2	9.0-9.3	4.2	<1.0			
Dried AER beads	135 sp1	9.0-9.3	7.6	6.8	6.8	6.7	N.D.
	135 sp2	9.0-9.3	5.7	4.2	2.7	1.2	N.D.
Dried AER powder	138	9.0-9.3	7.5	7.2	7.0	6.7	N.D.
	135 sp2	9.0-9.3	4.9	3.7	<1.0		
Dried XER	138	9.0-9.3	7.8-8.0	N.D.	6.0-7.3	N.D.	<1.6

N.D. not determined - C contaminated

XER was more easily contaminated, probably because the entrapping polymer (mixture of xanthan and carob gum) provided available substrate.

The number of living *Rhizobium* in semidried XER remained constant during the entire storage period of 300 days at 28°C. However, if not prepared carefully or if not stored in the cold, it was subject to contamination by fungi.

Drying the inoculants prevented the multiplication of contaminants but always affected dramatically the number of living cells entrapped in the polymer. The log of the colony-forming units of strains 138 and 135 sp1 decreased from 9.0–9.3 in the broth (column 1) before entrapping, to 6.9–7.6 after entrapping and immediately after desiccation (column 2). The much more desiccation-sensitive strain 135 sp2 was still more affected since the log of colony-forming units of this strain was only 4.2–5.7 after desiccation (column 2).

During storage following desiccation, the number of survivors decreased more rapidly in PER than in AER, suggesting that alginate might protect the *Rhizobium* from the effect of desiccation better than polyacrylamide. Attempts were made to improve the survival of the bacteria in the AER by adding clay (kaolinite) to the gel. Best results were obtained by incorporating 5 g of kaolinite to the slurry obtained by mixing the *Rhizobium* culture with alginate. Dried AER beads carrying strain 135 sp2 treated in this manner remained essentially unchanged in viability after 100 days' storage. In contrast, strain 138 did not benefit from the protective effect of the clay. Such results are reminiscent of those reported by Bushby and Marshall<sup>3</sup> who found that montmorillonite clay protected some strains but not others.

#### *Assessment of the performances of the inoculants*

Inoculant performance was evaluated primarily on the number and mass of nodules induced at 30–76 days (Senegal) or 105 days (France) after planting. Results were reported also in terms of the weight or N content of the shoots or as grain yield. These latter indices, however, were accorded secondary status since they are a function of many factors other than nodulation such as light<sup>10</sup>, soil water content or phosphorus nutrition of the host-plant. Thus, nodulation may not be correlated with growth or yield of the legume in some circumstances.

#### *Soil inoculation*

The results of experiment 1 (Table 3) conducted in Senegal indicate that there were no marked differences in performance among the three inoculants (semidried XER; dried PER; dried XER) and the reference treatment (liquid culture).

In all cases, the number of nodules and the nodule weight (ca. 4.0 g) was satisfactory. Non-inoculated plants were spontaneously nodulated, probably partly by native strains of cowpea rhizobia which can nodulate soybean<sup>1</sup>, and partly through contaminations with strain 138 used elsewhere for inoculation.

Table 3. Experiment 1: Comparison of semi-dried XER, dried PER and dried XER used for inoculating soil

Treatments	Nodules**		Shoots**		Grain yield***	
	No. plant <sup>-1</sup>	Fresh wt (g plant <sup>-1</sup> )	Fresh wt (g plant <sup>-1</sup> )	g N plant <sup>-1</sup>	Dry wt (kg ha <sup>-1</sup> )	kg N ha <sup>-1</sup>
No inoculation	21.9 a	1.6 a	187 a	1.09 a	1815 a	113 a
Soil inoculation						
Semidried XER (150 kg ha <sup>-1</sup> )	134.3 b	4.3 b	240 a	2.16 b	2300 a	169 de
Dried PER (15 kg ha <sup>-1</sup> )	126.2 b	4.3 b	220 a	2.11 b	2155 a	155 cde
Dried XER (15 kg ha <sup>-1</sup> )	200.4 c	4.6 b	213 a	2.08 b	1877 a	137 bc
Liquid culture (150 l ha <sup>-1</sup> )	169.6 c	5.0 b	238 a	1.94 b	2357 a	168 de
N fertilizer (urea: 200 kg N ha <sup>-1</sup> )	28.9 a	1.7 a	300 a	2.79 b	1990 a	139 bcd

\* Age of XER and PER, t = 30 days.

\*\* Mean of 40 plants sampled 85 days after planting.

\*\*\* Water content 13%.

Numbers with same letters in columns do not differ significantly,  $P = .01$ .



Table 4. Experiment 2: Influence of the rate of application of semi-dried XER to soil

Treatments	Nodules**		Shoots** fresh wt (g plant <sup>-1</sup> )	Grain yield***	
	No. plant <sup>-1</sup>	Fresh wt (g plant <sup>-1</sup> )		Dry wt† (g plant <sup>-1</sup> )	mg N plant <sup>-1</sup>
No inoculation	0.15 a	0.010 a	78.0 a	11.5 a	476 a
Soil inoculation*					
Semi-dried XER (26 kg ha <sup>-1</sup> )	63.2 cd	1.269 d	85.1 ab	24.1 c	1339 c
Semi-dried XER (34 kg ha <sup>-1</sup> )	89.9 c	1.490 cd	92.8 ab	26.4 c	1457 cd
Semi-dried XER (48 kg ha <sup>-1</sup> )	75.8 cd	1.788 cd	99.4 b	26.6 cd	1459 cd
Semi-dried XER (54 kg ha <sup>-1</sup> )	64.2 cd	1.673 cd	85.2 ab	29.2 d	1583 d
Seed inoculation with peat	27.1 b	0.779 b	92.7 ab	23.5 c	1208 c
N fertilizer (200 kg N ha <sup>-1</sup> )	0.03 a	0.000 a	78.1 ab	17.6 b	865 b

\* Age of XER, t = 15–30 days.

\*\* Mean of 35 plants sampled 105 days after planting.

\*\*\* Mean of 40 plants harvested by hand 190 days after planting.

† Water content 12%.

Numbers with the same letters in columns do not differ significantly,  $P = .01$ .

Application of urea (200 kg N ha<sup>-1</sup>) did not affect spontaneous nodulation in the non-inoculated plots.

Experiment 2 conducted in temperate conditions showed that the high rate of

Table 5. Experiment 3a: Influence of the age (t) and rate of application of semi-dried XER to soil.  
Experiment 3b: Influence of storage time for seeds inoculated with dried XER (T)

Treatments	No. of nodules plant <sup>-1</sup>	Nodule fresh wt (g plant <sup>-1</sup> )	Shoot fresh wt (g plant <sup>-1</sup> )
No inoculation	12 a	0.48 a	11.7 a
Experiment 3a: soil inoculation*			
Semidried XER (t = 20) 15 kg ha <sup>-1</sup>	42 b	1.68 bc	18.8 b
Semidried XER (t = 20) 15 kg ha <sup>-1</sup>	40 b	1.58 b	18.1 b
Semidried XER (t = 90) 30 kg ha <sup>-1</sup>	60 cd	1.91 bc	18.7 bc
Liquid culture 30 l ha <sup>-1</sup>	70 d	1.70 bc	18.7 b
Experiment 3b: seed inoculation**			
Dried XER (t = 0; T = 20) 0.6 kg ha <sup>-1</sup>	53 c	2.01 c	20.9 c
Dried XER (t = 0; T = 90) 0.6 kg ha <sup>-1</sup>	13 a	0.48 a	10.6 a
Peat (t = 100; T = 0) 0.4 kg ha <sup>-1</sup>	53 c	1.71 b	19.1 b

\* t = age of inoculant (days).

\*\* T = Storage time inoculated seeds (days); storage at 28°C.

Plants harvested on 30<sup>th</sup> day. Each figure is the mean of 30 replicates.

Numbers with same letters in columns do not differ significantly,  $P = .01$ .

Table 6. Experiment 4: Influence of different additives to AER used for inoculating seeds\*

Treatments	No. of nodules plant <sup>-1</sup>	Nodule dry wt (g plant <sup>-1</sup> )	Shoots	
			Dry wt (g plant <sup>-1</sup> )	g N plant <sup>-1</sup>
No inoculation	28 a	0.25 a	9.6 a	0.14
Seed inoculation**				
Dried AER	126 c	1.02 cd	16.9 bc	0.33
Dried AER + rock phosphate	101 c	0.84 c	12.1 ab	0.27
Dried AER + kaolinite	73 b	0.63 b	10.0 a	0.19
Dried XER	176 d	1.27 d	18.5 c	0.66

\* Plants harvested on 43<sup>rd</sup> day. Each figure is the mean of 7 replicates.

\*\* Age of inoculant: t = 9 days. Storage time for inoculated seeds: T = 28 days; storage at 28°C. Numbers with same letters in columns do not differ significantly, P = .01.

application of semi-dried XER (Table 3) could be decreased without significantly affecting nodulation (Table 4). In this experiment, seed inoculation with peat (reference treatment) was not satisfactory; the relatively poor nodulation was attributed to unfavorable physical soil properties of the experimental field.

Further work carried out in cylinders in Senegal (Experiment 3) confirmed that a low rate of application of XER to soil (15–30 kg ha<sup>-1</sup>) was still satisfactory (Table 5).

Table 7. Experiment 5: Comparison of AER and XER for seed inoculation

Treatments	Nodules**		Shoots <sup>(2)</sup> fresh wt (g plant <sup>-1</sup> )	Grain yield	
	No. plant <sup>-1</sup>	Fresh wt (g plant <sup>-1</sup> )		Dry wt*** (kg ha <sup>-1</sup> )	kg N ha <sup>-1</sup>
No inoculation	1.3 a	0.08 a	178.3 a	1919 ac	107 ac
Seed inoculation*					
Dried AER (0.4 kg ha <sup>-1</sup> )	55.8 b	1.6 b	138.6 b	2752 bc	190 b
Dried XER (0.4 kg ha <sup>-1</sup> )	179.3 c	5.0 c	338.5 c	2773 b	176 b
Soil inoculation					
Liquid inoculum (150 l ha <sup>-1</sup> )	62.5 b	2.2 b	136.0 b	2445 abc	160 b
N fertilizer (300 kg N ha <sup>-1</sup> )	0.5 e	0.02 d	177.8 a	2366 c	130 ac

\* t = 80 days; T = 0.

\*\* Mean of 40 plants sampled 85 days after planting.

\*\*\* Water content 13%.

Numbers with the same letters in columns do not differ significantly, P = .01.

Table 8. Experiment 6: Comparison of seed inoculation with AER and XER, influence of storage time on inoculated seeds

Treatments No.	Nodules**		Shoots** fresh wt	Grain yield***	
	Fresh wt plant <sup>-1</sup>	(g plant <sup>-1</sup> ) (g plant <sup>-1</sup> )	Dry wt†	mg N (g plant <sup>-1</sup> )	plant <sup>-1</sup>
No inoculation	9.6 a	0.146 a	41.4 a	12.1 a	582 a
Seed inoculation*					
AER (t = 9; T = 17)	92.1 bcd	1.411 bc	62.3 c	19.8 b	1039 b
AER (t = 15; T = 48)	62.0 bd	1.004 b	48.4 b	20.3 b	1058 b
XER (t = 9; T = 17)	113.9 cd	2.149 c	74.2 d	20.0 b	1137 b
XER (t = 15; T = 48)	114.9 cd	2.227 c	59.7 c	20.6 b	1091 b
Peat (t = 100; T = 0)	127.8 cd	1.300 bc	58.9 bc	21.6 b	1112 b

\* For definition of t and T, see Table 5.

\*\* Mean of 35 plants sampled 105 days after planting.

\*\*\* Mean of 40 plants harvested 190 days after planting.

† Water content 12%.

Numbers with the same letters in columns do not differ significantly,  $P = .01$ .

### Seed inoculation

Experiment 3 (Table 5, lower part) showed that when the storage time for seeds inoculated with freshly prepared XER was only 20 days ( $T = 20$ ), dried XER was as effective as peat which had just been applied on the seeds. When storage time was longer ( $T = 90$ ), however, nodulation was not different from that in the control, indicating that most Rhizobium on the seeds had died. This poor survival may have been associated with anti-rhizobial substances in soybean seeds<sup>20</sup> or to the increase in the water content of the inoculum. Experiment 6 (Table 8) confirmed that the survival of Rhizobium in AER and to a lesser degree in XER on the seed itself sharply decreased when the storage time for inoculated seeds ( $T$ ) was 48 days.

Experiments 4–6 (Tables 6–8) clearly indicated that dried XER was superior to AER in terms of nodule numbers and weight. Surprisingly, the use of rock phosphate or kaolinite as an additive to AER reduced the performance of this inoculant (Table 6). Comparisons of dried XER and peat (Tables 5, 8) showed that XER with storage time of up to 48 days ( $T = 17$ –48) was similar or even superior to inoculation with freshly applied peat so far as nodulation was concerned.

### Conclusion

Dommergues *et al.*<sup>5</sup> previously reported pot experiments which showed that the performance of wet polyacrylamide-entrapped Rhizobium (wet PER) was comparable to that of peat base inoculant. The field and cylinder experiments reported here indicate that polyacrylamide can be replaced by other polymers

(alginate, xanthan) as entrapping gels and that semi-dried or dried preparations of these gels serve successfully as inoculants.

In soil inoculation trials using preparations which had been stored for less than 90 days no marked differences were observed among semi-dried XER, dried AER, and dried XER. Since semi-dried XER could be prepared in a granular form and applied readily to the soil using common machinery, it was selected as the most appropriate form of inoculant for field application.

A number of experiments addressed the possibility of the use of gel entrapped rhizobia preparation of seeds. The main results of these experiments indicated that dried XER significantly outranked AER, probably for the following three reasons: (1) XER offered a better protection to the rhizobia while associated with seeds, (2) surviving rhizobia were more easily released into the soil from the XER, and (3) seeds pre-inoculated by up to 48 days with XER yielded plants which were comparable in nodulation and growth parameters to those derived from seeds receiving peat inoculation at the time of planting. This last aspect is encouraging since it holds forth the possibility not only that seeds may be preinoculated and stored at least for 30 days before planting, but also that the complication of damping off sometimes associated with the application of peat based inoculants at the time of seeding in the tropics, may be avoidable.

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