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Polymer-entrapped rhizobium as an inoculant for legumes

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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[2]. 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[4]. In 1979, Dommergues *et al.*[5], 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[17] designated as strain 235 spl, (3) another streptomycin-

of USDA strain 135 designated as strain 135 sp2 this last strain being desiccation-sensitive. *R. japonicum* was grown in yeast-mannitol medium[19] 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 sensity 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 $HgCl_2$ 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[5]; alginate, extracted from algae[15]; and a complex resulting from the association of locust bean (carob) gum[18] and xanthan gum[14].

Polyacrylamide-entrapped Rhizobium (PER) The Rhizobium cells were entrapped in polyacrylamide according to the method of Hicks and Updike[11] modified by Dommergues *et al.*[5]. 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 ± 3%, that of dried PER was 10 ± 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[6,9]. Gelation was achieved either with $CaCl_2$[8,12] or with $CaSO_4$[7].

Gelation with $CaCl_2$. 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) $CaCl_2$ 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 ± 2%.

Gelation with $CaSO_4$. 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) $CaSO_4.2H_2O$ 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[16]. A 1.5% suspension of xantham gum, a polysaccharide synthetized 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

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

	Guerina (South Senegal)	Bel Air ORSTROM Station (Dakar, Sengegal)	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^{-1} one month before sowing. A basal application of 30 kg P ha^{-1} (as triple superphosphate) and 100 kg K ha^{-1} (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^{-1}; inoculation with dried PER and with dried XER at the rate of 15 kg ha^{-1}; inoculation with liquid culture which had been carried from the laboratory under refrigeration, at the rate of 150 l ha^{-1}; application of urea, broadcast at sowing time (100 kg N ha^{-1}) and at flowering (100 kg N ha^{-1}). 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^{-1} of 0:25:25 fertilizer prior to seedbed preparation and 107 kg K ha^{-1} (as K_2SO_4) 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 × 10^7 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

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^{-1}, 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_{10} no. of Rhizobium per ml of broth before entrapping	Log_{10} 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

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^{-1}	Fresh wt (g plant^{-1})	Fresh wt (g plant^{-1})	g N plant^{-1}	Dry wt (kg ha^{-1})	kg N ha^{-1}
No inoculation	21.9 a	1.6 a	187 a	1.09 a	1815 a	113 a
Soil inoculation						
Semidried XER (150 kg ha^{-1})	134.3 b	4.3 b	240 a	2.16 b	2300 a	169 de
Dried PER (15 kg ha^{-1})	126.2 b	4.3 b	220 a	2.11 b	2155 a	155 cde
Dried XER (15 kg ha^{-1})	200.4 c	4.6 b	213 a	2.08 b	1877 a	137 bc
Liquid culture (150 l ha^{-1})	169.6 c	5.0 b	238 a	1.94 b	2357 a	168 de
N fertilizer (urea: 200 kg N ha^{-1})	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	Grain yield***	
	No. plant^{-1}	Fresh wt (g plant^{-1})	(g plant^{-1})	Dry wt† (g plant^{-1})	mg N plant^{-1}
No inoculation	0.15 a	0.010 a	78.0 a	11.5 a	476 a
Soil inoculation*					
Semi-dried XER (26 kg ha^{-1})	63.2 cd	1.269 d	85.1 ab	24.1 c	1339 c
Semi-dried XER (34 kg ha^{-1})	89.9 c	1.490 cd	92.8 ab	26.4 c	1457 cd
Semi-dried XER (48 kg ha^{-1})	75.8 cd	1.788 cd	99.4 b	26.6 cd	1459 cd
Semi-dried XER (54 kg ha^{-1})	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^{-1})	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^{-1}) did not affect spontaneous nodulation in the non-inoculated plots.

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

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

Treatments	No. of nodules plant^{-1}	Nodule dry wt (g plant^{-1})	Shoots	
			Dry wt	g N
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

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

Treatments	Nodules**		Shoots** fresh wt	Grain yield***	
No.	Fresh wt plant^{-1}	(g plant^{-1}) (g plant^{-1})	Dry wt†	mg N (g plant^{-1})	plant^{-1}
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[20] 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.*[5] 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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