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Use of bacteria protections in pelleting for preinoculation of bean seeds (*Phaseolus vulgaris* L.)

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Summary

Preinoculation of bean seeds (*Phaseolus vulgaris* L.) with *Rhizobium phaseoli* material has been carried out by incorporating the inoculum in seed pelleting. An inoculum was incorporated into a mixture of vermiculite and compost or wheat bran, and applied to seeds to produce a solid pellet. The pelleted seed contained a sufficient number of bacteria per seed for effective nodulation after 56 days storage of preinoculated pelleted seeds at 25°C. Those results are not achieved using a liquid inoculum of *Rhizobium*.

Introduction

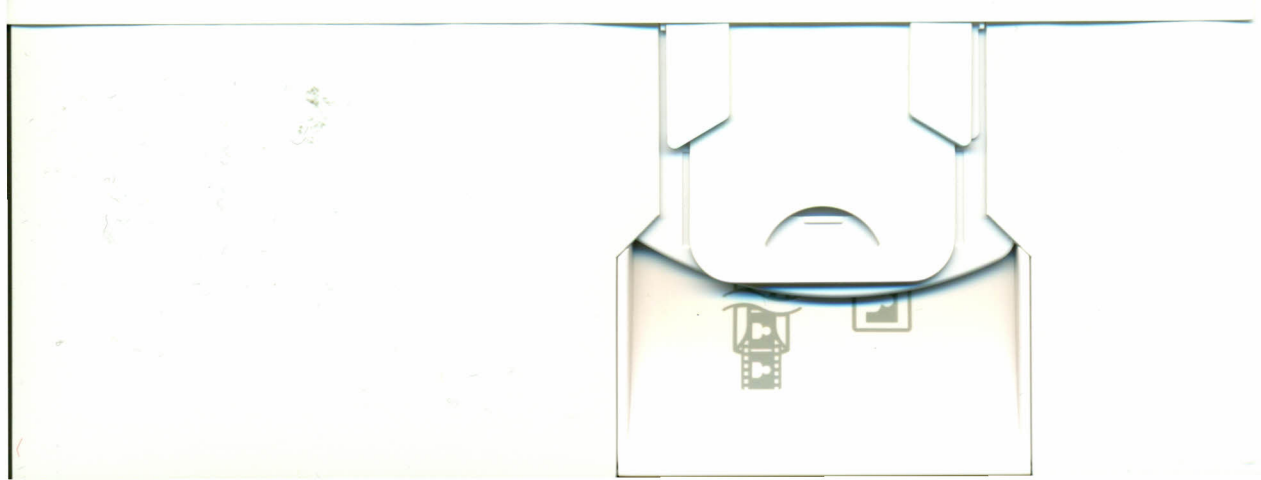
The economic benefit of atmospheric nitrogen fixation by the symbiotic relationship between Bean and *Rhizobium phaseoli* is, in many soils, no longer demonstrated. Absence of *Rhizobium* or presence of insufficiently active bacteria strains in some soils makes it necessary to inoculate seeds (Brakel, 1966). Among the different techniques utilized, preinoculation during seed pelleting, which does not require any special handling by the user, is economically most promising.

The major problem associated with preinoculation is the poor survival of *Rhizobium* during seed storage. Survival is possible only under very special conditions. According to Burton (1980), legume seeds preinoculation can be carried out but only in winter, when the seed is stored in a cool place (4 °C) before being sown in spring.

Mortality of *Rhizobium* during storage chiefly relates to its poor survival rate in dry conditions (Mary et al., 1985). It is claimed that some types of clays and some disaccharides play a part in the protection of bacteria against dessication (Busby et Marshall; 1977; Salema et al., 1982).

Seed coating in pellets can, in this field, bring about a significant contribution to reducing mortality of bacteria during storage. Previous work (Schiffers et al., 1982) showed the potential afforded by that technology. A strain of *Rhizobium japonicum* was incorporated in the coating of soybean seed, also treated with carbofuran. After 60 days storage, presence of live *Rhizobium* was demonstrated through the nodulation of plants grown form inoculated seed, that were sown in an artificial substrate free of bacteria. However, formulation and application techniques used remained rudimentary.

In accordance with those data, the goal of this experimentation was to develop a method to produce inoculum and to preinoculate bean seeds by pelleting, that will assure the survival of at least 10^5 bacteria on each seed, after storage at room temperature.



Materials and methods

Grading of seed

Bean seeds (*Phaseolus vulgaris* L., cv. 'Contender') were stored and graded through a sieve before the coating operation to obtain a uniform size of seed; this grading makes possible a more uniform application on each seed than for an ungraded batch. The mean weight of a thousand seeds (PMG) was 532 g after sieving (that is to say 470 seeds for a 250 g batch to process).

Selection of carriers

Pelleting forms a mould round the seed. It was made of a mixture of carriers and binding agents that guarantee the cohesion of the whole. When developing that coating formulation, it was necessary to keep in mind the epigeal germination of the bean: the rapid crumbling of the coating in the ground assures an immediate release of the bacteria at the rhizosphere level. This was essential to prevent the particles from being drawn up above the ground when germination ocurred.

Selection of components of the pelleting formulation (thinner/absorbent, adhesives, surfactants, form of the inoculum) was based on three main criteria: (1) capacity of sorption of weighting agents; (2) their compatibility towards *Rhizobium*; (3) their adhesiveness to the bean seeds testa.

Pelleting operation

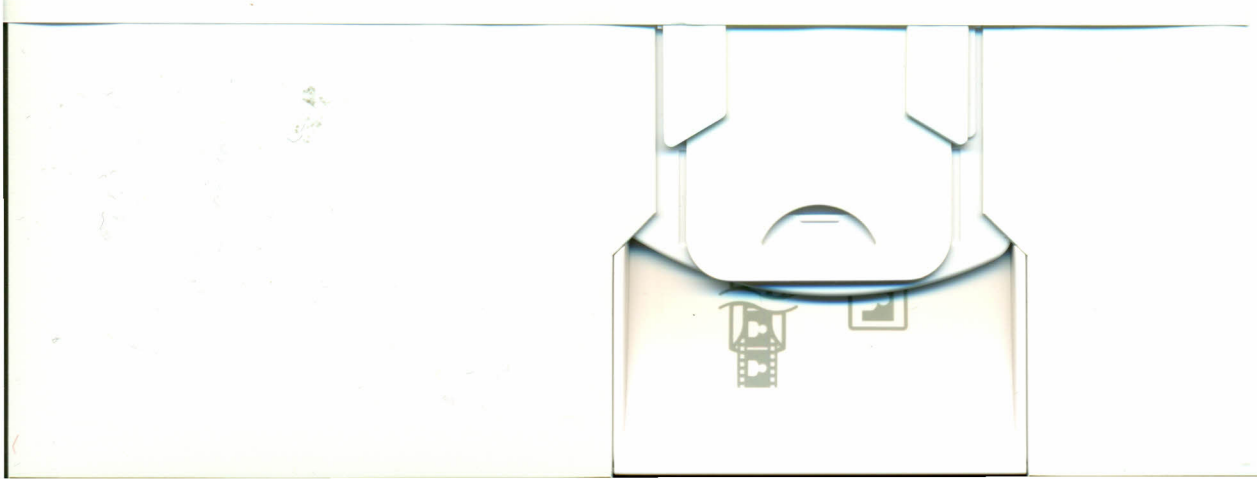
Graded seeds were pelleted and inoculated with three kinds of inocula. Four replications were used for each inoculum.

a) Preparation of carriers

The quantities of carriers necessary for the pelleting of the seeds (equal to 168 g/kg of seed) were mixed for 5 minutes with a mixer-homogenizer which produces a specific motion which assures a homogeneous mixture of powders with different granulometry. Before the incorporation of inoculum in the form of 425 µm in 850 µm vermiculite, the mixture was moistened with water to 15% dry weight basis.

The composition of the pelleting formulation, expressed in % of dry weight, is the following:

Sepiolite:	40.5%
Perlite:	14.3%
Fine vermiculite:	16.6%
Sodium lignosulfate:	4.8%
Hydroxypropylmethylcellulose:	4.8%
Inoculum:	19.0%
	100.0%



b) Incorporation of the inoculum
Sixteen grams of bacterial inoculum were incorporated to the previously mixed weighting agents. Liquid inoculum was constituted by absorption of 8 ml bacterial culture on 8 g of 425 µm–850 µm vermiculite, solid inocula were used as such (see below the production techniques). Before utilization, the whole was homogenized with a mixer-homogenizer for 5 minutes.

c) Pelleting working out
Pelleting was carried out following a rolling technique (Schiffers, 1988a and 1988b) with 250 g seed batches. Carriers and other weighting agents were deposited by dusting seeds in motion inside a turbine, and 25 to 30 ml of a 10% saccharose solution (w/v) were needed to moisten seeds during pelleting. After sticking on of the last part of weighting agents, 5 g talcum powder were added to the seeds in motion to dry them and improve their flow characteristics.

Pelleted seeds were dried in an air current (25 °C) for 15 minutes, then for 45 to 60 minutes at 30 °C in an aerated drying cabinet.

d) Storage
Pelleted and inoculated seeds were stored in paper bags for 56 days at 25 °C in a climatic chamber.

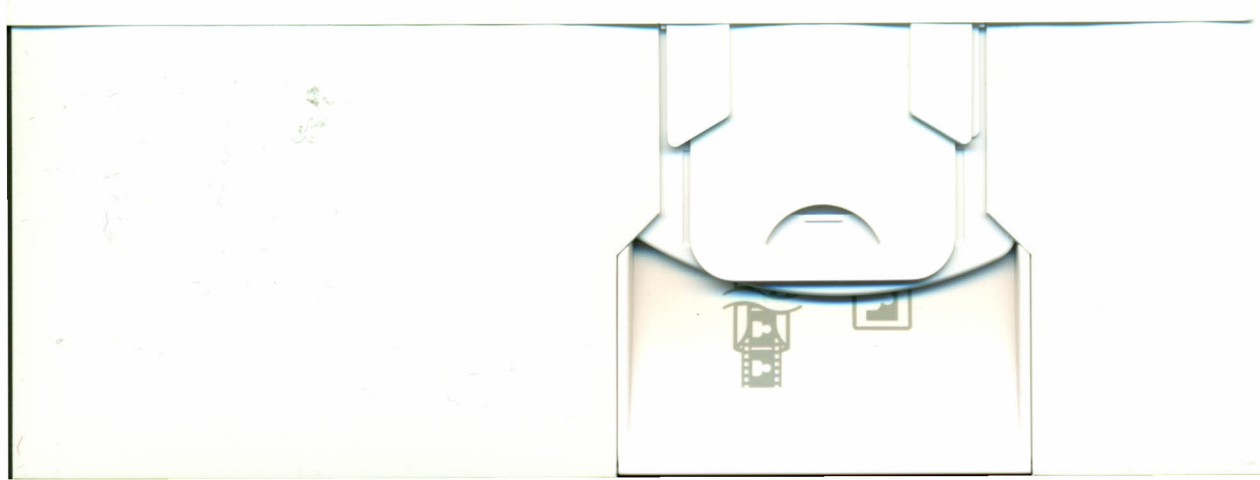
Inocula production
Rhizobium phaseoli used for the inoculation of bean seeds was the '9.R.53' strain, isolated at the Microbiology Laboratory of the Faculty in Gembloux. It was chosen for its activity and rapid growth among nine reisolated bacterial strains from nodules.

a) Liquid inocula
Rhizobium liquid inocula were obtained by suspension of the selected strain in an Erlenmeyer flask containing 100 ml Wright liquid medium (Bonnier and Brakel, 1969) and then incubating the mixture at 25 °C for a week.

b) Solid inocula
Three carriers of different compositions were used for the solid inocula production, which were:

Type of materials	Carrier 1	Carrier 2	Carrier 3
Vermiculite 425 µm–850 µm:	100.0 g	88.6 g	90.0 g
Wheat bran:	0.0 g	10.0 g	0.0 g
$CaCO_3$:	0.0 g	1.4 g	0.0 g
Compost[1]:	0.0 g	0.0 g	10.0 g
Total:	100.0 g	100.0 g	100.0 g

[1] Household rubbish composted, dried, crushed and sifted. Only partical sizes under 425 µm were used.



After sterilization (2 hours at 160 °C – dry heat), 10 g of carrier (1), (2) or (3) receive 9 ml of Wright liquid medium and 1 ml of *Rhizobium* culture on Wright liquid medium with a concentration of about 10^9 cfu/ml. Inocula were incubated at 30 °C for a week, while aerated and homogenized by a rotary motion on a shaker for two minutes every four hours.

Bacterial counts

One gram of solid inoculum was introduced into 100 ml Ringer medium and placed on a magnetic shaker for one minute. The same operation was repeated for the seeds by putting three pelleted and inoculated seeds on a magnetic shaker for one minute. The same operation was repeated for the seeds by putting three pelleted and inoculated seeds on a magnetic shaker for ten minutes.

Bacterial counts were carried out through the successive dilutions method and results are expressed in *'Colony Forming Unit'* (cfu) in ml or g of inoculum or seed unit. For each count, two serial dilutions were carried out and two Petri-dishes were seeded for each dilution.

Evaluation of the nitrogen fixation potential

Nitrogen fixation potential was evaluated by determining the protein content of aerial parts of the bean plants.

Plants were grown in a cold-house, in sterile conditions (technique described by Bonnier and Brakel, 1969). Pelleted preinoculated seeds were one day old, except 'wheat bran' solid inoculum pelleted seeds, that were stored for eight days before sowing.

Treatments consisted of seeds coated with the three pelleted inocula, controls of seeds soaked in Rhizobium liquid culture, and non inoculated seeds. Each treatment was represented by four seeds. The block of treatments was replicated four times except two of the replications did not contain controls. Sixty days after sowing, aerial parts of the plants were collected, dried and pounded. The crude protein content was obtained multiplying the total nitrogen content, determined by the Kjeldahl method, by the coefficient 6.25.

Results

Selection of the carriers and weighting agents according to their compatibility with Rhizobium

Properties and compatibility with *Rhizobium* of the various carriers and weighting agents considered in the formulation are shown in Table 1. Based on the chosen criteria of selection, selected carriers are shown in boldface in the table.

Inocula production

Results of the bacterial counts in three kinds of solid inocula after 1, 5, 10 and 15 incubation days are shown in Table 2. Three repetitions of the counting are carried out for each kind of carrier. Mean results are expressed in cfu/g of carrier.

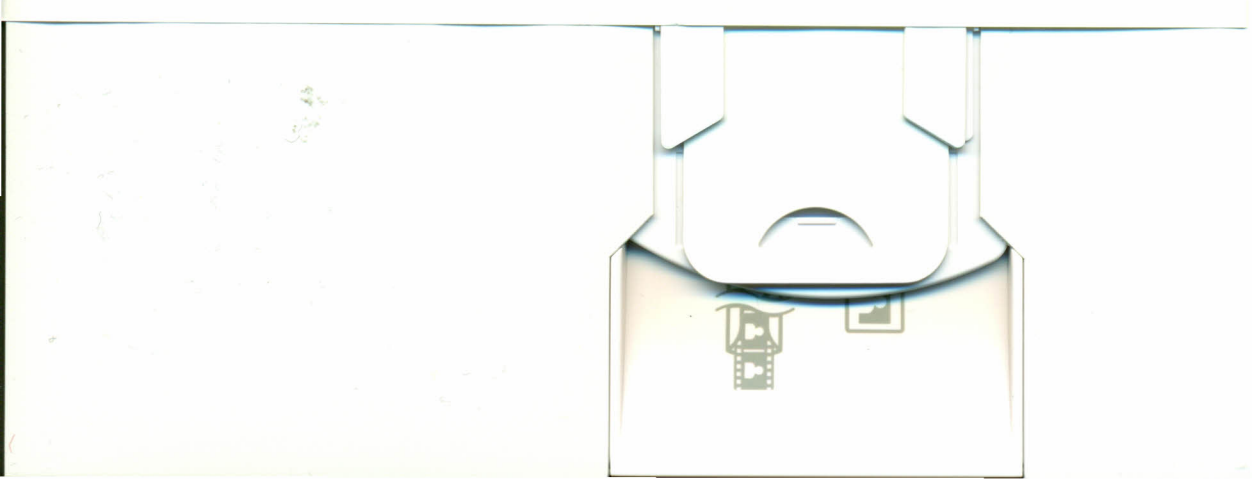


Table 1. Selection of the various carriers and weighting agents for pelleting based on several criteria (in bold, selection done and quality held).

Selection criteria	Sorption capacity	Bacterial compatibility	Testa adhesiveness
Carriers:			
Sépiolite	+	+	+
Attapulgite	+	–	+
Kaolinite	–		+
Vermiculite fine	–	+	+
Absorbents:			
Wessalon S	++		–
Perlite	++	+	–
Adhesives:			
Versicol S19		– –	++
HPMC		+	+
Surfactant:			
Lignosulfonate Na		+	

+ good – bad
++ very good – –very bad

Table 2. Evolution of the bacterial population in solid inocula after 1, 5, 10 and 15 incubation days. Results are expressed in cfu/g of carrier, mean of 3 repetitions ± standard deviation.

Incubation time	1 day	5 days	10 days	15 days
Vermiculite	$6.5 \times 10^8 \pm 0.25$	$7.5 \times 10^8 \pm 1.36$	$1.0 \times 10^9 \pm 0.97$	$3.5 \times 10^8 \pm 1.14$
Wheat bran	$3.3 \times 10^9 \pm 0.21$	$1.4 \times 10^{10} \pm 0.17$	$2.5 \times 10^{10} \pm 0.21$	$1.3 \times 10^{10} \pm 0.12$
Compost	$2.1 \times 10^9 \pm 0.16$	$3.7 \times 10^9 \pm 0.69$	$2.6 \times 10^9 \pm 0.21$	$1.0 \times 10^9 \pm 0.17$

Inoculation by seed pelleting

In the Table 3 appear the results of the bacterial counts (mean of four measurements and extreme figures):
– on inocula used for seed pelleting;
– on seeds directly after their pelleting and drying,
– on pelleted seeds after 7, 14, 28, 56 storage days at 25 °C.

Evaluation of nitrogen fixation potential

Protein production according to the kind of inoculation, expressed in mg per plant, is shown in Table 4. That table indicates also how many times seed pelleting has not completely crumbled away and has been partly drawn out of the substrate by the plant.

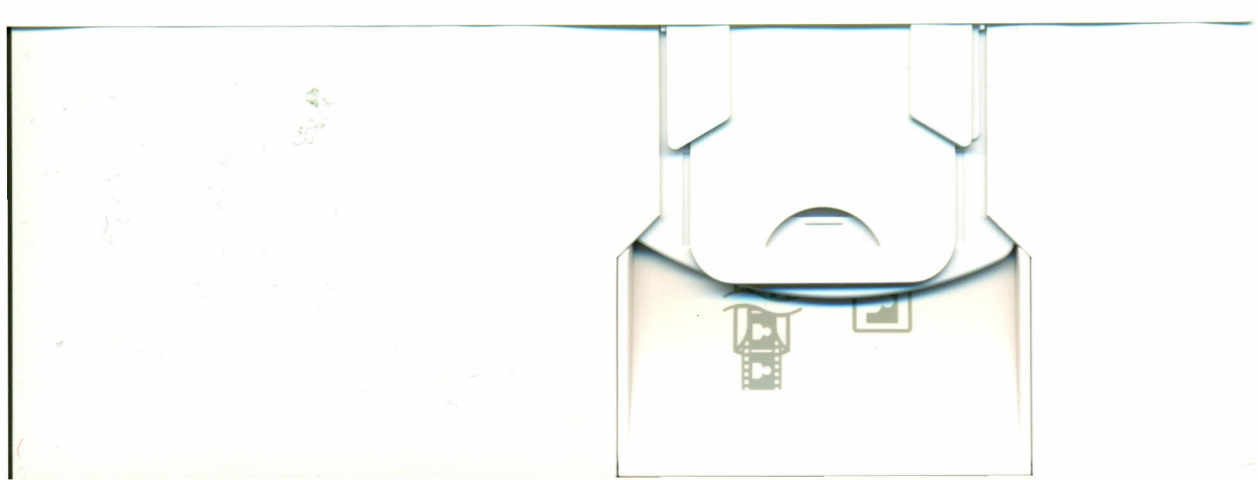


Table 3. Comparison of effectiveness of various pelleting formulations for incorporated bacterial populations survival: results after storage of pelleted seeds at 25 °C for 7, 14, 28 and 56 days (mean of 4 repetitions and extreme figures).

Inoculum content	Liquid inoculum		Solid inoculum with *'wheat bran'*		Solid inoculum with *'compost'*	
	Means	Extreme values	Means	Extreme values	Means	Extreme values
Before pelleting (cfu/ml)	5.9×10^8 *a*	$2.7 \times 10^8 - 7.2 \times 10^8$	4.6×10^9 *b*	$2.6 \times 10^9 - 5.7 \times 10^9$	2.7×10^9 *b*	$2.3 \times 10^9 - 3.4 \times 10^9$
After pelleting (cfu/seed)	2.4×10^4 *c*	$1.3 \times 10^4 - 4.1 \times 10^4$	1.5×10^7 *d*	$5.5 \times 10^6 - 2.8 \times 10^7$	3.5×10^7 *d*	$1.4 \times 10^7 - 5.8 \times 10^7$
(% nominal content)	(0.23%)		(9.2%)		(37.8%)	
After 7 days	9.8×10^2 *e*	$8.0 \times 10^2 - 1.1 \times 10^3$	1.1×10^6 *ff*	$7.0 \times 10^5 - 1.9 \times 10^6$	1.1×10^7 *dg*	$4.0 \times 10^6 - 1.2 \times 10^7$
After 14 days	–	–	4.1×10^5 *h*	$2.4 \times 10^5 - 7.8 \times 10^5$	5.1×10^6 *g*	$3.2 \times 10^6 - 9.0 \times 10^7$
After 28 days	–	–	1.9×10^5 *i*	$1.2 \times 10^5 - 4.0 \times 10^5$	1.2×10^6 *j*	$8.2 \times 10^5 - 2.0 \times 10^6$
After 56 days	–	–	1.1×10^5 *i*	$7.6 \times 10^4 - 1.9 \times 10^5$	8.2×10^5 *j*	$6.1 \times 10^5 - 1.1 \times 10^6$

a to j: values followed by the same letter are not significantly different according to Student's t-test at P = 0.05

Table 4. Evaluation of the nitrogen fixation potential according to the kind of inoculum, protein production, expressed in mg by plant (mean and standard deviation of 4 repetitions) and number of seeds of which pelleting was drawn out of the substrate.

	Protein production mean ± std deviation *(mg by plant)*	Number of seeds with pelleting partly drawn out of the substrate
Not inoculated	67 ± 6.0 *a*	–
Inoculated by soaking	264 ± 22.0 *b*	–
Liquid inoculum	150 ± 41.7 *ab*	2/16
Solid inoculum with 'wheat bran'	204 ± 67.8 *ab*	3/16
Solid inoculum with 'compost'	206 ± 60.3 *ab*	2/16

a,b: values followed by the same letter are not significantly different according to Student's t-test at P = 0.05.

Discussion

The utilization of 425 µm–850 µm vermiculite as a carrier for the production of solid inocula with the addition of either 'compost' or 'wheat bran' as a food base, allowed the obtaining of inocula in which the bacterial concentral concentration goes over 10^9 cfu/g, and even 10^{10} cfu/g for 'wheat bran'. The bacterial population reached a high between five and ten incubation days at 30 °C. 425 µm–850 µm vermiculite used without adding a solid food base never allowed the bacteria to reach those values, and therefore should not be used for pelleting.

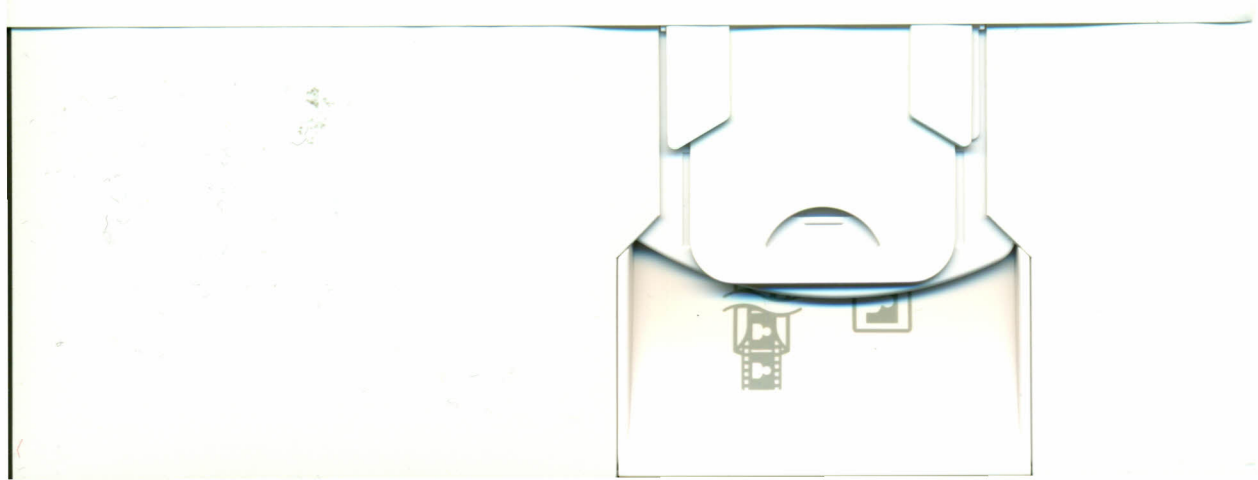
The use of inocula in a liquid form does not guarantee a population higher than 10^5 *Rhizobium* for one pelleted seed, which is necessary for an effective nodulation. Only 0.23% of the bacterial population is found again after pelleting and drying operations.

Bacterial population survival is clearly more important using an inoculum on a solid carrier made of 425 µm–850 µm vermiculite added with 'compost' or 'wheat bran' (respectively 37.8% and 9.2% of the initial population recovered after pelleting and drying). There is no significant difference between the solid inocula.

Using those two kinds of solid inocula, it is possible to guaranatee a bacterial population higher than 10^5 each seed after 56 storage days at 25 °C. The number of bacteria counted after that delay is statistically more important in the seed batches inoculated with the solid carrier added with 'compost' than those inoculated with the 'wheat bran' base solid carrier, notwithstanding a lower concentration of the inoculum at the beginning.

'Compost' appears then to play a part in the protection of *Rhizobium* during pelleting and storage of seeds, while 'wheat bran' shows a more pronounced multiplication role than 'compost'. It would therefore be possible to improve the preinoculation technique using 'wheat bran' and 'compost' together for solid inocula production.

The utilization of a solid kind of inocula for seed preinoculation seems to allow, in plants produced from those seeds, a higher protein production than in plants produced from preinoculated seeds with a liquid inoculum, even though the differences are not significant. That difference might be explained by the incomplete crumbling of the seed



pelleting, which makes a part of the inoculum ineffective for nodulation and protein production. That dragging out phenomenon is caused by the low density of the substrate used (vermiculite), and never happened when sowing in open ground (unpublished results). In any case, preinoculation obtains results clearly superior to an uninoculated control. Those results show that the various manipulations that the *Rhizobium* was subjected to did not affect the bacteria potential for nitrogen fixation.

It would be useful to compare, through an open field experiment, the performances of pelleted preinoculated seeds and 'classically' inoculated seeds.

Conclusions

Preinoculation of bean seeds by pelleting is possible using an inoculum made of 425 µm–850 µm vermiculite combined with 'compost' or 'wheat bran'. After 56 storage days at 25 °C, a bacterial population higher than 10^5 *Rhizobium* on a single pelleted seed was counted, assuring an effective nodulation. Most important a satisfactory bacterial population was obtained when unsing 'compost' made inocula as well as when using 'when bran' as a food base.

The different handlings needed for seed preinoculation do not affect the nitrogen fixation potential of the *Rhizobium* strain used.

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