VIABILITY OF NODULE FORMING BACTERIA ON STORED INOCULATED LEGUMINOUS SEED

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INTRODUCTION

It is generally accepted that legumes should be inoculated with their specific nitrogen fixing bacteria for good nodulation and nitrogen fixation (7, pp 247-249). Soil upon which legumes have recently been grown may contain a small number of Rhizobia. The seed naturally have a small number of the bacteria on them, but the organisms are either poor nitrogen fixing strains or are present in too small numbers to insure sufficient nodulation. It is therefore necessary to add superior commercial strains to obtain maximum nodulation and nitrogen fixation.

Although the beneficial results of seed inoculation has been established, it is difficult in practice to get the planter to cooperate in making such inoculations. Not only is there a great ignorance among farmers concerning seed inoculation of legume seed, but there is also a lack of confidence in its use. In some instances where inoculants are used, the most beneficial nodulation is not obtained because the inoculum is improperly applied. Yet not all the trouble originates with the farmer. The commercial inoculants often vary as to purity and numbers of organisms (10). (There is, however, an attempt on the part of three or four states to

rigidly control the identity and numbers of organisms of such cultures for commercial use.) Some cultures contain the proper numbers of organisms but have poor nitrogen fixing ability. Many are not dated or are left on the market after the expiration date (11). If the planter buys these inferior inoculants, he will not get full benefit for his added labors.

From this it can be seen that some means is needed to control the lack of uniformity. Effective batch inoculation by the commercial seed companies would indeed be a solution. If the seed distributor controlled this process, it would be possible to have all seed sold properly inoculated. It would save both time and trouble on the part of the planter. The seeds would be uniformly inoculated with pure cultures of good strains of Rhizobia.

If batch inoculation is to be possible, the Rhizobia must remain viable on the seed for a sufficient length of time to be effective after distribution. This period would probably be from six to nine months at the most.

Some remarkable reports of the longevity of Rhizobia have been made. Edwards (5) reported on the viability of clover and alfalfa organisms grown on wood ash maltose agar and sealed. They were found to produce nodules at 16 and 10 years respectively. Jones (12) also reported organisms alive after 15 years when stored at room temperature on Ashby's agar in sealed tubes. Even in water suspension under certain

conditions Rhizobia may remain viable for some time, as shown in the work of Albrecht and McCalla (2). It was found that viable nodulating organisms were alive in the tap water (which was rich in mineral salts) after three and one-half years of storage at room temperature. Rogers (18) found 1,000,000 organisms per gram of dried milk powder after storage for six months at room temperature. According to Albrecht (1) Rhizobia were found to be viable after seven years in moist soil under field conditions without the presence of their symbiont.

Previous to 1918 there had been no work done on the problem of batch inoculation. It is usually recommended that inoculated seed be planted as soon as possible in order that the bacteria may not die. Fellers (6) inoculated sterile alfalfa and soybean seed with Rhizobium cultures. He found the greatest loss of organisms occurred in the first few hours, after which they decreased more slowly. The organisms in very small numbers were viable after six months.

Richmond (17) inoculated seeds with muddy water prepared from soil upon which the legume crop had grown. Seed inoculated with muddy water from acid soil were free of Rhizobia within a week, while seed inoculated with muddy water from neutral soil retained viable organisms for almost a year.

In 1926 Allicante (3) made sugar, soil, and glue suspensions of the nodule bacteria for inoculation on legume seed. At the end of two months, when the experiment terminated,

nodules were observed on the plants with the sugar suspension treatment in greater numbers than on the plants with the soil or glue treatments. According to Alicante sugar protects against desiccation because of its moisture gathering characteristic. Soil was next best because of the film of moisture surrounding the soil particles.

In similar experiments Lochhead (13) used sweet skim milk to suspend organisms for inoculation. He found that he could get nodulation after six months and that refrigeration at 5° C favored longevity of the bacteria after drying.

Porges (15) has done the most recent work probably with the best results to date. He experimented with vetch and sweet clover, inoculating the sterile seed with a plant sap suspension of the organisms. He found that organisms when suspended with plant sap for inoculation were viable at the end of twenty-four weeks in greater numbers than those suspended in milk or the inoculum alone when all were stored at 5° C. Porges mentioned the possibilities of commercial distribution of inoculated seed but did not recommend such storage.

Fellers (6) used both a water and a gum tragacanth suspension of the organisms for the inoculation of the soybean and alfalfa seed. It had been shown by Giltner and Longeworthy (9) that Rhizobia survive a long time in air-dry soil because the moisture film surrounding the soil particle as well as the gum produced by the organisms themselves

helped to protect them. Since organisms naturally produce a gum capsule which helps protect the cell against adverse conditions, it is reasonable to think that if the organisms were imbedded in a coat of gum, there would be greater protection for the organisms enabling them to survive storage for longer periods. Fellers (6) did not find an appreciable difference between the effects of the water and gum inoculants. Even though there is some evidence against the use of the gum to help protect the organisms, it was thought advisable to reinvestigate the problem again using gum tragacanth.

For the most part the work mentioned in the literature to date has been done aseptically. This was done with the purpose of destroying all Rhizobia and other organisms on the seed coat so that the determination of organisms put on the seed coat would not be complicated by these contaminating organisms. The process of seed coat sterilization generally involves mercury and chlorine compounds. While both of these sterilize the surface of the seed effectively, they combine chemically with the organic matter on the seed coat so that it is practically impossible to entirely remove them by repeated washing. In certain cases the investigators have attempted to test for residual amounts of the sterilizing agent and found them to be negative. Such tests, however, would not detect these materials in the combined form. More than likely the organic mercury and chlorine compounds would have considerable effect on the longevity of the Rhizobia

when in contact with them over the storage period. For this reason it is felt that the probability of commercially inoculating the seed in large batches should be re-examined.

From Perkins' (14) work on soybean it was found that at least 25 to 50 organisms must be present on the seed to produce maximum nodulation for the plant. A more limited number of organisms were shown in turn to limit the number of nodules per plant. In a comparison of plate counts of bacteria on the seed with nodules on the plants, Hofer (10) showed that between 5 and 40 bacterial cells are required for the inoculation of an individual alfalfa and clover seed.

From such investigations it is apparent that larger numbers of organisms on the seed does not increase nodulation. Therefore, if at the end of nine months or a year there are at least 50 viable bacteria per seed, maximum nodulation will probably take place the same as if it had just been treated with a heavy inoculum of organisms. Consequently the object of this work is to reinvestigate the possibility of nitrogen fixing bacteria surviving on the seed in numbers greatly exceeding those required for good nodulation for a period of at least six to nine months. If this can be shown to be true, commercial inoculation of the legume seeds would be feasible.

METHODS

Two methods were used in the treatment of the seeds, the second being a modification of the first. The methods will be described as Experiment I and Experiment II.

Experiment I

Seed and organisms.—The seed of red clover, alfalfa, hairy winter vetch, and soybean (both Wilson and Manchu varieties) were used in this experiment. The respective nitrogen fixing symbionts, Rhizobium trifolii 205, Rhizobium meliloti 100 and 109, Rhizobium leguminosarum 301 and 302, Rhizobium japonicum 504, were taken from the V.P.I. stock cultures of proven strains. All four kinds of the above mentioned seed were treated in the same way. Where two strains of one species of organism are mentioned, a mixture of the two were used in the experiment.

Preliminary treatment.—The legume seed were first treated with 70 per cent alcohol for five minutes followed by two washings with sterile distilled water. The alcohol removes some of the lipid material on the seed so there is a better contact between the inoculum and the seed itself. With this removal of the lipid coating many of the contaminating organisms are also removed. Each kind of seed was then divided into six sets of at least 250 seeds.

Inoculation.—The six sets of seed (minimum of 250 seeds per set) were covered with water suspensions of their specific

nitrogen-fixing bacteria. These inocula contained approximately one billion organisms per ml. of water as determined by comparison with a barium sulfate turbidity standard. The bacterial suspension was removed from two sets of seed within thirty seconds, from the second two sets in twenty-four hours, and was not removed from the third two sets until the fourth day of contact.

Drying.—Air was filtered through cotton to remove dust particles thus removing bacteria and mold spores (Fig. 1A). For thirty minutes this filtered air was drawn over the seed which lay in the bottom of a glass suction flask (Fig. 1B).

Storage.—After drying, the seed were transferred asceptically to glass bottles. One set of each of the three inoculated treatments was placed at 5° C, and the duplicate sets were held at room temperature (approx. 23° C).

Controls.—For each of the four kinds of seed (clover, vetch, alfalfa, and soybean) three controls were kept. The first control was untreated seed which was kept at room temperature. The second was an alcohol washed, uninoculated seed, kept at 5° C and a duplicate of the latter kept at room temperature.

Medium.—The medium used for plate and dilution counts was Medium 79 as listed in Fred and Waksmen, Laboratory Manual of General Microbiology (8).

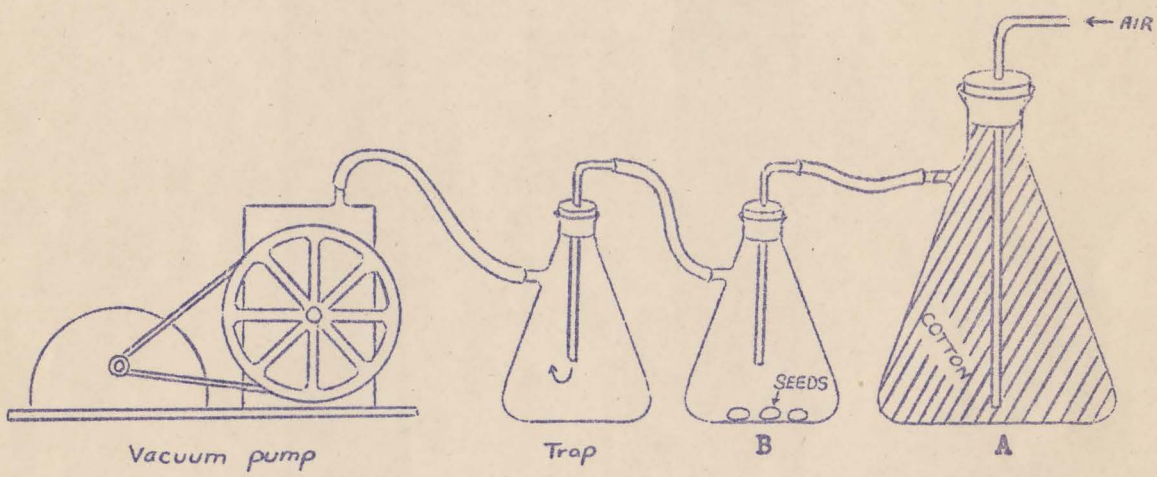


Diagram of the Drying Apparatus (Experiment I)

Figure 1

Medium 79

Mannitol	10.0 gm.
Dipotassium phosphate	0.5 gm.
Magnesium sulphate	0.2 gm.
Sodium chloride	0.1 gm.
Yeast water (single strength)	100.0 cc.
Distilled water	900.0 cc.

Single strength yeast extract

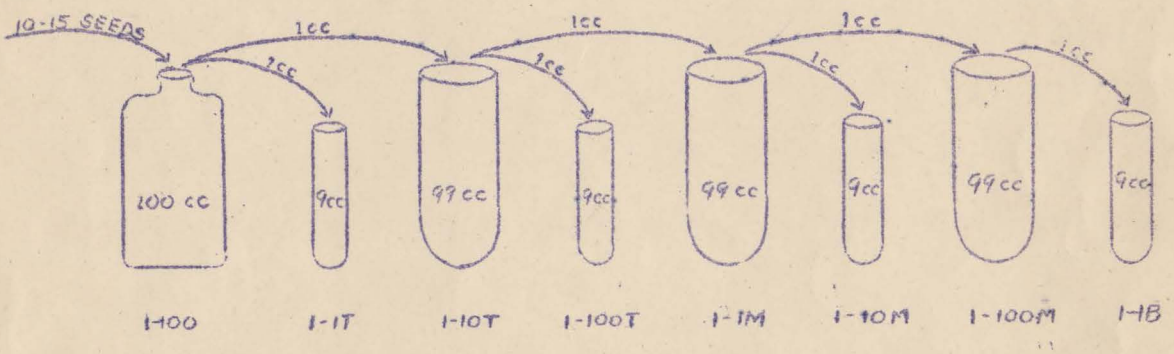
Yeast (dried starch free)	10.0 gm.
Distilled water	1000.0 cc.

The broth was used for dilution counts. Fifteen grams of agar was added to the broth for plate counting.

Counts.—Ten to fifteen seeds were shaken by a mechanical shaker in 100 cc. of sterile media for thirty minutes. From this, subsequent dilutions for plating were made. Figure 2 is the dilution plan used in the plating of all seeds.

Counts of the Rhizobia were made on all plates after ten days incubation at 30° C. Typical colonies were "fished" from the plates and inoculated into litmus milk. Rhizobia will not reduce or coagulate litmus milk while most other organisms likely to be present will reduce it (7 pp 84-85). Therefore, if the litmus was not reduced or coagulated, and the colonies were typical of Rhizobia it was considered in this case to be a positive check on the correct identification and count of only Rhizobia.

When dilution count method was used, the dilutions were made in nine cc. of Medium 79. The dilutions were made from the 100 cc. of medium in which the seed were shaken following



Dilution Plan for Plating

Figure 2

the dilution plan in Figure 3. Each dilution, made in triplicate, was shaken for three minutes before subsequent dilutions were made. They were then incubated for ten days at 30° C. All resulting growth was inoculated into litmus to check again for the presence of any contaminating organisms.

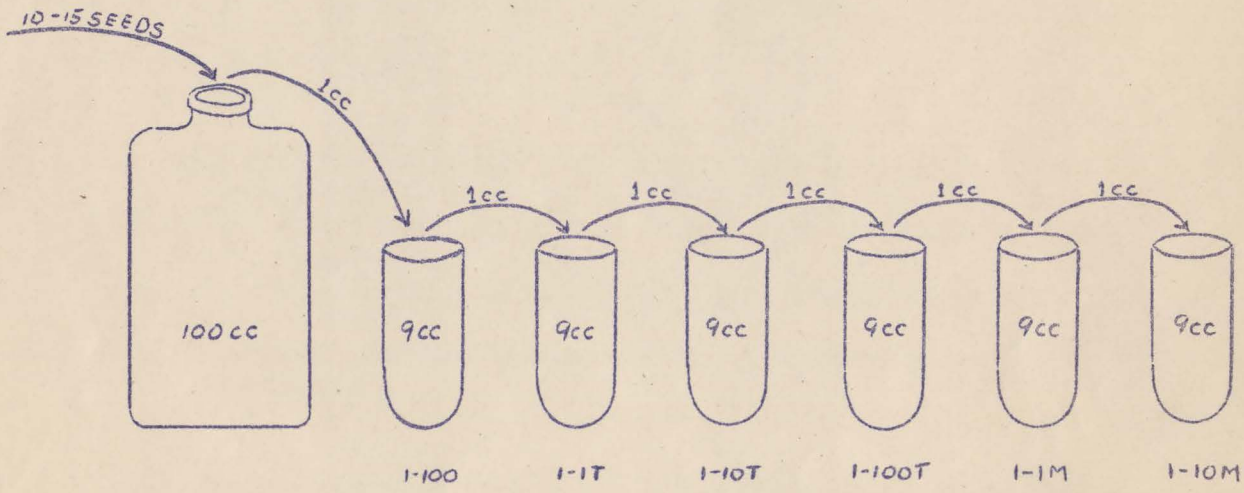
Nodulation.—From previous trials it was found that there was nearly 100 per cent germination of all seed. The seed were therefore planted directly in sterile, washed, white quartz sand. The nutrient solution was nitrogen free Crone's solution (4). To each flask of sand was added one milligram of nitrogen in the form of ammonium nitrate. A small amount of nitrogen has been shown to promote better nodulation (19). A paper cap was placed over each flask to prevent gross contamination. At the end of an eight-week period the plants were washed out of the sand and the average number of nodules per plant determined.

Experiment II

Seed and organisms.—The same seed and organisms were used in this experiment as were used in Experiment "I".

Preliminary treatment.—The seed were treated as before with 70 per cent alcohol for five minutes followed by two washings of sterile distilled water. They were then divided into eight sets, each containing 250 seeds.

Inoculation.—Two types of inoculants were used. Four sets of seed were covered with a water suspension inoculum



Plan for Dilution Count Method

Figure 3

which contained approximately one billion organisms per ml. of water as determined by comparison with a barium sulfate turbidity standard. The inoculum was removed from two sets of seed within thirty seconds, while the other two remained in contact with the inoculum for fifteen minutes.

The second group of four sets of alcohol washed seed were treated similarly except that the water inoculum was replaced by a one per cent gum tragacanth suspension of the organisms. The four treatments were:

1. Thirty second contact with water suspension of organisms
2. Fifteen minute contact with water suspension of organisms
3. Thirty second contact with gum suspension of organisms
4. Fifteen minute contact with gum suspension of organisms

Drying.--It was found that the drying method used in Experiment "I" did not thoroughly dry the seeds. The water absorbed by the seed during the treatment and inoculation was not sufficiently removed in all cases to prevent some growth of mold. Since the alcohol treatment did not sterilize the seed, the presence of one viable mold spore in a set of seed would be enough to contaminate the whole set with mold growth. With the exception of seven sets all in Experiment "I" were overgrown. For this reason the method was modified in favor of the following procedure:

Air was dried by passage through a sulfuric acid-calcium chloride air train (Fig. 4A and B). A seed rack of

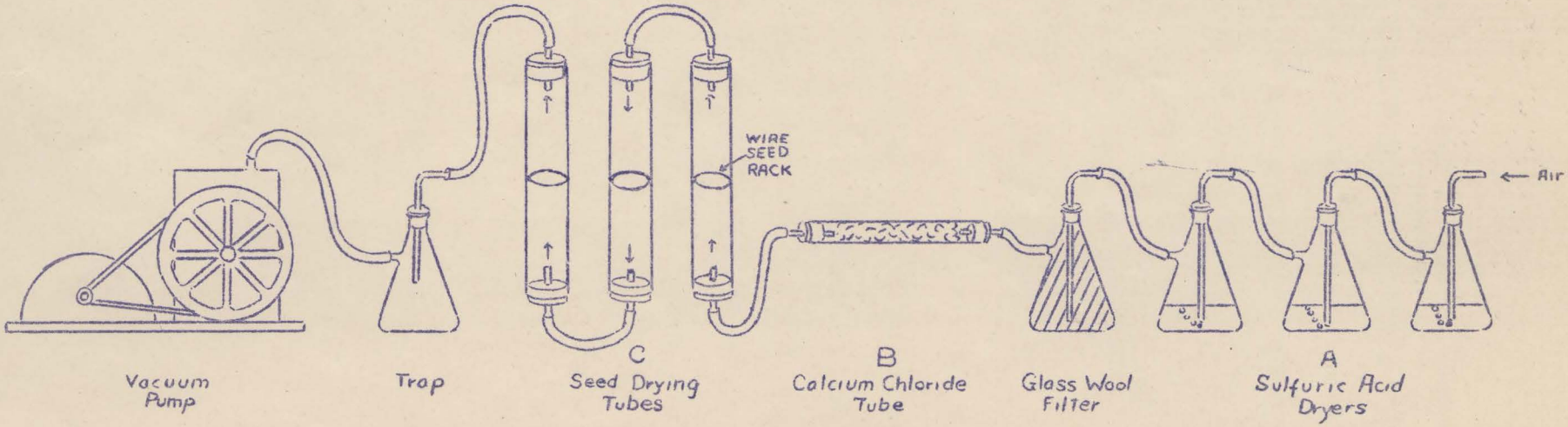


Diagram of the Drying Apparatus (Experiment II)

Figure 4

brass wire gauze covered with two layers of cheesecloth was placed in the middle of each drying tube (Fig. 4C). For thirty minutes dried air was drawn through the treated seed placed on these racks. The seed were then placed for two days in a calcium chloride desiccator which was held at 5° C.

Storage.--After drying the seed were transferred asceptically to glass bottles. One set of each of the four treatments was stored at 5° C, and the duplicate sets were held at room temperature.

Controls and medium.--Similar controls as those used in Experiment "I" were used in this experiment. The planting and dilution media were also the same in both procedures.

Counts and nodulation.--The method of counting the organisms and determining the nodulation was the same in both experiments.

DISCUSSION OF RESULTS

Previous work on batch inoculation, for the most part, has been done asceptically. The results in Table 1 show that sterilization of the seed before inoculation is not necessary.

Table 1.—Controls — Count of Bacteria on Stored Seed

Type of Control	Alfalfa Bacteria per seed	Soybean Bacteria per seed	Vetch Bacteria per seed	Clover Bacteria per seed
Untreated control Stored at 23° C	3.1 5.0 6.8	232 300 251	11 17 22	28 50 45
Alcohol washed Stored at 23° C	0.8 0.3 0.5	3.4 8.0 4.6	3.5 3.2 3.0	0.6 0.5 1.0
Alcohol washed Stored at 5° C	2.5 1.0 2.6	5.0 5.4 5.0	1.0 4.5 4.0	1.1 2.0 1.3

Plate counts of the seed used in the experiment showed no evidence of Rhizobia, so the above table is composed of the total number of contaminating organisms. These counts were made at intervals over a period of five months. Untreated seeds showed a relatively high contamination count, one which would possibly interfere with the counting of the test organisms. The alcohol probably removed some of the lipid coating of the seed and at the same time removed most of the contaminating organisms. Those organisms left are in such small numbers that there is little chance that they would

appear on plates above a 1 to 10 dilution. Only in a few cases were dilutions of less than 1 to 100 used in counting Rhizobia. Correct identification of colonies of these organisms was routinely checked by inoculating "fished" colonies into litmus milk. This makes possible the counting of only Rhizobia. With this method of checking and counting the test organisms there is no need for sterilization of the seed coat.

Table 2 is compiled from the results obtained in Experiment "I". Various investigations of Perkins and Hofer (14, 10) have shown that a maximum of 50 Rhizobia per seed are required for good nodulation. The initial bacterial count for all four types of seed is well over a million. Even after 10 months storage the inoculated Rhizobia remain on the seed in numbers greatly exceeding those required for good nodulation. After 11 months storage all treatments were still in excess of this number excepting clover which fell below the required number.

Tables 3 to 10 inclusive are compiled from the results of Experiment "II". In the third month microscopic colonies were observed by chance on the various plates of each treatment. These colonies range from 0.05 mm to 0.15 mm while the typical macro colonies ranged from 0.6 mm to 8.0 mm. Although the micro colonies are within the range of vision with the naked eye, they are so small that they are easily

Table 2.—Viability of *Rhizobia* on Seed Stored at 5° C (Experiment I)

Inoculation Time	Storage Time	Alfalfa Bacteria per seed	Clover Bacteria per seed	Vetch Bacteria per seed	Soybean Bacteria per seed
5 minutes	2 days	1,100,000	1,300,000	13,500,000	122,000,000
	10 months	3,250	320	2,290	17,200
	11 months	1,300	28	-----	117,000
4 days	2 days	1,400,000	-----	18,500,000*	169,000,000
	10 months	38,600	-----	2,710*	51,400
	11 months	36,100	-----	850*	42,000

*one day inoculation time

overlooked or mistaken for small precipitated particles in the medium. For correct count and identification of these colonies the plates were examined under a binocular microscope. The aging of the organisms in combination with the extreme dessication of the seeds is the probable cause of the very small colonies. It is not known whether or not the organisms that produce these colonies possess the ability to form nodules and fix nitrogen.

Temperature at which the inoculated seed is stored is an important factor in the survival of Rhizobia. From the results recorded in Tables 3 to 10 it is shown that these seeds stored at 5° C had greater numbers of viable organisms at 2, 3, 4, 5, and 6 months than the same seeds stored at room temperature for the same length of time. At first glance this does not appear to be completely true of the organisms on the vetch seeds of 30 second inoculation time (Table 4). Upon examination it is seen that the seeds stored at 5° C had a much lower initial organism count and that the percentage of dying off is really greater for those seeds stored at room temperature. At the fifth month the seeds stored at 5° C showed superiority of numbers.

The amount of moisture present during storage apparently is another important factor affecting the survival of the organisms. In almost every case the organisms embedded in gum tragacanth survived in greater numbers than those put on the seed by only a water suspension inoculum. Since the Rhizobia produce a large amount of gum naturally, it is probable that embedding the organisms in a gum coating would help to protect the Rhizobia

Table 3.-- Viability of *Rhizobia* on Stored Vetch Seed

Storage Temp.	30 seconds inoculation time				15 minutes inoculation time			
	23° C		5° C		23° C		5° C	
Storage Time	Organisms per seed		Organisms per seed		Organisms per seed		Organisms per seed	
Months	Macro colonies	Micro colonies	Macro colonies	Micro colonies	Macro colonies	Micro colonies	Macro colonies	Micro colonies
0	16.6 M	-	19.8 M	-	29.1 M	-	28.8 M	-
1	68 T	-	73 T	-	81 T	-	93 T	-
3	630	1100	-	-	-	-	-	-
4	<100	5850	1670	2520	<100	5850	70	420
5	25	137	3750	1530	11	39	-	-

T - Thousand M - Million

Table 4.-- Viability of *Rhizobia* on Stored Vetch Seed

Inoculum suspended in one per cent gum tragacanth

Storage Temp.	30 seconds inoculation time				15 minutes inoculation time			
	23° C		5° C		23° C		5° C	
Storage Time	Organisms per seed		Organisms per seed		Organisms per seed		Organisms per seed	
Months	Macro colonies	Micro colonies	Macro colonies	Micro colonies	Macro colonies	Micro colonies	Macro colonies	Micro colonies
0	11.5 M	-	12.3 M	-	36 M	-	41 M	-
1	55 T	-	59 T	-	96 T	-	13.8 M	-
3	4290	-	1786	-	1696	-	>24 T	-
4	1810	1310	1070	2860	3720	2430	54.2 T	-
5	25	1110	4540	660	160	680	40 T	23 T

T - Thousand M - Million

Table 5.-- Viability of Rhizobia on Stored Clover Seed

Storage Temp.	30 seconds inoculation time				15 minutes inoculation time			
	23° C		5° C		23° C		5° C	
Storage Time	Organisms per seed		Organisms per seed		Organisms per seed		Organisms per seed	
Months	Macro colonies	Micro colonies	Macro colonies	Micro colonies	Macro colonies	Micro colonies	Macro colonies	Micro colonies
0	1.15 M	-	.95 M	-	1.74 M	-	1.52 M	-
1	75.4 T	-	15.3 T	-	12.4 T	-	84 T	-
2	1080	-	1720	-	1280	-	3153	-
3.5	3	97	<100	214	6	149	690	-
4	213	-	500	1770	13	2000	173	193
5	<100	986	253	840	<100	853	753	740
6	1	116	680	413	1.5	68	1090	406

T - Thousand M - Million

Table 6.-- Viability of Rhizobia on Stored Clover Seed

Inoculum suspended in one per cent gum tragacanth

Storage Temp.	30 seconds inoculation time				15 minutes inoculation time			
	23° C		5° C		23° C		5° C	
Storage Time	Organisms per seed		Organisms per seed		Organisms per seed		Organisms per seed	
Months	Macro colonies	Micro colonies	Macro colonies	Micro colonies	Macro colonies	Micro colonies	Macro colonies	Micro colonies
0	2.09 M	-	1.91 M	-	1.47 M	-	1.65 M	-
1	9340	-	14.8 T	-	11.4 T	-	15.3 T	-
2.5	< 100	152	610	-	162	204	1240	-
3	1320	-	3300	-	< 100	1433	226	460
4	12	1353	1880	680	-	1213	280	1000
5	0.5	122	321	293	0.8	80	156	2650

T - Thousand M - Million

Table 7.-- Viability of *Rhizobia* on Stored Alfalfa Seed

Storage Temp.	30 seconds inoculation time				15 minutes inoculation time			
	23° C		5° C		23° C		5° C	
Storage Time	Organisms per seed		Organisms per seed		Organisms per seed		Organisms per seed	
Months	Macro colonies	Micro colonies	Macro colonies	Micro colonies	Macro colonies	Micro colonies	Macro colonies	Micro colonies
0	832 T	-	850 T	-	901 T	-	866 T	-
1	16.5 T	-	16.1 T	-	18.6 T	-	13.9 T	-
2	980	-	2800	-	2660	-	3540	-
3.5	4	-	393	-	5	-	1400	-
5	113	920	233	1126	4	873	853	1820
6	1.6	158	98	503	78	79	148	1786

T - Thousand M - Million

Table 8.-- Viability of Rhizobia on Stored Alfalfa Seed

Inoculum suspended in one per cent gum tragacanth

Storage Temp.	30 seconds inoculation time				15 minutes inoculation time			
	23° C		5° C		23° C		5° C	
Storage Time	Organisms per seed		Organisms per seed		Organisms per seed		Organisms per seed	
Months	Macro colonies	Micro colonies	Macro colonies	Micro colonies	Macro colonies	Micro colonies	Macro colonies	Micro colonies
0	918 T	-	1000 T	-	933 T	-	181 T	-
1	18.5 T	-	25.3 T	-	20.7 T	-	28.0 T	-
2	2720	-	4940	-	3130	-	5260	-
3.5	-	-	1460	-	693	-	1650	-
5	106	746	913	1066	560	880	1126	1446
6	3	52	196	1300	38	55	733	906

T - Thousand M - Million

Table 9.-- Viability of *Rhizobia* on Stored Soybean Seed

Storage Temp.	30 seconds inoculation time				15 minutes inoculation time			
	23° C		5° C		23° C		5° C	
Storage Time	Organisms per seed		Organisms per seed		Organisms per seed		Organisms per seed	
Months	Macro colonies	Micro colonies	Macro colonies	Micro colonies	Macro colonies	Micro colonies	Macro colonies	Micro colonies
0	181 M	-	218 M	-	159 M	-	224 M	-
1	10 M	-	84 M	-	41 M	-	97 M	-
2	69 T	0	28 M	0	29 T	0	36 M	0
3	40 T	0	4.7 M	0	1520	0	7500 T	0

T - Thousand M - Million

Table 10. -- Viability of Rhizobia on Stored Soybean Seed

Inoculum suspended in one per cent gum tragacanth

Storage Temp.	30 seconds inoculation time				15 minutes inoculation time			
	23° C		5° C		23° C		5° C	
Storage Time	Organisms per seed		Organisms per seed		Organisms per seed		Organisms per seed	
Months	Macro colonies	Micro colonies	Macro colonies	Micro colonies	Macro colonies	Micro colonies	Macro colonies	Micro colonies
0	162 M	-	170 M	-	154 M	-	119 M	-
1	16 M	-	25 M	-	21 M	-	30 M	-
2	710 T	0	1050 T	0	821 T	0	992 T	0
3	98 T	0	364 T	0	166 T	0	215 T	0

T - Thousand M - Million

Table 11.-- Comparison of Organisms per Seed with Nodules Produced per Plant

	Inoculum Suspension in	Inoculation Time	Storage Temp.	Storage Time Months	Rhizobia per seed* M ± 3 x P.E.	Nodules per plant** M ± 3 x P.E.
1.	water	30 sec.	5° C	9	2940 ± 39	11.3 ± 2.3
2.	"	4 days	5° C	9	27500 ± 550	18.6 ± 6.8
3.	"	30 sec.	5° C	3.5	394 ± 28	13.9 ± 4.1
4.	"	15 min.	5° C	3.5	1400 ± 118	14.5 ± 6.0
5.	gum	30 sec.	5° C	3.5	1460 ± 26	13.3 ± 2.9
6.	"	15 min.	5° C	3.5	1650 ± 44	12.1 ± 3.2
7.	water	30 sec.	23° C	3.5	4	0
8.	gum	30 sec.	23° C	3.5	--	0
9.		Alcohol washed control			0	0
10.		Untreated control			0	0

* average of five plates
** average of 8 to 10 plants

against desiccation. The number of surviving organisms in Experiment "II" stored for four months is about the same as the number of organisms stored for 10 months in Experiment "I". The use of a more severe method of drying (after inoculation) in Experiment "II" may account for the difference. It appears, therefore, that the amount of moisture is also an important factor in the survival of the organisms during storage.

The use of a protecting agent for the Rhizobia inoculated on seed and subsequently stored has already been tested by various investigators (3, 13, 15) and found to have some benefit. Gum tragacanth, on the other hand, was not found to aid in the survival of the organisms (6). The present investigation, however, as shown in Tables 3 to 10, demonstrated that a gum agent may be of value in protecting the Rhizobia when inoculated onto legume seeds. The organisms survived storage in greater numbers when protected by a coating of gum tragacanth than when applied to the seed as a water suspension. Although the margin was not large, the tendency for greater viability of the organisms with the use of the gum was marked.

Two treatments in inoculation time (30 seconds and 15 minutes) were used (Tables 3 to 10). The results were too inconsistant to be reliable, and a safe conclusion can not be drawn. When the inoculum was in contact with the seed for a period of four days (Table 2) considerably higher total counts of Rhizobia on the legume seeds were noted than when the

inoculum was applied for a shorter time.

Dilution counts made in conjunction with the plate counts of the organisms on the stored inoculated seeds checked in every case, or showed a count of approximately ten times the number found by plating. (This difference is a usual phenomenon observed in comparing the two methods.)

Because of the extreme desiccation of the organisms and seeds in experiment "II" the count of Rhizobia per seed dropped off much faster than the counts of the organisms in Experiment "I". The bacteria on seed stored at 23° C (Experiment "II") were reduced to numbers too small for good nodulation. However, those stored at 5° C still possessed numbers that greatly exceed that required for good nodulation.

The number of nodules produced on alfalfa when the treated seed was sprouted and grown under sterile conditions was compared with the number of organisms on the seed. To test the significance of any differences found, a range of plus or minus three times the probable error was chosen, the odds being 22 to 1 that the true value lies inside this range.

From the results shown in Table 11 it would appear that the second treatment (four days inoculation time with nine months storage) had the highest count of organisms per seed and, therefore, the highest nodule count. Closer examination will show that the probable error ranges of the nodule counts of all treatments overlap and that the error for the highest

count is large enough to overlap that of the lowest. There is then no significant difference between any of the nodule counts. The great excess of organisms on the seeds of the second treatment, therefore, has no greater effect in producing nodules than does the number of organisms of the third treatment (30 second inoculation time with four months storage) which has the lowest number of organisms per seed.

The alfalfa plants possessing nodules were tall, healthy, green plants as compared with the stunted, red stemmed, yellow nitrogen deficient plants of the control. The results show that Rhizobia inoculated on seed and stored are viable and still have the ability of forming nodules and fixing nitrogen after nine months.

In a trial experiment (30 seconds inoculation time of gum suspended inoculum, 4 month storage at 5° C) of both alfalfa and clover, seed were removed from the refrigerator and were placed at room temperature for a month, one part in the light and the other in the dark. The results showed that the organisms held for a month at room temperature were favored by the dark. The change of temperature did not seem to effect the death rate of the organisms in the case of alfalfa. The change to room temperature increased the death rate for the clover organisms, but did not lower the count below the maximum 50 needed for good inoculation. This small amount of evidence is not conclusive, but the application of this is readily seen. If the seed company employed batch inoculation

and stored their seed at the more favorable temperature of 5° C, it would be important to know how long a time could elapse for shipment, for storage by the grower and for planting before the bacteria would become reduced in numbers too small for maximum nodulation.

DISCUSSION OF THE PROBLEM

The results of this investigation have shown that inoculated Rhizobia may remain on the seed at least eleven months in numbers greatly exceeding those required for good inoculation. The temperature of storage and the amount of moisture present are both important factors in the survival of Rhizobia on seed. Commercial batch inoculation appears to be feasible, provided the temperature and moisture conditions of seed storage are controlled. With the knowledge that the organisms can be expected to survive long storage periods in large numbers, further work can be undertaken to make commercial inoculation practical. As the problem now stands there are probably other uncontrolled factors besides the storage temperature and humidity. When the proper control of such conditions has been worked out, large scale methods of inoculation could then be tested.

Moisture, which appears to be a very important factor, has not been sufficiently controlled in the present investigation to determine quantitatively the exact requirements for best longevity of Rhizobia. Optimum humidity for seed storage will probably increase the viability of the Rhizobia. (Optimum humidity in such work might be defined as the relative humidity low enough to prevent mold growth on the seed but high enough to maintain viability of the inoculated bacteria.)

Since the refrigeration temperature of 5° C (41° F) was the only low storage temperature available, it was used in this investigation. Ordinary warehouse temperatures probably would not be as low. Further experimentation should be carried out higher than 5° C (41° F) to determine the relative viability at temperatures that would more nearly correspond with those in the warehouse during the winter and early spring.

Another uncontrolled factor is the number of organisms in the inoculum and the size of the seed. The maximum number of Rhizobia required for good nodulation is about the same for all four of the seed sizes used in this experiment. The largest seeds would carry more organisms, and the larger the inoculum, the larger the initial count per seed. Therefore, an inoculum of a minimum number of organisms would have to be determined to insure nodulation after the storage period.

The results of this thesis definitely indicate the feasibility of commercial inoculation of legume seed. It would be necessary, however, to work out certain details previously discussed in this paper before such inoculation could be attempted commercially.

SUMMARY

Batch inoculation of legume seeds by commercial companies would remedy many of the difficulties encountered in getting proper and uniform inoculation by all planters. From the results of this investigation, which was to test the feasibility of commercial inoculation, the following statements appear to be justified.

1. Inoculated Rhizobia may remain on their specific legume seed when stored at 5° C at least 11 months in numbers greatly exceeding those required for good nodulation.

2. Sterilization of the seed before inoculation was not necessary in this experiment because of the low total organism count on the seed and because precautions were taken to check the identification of the test organisms.

3. Those treated legume seeds stored at 5° C had greater numbers of viable Rhizobia at 2, 3, 4, 5, and 6 months than the same treatments stored at room temperature for the same length of time.

4. It appeared that the amount of moisture present during storage is an important factor in the survival of the organisms regardless of storage temperature.

5. The Rhizobia on seeds inoculated with a suspension of the organisms in one per cent gum tragacanth solution withstood the desiccation and storage to a higher degree than organisms inoculated onto the seed suspended only in a water medium.

6. In the third month microscopic colonies ranging from 0.05 mm to 0.15 mm were observed on the various plates of each treatment. The significance of these small colonies is not known.

7. When the stored inoculated seed was sprouted and grown under otherwise sterile conditions, it was found that although the average number of organisms per seed varied considerably for the various seed treatments, there was no significant difference between the average nodules produced per plant for the same treatments.

The results of this thesis definitely indicate the feasibility of commercial inoculation of legume seed. It would be necessary, however, to work out certain details previously discussed in this paper before such inoculation could be attempted commercially.

REFERENCES CITED

1. Albrecht, W.A.

   Studies on the longevity of B. radicicola in the soil. Missouri Agri. Exp. Sta. Bul. 189: 54-56, 1921

2. Albrecht, W.A. and McCalla, T.M.

   Longevity of legume bacteria (Rhizobium) in water. Jour. Amer. Soc. Agron. 29: 76-79, 1937

3. Alicante, M.M.

   The viability of the nodule bacteria of legumes outside of the plant. Soil Sci. 21: 27-52, 1926

4. Bryan, O.C.

   Effect of different reactions on the growth and nodule formation of soybeans. Soil Sci. 13: 271-287, 1922

5. Edwards, S.F.

   A note on the longevity of some cultures of B. radicicola. Abs. Bact. 7: 9, 1923

6. Fellers, C.R.

   The longevity of B. radicicola on legume seeds. Soil Sci. 7: 217-232, 1918

7. Fred, E.B., Baldwin, I.L., and McCoy, Elizabeth

   Root Nodule Bacteria and Leguminous Plants. University of Wisconsin Press, Madison, 1932

8. Fred, E.B., and Waksman, S.A.

   Laboratory Manual of General Microbiology. McGraw-Hill Book Company, Inc., New York, 1928

9. Giltner, W. and Longworthy, H.V.

Some factors influencing the longevity of soil micro-organisms subjected to desiccation with special reference to soil solution. Jour. Agri. Research (U.S.) 5: 927-842, 1916

10. Hofer, A.W.

The number of legume bacteria in commercial cultures as related to nodule formation. Jour. Amer. Soc. Agron. 30: 451-460, 1938

11. Hofer, A.W. and Conn, H.J.

Legume inoculant tests in 1931. N.Y. State (Geneva) Agri. Exp. Sta. Bul. 602, 1-11, 1931

12. Jones, D.H.

The viability of Rhizobium legumenosum Frank and Rhizobium radicicola Beijerinck. Jour. Bact. 13: 55-56, 1927

13. Lochhead, A.G.

The longevity of legume bacteria on inoculated alfalfa seed. Sci. Agric. 7: 179-187, 1927

14. Perkins, A.T.

The effect of bacterial numbers on the nodulation of Virginia soybean. Jour. Agri. Research 30: 95-96, 1925

15. Porges, Nandor

The longevity of legume bacteria on seed as influenced by plant sap. Soil Sci. 32: 481-487, 1931

16. Reid, J.J., Fred, E.B., and Baldwin, I.L.

Effects of storage on Rhizobia. Jour. Bact. 29: 75-76, 1935

17. Richmond, T.E.

Longevity of the legume nodule organism. Jour. Amer. Soc. Agron. 18: 414-416, 1926

18. Rogers, L.A.

The preparation of dried cultures. Jour. Infect. Diseases 14: 100-123, 1914

19. Wilson, P.W., and Wagner, F.C.

Combined nitrogen and the nitrogen fixation process in leguminous plants. Wis. Acad. Sci. Trans. 30: 43-50, 1935