Circular No. 703

May 1944 • Washington, D. C.

UNITED STATES DEPARTMENT OF AGRICULTURE

Method of Testing Legume Bacteria Cultures and Results of Tests of Commercial Inoculants in 1943

By L. T. Leonard, *bacteriologist, Division of Soil and Fertilizer Investigations, Bureau of Plant Industry, Soils, and Agricultural Engineering, Agricultural Research Administration*

CONTENTS

NODULE BACTERIA form an association with the roots of leguminous plants through which nitrogen gas is taken from the air and made available for plant growth. These bacteria are widely distributed in soils, but as there are a number of species, each functioning satisfactorily on certain legumes only, and as strains of the same species vary in their ability to fix nitrogen, the proper nodule bacteria may be lacking in some field soils. They may be isolated from their nodules, tested for efficiency, and propagated to almost any degree under artificial conditions. Commercial laboratories prepare such materials for sale to planters. Whether it is advisable to use artificially prepared cultures of legume bacteria is a matter for local experience to determine.

COMMERCIAL INOCULANTS

At present 11 concerns are engaged in the production of nodule bacteria cultures, or legume inoculants, as they are sometimes called (table 1). For commercial distribution inoculating materials are prepared in "carriers," which are mainly agar (jelly) and moist peat powder. The use of the former within recent years has been declining.

Agar cultures are made by spraying under sterile conditions a suspension of pure nodule bacteria on the surface of hardened sterile special nutrient agar in glass bottles. Usually these bottles are capped with sterilized metal screw caps. The inoculated bottles are incubated at the proper temperature for the organisms they contain, and when good growth is obtained they are packaged and shipped to the merchandiser or consumer. The agar culture must be a pure culture of nodule bacteria, since the growth of contaminants under the extreme artificial conditions may injure the organisms and by appearance may affect the salability of the product.

TABLE 1.—*List of commercial seed and soil inoculants sold in the United States in the fiscal year 1943* [1]

Trade name	Carrier	Manufacturer and distributor	Address
Cal-Rhiz Legume Bacteria	Paste (?)	Nelson Laboratories	Stockton, Calif.
Farmogerm	Agar	Earp-Thomas Laboratories, Inc	Bloomfield, N. J.
Humogerm	Peat	do	Do.
G. L. F. Legume Inoculant	Charcoal	Cooperative G. L. F. Mills	Ithaca, N. Y.
Hansen's Humus Inoculator	Peat	Hansen Inoculator Co	Urbana, Ill.
Certigerm	do	Eastern States Milling Corp	Buffalo, N. Y.
Kelly's Humus Inoculator	Peat	Kelly Seed & Hardware Co	Peoria, Ill.
Kelly's Inoculator	Agar	do	Do.
National Cooperative Humus Inoculator.	Peat	Indiana Farm Bur. Coop. Assn	Indianapolis, Ind.
Legume-Aid	do	Agricultural Laboratories, Inc	Columbus, Ohio.
Blue Seal Grow Crop Inoculant.	do	Illinois Farm Bur. Purchasing Agency.	Chicago, Ill.
Sacco Legume Inoculant	do	Smith Agricultural Chemical Co	Columbus, Ohio.
Legumogen	do	The Legumogen Laboratories, Inc	Delphi, Ind.
Nitragin	do	The Nitragin Co	Milwaukee, Wis.
Nod-O-Gen Pretested Inoculator.	do	The Albert Dickinson Co	Chicago, Ill.
Badger Brand Inoculation	do	L. Teweles Seed Co., Inc	Milwaukee, Wis.
Cofer's Hitest Inoculator	do	Cofer Seed Co	Athens, Ga.
Gold Medal Inoculator	do	Louisville Seed Co	Louisville, Ky.
Hoffman's Inoculator	do	A. H. Hoffman, Inc	Landisville, Pa.
Kellogg's Rainbo Inoculator	do	Kellogg Seed Co	Milwaukee, Wis.
M-Inoculator	do	Ed. F. Mangelsdorf & Bro., Inc	St. Louis, Mo.
May-Bell Inoculator	do	Ross Seed Co	Louisville, Ky.
Ohio Farmers' Seed Inoculation.	do	Ohio Farmers' Grain & Supply Co	Fostoria, Ohio.
Red Bird Inoculator	do	Lewis Seed Co	Louisville, Ky.
Sioux Brand Inoculator	do	Sioux City Seed Co	Sioux City, Iowa.
Trinity Brand Inoculator	do	Belt Seed Co	Baltimore, Md.
Wood's Seed Inoculation	do	T. W. Wood & Sons	Richmond, Va.
Superyield Culture	Agar	Strasburger & Siegel	Baltimore, Md.
Uni-Culture	Peat	Kalo Inoculant Co	Quincy, Ill.
American Tubercle Germ	do	American Field Seed Co	Chicago, Ill.
Berry's Superior Nodule Germ.	do	Berry Seed Co	Clarinda, Iowa.
Blue Ribbon Inoculation	do	Allied Seed Co	Fort Wayne, Ind..
Corneli Keystone Inoculation	do	Corneli Seed Co	St. Louis, Mo.
Farm Master Nitrogen-Fixing Inoculant.	do	Sears, Roebuck & Co	Chicago, Ill.
Kalo Inoculation	do	Kalo Inoculant Co	Quincy, Ill.
Perfection Brand Legume Inoculant.	do	Geo. P. Sexauer & Son	Des Moines, Iowa.
Southern States Quality Inoculation.	do	Southern States Cooperative	Richmond, Va.
Sunfield Inoculant	do	Sunfield Seed Co	Chicago, Ill.
Urbana Culture	Agar	The Urbana Laboratories	Urbana, Ill.
Urbana Humus Inoculator	Peat	do	Do.

[1] Indented trade names indicate material originating in same laboratory as that of the unindented name above; indented names of firms following those of manufacturers denote distributors of subbrand inoculants.

Peat-powder inoculants, on the other hand, are usually prepared commercially by developing a heavy suspension of pure nodule bacteria in aerated liquid nutrients in protected tanks. When at the right stage of growth, the suspension is withdrawn and mixed thoroughly with neutralized peat powder. Further processing may include (1) curing (allowing to stand in a bin or tub for a few days), (2) additional mixing to obtain particle uniformity, and (3) packaging. It is then ready for the retail trade. Prepared in this manner inoculants are not pure cultures, but the presence of other organisms does not seem to affect their efficiency, and they are not sufficiently in evidence as a rule to affect the salability of the product.

The most important ingredient in an inoculant is its content of efficient living organisms. Under the conditions of transportation and storage these organisms may be gradually killed. The useful

period, therefore, is necessarily limited. This is usually less than a year, and the end of such period is ordinarily shown on the label of the package.

REGULATION OF SALE

For the protection of the planter against products of incompetents and from depreciated material some regulation of the industry is desirable. In addition to certain regulations by the United States Department of Agriculture, the following State institutions are charged with the regulation of the sale of inoculants within their State borders: Indiana Agricultural Experiment Station, at La Fayette; Kansas State Board of Agriculture, at Topeka; University of Maryland, at College Park; New Jersey Agricultural Experiment Station, at New Brunswick; Feed and Fertilizer Control Office, State College, N. Mex.; New York Department of Agriculture and Markets, at Albany; Ohio Department of Agriculture, at Columbus; and Wisconsin Department of Agriculture and Markets, at Madison. Some of these do not make tests of samples.

Where testing of samples is done, it is customary to collect each season sufficient material from each producer to give a satisfactory test of the product. Inoculants are sold by seed and hardware stores, grain elevators, mills, and farm agencies.

METHOD OF TESTING

The function of cultures of nodule bacteria is to produce stimulatory effects by furnishing nitrogen to the legumes to which they are applied. Under practical conditions commercial inoculants are used in the field, and it would seem advisable to test them under similar conditions. Field testing, however, is unsatisfactory on account of several factors, among which may be mentioned the almost universal presence of some species of nodule bacteria and varying climatic and soil conditions, some of which cannot be controlled. In view of these circumstances the greenhouse offers the most prompt, effective, and economical means of testing legume bacteria of the various groups under fairly well-controlled conditions of moisture, light, and temperature.

In greenhouse work of this kind various types of containers and apparatus for growing and watering plants have been employed. Sand is the material usually used to support root growth. A self-irrigating assembly recently has been devised for use in testing numerous samples in limited greenhouse space.[1] Briefly, as modified since its description was published, it consists of a 32-ounce screw-cap-finish bottle from which the bottom has been cut, as a container for a mixture of sand, dolomitic limestone, superphosphate, and potassium sulfate. The mouth end of the bottle is fitted with a plastic cap that has a hole in the center small enough to retain the sand. This end rests on the shoulders of a wide-mouthed pint cream

[1] LEONARD, L. T. A SIMPLE ASSEMBLY FOR USE IN TESTING CULTURES OF RHIZOBIA. Jour. Bact. 45:523–525, illus. 1943.

jar holding an acid nutrient solution containing zinc, copper, manganese, iron, and boron salts in very minute quantities. As prepared, the neck of the bottle with cap extends nearly to the bottom of the jar.

After the sand has been completely irrigated, holes are punched in its surface to receive seedlings. The cut end of the bottle is then covered with a Petri dish lid and the whole assembly subjected to steam sterilization in an autoclave. The sand is kept moist by the nutrient solution, which is drawn up by capillarity. The solution is sufficiently acid (pH 2.0) to preclude the survival of any common rhizobia that might drop into the jar when the solution is replenished. Upon reaching the sand the acid solution is neutralized and when carried farther up it becomes available as a nutrient for plants.

Preparation of Subsamples

Before testing moist peat materials, samples are weighed to obtain the net content of carrier in the package. The number of pounds the sample will treat is multiplied by 16, and the net weight of the sample in grams is divided by the number obtained to give the quantity of material in grams necessary for 1 ounce of seed. This is usually very small. Instead of subjecting the inoculant to the possibility of contamination in the process of direct weighing, an approximation of the quantity may be obtained by the use of disinfected, calibrated, narrow glass cylinders, from which the peat powder may be expelled by a disinfected piston of slightly smaller diameter than that of the cylinder. Cylinders and pistons are disinfected by 150° C. dry heat for 1 hour and are allowed to cool before using.

A flame is passed over the lid end before opening samples in cans with removable lids. Sealed cartons and crimped-end cans without a removable lid are opened by cutting with a disinfected knife, into the middle of the side of the container, 3 sides of a rectangular hole, the fourth side acting as a hinge. The carrier is poured from this opening into a properly labeled sterile Petri dish. Labeled sterile vials for subsamples from each package are arranged in holes on a wooden board. A glass cylinder with a piston set at the proper calibration point is tamped in the peat powder until it is filled to the end of the piston. Then, with the aid of an assistant, the subsample is discharged into a sterile vial. Usually five subsamples are taken, but occasionally more are desirable, especially from multiple-group cultures. Lids are replaced or holes are closed with masking tape as soon as possible after part of the contents is removed from the cans.

Agar cultures are not subsampled in this manner. They are prepared according to directions at the time of planting, and subsampling is done by means of sterile pipettes. The common method of preparing agar cultures for use is to add water and shake thoroughly to obtain a homogenized suspension of the nodule bacteria in the bottle.

Seed and its Treatment

Seed of the highest quality obtainable should be used in this work. It should be of uniform size, high in germinating ability, scarified when necessary, and free of debris and of defective, deformed, and broken seeds.

Since commercial legume bacteria cultures are customarily prepared for groups of legumes, each named on the package label, it is

necessary to have on hand a small stock of the various kinds of legume seeds. It is not always possible to test samples on all the species or varieties of legumes for which they are intended, but it is desirable to try them on some of the more common legumes mentioned. Legume seeds ordinarily used in testing cultures are listed below.

Group designation of bacterial sample:	*Plants upon which tests are made*
Alfalfa	Alfalfa and white sweetclover.
Alyce clover	Alyce clover.
Beans	Navy and green beans (Stringless Green Pod and dwarf varieties).
Clover	Crimson, red, and white clovers.
Cowpeas	Cowpea, crotalaria, kudzu, lima bean, and peanut.
Lespedeza	Annual (Kobe, Korean) and perennial species.
Lupine	Blue lupine and other species.
Soybeans	Biloxi, Laredo, Mammoth Yellow, Tokyo, Virginia, and Wilson varieties.
Vetch	Hairy vetch, garden pea (Alaska, Nott Excelsior), and Austrian Winter pea.

Seeds are first washed in sterilized weak peat water to remove clinging dirt particles, then agitated for 5 minutes in a 0.2 percent solution of mercuric chloride. After the disinfecting solution is thoroughly drained away, sterile peat water is used to remove soluble traces of the mercury salt, following which the mouth of the seed container (usually a 16-ounce, screw-capped, wide-mouthed bottle) is covered with a small blanket of sterile absorbent cotton and inverted so as to throw the seeds on the cotton and thereby dry them by draining.

As soon as the disinfected seeds pour easily they are ready to use. Petri dishes containing 0.6 percent sterile agar are prepared, and when hardened the seeds are sown on the surface for germination. Sometimes with large seeds it is desirable to compensate for excessive imbibition by adding sterile peat water. A good temperature for germinating most seed is 70° F.

The number of seedlings that can be planted in an assembly is determined by the growth habits of the resulting plant. Alfalfa, white sweetclover, red clover, crimson clover, and lespedeza may be planted six in a jar; cowpeas, soybeans, crotalaria, mung beans, and lima beans, three; and peanuts, one per jar. If it is desirable to conserve space, two of each of three species of the smaller seedlings may be planted in a jar, or, in the case of soybeans, two of each of two varieties.

Inoculation and Planting

Sterilized planting assemblies, germinated seeds, and subsamples of inoculating material are necessary at the start of the process of inoculation. Sterile Petri dishes and means of disinfecting the instruments (boiling water) should be at hand. A predetermined quantity of sterile water is added to each subsample, usually 20, in a series, and the vials shaken vigorously. Seedlings are placed in sterile Petri dishes in sufficient number to prepare at least 3 replicates of all treatments and 9 of no treatments. The series may also include 9 controls

inoculated with a known pure culture. The suspended samples are added to the seedlings and the Petri dish agitated to make good contact between seedlings and inoculum.

This method of treatment does not exactly conform to the directions given. The seedlings however, are bathed in a suspension of inoculating material prepared in quantities proportional to those prescribed, and therefore they receive approximately the same quantity as would ungerminated seeds plus those that adhere to the small rootlets.

The seedlings are transferred to previously made holes in the sand by using disinfected forceps. These plantings are usually made in a circle in the center of the bottle, the distance from the side depending on the kind; they are covered by depressing the sand in the middle by means of a ⅞-inch rounded metal tamper. A half inch of sterile gravel may then be added to prevent undue evaporation.

Assemblies are labeled on the bottle as to legume, treatment, and date of planting. Replicated treatments consisting of three assemblies are kept together in the greenhouse. Control sets of three are interspersed with the treated sets. Each assembly is placed on a number on the greenhouse bench. Under this number in a notebook the treatments and dates of planting are recorded on a form sheet upon which observations also are placed later. The number of the space used is also placed on the nutrient reservoir to prevent errors when the assemblies are rearranged or removed for observation.

In the greenhouse, control of insects and dust and isolation from ordinary greenhouse operations are necessary. As soon as assemblies are placed, lids may be removed to give better light, aeration, and physical conditions for growth, although the removal presents possibilities for contamination by foreign rhizobia. Since much better growth is obtained in the absence of the lids, however, it is preferable to dispense with them and maintain strict sanitation in the greenhouse.

Plants are usually allowed to grow for about 45 days, unless early maturity or a desire for information requires their earlier removal or it is apparent that a longer period would be advisable.

For some species, as cowpeas, peanuts, or soybeans, in short-day seasons it is desirable to furnish additional light. This is done by using electric bulbs with reflectors, beginning at sundown and continuing 4 or 5 hours into the night. Alfalfa, clovers, and vetch produce satisfactory results from the light coming through the greenhouse glass during the winter season.

In the case of vining or high-growing plants, supports are necessary to prevent falling or intertwining. This is done by means of disinfected hardware-cloth cylinders that will fit snugly around the sand bottles.

Observations and Performance

The main points to consider for effect of inoculating material are the differences between treated and control plants in nitrogen content, dry matter, color, size of plants, and nodule formation. In special cases, nitrogen analysis may be desirable, but for the ordinary estimation of bacterial stimulation color is a good index of nitrogen fixation or lack of it. Color may be observed more satisfactorily while the plants are in the greenhouse.

Observations on color and height are preferably made before the

plants are disturbed by removal from bottles. After roots are freed from sand by washing in water they are examined in a bottom-illuminated glass water bath, originally described by Marsh and Leonard [2] and later modified by Marsh to give side light. By means of this bath roots floating in water are easily measured and estimates made of nodule numbers and their location.

If observations on color show that plants from treated seeds are dark green and the corresponding control plants are light or yellowish green, it is a positive indication that organisms and the plant through symbiosis have produced satisfactory results.

TABLE 2.—*Summary of results of greenhouse tests on samples of legume bacteria cultures of commercial origin in the fiscal year 1943*

[U=unsatisfactory; S=satisfactory]

Trade name	Alfalfa		Clover		Cowpeas		Lespedeza		Soybeans		Beans		Vetch		Total		Source total[1]	
	U	S	U	S	U	S	U	S	U	S	U	S	U	S	U	S	U	S
Cal-Rhiz Legume Bacteria	0	5	0	3	0	2	---	---	0	2	0	2	0	10	0	24	0	24
Farmogerm	4	0	2	2	---	---	---	---	---	---	---	---	1	3	7	5	---	---
Humogerm	2	0	2	0	---	---	---	---	---	---	---	---	0	2	4	2	11	7
G. L. F. Legume Inoculant	2	2	4	0	---	---	---	---	---	---	---	---	3	1	9	3	9	3
Hansen's Humus Inoculator	0	2	4	2	---	---	---	---	---	---	---	---	0	6	4	10	---	---
Certigerm	0	3	0	3	---	---	3	0	1	2	---	---	0	3	4	11	---	---
Kelly's Humus Inoculator	0	2	---	---	---	---	---	---	---	---	---	---	---	---	0	2	---	---
Kelly's Inoculator	---	---	0	2	---	---	---	---	---	---	---	---	---	---	0	2	---	---
National Cooperative Humus Inoculator	0	2	0	2	---	---	2	0	0	2	---	---	---	---	2	6	10	31
Legume-Aid	0	2	0	2	0	2	0	2	0	6	1	4	0	11	1	29	---	---
Blue Seal Grow Crop Inoculant	0	7	0	7	---	---	---	---	0	1	---	---	---	---	0	15	---	---
Sacco Legume Inoculant	0	2	---	---	---	---	---	---	---	---	---	---	---	---	0	2	1	46
Legumogen	0	4	0	4	---	---	---	---	0	2	---	---	---	---	0	10	0	10
Nitragin	0	5	0	6	0	3	0	6	0	4	0	4	2	11	2	39	2	39
Nod-O-Gen Pretested Inoculator	0	6	0	6	0	3	---	---	0	5	0	5	0	10	0	35	---	---
Badger Brand Inoculation	0	4	0	2	---	---	---	---	---	---	---	---	---	---	0	6	---	---
Cofer's Hitest Inoculator	0	2	0	2	0	3	0	3	0	3	0	3	0	2	0	18	---	---
Gold Medal Inoculator	0	2	0	2	0	3	0	3	0	3	---	---	0	2	0	15	---	---
Hoffman's Inoculator	0	2	0	2	0	3	0	3	0	3	---	---	0	2	0	15	---	---
Kellogg's Rainbo Inoculator	0	2	0	2	---	---	---	---	0	3	---	---	0	2	0	9	---	---
M-Inoculator	0	2	0	3	0	2	0	3	0	2	---	---	0	2	0	14	---	---
May-Bell Inoculator	0	4	0	4	0	3	0	3	0	3	---	---	0	2	0	19	---	---
Ohio Farmers' Seed Inoculation	0	2	2	0	---	---	---	---	---	---	---	---	---	---	2	2	---	---
Red Bird Inoculator	0	2	0	2	---	---	---	---	---	---	---	---	0	2	0	6	---	---
Sioux Brand Inoculator	0	2	0	2	---	---	---	---	---	---	---	---	---	---	0	4	---	---
Trinity Brand Inoculator	0	3	0	2	---	---	0	6	0	2	---	---	0	2	0	15	---	---
Wood's Seed Inoculation	0	2	0	2	0	5	0	3	0	3	0	3	0	5	0	23	2	181
Superyield Culture	---	---	---	---	---	---	---	---	0	6	0	4	1	15	1	25	1	25
Uni-Culture	0	4	0	4	---	---	---	---	0	4	---	---	---	---	0	12	---	---
American Tubercle Germ	1	3	0	4	0	3	0	3	0	3	---	---	---	---	1	16	---	---
Berry's Superior Nodule Germ	0	2	0	4	0	3	0	3	0	1	---	---	0	3	0	16	---	---
Blue Ribbon Inoculation	3	0	---	---	---	---	---	---	0	6	---	---	---	---	3	6	---	---
Corneli Keystone Inoculation	0	4	0	4	0	6	0	3	0	3	---	---	0	2	0	22	---	---
Farm Master Nitrogen-Fixing Inoculant	0	2	2	0	---	---	---	---	0	1	---	---	---	---	2	3	---	---
Kalo Inoculation	---	---	---	---	---	---	---	---	0	1	---	---	0	4	0	5	---	---
Perfection Brand Legume Inoculant	0	3	0	3	---	---	3	0	2	4	---	---	---	---	5	10	---	---
Southern States Quality Inoculation	0	4	0	4	---	---	---	---	---	---	---	---	---	---	0	8	---	---
Sunfield Inoculant	0	4	0	4	---	---	---	---	---	---	---	---	---	---	0	8	11	106
Urbana Culture	0	2	0	2	5	0	0	6	0	6	0	6	0	2	5	24	---	---
Urbana Humus Inoculator	0	2	0	4	---	---	---	---	---	---	---	---	0	2	0	8	5	32
Total	12	101	16	97	5	41	8	47	3	81	1	31	7	106	52	504	---	---

[1] This column gives test results with products of individual manufacturers without particular reference to trade names.

[2] MARSH, F. W., and LEONARD, L. T. AN APPARATUS FOR THE SUPERFICIAL EXAMINATION OF ROOTS AND NODULES. Soil Sci. 26:403-404. illus. 1928.

If upon root examination nodules are found to be uniformly present on the dark-green plants from treated seeds and none or only a few on the control-plant roots, there is no doubt as to the effectiveness of the culture.

If plants are yellowish or not a normal green and no nodules are formed, it is evident that the treatment did not contain the proper organisms and is therefore unsatisfactory.

If foliage is yellow or not a normal green and nodules are observed uniformly on the roots, it shows that the culture is not adapted to the host and consequently is unsatisfactory. This condition, however, is found only occasionally.

TESTS OF COMMERCIAL INOCULANTS IN 1943

Using the method here described, the Bureau of Plant Industry, Soils, and Agricultural Engineering during the year ended June 30, 1943, tested numerous samples of inoculating materials seasonally collected in the open market from various parts of the country. This work was done in accordance with an act of Congress making appropriations for soil and fertilizer investigations "* * * including the testing of cultures procured in the open market for inoculating legumes, other crops, or soil * * *." The act provides also for publication of the results of the tests together with the names of the manufacturers "* * * if any such samples are found to be impure, nonviable, or misbranded * * *." Results of the 1943 tests are thus summarized in table 2, reference to table 1 showing the names of producers. The manufacturers of samples found to be unsatisfactory were notified of the results, so that steps could be taken to improve the product for the 1944 season.

U. S. GOVERNMENT PRINTING OFFICE: 1944

For sale by the Superintendent of Documents, U. S. Government Printing Office
Washington 25, D. C. - Price 5 cents