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RANGE-LEGUME INOCULATION AND NITROGEN FIXATION BY ROOT-NODULE BACTERIA

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CALIFORNIA AGRICULTURAL EXPERIMENT STATION

BULLETIN 842

The great importance of legumes in agriculture is that they add nitrogen to the soil and thereby save the costs of nitrogen fertilizer, both for the legume crop and for the associated nonlegume crop. Legumes obtain nitrogen from the air, which contains uncombined nitrogen—nearly 80 per cent—along with oxygen and other gases. Very few plants can utilize atmospheric nitrogen in its free form. However, if root-nodule bacteria of an effective strain have formed nodules on the roots of a legume, atmospheric nitrogen may be fixed within the nodules and converted to a utilizable compound.

The successful establishment of legumes, particularly in a pasture mix for grazing, depends on effective nodulation. This can be obtained by inoculating the seed with an appropriate strain of root-nodule bacteria. Methods of inoculating seed and measures that help to avoid inoculation failure are given in boxes on the following pages. All of the recommendations given here are of critical importance. There is no economical way to inoculate a field after planting.

Faulty inoculation usually results in failure or partial failure of the legume stand. From this cause alone, California growers waste many thousands of dollars' worth of range-legume seed every year and waste also the labor and the fertilizer used.

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AUGUST 1969

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INTRODUCTION

NITROGEN is an essential constituent of the amino acids and proteins and is the key element in the structure of all living cells. Legumes are specialized plants because of their faculty for fixing atmospheric nitrogen into compounds that can be utilized in plant metabolism. Actually, the legumes alone cannot accomplish this. The process takes place only in nitrogen-fixing nodules, which are formed on legume roots in symbiotic association with root-nodule bacteria of the genus *Rhizobium.* Both members of the symbiosis have their particular functions, and each step of the process involves a close interaction between them.

In this association both members derive advantages. The rhizobia live within the root tissues, where they are protected from competitors and receive nutrients, such as carbohydrates. The legumes receive amino acids, which they build into proteins. However, neither the legumes nor the rhizobia have any nutrient advantage without the symbiosis. Together they can fix surprisingly large quantities of atmospheric nitrogen.

Donald (1960) estimated that the world's annual income of biologically fixed nitrogen, mainly from symbiotic sources, was of the order of 100 million tons. Russell (1950) estimated that well-nodulated legumes could fix up to 500 pounds of nitrogen per acre per year—the equivalent of 2,352 pounds per acre of ammonium sulfate. Erdman (1959) gave 106 pounds per acre as the average amount of nitrogen fixed in a legume-grass pasture in North America.

Under California dryland conditions, effectively nodulated legumes are able to fix significant amounts of nitrogen, though the amounts do not approach Russell's estimate. A reasonable average for a good stand of range legumes might be at least 52 pounds per acre during one growing season—equivalent to 250 pounds per acre of ammonium sulfate. The cost of the inoculant is about 15 cents an acre. The following tabulation gives data from California. The amounts of atmospheric nitrogen fixed in the clover roots were estimated by comparing the yields from well-nodulated, thrifty legume stands with those from unthrifty stands in the same area. The amount of nitrogen in purple vetch was compared with that in barley, a nonlegume.

LEGUME, COUNTY, AND REFERENCE	NITROGEN GAIN LB./ACRE
Subterranean clover, Humboldt (Williams, Lenz, and Murphy, 1954)	45
Rose clover, Stanislaus (Williams, Love, and Berry, 1957)	50
Purple vetch, Santa Barbara (Williams, Ririe, *et al.,* 1954)	53
Rose clover, Placer (Martin, Williams, and Johnson, 1957)	60

[1] Submitted for publication September 29, 1964.

Cover photo: Kondinin rose clover plants 105 days old. Vigorous plant at left grown from pellet-inoculated seed; small plant at right from uninoculated seed, planted at the same time and only a few feet away. Yolo County, March 1, 1967.

ROOT NODULES AND NITROGEN FIXATION

Nodule development on a legume root

The first stage in the symbiosis between rhizobia and legumes is the multiplication of rod-shaped rhizobia in the soil in the region of a legume root. The multiplication is initiated and stimulated by substances secreted by the root and exuded from it. One of these exudates is tryptophan (Rovira, 1959). The rhizobia convert tryptophan to indoleacetic acid (Kefford, Brockwell, and Zwar, 1960), which causes curling of certain root hairs—a prelude to the invasion of these root hairs by the rhizobia. This process occurs only at certain specialized infection sites — foci — on a young root (Nutman, 1948 and 1949; Dart and Pate, 1959). The infection foci remain available for only a limited time. As the root elongates, the new infection foci are progressively more distant from the seed.

Many strains of rhizobia are able to multiply in the rhizosphere of any legume

SEED INOCULATION

1. Select a good commercial inoculant, prepared from a strain of root-nodule bacteria that is specific for the legume to be planted. Make sure the legume species is named on the label; no other inoculant will do. Mixtures of bacterial strains suitable for a great number of different species and varieties of legumes are not satisfactory.

2. Check the freshness of the inoculant by the expiration date stamped on the container. The inoculant is a living culture of root-nodule bacteria, which are killed by drying and also by high temperatures. Make sure this culture has been stored continuously under refrigeration. Poor storage conditions can cause nodulation failure by reducing the number of viable bacteria in the inoculant.

3. Use the inoculant before the expiration date. Do not carry it over to another year. The number of bacteria that survive will be too low for effective nodulation.

4. Use the inoculant at the rate recommended by the manufacturer and stamped on the container, or else at a higher rate—up to four times this amount (see paragraph 9, below). Never use a lower amount.

5. Mix inoculant with seed thoroughly. Follow closely the printed directions on the container for the wet-mix method, but do not use excess moisture, Make sure the inoculant sticks to all the seeds.

6. Hold inoculated seed in a cool, shady place, and plant it in a **moist seedbed.** Guard against drying, both before and after planting. Always remember that drying kills these bacteria quickly.

7. Plant as soon as possible. Reinoculate seed if it has been held longer that 24 hours after inoculating. The cost of inoculant is small in comparison with the costs of seed, fertilizer, soil preparation, and planting. Pellet the seed (see next paragraph) if planting must be delayed.

8. Pellet the inoculant on the seed to protect the bacteria from drying. Directions for pelleting are in the box on page 6. Always use pellet-inoculated seed if it must be sown in a dry seedbed, or broadcast without a soil cover, or sown from the air. Even with pelleting the protection is temporary, and the inoculant becomes valueless in three weeks under dry conditions.

9. Always use pellet-inoculated seed where native legumes are abundant. This includes many areas of California. Pellet with four times the amount of inoculant recommended by the manufacturer.

The root-nodule bacteria already in the soil in such areas often act as parasites on introduced legumes. These bacteria are ineffective nitrogen fixers with introduced legumes, but they do invade the young roots in great numbers and thus prevent adequate nodulation of a new legume by bacteria of the effective strain supplied in the inoculant. It requires a large amount of inoculant to compete and can initiate the preliminary stages of root-hair curling described above. However, because of the specific relationship between a host legume and a compatible strain of rhizobia, the bacteria that cause curling of root hairs may belong to a strain that cannot nodulate the available legume. If so, these rhizobia do not enter the root hairs.

The following reactions proceed only if the strain of *Rhizobium* is **invasive**, i.e., capable of stimulating nodule formation in a legume root. Rhizobia of an invasive strain produce an extracellular polysaccharide slime, which induces the legume to secrete polygalacturonase (Fåhraeus and Ljunggren, 1959). The function of polygalacturonase has not been fully determined. It may act with indoleacetic acid to increase the plasticity of the cellulose wall of a root hair and enable it to invaginate (fold inward). The root hair responds to the presence of invasive rhizobia by folding inward at some point near its tip to form a very fine tube, the **infection thread**, which extends inside the root hair (Nutman, 1963).

Concurrently with the formation of an infection thread, a specialized tetraploid cell forms in the adjacent root-cortex

AND FIELD PROBLEMS

with a large population of ineffective bacteria. The pellet concentrates the inoculant around the germinating seed and the emerging root, so that a nitrogen-fixing nodule is started immediately by the effective bacteria supplied in the inoculant.

10. Do not mix acid fertilizers with inoculated seed, and do not sow in contact with such fertilizers. The acidity may kill most or all of the root-nodule bacteria.

11. For the same reason, do not mix the seed with fertilizers that contain trace elements.

12. Check the acidity of the soil where inoculated seed is to be sown. Nodulation is usually defective where the soil is more acid than pH 5.2.

13. Do not use herbicides, fungicides, or any other pesticides when planting inoculated seed. Most of these poisons are highly toxic to root-nodule bacteria.

14. Make sure the soil contains adequate amounts of plant nutrients. A legume suffering from deficiency of any nutrient other than nitrogen cannot benefit fully from inoculation. To realize grazing potentials, the legumes must have an adequate supply of available phosphorus and sulfur—the elements most commonly deficient on California rangeland. Many pasture legumes—for example, subterranean clover—have poorer phosphorus-foraging ability than have the grasses.

15. Do **not** use nitrogenous fertilizers when establishing legumes in a pasture mix. Well-nodulated legumes do not need any nitrogen from the soil, and nitrogenous fertilizers usually give grasses a competitive advantage over the legumes. Moreover, nitrates and nitrites inhibit legume nodulation under most circumstances.

16. Maintain adequate pasture management. In a grass-dominant pasture, direct every effort toward making the environment favorable for the legume rather than for the grasses. A good legume stand should have at least 20 plants per square foot. Grazing the pasture reduces competition from grasses and forbs. Reduce grazing pressure only while the legumes are flowering and seeding, because abundant production of legume seed is essential. However, pastures must be grazed very heavily in summer after the legume seed is ripe.

17. Dig a few seedlings after the legumes have produced three or four leaves. The type and pattern of nodulation can give useful information.

a. Lack of nodulation usually indicates some fault in the inoculation or sowing technique. Review the above instructions.

b. A few large nodules on the crown or upper root indicate early, effective nodulation—provided these nodules are pink inside.

c. Many small, white nodules scattered over the entire root system indicate ineffective nodulation by root-nodule bacteria already in the soil (see paragraph 9, above).

SPECIAL PROCEDURE

PELLETING is an improved method of seed inoculation. Each pellet contains a legume seed, the inoculant in an adhesive, and a coating of calcium carbonate. Both the adhesive and the coating material assist the survival of the inoculant. The following recommendations are based on experiments in California.

Inoculant. Use peat inoculant prepared specifically for the legume to be planted. Mixed inoculants prepared for a great variety of legumes are not satisfactory. The inoculant must be fresh and of good quality. Use four times the amount recommended on the container. Review paragraphs 1, 2, 3, and 9 in the preceding box.

Adhesive. Use gum arabic ground to pass an eight-mesh screen—or finer. It must be without preservative. Do not dissolve more gum arabic than will be used in 36 hours. Without preservative, the gum arabic is decomposed rapidly by fungi and bacteria. These destroy the sticking properties of the gum and may make it toxic to the root-nodule bacteria.

Coating. Ground calcium carbonate, $CaCO_3$, is the most uniform and beneficial of the many materials tested. It is marketed under the labels: lime, calcium carbonate, calcite, enamel whiting, or 280 whiting. Do not use quicklime; it is highly toxic to root-nodule bacteria. The coating material should be finely ground, so that it does not flake off from the finished pellet: At least 80 per cent should pass through a 200-mesh screen.

Quantities of pelleting materials needed vary with the size of the seed. For the adhesive, use 4 pounds of gum arabic powder to 1 gallon of water. This amount makes about 5 quarts of solution.

- Vetch. For 100 pounds of seed, use 2½ quarts of gum arabic solution and 30 pounds of calcium carbonate.
- Subterranean, rose, or crimson clover. For 100 pounds of seed, use 5 quarts of gum arabic solution and 50 pounds of calcium carbonate.
- Alfalfa or bur clover. For 100 pounds of seed, use 5 quarts of gum arabic solution and 40 pounds of calcium carbonate.

tissue. As the infection thread elongates within the root hair and approaches this specialized cell, it stimulates cell division both in the tetraploid cell and in the neighboring diploid cells. The mass of cells so formed develops into a nodule. The infection thread branches and spreads throughout the nodule, in the central zone of the actively dividing tetraploid cells. Rod-shaped rhizobia invade the cytoplasm of the tetraploid cells by way of the infection thread (Goodchild and Bergerson, 1966). They multiply further and change into bacteroids.

The bacteroid is club-shaped. It is an incomplete growth stage in the sense that it has no cell wall but only a cytoplasmic membrane. Bacteroids are able to increase their numbers by cell division inside a nodule, but they never return to the rod shape.

After the nodule has formed and if hemoglobin has developed in the nodular cells, nitrogen fixation may proceed. Nitrogen is fixed only in tissue containing both bacteroids and hemoglobin. The presence of hemoglobin in a nodule is evidence of effective nitrogen fixation. It is indicated by the pinkish color of the nodule tissue, seen most clearly when the nodule is sliced open. Nutman (1963) gives further details of nodule formation and the factors involved.

Theory of symbiotic nitrogen fixation

Bergersen and Briggs (1958) studied mature, infected nodule cells by electron microscopy. They found that the cytoplasm of these cells is filled by a complex system of membrane envelopes, which are formed from folds of the outermost protoplasmic membrane of the host cytoplasm and enclose small groups of bacteroids. They suggested that these membranes play an important part in nitrogen fixation, be-

FOR SEED PELLETING

Preparing the pellets

1. Use a cement mixer for large quantities of seed. Small lots may be pelleted by hand in a tub or a bucket, or on a smooth floor.

2. Calculate the appropriate quantities of gum arabic and water to use with the desired quantity of the particular seed to be pelleted.

3. In a separate container, dissolve gum arabic in water. There are two possible methods: Either add the gum powder to the water slowly, while stirring vigorously, or else make a paste by adding half the water to the powder and then dilute with the remaining water. Gum arabic dissolves in cold water if left overnight, or in hot water in about 30 minutes. Do not let the solution boil.

4. Cool the gum arabic solution. Just before pelleting, add the appropriate amount of inoculant and stir to a smooth slurry. This mixture must not stand for more than 30 minutes. Some gum arabic is acid and will harm the bacteria unless the acid is neutralized by the calcium carbonate as soon as possible.

5. Pour the seed into the mixer. Add the gum-inoculant mixture and rotate the mixer at high speed, for good tumbling action, until all the seeds are coated.

6. Without stopping the mixer, dump in the calcium carbonate all at once and let the mixer run until all the seeds are pelleted.

7. Do not clean the mixer between loads. After the whole job is done, clean the mixer by running a load of water and gravel through it.

8. Pellets are firmer if they are allowed to season for 24 hours. They then work better for drill-seeding.

9. Screen the pelleted seed to remove lumps; they are likely to clog the seeding equipment. If there is an excess of calcium carbonate powder, screen or winnow it out so as not to clog the seeding equipment. The proportions given above allow for an excess of calcium carbonate, because the stickiness of the adhesive may vary slighty in different lots.

10. Remove vigorous agitators from seeding equipment, to prevent injury to the pellet coating.

cause nitrogen is fixed only inside the membranes. This research and the associated work of Appleby and Bergersen (1958), Bergersen (1958, 1960*a*, 1960*b*), and Bergersen and Wilson (1959) led to the formulation of a hypothesis to explain symbiotic nitrogen fixation. The process is summarized below. For more technical data see Vincent and Waters (1962).

In symbiotic nitrogen fixation, five primary reactions occur in the soluble cytoplasm inside the membrane envelopes—not on the membrane surface, as Bergersen proposed initially.

1. Free nitrogen occurs in this cytoplasm. Here it is activated—that is, ionized.

2. The legume supplies carbohydrates and other carbon compounds, which are partially oxidized by action of the bacteroids—releasing electrons, which are the energy source.

3. Hemoglobin, in solution in the cytoplasm, combines loosely and reversibly with these freed electrons, thus providing an essential linkage for their transport. The electron-transport train begins with the bacteroids. The ultimate acceptor of the electrons is activated nitrogen, which is thereby reduced to ammonia.

4. Products of the partial oxidation of the carbon compounds then serve as acceptors of ammonia in the process of amino acid production by the bacteroids.

5. Most of these amino acids are available to the host legume for production of protein, nucleic acids, and other nitrogenous plant products.

Breakdown of nodules

After some weeks or months of nitrogen-fixing activity, the host nodule breaks down. The bacteroids die and undergo lysis—a process of disintegration or dissolution. Rod-shaped rhizobia—previously dormant inside the infection thread (see nodule development, above) — then become active, invade the senescent nodular tissue, and multiply there but do not become bacteroids. When the nodule decays further, these new rods are released into the soil. They may start another cycle of infection on the same legume root or they may oversummer in the soil and start a new cycle in legumes in the next growing season.

Neither the rod form of rhizobia nor the bacteroid form produces spores or other specialized reproductive cells, as do many bacteria. Therefore, they are always in the vegetative state and are subject to rapid death on drying. If host legumes are not grown in the soil, the numbers of rhizobia decline gradually (Krasil'nikov, 1958; Rovira, 1961).

SPECIFIC RELATIONSHIPS BETWEEN HOST LEGUMES AND RHIZOBIA

The relationship of a species or strain of *Rhizobium* to certain legumes is fixed and usually specific.

Invasibility

Usually one species of *Rhizobium* can cause formation of nodules with many of the legumes in one or more genera. Thus, the agriculturally important legumes have been classified into seven so-called cross-inoculation groups—namely, the clover, alfalfa, pea and vetch, bean, lupine, soybean, and cowpea groups. Within each group of legumes there is commonly a mutual compatibility of the host plants with several related strains of one *Rhizobium* species. Moreover, rhizobia of this one species do not usually invade legumes outside their particular group.

The following list gives only the more important forage legumes in three of the cross-inoculation groups.

1. The clover group, nodulated by *Rhizobium trifolii*

Alsike clover, *Trifolium hybridum*
Berseem clover, *T. alexandrinum*
Crimson clover, *T. incarnatum*
Ladino clover, *T. repens giganteum*
Red clover, *T. pratense*
Rose clover, *T. hirtum*
Strawberry clover, *T. fragiferum*
Subterranean clover, *T. subterraneum*
White clover, *T. repens*

2. The alfalfa group, nodulated by *Rhizobium meliloti*

Alfalfa, *Medicago sativa*
Barrel medic, *M. tribuloides*
Black medic, *M. lupulina*
Bur clover, *M. hispida*
Fenugreek, *Trigonella foenumgraecum*
Hubam clover, *Melilotus alba annua*
Sourclover, *M. indica*
White sweetclover, *M. alba*
Yellow sweetclover, *M. officinalis*

3. The pea and vetch group, nodulated by *Rhizobium leguminosarum*

Common vetch, *Vicia sativa*
Field pea and garden pea, *Pisum sativum*
Hairy vetch, *Vicia villosa*
Horsebean, *V. faba*
Purple vetch, *V. benghalensis*
Woollypod vetch, *V. dasycarpa*

The classification into cross-inoculation groups is somewhat arbitrary, since rhizobial invasion can sometimes occur outside the accepted host groups and not every strain of the given species of *Rhizobium* invades all of the legumes in the group.

There are a number of legume species outside the seven recognized groups. Each of these legumes is dependent on its own particular strain of *Rhizobium* for effective nodulation. The following species among these ungrouped legumes are important in California:

Big trefoil, *Lotus uliginosus*
Birdsfoot trefoil, prostrate, *L. tenuis*
Birdsfoot trefoil, upright, *L. corniculatus*

Table 1

LABORATORY TEST OF SELECTED STRAINS OF *RHIZOBIUM TRIFOLII**

Host plant subterranean clover				Host plant rose clover			
Strain and origin	Foliage nitrogen content	Foliage weight, green†	Significance‡	Strain and origin	Foliage nitrogen content	Foliage weight, green†	Significance‡
	per cent	*mg/plant*			*per cent*	*mg/plant*	
None. Check plants with nitrogen	3.05	239	a	None. Check plants with nitrogen	2.67	317	a
RH 32, Sonoma County	3.09	213	a	RH 36, Yolo County	2.83	134	b
RH 24, Hopland Field Station	2.55	153	b	RH 39, Yolo County	1.93	115	c
RH 36, Yolo County	2.86	145	b	RH 28, Sonoma County	1.46	64	d
RH 45, Humboldt County	2.16	124	b	RH 24, Hopland Field Station	1.27	57	d
RH 51, Humboldt County	1.81	76	c	RH 21, Hopland Field Station	0.93	55	d
RH 48, Humboldt County	1.52	70	c	RH 48, Humboldt County	0.55	49	d
None. Check plants without nitrogen	0.94	54	c	None. Check plants without nitrogen	1.06	43	d

* Seedlings grown 42 days at 75° F in test tubes with nutrient solutions.

† Average of six replicate plants.

‡ In tests indicated by the same letter, the mean weights of the foliage are not significantly different from each other at the 1 per cent level but are significantly different from those in all other tests.

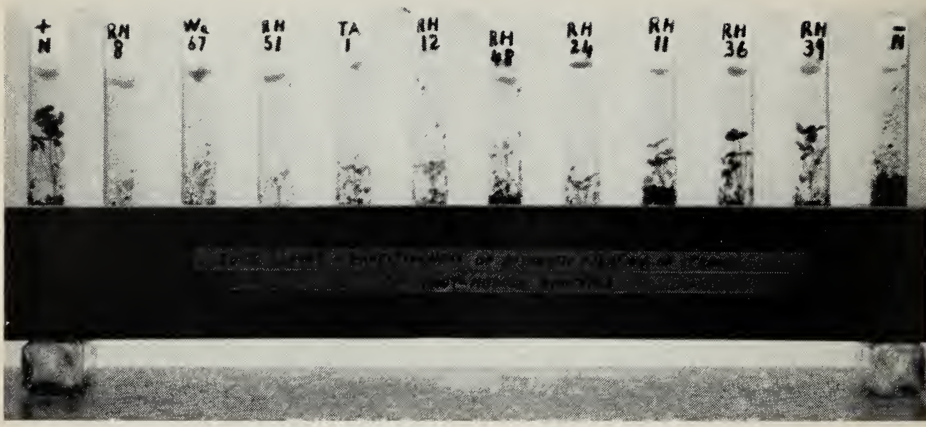

Fig. 1. Laboratory test of nine selected strains of *Rhizobium trifolii* on rose clover seedlings growing in nutrient solution under sterile conditions at controlled temperature. Test tubes at the two ends of the rack contain check plants without rhizobia: *left,* with added nitrogen; *right,* without nitrogen.

Effectiveness of rhizobial strains

As a further complication, each species of *Rhizobium* contains strains that differ in their ability to fix nitrogen in symbiosis with a given species or variety of legume. Also, a strain of rhizobia that fixes but little nitrogen with a certain legume may fix a large amount of nitrogen with another legume in the same cross-inoculation group. As an example, some strains of *Rhizobium trifolii* that nodulate both red clover and subterranean clover fix much more nitrogen with the red clover than with the subterranean clover. The reverse is true with some other strains of *R. trifolii.* Such variability in effectiveness of strains of rhizobia emphasizes the necessity for using a specific inoculant for each legume.

Selection of strains of rhizobia

Strains of rhizobia for use as inoculants are isolated from nodules on thrifty legumes in the field. Preferably, these strains should be selected from the general geographic region where the inoculants are to be used. Strains so selected are then compared with each other on seedlings grown aseptically under controlled conditions (fig. 1 and table 1). The best strains are tested further in the field.

Resident rhizobia in a soil

Root-nodule bacteria occur in the soil wherever legumes normally grow, but these resident rhizobia may not be effective nitrogen-fixers with a newly planted legume. An abundant soil population of invasive resident rhizobia will nodulate young legume roots as soon as they start to grow. If the legume seed carries only a few rhizobia of the desirable strain these few are not able to compete with the large numbers of resident soil rhizobia, and ineffectively nodulated plants result. Competition by resident rhizobia appears to be common on California rangelands, because of the prevalence of native legumes and their associated rhizobia. The following authors have reported on competition between strains of rhizobia: Vincent and Waters (1953); Harris (1954); Jenkins, Vincent, and Waters (1954); Purchase and Nutman (1957); Means, Johnson, and Erdman (1961); and Johnson and Means (1964). The following authors have reported on competition between rhizobia and other soil microorganisms: Hely, Ber-

Table 2
EFFECTS OF INOCULATION ON SUBTERRANEAN CLOVER*

Inoculant bacteria	Nodulation of 50 plants from each treatment†			Yield‡	
	On crown	Elsewhere	Nodules/ root system	Nitrogen content§	Foliage wgt., oven dry
no./seed	*no. of plants*	*no. of plants*	*av. no.*	*per cent*	*gm/plot*‖
0	0	50	56	1.9	3.3
9×10^2	0	50	43	2.0	4.2
21×10^3	43	7	12	3.7	27.3

* Before inoculation all the seeds were surface-sterilized with ethanol and hydrogen peroxide, drained, washed three times in sterile water, and dried under aseptic conditions. Plants were grown 120 days from seed (winter of 1963-64) in field plots 3 feet square, separated by buffer areas. Sutherlin soil series, Hopland Field Station, Mendocino County.

† Ten plants selected at random from each of five replicate plots.

‡ There were three 3-foot rows in a plot. Each treatment was replicated in five plots in a randomized block. The center row of each plot was harvested for the yield data.

§ Analysis of trifoliate leaves only.

‖ Difference for testing significance at the 1 per cent level = 19.9 grams.

Fig. 2. Subterranean clover plants 69 days after seeding. *Left,* no inoculant; plant ineffectively nodulated by resident rhizobia. *Right,* heavy application of a specific inoculant; plant effectively nodulated near crown. Sutherlin soil, Hopland Field Station, Mendocino County.

Fig. 3. Subterranean clover plot yields 120 days after seeding. *Left,* no inoculant; *center,* approximately 9×10^2 rhizobia per seed; *right,* approximately 21×10^3 rhizobia per seed. Sutherlin soil, Hopland Field Station, Mendocino County. Figure 2 shows younger plants from same experiment and table 2 gives the final data.

gersen, and Brockwell (1957); Cass Smith and Holland (1958); and Holland (1962).

Table 2 shows how competition between resident rhizobia and those in the inoculant affected a planting of subterranean clover. Rhizobia were abundant in the soil and produced many small, ineffective nodules scattered over the root system of the clover, both when the seed was not inoculated and when it was inoculated at the rate of 900 rhizobia per seed. However, a much heavier application of the same inoculant resulted in large and effective nodules on the upper parts of the root, near the seed (fig. 2). In this case, 12 effective nodules per plant fixed a far greater amount of nitrogen than did 43 to 56 ineffective nodules. Total forage protein in the heavily inoculated plot was 12 times that produced in the plot with light inoculum and 16 times that in the plot without inoculum (fig. 3). Vincent (1954) considered that it took at least 1,000 rhizobia per seed to form nodules in competition with soil microorganisms other than root-nodule bacteria.

Obviously the nodulation of a legume crop should not be left to chance, in view of the many possibilities of failure. It pays to inoculate seed; it is essential to use the correct inoculant for the particular legume being planted; and it is equally essential to use enough inoculant to ensure early and effective nodulation.

PRACTICAL CONSIDERATIONS

Care of the inoculant

Root-nodule bacteria **must be alive** when the seed is planted and when it germinates. The survival of the bacteria is the most important consideration at every step of handling and storing the inoculant, because it is a culture of living rhizobia. When seed is inoculated by the conventional water-slurry method, the inoculant begins to dry immediately, and drying is fatal to the rhizobia. Within 24 hours after inoculation, desiccation usually cuts down the number of viable rhizobia to less than the minimum needed for effective nodulation. Therefore, it is essential to sow into a **moist seedbed** as soon as possible—at least within the 24-hour limit. Death of rhizobia on the seed before germination is probably responsible for 99 out of 100 inoculation failures.

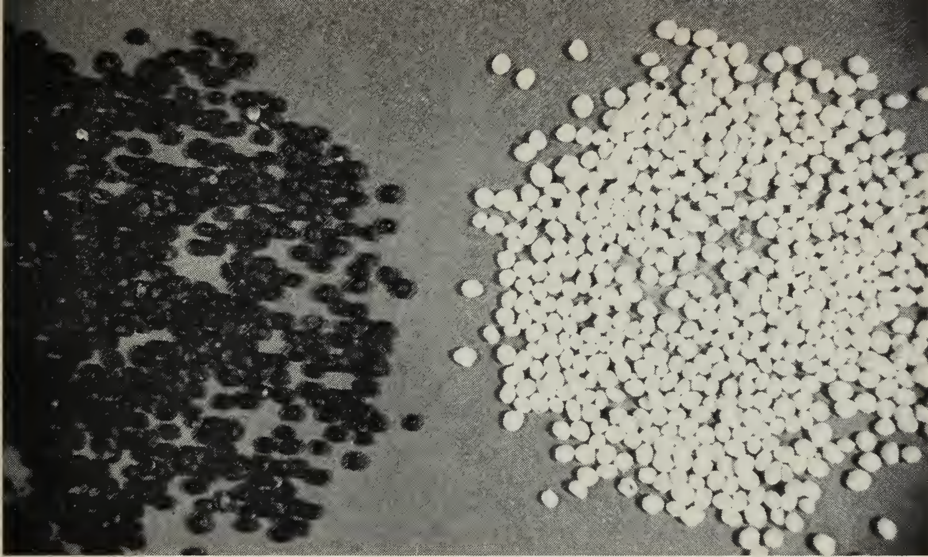

Fig. 4. Subterranean clover seed. *Left,* not inoculated; *right,* pellet-inoculated. Approximately actual size.

Pelleting seed (see box on page 6) prolongs the survival of the rhizobia (fig. 4). Brockwell (1962) found that the inoculant could be partially protected from rapid drying by mixing it with gum arabic, which was superior to the other adhesives he tested. Although the numbers of viable rhizobia dropped rapidly, even with this treatment, the survival after 10 days was 100 times as great with the gum arabic mixture as with the conventional water slurry. Brockwell found further that when he pelleted the gum arabic-treated seed with ground calcium carbonate he obtained reasonably satisfactory nodulation after storing the seed for 27 days at room temperature. Nevertheless, it is most important to avoid dry storage of any inoculated seed.

Soil conditions

Rhizobia multiply most successfully at a soil **pH** in the region of 6.4 to 7.2 (Vincent and Waters, 1954). Acid soils are unfavorable for their survival. Vincent and Waters (1954) and Vincent and Crofts (1958) found that a soil pH below 4.8—or 4.5, as the extreme—limited rhizobial multiplication. Loneragan *et al.* (1955) found faulty nodulation at pH 5.2 in a soil that was otherwise suitable for legume growth. In that instance, lime applied directly to the soil or pelleted on the seed made nodulation possible.

Acid fertilizers can kill rhizobia, whether the fertilizers are mixed with inoculated seed or come in contact with inoculated seed after planting. Fertilizers containing trace elements, particularly copper and zinc, likewise can kill rhizobia (Strong, 1938; Cass Smith and Pittman, 1939; Jenkins, Vincent, and Waters, 1954).

Other materials toxic to rhizobia are herbicides, seed fungicides, insecticides, and other pesticides. Williams, Harwood, and Hills (1960) showed that the fungicides Phygon, Spergon, Vancide, Arasan, Serenox, and Captan caused a highly significant decrease in the yield of legumes, because of their effect on the inoculants.

Nitrate and nitrite fertilizers inhibit legume nodulation, even at the low concentration of 6.5 ppm. Ammonia and urea fertilizers may not inhibit nodulation directly (Gibson and Nutman, 1960), but under field conditions nitrification may quickly convert ammonia and urea to nitrate and nitrite. There is another and extremely practical reason for limiting the use of nitrogenous fertilizers when establishing a pasture with legumes in the mixture: Readily available nitrogen in the soil encourages a strong growth of grasses and forbs, which compete with the seedling legumes.

Factors affecting nitrogen fixation and nitrogen utilization

Nutrient factors. Deficiencies of mineral nutrients have been studied in some detail because they affect the growth of a legume by affecting either the formation of nodules, or the fixation of nitrogen in nodules, or a plant's ability to utilize nitrogen when it is fixed and available.

1. Boron (Mulder, 1948) and calcium (Albrecht, 1937; Loneragan, 1959) have been found essential for nodule formation.

2. If a legume with structurally complete nodules responds to fertilizer nitrogen, this usually indicates that the nodules are not fixing nitrogen. Such a plant may lack adequate amounts of one or more of the elements needed for nitrogen fixation. These elements are calcium (Albrecht, 1937; Loneragan, 1959); cobalt (Delwiche, Johnson, and Reisenauer, 1961; Shaukat-Ahmed and Evans, 1961); copper (van Schreven, 1958); and molybdenum (Anderson, 1956).

3. On the other hand, if a well-nodulated but unthrifty legume fails to respond to fertilizer nitrogen, this usually indicates that the legume is unable to utilize nitrogen because of some fault in the metabolism of the plant itself, although probably the nodule is fixing nitrogen. Elements that are required for the healthy metabolism that enables a legume to utilize fixed nitrogen are boron (Mulder, 1948); calcium (Albrecht, 1937; Loneragan, 1959); copper (van Schreven, 1958); phosphorus (Trumble and Donald, 1938; Trumble and Shafter, 1937); potassium (Blaser and Brady, 1950); sulfur (Anderson and Spencer, 1950); and zinc (Mulder, 1948; Riceman and Jones, 1960).

Figure 5 illustrates how nitrogen utilization may depend on the nutrient status of the soil. A legume that lacks phosphorus or sulfur or both cannot synthesize protein even if it has an ample supply of available nitrogen. Table 3 gives another illustration. In an established pasture containing bur clover and grasses on phosphorus-deficient soil, a single application of treble superphosphate increased the forage yield and its protein content. The effect persisted for at least two years. The first year after phosphorus application, the treated field yielded 6 times as much dry forage and 10.4 times as much forage protein as the untreated field. The second year it yielded 4.1 times as much dry forage and 8 times as much forage protein. Neither nitrogenous fertilizer nor rhizobial inoculation was involved in this test, and it is not certain how much nitrogen was available in the soil.

Other factors. The following miscellaneous factors inhibit the efficient functioning of nitrogen-fixing nodules on legumes:

1. Ineffective strains of rhizobia. These bacteria may induce nodule formation on the roots of some legumes with which they are not specifically compatible. In such a case, the nodular tissue does not contain hemoglobin and the nodules do not fix nitrogen.

2. Low and high temperatures. Gibson (1963) found that nitrogen fixation was

Table 3
EFFECT OF PHOSPHORUS ON PROTEIN SYNTHESIS IN BUR CLOVER-DOMINANT FORAGE*

Phosphorus applied in 1955	Dry forage		Protein content		Nitrogen yield	
	1956	1957	1956	1957	1956	1957
lb./acre	*lb./acre*	*lb./acre*	*per cent*	*per cent*	*lb./acre*	*lb./acre*
0	640	1,140	7.8	7.7	8	14
106	3,840	4,700	13.5	14.9	83	112

* A single application of treble superphosphate, equivalent to 106 pounds per acre of phosphorus, was made in the autumn of 1955 to a resident stand of bur clover and grasses on Colusa fine sandy loam at Two Rock, Sonoma County. Farm Advisor Lloyd Harwood cooperated in this trial.

Fig. 5. Mixed pasture with subterranean clover, Marin County. *Left,* soil well supplied with phosphorus and sulfur; clover vigorous. *Right,* soil deficient in available phosphorus and sulfur; clover unthrifty although well nodulated.

greatest between 71° and 81° F. Above 86° it practically ceased. In low-temperature tests the amount of nitrogen fixed at 41° F was only 10 to 17 per cent of the amount fixed at 64° F.

3. Low light intensity. Nutman (1956) found that a decrease in light intensity during legume growth brought about a corresponding decrease in the amount of nitrogen fixed in a structurally complete and efficiently functioning nodule.

4. Certain genetic variations in a legume. This has been known to change the specific compatibility of a legume with a normally highly effective strain of rhizobium (Nutman, 1959). For example, Woogenellup subterranean clover, a field mutant, requires its own specific strain of *Rhizobium* and does not form effective nodules with the strains of rhizobia that are effective with other subterranean clovers.

5. Adverse moisture conditions in soil. Either waterlogging or soil dryness near the permanent wilting percentage decreases the amount of nitrogen fixed, even when an effective rhizobium is in symbiosis with the legume (Swaby and Noonan, 1946).

6. Toxic amounts of any element. The healthy metabolism of the host plant is essential for proper functioning of a nodule. For example, aluminum toxicity affects the host plant and consequently limits the amount of nitrogen fixed (Rorison, 1958).

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ABSTRACT

Successful seeding of range legumes can make dramatic increases in forage production. However, the legume must be effectively nodulated by seed-applied or soil-borne bacteria to be successful. Faulty inoculation can spell complete failure. This bulletin tells how legume roots are invaded by beneficial bacteria and outlines the vital processes of nodule formation and symbiotic nitrogen fixation. Field studies show the necessity for having large numbers of the proper strain of bacteria on the seed at the time of germination. An improved method of seed inoculation is described. Phosphorus and sulfur fertilization increases nitrogen fixation on most range soils. Fertilizer recommendations are outlined.

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