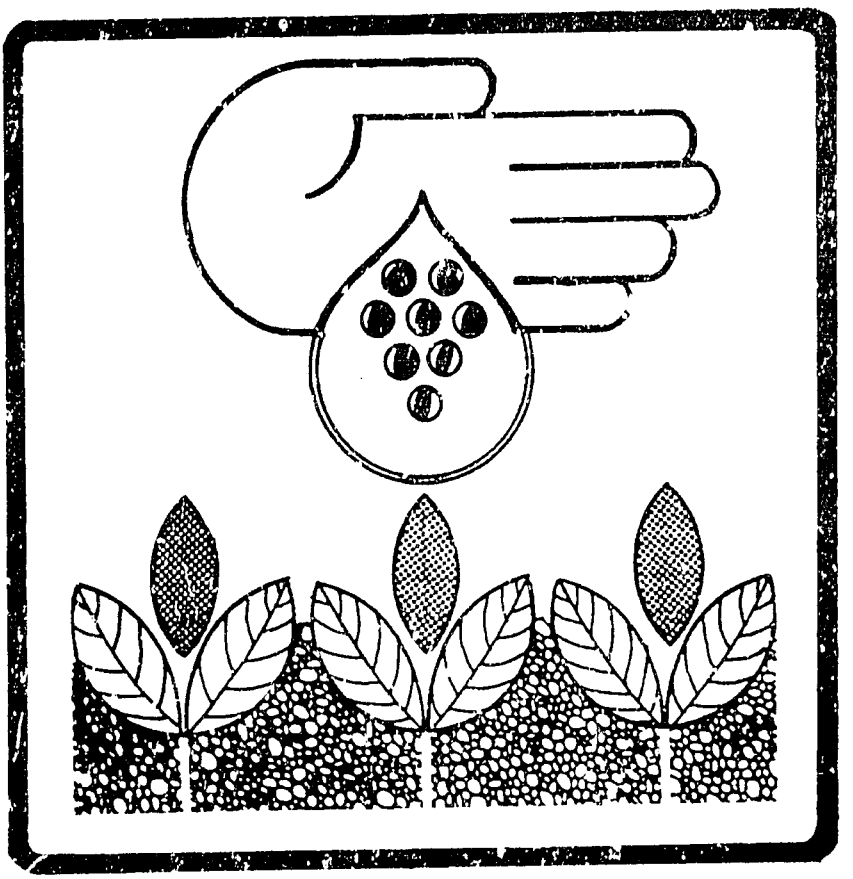
SYMBIOTIC NITROGEN FIXATION AND LEGUME INOCULATION MANUAL



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SYMBIOTIC NITROGEN FIXATION AND LEGUME INOCULATION

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EXERCISE 1 - SOIL SAMPLING

Background

Soil is an unique medium which contains a diverse community of organisms, representing many morphological and physiological types. In attempting to characterize these organisms, one must be cognizant of both numbers and activities. Some organisms exist at relatively low levels numerically but can exert profound influences on some soil biological processes.

Organisms in soils are never static in numbers or activity. Therefore the enumeration of some microbial population or activity represents only one point in time for that particular population which is in dynamic equilibrium with its physical, chemical, and biological environment. Variation of microbial numbers and activities can occur with depth and soil type. Soil structure, texture, and moisture levels can drastically affect aeration within the soil environment from one characterized as aerobic to one of anaerobic properties, thus influencing the distribution of physiological types.

At least as important as soil physical properties on microbial diversity are other factors, such as the season of the year. Soil fertility also plays a role in governing microbial development. Differences in numbers and diversity of actinomycetes, especially have bee noted to depend on soil acidity.

Finally, the diversity of organisms is dependent on the type of plant and the proximity of the organism to the plant

root itself. Numbers and activities of organisms vary with the distance from the root, with greatest numbers and activities in the soil most intimately associated with the root surface. The effect of the root on soil organisms decreases proportionally as the distance from the root increases.

Within this highly complex environment, studying soil organisms will be difficult. Close attention to soil sampling and handling procedures is critical to insure that the soil samples obtained are suitable for the objectives of the study. Before sampling, one must have a set of clearly defined study objectives. Without such a framework, data will be collected which are difficult to interpret and will not be as useful as they might be.

A. FIELD TRIP

If possible, the course should start with a field trip. The sites to be visited should be carefully selected in advance of the trip so that each stop has a specific purpose and that the students have ample opportunity to collect the materials that will be used for the rest of the laboratory, to take the appropriate notes describing each sample, to discuss problem situations, and to question the instructor(s) about the site being visited. This latter aspect is of utmost importance for at least two reasons; it helps foster an atmosphere of free and open discussion that will be essential to the laboratories that follow, and it serves to define the scope and direction of the course and create

interest in the practical value of what may appear to be an academic approach to day-to-day agricultural problems.

Permission of the farmer or owner should be obtained before observing or sampling fields. This also provides an opportunity to obtain field histories, planting dates, fertilizer and irrigation schedules, etc.

Objectives:

1. To gather soil, nodule and plant samples from defined environments that will provide experimental material for the laboratory exercises to follow.

2. To observe, define, and discuss in the field, the kinds of problems that are associated with symbiotic nitrogen-fixation.

Procedures:

To the extent possible, taking travel time and accessibility into account, a wide variety of sites should be chosen. The purpose is to have the trainees see as many different legume-growing situations as possible. Soil types and textures, different farming systems, croplands vs. pastures, and varying species of legumes all offer excellent opportunities for observation. What is actually chosen will vary from course to course. The occurrence of wild legumes should not be overlooked.

Many times, problems can be identified and diagnosed in the field. This increases the "practicality" of the course and makes the transition from field situation to laboratory session to application at the trainee's home station both

easier and more meaningful. For example, the identification of deficiency symptoms and discussion of how such a condition might affect symbiosis is a valuable field-teaching device.

If time permits, this same trip can be used to visit the previously-seeded demonstration plots. At this time, what has been done in anticipation of future observation can be explained.

Materials Needed:

Auger or sampling tube

Shovels

Knife

Scissors

Trowels

Insulated ice-chest or cooler

Plastic bucket

Cloth, paper, or plastic sample bags, as appropriate

Wide-mouth bottles for samples

Plastic garbage cans, or equivalent, for collecting sufficient volume of soil for later use

Marking pens for glass, plastic and paper

Notebooks for each student; this book will stay with the student through the course. It should, therefore, be of sturdy construction and easy use. Looseleaf notebooks are less desirable then spiral bindings; these open flat, yet pages are not easily lost.

A wide variety of perennial and annual legumes should be observed and sampled. Root samples, with intact nodules, should be taken. The site should be carefully identified and characterized with regard to field history, soil types, topography, status of the crop samples (vigor, color, pest problems, etc.), or anything else that may seem pertinent or interesting to the student. He should be encouraged to make his own observations, and to make them as complete as possible; too many notes are much more valuable than too few.

Different farm enterprises should be included. These might range from very intensive, such as a vegetable garden, to a grazing operation. Similarly, soils should be sampled from fields that are intensively cultivated, as well as those that may have been under grass or not tilled for many years. Finally, soils of as widely differing textures as possible should be sampled.

It is not necessary that each participant sample everything. It is desirable that each observe everything. If, for example, sampling is done by teams, depending on the number of samples to be taken, and if each team will ultimately report on the results obtained with its samples, the observations of the other teams may well be valuable to the discussion. Samples should be adequately protected from sun and heat. They will be used for microbiological studies and should not be permitted to lose moisture or become overheated.

EXERCISE 2 - SOIL PROPERTIES

Background:

Soil is made up of substances in three states--solid, liquid, and gas. The solid portion of soil is both inorganic (rock fragments) and organic (plant and animal materials, both living and dead).

Rocks are the source of the inorganic substances in soil. These substances are normally rock fragments and minerals. The fragments are remnants of larger rocks and are usually quite coarse. The minerals may be as large as the smallest rock fragment or too small to be seen with an ordinary microscope. These minerals can be coarse grained but are predominately fine grained. (Organic components of soil will be examined in the Biological section).

Most soils found in nature contain a mixture of sand, silt, and clay in different proportions.

Soil particles vary in size. Some are as large as rocks while others are smaller than sand particles. Particles of silt and clay are so small that they smooth and cannot be seen with the naked eye. Most soil particles support plant life.

In the following series of activities students will examine how rock fragments are formed, the various particles that comprise soil, and how the compaction of soil affects plant growth.

A. Soil Texture

Background

Soil texture is concerned with the size of mineral particles. Specifically, it refers to the relative proportion of the various size groups in a given soil. No less important is soil structure, which is the arrangement of soil particles into groups or aggregates. Together, these properties help determine, not only the nutrient-supplying ability of soil solids, but also the supply of water and air, so important to plant life.

Soil scientists classify soil particles as (1) sand, (2) silt, and (3) clay. Starting with the finest, clay particles are smaller than 0.002 mm (2 microns) in diameter. This would be too small to be seen with an ordinary light microscope. These clay particles, sometimes referred to as "micelles," are flat and offer maximum contact with the soil water because of their large surface area. Clay mixtures have a high absorptive capacity. The bound water will be strongly associated with the surface of the clay as absorbed moisture.

Silt particles are from 0.002 to 0.02 mm (2 to 20 microns) in diameter. They make a smaller contribution of nutrients to the plant due to a smaller surface area. Sand is largely quartz grains but may contain some particles of feldspar, offering a low level of nutrients for the plant. They range from 0.02 to 2.0 mm (20 to 2000 microns) in diameter. (Particles larger than 2.0 mm are commonly called "gravel" or "stones").

Objective

To determine the percentage of sand, silt, and clay (the mineral particles) in soil samples and to classify the sample by textural composition.

Materials Needed

Two 1000-ml graduated cylinders

2 rubber stoppers

12 grams sodium oxalate

50-gram soil sample

1 blender (or mixer)

1 hydrometer

Procedure

1. Secure a soil sample of 50 grams. Allow this sample to air dry overnight by spreading out thinly over a newspaper, etc.

2. Fill a 1000-ml graduated cylinder with water.

3. Pour some of the water (not critical, perhaps 500-600 ml) from the graduated cylinder into the blender.

4. Add 12 grams of sodium oxalate and the 50-gram soil sample to the blender or cylinder. Blend at high speed for approximately 1 minute or shake by inverting stoppered cylinder.

5. Pour contents from the blender into a second graduated 1000 ml cylinder.

6. Rinse blender with remaining clear water in the first cylinder, and pour into the second cylinder.

7. Agitate the soil suspension by inverting rapidly for approximately 30 seconds. Then let the cylinder and contents stand so the sand will settle to the bottom.

8. Place hydrometer in soil suspension and after 40 seconds read hydrometer. (Allow the cylinder to stand undisturbed so additional contents will settle). Carefully remove hydrometer from the soil suspension.

9. After 1 hour, repeat step 8 to determine the amount of clay in suspension.

10. To obtain the amount of sand in your sample, multiply the 40-second hydrometer reading by 2. The value is the % sand in the soil sample. Likewise, multiply the 1-hour reading by 2 to obtain the % clay in the sample.

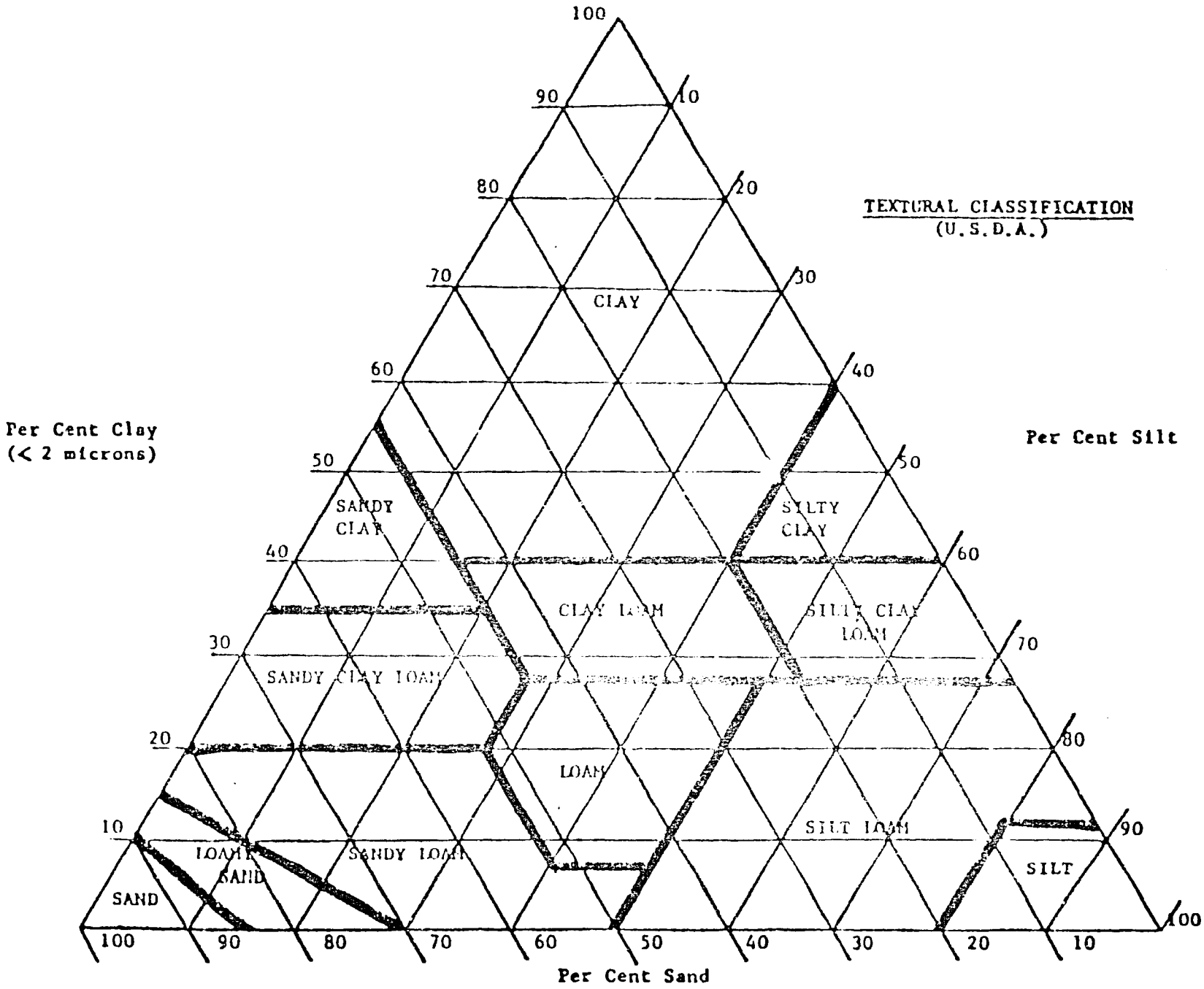
11. Calculate % silt as follows:

100 - [% sand + % clay] = % silt.

Interpretation of Data

After you have determined the relative percentages of mineral particles in your soil sample, you can classify your soil by referring to the textural classification chart of USDA.

To use this chart, follow the line parallel to the zero line of each soil particle. For example, suppose you found 20% clay in your sample and 40% sand. Therefore, your sample would contain 40% silt. Go up the left side of the triangle to the line which represents 20% clay. Follow this line to the right until it intersects the diagonal line (which runs from upper left to lower right) which represents



Follow line parallel to zero of each.

If on line read higher clay.

40% sand. Note: the diagonal which runs from upper right to lower left, representing 40% silt, intersects this same point. Therefore, you would classify this soil as loam.

As another example, suppose you had found that your sample contained 35% clay, 10% sand, and 55% silt. Find the point on the chart by using the directions as given in the previous example. Note that this point falls in the silty clay loam category.

Soil Texture (Alternative Procedure)

Materials Needed

Pint jars with lids

Water

5 x 8 index cards

Crayons

Hand lens

Tape

Soil samples

Procedure

1. Fill each jar one-fourth full of the soil sample.
2. Add water until the jar is three-fourths full and then cover.
3. Shake jars very thoroughly for 2 minutes.
4. Let the mixture stand for 15-20 minutes.
5. Observe and record the order in which the sediments settle in the jar. (Settling of each sediment may be timed and plotted on a bar graph if desired).
6. Examine each layer with fingertips and a hand lens.
7. Set some jars aside and observe over a 2-3 day period.

Observations

1. Note the size of the particles that settle to the bottom first.
2. How does the layer that settles above the first layer compare to the first layer?
3. Do all the soil particles settle out of the suspension? As the various particles, small stones, sand, silt, clay and organic materials separate and settle, the relative amounts of each should be compared. The thickness of each layer may be measured with a ruler. At the end of the observation period the groups should compare the results they obtained for the different soils. Further, any relationships with these results and observations made in the field should be discussed. For example:
4. Is there a relationship between wetness, as seen in the field, and texture?
5. Are there differences between soils from areas that are tilled frequently and those that are from grasslands or have not been tilled?
6. Do any of the observed differences relate to what the students think of as "good" soil?

B. What Determines the Rate at Which Water Moves Through Soil

Objective

To observe the rate at which water flows through the spaces consisting of large or small particles and to compare the spaces between large and small particles.

To determine and compare the water retention capacity of soil found in different sampling areas.

Materials Needed:

Cheesecloth

Rubber bands

Water

Food coloring

Small glass beads or fine-textured soil

Large beads (at least 1/2" in diam.) or coarse-textured soil

Straight-sided glass cylinders

or

Lamp chimneys or plastic tubing

2 pans or bowls

2 jars

Procedure

1. Cover one end of the cylinders (or tubing) with cheesecloth and fasten with a rubber band.
2. Put equal amounts of water in each jar and color with a few drops of food coloring.
3. Fill cylinder with small beads and the other with large beads or use two different soils.
4. Hold the cylinders over two pans at the same height.
5. Pour the colored water into the cylinders, through the beads or soil.
6. Pour the water in the pans back into their jars.

Questions/Discussion:

1. Through which cylinder does water move fastest?
2. Why do you think the water passed through the cylinder at different rates of speed?

3. Compare the amount of water returned to each container; are they different? Why or why not?

C. Capillary Action of Water in Soil:

Background

Soil texture refers to the amount of sand, silt, and clay in a soil sample. It has been found that water will move upward in soil by a process called capillary action (wicking). In this exercise we wish to demonstrate that the rate of movement of water in soils by capillary action will vary according to the amount of sand, silt, and clay in the sample.

Objectives:

1. To test the rate at which capillary action will cause water to move upward in soils of different textures.
2. To test the rate at which capillary action will cause water to move upward in soils of different structure.
3. To show, qualitatively, the amount of water which can be absorbed by soils of different texture or structure.

Materials Needed:

Three types of dry soil. For texture study--sand, silt, clay. For structure study--tilled soil, fence-line or meadow, packed soil from a field roadway.

Three tubes open on both ends with a minimum diameter of 1.5 cm. Drying tubes from a chemistry lab work nicely.

Three 250-ml gas bottles with mouths large enough for the tubes in no. 2 to enter freely. Cups of any kind will work.

Bottles for collecting soil samples.

Piece of window screen 10 cm x 10 cm.

Piece of cloth such as from an old sheet.

Rubber bands.

Timing device.

Meter stick.

Stand to support open-end tubes.

250-ml graduated cylinder (or similar size).

Procedure:

1. Collect and dry soil samples.
2. Set up a stand to support the open-end tubes.
3. Use a grease pencil to mark open-end tubes from bottom upward at 1-cm intervals to 12 cm.
4. Place the cloth over the end of the tubes which will be to the bottom and secure with a rubber band.
5. Fill open-end tubes with various soils to a depth of about 14 cm.
6. Place open tubes in the stand.
7. Put 200 ml of water, or a standardized amount, into each gas bottle, or cup of some kind.
8. Lower the three tubes of soil into the water of separate bottles to the second cm mark on the open-end tube.
9. Record time required for water to rise as a result of capillary action to each of the ten 1-cm marks above the water level on the tube.
10. Make a graph using height in cm on the vertical axis and time on the horizontal axis to show the relationship of capillarity to soil texture of the three samples.

11. Observe the amount of water taken up by each of the soil types by observing the level of the water remaining in each of the bottles. This can be measured since you started with 200 ml each.

Data table

Soil type (height)	Time water reaches each height		
1 cm			
2 cm			
3 cm			
4 cm			
5 cm			
6 cm			
7 cm			
8 cm			
9 cm			
10 cm			

Interpretation: You will find that the water will rise rapidly in the sand and overall about the same speed in the silt and clay. The speed with which capillarity occurs will be an indication of the amount of sand in the soil samples. More rapid capillary action indicates more sand.

Questions/Discussion:

1. What is capillarity?
2. What affect does soil structure and texture have on capillarity?
3. If you had two gardens, one in which the soil was mostly sand and the other mainly clay soil, which one would you have to water more often? Why?

D. Soil Texture and Aggregation:

Objective: To demonstrate the aggregating effect of microbial activity on soil.

Materials Needed:

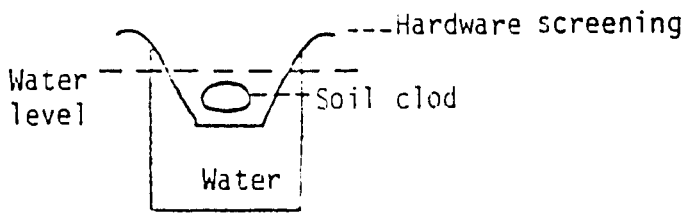
Three water glasses

Hardware screening with 3 mm openings, cut to fit inside the water glasses as shown in the diagram.

Soil samples (from sites such as an alfalfa field, corn field, and intensively cropped field).

Procedure:

1. Fill glasses with water to within 2 cm of the top. This water level should be about 3-5 cm above the lowest part of screen material. (See diagram).



2. Place a 2 cm diameter dry clod from each kind of soil on the three separate dry screens which have been bent to fit into glasses properly.
3. Place screens containing clods gently into water. (Be sure the alfalfa sod clod is not favored, place it in the water first.)
4. Follow with second-year corn sample and finally the intensively cropped sample. Do not shake glasses to speed up disintegration.
5. Observe the results.

Interpretation: The order of disintegration, in order of rapidity, should be the intensively cropped sample, second-year corn and finally the sod sample. Also note that the soil aggregates or granules from sod stay together and resist the complete dispersion which accompanied the other two samples.

The sod sample is being held together by a mass of tiny roots together with gums and resins which result from organic matter decomposition by microorganisms. These gums and resins are increased in the soil by growing meadow crops or by additions of crop residues which cause increased microbiological activity.

Soils with water-stable aggregates or granules brought about by the above processes are said to have good structure. This condition allows water to enter the soil more rapidly, thus helping to resist erosion. Air and water relationships are also improved in the root zone. These factors usually mean higher yields.

Questions/Discussion:

1. Which soil sample breaks up the quickest? Why?
2. What physical properties would cause some soils to hold together longer?
3. How would these properties affect potential water runoff losses and erosion damage?

E. Water-holding capacity

Background

Particle size is important when determining the amount of water soil will hold and how easily that water will move

through the soil. Water can enter the soil no faster than the water beneath the surface can move downward through the spaces between soil particles. If the spaces between soil particles are very small, like those in clay, the water will move slowly. The larger grains of sand will allow water to pass through much more quickly. Therefore, it takes a great deal of water and time to soak a clay soil, but sandy soils are saturated by relatively little moisture.

Soil-water relationships are important determinants of plant and microbial distribution. Plants or crops cannot live long in coarse, sandy soils low in clay because the spaces between the soil particles are so large.

Objective

To measure and compare the water-holding capacity of different soils.

Materials Needed

2 cans of equal size (juice cans)

Filter paper

Rubber bands

Scale balance

Water

Rulers or flat sticks

Tray

Soil samples

Procedure

1. Obtain two soil samples; one from a field that has been cultivated for many years and lacks organic matter (it

should be hard and cloddy) and one from a field or pasture where grasses or legumes have been grown (this should be crumbley and free from clods).

2. Remove the unopened end of the cans.
3. Secure a piece of filter paper over one end of the can with a rubber band.
4. Weight and record the weight of each can.
5. Fill one can about half full with one of the soil samples. Do likewise with the other soil sample. (Be sure soil samples are dry before starting this step).
6. Weigh the cans again and record the weights.
7. Fill the tray half full with water. Place the cans in the water and let stand overnight.
8. Remove cans from the water and drain for about one-half hour by placing rulers or sticks across the tray and standing the dripping cans on the rulers.
9. Before weighing cans again, make predictions about the new weight of the cans.
10. Weigh cans and record their weights. (Be sure to remove all excess moisture on cans before weighing).
11. Determine the percent of moisture-holding capacity by using the following formula:

Weight of dry soil = Weight of can and soil - Weight of can

Gain in weight due to immersion = Weight of can, soil and water - Weight of can and soil

$$\% \text{ Moisture-holding capacity} = \frac{\text{Gain in weight due to immersion} \times 100}{\text{Weight of dried soil}}$$

F. Soil Compaction

Objective

To determine soil compaction in various areas and to determine its effects on plants and animals.

Materials Needed

2 thumb tacks

1 wide rubber band

1 empty thread spool (wooded)

9-inch length of dowel (must slide easily inside the spool)

Procedure

1. Sharpen one end of the dowel in a pencil sharpener. With a ruler, measure 2 cm from the point and mark a dark line with a pencil.
2. Measuring from the unsharpened end of the dowel, mark off 12 lines, each 1 cm apart. Number the lines starting with 1 nearest the unsharpened end.
3. Fasten the wide rubber band to the top of the spool with a tack on each side (see diagram)

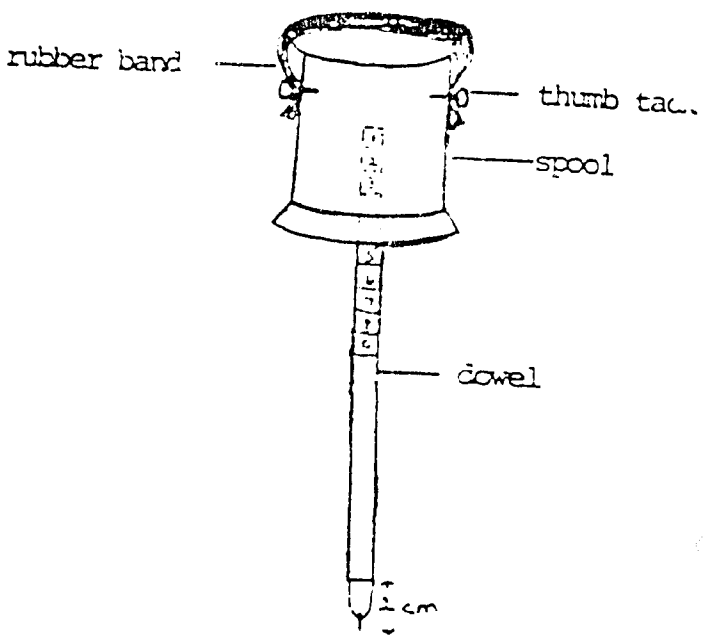
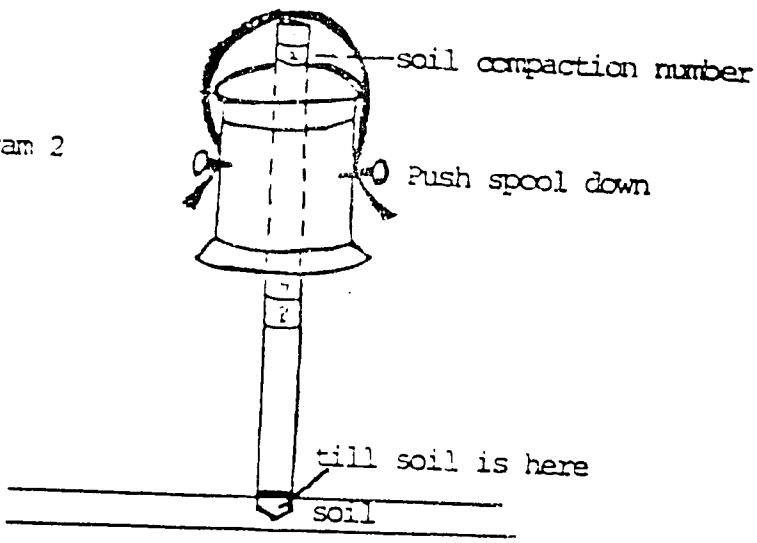


Diagram 1

4. Slip the dowel into the spool and pull down on the spool forcing the point of the dowel into the soil up to the dark line. When the pointed end is in this far, read the number at the top edge of the spool. This is the soil compaction for that location (see diagram 2).

Diagram 2



5. Select two sites 3' x 3' (or 1 square meter) where students congregate (path, foot of sliding board, etc.) and two other sites where student traffic is light or non-existent (shrub bed, middle of field, etc.).
6. Find and record the soil compaction for each site.

Interpretation

The compaction or hardness of soil is determined by how closely its particles are pressed together. For example, the soil under a balance bar is less compacted than the soil on either side of the bar. Water soaks slowly into compacted soil and this affects the plants that grow in the soil.

In one experiment, scientists found that pine trees growing in compacted soil grew only half as much as trees of the same kind growing in natural soil. Compacted soil is slowly loosened by the roots of plants and by animals such as earthworms. But the scientists estimated that it would take about 40 years for the compacted soil to become loose enough for the pine trees to grow at their usual rate.

G. Flocculation and Dispersion of Soil Clays.

As you know, most soil clays are negatively charged. This charge is neutralized in varying degrees by the absorbed cations. Floccules form most rapidly and are most stable where neutralization of the soil colloid by the absorbed cation is most effective. The amount of neutralization is related to ion size, valence, and concentration.

Objective

To determine the relationship between ion size and valence and flocculation and dispersion of soil clays.

Materials

1. Soil suspension (1 g soil/10 ml water)
2. 1 N solutions of $AlCl_3$, $CaCl_2$, NaCl, KCl, and NH_4Cl
3. Test tubes/test tube racks
4. Watch with sweep second hand or read out for seconds

Procedure

1. Add the prepared 1% clay suspension to each of the six test tubes. Fill the tubes so that each one contains the same amount.

2. Add 10 drops of 1 N $AlCl_3$ to test tube no. 1. Repeat this procedure using 10 drops of 1 N KCl, $NaCl_2$ and NH_4Cl in test tubes 2, 3, 4 and 5 respectively. Tube 6 containing the original clay suspension will be used as a check.

3. Shake each suspension. Hold each tube up to the light in order to see when the flocculation starts. Record the time that flocculation starts in each tube.

Observations

1. Wait 15 minutes. In that time, any floccules that form will sediment in the tube. Observe the appearance and size of the sedimented floccules. Are they loose and fluffy; or small and tightly grouped? Fill in the table:

Cation added	How soon did flocculation start? (1 min., 2 min., etc.)	Floccule size Small, Med., Large
NH_4^+		
K^+		
Na^+		
Ca^{++}		
Al^{+++}		
Check	No floccules, remains dispersed	

2. Rank the cations in order (fastest to slowest) in which they caused flocculation to occur

3. Did any cation not cause flocculation _____

Which one _____.

H. Ion Exchange In Soils

The charge on soil clay and organic matter can also be demonstrated by adding dyes containing colored cations or anions to soil and observing the color of the solution after it passes through the soil. The charge on the soil clay or soil organic matter must be opposite in sign to that of the adsorbed dye ion if a clear solution results. The colored in Gentian violet is positively charged, whereas the eosin red contains negatively charged ions (Fig. 1).

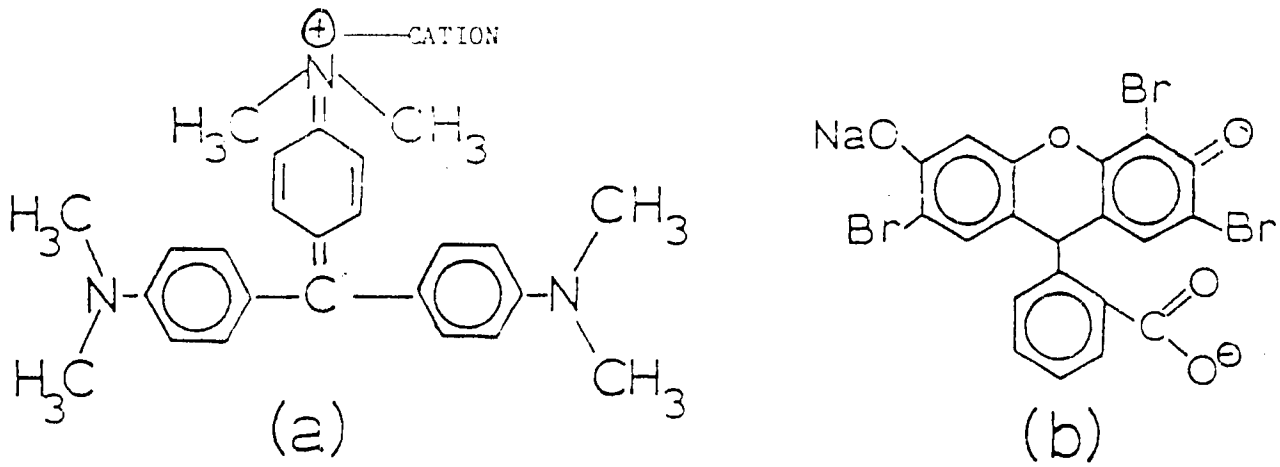


Fig. 1. Gentian violet (a) and eosin red (b). These dyes, one cationic, the other anionic, are used to demonstrate ion exchange.

Objective

To determine the charge characteristics of soil.

Materials

1. Funnels/funnel rack
2. Solutions of gentian violet and eosin red
3. Filter paper

4. Soil

5. Scoop

Procedure

1. Place two funnels in the rack, fit them with filter paper, and insert test tubes under each funnel.
2. Place two or three teaspoons of one of the soils in a filter funnel; place a similar amount of the other soil in the second funnel. Make a depression in the soil, and add Gentian violet dye in small portions so that the dye goes through the soil and not down the side of the filter. Add eosin red to the other sample in a similar fashion.

Observations

1. What is the color of the solution that leaches through the soil from the Gentian violet, from the eosin red preparations?

I. Soil Acidity

Background

The proper soil reaction or soil pH factor is extremely important to plants because it directly affects the availability of the plant food nutrients which the plants need for efficient growth. Soils that are too acid or too alkaline will not favor the solution of compounds and restrict the presence of ions of essential plant nutrients, as many of these elements do not dissolve easily in these extreme soil conditions. Because of this, most plants prefer a soil reaction that is neutral or near the neutral point. Soils

in the pH range of 6.0 to 8.0 are generally satisfactory except for certain acid-loving plants that grow best in soils that have pH values in the range of pH 4.0 to 5.0. The pH value of a soil sample indicates the 'chemical climate' of the soil (Fig. 1).

Objective

To determine the pH of the collected soil samples.

Materials

Beakers

pH 7.0 buffer

pH paper

Glass stirring rods

Procedure

1. Add about 10 grams of soil to a 50-ml beaker; add 10 ml of distilled water; stir thoroughly.
2. Let stand for at least 30 minutes, stirring 2 or 3 times.
3. Read with a pH meter using a glass electrode, adjusted originally to pH reading of 7.0 with the pH 7.0 buffer. The instructor will demonstrate the proper use of the pH meter and electrode.
4. Tear off piece of pH paper and dip into soil suspension. Compare reaction color with standard supplied with paper.

Interpretation

The chemical characteristics of a soil are dependent, in part, upon the nature of the rock from which the soil

formed. The weathering of granite and many sandstones produces acid soils. The weathering of limestone commonly results in alkaline soils. In the humid regions, however, part of the rain water that falls on the land moves through the soil and carries away the most soluble constituents. Thus, in time a soil formed from limestone can become acid after the lime has been removed.

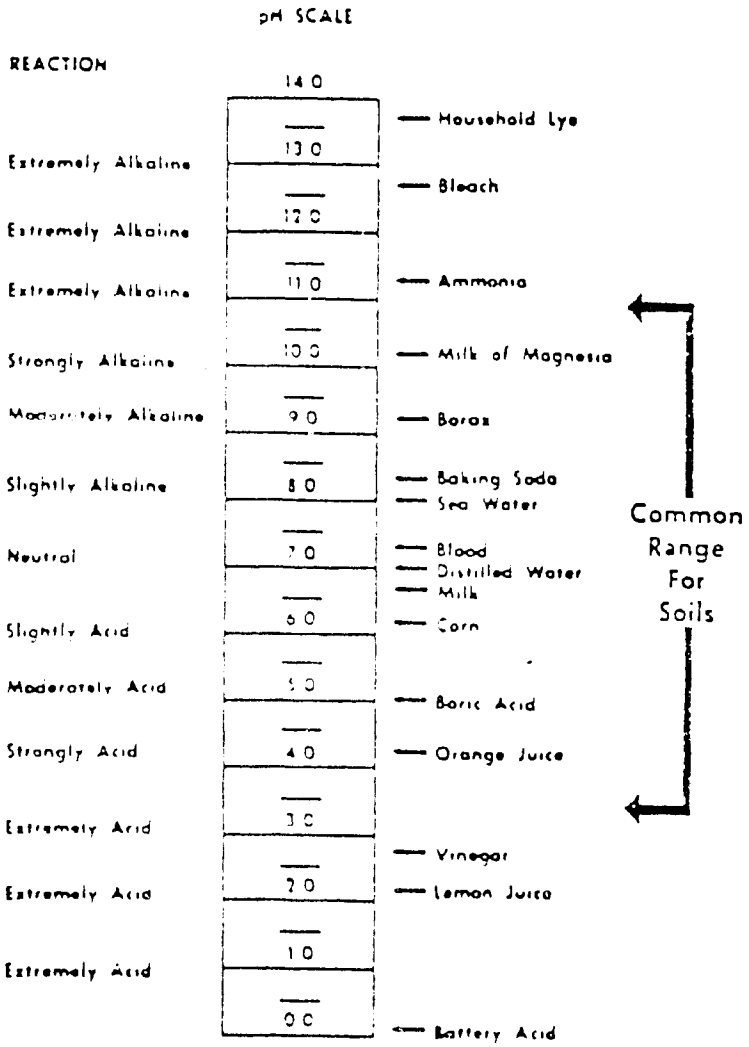
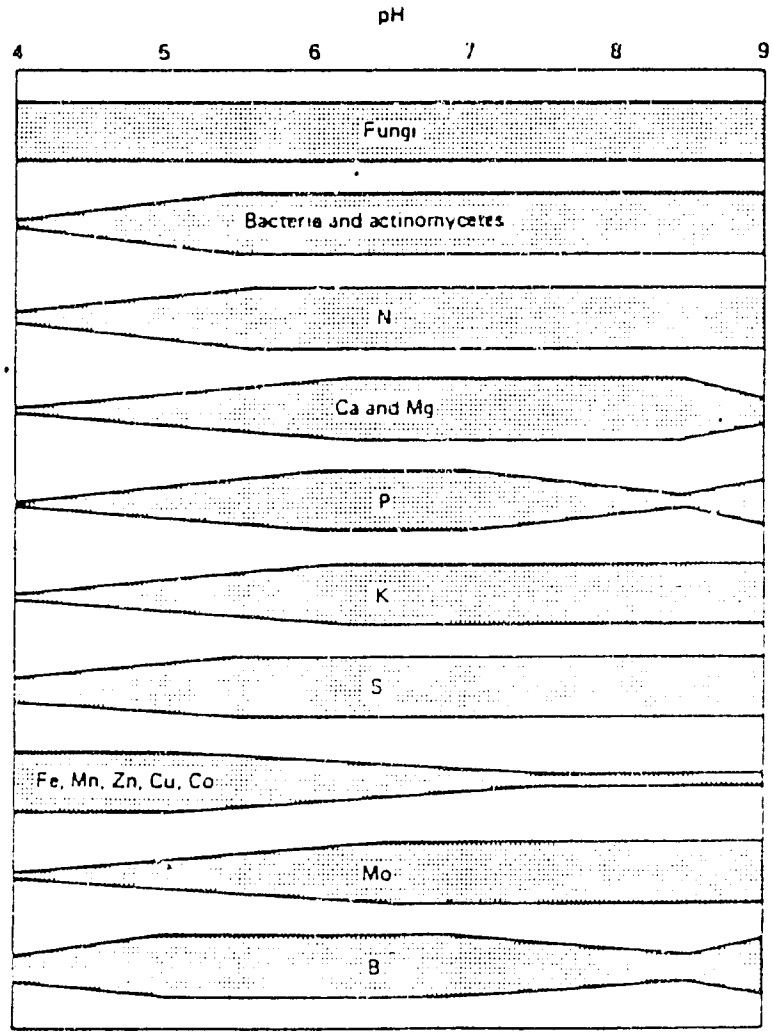


Fig. 1 The approximate pH values of some common substances, including soil.

The direct effect of pH on both soil microorganisms and on the availability of several essential elements is shown in the diagram below. It is apparent that several nutrient elements important to good legume growth have limited availability at pH levels very common in the tropics.



Relationships existing in mineral soils between pH on the one hand and the activity of microorganisms and the availability of plant nutrients on the other. The wide portions of the bands indicate the zones of greatest microbial activity and the most ready availability of nutrients. Considering the correlations as a whole, a pH range of approximately 6 to 7 seems to promote the most ready availability of plant nutrients. In short, if soil pH is suitably adjusted for phosphorus, other plant nutrients, if present in adequate amounts, will be satisfactorily available in most cases.

J. Major Soil Nutrients

Background

Plants absorb many elements from the soil but not all are required for growth. The elements required by plants in order to complete vegetative and reproductive cycles are called essential elements. There are about 17 essential elements, but not all are required in equal amounts. Of those required for growth, nitrogen, phosphorus, potassium, calcium, magnesium and sulfur are used in relatively large quantities. Some of the nutrients, even though required in small amounts are very important for normal plant development. For instance, for the leguminous plants these nutrients are generally thought to be iron, cobalt and molybdenum.

Nitrogen, phosphorus and sulfur are important in the elaboration of amino acids and proteins. For the leguminous plants nitrogen may be obtained through the rhizobia that cause and inhabit the nodules found on the roots. Nitrogen gas from the soil atmosphere is enzymatically converted by the rhizobia to ammonia and used by the plant. The other nutrients required for growth must be supplied from the soil or applied as fertilizers.

Phosphorus is also important in energy transfer processes in the plant and if available in small quantities will prevent the conversion of light energy into protoplasm. Some of the other nutrients are important as cofactors for the many enzymatic reactions that occur within the plant and

without an adequate supply normal growth will not take place.

Objective:

To determine the effect of different soil nutrients on the growth of leguminous plants and observe deficiency symptoms of various nutrients.

Materials

1. Seed of appropriate leguminous plants (peanuts work well).
2. 24 - 4 liter containers.
3. Nutrient solution stocks as indicated in table on next page.
4. Alcohol for disinfecting seed.
5. Air pressure pump.
6. Tubing - flexible.
7. Tubing - glass.
8. Large bore syringe needles.
9. Filter paper.

Procedure

1. Arrange plant growing containers on bench in glasshouse.
2. Attach flexible tubing to 10 cm pieces of glass tubing. Place glass tubing in container and connect other end flexible tubing to syringe needle. Insert needle into tubing connected to air pressure port on air pump.
3. Add nutrient solutions to containers as indicated in following table. Prepare three replicates for each treatment. Make to 4 liters with distilled water.

Volumes of Stock Solutions to be Used

for Various Nutrient Solution Treatments When

Growing Leguminous Plants

Stock Solution	Complete	N	P	K	Ca	Mg	S	Fe
	---------- milliliters per fourliters ----------							
1 M $Ca(NO_3)_2$	16	0	16	16	0	16	16	16
1 M $MgSO_4 \cdot 7H_2O$	8	8	8	8	8	0	0	8
1 M $NH_4H_2PO_4$	4	0	0	4	4	4	4	4
1 M KNO_3	24	0	24	0	24	24	24	24
1 M $CaCl_2$	0	8	0	0	0	0	0	0
1 M KCl	0	12	0	0	0	0	0	0
1 M NaH_2PO_4	0	4	0	0	0	0	0	0
1 M $NaNO_3$	0	0	0	24	32	0	0	0
1 M Na_2SO_4	0	0	0	0	0	8	0	0
1 M $MgCl_2$	0	0	0	0	0	0	8	0
1 M NH_4Cl	0	0	4	0	0	0	0	0
1000 PPM Fe as FeEDDHA	4	4	4	4	4	4	4	0

4. Soak seeds in alcohol for 10 minutes and then rinse with 5 changes of sterile water.
5. Germinate seed in filter paper moistened with complete nutrient solution. Keep in humid chamber in the dark.
6. When radicals are 7 to 12 cm long select uniformly developed seed and suspend in 24 nutrient solution containers.
7. Start pump for aerating nutrient solutions.
8. Change nutrient solutions after 7 days and again 7 days later. Subsequently change nutrient solutions every 3 days until the end of the experiment (from 35 to 42 days depending on plant growth).

Observations

1. Note the comparative growth rate of the plants of different treatments.
2. Note time for yellowing to begin, necrotic spots to develop on leaves and petioles.
3. Note other abnormalities of growth as compared to the "complete" treatment.
4. Where on the plants were the various deficiency symptoms expressed (top, middle, or lower leaves)?
5. On which plants did the lower leaves fall off first?
6. In your observations of field growth plants have you noted similar plant symptoms? Based on what you have now seen, what do you think the nutrient deficiencies were?

EXERCISE 3 - BACTERIA AND RHIZOBIA

A. Introduction

Nowadays we readily accept the notion that a man, or a cow, can be the prey of creatures smaller than you can see with the naked eye. After all, who has not heard of bacteria? But there was a time, and not so long ago, when people were not ready to accept the existence of these minute organisms. If you had lived at that time and were told that...

> almost 2,000 bacteria placed side by side can fit on the head of a pin; and, there are about a trillion bacteria (1,000,000,000,000) in a quart of sour milk; and, a cow weighs about 100 billion times more than a single bacterium

...would you have believed this was so?

You could not be blamed even now if you did not believe these statements. They do force us to stretch our imagination. However, a significant part of science is exercising the imagination, but in a critical way, so that the truth of any statement can be tested. If you don't know whether or not you want to accept the statements above as true, you will be better able to judge for yourself after doing the work in this Block.

Why Study Microbes?

Why study microbes when it seems so much easier to work with larger creatures? First, microbes have great importance to man and to other living organisms.

There is hardly any aspect of our lives, including our food, shelter, or health, that is not directly or indirectly affected by the growth of microbes. We can name the production of cheese, beverages, antibiotics, industrial chemicals, and fuels as examples. On the other hand, microbes cause diseases that afflict us all. Microbes rot buildings and food. So, whether these small organisms help man or hinder him, they

cannot be ignored and must be understood. More directly, in 1886, two German scientists discovered a symbiotic relationship existed between rhizobia bacteria and legume plants within the nodules of the legume root systems. The rhizobia bacteria were isolated, and in 1890 the advantage of adding pure bacteria to the seed prior to planting was demonstrated.

Nitrogen-fixing bacteria (rhizobia) are introduced into the soil either directly or by applying them to the seed. When the seed germinates, the bacteria invade the root hairs of the seedling and begin to multiply. Nodules (lumps) form on the roots, which house the bacteria. The symbiotic relationship occurs, as the plant provides food and a supply system for the bacteria--and the bacteria convert nitrogen (N_2) to ammonia nitrogen (NH_3) for their host. This biochemical conversion process thus makes unuseable atmospheric nitrogen available to the legume plant in a useable state.

The legume is able to translocate the useable nitrogen through its vascular system to its fruit and leafy tissue. The plant supplies the rhizobia with photosynthate, a food source the bacteria use as an energy source.

Rhizobium is the genus or type of bacteria that will enter into a symbiotic relationship with legume plants. Within the genus there are different species. From these species scientists are able to identify thousands of strains of rhizobia bacteria, each possessing some unique characteristics from other strains.

The major nonleguminous food crops of the world do not enter into a symbiotic relationship with any genus of bacteria. Their major source of nitrogen comes from commercially produced synthetic fertilizers.

Current research projects throughout the world are looking at the unique relationship of rhizobia to legumes with the objective to someday be able to transfer these characteristics to nonleguminous plants.

Most of the methods used to study microorganisms were first developed for the study of bacteria, the smallest free-living microorganisms. Although the methods may seem complicated at first, actually they are simple and easy to learn. The techniques can be mastered with a little practice, especially if the following points are kept in mind.

1. Types of instruments. The inoculating loop, the needle, and the pipette are the most common instruments--they are the hallmarks of the microbiologist. These simple tools are designed to handle organisms of minute size. Actually, individual microbes are seldom studied separately. Instead, they are usually handled in large numbers. From our point of view, however, the amount of microbial growth may be very small. It may be barely visible, so we need these special instruments. Before these instruments are used to transfer microbes, they are sterilized; the wire loop and needle in the flame, the pipette in the autoclave.

2. Microbial cultures. In nature, many kinds of microorganisms are found growing together. But when we grow ("culture") microbes in the laboratory we usually want to separate the different kinds and grow each kind ("species") as a pure culture. To make a pure culture we must sterilize the culture media and containers. We must, moreover, keep the culture media free of foreign microbes by providing barriers to their introduction. The use of cotton to strain out microbes has been practiced since 1855 when a pharmacist used a cotton plug to keep molds out of his drug preparations. Making media, preparing culture containers with cotton

plugs, sterilizing glassware and media are necessary preparations for growing microorganisms.

3. Aseptic techniques. When we transfer microorganisms from one piece of equipment to another, we do so in an environment in which there are unwanted microbes. If they are introduced, we call them contaminants. We cannot see them with our eyes so we must "see" them with our minds! We must visualize how unwanted microbes can get into cultures. Then we handle the cultures in such a way as to prevent the entrance of the contaminants. This requires: (1) disinfecting working surfaces with chemical solutions; (2) heating openings of culture containers when the cotton plug is removed; (3) sterilizing loops and needles before transferring microbes; (4) removing covers from petri dishes just far enough and only long enough to introduce the culture medium and the microbes.

4. Preventive measures. Finally, when we handle microbes we must be careful not to distribute them carelessly about the working area. This is especially important when microbes that cause disease are studied in hospital and research laboratories. Even though you will not be handling disease agents, you should learn to handle cultures as if they were. Develop a steady hand for the careful transfer of microbial growth. Don't leave a trail of microbes behind! Sterilize the inoculating loop and needle before putting it down. Put all cultures and other materials containing living microbes in containers to be sterilized. Use disinfectant solutions to clean work areas. All these practices are designed for everyone's welfare.

The basic microbiological techniques will be demonstrated. A film on techniques will be shown. You will practice these techniques until you too become skilled at working with the microorganisms.

B. Basic Techniques

How to Observe Bacteria

Preparation of Wet Mounts: A technique basic to many procedures in preparing bacteria for microscopic observation.

1. Place a drop of water in the center of a slide.
2. Flame a transfer needle and wait until it cools.
3. Remove a small amount of bacteria from a culture and mix it with the water.
4. Flame the needle again.
5. Apply the cover slip and observe.

Preparation of a Smear: Another basic technique that you must perform with precision, as illustrated in Figure I-8.

1. Carefully clean a slide by washing it with soap and water, drying, and gently heating one side in the flame of a Bunsen burner. These precautions are necessary to remove any fatty materials from the slide. If your slide is clean, a drop of water will spread evenly over the slide instead of forming discrete drops.
2. Handle the slide by grasping the edges and placing it on the desk, flamed side up.
3. Flame the transfer loop and use it to place a drop of water in the center of the slide. Flame the loop again, and set it down.
4. Flame the needle. Remove the cotton plug from the mouth of a culture tube by grasping it in the right hand and removing and holding the plug in your right hand.
5. Flame the neck of the tube and remove a minute amount of the material from the culture. The amount of inoculum needed is very small.

6. Flame the neck of the tube again, replace the cotton plug, and set the culture in the test-tube rack.
7. Spread the material on the needle in the drop of water over an area about one-inch square.
8. Sterilize the needle by flaming it and set it down. Always make it a rule to flame the needle or loop before and after use.
9. Dry the film on the slide by holding it high over a low gas flame. Do not steam the liquid.
10. Fix the film by passing the slide five or six times through the upper portion of the Bunsen flame. With the completion of this final step the smear is ready to observe.

Variation: If you want to make a smear of a bacterium in liquid culture, proceed as described above except that the bacterial suspension is applied directly to the slide instead of using a drop of water.

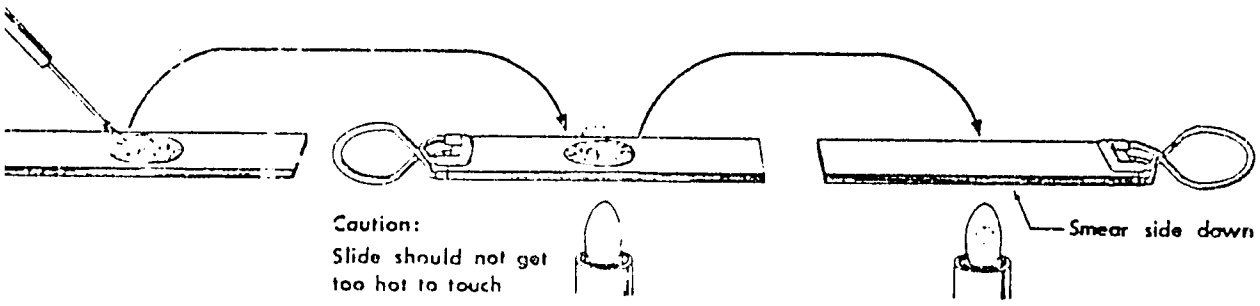


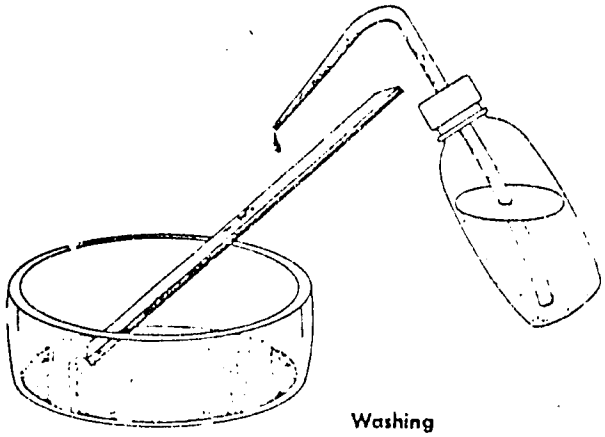
FIGURE I–8

A Simple Stain: Most routine stained preparations of bacteria are prepared by the use of a dye like methylene blue, gentian violet, or carbol fuchsin. To stain in this way, the dye is poured onto a slide which has been smeared as described previously. The time of exposure depends on the stain used, and sometimes on the bacterium. After the dye is washed off the slide, the smear is dried. The most frequent mistake is having the organisms too crowded on the smear.

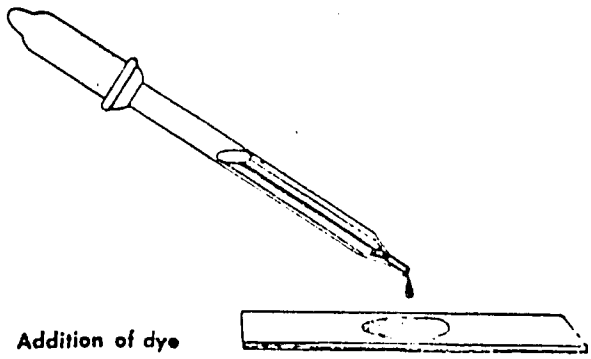
1. Cover a smear with methylene blue stain.
2. Allow the stain to remain for 5 minutes.
3. Wash in tap water, drain, blot, and air-dry, or dry high over a Bunsen flame.

For practice, to make sure you understand the staining technique before beginning your experiments, prepare a test slide as follows:

4. Cover with a cover slip and examine under your microscope.
5. If a permanent slide is desired, a cover slip may be mounted in a drop of balsam.



Washing



Addition of dye

FIGURE I-9

The Gram Stain: A separate category is retained for this stain, for it is probably the single most important bacterial stain. A Danish physician named Gram worked out its details when he noted that certain bacteria, after being stained with gentian violet, become decolorized

when washed in an iodine solution followed by ethyl alcohol. These bacteria are referred to as being Gram-negative, while those that retain the stain under these conditions are called Gram-positive. The Gram stain is significant because many other characteristics appear to be correlated with this reaction, including types of flagellation, response to certain poisons, etc.

1. Prepare a smear.
2. Cover the smear with Hucker's ammonium oxalate crystal violet stain and allow to remain for 1 minute.
3. Drain the excess stain and gently wash the slide in tap water.
4. Flood with Gram's iodine solution and leave for 1 minute.
5. Rinse with 95% ethyl alcohol until no more dye flows away from the smear (about 30 sec to 1 minute).
6. Wash in tap water.
7. Counterstain with safranine for 10 to 30 seconds.
8. Wash in tap water, drain off the excess water, blot, and allow to air-dry.

Spore-Stain: Certain bacteria produce resistant cells, called spores, which are characterized by their heavy wall and by their lack of affinity for stains. As in the case of acid-fast organisms, special techniques are required in order to stain these cells.

1. Prepare a smear in the usual way except that the slide should be passed through a flame 20 times.
2. Immediately flood the warm slide with 6% aqueous malachite green and stain for 10 minutes.
3. Rinse with tap water for 10 seconds.
4. Counterstain with 0.25% aqueous safranine for 10 seconds.
5. Rinse with tap water, blot, and air-dry.

C. Air-Borne Microbes

Microorganisms are all around us even though we are not conscious of their presence. They are spread far and wide from those places where they grow (habitats) to all parts of our environment. Often they go along for a free ride with anything that moves. So the questions of where microorganisms are found and how they are spread are appropriate ones to ask.

Materials

1. Eight or 10 tubes with 15 ml of sterile nutrient agar in each
2. Eight or 10 sterile petri dishes
3. Water bath

Procedure

Preliminary Preparation: Prepare the medium and sterilize the petri dishes before the day of the experiment.

Preparation of the Air-Exposure Plates: Melt the agar in a boiling water bath and cool to 50 C.

Pour the nutrient agar into the set of petri dishes and let it solidify.

Before the students assemble for classes in the morning the teacher or an appointed student will remove the cover from one agar plate, leaving the cover off for 10 minutes. Replace the cover. Label the dish.

During each class period throughout a day a student will expose 1 plate to the air of the classroom for 10 minutes.

After classes are over and while the room is being cleaned the teacher or a student will expose another plate. All plates should be exposed at the same location in the room.

The next morning before classes begin, the last plate will be exposed.

All the plates are labeled, inverted, and incubated for 3 or 4 days.

Observations

The assigned students will count the numbers of bacterial and mold colonies on the plates and will arrange the plates as a demonstration for all the classes to observe.

The colony counts on each plate are reported to the classes and recorded on the chart.

Make a bar graph of the data.

CHART FOR AIR-BORNE MICROBES

Time	Numbers of Colonies		
	Bacteria	Molds	Total
Before Classes			
First Period			
Second Period			
Third Period			
Fourth Period			
Fifth Period			
Sixth Period			
Seventh Period			
Eighth Period			
After Classroom Clean-up			
Next Day Before Classes			

D. Microscopic Appearance of Bacteria

In 1676 Anton van Leeuwenhoek, a Dutch lens grinder, mixed peppercorns (unground pepper) with water and looked at a drop of this "infusion" with his simple microscope. In the infusion he saw bacteria for the first time.

Now we use high-powered oil immersion microscopes to observe bacteria. With such instruments we see bacteria magnified almost 1,000 times. We look at living bacteria in wet mounts, or more often we stain them to see them more easily.

In this exercise the staining technique will be used to reveal a few characteristics of bacteria in a peppercorn infusion.

Materials

1. Air-exposed agar petri plates from previous exercise.
2. Microscope slide and cover slips.
3. Crystal-violet stain (see section on stains in Appendix).
4. Paper towel.
5. Compound microscope with oil immersion objective.
6. Immersion oil.

Procedure

A. Technique for Staining Bacteria: There are three main parts to this technique, each with several steps as follows:

Preparing the bacterial film on the microscope slide:

1. Using a very clean slide, free of oily or greasy material, heat gently by passing it above the blue cone of a burner three times.
2. When the slide is cool, place a loop of the peppercorn infusion on the slide and spread it over an area the size of a nickel.
3. Let the film dry in the air.

Fixing the bacterial film onto the slide:

1. Quickly pass the slide three or four times through the flame to fix or stick the bacteria onto the slide.
2. Let the slide cool to room temperature.

Staining the bacterial film:

1. Dip the slide film into a glass of clean water.
2. While the film is wet cover it with several (3 or 4 usually) drops of crystal violet.
3. Leave the dye on the film for 15 to 20 seconds before pouring off the excess.
4. Gently wash the slide in a tumbler of clean water. Repeat.
5. Drain the water from a corner of the slide.
6. Remove the remaining water with a piece of paper toweling by blotting. CAUTION: DO NOT RUB THE FILM.
7. When the film is dry, it is ready to be examined microscopically.

B. Observing the Bacterial Film with the Compound Microscope: Use the low-power objective to focus on the stained bacteria. What do you see?

Now swing the high-power objective into place. If necessary, re-focus with the fine adjustment. What do you see?

C. Technique for Observing Bacteria with the Oil-Immersion Objective: Swing the high-power objective out of place.

Put one drop of immersion oil directly onto the stained bacterial film.

Swing the oil-immersion objective into place at the bottom of the tube. The tip of the objective will go into the drop of oil. The oil makes a continuous medium between the slide and the lens system of the objective.

You are now ready to focus the microscope on the stained bacteria. CAUTION: Focusing should be done with very great care to prevent breaking slides and damaging the objective. Use only the fine adjustment and turn it slowly to bring the colored film into clear focus. Usually you turn the fine adjustment toward you. This moves the tube of the microscope upward.

Observations

Observe the different kinds of bacteria, their shapes, and their arrangements.

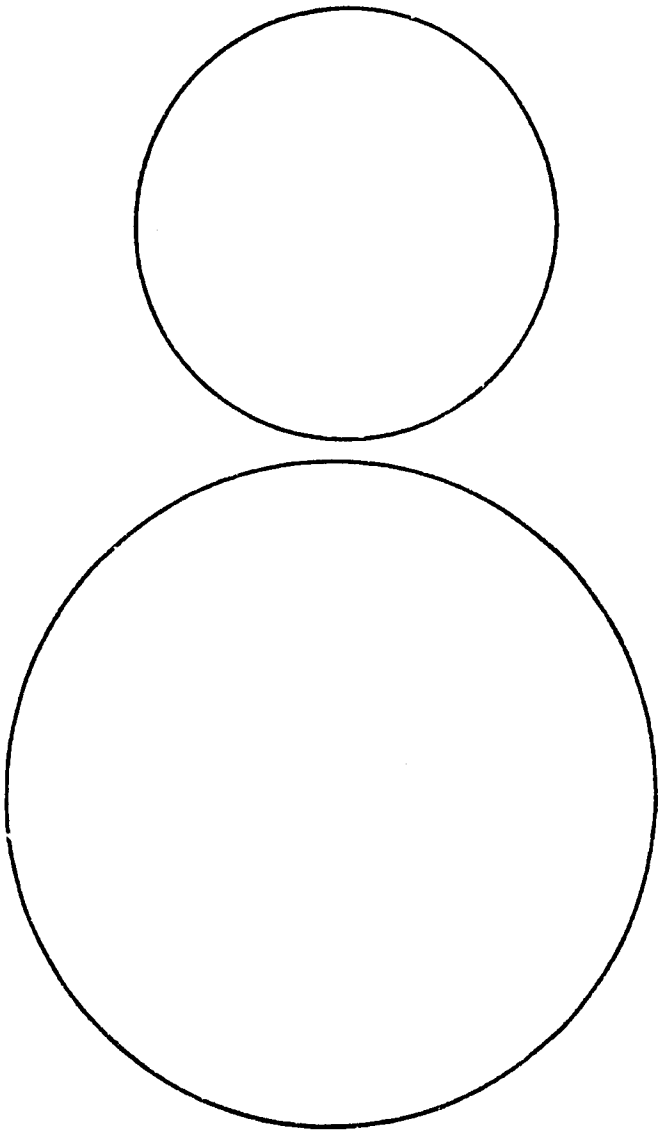
Draw representative bacteria, in their characteristic groups, which you have observed on the stained slide. Make your drawings on the chart provided.

Even though you are seeing the bacteria greatly magnified, still they appear very small. Make an enlarged drawing of each bacterial type to show its shape and proportions.

Determine the approximate dimensions of the different kinds of bacteria. Record in microns on the chart.

CHART FOR OBSERVATIONS

Representative Bacteria



Kinds of Bacteria	Approximate Dimensions (in microns)

E. How To Get Pure Cultures of Bacteria

The need for pure cultures of bacteria was especially evident to Robert Koch when he studied infectious diseases. The problem was to determine which bacterium, of the many associated with the diseased animal or plant and its environment, was the causative agent of a given disease. To do this would require the separation of the different kinds of microorganisms present.

Since microbes are so minute and so closely intermingled in their natural habitats, the separation of different kinds cannot be done as easily as one can separate dandelions from grass in a lawn. Yet it is possible to make these separations to obtain pure cultures--cultures containing only one kind of microorganism.

How are microorganisms separated? You will use techniques which Koch and his coworkers developed and which are still used today to isolate pure cultures of all types of microbes.

Purpose

Pure cultures of microorganisms will be prepared by using two methods: the "streak plate" method and the "pour plate" method.

Materials (per team)

1. 40 ml nutrient agar or beef bouillon.
2. Water bath (double boiler)
3. One transfer loop.
4. One 1-liter flask.
5. 8 test tubes.
6. 3 petri plates.
7. 1 graduated cylinder.
8. 10-ml pipettes.
9. Soil suspension (1 g garden soil in 5 ml water).
10. Mixed suspension of *Rhizobium*-*Serratia marceseus*.

Procedure

First Day

A. Preparation of Culture Medium (per team): Select 5 clean test tubes of approximately equal diameter. Mark the 15 ml level on all 5 as follows: Place 15 ml of water in 1 tube and mark the water level with a wax pencil. Pour out the water and use this tube as a measure to mark the 15 ml line with wax pencil on the other tubes.

Fill all tubes to the 15-ml mark with nutrient agar medium or beef bouillon agar medium made according to directions in Appendix, and plug with cotton.

Select 5 more clean test tubes. Using a 10-ml pipette, add 10 ml of distilled water to each of the test tubes and plug with cotton.

Sterilize all tubes containing water and agar in an autoclave or pressure cooker at 15 lb pressure for 15 minutes (121.5 C). Each pair of students should have three tubes of water and five tubes of agar.

Second Day

B. Preparation of Plate Cultures: Each pair of students will use one tube of mixed suspension of *Rhizobium* and *Serratia marceseus*. Set up a boiling water bath to melt the tubes of media. While agar is melting make dilutions for the following methods.

Pour Plate Method

Arrange the three test tubes of sterile water in a rack and number tubes 1 through 3. Place 5 sterile petri plates on desk and number 1 through 5.

Sterilize a bacteriological loop in flame, cool, and take 2 loops of the source material and inoculate Tube 1. Mix by tapping gently or rolling tube between palms of hands. Flame loop, cool, and transfer 2

Take 2 loops of culture from Tube 1 and add to Tube 2. Mix and transfer 2 loops from Tube 2 to Plate 2 and to Tube 3.

Mix Tube 3 and transfer 2 loops of culture to Plate 3. (Caution: Flame loop and tube lip before and after each transfer. Also replace cover of petri plate as soon as plate is inoculated.)

When agar in tubes is melted, cool in the water bath to 45 C and pour 1 tube of agar into each numbered plate. Dry tube with towel and flame mouth of tube before pouring plates. Do not allow tube lip to touch inside of plate. Plate 4 is a control containing agar but no source of microorganisms.

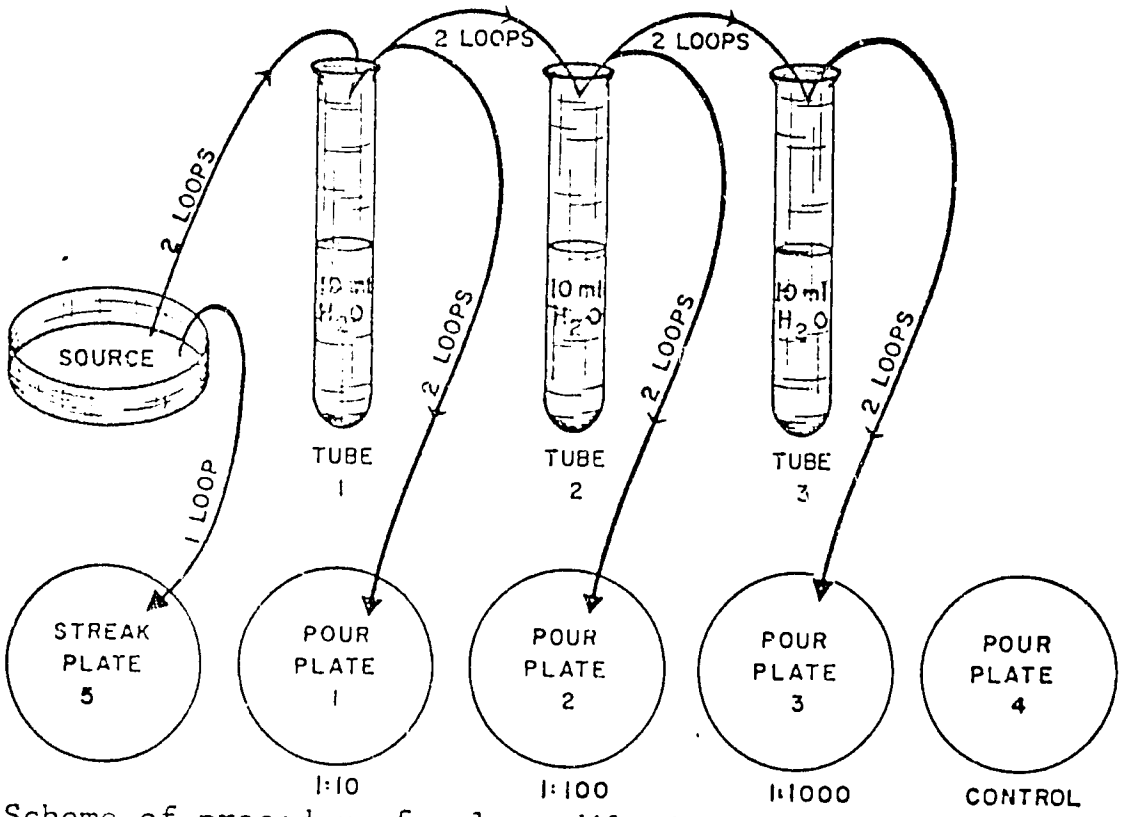


FIG. 20 Scheme of procedure for loop dilution with pour plates.

Streak Plate Method

When Plate 5 has solidified, flame loop, cool, and take a loop of the original source material and streak the surface of the agar. Do not

dig or cut into the agar. Use one of the methods illustrated in Figure 21 to streak the plate.

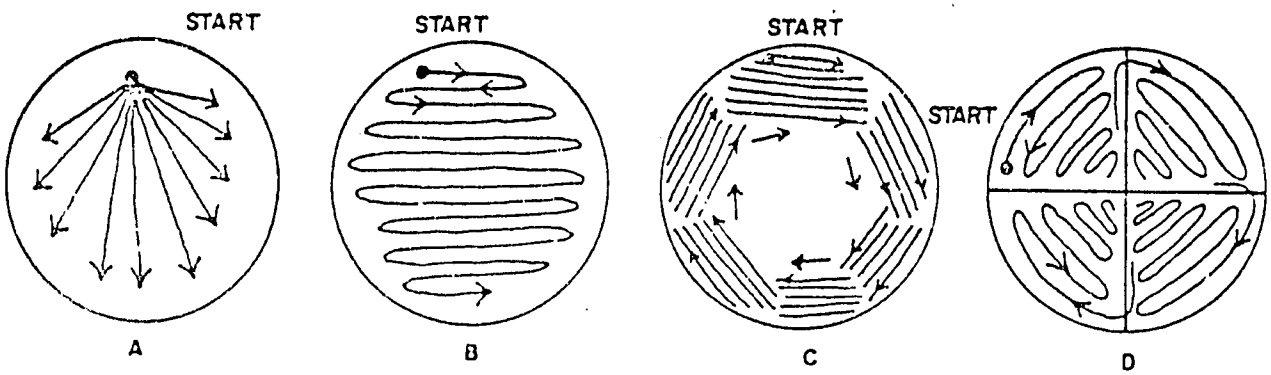


FIG. 21 Four different methods of streaking plates.

Incubation of Plates

When the plates have solidified, incubate them upside down at room temperature (do not incubate plates near a radiator or in direct sunlight. 25 C is a good temperature). Observe daily for 3 days after which time final observations are to be recorded.

Observations

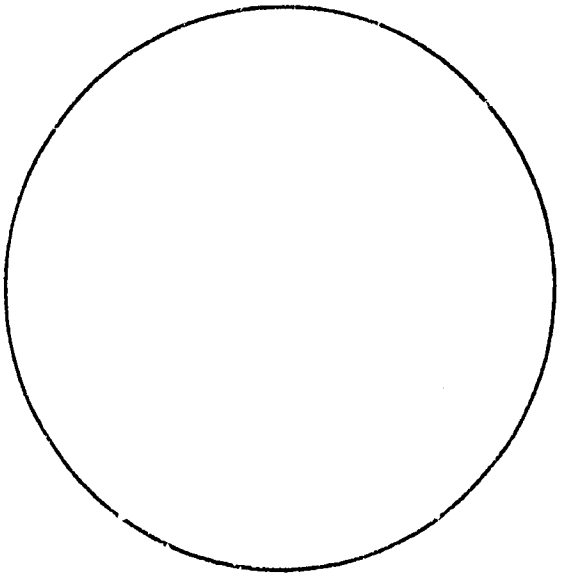
Record your results diagrammatically on the following chart.

Divide each plate into sections and count the number of colonies. Assume that each colony came from a single bacterial cell. Hence, the number of viable organisms in the original source material equals (number of colonies per section) x (number of sections) x (dilution). Instead of dividing the plate into sections, you may use plastic counting discs if available.

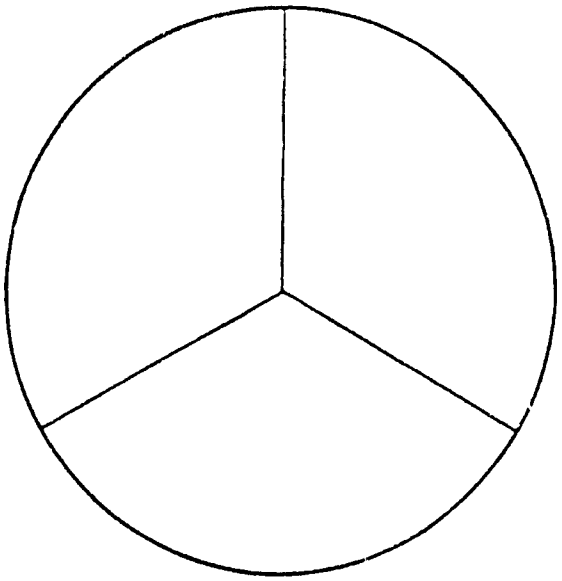
Estimate the number of bacteria present in the first loopful of source material assuming that (1) the volume of the material transferred by a loop is 1/100 ml, or 10^{-2} ml; and (2) the diameter of the petri dish is 10 cm. In your calculations try to use plates containing from 30 to 300 colonies.

CHART OF OBSERVATIONS

Plates Inoculated with



Agar Streak Plate



Composite Loop Dilution Agar Plate
(The three sectors represent plates 1, 2, and 3).

EXERCISE 4 - COLLECTION OF NODULES AND ISOLATION OF *RHIZOBIUM*

A. Introduction

A successful legume plant at a particular site is frequently a manifestation of an especially effective symbiotic association between host plant and strain of *Rhizobium*. A plant explorer who collects seed alone should not expect to see the full potential of the plant line in subsequent evaluations unless some attempt is made to reconstitute an equally effective symbiosis. Commonly, an introduced legume fails to encounter effective native strains, and/or nodulate effectively with available inoculants, in a new environment. Experience shows that this situation can be avoided if strains of *Rhizobium* which are genetically and geographically affiliated with that host can be made available for use as inoculants. Thus nodule (*Rhizobium*) collection should be undertaken as a routine procedure by plant collectors.

This section is directed specifically at the plant collector who has neither microbiological training nor legume bacteriology back-stopping from within his research organization. The step-by-step instructions for collecting nodules in the field maximize the possibility of successful isolation of a *Rhizobium* strain by the laboratory which undertakes processing of the samples. A companion volume of

this manual describes field and laboratory methods for isolation of Rhizobium from nodules, for characterizing the strains in a pure culture and for conserving strains in a Rhizobium collection. It is sufficiently comprehensive to permit a research group to initiate work in this area and would be particularly useful to a plant pathology laboratory with no prior experience with Rhizobium if they were to undertake to back-stop a legume collection program.

B. Nodule Collection

In this exercise you will have the opportunity to become familiar with legumes in the field, examine their nodules, isolate presumptive rhizobia from such nodules, verify the purity of the isolates and then authenticate them as *Rhizobium*.

Materials

Legumes with roots from field trip.

Razor blade.

Forceps.

Transfer needle.

Compound microscope.

Slides and cover slips.

Spot plates.

Carbol fuchsin stain (appendix).

95% ethanol.

1% acidified mercuric chloride solution ($HgCl_2$ 1 g; HCl 5 ml; water to make 1 liter).

Procedure

Preparation for the Collection Trip: Timing of a trip to collect nodules for isolation of *Rhizobium* should coincide with the season of vegetative plant growth and adequate soil moisture. This may not always be possible, since combined *Rhizobium*/plant collecting expeditions usually are made at plant maturity to facilitate seed collection. Unfortunately, at this time most legumes carry only a

reduced nodule complement (if any) and the dry, baked soil conditions impede excavation of roots.

It may be necessary to undertake a separate expedition specifically to collect nodules because of non-coincidence of the nodule- and seed-collecting seasons. Therefore, it is imperative that plant collection site documentation be sufficiently explicit to permit the exact location (and ideally, the same plant) to be re-visited in a different season.

Materials and Equipment Need for the Collection Trip:

The materials required during a collection trip depend on its duration:

Short term

-- spade;
-- knife;
-- permanent marker;
-- field book;
-- selection of polyethylene bags;

Medium term

-- all above;
-- collection vessels (Figure 1);

Long term

-- all above;
-- packing materials for dispatching samples to collaborators
-- sterilizing solution (1% acidified $HgCl_2$, see above).

Collection of Nodules: When collecting from roadsides and other disturbed sites, note that soil conditions, plant performance and nodulation may not be typical of the region. Seek out legumes that appear successful in an ecosystem pertinent to your interests, such as native pastures and/or stable natural ecosystems. Having located a plant from which to collect nodules, subsequent procedures depend very much on duration of the collecting trip.

When it is possible to deposit the sample on the same day with the laboratory which will perform the isolation, it is simplest to excavate the whole plant and its nodulated root system in soil and pack it firmly into a polyethylene bag for transport to the laboratory.

For trips of one to 14 days, collected nodules must be prevented from decomposing and protected from invasion by soil microorganisms which interfere with subsequent isolation procedures. Segments of the nodulated root (be sure it belongs to the proper plant) may be collected in a plastic bag with moist soil.

The nodules may be conserved in a vial containing a desiccant. Collection vessels (Figure 1) must have caps that provide an air-tight seal. Desiccant (anhydrous calcium chloride or silica gel) occupies one-quarter to one-third of the volume of the container. It is held in place and must be prevented from coming in contact with the nodule samples by a cotton wool plug. The volume of nodules and/or roots should not exceed the volume of dessicant.

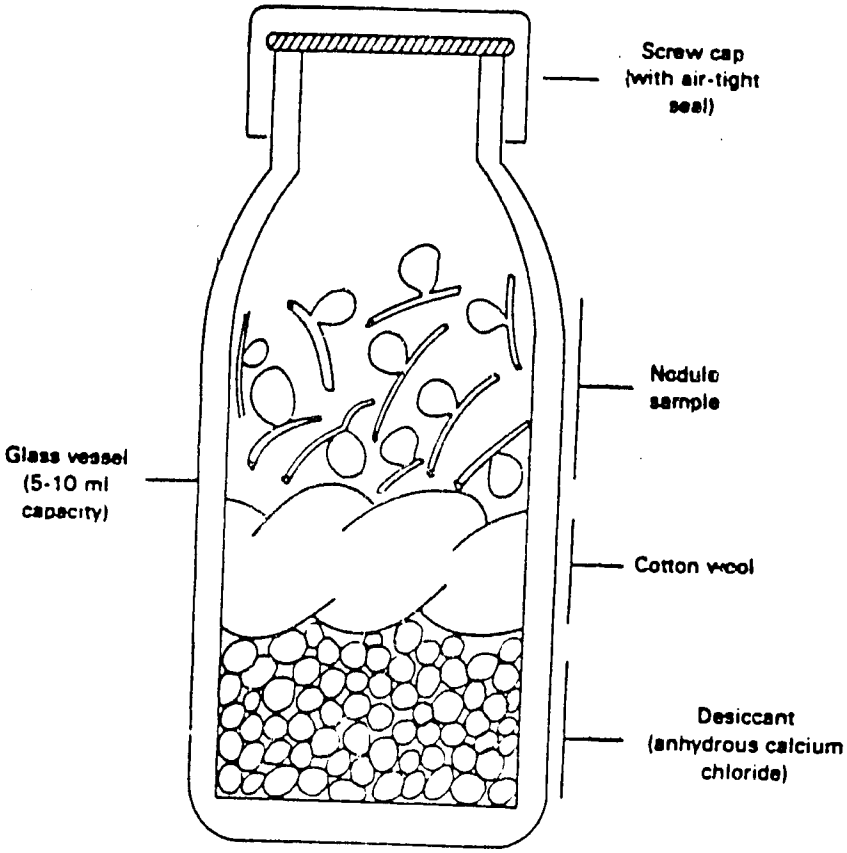


Figure 1 Vessel for storing nodules.

Seldom is it necessary to excavate whole plants to retrieve nodule samples. Careful paring away of soil around the root crown or stolon nodes to locate young adventitious roots and then carefully excavating these with a penknife often yields best results. If this method proves unsuccessful, (and it depends very much on the species involved), deeper excavation of the root system may be necessary. Insert a spade vertically into the ground some 15-20 cm from the base of the plant and on all four sides so that an intact sod of soil containing the bulk of the plant root can

be pried free. Extraction of the root system is made easier by immersing the sod in water and allowing the soil to fall away. Even without water, it should be possible to examine the root system for nodules by sequentially fragmenting the soil by hand.

The location of nodules on the root system is very species dependent. In a sward situation, the nodules of stoloniferous legumes usually are concentrated superficially, within 1-2 cm of the soil surface, attached to adventitious roots. In most Stylosanthes species they are equally distributed along the length of the primary and lateral roots in the top 10-15 cm, although in Stylosanthes capitate they tend to be found only at a depth of 15-25 cm.

In Leucaena, nodules seldom can be found under mature stands and the best hope of success is with isolated seedlings and young plants growing in full sunlight.

Do not expect to locate nodules on the main rootstock of perennial species. Nodules of most legumes have a finite life span, and a maturing root that may have borne nodules when younger, no longer has the anatomy that would permit reinfection and nodulation. Nodules on perennial species sometimes can be located by following a root out to the zone of new growth; this zone may be many centimeters from the crown. Do not expect to find nodules on a plant that has been pulled out of the ground by force. In most species, the connection between the nodule and root is extremely tenuous.

Sample only fresh, firm nodules and avoid taking samples of those which are damaged or decaying, since routine isolation procedures are unsuccessful with such material. If the root to be sampled is abundantly nodulated, section some of the nodules to check for white, pink or green pigmentation (sectioned nodules are of no value for subsequent isolation).

The inner pigmentation often is visible throughout the semi-opaque nodule periderm, making it easy to pick active nodules for sampling. Use a sharp knife or small scissors to sever the root about 0.5 cm on either side of the site of nodule attachment. Even very fine roots can be amazingly resistant to attempts to break them with the fingers. Such efforts usually result in compression damage to the nodules.

If the root to be sampled bears only a few healthy nodules, it is more important to collect these than to section them to describe their pigmentation. Try to collect at least 10 nodules per plant. There are two reasons for this. First, it increases the chances of obtaining a viable isolate for a particular plant. Second, the proportion of nodules from a single plant that yields strains which are subsequently found effective in nitrogen fixation with the homologous host, can be quite low, as is the case with Stylosanthes. This is not surprising, given the range of effectiveness of strains in the soil population of Rhizobium that may infect a native legume species. All nodules from a single plant represent one unit of collected material and

may be stored in the same desiccating tube. Under no circumstances should nodule samples from different plants be combined. Label the sample with a specimen number that relates it to the documentation of its plant accession and site of origin.

C. Microscopic Examination of Rhizobia Nodules: When few other organisms are present, rhizobia may be isolated directly from soil, peat cultures or inoculated seed by streaking a suspension of the material to a suitable culture medium. However, in the presence of other organisms the suspension is used to inoculate a seedling host growing under bacteriological control, isolations being made subsequently from fresh nodules as described below.

During the field trips you became familiarized with the general toxonomic characters of the Leguminosae. The course followed by most collectors is to recover a good plant specimen, dry and press it and forward it to a reliable botanical source for precise identification. Nodule size, shape, distribution, and other characters are species-dependent, and this will be illustrated for you. Carefully excavate nodulated roots from the soil and place in polythene bags for return to the laboratory. If the legume is in seed, collect seeds for authentication of isolates as Rhizobium.

Cut thin sections of the nodules by hand and float them out in a little water on a microscope slide, cover with a cover glass and examine under low- (10x) and high- (40x) power objectives. The presence of leghaemoglobin can be

noted as pink, red or brown coloration. White, green and (rarely) black nodules may also be found. Ask your instructor to explain the significance of these color differences.

Rub the cut surface of a nodule on a dry microscope slide thereby making a smear. Allow the smear to dry in the air, then pass the slide through a flame momentarily. Cool the slide and stain the smear with dilute carbol fuschin for 10-20 seconds. Wash in water, blot off excess moisture and dry in air. Examine under the high power, oil immersion objective. Note the difference in morphology between the "bacteroids" in this smear from a nodule and bacteria of the same rhizobial species but grown in pure culture in the laboratory.

D. Isolating Rhizobium from a Nodule: The first step is surface sterilization. Wash roots thoroughly to remove soil and gross surface contamination. Sample nodules (preferably as many as 10) from the root by severing the root about 0.5 cm on either side of the site of the nodule attachment. These root tails can then be used to manipulate the nodules with forceps, thereby reducing the risk of damage to the nodule itself. Immerse briefly (1-5 seconds) in 95% ethanol, transfer to 0-1% acidified mercuric chloride solution ($HgCl_2$ 1 g; HCl 5 ml; water to 1 liter) and leave for 4 minutes. Rinse in five changes of sterile water, making transfers with alcohol flamed forceps. If the surface sterilization is performed with desiccated nodules, these

should be allowed to rehydrate in sterile water for 5 minutes after the final rinse.

Crush the nodule in a minimal volume of sterile water with fine-pointed forceps to give a milky suspension. Streak out one loopful of this on yeast-mannitol agar (YMA) plates. The most satisfactory means of coping with contamination is to vary the time of sterilization in mercuric chloride, emphasizing the need for a large initial nodule sample to allow repetition if the first attempt fails.

The procedure lends itself well to improvization, and many variations exist basically because they are preferred by individual rhizobiologists. Here are some variations--try them and compare your success at isolating by at least two methods.

The needle method of isolation is useful for large (diameter greater than 2 mm), freshly harvested nodules. Wash the nodule well with water, dip it in 95% ethanol and flame it momentarily. Holding the nodule with forceps, slide off a segment with a sterile scalpel. Insert a sterile inoculation needle, the tip of which is bent to a tiny 1 mm loop, into the cut surface. Streak the moistened loop directly onto YMA. An additional isolative procedure is illustrated in Appendix 2.

E. <u>Obtaining Single Colony Isolates from Primary Isolation Plates</u>: The plates you have prepared from the nodule homogenates are referred to as the primary isolation plates. Incubate these at 26°C and check for growth typical of

Rhizobium along the streak lines. Well isolated single colonies should be picked off and restreaked on clean plates to obtain pure cultures of the presumptive Rhizobium strain. It is possible that more than one "typical" colony type may appear on a plate streaked from a single nodule, and each of these should be taken to pure culture and held as stock cultures on agar slants in screw-top tubes.

Inoculate your presumptive rhizobia into flasks (two per isolate) containing 50 ml yeast-mannitol broth (see Appendix VII). Authentication will be performed using these cultures.

The isolation techniques used are the same as those listed in the earlier exercises. Yeast extract mannitol medium (for composition see Appendix VII) can be modified for use as a differential medium useful for authentication of the isolates. This is done by adding brom thymol blue (10 ml/liter of a solution made by dissolving 1.25 grams in 40 ml of .05 normal Sodium Hydroxide and diluting to 250 ml. with water). The majority of isolates from tropical legumes produce alkaline end products which turn this medium blue. Acid producers turn it yellow. While strains producing acid are associated characteristically with the Trifolieae-Vicieae, they occur also with other unrelated groups of legumes, including a few tropical types. Since the vast majority of tropical Rhizobium isolates produce these alkaline end products, blue color of the medium surrounding the bacterial colonies gives good venfication that rhizobia are present.

F. Authenticating the Isolates as Rhizobium: The importance of testing that the isolate is a pure culture of a single organism which can form nodules on legume roots cannot be over-stressed, and is an indispensible prerequisite for the organism to be referred to as Rhizobium.

Prepare four plastic cup assemblies for each of the isolates (see Appendix 1).

Surface sterilize seeds by momentary immersion in 95% ethanol followed by 2-3 minutes in 0.5% $HgCl_2$. For hard-seeded species (e.g., Stylosanthes spp.) immersion for 10-20 minutes in concentrated sulphuric acid will simultaneously scarify and sterilize the seed surface. In both cases sterilization treatment is followed by at least five washes in sterile water. Seed is allowed to imbibe by soaking in sterile water for about an hour, and is then transferred to 1% sterile water-agar plates. These are incubated in an inverted position so that the emerging radicles grow straight down. Germinated seeds are transferred to the tubes when radicles are about 1 cm long and are allowed to grow for about 5-7 days before inoculation.

Plant three pregerminated seeds of the appropriate host in each of four sterilized cup assemblies containing a nitrogen-free nutrient solution. Apply 1 ml of each broth onto each of the seeds in two cups. The other two are not inoculated and will serve as controls. These operations should be performed in the glasshouse or growth room where the plants will be cultured. Take adequate precautions against cross-contamination between treatments.

Examine for differences in vigor between the inoculated and uninoculated plants. These should become apparent 15-30 days after sowing. Remove the plants from the rooting medium and record the presence or absence of nodules. The presence of nodules in the uninoculated treatment invalidates the authentication and it must be repeated with better bacteriological control. Record the nodule number, nodule fresh weight and total plant dry weight in each treatment.

EXERCISE 5 - THE SYMBIOSIS

A. To Test For Genetic Compatibility Between Rhizobia and Legumes

In this exercise you will use methods that detect strains of *Rhizobium* able to nodulate particular legumes. You will become familiar with specificity and promiscuity of infectiveness in rhizobia, and also learn of the specific requirements of certain legumes for particular rhizobia.

Materials

Infiltration irrigation system for leguminous plant infectivity testing.
Nitrogen-free nutrient solution (Appendix 9).
Yeast extract mannitol broth.
Water agar (water plus .75% w/v agar).
Petri dishes.
Rhizobia and legumes as shown on Table 1.
3% solution of sodium hypochlorite (clorox).
Sterile water.
Forceps.

A. Preparing Seedling Agar Tubes and Plastic Cups: Prepare seedling agar slants in 22 x 250-mm test tubes (Appendix 4). Set up cups as explained in Appendix 1.

B. Preliminary Culturing of Strains of Rhizobium: Cultures of each of the strains listed in Table 1 set up in 100 ml of YM broth in 200 ml Erlenmeyer flasks. This is done as advance preparation for this exercise.

C. Preparing Germination Plates: Make 300 ml of 0.75% (w/v) water-agar in a 500-ml flask and sterilize. Pour 25 ml of molten water-agar into each of 12 petri dishes and allow to cool. Seeds will be pre-germinated in these dishes.

Table 1. Strains of Rhizobium and hosts with which they will be tested.

Rhizobium species	Legumes
Rhizobium japonicum	Glycine max (soybean)
Rhizobium sp.	Vigna unguiculata (cowpea)
Rhizobium phaseoli	Phaseolus vulgaris (bean)
Rhizobium leguminosarum	Cicer arietinum (chickpea)
Rhizobium lupini	Lupinus sp. (lupin)
Rhizobium meliloti	Medicago sp. (medic)
Rhizobium trifolii	Trifolium sp. (clover)

D. Surface Sterilizing Seeds: Pre-check the percentage germination of the seeds and surface sterilize enough to give 100 viable seeds for this exercise. Surface sterilize the seeds by immersion in a 3% solution of sodium hypochlorite for 10 minutes. Rinse seeds with 6-8 changes of sterile water after surface sterilization. Allow the seeds to imbibe in sterile water for 1 hour and then rinse twice. Transfer the seeds aseptically to agar plates with a spoon-shaped spatula. Each batch of 100 seeds should be dispersed evenly in two water-agar plates and incubated at 26°C. The small-seeded species should be inverted while incubating. This will give straight radicles that are much easier to handle in later steps of the exercise.

E. Planting and Inoculation: Soybean, bean, chickpea, and lupin seeds will be planted in cups. Make three well spaced holes in the rooting medium to a depth that will accommodate the pregerminated seeds 1 cm below the surface. Add sterile gravel over the surface of the rooting medium. Set up three cups for each species.

Clover and medic will be cultured on agar slants in tubes. Plant one pregerminated seedling in the agar surface. This is easiest when the

radicles are 0.5-1.0 cm long and straight. Wrap aluminum foil around the lower part of the tubes to shield the roots from light.

After 5 days, thin the cups to two uniform plants per cup by excising the shoot of the unwanted plant aseptically, leaving the rooting medium undisturbed. Dispense drops of rhizobial culture (totalling 1 ml) around the sites of emergence of the two remaining plants. Also as this time dispense 1 ml of Rhizobium culture over the roots of the seedlings in the agar slants.

Avoid cross contamination between strain treatments.

Examine the plants over a period of 5 weeks after inoculation. Note their color and whether nodulated. Replenish the tubes and cups with sterile water as required. At the end of the 5th week, excise the plant tops and determine their dry weight (dry for 48 hours at 60-70°C). Remove roots from the cups and tubes and wash free of rooting medium. Record the presence or absence of nodules in Table 2 and describe the nodule pigmentation.

Look for incidences of cross-inoculation revealed in the table and discuss the infectiveness and effectiveness of each strain/host combination. Effectiveness will be apparent from the dry matter produced by the associations.

After 5 days the plant population is thinned to two plants/cup by excising the shoot of the unwanted plant with sterilized scissors.

Make daily observations on plant growth. Plants may "green-up" quite rapidly at the time that nodules begin to function, delivering fixed nitrogen for plant metabolism. Plants inoculated with ineffective strains of Rhizobium, and also the uninoculated controls, will remain yellow and stunted.

Rhizobia \ Legumes	soybean	cowpea	bean	chickpea	lupin	alfalfa	clover	siratro
R. japonicum								
Rhizobium sp.								
R. phaseoli								
R. leguminosarum								
R. lupini								
R. meliloti								
R. trifolii								
Noninoculated								

Table 2. For recording presence (+) or absence (-) of nodules in each *Rhizobium*/legume combination.

B. To Screen Rhizobia for Nitrogen Fixation Potential

In this exercise you will compare the nitrogen fixation potential of a range of strains of *Rhizobium* in symbiotic associations with soybean. The most effective strains in this exercise will be compared later in a field trial.

Materials

Infiltration irrigation system for leguminous plant infectivity testing (Appendix 1)
3% sodium hypochlorite solution (clorox)
Petri dishes with water agar

A. Preparing Cups: A total of 51 cup assemblies will be required. Set these up as explained in Appendix 1.

B. Culturing Strains for Testing: Each of the 15 *Rhizobium japonicum* strains that will be evaluated should be cultured 7-9 days in advance of the exercise by inoculating them into 20 ml of YM broth contained in 100 ml conical flasks. Incubate these at 26°C on a rotary shaker.

C. Surface Sterilizing the Seeds: Pre-check the percent germination of the soybean seeds and surface sterilize a sufficient number of uniform, undamaged seeds to give about 200 viable seeds for the exercise. Surface sterilization is accomplished by immersion in 3% sodium hypochlorite as outlined in Exercise . Germinate the seeds on sterile water-agar [0.75% (w/v)] until the radicles are 0.5-1.0 cm long.

D. Planting and Inoculating the Cups: Follow the method given in Exercise . Take precautions against cross-contamination. The plus nitrogen control is amended with 70 ppm N (0.05% potassium nitrate). Do not inoculate the plus N and uninoculated controls. The 15 strain

treatments and two controls are replicated three times, giving 51 vessels in all. The cups should be randomized on the growth room (or glasshouse) bench.

Bear in mind that growth conditions such as temperature and light intensity during this experiment must be in the range to which the species are adapted. Excessive root temperatures in legumes cultured i greenhouses are particularly damaging. Infection, nodule development and nodule function are severely impaired by this stress. If the plus nitrogen control plants in a strain selection experiment have not grown well, the strain differences in nitrogen fixation effectiveness cannot be expressed because of other limiting factors.

C. Nodulation and Plant Growth

Background

Increased demands for food and fiber by an increasing world population have strained the world's existing natural resources. This demand is expressed as a dependency on fast dwindling supplies of natural gas and has focused much attention to conservation practices. For example, only 2.5% of the United States annual consumption of natural gas is utilized for the commercial synthesis of nitrogen (N) fertilizer, but the scarcity and cost of this source of energy have stimulated an interest in alternatives to synthetic N fertilizers. Fortunately, there is a great potential to increase soil and plant N supplies without depleting our energy resources, through biological N fixation. This ability resides with some free-living microorganisms as well as some microorganisms in a symbiotic relationship. Perhaps the best known association is between the bacterium, *Rhizobium* and leguminous plants. Initial recognition of this important relationship is attributed to Hellreigel and Wilfarth. However, the soil-enriching quality of leguminous crops was known during the time of the Roman Empire when rotational cropping systems were introduced.

"Symbiosis" may be defined as two or more dissimilar species which live together in an intimate relationship and exert mutually beneficial effects.

The Symbionts

Species in the family Fabaceae (Leguminosae) are second in number only to Asteraceae (Compositae) among the dicotyledons. It has been estimated that there are some 700 genera encompassing over 14,000

different species. While the family is considered tropical in origin, it is diverse both in habitat and characteristics and is divided into three subfamilies. The largest of the subfamilies, Papiloneideae, occupies a wide range of econiches from tropical, temperate, and arid, to even arctic.

Approximately 10 to 20% of the family Fabaceae have been examined for the presence of nodules, and of those examined about 90% were observed to be nodulated. However, the absence of nodules at one location is no guarantee that a species is a non-nodulating type.

Rhizobia, the other partner in the symbiosis, are gram-negative, rod-shaped, nonspore-forming, aerobic, chemoorganotropic, and motile bacteria. Their temperature optima are between 25 and 30°C and pH range is 5.0 to 8.5. They have been classified on the basis of ability to infect a single leguminous species, primarily on temperature zone hosts (Table 1).

Table 1. Designated species of *Rhizobium* based on representative preferred hosts.

Rhizobium species	Representative preferred hosts
leguminosarum	*Pisum*, *Vicia*, *Lathyrus*, *Lens*
trifolii	*Trifolium*
phaseolus	*Phaseolus vulgaris*
meliloti	*Medicago*, *Melilotus*, *Triogonella*
lupini	*Lupinus*, *Ornithopeus*
japonicum	*Glycine max*

J. M. Vincent, 1974.

Biological Nitrogen Fixation

The transformation of the dinitrogen molecule to ammonia is a reductive process and is thought to occur through its binding of the N molecule to the surface of the enzyme nitrogenase. Microorganisms which

possess nitrogenase ordinarily require no other N source; however, those organisms which lack nitrogenase are dependent either on available soil N forms or synthetic N fertilizers.

Nitrogenase occurs in a variety of microorganisms, some of which are free-living and some of which occur in symbiosis with another organism. It is the latter group that are of potential significance in crop production. Bacteria of the genus Rhizobium can form tumors called nodules on the roots of specific species of the leguminous plant family. Rhizobia are not obligate symbionts, but probably fix only small quantities of N saprophytically. By far the greatest amount of N is fixed when the organism is growing symbiotically with the appropriate host. The amount of N fixed varies depending upon the crop and climate conditions but quantities of up to 300 kg N/ha/year have been reported. Estimates of 50 to 100 kg N/ha/year represent the average N fixed for an average leguminous crop. On a world-wide basis this amounts to about 100 million tons N fixed each year.

Objectives

1. Demonstrate the importance of N for adequate growth of plants.

2. Demonstrate that for some plants their N requirements may be satisfied by root nodule-forming bacteria.

Materials

Seeds of nonleguminous plants (may be corn, sorghum, grass, etc.)
Seeds from leguminous plant (may be alfalfa, soybean, beans, peas)
Sterile soil (autoclaved for 1 hour, each of three successive days)
Nonsterile soil from field with a history of growing the legume to be used
Pots (12) (holes in bottom)
Nitrogen solution (9.0 g KNO_3 · 500 ml^{-1} N as KNO_3)
Marked stakes
Brown paper

Procedure

1. Fill 12 pots with sterilized soil.

2. Fill 4 pots with nonsterile soil.

3. Place enough sterile soil on brown paper to fill 6 pots. Add additional nonsterile soil (enough to fill 6 pots). Thoroughly mix soil. Fill 12 pots with mixed soil.

4. Moisten with sterile water.

5. Plant half of the pots containing sterile soil with 5 legume seeds per pot by making small hole in surface with alcohol-flamed glass rod. Plant remaining pots of sterile soil with 5 nonlegume seed per pot in a similar fashion.

6. Label these pots with 0, 25, 50, 100, 200, or inoculated (I).

7. Repeat planting operation for pots containing nonsterile soil and for pots containing the mixture of sterile and nonsterile soil.

8. Cover seeds in all pots except those marked with I (for inoculated). Use alcohol-flamed glass rod starting with pots containing sterile soil only.

9. Use about 0.5 ml of the appropriate Rhizobium species to inoculate each seed in I marked pots. Cover seed as before.

10. Keep surface of pots moist with sterile water but not saturated until germination is complete.

11. Water pots periodically with sterile water to maintain soil moisture at an optimum condition.

12. Four days after germination is complete, add N solution to pots as follows:

Pot designation	Amount	
	KNO_3 solution	Water
	ml pot^{-1}	
0	0	40
25	5	35
50	10	30
100	20	20
200	40	0
I	0	40

13. Thin seedlings in pots to same number (3 · pot^{-1}).

14. Resume normal watering schedule until seedlings are harvested 28 days later.

Observations

1. Harvest tops of all plants; place in bags and dry.

2. Weigh contents of each bag.

3. Look for presence of nodules on roots of legumes; if pots other than I pots have roots with nodules, estimate nodule number and approximate mass.

4. Plot data on graph paper using Y axis as top biomass and X axis as the amount of N added. The levels used corresponded to about 0, 25, 50, 100 and 200 kg N · ha^{-1}. Plot biomass for I pot to the right of the 200 kg N point.

5. Compare growth of legume and nonlegume for each soil condition (i.e., sterile, nonsterile and sterile + nonsterile).

6. Also compare growth within a type of plant (legume or nonlegume) across all soil conditions.

D. To Test the Survival of Rhizobia on Inoculated Seed

In this exercise you will be monitoring the survival of rhizobia during the time at which they are at greatest risk, *i.e.*, while on the seed coat awaiting the emergence of the radicle.

Preparing Inoculants

Inoculate two 250-ml flasks containing 100 ml of YEM broth with a *Rhizobium japonicum* strain. Incubate at 26°C on the rotary shaker.

Sample after 7 days and test for purity by Gram stain, pH determination, plating on peptone glucose agar plates, and on YEM plates. Determine the viable count by the spread plate method (Exercise).

Inject 38.6 ml of culture into a sealed bag containing 41.4 g of sterile neutralized peat. Incubate at 26°C for 2 weeks.

Inoculate two additional flasks each containing 50 ml YEM broth with *R. japonicum* after 1 week. Conserve to be used as liquid inoculum at end of exponential phase (approximately 7 days).

Preparing of Adhesives

You will require a 40% solution of gum arabic in water, a 5% solution methyl ethyl cellulose (Cellofas A) in water, undiluted honey and precipitated calcium carbonate.

Coating and Pelletting Seeds

Inoculate soybean seeds (100 g/batch) with each of the following inoculant preparations (Table 1). Containers, spatulas, glass rods, and other instruments should be sterile. Seeds are not surface sterilized for this exercise.

Treatment no.	Inoculation technique
1	Seeds wetted with 1.5 ml of a 10^9 cells/ml suspension of rhizobia in YM broth
2	Seeds wetted with 1.5 ml of a mixture of 3 ml of YM broth mixed with 3 ml of gum arabic
3	Seeds coated with 1.5 ml of a slurry formed by mixing 1.5 g of peat inoculant with 5 ml water
4	Seeds coated with 1.5 ml of a slurry formed by mixing 1.5 g of peat inoculant with 5 ml gum arabic
5	Seeds coated with 1.5 ml of a slurry formed by mixing 1.5 g of peat inoculant with 5 ml of Cellofas A
6	Seeds coated with 1.5 ml of a slurry formed by mixing 1.5 g of peat inoculant with 5 ml honey
7	Seeds wetted with 1.5 ml gum arabic then coated with peat inoculum
8	Seeds coated with 2 ml of a slurry formed by mixing 1.5 g peat inoculant and 5 ml gum arabic and then pellet

The coating material is pipetted into a 2-liter flask, then the seeds are added and agitated until evenly coated.

For the pelleting required in Treatment 8, add 6 g of calcium carbonate immediately after coating. Shake the flask with a rotating action, until a white layer forms around each seed. This procedure should be practiced. Insufficient shaking allows seeds to clump, too much shaking causes pellets to break down.

You will also realize that gauging the correct quantity of gum is important and is based more on experience than any specific recipe. Satisfactory pellets will not form if there is too little, or too much gum.

Half of each batch of inoculated seeds is to be stored at 4°C and the other half at 26°C.

Determining the Number of Viable Rhizobia on Seeds

The number of rhizobia on the seeds of each treatment will be determined at 0, 1, 7, 14, 21 and 28 days after coating. Place 20 seeds into a 50-ml flask containing 20 ml of sterile diluent. Shake vigorously for 5 minutes to wash the rhizobia off the seeds. One ml of the resultant suspension therefore contains the rhizobia from one seed.

Make a serial dilution for 10^{-1} to 10^{-4} as described in Exercise 3-E. Plate 0.1 ml of each dilution by the spread plate method in triplicates on YMA plates containing brilliant green (1.25 μg/ml).

Record the results of all counts. Plot the logarithm of cell number against storage time for each treatment. Compare and discuss the relative survival rates of rhizobia inoculated by the different techniques.

E. To Test the Field Response by a Tropical Legume to Inoculation with Rhizobium

In this exercise you will initiate a field experiment and you will be confronted with the practical problems of setting up a multiplot field experiment in which the "treatments" are strains of living micro-organisms.

Preparing the Site

The experimental site has already been selected and cultivated for this exercise. Mark out the plots in a randomized complete-block design of nine treatments replicated three times. Plots should be 3 m x 7.5 m (Fig. 1). Only one fertility level (optimal) is employed in this exercise and this was therefore imposed as a blanket treatment 2 weeks in advance on the basis of soil analysis. You will receive details of the soil amendments from your instructors. You will, however, have to apply nitrogen, as urea, to the designated plus nitrogen plots. Apply the equivalent of 30 kg N/ha in furrows some 4-5 cm from, and parallel to, the planting furrow.

Making the Inoculants

Matured, peat-based inoculants containing the seven *Rhizobium japonicum* strains to be used in the exercise are prepared in advance by the methods described in the lectures.

Sowing the Trial

The experimental site should be irrigated the evening prior to the day of sowing. Mark plots clearly with their respective treatments to assist in the planting. Make furrows for only a few plots at a time so that open furrows are not subjected to drying out from a prolonged

exposure in the sun. You will be sowing seeds inoculated with a slurry of peat inoculant mixed with gum arabic. The method is described in Exercise . The noninoculated treatments (i.e., the plus N control and the noninoculated control) are sown first and covered over as soon as each row is completed.

Next, sow the three replicates of the treatment having the first strain and then sterilize hands, footwear and all implements before sowing the seeds of the next treatment.

Determining the Number of Viable Rhizobia per Seed

It will be impossible to make a proper interpretation of the results of the trial unless the number of viable rhizobia inoculated onto the seeds is known. Determine the number of viable cells per seed by the method described in Exercise

Monitoring the Trial

Make frequent observations of plant vigor and foliage color. Make further applications of 40 kg N/ha and 30 kg N/ha after 35 and 60 days, respectively, to plus N plots. At about 3-4 weeks, or at a time when there are visual differences between plots, harvest the plants designated for the early sampling (Fig. 1). Record nodule size, number, color and distribution. Detach representative samples of nodules for serotyping. Wash roots and determine total plant dry weights.

An acetylene reduction determination can be performed at this stage if desired and will give an indication of the relative nitrogenase activity in the different treatments at the time of the assay.

When the plants are mature, they will be harvested from the designated area in the plot (Fig. 1) and the grain yield determined. This will be expressed on a kg/ha basis. (These results will be forwarded to

you.) Compare the grain yield between treatments and comments on the extent to which inoculation responses apparent early in the trial were translated into grain yield. Do the data indicate that soybean needs to be inoculated at that particular site?

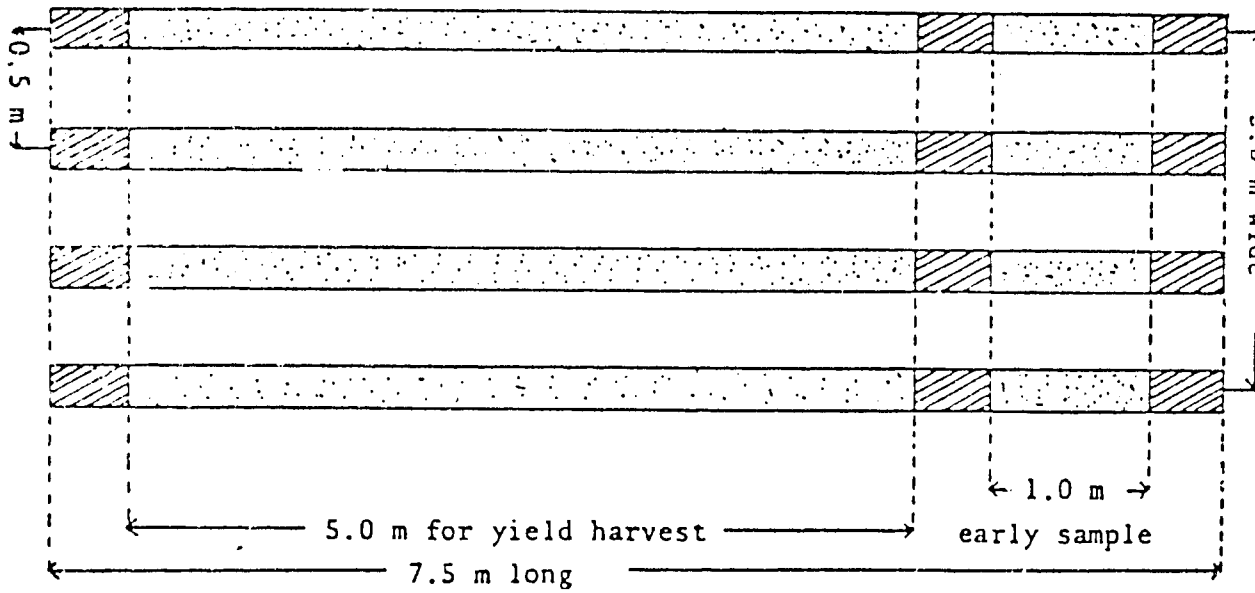


Figure 1. Plot diagram. The center rows only are harvested.

F. To Count Rhizobia By a Plant Infection Method

In this exercise you will perform the only satisfactory method for quantifying the occurrence of a particular strain of *Rhizobium* in the presence of other organisms and nonseparable particulate matter. The most common use of this method is in determining the quality (viable count and nitrogen fixation effectiveness) of nonsterile inoculants.

Preparing Inoculants

Start duplicate 100-ml broth cultures of *Rhizobium japonicum* in 250-ml flasks. Aerate on a rotary shaker for 7 days.

Prepare or obtain one sealed polyethylene bag of neutralized sterile peat and one sealed polyethylene bag of neutralized nonsterile peat.

Confirm the purity of the 7-day broth cultures of the *R. japonicum* used in the previous exercise by pH measurement on the YEM bromthymol blue medium, Gram stain (Exercise). Inject sufficient inoculum into each sterile and nonsterile peat to give a final moisture content of 50%. Allow to mature at 26°C for at least 2 weeks.

Setting Up Plastic Growth Pouches

The pouches used in this exercise are of 16 x 18-cm polypropylene with paper-wick liners, obtainable from Scientific Products, Evanston, IL, USA. Place 20 ml of nutrient solution (Appendix 5) in each of 36 growth pouches, arrange on a wire rack in an upright position, and autoclave at 121°C for 20 minutes. As a substitute for these growth pouches, autoclavable plastic or glass tubes may be used with N-free agar (nutrient solution, Appendix 5, plus 1.5% agar) slants as demonstrated.

Growth pouches serve well as an inexpensive, space-saving substitute for Leonard jars and conventional tube culture. They are quite susceptible

to air-borne contamination, however, and are not shielded against radiated heat. Their use is therefore restricted to growth chambers. A disadvantage of pouch culture is that plants cannot be grown to maturity in such a system.

Surface sterilize and pregerminate approximately 100 soybean seeds as explained in Exercise . Select 36 seeds that germinated together and transfer one seed to each growth pouch aseptically. Locate each seed in the trough in the paper wick of the growth pouch.

Transfer all of the sterile-peat based inoculant into a sterile 2-liter flask. Dilute 1:9 (weight:volume) with sterile water, e.g., 80 g peat inoculant would be diluted with 720 ml water. Remove the peat through a cut at one corner of the bag as described in Exercise . Similarly, make a 10^{-1} dilution of the nonsterile peat inoculant. Shake each flask vigorously for 3 minutes. Make a dilution series from each of the peat samples from 10^{-1} to 10^{-9}.

Plate the 10^{-6}, 10^{-7} and 10^{-8} dilutions of each inoculant in triplicate on agar plates by the spread plate method (Exercise). Use YMA plates for sterile-peat based inoculant and YMA plates containing brilliant green for the nonsterile peat inoculant. Include one plate of YMA containing congo red for each dilution as a check for contaminants. Incubate for 7 days at 26°C. Using the same dilution series, apply 1 ml of the 10^{-5} to 10^{-9} dilutions of the sterile peat inoculant over the seeds in the plastic growth pouches. Set up three growth pouches per dilution. Similarly inoculate three growth pouches with each dilution of the inoculant made from nonsterile peat.

Observe growth pouches periodically, and irrigate aseptically with sterile water.

Determining of the Most Likely Number (MLN)

After 3 weeks of growth, harvest the growth pouches and record the presence (+) or absence (-) of nodules. Refer to the table in Vincent (1970) for estimation of the number of rhizobia by the plant infection method. The number of replications is indicated by "n", and "s" signifies the number of samples used (dilution steps). The MLN is given for the least dilute member of a dilution series. Dilutions may be made in duplicate or quadruplicate. Each series should end with a dilution at which no nodules formed.

The MLN is determined by counting the total number of + pouches, counting the number of dilution steps used, locating the number of + pouches in the "n" column (make sure it is the column for the replication used) and reading off the MLN in the parallel row of the "s" column for the number of replications used.

The estimated number of rhizobia per gram, or per ml is given by:

$$X = \frac{m \times d}{v \times g}$$

Where "m" = likely number at the lowest dilution of the series; "d" is the dilution of the first pouch; "v" = the volume of the added sample; and "g" = the weight of peat from which the aliquot was prepared.

Example: The samples were inoculated with 1.0 ml based on 1-g sample.

A (from peat)		B (from broth)	
Dilution	+ Pouches	Dilution	+ Pouches
10^6	2	10^6	2
10^7	1	10^7	2
10^8	0	10^8	1
10^9	0	10^9	0
	3		5

Replications: n = 2. Dilutions: s = 4.

The value corresponding to three + pouches (column n_2) is 5.8 x 1 for 4 replicates as shown in column s = 4. Since the least diluted sample is 10^{-6}, the total bacterial count is:

$$\frac{5.8 \times 10^6}{1 \times 1} = \underline{5.8 \times 10^6}$$

For five positives, the table shows a value of 5.9×10^1. The total bacterial count therefore is:

$$\frac{5.9 \times 10^6}{1 \times 1} = \underline{5.9 \times 10^6}$$

Compare the results of the plate counts and the MLN counts of the rhizobia in the sterile peat and nonsterile peat inoculants.

EXERCISE 6 - PLANT PROCESSES

A. The Extraction and Separation of Pigments in a Green Leaf:

In this exercise you will take some green leaves, extract the pigments, and see how they function in photosynthesis. We have to find leaves from which we can easily remove the pigments. Bean leaves or leaves of geraniums will provide suitable material.

Materials:

1. Twelve living green leaves.
2. Hot plate.
3. Two 600-ml beakers.
4. One 8-oz capped medicine bottle.
5. 250-ml graduated cylinder.
6. One medicine dropper.
7. One 100-ml flask.
8. Carbon tetrachloride.
9. 200 cc of 95% ethyl alcohol.
10. Large forceps.
11. Hot dish holder.

Procedure: Turn on the hot plate. While it is warming up, half fill one of the 600-ml beakers with water and start it heating. Take the other 600-ml beaker and measure 200 cc of 95% ethyl alcohol into it.

Some words of warning are required in this and other exercises. Ethyl alcohol will be used in several exercises as a solvent for leaf pigments. The alcohol must be boiling to do the job properly. Never heat alcohol over a flame of any kind. Use instead an electric hot plate in good working order. Have a CO_2 fire extinguisher handy to smother a fire if one should get started. Cover it with a glass plate and put it on to heat. Which liquid starts boiling first? (1) Why did you put a cover on the beaker with the alcohol? (2)

While you are waiting for the water to boil, take about 8 leaves, crumple them up in your hands, and put them in the boiling water. Don't leave them in the hot water too long. A minute or two will be sufficient. Did any pigment come out into the water? (3) What can you conclude about the solubility of the green pigments? (4)

With the large forceps or tongs move the wilted leaves from the boiling water into the hot alcohol. Boil the leaves for several minutes and at the same time stir them with the tongs. Now what can we say about the solubility of leaf pigments? (5) What color are the leaves after about 5 minutes of boiling? (6)

At the end of 5 minutes you should have extracted most of the pigments. Allow the alcohol extract to cool a little. Using a holder to pick up the beaker of hot extract, measure 100 cc of extract and put it in the 8-oz medicine bottle. Put the balance of the extract in the flask, stopper it with a cotton plug, and store it in a refrigerator for future use.

Different substances show different degrees of solubility in the same solvent. For example, we wouldn't think of trying to remove a grease stain from out clothes with water; we would use carbon tetrachloride instead. Because of differential solubility it is possible to separate substances which are mixed together in the same solution.

When you look at the pigment extract obtained from the leaves, its color would seem to indicate that it is composed of green chlorophyll. But is it really all chlorophyll? Let's use the principle of differential solubility and find out.

To the 100 cc of pigment extract in the 8-oz medicine bottle, add 130 cc of carbon tetrachloride. Do not inhale the carbon tetrachloride

fumes. They are poisonous. Shake the contents of the bottle and if there is no sign of a separation into two layers, take 2 or 3 medicine droppers full of water and add this to the solution. When you shake the bottle of extract, it should have a cloudy appearance. If it turns clear and there is no sign of a separation, add a little more water. What does the water do in the solution? (7) If the cloudy appearance remains after shaking the solution, put the bottle down. You are ready to make some observations.

Observations: This time the cloudiness will disappear rather slowly. When the separation is complete, you will observe two layers in the bottle. Describe the colors of the layers. (8) What are your conclusions about the number of pigments present in the extract? (9) What pigments are more soluble in carbon tetrachloride? (10) Which ones are more soluble in alcohol? (11)

B. Chlorophyll and Photosynthesis:

You may have noticed that some plants have variegated leaves. This is a leaf in which chlorophyll is lacking in some parts and present in others. The silver-leaf geranium and certain kinds of *Coleus* have variegated leaves. If we experiment with the leaves of one of these plants, we may be able to determine whether or not the presence of chlorophyll in the leaf is really important in photosynthesis.

Starch is the first visible product of photosynthesis for which we can easily test. Sugars are present but they are soluble and not easily detected in the leaf.

Materials:

1. Plant with variegated leaves.
2. Hot plate.
3. Two 600-ml beakers.

4. 95% ethyl alcohol.
5. Tongs.
6. Petri dishes.
7. Iodine solution.

Procedure: Pick a fresh, living, variegated leaf and make an outline drawing showing the distribution of green pigments. You will want this later for comparison.

Next, we want to remove the pigment from the leaf using the same procedure described in Exercise 4 where the leaf is wilted in boiling water and the pigments are removed in boiling alcohol. When the leaf is quite well bleached, remove it from the hot alcohol and put it in a petri dish. Flood the leaf with iodine solution. If starch is present, the leaf tissue will turn brown to purple in color.

Observations: Compare the distribution of starch in the leaf with the distribution of chlorophyll. What is the relationship of the two patterns? (1) Keep in mind that starch is an indirect product of photosynthesis derived from the combining of glucose molecules. Why don't we test for the sugar instead of starch? (2) What can we conclude about the importance of chlorophyll in photosynthesis? (3)

C. The Absorption of Light Energy by the Green Pigments:

We now know that there are different kinds of chlorophylls in green leaves and these are related in some way to photosynthesis. We have stressed again and again that light energy (ultimately solar energy) is required to carry out all the life functions of the photosynthesizing plant. But how does the energy of white light get into the green leaf to be transformed into other kinds of energy to carry on the work of the plant?

Before we can understand how this is done, we will have to learn a little something about the composition of white light, its reflection and absorption.

Observations and Demonstrations:

I. A beam of white light is projected through a glass prism onto a screen. What does the prism do to the beam of white light? (1) How many colors can you identify? (2) List the colors in the order they occur. (3)

One of the easiest ways to get a satisfactory spectrum is to use a 2 x 2 slide projector as the light source. The brighter the light, the better the spectrum, of course. Use a 1000-w projector if available; otherwise even a 300-w projector can be used in a darkened room.

Materials:

1. Projector.
2. Prism (60° or 45°), as high a quality as possible.
3. Slide mounts, preferably the metal or plastic kind where the film is held between two pieces of glass.
4. Aluminum foil.
5. Razor blade.
6. Projection screen.

Cut a piece of aluminum foil about the size of a 35-mm transparency. With the razor blade cut 2 parallel lines in the center of the foil, about 1.5 to 2 cm long and not more than 1 mm apart. Take out the foil between the lines. Mount the foil with its slit in the slide mount, and insert in the projector as you would a regular slide. Focus the image of the slit on the screen. Now you can place the prism in the beam of light to produce the spectrum, but you will need to rotate the projector to bring the spectrum onto the screen. Other materials can be used to make the slit, but aluminum foil is thin enough to have very sharp edges and produces a superior image. You may want to try slits of different widths.

II. In the second demonstration we will shine a bright light on 2 squares of cloth or paper. One square is black; the other is white. A thermometer is placed under each square and one is left uncovered. After a suitable interval of time, say 10 minutes, read the thermometers and record the temperatures. On the assumption that light absorption and heat absorption are related, which of the squares absorbed more light? (4) Which reflected more light? (5) On the basis of what you have seen in this demonstration, which colored garment do you think will absorb more light on a sunny day, a white one or a black one? (6) What part or parts of the spectrum will the white garment reflect? (7) What part or parts of the spectrum will the black garment reflect? (8) What does the uncovered thermometer demonstrate? (9)

Materials:

1. Two squares of cloth of equal dimensions (appox. 20 cm square) of the same thickness and type; one square white, the other black. White and black art paper may be substituted for cloth.
2. Three centigrade thermometers.
3. One strong incandescent lamp.
4. Scotch tape.

Place the 2 squares of cloth side by side on a surface such as the top of a demonstration table. Under each square of cloth place a thermometer, with the tip of the thermometer at the center of the square. The thermometers should also be taped in place to the surface of the demonstration table.

Illuminate the cloth squares with as strong an incandescent lamp as possible. The lamp must be placed the same distance from both cloth squares so that each will receive the same amount of illumination. It may be necessary to check this with a light meter, or place the thermometers on the table under the light and make sure they read the

same before they are covered with the cloth. After 10 minutes, have the students check the thermometers and record the observed temperatures.

III. In this demonstration we will shine lights of various colors, separately, on a white surface. What color does the white surface seem to be in each case? (9) What part or parts of the spectrum does a red sweater reflect? (10) What part or parts of the spectrum does a green leaf reflect? (11)

Materials:

1. Cellophane of several colors: red, green, blue, etc.
2. Slide mounts.

Cut pieces of colored cellophane to fit the slide mounts, and mount as you would a transparency. Most cellophane is rather lightly colored, so 2 or 3 layers may be required. Place these "filters" in the projector and project on the screen. Place variously colored objects next to the screen: red sweater, green leaf, etc.

IV. In the next demonstration we will shine the various colored lights through a prism to the screen. The red light, for example, was obtained by letting white light pass through red cellophane. What color light does red cellophane transmit? (12) When the red light is broken up into its spectrum by the prism, do you see pure red light? (13) Do the other colors of cellophane transmit only one color of light? (14)

V. Next, the spectrum of the light which has passed through the chlorophyll extract from a previous experiment will be allowed to fall on the screen. What colors of light does the chlorophyll extract transmit? (15) What colors of light does it absorb? (16) What colors of light would a green leaf transmit and what colors would it absorb? (17)

A demonstration of light absorption and transmission by chlorophylls. This can be demonstrated by holding a test tube of a relatively dilute

solution of chlorophyll in the light path. A glass container with two parallel sides produces less distortion. If the slit in the foil is really narrow, a test tube will work satisfactorily. Use varying concentrations of pigment extract until you find the best one.

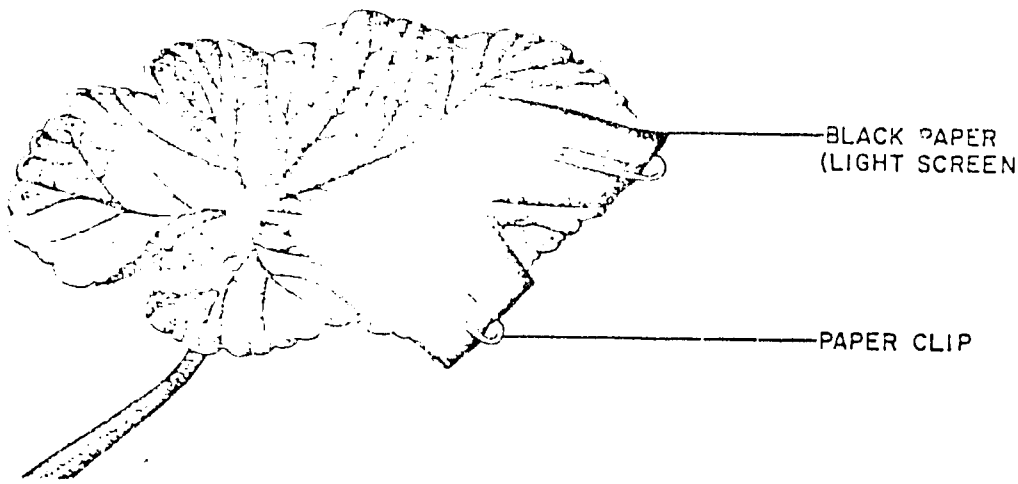
VI. You may have noticed that the chlorophyll extract doesn't always appear to be green. When you look at the extract from the side, in front of a window or a bright light, you will see a deep red instead of a green color. Does this give us an additional clue about the part of the spectrum absorbed by the chlorophyll? (18) Only a small amount of light energy absorbed by the chlorophyll is lost in this way. It gives the chlorophyll the property of fluorescence.

D. Light--A Factor in Carbohydrate Synthesis:

From previous experiments we learned that green leaves absorb certain wavelengths of light and reflect or transmit others. We also discovered that in the presence of the absorbed light, living green cells with a sufficient supply of carbon dioxide and water synthesize carbohydrates. One of the first visible products of carbohydrate production is starch in the form of small grains. You may have already seen these grains in potato or some other plant part. Starch, as a product of photosynthesis, is easy to demonstrate with an iodine test. Its presence or absence in the green leaf will serve as an indication of photosynthetic activity. With this in mind we can go on to an experiment designed to see what effect light has on green plant parts.

Preparation for this experiment must begin at least 4 days before the exercise is given in class. It starts with destarching several geranium plants by placing them in a dark room for 3 days. Sometime during this interval prepare the light screen of black construction

paper. The screen must cover the lower as well as the upper surface of the leaf. Cut a design in the paper. This will be reproduced in the leaf when the student tests the leaf for starch distribution. A good contrasty negative will work if the lower surface of the leaf is backed with black paper, so that the only light reaching the leaf must pass through the negative which is placed on the upper surface of the leaf. Make enough light screens so that each student can have a leaf to test.



Leaf with light screen attached.

Just before removing the plants from the darkroom, put the light screens in place as shown above. Now bring the plants out into the light for at least 12 hours before using the plants in class.

The method for removal of chlorophyll is explained in an earlier exercise.

Materials:

1. Geranium plants with light screens.
2. Hot plate.
3. Two 600-ml beakers.
4. 95% ethyl alcohol.
5. Tongs.
6. Petri dishes.
7. Iodine solution.

Procedure: The plants with the light screens attached to their leaves have been prepared ahead of time by your teacher. We have to tell you how they were treated before you will be able to draw any conclusions at the end of the experiment.

Your teacher put the geranium plants, minus the light screens, in a dark, light-tight room for 36 hours. What effect will this have on photosynthesis? (1) What will the plant use as a source of food? (2) At the end of the period in the dark the light screens were put in place and the plants exposed to light for 12 hours. They are ready for your experiment at this stage.

Pick two leaves from the plant, one with and one without a light screen. Remove the light screen, then put both leaves in boiling water followed by boiling in alcohol to remove the chlorophyll. Test both leaves for the presence of starch by flooding them with iodine solution.

Observations: Compare the two leaves. What indication is there that light is required for the synthesis of food in the leaf? (3)

E. The Root--An Aid to Plant Nutrition:

If you know that plants make their own food by photosynthesis and don't absorb it through their roots, you are doing better than Aristotle, his students, and 30% of the students in a freshman class tested at a university.

Since roots don't absorb food for the plant, what are their functions? Are these functions related to the nutrition of the plant? How are roots designed to accomplish their functions? You will need the following materials to find some of the answers.

Materials:

1. Dissecting microscope.
2. Radish seedlings germinating in a petri dish.
3. Grass seedlings germinating on water.
4. Prepared slide of a root cross section.
5. Root system of a grass plant.
6. A bunch of carrots.

Absorption of Water: The absorption of water with minerals dissolved in it is one of the obvious and important functions of the root. Without water the processes of the plant, including photosynthesis, would soon stop. Whenever we use the word absorption, a sponge, blotter, or dish towel comes to mind. Each of these objects is composed of many small particles of skeleton, cellulose, fibers, etc., which provide a great deal of surface to the liquid for absorption. The more surface, the greater the possibility for absorption. We can see right away that the fibrous root system of a grass is composed of many small slender roots with a great deal of surface for absorption. How does this compare with a root of a carrot? (1) The carrot seems to have a relatively small

amount of surface for absorption. This is because you are unable to see some of the smaller branch or secondary roots and root hairs which increase the surface for absorption hundreds of times. Look at some roots that have root hairs.

First is a demonstration of radish seedlings your teacher has germinated on an absorbent paper in a petri dish. (CAUTION: Don't remove the covers from the dishes. The drying effect of the air will ruin the demonstration.)

Look at the cottony mass of root hairs under the dissecting microscope. Do the root hairs extend all the way to the tip of the radish root? (2) On what part of the root are the root hairs the longest? (3) What would happen to the hairs if the plant had been growing in the soil and we pulled it up? (4) You can see that root hairs are delicate structures but it is difficult to tell whether they are multicellular or unicellular.

Take some forceps and pick a young grass seedling from the water in the dish where they are growing. Mount the seedling in a drop of water on a clean glass slide. Don't put a cover slip on the specimen at this time. With the low power of your microscope, look for the root tip. Don't confuse the young green shoot with the root tip. When you find it, look along the root until you find some root hairs. Are they multicellular or unicellular? (5) What relationship do they have to the epidermis of the young root? (6) To answer this last question, you may have to put a cover slip on the specimen and examine it under high power. Are root hair cells living or dead? (7) How does the function of the root epidermis with its root hairs compare with the function of the epidermis of stem and leaf? (8) What process is involved in the

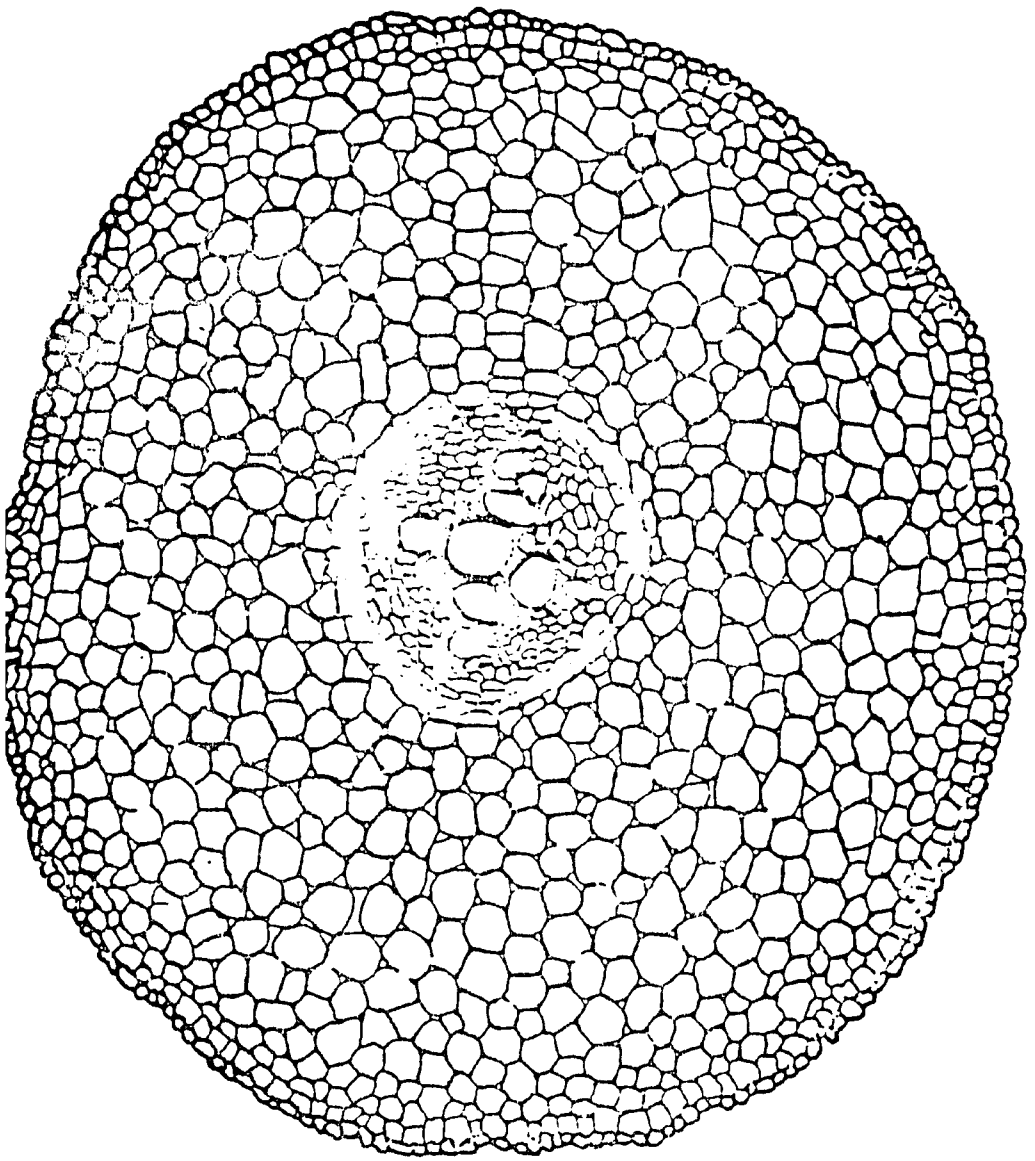


FIG. 1. Diagram of cross section of buttercup root.

F. Conduction of Materials Through a Plant:

As you have observed in previous exercises, stems, leaves, and roots are all equipped to conduct. The conducting tissues (vascular tissues) are composed of xylem and phloem which form a continuous pathway for movement of water, minerals, and soluble food throughout the plant.

In this exercise we want to (1) get the idea of continuity of the vascular system in a plant, and (2) the speed of movement of water through a plant part.

Materials:

1. Two fresh celery plants.
2. Eosin stain or red food coloring.
3. Sharp razor blade.
4. One 1-liter beaker.

When you buy celery at the market, you get a whole plant minus its root system. Look at the base of the celery plant and you can see where the stem was cut in harvesting. The parts of the celery we eat, called the stalks, are really fleshy petioles of leaves. The leaflike parts that we trim off when we prepare the celery for eating are leaflets.

Procedure: Place a stem of celery in a liter of water. With the base of the celery stem underwater, use the razor blade to slice a short section from the end of the stem. Why is the cut made underwater? (1) Add enough eosin stain or red food color to the solution to make a dark red color. Observe periodically, over a period of 24 hours, the path of the red solution through the bundles of the petiole and the veins of the leaflets.

Determine the effect of different factors upon the rate of movement of water in plant parts.

1. Trim the tops from each of the four celery leaves so that each petiole is 12 cm in length.

2. Place one petiole in colored water heated to 50 C.

3. Place one petiole in colored water which is chilled to 5 C with ice cubes.

4. Place one petiole in colored water which remains at room temperature. This will serve as a control.

5. Record the time when petioles are placed in H_2O. Observe the four petioles over a period of 30 minutes and record the time at which the red color appears in the vascular bundles at the tops of the stems. What conclusions can you make regarding the effect of temperature on the rate of movement of solutions through a stem? (2)

Pull a bundle from a petiole and cut it into sections approximately 5 mm long. Place one section in a drop of water on a clean slide. Cover with a second slide and squash the section with enough pressure to flatten and spread the tissue. Remove the second slide; cover the tissue with another drop of water and with a cover slip.

Find the xylem cells which show spiral thickenings in the walls of the cells.

Cut a very thin cross section of the celery petiole, place it under the stereoscopic microscope, and observe the location of the red dye. Where did you find the most red dye? (3) What does this indicate about the path of dye conduction in stems and leaves? (4)

G. Transpiration in Plants:

Previous exercises have demonstrated that water is absorbed by roots and moves with some rapidity into the stems and leaves. This supply of water carries dissolved minerals from the soil through the plant; it is

used in photosynthesis; it keeps living cells supplied with water, maintaining their rigidity--in general, a supply of water is required for all the functions of the plant. But how does the water move through the plant? How does it get to the topmost leaves of a tree 300 feet tall? Is it pushed up? Could it be pulled up? We will agree that a tremendous force of some kind is required to move water to such a height.

The movement of water upward in a plant is related to the loss of water from the above ground parts of the plant in the form of water vapor. The loss of water vapor is called transpiration. Most of the water vapor is lost through the stomata in the leaves; however, some is lost through the cuticle of leaves and stem. Of all the large quantities of water absorbed by a plant, only a very small part, often less than 5%, is used by the plant in photosynthesis and other processes involved in nutrition. The rest is lost by transpiration. Does a plant make efficient use of the water it absorbs? (1)

Part I. Water Loss from Leaves

A good way of detecting the presence of water vapor is by using cobalt-chloride paper. In the presence of moisture this blue paper turns pink. It is sensitive to relatively small amounts of moisture that may be in the atmosphere. Because of this, it is best to keep the paper in a calcium-chloride desiccator.

Materials:

1. Cobalt-chloride paper.
2. Cellophane.
3. Paper clips.
4. Two geranium plants.

Procedure: Cut two 1-in. squares of blue cobalt-chloride paper. Put one square on top of a leaf and the other on the bottom. Fold a strip

of cellophane over the leaf and papers and fasten cellophane, papers, and leaf together with a clip. If the cobalt-chloride paper is pink, heat it over a flame for a minute or two until it turns blue again.

Observations: Look for color changes in the cobalt-chloride paper. What does the pink color indicate is happening to the leaf? (1) Why does the paper on the lower surface of the leaf turn pink first? (2) To answer this question, think back to the structures of a leaf.

Part II. Measuring the Volume of Water Lost

A simple, graphic demonstration can be constructed to show how much water a geranium plant will lose.

Materials:

1. Balance which will weigh to the nearest gram.
2. One potted geranium plant.
3. One pot filled with soil.
4. Two plastic bags (big enough to cover the pots).
5. Graduated cylinder (250 cc).

Procedure: Water both the potted geranium plant and the pot filled with soil; then cover each with a plastic bag. Tie the plastic bag snugly around the stem of the potted plant so that no water can evaporate from the pot. Be sure the pot with soil is also tightly sealed in the plastic bag.

Observations: Now weigh the pot containing the plant and also weigh the other pot containing soil only. Weigh to the nearest gram. Record the weights of each one in your notebook. Each day for the next 5 days record the weight of each. If the plant needs watering, measure the amount of water you add and add a similar amount to the pot of soil. As soon as the watering is done, weigh the two pots again and record. Which loses the greater amount of water in the 5-day period--the pot with or

without the plant? (1) How can you account for the differences in water loss between the two pots? (2) List all of the processes in the plant that account for the differences. (3)

Explain how transpiration regulates the movement of water in a plant. (4) What factors of the environment regulate the rate of transpiration? (5) When would a plant lose the greater amount of water: on a dry, bright day or a dull, humid day? (6) Be sure you can explain.

Part III. Can You Beat the Force Developed by a Plant?

The demonstration will be set up by your teacher prior to class. It consists of two parts: one part to illustrate the force generated by the plant in moving a column of a liquid; and the second, an apparatus that you can use in trying to duplicate the force exerted by the plant.

This experiment is also a demonstration. Although preparing for the experiment may seem a little complicated, it is worth the effort because it provides a very graphic demonstration of the great force of transpiration pull and the cohesion of water molecules.

The following is a detailed account of the materials and methods of assembling of the potometer.

Materials:

1. Two ring stands with 36" supporting rods.
2. Eight utility clamps with extensions.
3. Two 36" sections of .25-mm bore capillary tubing.
4. Three finger bowls.
5. Mercury.
6. Two watch glasses.
7. Two 125-ml wide-mouthed bottles.
8. Two 2-holed #8 rubber stoppers.
9. One 6" section of glass tubing.
10. One 24" section of rubber tubing (5-mm diameter)

11. Ten glass mouthpieces.
12. One pine branch.
13. 70% ethyl alcohol.
14. Two meter sticks.
15. Sharp knife.
16. One liter of boiled water.

Preparing the Apparatus (see illustration): 1. Both pieces of capillary tubing must be bent into a U at one end. While you are bending glass, take the 6" glass tubing and bend it into a U. It should be inserted into one of the holes of the 2-hole rubber stoppers.

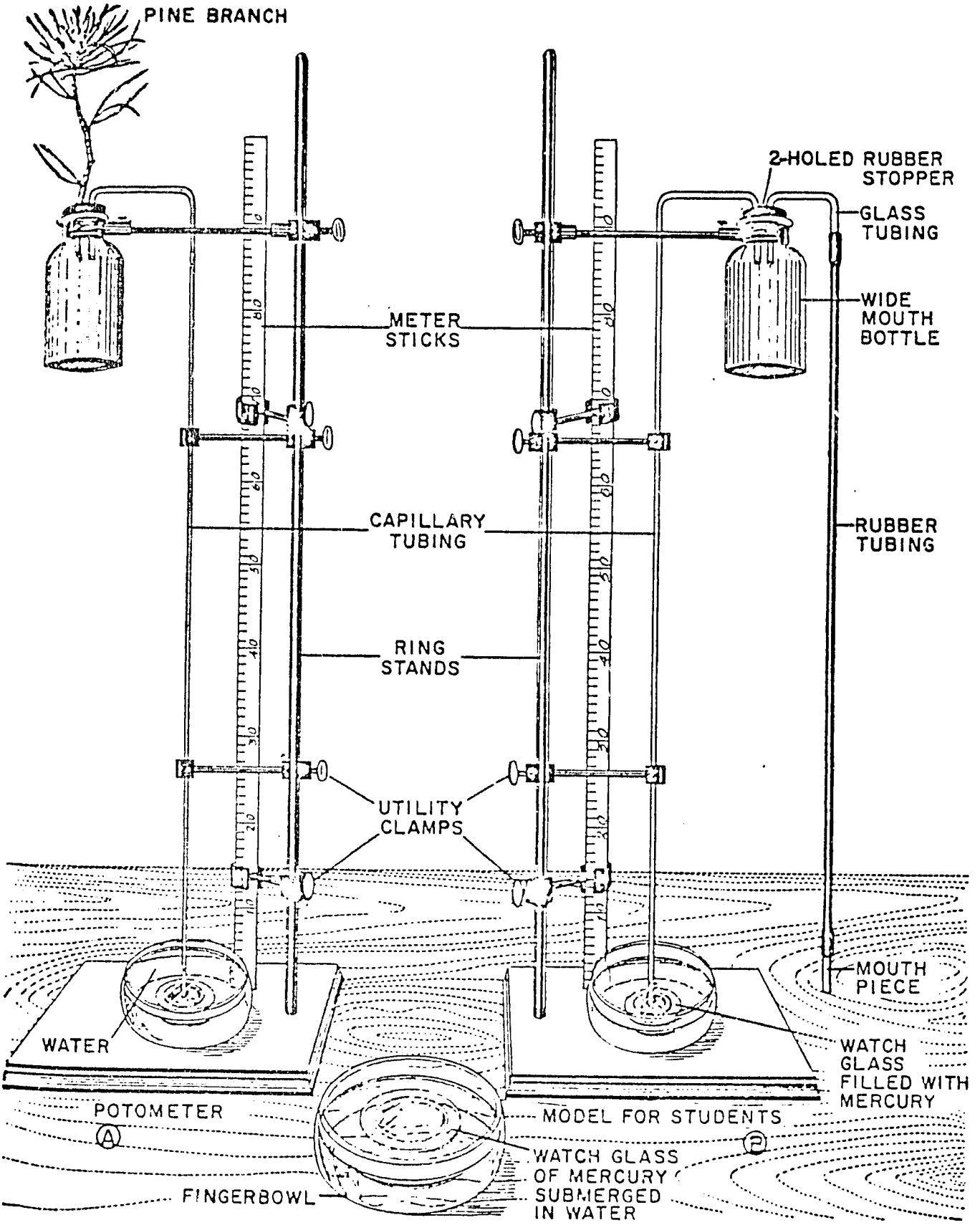
Before we get the pine branch, set up the rest of the apparatus using the illustration on the following page as your guide. You will have to make adjustments in the clamps to get the bottle and cork lined up.

2. Boil at least a liter of water. Boil it hard. Why?

3. Now is the time to get the branch. White pine branches work the best. In any event a branch of some kind of pine is best because they transpire more rapidly than the branches of broadleaved trees. Preparing the pine branch is one of the most critical steps in preparing the experiment. To collect branches we need sharp pruning shears and a small bucket of water. The branches should be collected no longer than a day before they are to be used.

When you cut a branch, keep in mind that the end of the branch must be pushed through and fit very tightly in the hole of the rubber cork. As you cut the branch, plunge the cut end into the bucket. Get three or four branches.

4. While it is still submerged in the bucket, trim about 1" of bark from the cut end of the branch. Still underwater, work the trimmed end through the hole in the top of the rubber stopper. Try not to peel the


PINE BRANCH
2-HOLED RUBBER STOPPER
GLASS TUBING
WIDE MOUTH BOTTLE
METER STICKS
CAPILLARY TUBING
RUBBER TUBING
RING STANDS
UTILITY CLAMPS
MOUTH PIECE
WATER
WATCH GLASS FILLED WITH MERCURY
POTOMETER
A
MODEL FOR STUDENTS
B
WATCH GLASS OF MERCURY SUBMERGED IN WATER
FINGERBOWL

bark back but work it down into the hole. The trimmed end of the branch should extend an inch beyond the bottom of the cork. This should be a tight fit! Leave the branch and cork in the water.

5. Fill the wide-mouthed bottle and the finger bowl of the potometer with boiled water. Fill two watch glasses with mercury submerging one in the water contained in the potometer finger bowl and placing the other in the empty finger bowl of the model for the students.

6. Quickly lift the pine branch, inserted into the stopper, out of the bucket and fit it into the opening of the wide-mouthed bottle of the potometer. Don't squeeze the stopper in place yet. Now work the end of the capillary tube back into the second hole of the rubber stopper. It must fit tightly! Be sure the lower end of the capillary tube is in the water of the finger bowl. Now squeeze the rubber stopper tightly into the wide-mouthed bottle. As you squeeze, you will force all of the air out of the capillary tube. If there are any air bubbles in any part of the system, the potometer will not work properly. Remember we are depending on the cohesive force of molecules to raise an uninterrupted column of water and mercury.

If a bubble does appear or you wish to start the potometer over again, loosen the rubber stopper, fill the bottle once again with boiled water, and squeeze the cork back into place. If the mercury stays up in the capillary tube you can blow it out before you start the apparatus going. (CAUTION: If students are working with mercury, warn them not to get it on their hands or inhale the fumes.)

7. Care must be taken not to let air into the end of the capillary tube when you move it into the submerged dish of mercury.

8. An electric fan blowing on the pine branch will hasten the rate of transpiration. Time the rate of rise of the mercury column both with and without the fan.

9. The model of the potometer for the students to use is the same as the potometer with the plant except there is no water in the system. Any student who wants to should be given a chance to see how high he can raise the column of mercury by sucking on the mouthpiece at the end of the rubber tube. There is no danger from mercury fumes. The air that they inhale comes from the wide-mouthed bottle which will trap any mercury that by some chance comes all the way up the tube.

Have about 10 glass tubing mouthpieces with flamed ends in a dish of 70% alcohol. Each time a student tries to raise the column, he should use a fresh mouthpiece.

To flame the ends, heat them, using a gas flame, until the ends begin to melt removing the rough edges.

10. After you have set this up the first time, it is easy to do later. Leave the apparatus intact so that in the future all you will have to do is get new branches.

Observations: Look at the potometer with the pine branch and see how it is constructed. You can see that the branch has its cut end inserted through a rubber cork into a bottle filled with water. The water-filled bottle in turn is connected by a long capillary tube (a tube with a very small bore or hole through it) to a finger bowl also filled with water. The capillary tube substitutes for what part or parts of the plant? (1) Is there a water column in the capillary tube or is it empty? (2) Take a close look because what you observe is important to understanding the factors involved in the upward movement of liquids.

What part of the plant's natural environment is represented by the water in the finger bowl? (3)

Look in the finger bowl. Submerged in the water you can see a watch glass filled with mercury. Carefully help your teacher to raise the end of the capillary tube in the water and lower it into the submerged dish of mercury. [CAUTION: You must be very careful not to let the end of the capillary tube come above the water level in the finger bowl. If you do, an air bubble will enter the system, interrupting the continuity of the liquid. This will spoil the experiment. Can you explain why? (4)]

Now your teacher will let the electric fan blow on the pine branch. What happens to the mercury column when the fan is on? (5) When it is off? (6) Before the end of the class period your teacher will measure, with a meter stick, the maximum height the mercury ascended in the tube. Mercury is 13.6 times as heavy as water. Knowing this, how high would the plant have moved a water column during the class period? (7) Was the mercury column pushed or pulled up the capillary tube? (8) What process going on in the pine branch caused the water and mercury to move in the capillary tube? (9) What do we mean by cohesive force? (10) Explain why the column of water or mercury would not move without this force. (11) What happens to the cohesive force of water if an air bubble gets into the system? (12)

While you are waiting for the mercury column to ascend in the potometer with the pine branch, let's see how "easy" it is to pull a column of mercury into a capillary tube of the same size used by the plants. Take a sterilized glass mouthpiece and insert it into the end of the rubber tube. Suck on it as hard as you can. Record the maximum

height you could get the mercury column to ascend and multiply this by 13.6. How many meters high could you pull a column of water? Compare this with the plant. Now can you explain how water gets to the uppermost leaves of a 300-ft tree? Does it take a great deal of force?

APPENDIX I. AN INFILTRATION IRRIGATION SYSTEM FOR LEGUMINOUS PLANT INFECTIVITY TESTING

1. Materials

   1.1 Prepared dilutions of suspended samples

   1.2 Disinfected seeds of appropriate host

   1.3 Appropriate rhizobial culture for host (for positive control)

   1.4 Nutrient solution (Ahmed and Evans, 1960; McClure and Israel, 1979) (Table 1)

   1.5 Pipettes, forceps, alcohol

   1.6 Plastic/plasticized paper cups (9.8 cm diameter, 450 cm^3 volume)

   1.7 Planting medium

2. Procedures

   2.1 Punch three holes in the bottoms of the cups.

   2.2 Distribute planting medium in cups to within 2 cm of the top. Suspend filled cups in a wooden rack over the glasshouse bench (Fig. 1). Thus, cups are equally spaced and the excess nutrient solution that flows from each cup does not contaminate the others. A bench 125 x 500 cm supports 500 cups. Vermiculite or perlite is convenient as a planting medium as it generally requires no prior sterilization. Other media such as sand may contain viable rhizobia which could confound test results. Therefore, the planting medium should be tested for presence of rhizobia before adoption into large-scale testing program. Materials with high counts of rhizobia should not be used or should be sterilized prior to use. To sterilize planting medium, place in autoclave on three consecutive days at 1.05 $kg \cdot cm^{-2}$ pressure and 121°C for a minimum of 3 hours each day.

2.3 Add excess nutrient solution to cups and allow to drain.

2.4 Make four small holes in the planting medium about 1 cm deep using a sterile pipette.

2.5 Use alcohol-flamed forceps to place one disinfected seed in each hole, add 0.25 ml of the appropriate sample dilution to each seed. Use the pipette to cover the seed with planting medium. Do not use fingers to cover seeds as this may transfer inoculant from pot to pot. Be careful not to drip the inoculum from pot to pot. Use a fresh, sterile pipette for each sample or dilution.

2.6 Duplicate each dilution with five individual cups, with the last set representing at least one dilution beyond what is expected to be the extinction point (last dilution at which nodules will form).

2.7 Add excess nutrient solution to the cups every 2 days to replenish moisture losses. Once a week flush cups with water.

2.8 Score root systems at the end of the 21 days for presence (+) or absence (-) of nodules. Calculate most probable number using the appropriate MPN tables (Speidel and Wollum, 1980).

3. Precautions

3.1 Controls: Several types of control treatments should be included in every run. A minimum of five cups should be maintained for each sample tested.

3.1.1 Noninoculated N-free plants: If noninoculated plants nodulate, there has been contamination and the trial should be rerun.

3.1.2 Noninoculated N-containing control: Seventy ppm N

Table 1. Nutrient solution used for plant infectivity strain testing.

Stock	Concentration	Stock solution	Nutrient solution
		(g/l stock)	(amount/liter)
1. $CaSO_4 \cdot 2H_2O$			1.12 g
2. Fe EDTA			1.0 ml
Na_2H_2 EDTA	0.0177 M	6.59	
$FeCl_3 \cdot 6H_2O$	0.0177 M	4.8	
3. K_2SO_4	0.5 M	87.13	2.0 ml
4. KH_2PO_4	1.0 M	136.09	0.25 ml
5. K_2HPO_4	1.0 M	174.18	0.625 ml
6. Micronutrients			1.0 ml
$MnSO_4 \cdot H_2O$	2.7 mM	0.453	
$CuSO_4 \cdot 5H_2O$	0.157 mM	0.0393	
H_3BO_3	1.70 mM	0.105	
$NaMoO_4 \cdot 2H_2O$	0.12 mM	0.0302	
NaCl	5.60 mM	0.329	
$CoCl_2 \cdot 6H_2O$	0.017 mM	0.004	
7. $MgSO_4 \cdot 7H_2O$	1.0 M	246.48	2.0 ml

Comments: The reagents and stock solutions must be added in the order given to at least 75% of the final volume of distilled water to avoid precipitation of Ca and Mg salts of HPO_4. Stir solution vigorously while adding stock solutions.

Due to the relatively low solubility of $CaSO_4 \cdot 2H_2O$ it is not feasible to make a concentrated stock solution. Dissolve appropriate amount in 75% of final volume of solution just before adding the other stock solutions.

Geigy sequestrene Fe138 can be used as the source of iron. EDDHA (ethylene diamine di-o-hydroxy phenyl acetate) is the chelator in this material. Solubilize Na_2H_2 EDTA before adding $FeCl_3$ to stock solution.

Unless highly purified salts are used, do not include $CoCl_2 \cdot 6H_2O$.

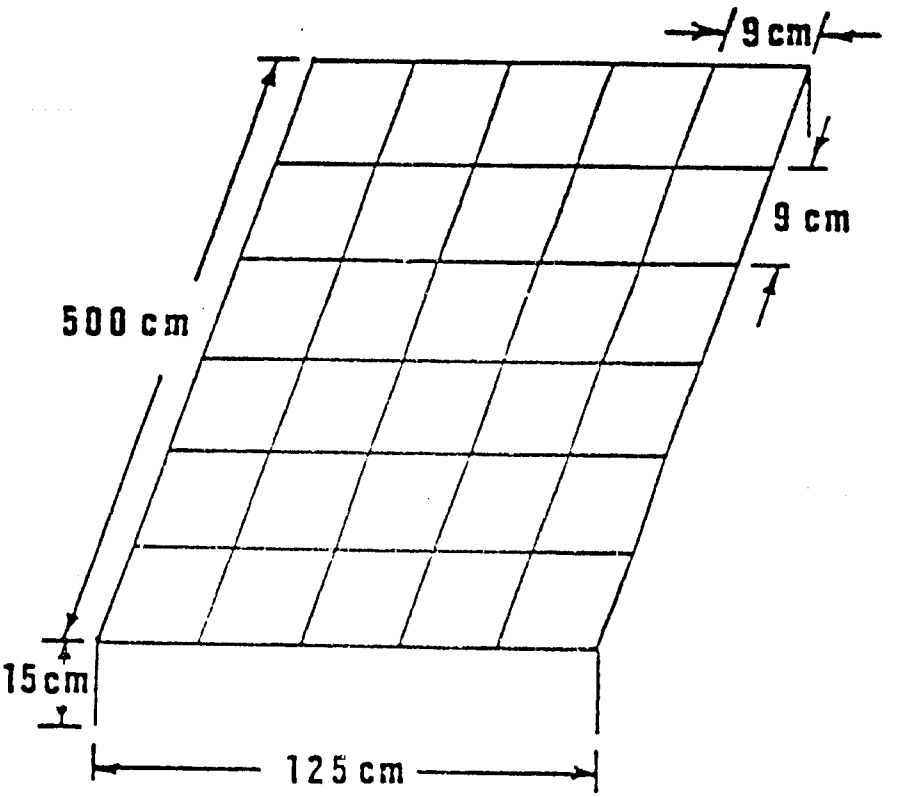


Fig. 1. Schematic representation of a wooden rack used to support cups for plant infestivity testing.

these plants only. These plants will be used for comparison to evaluate the efficiency of rhizobial strains.

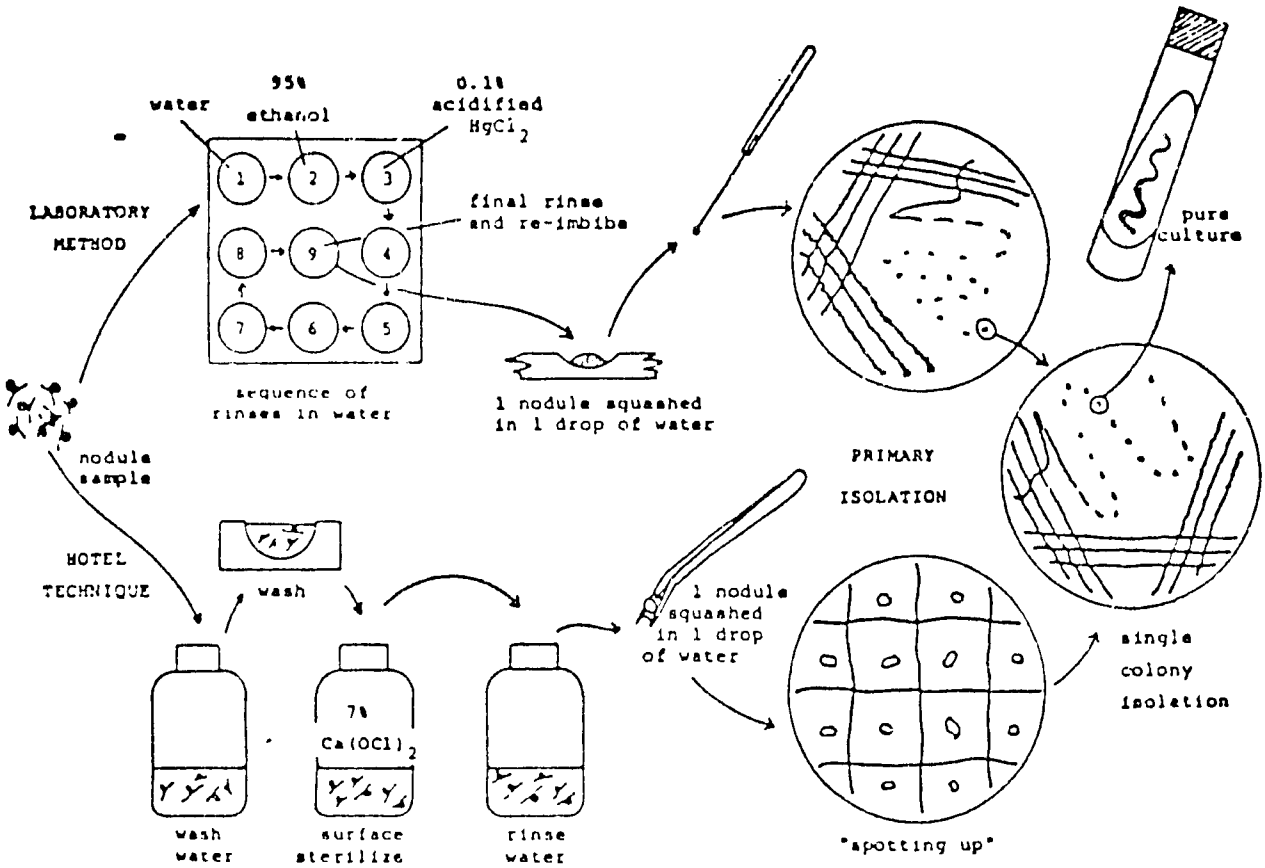
3.1.3 A standard host x strain pair which does not vary from run to run: If this symbiotic relationship produced results significantly different from previous runs, control of environmental conditions should be suspected and the trial should be rerun.

3.2 Dilutions: Generally the decimal or tenfold dilution is adequate to estimate rhizobial numbers in a sample. If additional precision is desired, use either a four- or twofold dilution series. Extend the dilution series to a point where no rhizobia are likely to occur. Five replicates from each dilution will provide a satisfactory level or precision without undue expense.

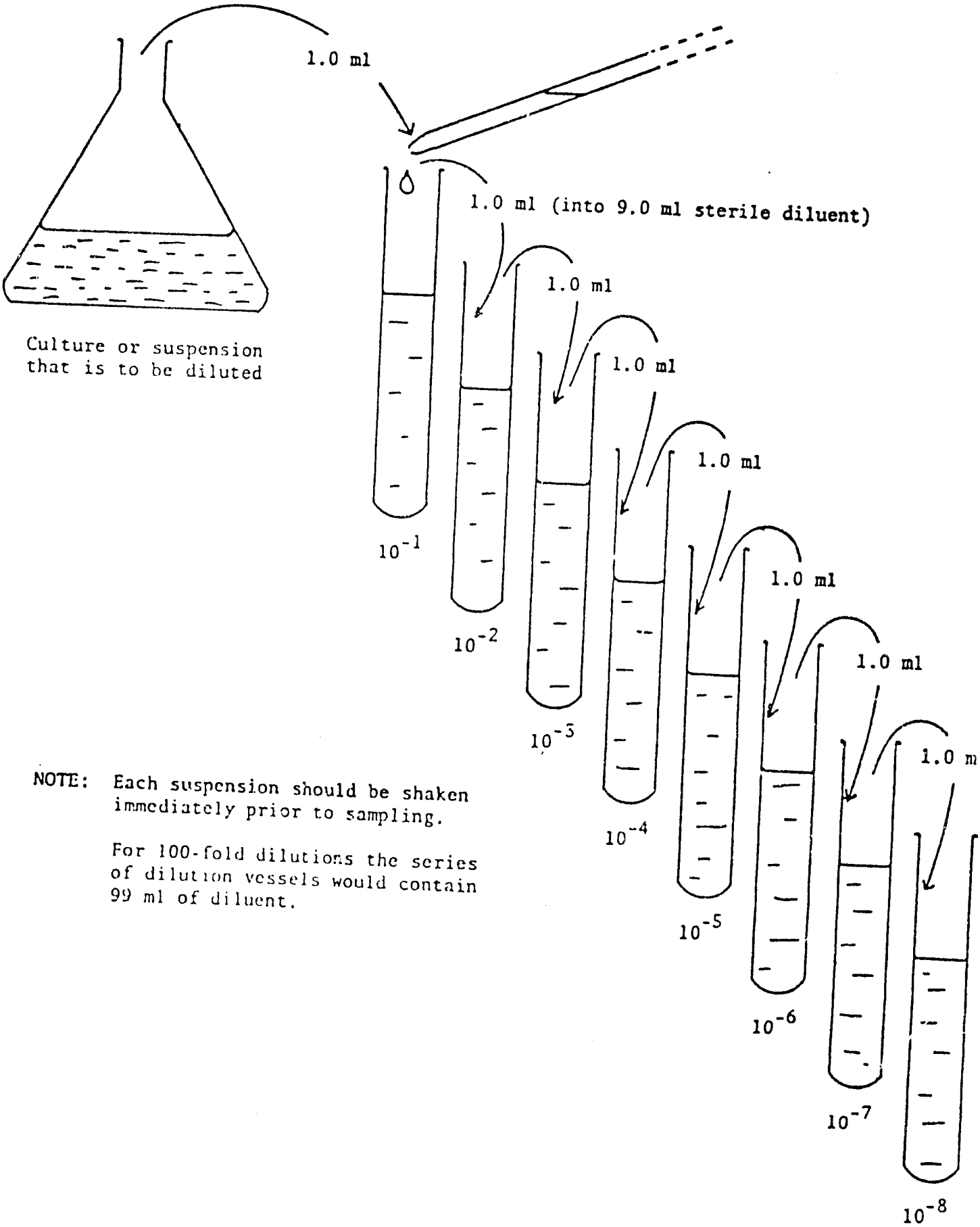
Two samples for sample inoculant test planning follow:

1. Number difficult to predict or unknown. Use tenfold dilutions to 10^{-10} as previously described.
2. Number likely to be in the 10^3/g to 10^6/g range, fair precision required. Use tenfold dilution to 10^{-3} and then twofold steps to 2.1×10^{-6}.

APPENDIX II. ISOLATION PROCEDURES AS USED BY J. HALLIDAY AND R. A. DATE



APPENDIX III. SERIAL DILUTIONS



NOTE: Each suspension should be shaken immediately prior to sampling.

For 100-fold dilutions the series of dilution vessels would contain 99 ml of diluent.

APPENDIX IV. SEEDLING AGAR SLANTS

$CaHPO_4$	1.0 g
K_2HPO_4	0.2 g
$MgSO_2.7H_2O$	0.2 g
NaCl	0.2 g
$FeCl_3$	0.1 g
Water	1.0 g
Agar	
Microclements*	1.0 ml

*from stock containing: 0.05% Bo; 0.05% Mn; 0.005% Zn; 0.005% Mo; and 0.002% Cu

Autoclave at 121°C for 15 minutes and dispense equal volumes into tubes (tube size depends on plant species). An appropriate amount of molten agar is dispensed so that after solidifying in inclined tubes, a 5-10 cm long agar face is presented for seedling growth.

APPENDIX V. NUTRIENT SOLUTIONS

N-free nutrient solution

Stock Solutions	Element	µM	Form	MW	g/l	M
1	Ca	1000	$CaCl_2.2H_2O$	147.03	294.1	2.0
2	P	500	KH_2PO_4	136.09	136.1	1.0
3	Fe	10	Fe Citrate	355.04	6.7	0.02
4	Mg	250	$MgSO_4.7H_2O$	246.5	123.3	0.5
	K	1500	K_2SO_4	174.06	87.0	0.5
	S	500				
	Mn	1	$MnSO_4.H_2O$	169.02	0.338	0.002
	B	2	H_3BO_3	61.84	0.247	0.004
	ZN	0.5	$ZnSO_4.7H_2O$	287.56	0.288	0.001
	Cu	0.2	$CuSO_4.5H_2O$	249.69	0.100	0.0004
	Co	0.1	$CoSo_4.7H_2O$	281.12	0.056	0.0002
	Mo	0.1	$Na_2MoO_2.2H_2O$	241.98	0.048	0.0002

For each 10 l of full-strength culture solution, take 5.0 ml each of solutions 1 to 4, and add to 5.0 l of water, then dilute to 10 l.

For plus N control treatments, KNO_3 0.05% is added giving an N concentration of 70 ppm.

APPENDIX VI. STAINS AND REAGENTS

A. Crystal Violet Stain*

2 g crystal violet (gentian violet) dissolved in

20 ml ethyl alcohol (95%), add

180 ml distilled water

B. Methylene-Blue Stain

0.3 g methylene blue dissolved in

30 ml ethyl alcohol (95%), add

100 ml distilled water

C. Congo-Red Vital Stain

0.1 g Congo red

100 ml distilled water

Add Congo red to the water and stir thoroughly so that all the dye is dissolved. Filter, if necessary, to remove undissolved dye.

D. Reagents for Gram's Staining Technique

I. Ammonium oxalate-crystal violet stain

Solution A:

2 g crystal violet (gentian violet) dissolved in

20 ml ethyl alcohol (95%)

Solution B:

1.0 g ammonium oxalate dissolved in

100 ml distilled water

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*This stain can be used in place of ammonium oxalate crystal violet stain for the Gram's stain technique (see "D") if ammonium oxalate is not available. Results, however, may not always be consistent.

Mix solutions A and B. It is sometimes found, however, that this gives so concentrated a stain that some Gram-negative bacteria do not decolorize properly. To avoid this, solution A may contain half the amount of dye.

II. Gram's iodine

1 g iodine

3.5 g potassium iodide

300 ml distilled water

To the iodine and potassium iodide add a few ml water. Be sure the iodine is in solution before adding the remaining water.

III. Decolorizing agent

Ethyl alcohol (95%)

IV. Safranin counterstain

0.5 g safranin dissolved in

20 ml ethyl alcohol (95%), add

200 ml distilled water

APPENDIX VII. FORMULAS FOR MEDIA

A. Beef Bouillon

4 beef bouillon cubes

1000 ml water

Filter to clarify

B. Nutrient Broth

5 g peptone

3 g beef extract

1000 ml distilled water

C. Sugar Nutrient Broth

10 g specific sugar, glucose (dextrose) or lactose

1000 ml nutrient broth

D. Nutrient Agar

5 g peptone

3 g beef extract

15 g agar

1000 ml distilled water

E. Sugar Nutrient Agar

10 g specific sugar, sucrose (cane sugar), glucose, or lactose (milk sugar)

1000 ml nutrient agar (liquefied)

F. Yeast Extract Mannitol Agar

0.5 g K_2HPO_4

0.2 g $MgSO_4 \cdot 7H_2O$

0.1 g NaCl

10 g Mannitol

100 ml yeast water

900 ml distilled water

Autoclave at 120°C for 15 minutes. The solid medium (yeast mannitol agar) contains 15 g agar/liter. 3 g $CaCO_3$ per liter can be added when excess neutralizing agent is required.

G. Potato Dextrose Agar

200 ml potato infusion

20 g dextrose

15 g agar

1000 ml distilled water

H. Formula for N-Free Agar: Stock Salt Mixture

10 g KCl

2.5 g K_2HPO_4

2.5 g $CaSO_4$ $2H_2O$

2.5 g $MgSO_4$ $7H_2O$

2.5 g $Ca_3(PO_4)_2$

0.1 g $FeSO_4$

Mix salts, grind to powder, place 1.5 g in a liter of water. Use 8 g of agar per liter for growth of plants.

I. Formula for Nitrogen-Free Nutrient Solution

0.8 g K_2HPO_4

0.2 g KH_2PO_4

0.2 g $MgSO_4$ $7H_2O$

0.1 g $CaSO_4$

0.01 g $Fe_2(SO_4)_3$

1000 ml distilled water

APPENDIX VIII. PREPARATION OF MEDIA AND OTHER MATERIALS

1. For making an agar plate, 15-20 ml agar is required. If individual tubes of agar are to be melted and used for pouring plates by each student as in the experiments on "How to Get Pure Cultures" ( ) and "Microbial Antagonism" ( ), put that amount in separate test tubes. If the plates can be poured with agar before their use, as in the majority of the experiments, the agar may be put into flasks before sterilization and several plates subsequently poured. For example, three plates can be poured from a 125-ml flask containing 50 ml agar.

2. Sterilize all media in the autoclave or pressure cooker at 15 lb pressure for 15 minutes. Directions for using the autoclave or pressure cooker should be available from the manufacturer. If instructions are not available, consult a home demonstration agent or any home economics specialist.

3. Sterilize the petri dishes in a hot air oven at 170 C for 2 hours.

4. Wrap pipettes in paper, and sterilize them in the autoclave.

APPENDIX IX. CLEAN-UP PROCEDURE

Disinfecting solutions for general laboratory use are:

1. 1% phenol, or 2% Lysol, or 2% clorox
2. 5% phenol

Make up liter quantities. Mark the bottles POISON.

Laboratory work areas should be cleaned and disinfected with solution (1) at the beginning and the end of the laboratory period. Students should wash their hands immediately after using the disinfecting solutions.

When microbial cultures are accidentally broken or contents spilled, pour 5% phenol liberally over them and allow it to stand 10 minutes to kill the microbes before cleaning up.

To prevent gross contamination of the laboratory and the preparation room, all materials after use should be sterilized in the pressure cooker or autoclaved at 15 lb pressure for 15 minutes before discarding or washing.

All glassware should be thoroughly washed with a good detergent in warm water. Rinse well, at least five times, in warm water. Drain and dry, and return to storage place.

APPENDIX X. MODEL SCHEDULE

Day		
-28	Several exercises and demonstrations will have to be established in advance of the start of the course. These include: Genetic Compatibility study Nitrogen-fixation Potential study Nodulation and Growth study Rhizobium Survival study Field Response study	
-21	Establish second block of the Rhizobium Survival study Start the Counting study	
-14	Establish third block of the Rhizobium Survival study	
-7	Establish fourth block of Rhizobium Survival study Choose sites for Field-trip stops Make apparatus for Transpiration study	
0	Students arrive Establish fifth block of Rhizobium Survival study	
	Morning	Afternoon
1	Opening ceremony	
	---------- Field Trip ----------	
2	Complete Soil Sampling work. Begin Soil Properties.	Basic microbiological techniques. Start Air-borne microbes. Start Authentication study
3	Complete Soil Texture and Capillary Action.	Finish Basic Techniques. Start Bacteria. Start Pure Culture. Observe "air-borne" and other plates.
4	Complete Aggregation and Soil Organisms.	Soil Sampling. Observe previously prepared materials.
5	Water-holding Capacity and Compaction	Complete data collection on Genetic Compatability
6	Soil Clays, pH and Ion Exchange. Start Light experiment and Root study.	Complete unfinished work. Observe Pure Cultures.
7	---------- Rest Day ----------	

	Morning	Afternoon
8	Plant Pigments and Photosynthesis. Begin Water Loss.	Complete Nodule Authentication. Explain next day's work.
9	Complete Energy Absorption.	Complete N-fixation Potential. Explain next day's work.
10	Complete Light study and Root work.	Complete Nodulation and Growth. Explain next day's work.
11	Plant Conduction, Transpiration, and Water Loss.	Complete Survival exercise.
12	Complete all plant work.	Complete Field Response exercise.
13	Wrap-up discussion.	Complete Rhizobium counts. GRADUATION!