Nitrogen Fixation and Legumes
Experiment Instructions

Prior to starting the activity and planting, students should be split up into groups of 3-4; each group will be responsible for examining nodule growth on growing soybeans or their own 5-gallon bucket of 3-4 mature soybeans at the end of the experiment. If green/glasshouse is not available, teachers will need to have an adjustable timed growth light shelf. It is recommended that the growing shelves have light fixtures totaling at the least 6 fluorescent bulbs (the more lights the better), and that the fixtures be suspended in a way that allows for light height adjustment as the plants grow taller (shop light fixtures work well).

Prepare for Planting

Use soil made for containers. (Do not use pure garden soil, because it will clump and will not drain properly. Topsoil will have many organic pieces (wood, etc) that will continue to break down and stimulate formation of nodules. Soybeans need a container mix that will drain well. A standard container soil should suffice.

-Make your own container mix by combining peat moss, perlite (75%) and garden soil (25%).

Heat sterilize to kill any microbes already existing in the soil. This can be done using an autoclave or oven. If using an autoclave, use ceramic pots or a large autoclavable container; fill completely with soil mix, cover with aluminum foil and run autoclave.

Oven method:

- Spread soil not more than four inches deep in non-plastic containers, such as seed flats, clay pots and glass or metal baking pans.
- Cover each container tightly with aluminum foil. Insert a meat or candy thermometer through the foil into the center of the soil.
- Set the oven between 180° and 200° F. Heat the soil to at least 180° F; keep at this temperature for 30 minutes. (Do not allow the temperature to go above 200° F.)
- After heating, cool, remove containers from the oven and leave aluminum foil in place until ready to use. The heated soil may give off an odor. Keep the sterilized container/pots in a cool and clean spot where contamination from insects and airborne rhizobia can be controlled. Pots should be prepared two days before planting.

Inoculant under the trade name N-DURE is available from eBay or Amazon. The directions included have taken into consideration the smaller amounts of soil and seed being used in the classroom.

Use a fertilizer mix that is slow release and includes equal amounts of N, P, K

Nodule formation will occur within 3-4 weeks of planting. If a lesson is planned to observe results of the various treatments, the teacher can prepare the soil and seeds for planting in advance.

Student Planting
The initial planting will take a full 60 minutes; students should subsequently make observations for 10 to 15 minutes twice a week until tissues or seeds have been harvested.

Day 1:
Activity – Planting

- Prior to planting, seeds must be inoculated and dried
    - Weigh 2 g of inoculant and place it into a container
    - Add 6 ml of water and mix until a uniform mixture is achieved
    - Weigh 100 g of seeds and place them into a separate container
    - Add 2 ml of the slurry to the seeds and stir until they are uniformly coated with the inoculant slurry
    - Remove seeds by straining them from the container and spread them on a piece of clean paper to dry
    - To reduce variables in the experiment, seeds in (-) soil inoculum regimes should still go through the slurry and drying process the same as inoculated seeds only without the addition of the inoculum
- In large plastic bins, soak sterile potting mix using water until slightly moist. Fill pots with slightly moistened growing media to about 3/4s up the soil line
- Spread slow release fertilizer evenly on top
    - 1X treatments should add a cup of fertilizer
    - 0.25X treatments should add a quarter cup of fertilizer
    - 0X treatments should skip the fertilizer step
- Continue to fill the pots to the soil line with slightly moistened soil
- Poke 8 one inch deep hole in the top of the growing media using your finger
- Place a soybean in each hole and lightly cover with dry growing media
- Using a pipette or dropper, gently apply water to each seed (10-20 drops)
- Label your groups pot properly and place it on the proper lighted shelf

Growing Tips

- Adjust lights as the plants grow so that the lights are 10-15 cm from the growing meristem
- Keep room temperature above 60°F
- Check on plant hydration everyday and water as needed
    - Plants will need watered more often as they grow in size
    - If you lift the pot and it feels light, it may need to be watered
- Thin to 3-4 plants per pot; transplant from other pots if necessary
    - Thin and transplant as soon as you are confident in the establishment of the seedlings

Discussion

- Prior to planting, give students an overview of the experiment.
- Explain to the students what the control and experimental groups are

Days 2-7: Germination

- Have students make observations and record the dates of germination
    - Include germination rates and note any unusual features
    - Do the germination rates and dates differ between the groups?
- After the first week, seedlings will appear. Seedlings should be thinned using scissors or forceps so that only 3-4 plants remain per pot
    - If needed, transplant seedlings from crowded pots and plant them in pots that need them
- The seed coat may not fully fall off the emerging seedling, causing the cotyledons to not fully expand. The remainder of the seed coat should be removed carefully with forceps

Discussion Points

- Germination starts with seed and ends with a fully expanded seedling
- Each seed contains a single embryo and enough stored energy/nutrients to give the embryo a head-start in its growth
- Germination is initiated when the seed **imbibes** water from the growing media (why we need to use moist soil); this intake of water causes the dried and dormant tissues of the seed to swell and crack the outside layer of the seed, **seed coat**
- An embryonic root, the **radicle**, will soon emerge and grow downwards
    - The radicle soon develops fine root hairs that greatly increase the roots surface area; in turn, increasing the water uptake of the whole plant
- The new uptake of water causes the cell of the embryonic stem, **hypocotyl**, to swell and elongate, causing the seed to become upright and emerge from the soil
- As the seedling emerges and casts off the seed coat, two embryonic leaves, **cotyledons**, fully expand and begin to produce chlorophyll.
    - The cotyledons will begin photosynthesizing, but will look different than the future leaves. That is because the cotyledons primarily act as storage for essential nutrients and energy the seedling needs to quickly grow
- While all **angiosperms**, flowering plants, germinate from seeds, they don't all follow the previously outline steps.
    - For example, soybeans are **dicots** and germinate with two cotyledons; while corn and rice, being **monocots**, germinate with one cotyledon.
- Despite having a great understanding of germination in higher plants, scientist continue to have question and perform research to better understand the mechanisms and environmental factors that control germination.

Days 10-30: Growth and Development

- Weekly, have students make observations and record the data on the progress of their plants
    - Measure and/or record the following for each plant:
        - Number of leaves
        - Plant height from soil to **apical meristem**, growing point
        - Number of leaf attachment areas, **nodes**
        - Date of first flower
        - Total number of flowers
        - Number of flowering nodes
    - Items and changes to notice:
        - Leaf shape, **morphology**, will change as plants grow
        - Length of stem segments between leaves, **internodes**
        - Developing expansion of new leaves
- By day 30, there should be visible differences between fertilizer regimes
- The data collected throughout the weeks will help students quantify these changes
- Students should also become familiar with the vegetative and reproductive stages of the soybean, more information and pictures of these stages can be found at the OSU crop extensions sites: http://corn.osu.edu/newsletters/2013/2013-27/soybean-reproductive-stages
- As these stages come about, their dates of occurrence should be recorded
- As plants grow, they will require staking

Discussion Points

- As the plant matures, it undergoes **growth**, the additions of new cells and elongation of cells, and **development**, the arise of different tissue and organs
- As the soybean grows, it adds new nodes from the apical meristem and elongates internodes, both causing the plant to increase in height
    - New leaves and nodes always arrive from a meristem
    - Each new organ emerges and overtime grows larger
- Plants may also start to form branches at lower nodes as the apical meristem gets further from the lower nodes
    - This branching is caused by the gaining and losing of plant **hormones**, chemical signals, from the apical meristem
- Growth allows plants to “reach” new areas for photosynthesis and place their leaves and flowers in positions that are conducive to their purpose
- Leaves produce carbohydrates by combining carbon dioxide and water in the presence of light, this process is called **photosynthesis**
- These sugars are then transported throughout the plant via the stem; simultaneously the stem also transports water from the roots to the leaves
- The emergence of flowers denotes a change from the plants vegetative stage to a reproductive one
    - From then on out, the plant is solely focused on creating more progeny and producing healthy seeds

Days 30-45: Flowers and Pollination

- Weekly, have students make observations and record the data on the progress of their plants
    - Measure and/or record the following for each plant:
        - Number of nodes with flowers
        - Plant height from soil to apical meristem
        - Total number of flowers
        - Number of flowering nodes
    - Items and changes to notice:
        - Leaf shape, **morphology**, will change as plants grow
        - Length of stem segments between leaves, **internodes**
        - Developing expansion of new leaves
- By day 30, there should be visible differences between fertilizer regimes
- The data collected throughout the weeks will help students quantify these changes

Days 45-90: Fertilization and Seed Development

- Weekly, have students make observations and record the data on the progress of their plants
    - Measure and/or record the following for each plant:
        - Number of leaves
        - Plant height from soil to **apical meristem**, growing point
        - Number of leaf attachment areas, **nodes**
        - Date of first flower
        - Total number of flowers
        - Number of flowering nodes
    - Items and changes to notice:
        - Leaf shape, **morphology**, will change as plants grow
        - Length of stem segments between leaves, **internodes**
        - Developing expansion of new leaves
- By day 30, there should be visible differences between fertilizer regimes
- The data collected throughout the weeks will help students quantify changes
- Instructors may choose to end the experiment at any point after 45 days
    - It is advised to wait till there is a visible significance difference between the fertilizer regimes
    - Plants can be grown through maturity and all the way to harvestable dry seeds, or the experiment can be finished after pod formation and embryos are counted
        - The longer an instructor waits the more data there is to collect, but this additional time also allows for problems to arise (plants dying early, pest build up, etc.)
- After data collection, instructors can compile data from all groups and present the total data to the class
    - Look at which traits and characteristics have the most variability between and within the different fertilizer regimes