Inoculation of Leguminous Crops and Trees

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Soil nitrogen is one of the most limiting factors in agriculture. Nitrogen is essential for plant growth, being a key component of protein, chlorophyll, enzymes, and genetic material. Though nitrogen is abundant in nature—cycling between the atmosphere, soil and living organisms—it is only directly available to plants when converted through biological or industrial processes to certain forms, primarily ammonium (NH_4^+) and nitrate (NO_3^-). Some nitrogen is made available to plants through the decay of existing soil organic matter (e.g. manures and plant residues) or residues of the current crop. A small amount of atmospheric nitrogen is also "fixed" and converted to NO_3 through lightning. Generally, however, these inputs are not sufficient to replenish the nitrogen taken up by the crop and removed with the harvest. Adequate replacement of plant-available nitrogen in the soil, then, is accomplished by applying fertilizers (inorganic, manure, etc.) and through biological nitrogen fixation

Nitrogen fixation in the soil is primarily accomplished through a symbiotic relationship between plants in the legume family and specialized bacteria (rhizobia, bradyrhizobia, or azorhizobia). Most legumes can be identified by their doubleseamed seed pods. Examples include cowpea, peanut, clover, crotalaria, leucaena trees, and velvet bean. When the appropriate bacteria are present in the soil, the bacteria infect the roots of the leguminous plant, causing the plant to develop a nodule. This nodule houses the bacteria and supplies sugars for their growth. In exchange, the bacteria trap atmospheric nitrogen (N_2) and convert it into ammonia (NH_3), which is ultimately converted into NH_4^+ and amino acids that are utilized by the plant. Different rhizobial bacteria nodulate different legume species, so it is important to match the two.

When a legume is growing in its native habitat, it is likely that the appropriate bacteria are present in the soil. If nodules are present on the roots, especially along the taproot near the crown, and if the nodules are pink or red inside, then an appropriate bacterial strain is living in the soil and actively fixing nitrogen in symbiosis with that legume (Figure 3). If not, it is necessary to inoculate with the matching bacteria. Note that sometimes a crop growing where it has not been grown in recent years may still form nodules and even fix some nitrogen. Some rhizobial bacteria are generalists and can fix nitrogen in relationship with a range of legumes. But it is possible that more nitrogen is fixed if specific legumes are matched to the bacteria that nodulate them best. One way to encourage this is to introduce to the soil rhizobia from the crop's native habitat.

Figure 3: Nodules on plant (Aeschynomene sp.) roots, indicating colonization by rhizobial bacteria. Photo by Tim Motis

The benefits of having the best kind of rhizobia in the soil are many. Reduced need for fertilizer application (specifically inorganic nitrogen) is the most obvious. This means reduced costs as well as improved nutrient efficiency. Nitrogen fixed by bacteria is not as quickly leached from the soil as is applied nitrogen, resulting in a more stable nitrogen pool. Soil quality is also enhanced by inoculation, which fosters microbe diversity and residual nitrogen build-up over time. Finally, forage quality is improved when legumes are inoculated, since the increase in nitrogen levels promotes an increase in the legume's protein content.

Just because a legume can fix nitrogen doesn't mean that some nitrogen fertilization might not be helpful. An initial application of inorganic fertilizer or manure can help stimulate plant growth just after germination until nodules are developed. Adding too much nitrogen to the soil, however, can cause the rhizobia to slow down or stop producing nitrogen. More important than adding nitrogen is remedying deficiencies of other essential nutrients. The process of fixing nitrogen is complex, requiring input from the plant as well as the bacteria. A healthy plant will fix more nitrogen than a stressed one, so it is important to prevent nutrient deficiencies, drought stress, and other stresses.

How to inoculate

In most cases, the best method of inoculating legumes is to apply the inoculant to the seed. Commercial preparations of live rhizobial bacteria come in various forms, the most common of which is in a peat or humus mix. The peat keeps the rhizobia moist and helps the bacteria stick to the seeds until they germinate. Inoculants also are sold in liquid and granular form.

With peat-based inoculants, it is helpful to add a "sticking gent." Mix one part sugar, honey, or syrup with nine parts water, and then sprinkle this mixture on the seed. Do not make the seed too wet, or the inoculant will wash off. The sugar in the sticking agent also provides a temporary food source for the bacteria until the seed has germinated and begins producing its own sugars. Once the seed is damp, add the inoculant. If the packaging did not provide application recommendations, adding about six teaspoons of inoculant to one kilogram of seed should provide sufficient inoculation.

Rhizobia thrive in cool, moist, aerobic conditions, so take care when planting inoculated seed. The seed bed should be thoroughly moistened before sowing; it is also helpful to sow during the cooler parts of the day (early morning or evening). Do not allow inoculant or inoculated seed to dry out or be exposed to the sun. In the tropics, it may also be necessary to increase the amount of inoculant used. According to the University of Florida Extension Service, most North American manufacturers do not take into consideration tropical conditions when recommending the amount of inoculant to apply. High temperatures and dry conditions (especially in the soil) might make it necessary to apply twice the recommended amount for large seeds and five times the recommended amount for small seeds. If inoculation does not appear to be successful, increasing the inoculation rate may solve the problem.

Sometimes it is better to apply the inoculant to the soil after seeding. If the soil is hot, dry or acidic, or if the seed is pelleted or treated with a chemical, the rhizobia may survive better if applied directly to the soil. This can be done in a water solution or as a band of peat-based inoculant shallowly incorporated into the seed bed. Do not apply inorganic fertilizer, pesticide, or any other chemical directly after inoculation, as these may kill the rhizobia.

Check for good root nodulation by digging up (rather than pulling up) the plants to avoid stripping off the nodules. Nodules should appear on the taproot and lateral roots near the crown of the legume within 21-28 days. Effective nodules are large and, when mature, show pink or red coloration inside. This color is produced by leghemoglobin, a compound similar to hemoglobin in human blood, which carries oxygen. Oxygen actually inhibits nitrogen fixation, so the leghemoglobin traps any oxygen that may interfere with the process.

Just because a plant is producing nodules does not mean those nodules are effective. Unproductive nodules are small and distributed throughout the entire root system. When cut open, they are white or grey to pale green. However, immature effective nodules can also have white or green coloration. The difference lies in the number of non-pink nodules: a plant that is properly inoculated will have a small percentage of white or green (immature) nodules; an improperly inoculated plant will have nearly all white or green (ineffective) nodules.

Trouble-shooting: why does inoculation fail?

There are several reasons why inoculation may be unsuccessful.

Wrong inoculant: as mentioned earlier, it is important to match the legume species to the appropriate bacteria that can effectively nodulate that plant. Legumes have been divided into groups based on what bacteria colonize them. These are called cross-inoculation groups. Most manufacturers produce rhizobia mixes for each cross-inoculation group, so it is not essential to know exactly which bacteria species you need. Nevertheless, Table 2 contains a list of the cross-inoculation groups and the rhizobia that colonize them.

Poor soil conditions: Rhizobial bacteria grow best at a emperature of 28-30°C (82-86°F). If soil temperatures are too high, especially during establishment, the bacteria will not survive. A pH of 6-7 is optimal for rhizobial growth, while acidic conditions inhibit growth. Most likely, however, if the soil is suitable for plant growth, it will also support rhizobia.

Dead inoculant: inoculant is a biological product, meaning it contains living organisms. Consequently it does not have a long shelf life. A quality inoculant should last six months if stored properly. This makes the expiration date printed on the packaging very important. If the expiration date is past, discard the inoculant and purchase fresh material. Even if the expiration is still current, poor storage and handling during shipment may have killed the bacteria. Buying from a local source, if possible, may be better, though storage conditions can also be unreliable in this case.

Table 2. This is a generalized list of cross-inoculation groups and the corresponding rhizobia, collected from several sources. The list changes as new relationships and bacteria are researched. When purchasing inoculant, the most important information to bring to the manufacturer is what legume species you wish to inoculate.

Group/ Rhizobia	**Host species**
Alfalfa *Rhizobium meliloti*	*Medicago sativa* (alfalfa) *M. lupulina* (black medic) *M. polymorpha* (bur clover) *M. orbicularis* (button clover) *Melilotus* spp. (sweet clovers; e.g. white and yellow)
Clover *Rhizobium trifolii*	*Trifolium* spp. (clovers) *T. alexandrinum* (berseem clover) *T. grandiflorum, T. campestre* (hop clover) *T. hirtum* (rose clover) *T. hybridum* (alsike clover) *T. incarnatum* (crimson clover) *T. nigrescens* (ball clover) *T. pratense* (red clover) *T. repens* (white clover) *T. resupinatum* *T. subterraneum* (subterranean clover)
Cowpea "Cowpea rhizobia" group or *Rhizobium* sp.	*Aeschynomene* spp. *Albizia* spp. *Arachis hypogaea* (peanut) *Alysicarpus ovalifolius* (alyce clover) *Cajanus cajan* (pigeon pea) *Crotalaria* spp. *Indigofera hirsuta* (hairy indigo) *Lespedeza* spp. *Leucaena* spp. *Mucuna pruriens* (velvet bean) *Phaseolus lunatus* (lima bean) *Pueraria montana* var. *lobata* (kudzu) *Stylosanthes humilis* *Vigna mungo* (mung bean) *Vigna subterranea* (Bambara groundnut) *Vigna unguiculata* (cowpea)
Lupine	*Lupin* spp. (e.g. blue, white lupine)

Rhizobium lupin	*Lotus* spp.
Pea and Vetch *Rhizobium leguminosarum*	*Lathyrus* spp. *Lens* spp. *Pisum sativum (peas)* *Vicia faba (broad bean)* *Vicia grandiflora (bigflower vetch)* *Vicia sativa (common vetch)* *Vicia villosa (hairy vetch)*
Bean *Rhizobium phaseoli*	*Phaseolus vulgaris* (common bean)
Soybean *Bradyrhizobium japonicum*	*Glycine max* (soybean)
Rhizobium loti	*Lotus corniculatus* (bird's-foot trefoil)
Azarhizobium caulinodans	*Sesbania* spp. (e.g. *Sesbania rostrata*)

Sources: "Inoculation of Forage Legumes," University of Kentucky Cooperative Extension Service; "Technical Paper 2: Biological Nitrogen Fixation," FAO; "Legume inoculation in Florida," University of Florida, IFAS.

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How to store inoculant

As mentioned earlier, rhizobial bacteria thrive in cool moist conditions. A good rule of thumb, therefore, is to keep the inoculant cool and moist: 20°C/68°F in a tightly sealed container. Keep out of direct sunlight and away from high temperatures. If you do not have access to refrigeration, bury the inoculant in a sealed container in a shady location [see *EDN* 86-3 for details about this storage method].

