HAITI AGROFORESTRY RESEARCH PROJECT

SOUTH-EAST CONSORTIUM FOR INTERNATIONAL DEVELOPMENT/

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MICROSYMBIONT COLONIZATION AND SEEDLING DEVELOPMENT AS INFLUENCED BY INOCULATION METHOD: RHIZOBIUM AND FRANKIA

by R. Kent Reid

Nursery Specialist

Summary

This investigation tested the various methods used in Haitian nurseries for inoculating tree seedlings with nitrogen-fixing organisms. Acacia, leucaena, and frene were inoculated with Rhizobium powder by oil-coat, water-coat, water-drench, and gum-arabic methods, and by soil. Casuarina was inoculated with Frankia powder by water-drench and salt-shaker methods, and by soil. Variables tested were seedling height, root collar diameter, and numbers of total and red nodules per tree.

Only a few differences were seen among the inoculation methods tested on acacia, casuarina, and leucaena. When differences were found, they were not large. More differences may have been found if the inoculum used on these species had been better. For these species, only about two nodules per tree were found on the inoculated seedlings. This low number indicates low viability of the inoculum. The inoculum strain used on frene appeared to be good, however, and produced about 21 nodules per inoculated tree. Inoculation by soil produced only 5.4 nodules per tree, not different from the controls at 3.4 per tree. Other variables were not affected significantly.

The major conclusions of this study are that inoculum must be viable to produce nodules, but that when the inoculum is viable, any inoculation method may be used without compromising nodulation or seedling development. Soil can also be used as a inoculum source, but it must be recently gathered and probably should be used at a ratio of about one part soil to two parts Gromix.

Rezime Kreyol

Esperyans sa-a te fèt pou eseye kèk metòd inokilasyon ki kapab sevi pepinyeris pou inokile ti pyebwa ak Rizobyom ou Frankya. Nou sevi ak plizyè metòd. Nou mouye grenn yo ak lwil kwizin, ak dlo, e ak gom arabik. Nou wouze grenn yo ak yon melanj dlo e poud Rizobyom. Nou pran tè ki gen Rizobyom landan-l e nou melanje-l ak Gromiks. Tout metòd sa yo te sevi pou inokile akasya, lesena, epi frèn. Pou Frankya, nou sevi ak metòd dlo wouze, simen poud, ak Frankya melanje nan tè pou inokile kazawarina. Nou mezire wotè ak lage chak plantil, epi nou konte tout ti boul ak ti boul wouj chak plantil pouse sou rasin li.

Nou prèske pat jwenn diferans ent metòd inokilasyon nou te esaye sou akasya, kazwarina, oubyen lesena. Lè nou te jwenn yon diferans, li pat gwo. Petet nou ta ka jwenn plis diferans si inokilan yo te pi bon. Nou jwenn de ti boul nan chak plantil inokile. Yon ti valè konsa vle di inokilan sa yo pat bon.

Kalite inokilan ki te sevi sou frèn te byèn mache. Li fè plis pase vèn ti boul pouse nan chak pyebwa inokile. Inokilasyon nan tè fe senk ti boul pouse nan chak plantil, un kantite ti boul prèske egal kantite ti boul nan plantil pa inokile yo. Metòd inokilasyon pa fè gwo diferans ent lot mezi yo.

Avec esperyans sa-a, nou wè anko fok gen bon inokilan pou fè ti boul sou rasin plantil pouse. Pepinyeris kapab sevi ak nenpòt metòd inokilasyon lè inokilan-an bon. Pepinyeris kapab sevi ak tè tankou sous inokilan, men fok tè-a frè. Li kap melanje yon bokit tè ak chak de bokit Gromiks.

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PADF and Scott Josiah provided seed, NifTAL *Rhizobium* inoculum, and *Frankia* powder inoculum.

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SECID/ Auburn team members critically reviewed an earlier draft of this report. Sigrid d'Aquin helped with the Rezime Kreyol translation.

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Introduction

Farmers around the world are recognizing the need to inoculate crop legumes such as alfalfa and soybeans in order to save on fertilizer costs and simultaneously obtain maximum yield (NifTAL/FAO 1984). This need also is recognized in nursery culture of tree seedlings. In fact, strains of root-colonizing symbiotic microorganisms are being isolated that are specifically tailored for trees. However, information on inoculating nursery seedlings is difficult to obtain, and methods developed for the temperate zone may be inappropriate for the tropics. How to best inoculate our nursery seedlings is a concern of all AOP organizations.

Two nitrogen-fixing root microsymbionts, Rhizobium spp. on legumes and Frankia spp. on Casuarina, are important in Haitian tree nurseries. Several different methods are used to inoculate Haitian nursery stock with the appropriate commercially-prepared microorganism. Concerns about long-term sustainability have sparked interest in alternative inoculation methods such as incorporation into the potting mix of soil thought to contain the appropriate microorganism, but alternative methods have not been investigated thoroughly.

The study described in this report had as its objective to determine the best methods for inoculating selected tree species with appropriate microsymbionts. This study was limited to nitrogen-fixing associations; mycorrhizal colonization will be tested at a later time.

Methods

Seedlings for this study were grown in commercial peat-vermiculite (Gromix) in Rootrainer Deep 5s. All seed received standard pre-sowing treatments (Josiah 1989). Three leguminous species and one Casuarina species were tested (Table 1).

Inoculation methods used in this study are listed in Table 1. They are described in Josiah (1989) for nursery operations, and below as they were used in this study. Note that because the different inoculation methods were developed to treat different amounts of seed, they call for different amounts of inoculum. For this study, however, the same amount of legume inoculum was used for each method. The amount used for the legumes was the amount specifed for the seed-coat methods, which called for the equivalent of 0.5 g inoculum per 35 seedlings. The amount used for Frankia was about 1.5 g per 35 seedlings for the water-drenches and 3 g per 35 seedlings for the Shaker.

The Rhizobium strains used were from the NifTAL Project, supplied by PADF, and were matched with the proper tree species. The production date was 17 October 1988 for all the Rhizobium strains. Soil inocula for both Rhizobium and Frankia was supplied by CARE, and were collected under trees showing active nodules. The legume source trees were known to have been inoculated with NifTAL inoculum before they were outplanted. The four soils used as inoculum sources were transported on 27 April 1989 from CARE in Gonaives to SECID/Auburn in Petionville, where they were stored outside under shade until used. Frankia

inoculum was supplied by PADF and had no specified production or expiration date.

Non-inoculated (Control) seeds were sown after the proper pre-germination treatment. They received no additional treatment. Approximately 150 seeds were sown in the Control treatment, and in each of the other treatments.

Oil-coat inoculation consisted of pouring enough corn oil on the seeds to coat them thoroughly, then sprinkling the inoculum over them. The seeds and inoculum were mixed until the seeds were evenly coated.

Water-coat inoculation was carried out in much the same way. Seeds damp from the pre-germination treatment were sprinkled with inoculum and mixed until evenly coated.

The Gum-arabic method coats the treated seed with a sticker before applying the inoculum. The sticker was mixed as 4 g granular gum arabic : 10 ml water : 0.24 g $CaCO_3$. This mixture was applied to the seeds, inoculum was sprinkled onto the surface and evenly distributed, and the coated seeds were placed in the shade to dry before planting. This was the only method where seeds were allowed to dry between inoculation and planting.

The Soil method mixed the appropriate soil with the Gromix (1:10, v:v). Deep 5s were filled with this mix and seed was planted in it.

Water-drench inoculation was the only post-planting method tested. One-half gram inoculum was stirred into 750 ml water and poured over the planted seed. Seed inoculated by water-drench

were treated twice, at sowing and two weeks afterwards.

The above methods were used to inoculate legumes. Besides the Control, Casuarina was inoculated with Frankia by the Soil method as described above, and by three other methods described below.

The method not used with Rhizobium but used with Frankia was the Shaker method. Approximately 1 ml Frankia inoculum was shaken from a container into each Deep 5 cell, and the Casuarina seed was planted on top of it.

Two water-drench methods were tested with Frankia. The first mixed 5 g Frankia inoculum with 750 ml untreated tap water (Low pH). The second mixed the same amount of Frankia with 750 ml tap water containing 5 ml household ammonia (Torrey 1981) (High pH). These solutions were used to water Casuarina seed at sowing and two weeks afterwards.

Seven Deep 5s were planted for each inoculation method (including control) for each species. Casuarina was sown 4 May and harvested 25 October 1989. Sesbania was sown 5 and 6 May and harvested 8 and 9 August. Leucaena was sown 5 May and harvested 22 and 23 August. Acacia was sown first on 6 May. That sowing failed, and Acacia was resown on 21 and 22 June. Acacia was harvested on 25 and 26 October. Soluble 20-20-20 fertilizer (1.5 Tbs / 3 gal water for 750 trees) was supplied once a week during the time in the nursery.

Shoot length (height) and root collar diameter (caliper) were measured at harvest. Colonization was evaluated for the

legumes by counting total nodules and the number of nodules which were red inside. Colonization was evaluated for Casuarina by counting total nodules, with some allowance for size differences among nodules.

A single Deep 5 was considered the experimental unit; thus, measurements from the seedlings in each Deep 5 were averaged. The seven replications of each method were were then tested for inoculation effects by analysis of variance of a completely random design. When inoculation effects were found to differ, treatment means were separated using contrast statements (Snedecor and Cochran 1967). Protection against Type I errors was set at 5%, or α=0.05.

Results

No significant effects due to inoculation method were seen with Acacia (Table 2). Most trees had no nodules. Two of the six trees which had nodules were in the control group, and one of those two had a red nodule, indicating activity.

Total nodule number was low but varied significantly with inoculation method on Leucaena (Table 3). Inoculation with soil produced the most nodules, water-drench and the control the least, and the other methods produced intermediate numbers and did not differ among themselves. An average of just over two nodules per tree were found on the seedlings inoculated by the seed-coat methods, and a red nodule was found on only one tree. Morphological measurements did not differ significantly, although

the tallest seedlings were those inoculated with soil.

The powdered Rhizobium inoculum strain used on Sesbania performed well and produced about 21 nodules per root system (Table 4). The soil method produced only 5.4 nodules per tree, not different from the controls at 3.4 per tree. Very few of the nodules were red, however. Other variables were not affected significantly.

Root collar diameter and number of nodules per tree did not differ for Casuarina inoculated with Frankia (Table 5). Seedling height differed with inoculation method for Casuarina, with the high pH and soil methods producing the tallest seedlings and the control the smallest. Seedlings inoculated by the other methods did not differ from these extremes. Note that most trees did not have nodules, and that average nodule number was less than one.

Discussion

One obvious problem with drawing conclusions based on these data is that nodulation reached expected levels only for Sesbania. Another problem is that few red nodules were found, something that was true even in the Sesbania. For species that are as susceptible to nodulation as are the three legume species used here, nodulation this poor is unusual. Four factors typically contribute to poor nodulation in the nursery: old inoculum, weak seedlings, inhibition by fungicide, and excessive nitrogen (Joe Rourke, personal communication). Each of these factors and their influence on this study are examined below.

When this study was begun, the expiration date of the packaged NifTAL inoculum that was used was thought to be at some indefinite time in the future. Since then, additional information indicates the expiration date had passed and suggests that the Rhizobium was no longer alive. Indeed, research cultures are maintained under ideal conditions for no longer than 6 months (Joe Rourke, personal communication), and these packages were almost 7 months old when the inoculum was used. Thus, possibly the biggest reason for the poor nodulation was old inoculum. The good nodulation seen with Sesbania was a fortunate accident.

Another reason for poor nodulation is poor seedling health. If a seedling is not healthy, either it can not be induced to form nodules, or bacteria is not stimulated in its rhizosphere, or both. Healthy seedlings are needed for nodulation to occur (Joe Rourke, personal communication). This study's seedlings were healthy during the period colonization would have occurred.

Fungicides such as Captan or Benlate have been shown to be detrimental to the effectiveness of Rhizobium inoculum. Fungicide and Rhizobium contact can be minimized by applying fungicide only until the seed coat emerges from the potting mix; if no additional application is made, the residual fungicide will become progressively less active in the mix and should not harm the inoculum. Normal fungicide treatments may also be applied safely after some nodulation has occurred (Joe Rourke, personal communication). Perversely, seedlings are most susceptible to infection by damping-off fungi and to colonization by Rhizobium

at the same time, about ten days after emergence. Thus, the best procedure to follow in an AOP nursery, as always, is to avoid the use of fungicides to control damping-off on legumes by carefully controlling watering around the time of emergence. Fungicide was never applied to this study.

Too much nitrogen in the planting mix can inhibit nodule formation (NifTAL/FAO 1984). Situations also can exist where nodules are formed but excess nitrogen represses their ability to fix nitrogen. Although nodules may be present, the bacteria inside them depend on an unidentified signal from the plant to turn on their nitrogen-fixing mechanism, and that signal is not released as long as the plant receives adequate nitrogen from the soil. Currently, NifTAL uses an initial concentration of 70 ppm N in the growth medium of its experimental plants. This nitrogen level satisfies plant requirements for a few days, but becomes exhausted in time to allow nodule activity to begin (Joe Rourke, personal communication).

Nitrogen level in the potting mix may have affected this study, and has implications for successful nodulation in AOP nurseries. The Gromix used in this study has 47 ppm N (Reid 1989), a background level which probably requires amendment during seedling establishment. This study's nitrogen fertilizer was added at a solution concentration of 278 ppm. This N level was probably greater than needed, although it was necessary to maintain P and K levels. More of a problem than the N level, however, was the timing of the application. Regular fertilizer

addition was begun about a week after emergence. That addition plus the relatively high weekly input thereafter may have contributed to nodulation inhibition in Acacia and Leucaena, and repressed Rhizobium activity within the nodules of Sesbania (Joe Rourke, personal communication).

The surprise in these data is found in the results from the water-drench method. Since the plant is most susceptible to colonization by the Rhizobium bacteria about two weeks after emergence, NifTAL currently recommends inoculating seedlings with an aqueous suspension of inoculum at that time (Joe Rourke, personal communication). Thus, even though here seed was inoculated two weeks after sowing and not two weeks after emergence, nodulation would be expected to be at least as good for the water-drench method as it would be for the other methods using packaged inoculum. However, water-drench produced more nodules only for Sesbania, and then the difference was not significant. For Leucaena, water-drench inoculation produced fewer nodules, and that difference was significant.

The most important lesson to be drawn from this study is that if the inoculum is viable, how it is applied to the new root system is not a concern, since nodulation will be good for all inoculation methods. However, management factors such as those discussed above can affect nodulation even when inoculum is viable. Nurserymen should pay particular attention to fungicide and nitrogen fertilizer applications.

Since no Frankia inoculation method produced a significant

number of nodules on Casuarina, those results are not as easily interpreted. One possible conclusion is that none of the methods used here are suitable for inoculating Casuarina. However, a water-drench method is recommended (Josiah 1989) and used operationally in AOP nurseries. Since that method consistently produces nodules on Casuarina, and a similar method (low pH) was used here, more likely conclusions are that this Frankia inoculum was not viable or was inhibited by high nitrogen concentration. The other water-drench, the high pH method, was tested because Torrey (1981) found inoculation using a suspension of ground nodules produced the best nodulation when the suspension water was made alkaline with ammonium hydroxide. That finding might also be true for Haitian conditions, but was not true when tested with this inoculum.

Soil can be used as a Rhizobium inoculum source, but it must be properly collected and used quickly. The soils used here were kept at least a week before use, a period of time that should be shortened to one or two days. From a sustainability standpoint, collection of soil colonized with the proper strain of Rhizobium should be encouraged. Practically, however, the establishment of a Rhizobium production facility here in Haiti and the furnishing of inoculum by USAID to the AOP nurseries eases the nurseryman's burden. Packaged inoculum is easy to use and should no longer have a viability problem when properly handled. Knowledge that locally-collected soil can substitute for packaged inoculum is useful, however, and can be drawn upon if the source of packaged

inoculum should ever dry up.

When soil is used as a inoculum source, it probably should be used at a greater ratio than the 1:10 used here. When properly aerated, pure soil colonized by a microsymbiont would be best to insure good nodulation, but pure soil should not be used in a plastic container. The 15% soil used in Neg mix theoretically should be enough to cause good nodulation; however, in this experiment, nodulation using soil was never as good as nodulation using powdered inoculum. Use of about 30% colonized soil by weight in the potting mix should produce an acceptable compromise between good nodulation and good aeration.

Recommendations

1. Nurserymen and other field people should be certain powdered inoculum is never allowed to heat up or dry out. Powdered inoculum should be used within 5 months of production, or within the expiration date on the package label.

2. A nurseryman can use the easiest possible method to inoculate his seedlings. He must pay careful attention to seedling health, fungicide use, and nitrogen fertilization to avoid inhibiting nodule formation.

3. Packaged inoculum generally is preferable to soil inoculum, and should be used when possible. When soil is used as a source of inoculum, it should be gathered properly, used quickly, and make up approximately 30% by volume of the potting mix.

Table 1. Nitrogen-fixing microsymbiont, inoculation technique, and tree species evaluated for colonization.

Microsymbiont	Inoculation method	Tree species
Rhizobium	seed-coat with oil seed-coat with water water-drench gum arabic soil	Acacia auriculiformis Leucaena leucocephala Sesbania grandiflora
Frankia	salt-shaker low-pH water-drench high-pH water-drench soil	Casuarina equisetifolia

Table 2. Mean values for measurements of acacia (Acacia auriculiformis) inoculated with Rhizobium by various methods.

Treatment Method	Height	Root Collar Diameter	Nodules per Tree	Red Nodules per Tree
	-cm-	-mm-	- # -	- # -
control	11.8a	1.08a	0.13a	0.04a
oil coat	12.3a	1.32a	0.10a	0.00a
water coat	12.9a	1.43a	0.28a	0.03a
water drench	11.1a	0.98a	0.00a	0.00a
soil	11.7a	1.00a	0.00a	0.00a
gum arabic	12.3a	1.09a	0.00a	0.00a

Values followed by the same letter are not different (α=0.05)

Table 3. Mean values for measurements of leucaena (Leucaena leucocephala) inoculated with Rhizobium by various methods.

Treatment Method	Height	Root Collar Diameter	Nodules per Tree	Red Nodules per Tree
	-cm-	-mm-	- # -	- # -
control	20.5a	2.69a	0.00c	0.00a
oil coat	20.2a	2.67a	2.30b	0.00a
water coat	21.6a	2.64a	2.03b	0.00a
water drench	19.9a	2.69a	0.33c	0.00a
soil	22.7a	2.58a	3.69a	0.00a
gum arabic	24.2a	2.66a	2.21b	0.06a

Values followed by the same letter are not different (α=0.05)

Table 4. Mean values for measurements of frene (Sesbania grandiflora) inoculated with Rhizobium by various methods.

Treatment Method	Height	Root Collar Diameter	Nodules per Tree	Red Nodules per Tree
	-cm-	-mm-	- # -	- # -
control	24.8a	2.71a	3.4b	0.43a
oil coat	22.0a	2.44a	20.8a	0.62a
water coat	20.4a	2.52a	20.5a	0.38a
water drench	20.7a	2.56a	23.2a	0.74a
soil	21.5a	2.63a	5.4b	0.24a
gum arabic	20.4a	2.56a	21.3a	0.28a

Values followed by the same letter are not different (α=0.05)

Table 5. Mean values for measurements of casuarina (Casuarina equisetifolia) inoculated with Frankia by various methods.

Treatment Method	Height	Root Collar Diameter	Nodules per Tree
	-cm-	-mm-	- # -
control	21.6b	1.67a	0.00a
low pH drench	24.9ab	1.34a	0.48a
high pH drench	26.7a	1.47a	0.87a
shaker	24.6ab	1.32a	0.48a
soil	26.6a	1.40a	0.00a

Values followed by the same letter are not different (α=0.05)

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