PROJECT NO. RM-6

2008 ANNUAL REPORT
COMPREHENSIVE RICE RESEARCH
(January 1, 2008 - December 31, 2008)

PROJECT TITLE: Salt Tolerance and Yield Enhancement in Rice Plants via Fungal Symbiosis

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Research Goals: Our previous laboratory, greenhouse and small field trials (see 2007 CRRB Annual Report) indicated that we can achieve salt and temperature stress, growth response, and yield enhancement in symbiotically colonized M206 rice plants when compared to non-symbiotic (NS) plants. Here, we propose to use fungal symbionts to confer salt and cold tolerance to rice under larger field conditions. In addition, the effects of symbiosis on plant biomass and seed yields will be assessed. Rice plants were chosen because it is an economically important agricultural crop used to feed much of the planet. In addition, rice is a crop that consumes major amounts of water and grown often in areas where salinity in the soil or water is an issue. Furthermore, rice is a model plant making it an attractive system to study the potential molecular and genetic basis of symbiotically conferred stress-tolerance for future studies.

We propose to test the following hypotheses:

$\mathbf{H_1}$ – Symbiotic plants will exhibit higher salt and cold tolerance than non-symbiotic plants.

$\mathbf{H_2}$ – Symbiotic plants will exhibit greater plant biomass and seed yields than non-symbiotic plants

$\mathbf{H_3}$– Endophytes will be present in rice seed husks from CRRF seed bank and confer stress tolerance (salt and cold) and growth and yield enhancement to M-206 rice plants under greenhouse conditions

INTELLECTUAL PROPERTY STATEMENT: This project has the potential to be developed as a feasible and realistic tool that can be provided to rice farmers to ultimately obtain yield enhancement and stress tolerance (salt and cold). As such, the merits on an academic and commercial level could potentially be significant. Increased plant survival and seed yields would afford collectively for the obtainment of a higher quality product and monetary return.

2008 OBJECTIVES AND RESULTS OF PROPOSED RESEARCH:

1. Development of an effective rice seed-endophyte colonization protocol: Here, we propose to utilize a newly developed symbiotic imbibing colonization protocol (developed in the Redman/Rodriguez lab in 2007) to feasibly generate large numbers (approx. 20 lbs of seed) of symbiotic (S) and non-symbiotic (NS) plants. Three different endophytes will be used to confer different stress tolerances to M-206 rice plants under field conditions. In each instance, growth and yield enhancement of S plants will be directly compared to NS plants. The “Salt Symbiont (Salt sym)” will be tested to confer salt tolerance in identified field sites that have +/- elevated levels of salt, and the “Cold Symbiont (Cold sym)” used to confer cold tolerance in identified field sites that have cold water exposure issues. M-206 rice plants were chosen because it is an economically important agricultural crop of interest to California rice growers. All field studies will be conducted in collaboration with Dr’s Chris Greer, Randall “Cass” Mutters, and Luis Espino, UCD Cooperative Extension, and Dr's Regina Redman, Sharon Doty, Yong Ok Kim, and Rusty Rodriguez at the University of Washington. Field trials will be performed over a single growing season (2008) at various experimental sites in Northern

California. In addition, if the above mentioned field trials in 2008 gives positive results, preparatory studies may then begin such that a large scale field trial in future will be feasibly employed in this rice system. The eventual goal being to developed this system as a tool that rice farmers can realistically use (aerial plane dispersal). Furthermore, rice is a model plant making it an attractive system to study the potential molecular and genetic basis of symbiotically conferred stress-tolerance to be addressed for future studies.

Objective 1 Experimental Design and Results:

Experimental Design: We utilized three different endophytes (Cold symbiont 1 = Cold Sym1; Cold Symbiont 2 = Cold sym2; and Salt Symbiont = Salt sym - see previous proposal RM-6 for details) to confer different stress tolerances to M-206 rice plants under field conditions. In each instance, growth and yield enhancement of S plants were directly compared to NS plants. The "Salt Symbiont" was tested for it's ability to confer salt tolerance in an identified field sites that had slightly elevated levels of salt (EC readings range: 0.47-1.30; avg=0.66 dS/m), and the "Cold Symbiont" used to confer cold tolerance in an identified field sites that has cold water exposure issues (temperature range throughout growing season: 12 C - 30 C, avg =25 C), and endophytes assessed in general for potential positive effects in the form of growth and yield enhancement in an identified non-stressed site. Field trials were conducted over a single growing season (planted in late May and harvested in late October, 2008) in three different experimental sites (Site #1 imposing salt stress, Site #2 imposing cold stress, Site #3 imposing no-stress; Table 1).

Sterile NS and S plants were be generated in the Dr's Redman/Doty/Rodriguez lab using a fluid imbibing process recently developed in our lab by Dr. Yong Ok Kim.
<u>Salt stress experiments:</u> S plants will be colonized with the "Salt Symbiont";
<u>Cold stress experiments:</u> S plants will be colonized with the "Cold Symbiont 1&2";
<u>Seed Yield enhancement:</u> S plants will be colonized with the "Salt Symbiont, and Cold symbiont 1" as preliminary results indicate this is achievable with these endophytes (Table 1).

Plant seeds were driven and/or shipped to one of the UCD collaborators and planted into 10'x20' field plots: x2 treatments (S & NS) x 4 reps, at x1 unique locations per site, x3 conditions (salt stress, cold stress and no stress field sites). This will equate to 310-350 g seed/plot = 8-12 plots each for salt, cold and non stress conditions = 32 plots total. This will equate to the required production total of 16 lbs of S seeds (approximately 3-6 lbs per symbiotic treatment), and over 9 lbs of sterile NS seeds. The salinity (EC) water and air temperatures will be monitored throughout the growing season. S and NS treatments will be separated by a 10' buffer to minimize cross contamination. At the end of the season, plant seed biomass will assessed (Figure 2) and 5-10 random plants per plot assayed for +/- S colonization, and data analyzed by ANOVA to assess significance differences between treatments.

Results: A statistically significant increase in plant biomass at the early seedling stage was observed under field and laboratory conditions (Figures 1 & 2,respectively). Under field conditions, observations were made at the cold stress site (Site #2) that symbiotic plants (both Cold sym1 & Cold sym2) were larger, with Cold sym1 having the largest and thickest onset of rice seedlings, compared to Non-Symbiotic (NS) plants (Figure 1; Table 1). It is possible that symbiotically conferred stress tolerance is most important during a portion (the early seedling stage perhaps) of the growing season or at specific developmental stages. Therefore, it will be necessary in future, to be much more diligent especially during the early stages and monitor plant growth and health, as well as continue to do so throughout the growing season. In addition, our laboratory studies have shown that the on-set of stress to plant seedlings often results in the biggest symbiotic effects. That is to say, positive effects in the form of growth response is most acute when sufficient stress is imposed. In the absence of stress during the early growth period, and lack of sufficient stress is continued throughout the growing season, often, no differences in growth response and yields are observed (Figure 3). However, in the presence of stress, a early seedling growth response is observed (Figure 1), and if adequate stress is imposed throughout the growing season, enhanced growth response and yields are observed (data not shown, in barley system, in collaboration with Dr. Rodriguez and Precision Labs).

At the end of the season when yields were assessed, no negative effects due to the symbiosis was observed in any of the three sites, for any of the treatments. As our previous studies indicated, in the absence of stress, no differences are generally observed between treatments and as such, was the expected result in our no-stress site (Site #3), However, this was not the expected result for the salt stress (Site #1) and cold stress (Site #2) sites (Table 1; Figure 3). Analysis of the salinity and water temperatures at these sites, respectively, indicated that the stresses imposed were marginal at best. The salinity level average was 0.66 dS/m which equals to approximately 6-7 mM NaCl, little to no salt stress. In our previous laboratory and 2007 field studies, plants were subjected to a minimum of 100 mM (=15 dS/m) and up to 500 mM (=55 dS/ml) NaCl. Similarly, water temperatures in the Cold stress site began rather cool (21 C), but then increased and warmed throughout the growing season (to 30 C high, avg = 25 C). These results suggest that the site specific stresses were not sufficient enough to see the benefits of the symbiosis clearly. This was not surprising as previous laboratory and field studies (Rodriguez et al., 2008; & personal communication and observations) indicated that in the absence of stress, differences between symbiotic and non-symbiotic treatments (survival and yields) were not observed. In contrast, when stress is imposed, particularly if it begins from the time of seed planting, a measurable, and often dramatic increase in survival and plant yields will result under both field and laboratory conditions (Rodriguez et al.,1997, 2008; Redman et al., 1999, 2002; personal communication). Other higher stress salt and cold sites will need to be identifies for future field studies.

Having just stated the importance of maintaining steady-stress-pressure under field conditions to observed maximum benefits of symbiosis, under laboratory conditions, we have observed dramatic growth response in seedlings even in the absence of stress (Figure 2, Table 2). Seedlings (after 8 days) showed more than a x5 increase in plant biomass. The root system in symbiotic plants are much more extensive and an obvious increase in fine root hairs are observed ($P \leq 0.01$, Table 2). These results are suggestive that symbiosis, particularly in young plant seedlings, may be quite beneficial for the early establishment and thus, survival of plants.

Figure 1: M206 Rice Cold Stress Site#2
(P≤0.05)

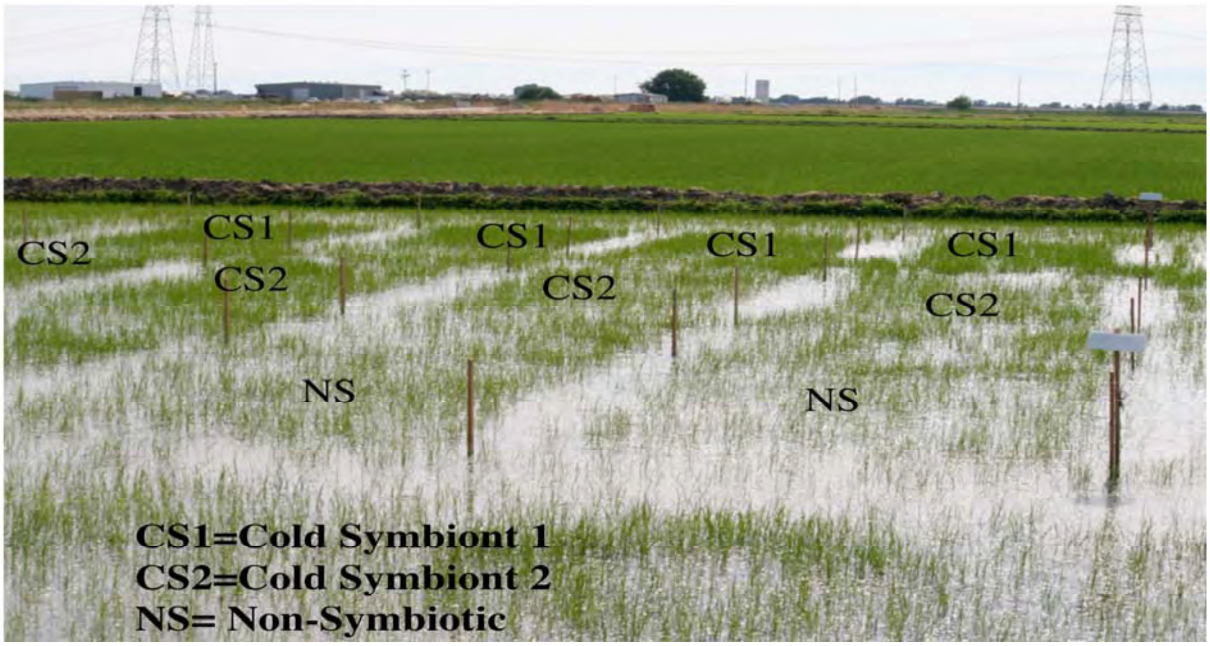
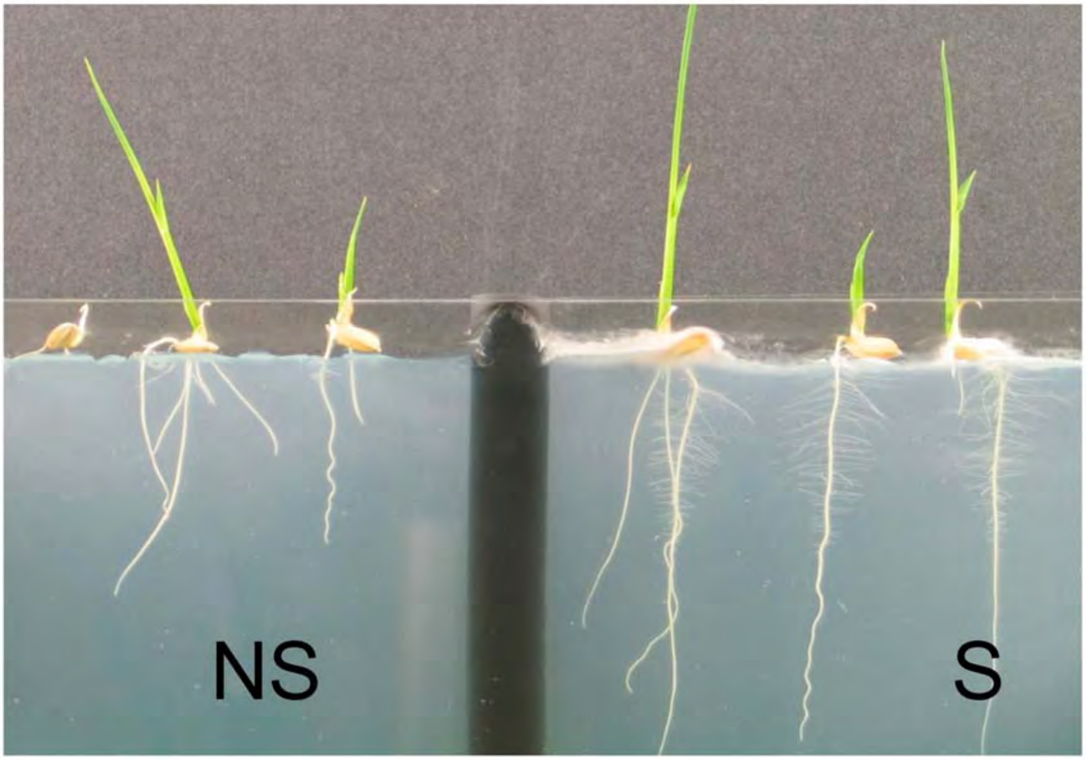


Figure 2



8 day old M206 rice seedlings. NS=Non-Symbiotic, S=symbiotic with Salt Symbiont

Table 1: Field Site Design:

Site 1: M206 Rice Salt Stress Experimental Plot Design

Treatments 1	Buffer plot	**Treatment 2**
NS rep1	rep1	Salt S rep1
NS rep2	rep2	Salt S rep2
NS rep3	rep3	Salt S rep3
NS rep4	rep4	Salt S rep4

Site 2: M206 Rice Cold Stress Experimental Plot Design

Treatments 1	Buffer plot	**Treatment 2**	Buffer plot	**Treatment 3**
NS rep1	rep1	Cold S1 rep1	rep1	Cold S2 rep1
NS rep2	rep2	Cold S 1 rep2	rep2	Cold S2 rep2
NS rep3	rep3	Cold S1 rep3	rep3	Cold S2 rep3
NS rep4	rep4	Cold S1 rep4	rep4	Cold S2 rep4

Site 3: M206 Rice No-Stress Experimental Plot Design

Treatments 1	Buffer plot	**Treatment 2**	Buffer plot	**Treatment 3**
NS rep1	rep1	Salt S rep1	rep1	Cold S1 rep1
NS rep2	rep2	Salt S rep2	rep2	Cold S 1 rep2
NS rep3	rep3	Salt S rep3	rep3	Cold S1 rep3
NS rep4	rep4	Salt S rep4	rep4	Cold S1 rep4

NS= non-symbiotic, S= symbiotic. 350 grams of symbiotically colonized and NS seeds were planted in each plot (10'x20' each). Buffer plots were included between treatments to help minimize cross contamination.

Table 2: M206 Rice Seedling Growth Response

Treatment	**Rep 1**	**Rep2**	**Rep3**
Non-Symbiotic	0.065	0.067	0.054
Salt Symbiont	0.101	0.108	0.105
Cold Symbiont	0.074	0.082	0.071

A total of thirty plant seedlings (N=10 plants/rep) were measured and both salt and cold symbiotically colonized plants found to be significantly ($P<0.05$) larger than non-symbiotic plants.

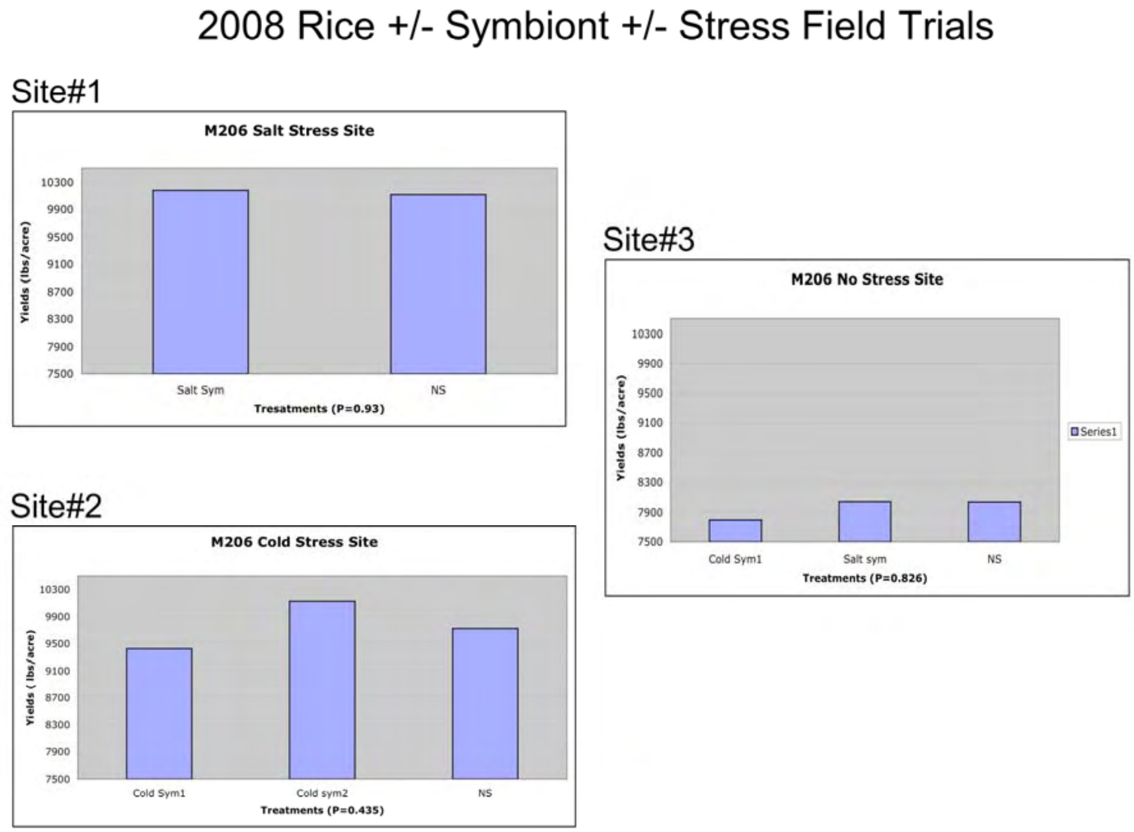


Figure 3

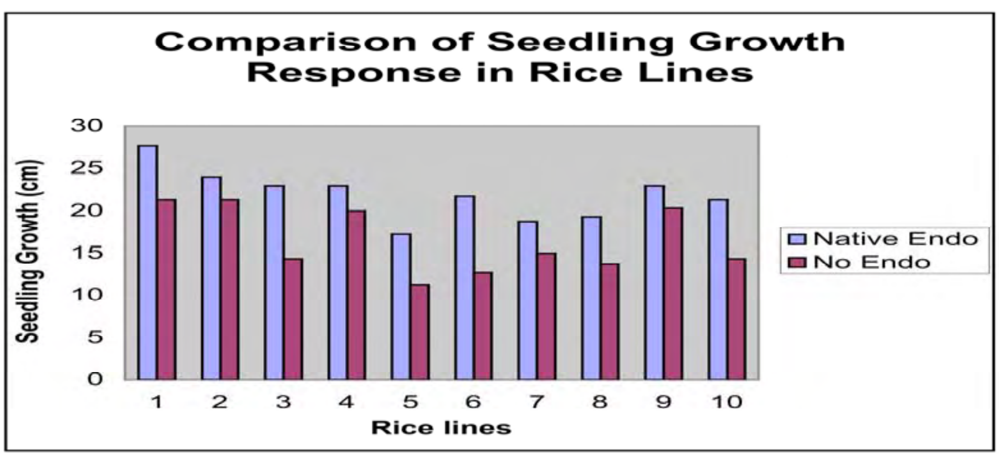
Objective 2. Analysis and isolation of endophytes from rice seeds obtained from the Rice Experiment Station (RES) seed bank in collaboration with Dr’s Kent McKenzie (CRRF, UCD) and Dr Harold Bockelman (USDA), and Dr's Regina Redman, Sharon Doty & Rusty Rodriguez (PI/Co-PI's) and Research Scientist, Yong Ok Kim (UW). Specific lines of seed were chosen that have stress (cold and salt stress) or yield tolerance benefits of interest. The basic idea being that endophytes will be associated with the seed husks of these rice lines of interest that can then be isolated and employed for functional testing under laboratory and greenhouse conditions. Endophytes that impart yield enhancement or stress tolerance to rice plants, can then be utilized in future field studies (2009+). Increasing the possibilities of symbionts to utilize in an agricultural setting will be beneficial as it will provide a bank of endophytic symbionts that can be employed in all or specific cultivars of rice. In addition, since endophytes may confer habitat-specific stress tolerance, these indigenous symbionts may prove to show enhanced benefits.

Results:

A total of 41 seed lines were obtained and endophyte profiles obtained and tested for growth enhancement. Microscopic and ribosomal DNA sequence analysis of representative morphotypes indicated that >90% of the endophytes obtained were *Alternaria* & *Curvularia* sp., and >5% *Fusarium* sp. Symbiotic benefits were functionally assessed by measuring rice seedling growth rates of the 41 lines that were either surface sterilized to remove their indigenous endophytes from the rice seed husks, or not sterilized to assess for the effects of the resident endophyte. In each instance, all of the indigenous endophytes conferred some level of growth response when compare to their non-symbiotic counterparts (Figure 4). In addition, >80% of the growth response measured were statistically significant ($P \leq 0.10$; ANOVA). Select representative endophytes were then tested on M206 rice. Rice seedlings were +/- symbiotically colonized and are presently being assessed for growth response, yield enhancement, fluid usage, salt and cold tolerance, and decrease nitrogen usage experiments under laboratory and greenhouse conditions.

These results were unexpected as although we anticipated that rice seed husks would have endophytes associated with them, it was surprising that *Alternaria* sp. was the dominant endophyte. It was expected that the salt and cold tolerant lines would share some similar endophytes (which they do), but, also have one or more unique endophytes dominantly associated with them (which they do not appear to). It may be that *Alternaria* sp. is just simply able to survive better in the patty rice field sites. Alternatively, the *Alternaria* sp. may be present in larger quantities (perhaps more epiphytically) on the seed coat, and is swamping out the presence of other endophytes. Perhaps addition of a light surface sterilization protocol needs to be added to determine more accurately the true endophyte-seed profile. Additional studies incorporating both microscopy and a surface sterilization protocol will be repeated with the 41 lines of rice in near future. Our present ongoing studies will address if *Alternaria* sp. can confer stress tolerance (salt and cold tolerance) to M-206 rice. If *Alternaria* sp. are the true seed endophytes, we anticipate that these endophytes will confer some level of growth and seed yield enhancement.

Figure 4: Representative 10 day old rice lines seedlings +/- indigenous endophytes



Problems that occurred and solutions/discussion:

1. **Problem:** There were no differences statistically in plant yields in symbiotic versus non-symbiotic plants. **Solution:** Locate additional salt and cold stress sites that will provide higher and more constant stress to plants throughout the entire growing season. Alternatively, screen and utilize other new symbionts (to be tested functionally in the laboratory prior to May 2009 planting).
2. **Problem:** Again, although there were no negative effects to the symbiosis, enhanced yields (ultimate goal) was not observed in Symbiotic vs. Non-Symbiotic treatments to justify large scale aerial application. **Solution:** Focus on committing to more 10x20 plot field trials testing more endophytes, and testing for additional stresses that are of concern to the rice grower (eg., decreasing nitrogen use via symbiosis, and/or possibly looking at pest disease protection via symbiosis as well). Plan to test for the large aerial dispersion in 2010. Alternatively, test just one endophyte of choice for large scale aerial dispersion.
3. **Problem:** Is *Alternaria* sp. the true dominant endophyte(s) from the 41 rice lines representing salt & cold tolerance lines, and enhanced yield lines? **Solution:** Include a surface sterilization protocol that will eliminate epiphytic endophytes and allow for a more accurate assessment of endophyte profiles. Basically repeat endophyte profiles with 41 lines and either confirm or deny the previous results. Functionally test representative *Alternaria* sp. isolates for cold and salt tolerance, and yield enhancement under laboratory and greenhouse conditions. If positive results are obtained, utilize representative endophyte in 2009 field trials (10'x 20' plots, as previously described). Obtain additional rice lines from USDA that are cold & salt tolerance, or have enhanced yields and screen for endophyte profiles.

Overview of Meeting Proposal Goals:

Objective 1 completed. Filed trials were successfully completed with several endophytes in 3 locales imposing different levels of stress. Parallel rice experiments were conducted under laboratory and greenhouse studies in 2007-2008. Collectively, these data are now being written-up as a joint publication-to be submitted to a peer reviewed journal.

Objective 2 completed. Endophyte profiles from 41 rice lines received were completed. Microscopic and molecular analysis identified the fungal species. Endophytes from 41 of the lines were functionally tested for symbiotic benefits via measuring growth response in rice seedlings. Some level of growth response was observed in all 41 symbiotic lines when compared to non-symbiotic counterparts. Presently, representative endophytes are being tested for positive functional benefits in the desired rice line M206.

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