PROJECT NO. RM-6

ANNUAL REPORT
COMPREHENSIVE RICE RESEARCH
(January 1, 2007 - December 31, 2007)

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

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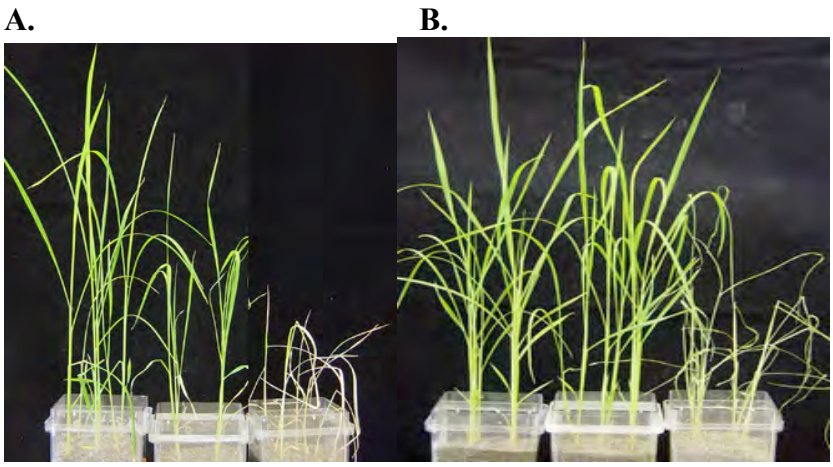
Research Goals: Here, we propose to use symbiont B to confer salt tolerance to rice. In addition, the effects of symbiosis on plant biomass and seed yields will be assessed. Rice plants were chosen because it is an economically important agricultural crop used to feed much of the planet. In addition, 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.

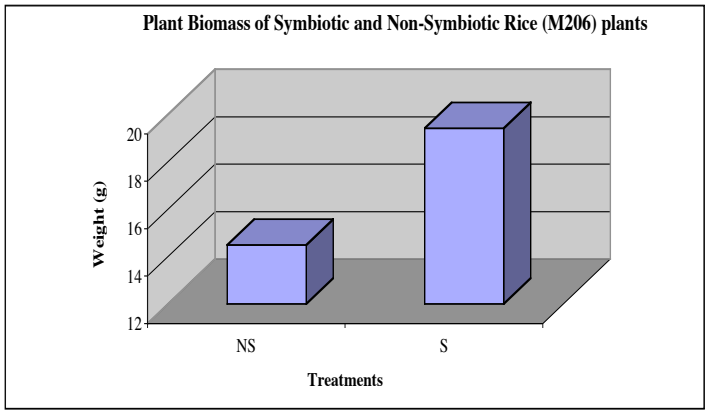
Objective 1: Pilot Laboratory and Greenhouse Experiments in Rice Plants
We have conducted growth chamber and greenhouse studies with 16 different rice cultivars grown commercially in USA and Korea. Collectively, our studies have shown that we can achieve salt (≤300 mM NaCl) and drought tolerance (≤15 days in the absence of water), and increased plant biomass (≤25%) and seed yield (≤40%) in symbiotic plants compared to non-symbiotic plants (Fig. 1 & 2). In addition, water usage in symbiotic plants was ≤30% less when compared to non-symbiotic plants (Fig. 3).
These studies were conducted to:
1. Screen our existing endophyte library to see which isolates worked best in the rice system.
Results: Identifies two endophytes that symbiotically conferred salt stress and/or yield enhancement in rice.

2. Identify isolates that positively benefited (namely salt tolerance and plant and seed yield enhancement) rice plants via plant-fungal symbiosis, and the best candidate endophyte selected.
Results: The best candidate endophyte was identified as *Fusarium* sp., which will be referred to as symbiont B and utilized in greenhouse and field studies.
3) Apply the endophyte to a California variety of rice that is of research and commercial interest.
Results: California rice M206 was found to be the best candidate.

Figure 1. Representative photos of the effects of symbiosis on salt and drought tolerance in rice plants[N=18 (cultivar M206)]. All descriptions are from left to right. **A.** Non-stressed hydrated controls exposed to 0 NaCl, plants colonized with symbiont B (SB) and symbiont A (SA) exposed to 300 mM NaCl for 14 days. **B.** Plants colonized with symbiont B, symbiont A and non-symbiotic (NS) plants exposed to drought stress for 15 days.



A.



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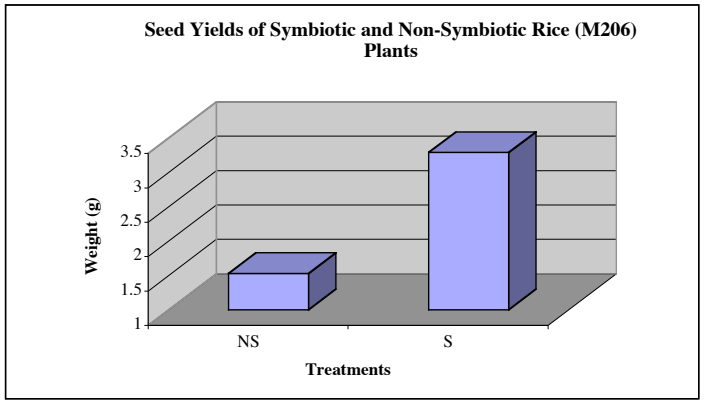


Figure 2. Rice plants (N=18) were symbiotically colonized with symbiont B (S) or non-symbiotic (NS) and assessed for: **A.** Total fresh weight plant biomass, and **B.** Seed yield. SE = ≤0.9 and ≤0.08 for plant biomass and seed yields, respectively.

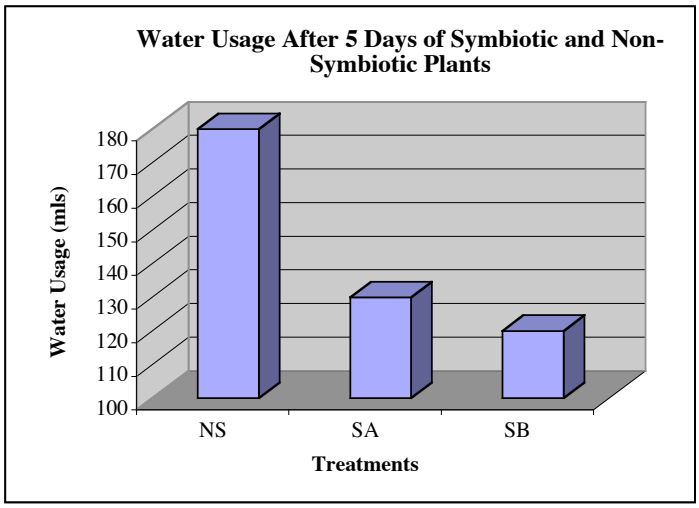


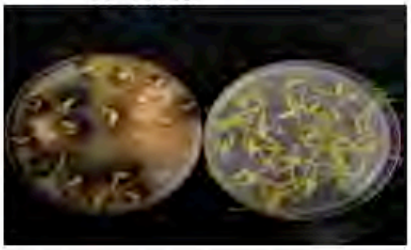
Figure 3. Water usage of Rice [cultivar M206 (N=18)] plants after 5 days of non-symbiotic (NS) or symbiont A (SA) and symbiont B (SB) colonized plants. SE = ≤15 ml.

Objective 2: Surface Sterilization and Imbibing/Colonization Protocol Development
After the original submission of the 2007 RRB grant, the principal investigator was asked to develop an effective symbiont colonization protocol incorporating a liquid imbibing process such that the system could be developed to mimic commercial type planting practices. To address this objective:
1. An effective surface sterilization protocol of seeds needed to be developed as the seed husks contained a high level of fungi.
2. An effective liquid imbibing/colonization technique needed to be developed.
Results: To date, a surface sterilization protocol of 85-90% efficacy has been achieved using a combination of sodium hypochlorite, ethanol and "Spotack" (prochloraz). In addition, an effective (100%) liquid imbibing/colonization was achieved after several parameters were optimized (a range of fungal spore concentrations were investigated in combination with varying times of imbibing, and optimal seedling germination obtained) (Fig. 4).

Figure 4. Optimization of surface sterilization and imbibing/colonization

Surface Sterilization of M206 Rice Seeds + Seed coat:

1. 75% EtOH, Bleach (100% = 6.15% Sodium Hypochlorite) for 60' = 30% clean
2. Spotack 24 Hr (1 ml/2L sterile water; Dr An Pohang University, South Korea), 6.15% Bleach 30" = 80-85% Clean
3. 5% EtOH, Bleach (100% = 6.15% Sodium Hypochlorite) for 60'; Spotack 24 Hr (1 ml/2L sterile water; Dr An Pohang University, South Korea), 6.15% Bleach 30" = 90+%
4. Presently, doing a heat treatment + all the above: optimal goal 95-100%!



A. In the absence(Left plate) and in presence (Right plate) of surface sterilization of seeds and plated on fungal growth media. Dark coloration (Left) is fungi.

B. Surface sterilized rice seedlings(M206) in the absence (Left plate) and presence (Right plate) of symbiont colonization utilizing a liquid imbibing/colonization protocol. The solid white cloudy color (Right plate) is the fungal endophyte of interest.

Objective 3: Greenhouse and Field Experiment Objectives
Application and comparison of non-symbiotic versus plants colonized with Symbionts B (California rice cultivar M206) under greenhouse field conditions as previously described (Redman et al., 1999, 2001). Plants will be tested for salt tolerance as well as plant biomass and seed yields. The greenhouse studies will be performed by Dr. Regina

Redman and Dr. Yong Ok Kim (Research Scientist) at Montana State University (MSU) and University of Washington (UW), and the field studies will be performed in collaboration with Dr. Mike Davis, University of California Davis (UCD), Dr Chris Greer, Farm Advisor, Cooperative Extension (UCD), and Dr's Regina Redman and Yong Ok Kim (MSU/UW). Field trials will be performed over a single growing season (2007) at the UCD experimental farm.

We propose to test the following hypotheses:
H_1 – Symbiotic plants will exhibit higher salt tolerance than non-symbiotic plants.
H_2 – Symbiotic plants will exhibit greater plant biomass and seed yields than non-symbiotic plants

Overview of greenhouse and filed plant preparation
Preparation of plants (began with approximately 20,000 seeds) for field and parallel greenhouse experiments were conducted by surface sterilizing seeds (at the time the sterilization technique was generating only 30-50% sterile seeds), plating seeds onto fungal growth media, and removing any seeds and/or seedlings that had evidence of fungal contamination. One-half of the sterile seeds were dip inoculated into a Symbiont B fungal spore suspension (1-5 E5 spores/ml) and planted into cell packs (x72/tray) containing 1/2 clay and 2/3 Sunshine Mix soil and grown in the greenhouse for 7 days, and re-inoculated with the same fungal spore suspension by the addition of 1-2 ml of the spore solution to seedlings, and let to grow and acclimate until shipped for field planning at UCD. The other half of the seedlings (non-symbiotic) were planted in cell packs and allowed to grow in the greenhouse until shipped to UCD for field planting. For the parallel greenhouse studies, a small subset (approximately 100+ plants each of symbiotic and non-symbiotic) were reserved and assayed for salt and plant biomass and yield studies.

Greenhouse salt tolerance study design: Non-symbiotic and Symbiont B colonized plants [approximately 15/tub, x 2 reps, x4 conditions (0, 100, 200, 300 mM NaCl)] were planted into large sterile plastic tubs (2'x2.5'), or 5 plants/8" pot [x 6 reps, x4 conditions (0, 100, 200, 300 mM NaCl)] containing a clay/soil mixture, and exposed to a gradual increase of salt over a 3 month period. That is to say, with the exception of 0 NaCl control plants, all other plants were exposed to 100 mM NaCl, for 2-3 weeks, 1/3 of them maintained at 100 mM NaCl exposure and the other 2/3 if the plants exposed to 200 mM NaCl for another 2-3 weeks. Lastly, of the plants exposed to 200 mM NaCl, 1/2 were maintained at 200 mM NaCl exposure and the other 1/2 exposed to 300 mM NaCl until the duration of the experiment and which point, the root, stem length, # shoots, root, and stem biomass of plants and seed yields were assessed, recorded and photo documented (Fig. 5).

Greenhouse salt stress studies results: Symbiont B induced growth response, enhanced seed yields and plant biomass was observed in both young seedlings (Fig. 5A) and mature plants (Fig. 5B & C) which was found to be statistically significant. Significant differences occurred in the shoot #, weight, and root weight with the highest significant differences in the seed yield with symbiotically colonized plants out performing their non-symbiotic counterparts (see Fig. 5C figure legend for P values).

Figure 5A. Growth response of symbiotic (S) and non-symbiotic (NS) M206 seedlings (N=100) grown on agar water (Left pane) and bare root (Right panel) conditions.

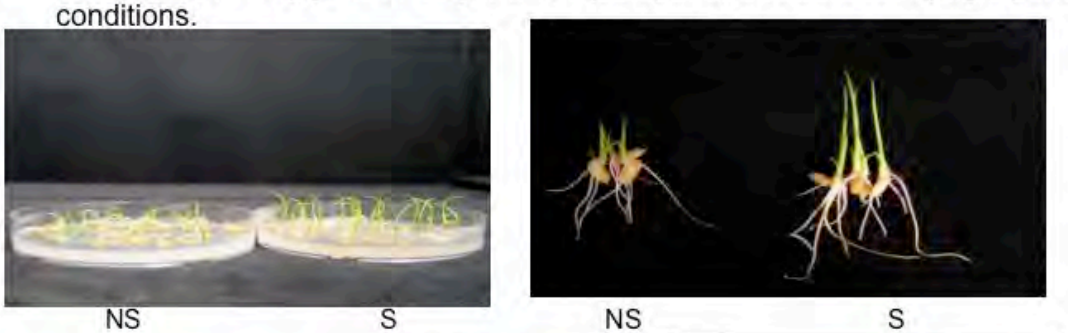
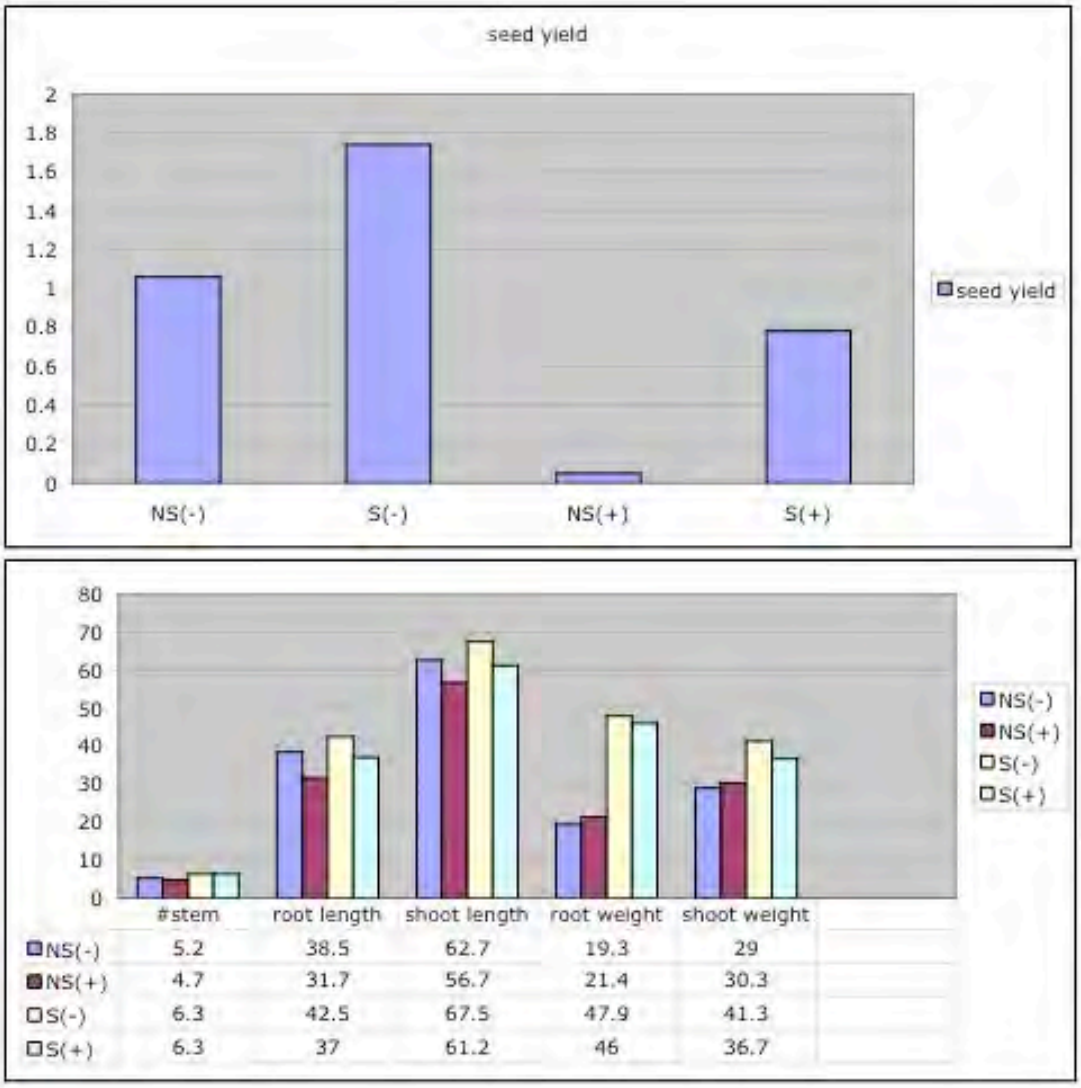


Figure 5B. Growth response and salt tolerance of S and NS M206 rice plants(N=100) under greenhouse conditions +/- salt stress (300 mM NaCl) over 3 months.

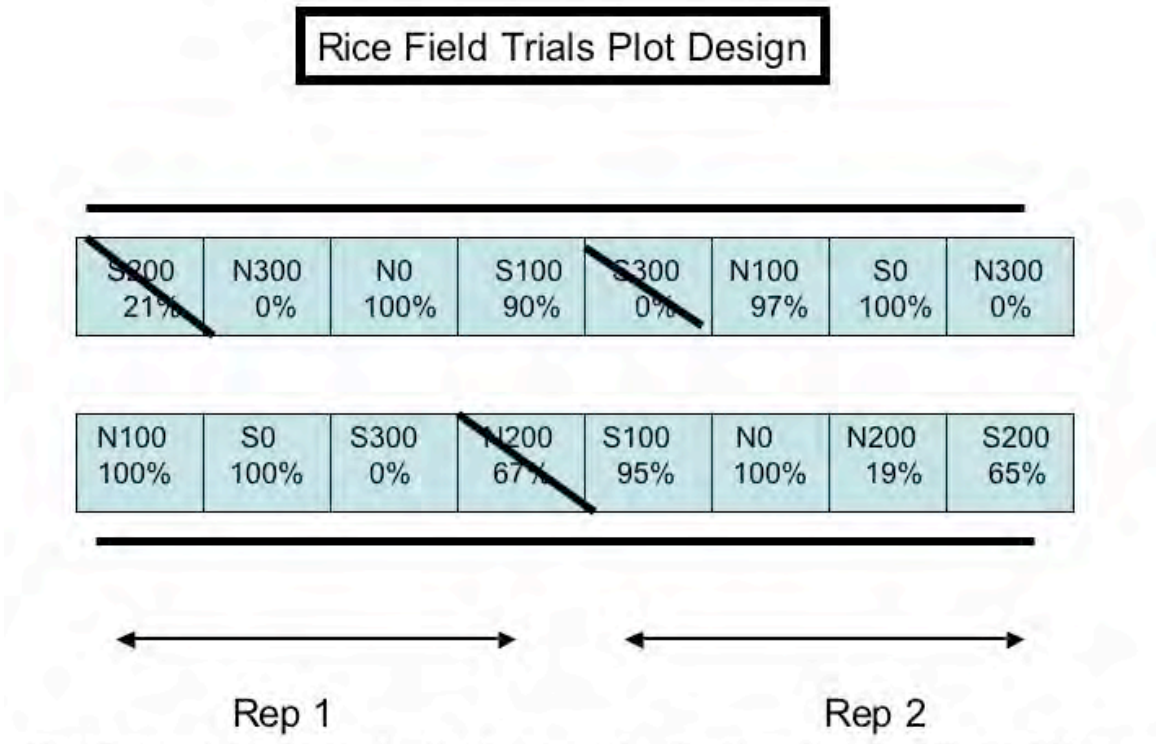


	#stem	root length	shoot length	root weight	shoot weight
NS(-)	5.2	38.5	62.7	19.3	29
NS(+)	4.7	31.7	56.7	21.4	30.3
S(-)	6.3	42.5	67.5	47.9	41.3
S(+)	6.3	37	61.2	46	36.7

Figure 5C. Seed yield ((grams; upper panel), # of shoots, shoot and root length and root and shoot biomass (grams, lower panel) of symbiotic (S) and non-symbiotic (NS) mature M206 rice plants (N=30) +/- salt stress (300 mM NaCl) under greenhouse conditions. Plants assessed after approximately 3+ months. Statistical analysis (ANOVA) indicated significant differences between treatment S versus NS plants for # stems +/-salt stress(P=0.097 & P= 0.049, respectively); # shoots in the absence of salt stress (P=0.68); significant differences were also observed in root weight +/- salt stress (P=0.003 & P=0.03, respectively) and highly significant differences in seed yields+/- salt stress (P=0.07 & P=0.057 E-07, respectively). No significant difference were observed in root length +/- salt stress(P=.0.134-0.234), shoot length + salt stress(P=0.56) and shoot weight +salt stress (P=0.28).

Field salt tolerance study design: Salinity experiments were performed under paddy rice conditions in 3m x 3m independently irrigated plots lined with plastic and filled with soil. Cultivar M206, a commonly used rice cultivar in California, was transplanted for a final plant density of 150 plants m^{-2}. Plots received an initial concentration irrigation at either 0, 100, 200 or 300 mM NaCl. Salt concentrations were measured periodically through the season. Treatments were replicated four times. Symbiotic plants were colonized with Symbiont B which has been shown to confer salt tolerance to rice under laboratory conditions (see preliminary data). When plants reached maturity, a small subset (a few stems /bunch of a plant) from 10 random plants/plot (a total of 16 plots, Fig. 6A) were surface sterilized using standard protocols (Redman et al., 2002) and assessed for +/- presence of symbiont B. The dry weight biomass (of 20 random plants/plot) was assessed (Fig. 6B) as was the % viability (dead vs. live = brown vs. green plants). Data analyzed by ANOVA to assess significance differences between treatments.

Figure 6A.



N=150 plants/plot. Eliminate plots that are not well colonized and a NS plot that was found to be colonized.

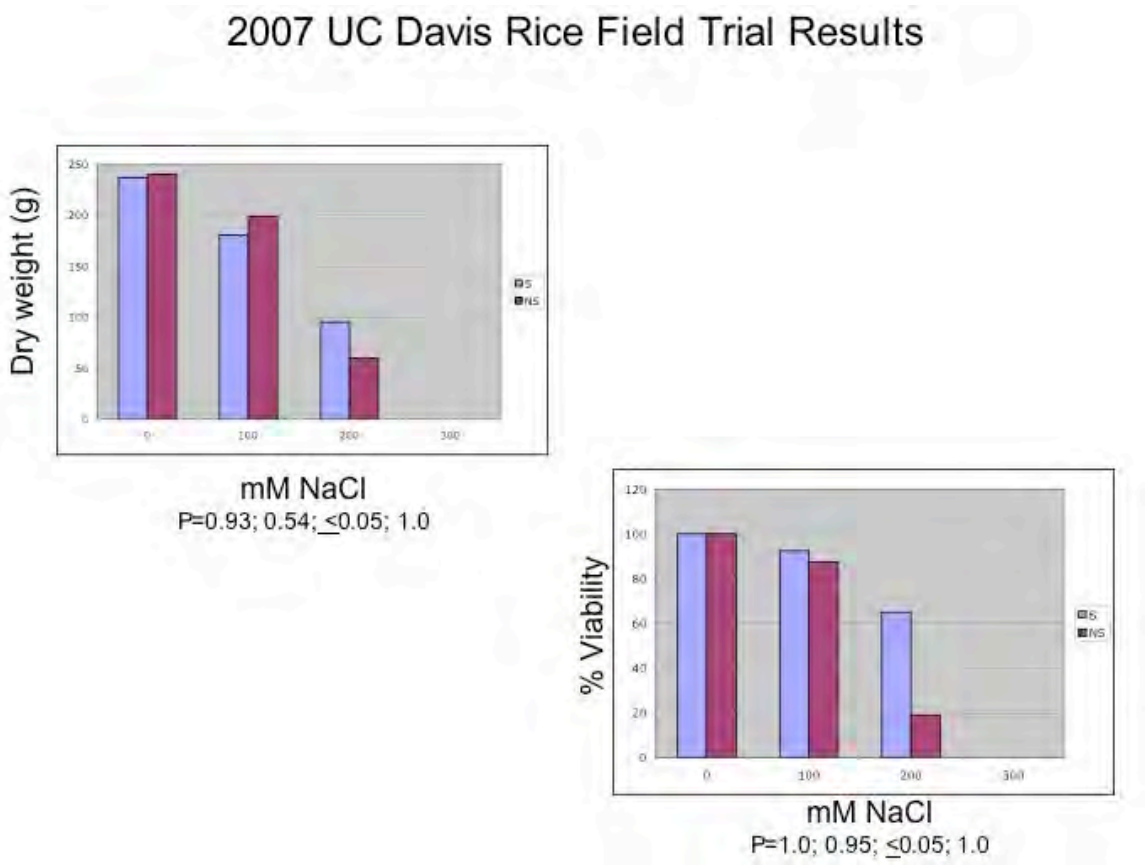


Figure 6B. Dry weight (N=20) and % viability (N=300) of mature M206 plants grown under field conditions in the summer of 2007 at UCD. Symbiotic (S) and non-symbiotic (NS) plants were generated in the greenhouse, planted in the field after 6 weeks, and plants harvested after an additional 2 months. Plants (N=150/treatment; x2 reps = 300 total plants/treatment) were exposed to constant level of salt stress ranging from 0, 100, 200, and 300 mM NaCl. Verification of colonization of S plants and absence of colonization in NS plants was assessed near the end of the growing season. Plots in which S plant were not colonized significantly (<50%), and NS plots that were cross contaminated and found to be colonized, were discounted (x2 S and x1 NS plot). All of the S and NS plants exposed to 300 mM NaCl died. Statistical analysis (ANOVA) revealed there were significant differences in % viability and biomass (P=≤0.10) of plants exposed to 200 mM NaCl, with S plants achieving higher viability and biomass compared to NS plants. No significant differences between treatments were seen at 0 and 100 mM NaCl treatments.

Results of field salt stress studies: At the end of the field season, colonization studies revealed that two of the symbiont B colonized plots were not effectively colonized (less than 50%) and one of the NS plots was colonized with symbiont B (miss labeling or cross contamination event most likely). These 3 plots were therefore eliminated from the statistical analysis (Fig. 6A). Statistical analysis of the remaining plots then revealed that there were statistically significant differences in plant biomass and % viability of plants exposed to 200 mM NaCl, with Symbiotic B plants having approximately 35% higher viability and 60% higher biomass compared to non-symbiotic plants (Fig. 6B). No

significant differences in 0 and 100 mM NaCl treatments for symbiotic and non-symbiotic plants were observed for biomass. Unfortunately, due to the late nature of planting into the field (see list of experimental problems below), no seed yield biomass was possible, but as the parallel greenhouse experiments showed, it is likely that symbiotic plants exposed to 100 and 200 mM NaCl would have out performed non-symbiotic plants. Lastly, 300 mM exposed Field plants all died. This extreme, rapid and continuous exposure of the rice seedlings (unlike the gradual exposure to salt stress imposed in our greenhouse experiments where symbiotic plants survived and out competed non-symbiotic plants) caused the rapid demise to both symbiotic and non-symbiotic treatments. In future, field salt stress experiments of these elevated levels will need to incorporate a gradual gradational exposure to salt as described in the greenhouse studies for optimal success.

Interestingly, field plants (150 plant x2 reps =300 total each for S and NS) in the absence of salt stress (just exposed to water, no salt stress, no extra nitrogen) had notable visual differences in appearance. The S plants were visibly greener, more robust and had a higher number stems/plant compared to NS plants (Fig. 7). It appears as though the symbiotic plants are healthier than their non-symbiotic plants. Future studies looking at the physiological, biochemical, molecular, and nutritional differences in S and NS plants will be addressed to address this phenomenon.

Figure 7. Rice M206 field plants (N=300) in the absence of salt stress (-) Showed notable green color and density differences between symbiotic (S) and non-symbiotic (NS) plants with S plants being more robust, greener, with more branching compared to NS plants.

NS(-) S(-)

Problems that occurred and solutions:

1. Problem: The M206 seeds had many fungi associated with the seed husks that made generation of sterile plants difficult. Solution: Utilizing different chemicals, we have developed an effective surface sterilization protocol to obtain sterile seeds and/or plants that has minimal impact on germination.
2. Problem: Lack of an existing liquid imbibing/fungal colonization protocol. Solution: We have developed an effective imbibing/colonization protocol.
3. Problem: Due to a variety of issues, the PI grossly underestimated the amount of time, effort and funds for plant generation and shipping of plants for field studies. These issues included: seed contamination; low plant germination; lack of an imbibing/colonization protocol during this time; miscalculation of amount of personnel time, effort and funds for supplies, to generate the multiple thousands of symbiotic and sterile non-symbiotic plants by hand. Solution: In future proposals, funds required for the shipping, supply and personnel time will be more accurately assessed. In addition, utilization of the effective surface sterilization and imbibing/colonization procedure developed will facilitate the researcher to effectively mass produced plant seeds, seedlings or plants as required for field trials.
4. Problem: 2007 Field trials of constant and elevated levels of salt exposure to plants resulted in less than optimal results. Solution: A gradual increase in salt exposure to plants will be used in future field trials of this nature. In addition, salinity levels that more accurately reflect the needs and problems of the California rice growers will be the focus. Levels in the range 2 dS/m –18 dS/m (approximately 20-200 mM NaCl) are of more concern where our field trial plants were exposed elevated levels ranging from 100-300 mM NaCl levels.

Overview of Meeting Proposal Goals:

Objective 1 completed. Pilot experiment were conducted and an optimal fungal endophyte candidate identified that imparted salt stress tolerance and enhanced plant and seed biomass.

Objective 2 completed. An effective surface sterilization and imbibing/colonization protocol was developed that collectively will be able to generate sterile non-symbiotic plants and endophyte colonized plants for future larger scaled field trials.

Objective 3 completed. Greenhouse and field trials collectively indicated that symbiotic plants out competed non-symbiotic plants with symbiotic plants showing higher %viability, biomass and seed yield in the presence of salt stress.

References:

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