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Technical Report. DOE DEFG0395ER20201 The rhizosphere association of the nitrogen fixing bacterial species *Azotobacter paspali* with the tropical grass *Paspalum notatum*: Specificity of colonization and contribution to plant nutrition (\$142,500) 7/1/95 - 2/14/97

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The significance of the rhizosphere association of the nitrogen fixing bacteria *Azotobacter paspali* and the sub-tropical grass *Paspalum notatum* in supplying nitrogen to the grass.

The nitrogen fixing bacterium *Azotobacter paspali* was first isolated from the roots of the sub-tropical grass, *Paspalum notatum*, and added to the genus in 1996, by Dr. J. Döbereiner (Brazil). It is mentioned that this root associating bacteria shows remarkable signs of host-plant specificity to one eco-type of this grass, namely Batatais from which it has been isolated. This specificity is rare in non-symbiotic plant-microbe interactions so far identified. It is considered that the grass is benefited by biologically fixed N supplied by *Azotobacter paspali*, although this has not so far been proven. Yet, there is a great importance in studying this association as this presents an ideal model system to investigate how a monocot might associate with a nitrogen fixing rhizosphere bacterium. The findings may in the future, lead to develop important monocot crops that could benefit from biologically fixed nitrogen.

The main objective of this project was to study whether the rhizosphere associative *Azotobacter paspali* provides biologically fixed N to the grass to support its growth. This study was initiated in December 1995. Prior to initiation of experiments, following preliminary steps were taken to set up methods as follows:

Obtaining sterile seedlings.

P. notatum Batatais seeds (harvested from the field and stored at 4°C) were subjected to hot water (54°C) and conc. H₂SO₄ acid scarification to reduce the hard seed coat, and surface-sterilized by treating with 70% ethanol and 5-10% bleach followed by several washings with double distilled sterile water. Seeds were plated on MX- medium and incubated at 30°C for germination. Six to 14 days old seedlings were used in experiments. Seedlings developed this way were found to be sterile. Sometimes the seedlings were transferred from MX- medium to 4mL test tubes containing sterile MS nutrient solution for further growth prior to using in the experiments. This was done in order to avoid the spread of fungal infestations that sometimes occurred in the agar medium to seedlings, and to allow a better root development before planting.

Selection of plant growth medium

In order to find a suitable growth medium that can be sterilized as well as that does not contain significant amounts of N and other nutrients, vermiculite, sand (#30) and a mixture of both (1:1) were tested in a replicated experiment. Although the mixture of sand and vermiculite proved to be the best medium that gave significantly higher growth in *Paspalum notatum* Batatais after 40 days, recovery of the root system without damage and loss was found difficult with vermiculite, as roots penetrated vermiculite particles. Thus, it was decided to use sand with a slightly large grain size (#20) as the medium in all experiments.

Containers and incubation of experiments

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Eight oz. styrofoam cups were selected to prepare experimental 'pots' or containers, as these were autoclavable. Three cups were used/container. The base of one was removed and placed inside another which had a hole pierced in the center of its base. A cotton plug was passed through the hole and the 2 cups were placed inside an autoclavable (250mL) plastic beaker. Already autoclaved (for 40 minutes at 121°C) sand (~300g) was placed inside the styrofoam cup set-up, covered with aluminum foil and were autoclaved for 15 minutes. A styrofoam ring cut out of the top part of another cup was passed around the outside of the two sterilized cups to hold them lifted up on the beaker, allowing some space at the bottom of the beaker. This space was filled with the sterile nutrient solution/water. MS nutrient solution (-N) was used at the rate 175mL/container, before planting. N was added at required concentrations using NH_4NO_3 in 3 splits 4 days apart. In experiments each container had 1-3 plants. A minimum of 3 replicates/treatment were used. Experiments were incubated at room temperature, under fluorescent lights (12 hr light, 12 hr dark).

Experiments were conducted to test the following: N nutrition of the grass, effects of inoculation on the growth of the grass, survival of the inoculated bacteria on the roots.

N nutrition of *Paspalum notatum*.

It was important to know the 'critical' N level, or the lowest level of N that can be added to the grass that will prevent it from being deficient in N. When the seedlings are inoculated the bacteria will depend on the carbon supplied by the roots. If the plant is deficient in N, this supply will be affected and the establishment of the inoculum on the roots will fail. On the otherhand, addition of higher levels of N will prevent the detection of any growth due to biologically fixed N.

Three replicated experiments were conducted to test the critical N level for *P. notatum* Batatais, using a range of N levels from zero to 6.75 mM N. The effect of added N was visible from the 12th day after planting. The growth increased linearly with every increment of N up to 3-4 mM N. Above this, a decrease in growth indicated the adverse effects of high N levels. Below 0.50 mM N, the plants showed clear N deficiency symptoms with stunted growth, yellowing to severe browning of leaf blades. When N was not added the growth was completely affected with no significant growth visible. From 0.50 - 1.00 mM N, yellowing of leaves was visible but not severe. As the growth was severely affected below 0.50 mM N, the lowest critical N level for the grass may fall within 0.50-1.00 mM N. These experiments indicated that *P. notatum* is a very sensitive plant to N.

Inoculation with *Azotobacter paspali* (strains Ap 11562 and Ap 12095)

Two strains, Ap 11562 and Ap 12095 which have been stored in glycerol in the freezer for many years were used initially. Both were first grown on BSN medium and transferred to BS. After an initial slow growth both began to grow normally in BS after few repeated streakings on BS plates.

Bacterial cultures multiplied in BSN (sucrose) for 24 hr and transferred to BS medium (sucrose) for 24 hr. were washed in p-buffer and were suspended in p-buffer to use as the inoculum. A serial dilution and a plate count were done to find the cell concentration in the inoculum before inoculation. Inoculation was done by immersing the root of the seedlings in the bacterial culture for 3-4 hr. Roots were washed before planting and a 0.50-1.00 mL of

the inoculum was also added to the sand medium at planting. These inoculation experiments were carried out in combination with treatments with and without sucrose and N to test the combined effects of these on the growth. Repeated experiments failed to show any significant effect due to inoculation. Sucrose alone did not show any growth stimulation. Sand grains from the growth medium and root pieces taken at the end of the experiments (35-40 days) when cultured in BS agar medium, inoculated treatments showed some colonies similar to laboratory cultures of *Azotobacter paspali* (creamy to off-white, dense). On semi-solid BS agar, these showed C_2H_2 reduction. Colonies that grew from not inoculated treatments were different in appearance (transparent to white and slimy) and did not indicate C_2H_2 reduction. Although there was no effect on inoculation on the grass, the inoculated bacteria species did not survive in the growth medium and retained the ability to reduce C_2H_2 . The root-wash from the inoculated plants (roots were sonicated and shaken in p-buffer) however, when plated on BS medium, did not show colonies similar to *Azotobacter paspali*.

Inoculation with the strain Ap 1

As Ap 11562 and Ap 12095 have been stored for a long time in the freezer, their ability to colonize roots and fix N may have been affected. Therefore it was decided to use a recently isolated *Azotobacter paspali* strain (Ap1) from soil (isolated and sent by the laboratory of Dr. J. Döbereiner in Brazil).

This strain was first grown on BSN followed by BS medium for 24 hr periods, washed in p-buffer and the final inoculant was made in p-buffer. The cell concentration of the inoculant was determined by serial dilution and plate counts (5×10^5 and 3×10^6). The effect of inoculation was tested with and without N and added sucrose. N effect was dramatic after 3 weeks but no effect of inoculation was observed. Sand grains taken from pots showed colonies similar to *Azotobacter paspali* in BS agar media and these showed C_2H_2 reduction. Few of the containers with whole plants when placed in wide-mouth 4L bottles and subjected to C_2H_2 reduction and the results were negative. After 40 days, plant weight determinations showed no significant effect of inoculation though N effect was significant. The new isolate (Ap1) too showed viability in the sand and retained the ability to reduce C_2H_2 to the end of the experiment.

******At the beginning of this project, there was a stock of *Paspalum notatum* seeds (300-400 g) stored in the cold room (4 °C) which were harvested from the field and the green-house (1994 - 1995). At the beginning of the project, seeds showed 85-90% germination. With time however, the germination % went down to ~ 25-30%, and further, a fungal infestation started to occur in the germinating plates. As the seed quality reduced with time it was found difficult to obtain the required number of good seedlings for a planned experiment. Sometimes, the number of replicates had to be reduced. Further, the recovery after planting of seedlings produced from aging seeds became low. An effort was taken to find good quality *Paspalum notatum* seeds. Dr. Glenn Burton, USDA/ARS, Georgia kindly sent ~200 g of *Paspalum notatum* var. Argentine. As this is also a tetraploid (Dr. Glenn Burton, personal communication) as in the case of the Batatais variety, it was decided to test this in inoculation experiments.

Inoculation of *P. notatum* Argentine with strain, Ap 12095.

An inoculation in p-buffer was prepared using Ap 12095 strain as described above. Four weeks old *P. notatum* Argentine seedling roots were immersed in a BS solution (0.2% sucrose) and the inoculum (7×10^7 cfu/mL) was added to the solution and incubated for 4

days. The BS solution and the inoculum was replenished and re-incubated for another 4 days before planting with 0.15 and 0.50 mM N levels. Experiment was carried out for a longer period, and after 12 weeks a clear difference in growth between the inoculated and not inoculated plants was observed at both N levels. Inoculated treatments showed a better growth with more greener and somewhat larger plants. Dry weights of aboveground plant parts, fresh and dry weights of roots showed a significantly better growth in inoculated plants at lower N level (0.15 mM N). A serial dilution and a plate count of the root-wash from inoculated treatments showed colonies similar to laboratory cultures of Ap 12095. These (both N levels) showed C_2H_2 reduction. The number of colonies/g roots however was very low. The same from surface sterilized and macerated roots too showed many colonies but were not similar to Ap 12095 laboratory cultures and no C_2H_2 reduction was detected. Sand from inoculated treatments showed colonies similar to laboratory Ap 12095 cultures and indicated C_2H_2 reduction. The strain Ap 12095 seem to have survived in the sand and the root surface for 14 weeks after inoculation although the cell number/g roots was low. Further it has retained the C_2H_2 reduction ability. The better growth observed specifically in the roots of *Paspalum* may have been due to fixed N or growth stimulating effects of the inoculated Ap 12095 strain.

Survival of the inoculum in roots of *Paspalum notatum* Batatais

Many attempts at inoculation of *Paspalum notatum* Batatais during this study failed to show any positive effects on the growth of the grass. A study was undertaken to test the survival of the inoculum on the roots of *Paspalum notatum* Batatais following inoculation. This was done by comparing with other known root colonizing bacteria and a free-living bacteria as follows: Ap1, Ap 12095, Ap 11562, *Pseudomonas aureofaciens* (a root colonizer) and *A. vinelandii* (free living). All were grown in BSN and transferred to BS except *Pseudomonas* (in BSN) and multiplied for 48 hr. A serial dilution and plate counts determined the concentration of each (4×10^7 and 7×10^8 cfu/mL). Twelve days old seedling roots were immersed in the inoculum for 4 hr and planted in the sand medium. One mL of the appropriate bacterial culture was also added to the sand before planting. N was added at 1 mM N/container. Sampling and estimation of the number of bacteria/g roots was done every 5 - 6 days. A p-buffer containing the separated roots was sonicated and vortexed (1 minute), and subjected to a serial dilution and plate counts. All strains survived on the roots (for 44 days) but the numbers fluctuated over time. Except for Ap1 (which showed an increase), all other strains showed a steady decline with time from the initial numbers observed after 5 days of inoculation. As the inoculation rate was high in all strains, the decline may be due to lack of sufficient carbon supply from the roots. Soon after planting, roots may not have supplied to the demand of the inoculated bacteria.

Above experiment was repeated using all the conditions described basically unchanged. The inoculation of all strains was $\sim 1 \times 10^8$ cfu/mL. Sampling and estimation of cells/g root was however, done more frequently soon after inoculation, starting from 12 hours. Further, sucrose-C (100 ppm C) was added to all treatments at the time of planting. The cells/g roots varied with time in all strains and they survived without a significant decline in number as previously observed, until the end of the experiment at 35 days. The experiment was not carried out for a further period of time due lack of sufficient number of plants in some treatments. The inoculum survived on the roots for 35-40 days after inoculation and it seem

beneficial to add carbon at the time of inoculation.

Overall observations

Inoculation attempts of *Palpalum notatum* Batatais failed in all experiments, although the inoculated bacteria was viable and retained C_2H_2 reduction ability after inoculation. Further, the inoculated bacteria survived in the roots for over 6 weeks. It is not clear if there was a condition that was not conducive for effective colonization and N fixation in these experiments. The strains used may not have been sufficiently effective in colonization and/or N fixation in the roots. As described by Boddy et al. (1983) using N^{15} dilution technique, this system has so far shown to provide 11% of the N in the grass by fixation. As we were unable to have large number of replicates/treatment, if the N obtained by N fixation was small, it may not have been detected by the experiment. One reason for the positive growth effects shown by inoculation on *P. notatum* Argentine may have been due to the inoculation method used. The roots were allowed to grow for 4 weeks and were immersed in the inoculum for 8 days, which may have caused more cells to colonize the roots. The positive effects observed may also have been due to growth regulators produced by the inoculated bacteria.

A paper describing this work is ready for submission to the journal Plant and Soil.