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Honors Thesis

Effect of Velvetleaf Root Exudates on Indole-3-Acetic Acid Production by *Rhizobium rubi* AT3-4RS/6

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2 April 2012

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Primary Advisor Signature: _____

Department: _____

Abstract

Rhizobium rubi AT3-4RS/6 was isolated from the Velvetleaf rhizosphere and is being tested as a potential biological control of Velvetleaf. The purpose of this research is to test the levels of indole-3-acetic acid (IAA) produced by *Rhizobium rubi* AT3-4RS/6 after exposure to and incubation in Velvetleaf root exudates. IAA production has been found in other rhizobacteria that are deleterious to weed growth. Velvetleaf seeds were sprouted and the roots suspended in a sterile mineral solution, which was then recovered and inoculated with *Rhizobium rubi*. Levels of IAA and numbers of viable bacterial colonies were then determined. The average level of IAA was .042 $\mu\text{g}/10^6$ CFUs in the presence of root exudates and .1196 $\mu\text{g}/10^6$ CFUs in the absence of root exudates. The average number of viable bacterial colonies in the presence and absence of root exudates were 32.69 and 9.49 CFUs ($\times 10^6$)/ml, respectively. Results show that the presence of Velvetleaf root exudates increased bacterial growth and reduced IAA production significantly ($p < .05$).

Introduction

The Velvetleaf weed (*Abutilon theophrasti*), originally from southern Asia, was originally thought to be a good potential fiber crop. However, it has become a problematic invader for many agricultural crops such as corn, soybeans, cotton and other such crops. Not only is its large size (growing to at least 5 feet) a problem by blocking out sunlight for plants, but it is also a harbor for diseases and pests that attack the very crops it inhabits. With seeds remaining viable for 50 years in soil, it is important that the growth of Velvetleaf be stopped early in its life (Anonymous 2011). With many plants developing tolerance to herbicides, other sources of weed control are being sought out, which is where naturally occurring plant-bacteria interactions come in (Tranel and Wright 2002).

Plant-bacteria interactions play an important role in the growth of plants. One such interaction is that of *Rhizobium rubi* AT3-4R/6 and the Velvetleaf weed. The potential for this bacterium to be harmful to Velvetleaf plants lies in the possibility of its production of indole-3-acetic acid (IAA), which has been found to be produced by 22.8% of deleterious bacteria tested (Sarwar and Kremer 1995). Depending on the concentration of IAA present (which is dependent on the amount of bacteria surrounding the plant roots), the effects can range from beneficial to harmful (Lambrecht et al 2000, Parsello-Cartieaux et al 2003). There are many pathways found that bacteria use to produce IAA, and though one specific one cannot be pinpointed for each bacteria, the majority of the pathways use tryptophan as a precursor to IAA production (Spaepen et al 2007). Previous research done by Brubaker and Zdor (2009) showed that the ability of *R. rubi* to reduce the growth of Velvetleaf occurred under soil conditions that promoted IAA production. The purpose of this research was to determine whether or not root exudates can affect bacterial growth and IAA production.

In order to grow Velvetleaf and isolate its root exudates in a sterile environment, hydroponics were used. This allowed for the fluid containing root exudates to be inoculated with *R. rubi* and subsequently tested for the production of IAA without any other factors involved. Previous work done by Simons et al (1997) used hydroponics to grow tomato plants in order to analyze root exudates and culture plant roots with rhizobacteria.

Methodology

Two complete, independent trials were run with data collection from both.

Standard procedures were used in making 1% water agar plates, tryptic soy agar (TSA) plates and Jensen's solution. Jensen's solution was made of 1g CaHPO₄, 0.2g K₂HPO₄, 0.2g MgSO₄·7H₂O, 0.2g NaCl, 0.1g FeCl₃, and 0.1g NH₄NO₃ per liter of distilled water. 500ml of Jensen's solution was made and pre-filtered with No. 1 filter paper and then sterile filtered (.2 micron pore size) and stored at 4 C.

Magenta containers were obtained and aluminum wire mesh was cut and fit to each container to make a platform for the velvetleaf seeds to grow in (Simons et al 1997, Appendix A). Strips of wire mesh that measured 25cm by 6cm were cut and then bent over a metal edge 9 cm from the end. Measuring 6cm from the first bend, the mesh was bent again and placed into the magenta container, folding the ends over the top edge and placing the lid on the container. A total of six containers were prepared and autoclaved for 25 min.

In order to surface disinfect the seeds about 3g of velvetleaf seeds were weighed and transferred to a beaker with 50% bleach solution and 3 drops of Liquinox soap which was placed in a hot water bath (65 C) for 10 minutes with occasional stirring. Then the liquid was poured off and the seeds were rinsed one time with sterile water. Another 100ml 50% bleach solution was added to the seeds along with a magnetic stir rod and 3 drops of Liquinox and the covered beaker was placed on a stirrer for 30min. The liquid was then poured off and the seeds rinsed 4 times with sterile water. Under a sterile hood, the seeds were spread out onto three 1% water agar plates and placed in a 28 C incubator for 24-28 hours.

The seedlings were aseptically placed with their roots sticking through the wire mesh as evenly spaced as possible into three magenta containers. Approximately 40-60 seeds were planted in each Magenta container and the same numbers of seeds were planted in the three containers within each trial. Three control containers were left without seeds. Then 70ml of Jensen's solution were pipetted into all 6 Magenta containers, letting the fluid flow down the side of the container to avoid contact with the seeds. The containers were then placed under a growth light (12 hrs of light) and grown for 7 days at 23 C.

A streak TSA plate of *R. rubi* was prepared and incubated for two days prior to the completion of the seedling growth.

A bacterial suspension was then prepared by mixing .5ml of Jensen's solution in a microcentrifuge tube with 1 colony of bacteria and centrifuged for 5 minutes at top speed. The supernatant was then poured off and then suspended a second time in .5ml of Jensen's. The supernatant was then removed and the bacteria suspended in 750µl of Jensen's. This inoculum was then serial diluted and .1ml of dilutions 3, 4, and 5 were spread plated in triplicate on TSA plates and incubated for 48 hours after which the number of viable cells in the inoculum were determined.

On day 7, the wire mesh was removed from each container and approximately 12ml of fluid was poured into a syringe and filtered through a .2µm syringe filter into a sterile test tube.

Then 10ml of fluid was transferred to each of two sterile glass test tubes and inoculated with 10 μ l (trial 1: 10 μ l= 8.64x10⁶ CFU *R. rubi*, trial 2: 10 μ l= 3.63x10⁵ CFU *R. rubi*) of bacterial suspension. The labeled test tubes were then placed in a shaker bath with 28 C water and incubated for 6 days.

For each of the Magenta containers, 10 μ l of from each pre- and post-filtered Jensen's solution was deposited onto TSA plates and left to soak in, then placed in an incubator for 48 hours to verify sterility.

After incubation, the fluid from each test tube was serial diluted 4 times and .1ml of the 2nd, 3rd and 4th dilutions was spread plated in triplicate on TSA plates. The plates were incubated for 48 hours and the number of colonies determined. The remaining Jensen's solution from the Magenta containers was poured into clean plastic test tubes and placed in the freezer.

A standard curve for IAA levels was determined using concentrations of .2, .5, 1, 5, 10, and 20 μ g of IAA/ml of Jensen's solution. A Salkowski test was run on thawed Jensen's solution from each container in duplicate and IAA levels were quantified by spectrophotometric absorption using standard procedure.

In order to determine dry weight of the roots growing in the solution, three aluminum squares were pre-weighed, then the roots from the three containers were cut off onto the aluminum and placed in an oven (80 C) and left for at least 48 hours. The aluminum with the dry roots was then weighed and the weight of the roots determined by taking the pre- and post-weight differences. Average root weight per trial was then determined.

Results

Figure 1.1

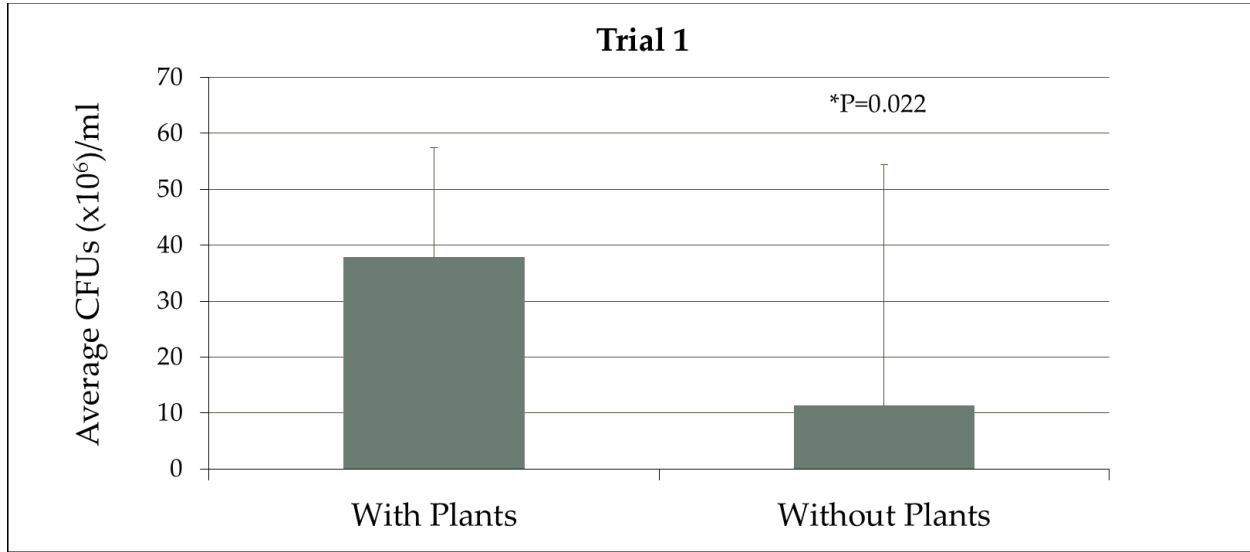


Figure 1.2

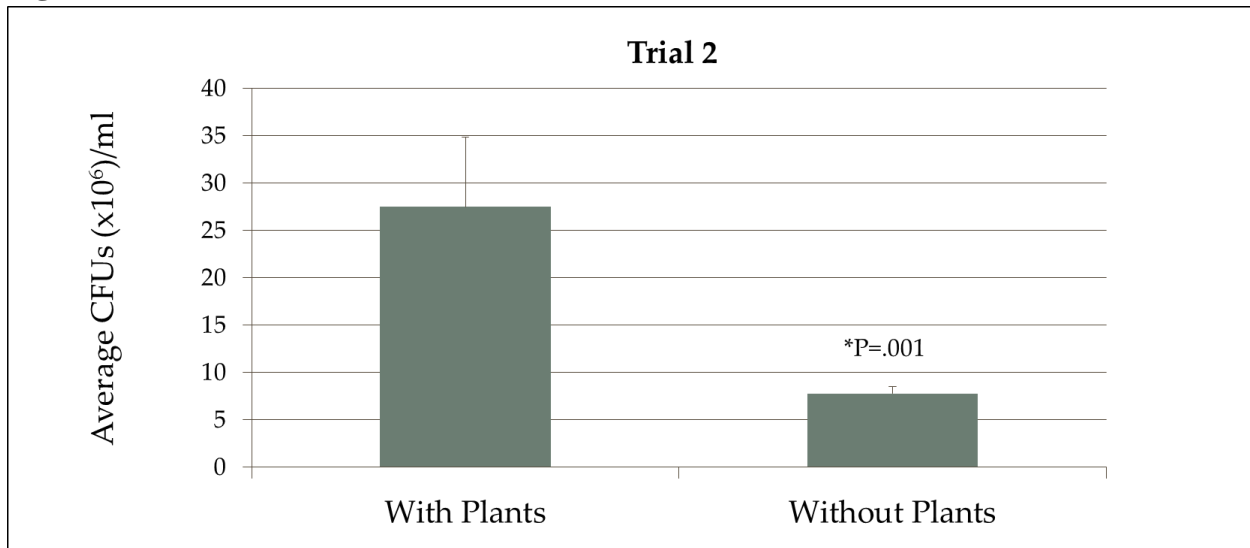


Figure 1.1 and 1.2: Viable *Rhizobium rubi* populations in Jensen's solution in the presence and absence of Velvetleaf root exudates. Each bar is the average of 18 determinations. The initial CFU/ml in Jensen's solution was 8.64×10^4 and 3.63×10^3 for trials 1 and 2, respectively. Error bars represent 1 standard deviation. The number of bacteria that grew in the presence of root exudates was significantly higher ($p < .05$) than those not exposed to root exudates.

Figure 2.1

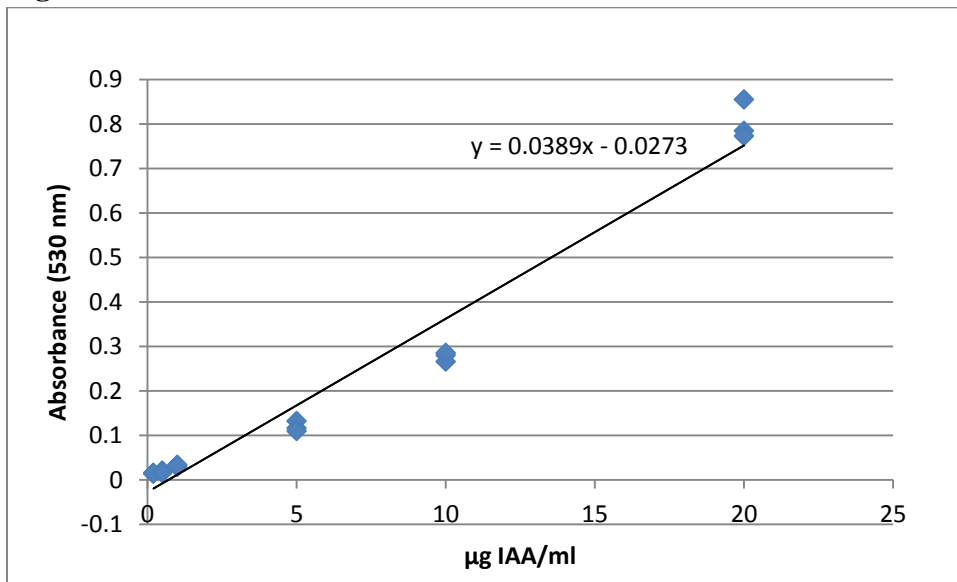


Figure 2.1: Standard curve for known levels of IAA using Salkowski reagent.

Figure 2.2

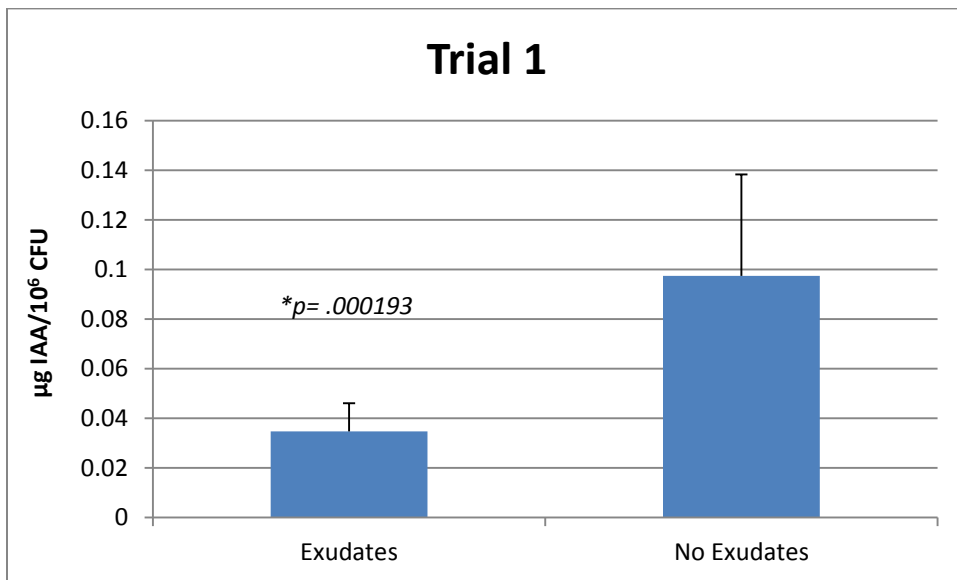


Figure 2.3

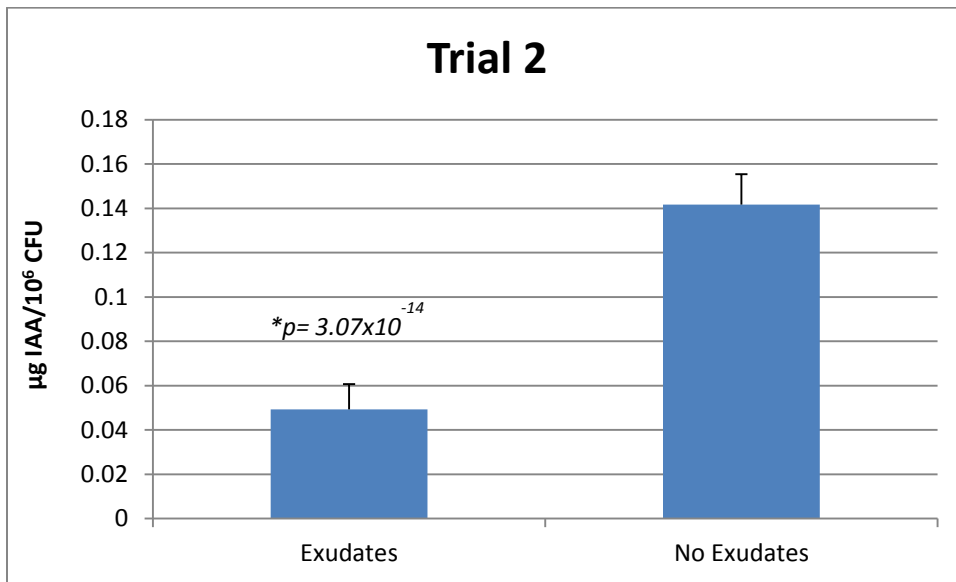


Figure 2.2 and 2.3: IAA production by *Rhizobium rubi* in the presence and absence of Velvetleaf root exudates. Each bar is the average of 12 determinations. Error bars represent 1 standard deviation. The level of IAA produced by bacteria grown in the presence of root exudates was significantly lower ($p < .05$) than those that were not exposed to root exudates.

The average number of bacterial colonies that grew in the presence and absence of root exudates for trial 1 was 37.88 and 11.22 CFUs ($\times 10^6$)/ml Jensen's solution, respectively (Fig 1.1). For trial 2, the average was 27.5 in the presence of and 7.76 CFUs ($\times 10^6$)/ml in the absence of root exudates (Fig 1.2). Initial levels of *R. rubi* cells/ml Jensen's solution were 8.64×10^4 and 3.63×10^3 for trials 1 and 2, respectively. For both trials there was a significant difference ($p < .05$) between the number of viable colonies in the presence of root exudates than in the absence of them (trial 1: $p = .023$ and trial 2: $p = .001$).

Levels of IAA were determined using an experimentally determined standard curve ($y = 0.0389x - 0.0273$) and found to be, on average, .0347 and .0493 $\mu\text{g}/10^6$ CFUs for trials 1 and 2, respectively, in the presence of root exudates (Fig 2.1, 2.2, 2.3). In the absence of root exudates, the average $\mu\text{g}/10^6$ CFUs for trials 1 and 2, respectively, were .0974 and .142 (Fig 2.2, 2.3). There was a significant difference ($p < .05$) between the level of IAA produced in the absence and presence of root exudates (trial 1: $p = 1.93 \times 10^{-4}$ and trial 2: $p = 3.07 \times 10^{-14}$).

Average weight of dry roots for trial 1 was found to be .0665g and for trial 2, .0303g.

Conclusion

In a sterile, hydroponic growth environment, it was found that the presence of Velvetleaf root exudates stimulates *R. rubi* growth and reduces the production of IAA. *R. rubi* populations increased more than 100 fold during incubation in Jensen's solution showing that this mineral solution contained adequate nutrients to support growth. However, velvetleaf roots appear to produce substances that resulted in higher bacterial levels. Work done by Simons et al (1997) found that bacterial colonization of plants roots was dependent on the presence of amino acids that the plant produces. One important amino acid to the production of IAA is tryptophan, which is a major precursor in IAA synthesis (Spaepen et al 2007). It has been found in plant root exudates and has also been found to increase microbial IAA yield in vitro (Tsavkelova et al 2007). These do not match results found in this experiment since the amount of IAA produced by each bacterium was significantly lower when exposed to root exudates, suggesting that Velvetleaf root exudates decrease bacterial production of IAA. A possible explanation could be that one or more substances that Velvetleaf seedlings produce have an adverse effect on *R. rubi*'s ability to produce IAA. Anthranilate (a precursor to tryptophan) has been found to reduce IAA production, but can also be regulated by tryptophan, keeping IAA levels in balance (Spaepen et al 2007). If Velvetleaf were to produce more anthranilate than tryptophan, for instance, IAA production could be decreased by this mechanism. Future beneficial research would be to test for the presence of amino acids and more specifically, tryptophan along with anthranilate in the root exudates of Velvetleaf plants.

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Appendix A



Magenta container with Velvetleaf seedlings after 7 days of growth in Jensen's solution.