ENVIROTECH

EVALUATION STUDY

TINA RODRIGUES, MICROBIOLOGIST B.S.
RAMSENA DADESHO B.S.

The Efficacy of Peroxyacetic Acid (PAA) (PerasanTM) Against Aerobic Bacteria and Coliforms on Various Types of Germinated Seeds and During the Process of Seed Germination

Background

The contamination of germinated, organic seeds and beans by various types of bacteria, such as coliforms, can have a devastating impact upon the industry that produces and distributes these products to grocers and health food stores. The seeds undergo a two day germination process in which they are subjected to a dark, moist, warm (80°F) environment which subsequently allows the seeds to sprout. It is well known that under these conditions, exponential growth of the microorganisms present on the seeds is likely to occur. A processor of organic seeds sought to improve the safety and quality of their products and wanted to know whether treatment of their products with a solution of peroxyacetic acid (from BioSide HS 15%) prior to the germination process would be an effective means of eradicating the majority of the microorganisms before they have the opportunity to multiply exponentially during germination. Enviro Tech Chemical Services offered to provide microbiological testing on several types of seeds, providing they send samples of these seeds that have not been chemically treated. This study was conducted in two separate experiments. Experiment 1 was performed to determine the efficacy of peroxyacetic acid (PAA) against aerobic bacteria and coliforms on germinated seeds that had been dried and packaged ready for use. Experiment 2 consisted of seeds that had not yet been germinated that were subjected to a six hour PAA treatment followed by a two day germination. Thereafter, the efficacy of PAA against aerobic bacteria and coliforms was measured.

Methods

EXPERIMENT 1

Three plastic bags of germinated seeds that had not been exposed to chemical treatment were received at Enviro Tech Chemical Services on the afternoon of December 8, 2009. They were immediately placed in the refrigerator and stored until the start of testing the following day. The plastic bags that contained the germinated seeds were labeled as follows:

1) Organic Sprouted Green Lentils
2) Organic Sprouted Mung Beans
3) Organic Germinated Brown Rice

To simulate a short peroxyacetic acid (PAA) rinse, test and control solutions were introduced to new zip- lock bags containing the germinated seeds. The bags were sealed and gently tumbled for two minutes to dislodge any microorganisms present, after which the rinse solutions were enumerated for aerobic bacteria and total coliforms. The test solutions were prepared using hard water (city water at 180 ppm hardness). Each test group is reported below:

a) 100 g organic sprouted green lentils in 250 ml of hard water to serve as the control.
b) 100 g organic sprouted green lentils in 250 ml of 80 ppm PAA prepared in hard water.
c) 100 g organic sprouted mung beans in 250 ml of hard water to serve as the control.
d) 100 g organic sprouted mung beans in 250 ml of 80 ppm PAA prepared in hard water.
e) 100 g organic germinated brown rice in 250 ml of hard water to serve as the control.
f) 100 g organic germinated brown rice in 250 ml of 80 ppm PAA prepared in hard water.

The PAA used to prepare the test solutions were made from PerasanTM that measured 22.5% Hydrogen Peroxide and 15.22% PAA.

After each zip-lock bag was tumbled in the test solution for two minutes, the rinse water was plated on 3M Aerobic Petrifilm Plates and 3M Total Coliform Petrifilm Plates. The 3M Aerobic Plates were incubated at 32°C for 48 hours and the 3M Total Coliform Plates were incubated at the same temperature for 24 hours, after which the plates were enumerated.

EXPERIMENT 2:

After obtaining results and discussing the data from Experiment 1, it was decided that Enviro Tech Chemical Services continue this seed study by executing a second experiment which differs from the first because it includes the whole germination process. This scenario involves test conditions similar to those in practice by facilities that process and germinate seeds. Subsequently, four plastic bags of seeds that had not been germinated nor exposed to chemical treatment were received at Enviro Tech Chemical Services on December 14, 2009. They were immediately placed in the refrigerator and stored until the start of testing two days later. The plastic bags that contained the seeds were labeled as follows:

1) Organic Green Lentils
2) Organic Mung Beans
3) Organic Brown Rice
4) Organic Quinoa

Each test solution was made with Modesto city water immediately prior to use. 100 g of seeds were placed in each of the 1 quart storage containers. 300 g of each test solution was placed in the bin to accompany the seeds. The seeds were allowed to sit in the solution for six hours at room temperature (approximately 70° F), gently agitating them every 30 minutes. See Images 1-4. All Page 3 of 16 3 containers were covered using aluminum foil to prevent degradation of the active ingredients by UV light.

IN SUMMARY:

a) 100 g organic green lentils in 300 ml of hard water to serve as the control.
b) 100 g organic green lentils in 300 ml of 80 ppm PAA prepared in hard water.
c) 100 g organic mung beans in 300 ml of hard water to serve as the control.
d) 100 g organic mung beans in 300 ml of 80 ppm PAA prepared in hard water.
e) 100 g organic brown rice in 300 ml of hard water to serve as the control.
f) 100 g organic brown rice in 300 ml of 80 ppm PAA prepared in hard water.
g) 100 g organic quinoa in 300 ml of hard water to serve as the control.
h) 100 g organic quinoa in 300 ml of 80 ppm PAA prepared in hard water.

The PAA used to prepare the test solutions were made from PerasanTM that measured 22.4% Hydrogen Peroxide and 15.21% PAA.

The PAA concentration in the test solutions was measured every hour by using a HACH DR/700 Colorimeter and HACH 10 mL Total Chlorine pillow packets. The results were then multiplied by the dilution used and 1.07 (the molecular weight difference between chlorine and PAA). There was some demand reactions which caused degradation of the PAA solutions. Therefore, after the second and fourth hour, the PAA solutions were adjusted back up to a nominal 80 ppm. After the seeds soaked in the solutions for six hours, the solutions were plated on 3M Aerobic Petrifilm Plates and 3M Total Coliform Petrifilm Plates. The 3M Aerobic Plates were incubated at 32° C for 48 hours and the 3M Total Coliform Plated were incubated at the same temperature for 24 hours, after which the plates were enumerated.

IMAGE 1: Green lentils (control solution on left, PAA solution on right)

IMAGE 2: Mung beans (control solution on left, PAA solution on right)

IMAGE 3: Brown rice (control solution on left and PAA solution on right)

IMAGE 4: Quinoa (control solution on left, PAA solution on right)

Each set of seeds were then drained off and placed on clean paper towels, wrapped in terry cloth towels and placed in an 80° F incubator for two days which allowed them to germinate. After two days, the germinated seeds were removed and tumbled in 300 g of city water for two minutes to dislodge any bacteria that might have been present. The solutions were plated the same manner as Experiment 1.

Visual observations were noted before performing any microbiological testing. Surprisingly, a large amount of black mold was present on the control samples after the two day germination period. There appeared to be no other visual differences between the control and PAA seeds. These observations can be seen in Images 5-8.

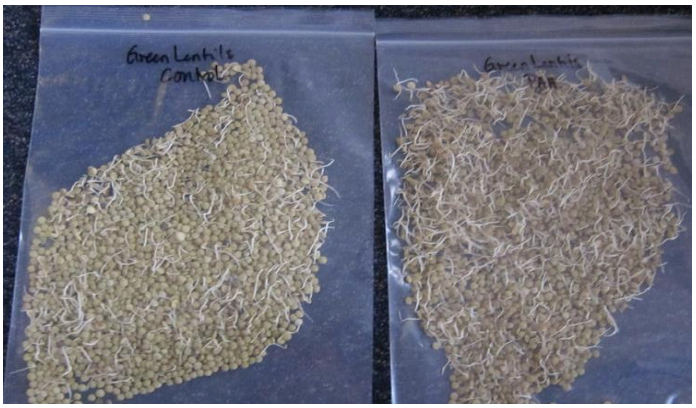


IMAGE 5: Green lentils after germination (control on left and PAA treated on right)

IMAGE 6: Mung beans after germination (control on left and PAA treated on right)

Efficacy of Bioside HS 15% and Reflex Against Planktonic Alicyclobacillus acidoterrestris at Three Different Concentrations

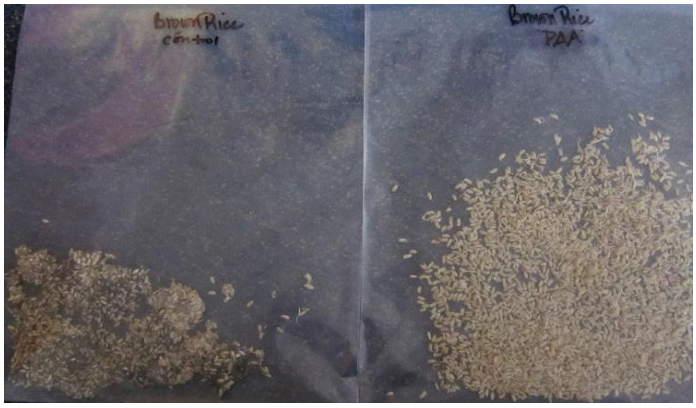


IMAGE 7: Brown rice after germination (control on left and PAA treated on right)

IMAGE 8: Quinoa after germination (PAA treated on right and control on left)

Results and Discussion

Experiment 1:

This experiment was conducted on seed samples that had already been germinated and were provided by a company which processes and germinates these types of organic seeds. The samples used had not undergone any chemical treatment prior to the testing conducted in Experiment 1.

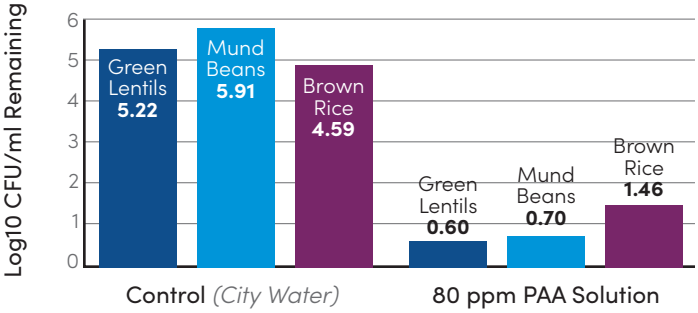
Table 1 and **Figure 1** show the log10 CFU/ml aerobic bacteria remaining on the seeds after gently tumbling for two minutes in either city water or 80 ppm PAA solution made from city water. All three sets of seeds contained log10 CFU/ml between approximately 5–6 of aerobic bacteria when treated with water only. This large bacteria load is most likely from the two day germination process, in which the seeds were kept in a warm, moist environment which is optimal conditions for microbial growth. The PAA treated samples contained log10 between 0.60 – 1.46 CFU/ml aerobic bacteria, which resulted in 99.971% reduction for brown rice and >99.998% reduction in aerobic bacteria for both green lentils and mung beans.

Table 1

EXPERIMENT 1: Aerobic Plate Results On Germinated Seeds After 2 min. Tumble

Description	log10 (average)	log10 reduction	% reduction
Control Green Lentils	5.22	N/A	N/A
PAA Green lentils	0.60	4.62	99.998
Control Mung Beans	5.91	N/A	N/A
PAA Mung Beans	0.70	5.21	99.999
Control Brown Rice	4.99	N/A	N/A
PAA Brown Rice	1.46	3.53	99.971

FIGURE 1: Log10 CFU/ml Aerobic Bacteria Remaining on Germinated Seeds after Two Minute Wash



In Experiment 1, not only was general aerobic bacteria measured but the number of coliforms present were also measured and can be seen in **Table 2.** Although there was not a tremendous amount of coliforms present in the control samples, it is well know that several virulent species of bacteria are coliforms and it doesn't take a significant amount of these coliform cells to cause illness in humans, depending on the species. Fortunately, this table shows that after the two minute PAA wash (at 80 ppm), there were no coliform colonies present, indicating that there was a >99.999% reduction.

Efficacy of Bioside HS 15% and Reflex Against Planktonic Alicyclobacillus acidoterrestris at Three Different Concentrations

Table 2
EXPERIMENT 1: Total Coliform Plate Results On Germinated Seeds After 2 min. Tumble

Description	log10 (average)	log10 reduction	% reduction
Control Green Lentils	1.57	N/A	N/A
PAA Green lentils	0.00	>1.57	>99.999
Control Mung Beans	2.60	N/A	N/A
PAA Mung Beans	0.00	>2.60	>99.999
Control Brown Rice	2.34	N/A	N/A
PAA Brown Rice	0.00	>2.34	>99.999

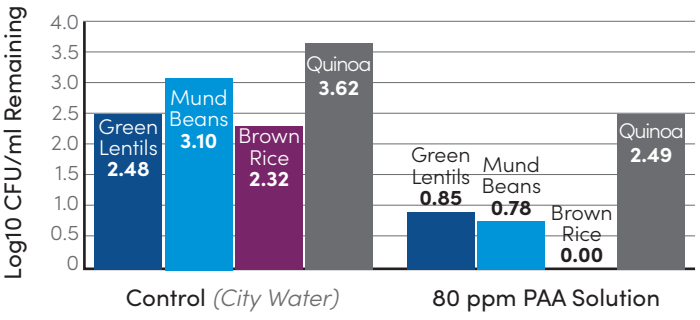
EXPERIMENT 2:
It seemed logical to perform Experiment 2 because this experiment includes the whole germination process, which resembles a typical scenario that may occur at a facility that processes and germinates these types of seeds. This experiment started with four types of untreated seeds that had not been germinated and concluded with these four seed types fully sprouted. The process was conducted in two parts. First, the seeds were submerged in either city water (control) or the 80 ppm PAA solution for six hours to initiate germination. The actual PAA concentration was tracked and adjusted every two hours. The solutions which contained the seeds were then measured for aerobic bacteria and total coliforms. This data can be seen in **Tables 3-4 and Figures 2-3**. After the seeds were drained off, placed in clean towels, and allowed to sprout for two days in an incubator, the now germinated seeds were then measured for aerobic bacteria and total coliforms by tumbling them in water to dislodge any microorganisms and plating the rinse water. The data from this part of the experiment can be seen in **Tables 5-6 and Figure 4**.

The data in **Table 3** show that after the six hour submersion, the control seeds (water only) contained a log10 CFU/ml range of 2.32 (brown rice) and 3.62 (Quinoa) aerobic bacteria. Treating the green lentil seeds with 80 ppm PAA for six hours produced a log10 reduction in aerobic bacteria of 1.63 CFU/ml, corresponding to a 97.682% reduction. The mung bean seeds and quinoa seeds had log10 CFU/ml reductions of 2.32 (99.523%) and 1.13 (92.588%), respectively. The brown rice had the largest reduction, which was >99.999%, meaning that there were no aerobic bacteria present on the 3M Aerobic Petrifilms after PAA treatment. This data shows that PAA at 80 ppm is very effective in eliminating aerobic bacteria.

Table 3
EXPERIMENT 2: Aerobic Plate Results: Seeds After 6 Hour Submersion

Description	log10 (average)	log10 reduction	% reduction
Control Green Lentils	2.48	N/A	N/A
PAA Green lentils	0.85	1.63	97.682
Control Mung Beans	3.10	N/A	N/A
PAA Mung Beans	0.78	2.32	99.523
Control Brown Rice	2.32	N/A	N/A
PAA Brown Rice	0.00	>2.32	>99.999
Control Quinoa	3.62	N/A	N/A
PAA Quinoa	2.49	1.13	92.588

FIGURE 2: Log10 CFU/ml Aerobic Bacteria Remaining on Seeds after Six Hour Submersion



Efficacy of Bioside HS 15% and Reflex Against Planktonic Alicyclobacillus acidoterrestris at Three Different Concentrations

The data demonstrated in **Table 4** show that the seeds submerged in the 80 ppm PAA solution for six hours contained log10 reductions of >99.999% in total coliforms, which means that there were no coliforms present on the 3M Total Coliform Petrifilms used in the microbiological analysis of these test solutions. These results are great; however, there were not very many coliform colonies to begin with.

Table 4
EXPERIMENT 2: Total Coliform Plate Results: Seeds After 6 Hour Submersion

Description	log10 (average)	log10 reduction	% reduction
Control Green Lentils	None present	N/A	N/A
PAA Green lentils	None present	>0.30	>99.999
Control Mung Beans	0.30	N/A	N/A
PAA Mung Beans	0.00	>0.30	>99.999
Control Brown Rice	0.30	N/A	N/A
PAA Brown Rice	0.00	>0.30	>99.999
Control Quinoa	0.30	N/A	N/A
PAA Quinoa	0.00	>0.30	>99.999

Upon completion of the six hour soak, visual observations were made to compare the differences between the seeds that had been submerged in the PAA solution and the seeds that were submerged in city water. There was no difference between in color, smell, or size of seeds that had been in just water or the 80 ppm PAA solution.

The PAA solution with the four seed types had been tracked for six hours to measure the residual ppm PAA left in the solution. There was some degradatio n in PAA throughout the six hour submersion so therefore, from a 1000 ppm PAA stock solution; more was added to each test solution after the second and fourth hour to keep up the PAA concentration. These measurements, along with the two and four hour adjustments, are charted in **Figure 3.**

After two days, the seeds were fully germinated and completely sprouting. It was noted that all the germinated 'control' seeds were heavily contaminated with a black mold. This unexpected observation can be seen in **Images 5-8,** in the **"Methods"** portion of this report. There was very little to no black mold present on the seeds that had been submerged for six hours in the 80 ppm PAA solution two days prior. The images also show that there was no difference in the amount of sprouting or the size of each sprout when comparing the control and PAA treated seeds.

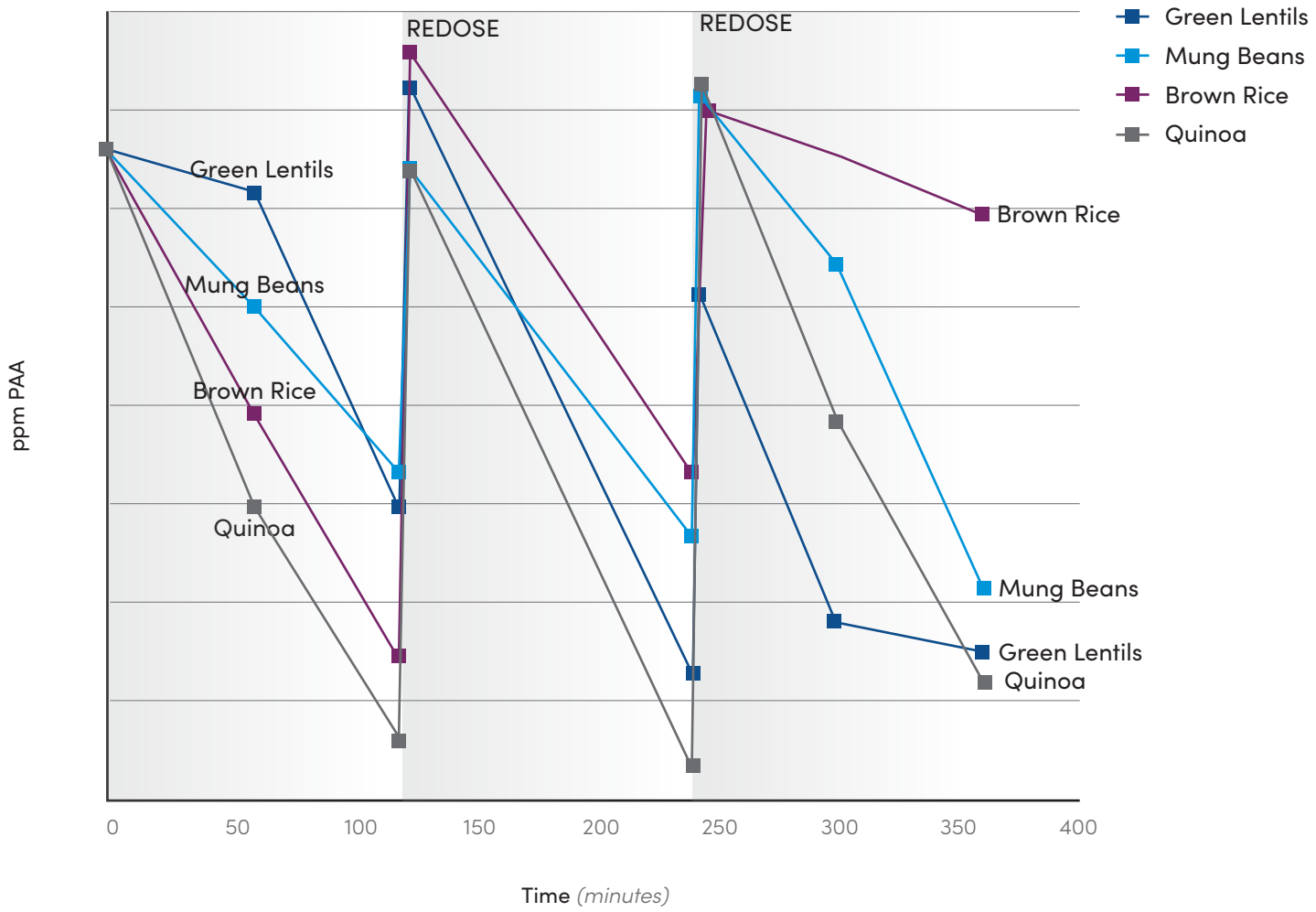
The data in **Table 5** show that the two day germination period provided optimal growth conditions for aerobic bacteria left from the six hour submersion two days prior, to rebound and multiply exponentially. The control seeds (water only) contained a log10 CFU/ml range of 6.82 (mung beans) to an astonishing 9.21 (Quinoa). Of the four types of seeds treated with 80 ppm PAA for six hours and then allowed to germinate for two days, the quinoa resulted in the lowest reduction of aerobic bacteria when compared to the control, which was a log10 of 0.59 CFU/ml, corresponding to 74.30%. The sprouted green lentils and brown rice had log10 CFU/ml reductions of 0.70 (80.05%) and 0.92 (87.98%), respectively. The mung beans had the largest reduction, which was 98.00% compared to the control. This data can also be seen in **Figure 4.**

Table 5
EXPERIMENT 2: Aerobic Plate Results: Seeds After 2 Day Germination

Description	log10 (average)	log10 reduction	% reduction
Control Green Lentils	7.11	N/A	N/A
PAA Green lentils	6.41	0.70	80.05
Control Mung Beans	6.82	N/A	N/A
PAA Mung Beans	5.12	1.70	98.00
Control Brown Rice	8.27	N/A	N/A
PAA Brown Rice	7.35	0.92	87.98
Control Quinoa	9.21	N/A	N/A
PAA Quinoa	8.62	0.59	74.30

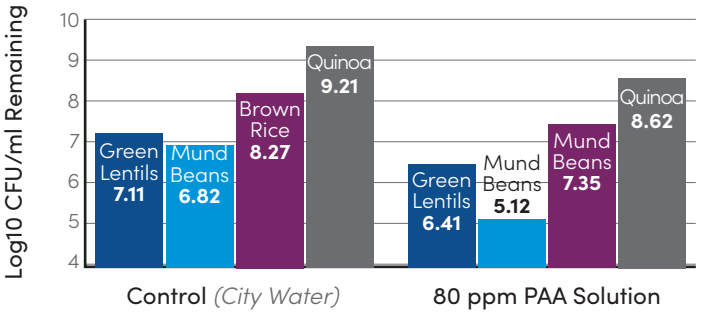
Figure 3

EXPERIMENT 2- PAA Tracking of Nominal 80 ppm PAA Test Solutions with 4 Types of Seeds for 6 Hours



Efficacy of Bioside HS 15% and Reflex Against Planktonic Alicyclobacillus acidoterrestris at Three Different Concentrations

FIGURE 4: Log10 CFU/ml Aerobic Bacteria Remaining on Germinated Seeds after Two Day Incubation



Although there were no coliforms present on the 3M Total Coliform Petrifilm after the six hour submersion, there were few present in the control samples after the two day germination period. This indicates that initially there may have been some coliform cells present, but not enough that resulted in positive readings upon microbiological analysis. However, there was a reduction of >99.999% in coliforms when compared to the respective controls for all four types of germinated seeds that had been submerged in the 80 ppm PAA solutions two days prior. This can be seen in **Table 6** below.

Table 6

EXPERIMENT 2: Total Coliform Plate Results: Seeds After 2 Day Germination

Description	log10 (average)	log10 reduction	% reduction
Control Green Lentils	2.48	N/A	N/A
PAA Green lentils	0.00	>2.48	>99.999
Control Mung Beans	2.30	N/A	N/A
PAA Mung Beans	0.00	>2.30	>99.999
Control Brown Rice	3.00	N/A	N/A
PAA Brown Rice	0.00	>3.00	>99.999
Control Quinoa	2.70	N/A	N/A
PAA Quinoa	0.00	>2.70	>99.999

Conclusions

- The contamination by various types of microorganisms on germinated, organic seeds can have a devastating impact upon the industry that produces and distributes these products to grocers and health food stores. Therefore, the purpose of this study was to determine the efficacy of peroxyacetic acid (from Perasan™) at 80 ppm against aerobic bacteria and coliforms on germinated seeds and during the germination process.
- Experiment 1 was conducted to determine the efficacy of 80 ppm PAA against aerobic bacteria and coliforms on germinated green lentils, germinated mung beans and germinated brown rice. Four seed samples that had not yet been germinated, which included green lentils, mung beans, brown rice and quinoa, were tested in Experiment 2. This experiment was conducted in two parts. First, microbiological testing was performed after the seeds were submerged in either Modesto city water or the 80 ppm PAA solution for six hours. The second portion included microbiological analysis of the seeds that had sprouted after being incubated for two days at 80° F.
- **Table1, Table 2, and Figure 1** of Experiment 1 show a dramatic decrease in aerobic bacteria and coliforms when the germinated seeds were treated with 80 ppm PAA. The use Page 16 of 16 16 of PAA provided >99.971% reduction in aerobic bacteria and >99.999% reduction in coliforms washed from the three types of germinated seeds and into the aqueous phase, when compared to the control.
- Experiment 2 involved the whole germination process. This portion of the study was conducted to determine how efficacious the use of PAA was on seeds treated prior to germination. There was a significant reduction in the number of aerobic bacteria and coliforms when comparing the control and the PAA solutions in the wash water after the six hour submersion. However, as shown in **Table 5,** and **Figure 4,** there were relatively lower reductions in the number of aerobic bacteria present when comparing the control and PAA treated germinated seeds after two days. It is well know that microbes flourish under warm, moist conditions. These are the exact conditions the seeds were kept during the germination process. On a positive note, if there were any coliforms left on the seeds after the six hour submersion in the PAA solutions, they would have more than likely rebounded under these optimal conditions. But as seen in **Table 6,** there were no coliforms present on any of the PAA treated seeds after two days.
- After the two day germination period there was a significant amount of black mold present on the seeds that had not been treated with PAA and therefore, it is recommended that PAA be used at 80 ppm at the start of germination, when the seeds are submerged for several hours. Because the process of germination provided optimal conditions for the growth of microbes, even the seeds treated with PAA, which contained very few bacteria after submersion, are vulnerable for an exponential rebound of the small amount of bacteria that had been left on them. Therefore, in order to achieve superior reduction in aerobic bacteria on the finished product, and inhibit re-contamination, it is suggested that PAA from Perasan™ be applied after germination also.