Grasping Water: Exploring a Novel Method of Inducing Precipitation using the Ice Nucleating Protein

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INTRODUCTION

Global warming has been rapidly disturbing the natural global precipitation distribution patterns. The annual precipitation in the Northern hemisphere is projected to decrease by 9%-17% over the next century. In addition, regions in central Asia and Africa are suffering from frequent drought and insufficient water supply. Currently, cloud seeding with the ice nucleator silver iodide (AgI) can be used to induce precipitation. However, finding a suitable replacement for AgI is crucial because regions cloud seeded with AgI receive uneven distribution of rainfall that results in excess silver ions in the soil. Silver ions inactivate the sulfhydryl groups of proteins and strongly inhibit the metabolism of microorganisms. Experiments suggest that the silver is concentrated in the plant root zone. This indicates that the silver ions will come into contact with the microorganisms at the roots of plants. Many plants depend on the nitrogen fixing bacteria, which fixates nitrogen at the root nodules, to intake nitrogen. If AgI could be replaced with an organic substance, such as the Ice Nucleating Protein (INP), that reduces the adverse ecological effects of cloud-seeding, then cloud seeding could be more broadly used. With an ice nuclei that causes less ecological consequences, cloud seeding could have major implications such as inducing precipitation in regions suffering from drought, giving dryer regions access to more water, or clearing smog in cities.

INP is synthesized by the ice nucleation-active (*inaZ*) gene in the surface cell layers of the epiphytic bacteria *Pseudomonas syringae (P. syringae)*. Due to the presence of INP, *P. syringae* is an ice-nucleating bacteria that possesses the strongest ice nucleating capacity among the known organic nucleating agents. The tertiary structure of INP serves as a template for ice-crystal formation, attracting water molecules into a lattice structure similar to the crystalline structure of ice. INP can be easily extracted from the bacteria using gel filtration chromatography and replicated in large quantities.

OBJECTIVE AND SCOPE OF PROJECT

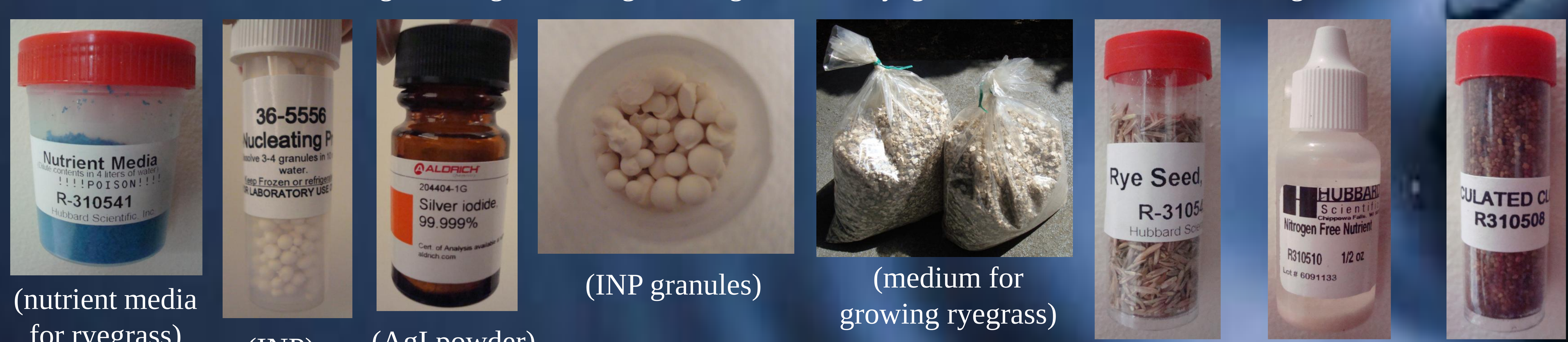
This project explores the potential of replacing AgI with INP as an ice nucleating agent to decrease the ecological ramification of cloud seeding. The project is divided into two main phases of experimentation, focusing on comparing both the ecological effects of AgI and INP and their abilities in catalyzing ice nucleation.

Phase A : comparing AgI and INP's effects on the metabolism and growth of the nitrogen-fixing bacteria at the root nodule of ryegrass and, consequentially, their effects on the nitrogen intake of ryegrass.
The question of primary importance is whether INP has less effect on the nitrogen intake of ryegrass.

Phase B : quantifying and comparing the abilities of AgI and INP in catalyzing ice nucleation of water. The droplet-freezing assay was employed to quantify the number of ice nuclei present in the water. The effects of the presence of particulate matter (PM) on the abilities of AgI and INP are also observed due to the constant presence of PM in the atmosphere.
Subsequent experiments were conducted to find possible explanations for the effects of PM on AgI and INP's abilities in catalyzing ice nucleation of water.

MATERIALS AND METHODS

AgI sample: commercial powder preparation, density 5.68 g/mL at 25°C with a 99.999% trace metals basis
INP: from *P. syringae* bacteria strain T2304, obtained from Boreal Northwest science supplyhouse as extracted granules
Ryegrass: short germination period of 5-7 days, dependent on nitrogen-fixing bacteria for nitrogen intake
Clover inoculated with the nitrogen-fixing bacteria, grown together with ryegrass to control amount of nitrogen intake



(nutrient media for ryegrass) (INP) (AgI powder) (INP granules) (medium for growing ryegrass) (ryegrass seeds) (nitrogen-free nutrient) (inoculated clover seeds)

Figure 1.

Phase A : Nitrogen-Fixation

1. Growth rates of 900 ryegrass were observed over a 4-week period
2. 30 plant pots each containing 30 seeds of ryegrass and 20 seeds of clover inoculated with the nitrogen-fixing bacteria, were placed under constant lighting conditions and at 22°C (optimum temperature=20°C to 24° C) in the laboratory. Nitrogen-free soil and fertilizer were used to control the amount of nitrogen available to the ryegrass.
3. Ryegrass seeds were placed at 2.0 cm under the soil line for optimum germination, watered daily with 10 mL of water.
4. The amounts of change in the rates of growth in height and in mass of the ryegrass, watered with distilled water, were observed over a 2-week period.
5. The samples were then equally divided into three groups (controlled, AgI, INP). The samples were exposed to the ice nuleators for 2 weeks through the addition of 0.01 g of INP or AgI into the water used to water the corresponding test groups. All other factors were kept constant. Growth rates after exposure to ice nuclei were compared the rates before the exposure to ice nuclei.

Phase B: Catalyzing Ice Nucleation

Droplet Freezing Assay

1. AgI, INP, and PM were each suspended at 1% concentration in distilled water. PM was obtained by placing Petri dishes, covered by plastic wrap with 5 one-centimeter holes, at two major street intersections over a 48-hour period.
2. Six test groups (controlled, PM, AgI, INP, AgI+PM, INP+PM) were prepared. Each test group had 1% concentration of the corresponding ice nuclei suspended in 10 mL of distilled water. The controlled group contained only distilled water. The group containing PM simulates the state of water in the atmosphere, where PM is constantly present.
3. Using plastic pipettes, 30 drops of 10 μL from each of the suspensions were transferred into 40 wells of 6 corresponding microplates, labeled with the names of the test groups. All six microplates were sealed using sterilized transparent plastic wrap.
4. The microplates were then placed into a temperature of -20°C, for 20 minutes, after of which the number of frozen drops were determined. The number of ice nuclei that were active in catalyzing ice nucleation was determined as follows:

Equation 1.

$$(IN/mL) = \log\left[\frac{In(Nt/Nu)}{ds} \times \frac{1}{d}\right]$$

Where Nt=total drops per test group (40) Nu=number of unfrozen drops
ds=droplet size (10μL) d=dilution (10-fold)

5. The droplet-freezing assay was repeated three times to obtain 160 sample drops for each test group. The difference between the results for the AgI and INP groups without the presence of PM and the difference with PM present were compared.

Cooling Curves and Heat of Crystallization

To further explore the effect of PM on AgI and INP's abilities to catalyze ice nucleation, subsequent experiments were conducted.

1. Six test groups (controlled, PM, AgI, INP, AgI+PM, INP+PM) were prepared using a similar method as the one outlined for the droplet-freezing assay
2. The ice nuclei were each suspended at 1% concentration in 10 mL of distilled water. All 6 test groups were placed into test tubes, which were sealed using silicon caps.
3. A thermocouple was inserted through the cap to allow efficient measurement of temperature.
4. All 6 microplates were placed into a -20°C temperature for 60 minutes, the temperatures of the samples noted every 5 minutes.
5. Using the data, the cooling curves, rate of cooling, and amount of heat of crystallization for each test group were determined. The results for the AgI and INP groups, with PM absent, were compared to the results for when PM was present.

RESULTS

Phase A: Nitrogen Fixation

Part A of the project investigated the effects of AgI and INP on the nitrogen-intake of ryegrass, using the rate of growth in height and in mass as indicators of nitrogen-intake.

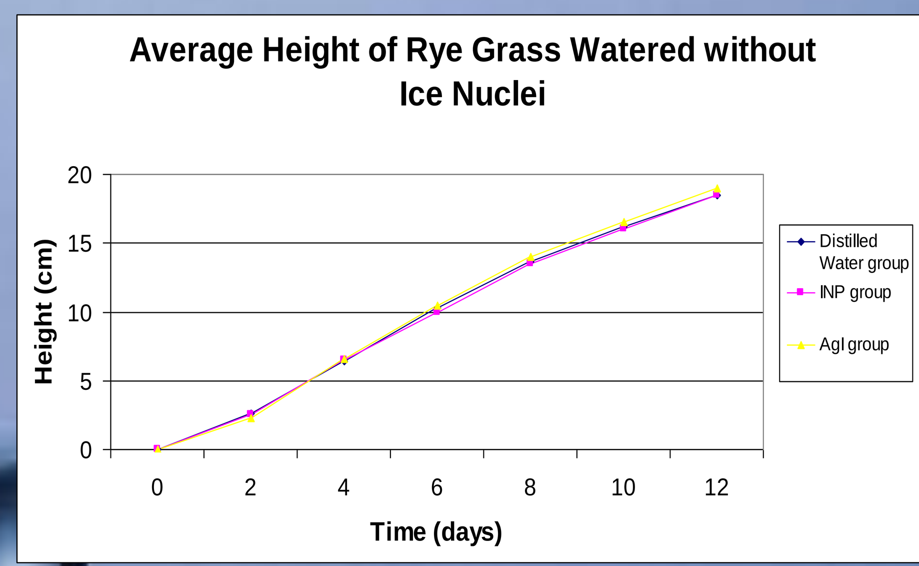


Figure 2. Average height of samples of ryegrass over two weeks of no exposure to ice nuclei. Measurements taken with uncertainty range of +/- 0.5 cm.

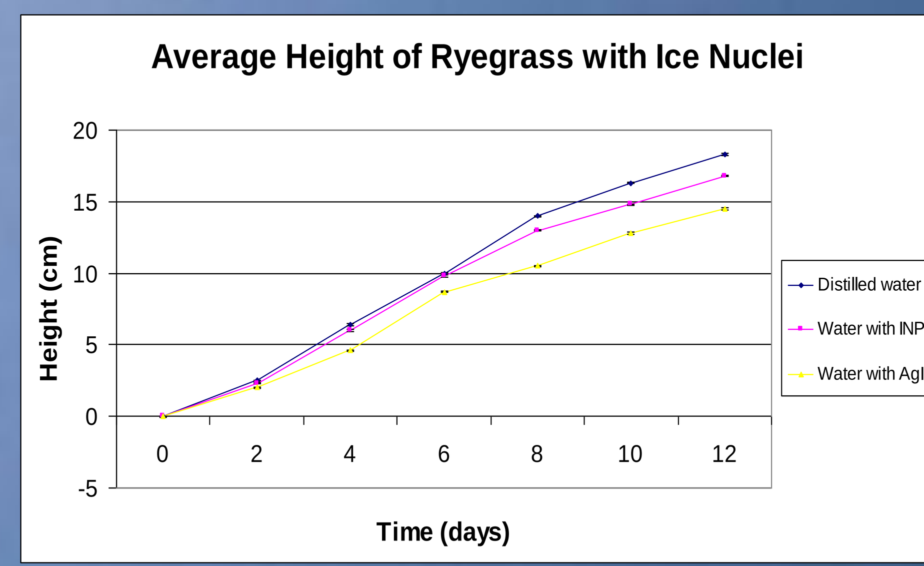


Figure 3. Average height of samples of ryegrass over two weeks of exposure to AgI or INP. Measurements taken with uncertainty range of +/- 0.5 cm. Portrays decrease in growth rates.

Test Group	Growth Rate without Ice Nuclei (cm/day) +/- 0.05 cm	Growth Rate with Ice Nuclei (cm/day) +/- 0.05 cm
INP	1.54	1.40
AgI	1.58	1.21
Water	1.54	1.53

Table 1. Average Growth Rate in Height
The growth rates shown in table 1 are the average growth rates in height of the samples of ryegrass (sample size=300 per test group). The period of exposure to ice nuclei was 2 weeks, after a 2-week period of no exposure.

The rates of growth in height for the AgI and INP groups decreased by 10.0% and 31.0% (+/-0.5%) after exposure to ice nuclei. The rates of growth in mass decreased by 12.7% and 15.2% (+/- 0.5%) for the AgI and INP groups.

Test Group	Mass Gained without Ice Nuclei (grams) +/- 0.1 g	Mass Gained with Ice Nuclei (grams) +/- 0.1 g
INP	30.2	26.2
AgI	31.1	27.6
Water	30.3	29.8

Table 2. Average Growth Rate in Mass
The mass was observed as an indication of primary growth of the ryegrass, and was compared to the changes in secondary growth.

The T-test results show a strong correlation, at 95% ($p<0.05$) confidence interval, between the exposure to AgI and the decrease in growth of the ryegrass.

Phase B: Catalyzing Ice Nucleation

Droplet-Freezing Method

The droplet-freezing assay was employed to quantify the abilities of AgI and INP in catalyzing ice nucleation. Four trials were conducted to obtain a sample size of 160 per test group. The graphs below portray the arithmetic mean of the results from the four trials.

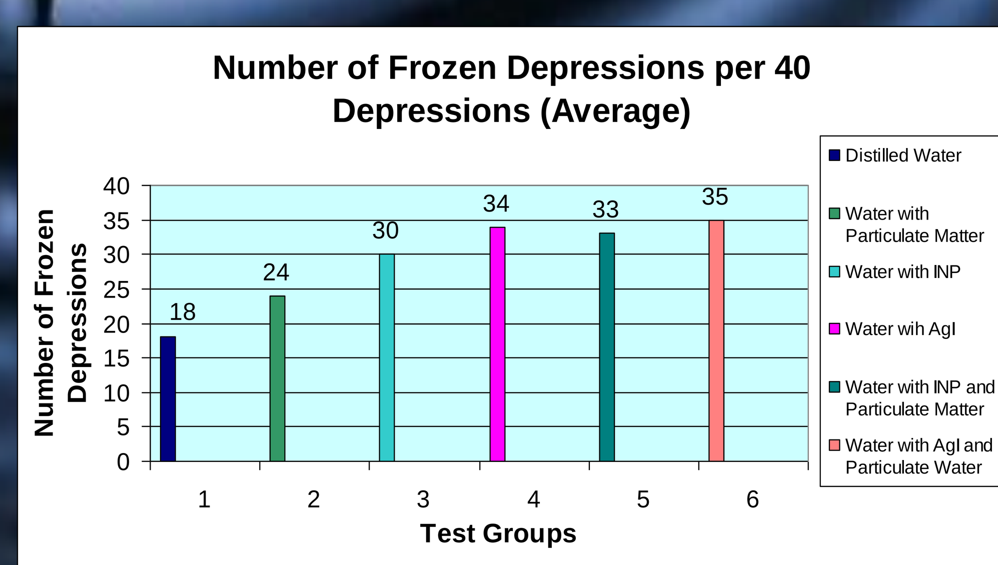


Figure 4. Values shown are the arithmetic means of the results for the four trials of the droplet-freezing assay. Sample size per test group for each trial was 40.

Figure 5 indicates that, with the presence of PM, the AgI test group had 6.0% more ice nuclei than the INP group. When PM was absent, the AgI group had only 13% more ice nuclei than the INP group.

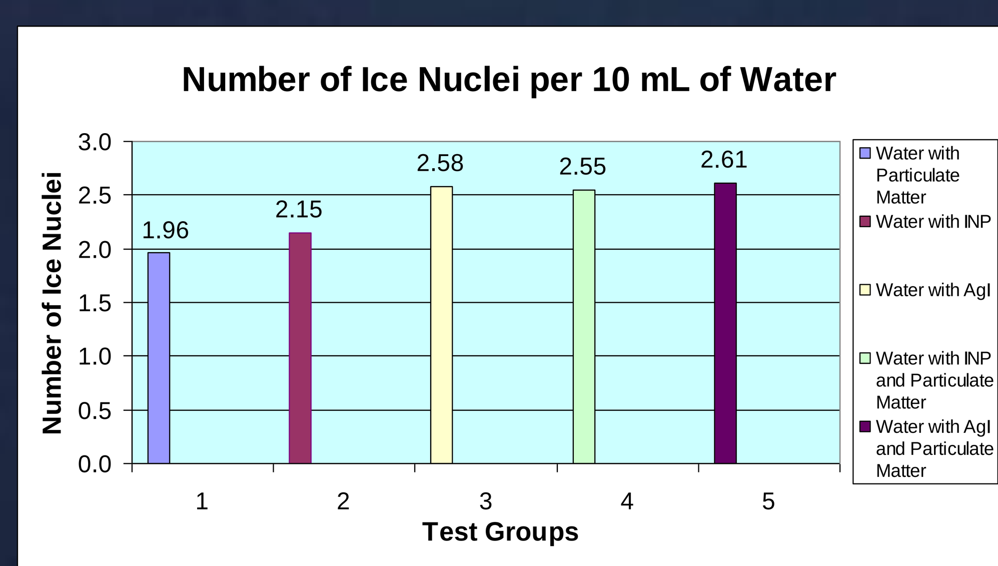


Figure 5. Calculated using the results shown in Figure 4, with Equation 1. :

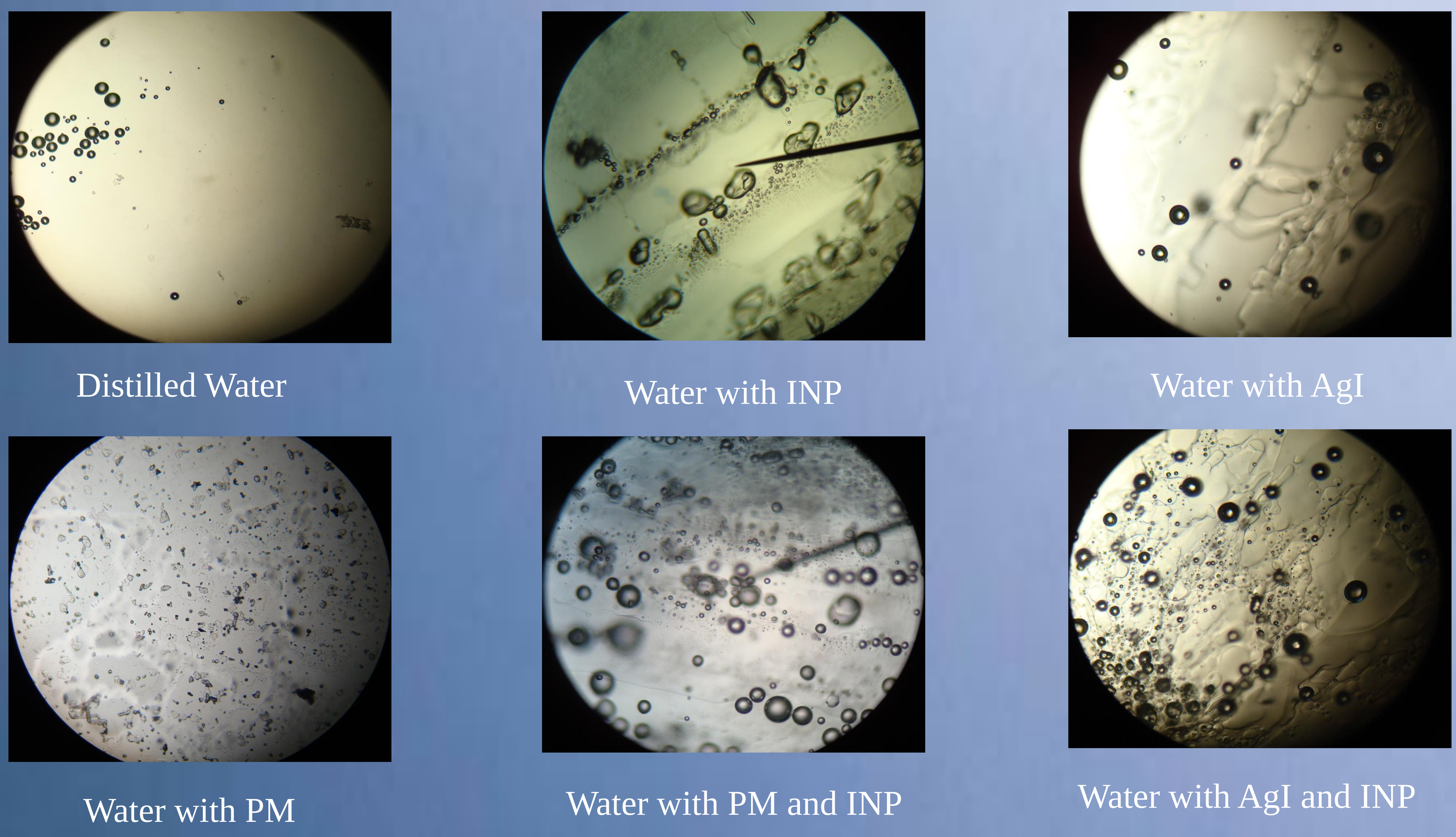
$$(IN/mL) = \log\left[\frac{In(Nt/Nu)}{ds} \times \frac{1}{d}\right]$$

Using the T-test, the difference in the number of ice nuclei in the INP and AgI groups, when PM was present, was determined to be not statistically significant, as opposed to when PM was absent.

The standard deviations of the four trials were 0.71 for the controlled, PM, and AgI+PM groups, and 0.77 for the INP, INP+PM, and AgI groups.

The F-test was employed and the null hypothesis was accepted at the 95% confidence interval, indicating that the trials yielded results with the same level of precision; the difference in the standard deviations was not statistically significant.

Figure 7. (Below) Frozen test samples viewed under light compound microscope (400X magnification, diameter of field 400 µm). Pictures suggest that ice that formed on different ice nuclei have different formations that contribute to the speed of ice nucleation. The picture for distilled water suggests that, due to exposure to the air, the test sample may have had particulate matter when it was frozen



Cooling Curves and Heat of Crystallization

The cooling curves and the heat of crystallization of the test groups were observed to further investigate the effect of PM on the abilities of INP and AgI in catalyzing ice nucleation.

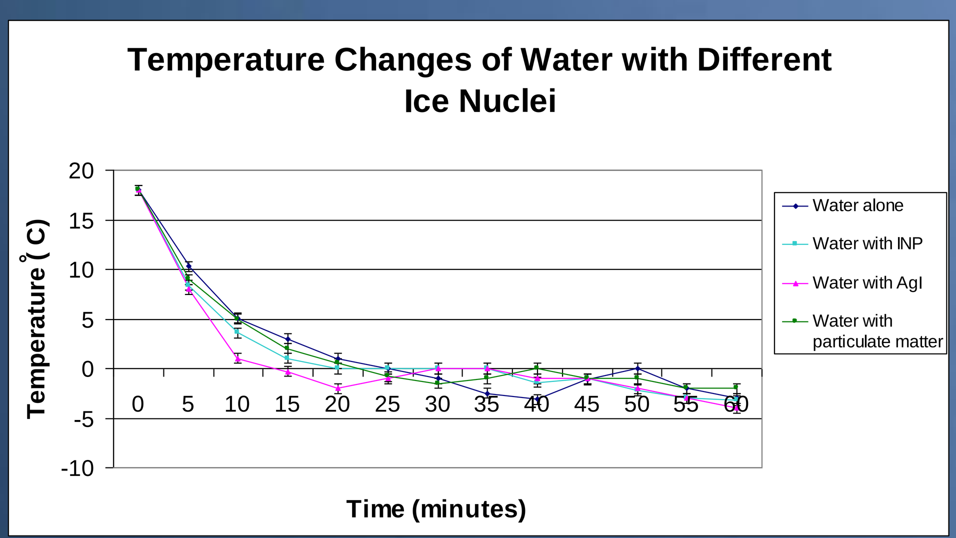


Figure 8 Temperature changes of test groups

Figure 8 shows the temperatures changes of the groups during the 60-minute duration in -20 degrees Celsius temperature.

Without PM, the AgI test group reaches 0 degrees Celsius at approximate 15 minutes and the INP group at 20 minutes.

With PM, the AgI test group reaches 0 degrees Celsius at 25 minutes., while the INP group stays at 20 minutes.

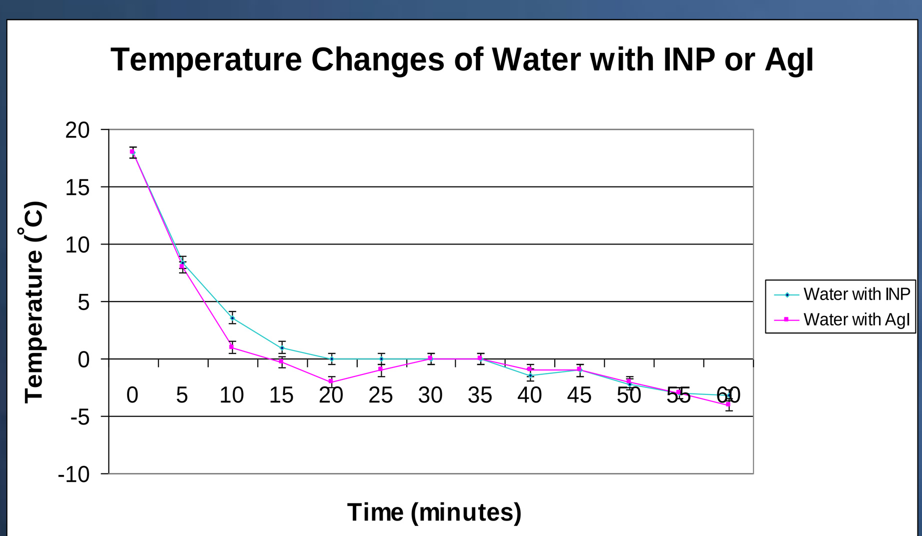


Figure 9. The temperature changes of the test groups containing INP or AgI, without PM, over a 60-minute period.

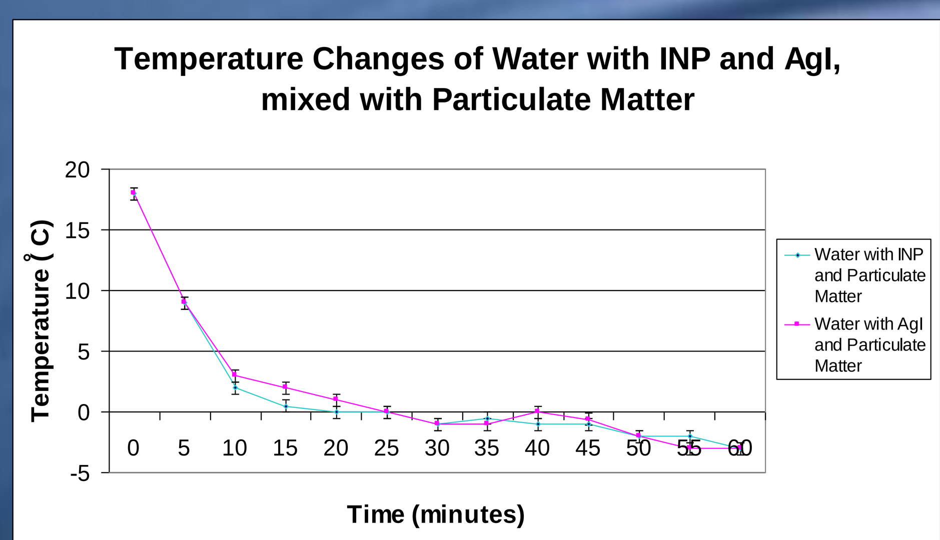


Figure 10. The temperature changes of the test groups containing INP or AgI, with PM, over a 60-minute period

Test Group	Rate of Cooling (°C/min., +/-0.05 °C/min.)
Controlled	-0.38
PM	-0.24
INP	-0.35
AgI	-0.35
INP+PM	-0.35
AgI+PM	-0.34

Table 3. Average Rates of Cooling over 60-minute Period

The values shown are calculated using the results portrayed in figures 9 and 10. The rates of cooling were determined by dividing the total amount of decrease in temperature for each test group by the total amount of time, 60 minutes.

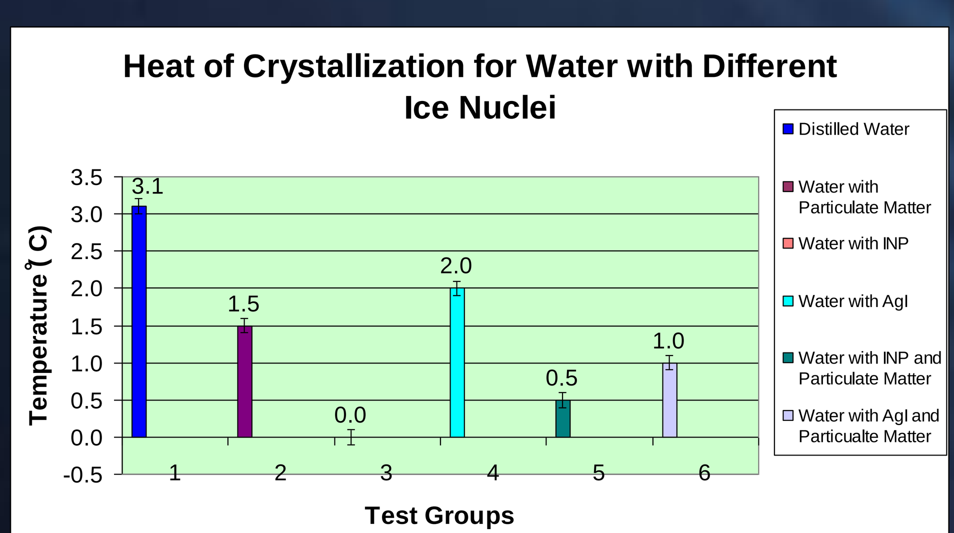


Figure 11. The heat of crystallization was released between the first and the second time that the graphs of the cooling curves reach 0 degrees Celsius. The heat of crystallization for the groups containing AgI was consistently greater than that of the groups containing INP. With the addition of PM, the heat of crystallization for the group with AgI was 50% greater than that of the group with INP.

F-Test: Comparing σ of Distilled Water with INP and Particulate Matter, and Distilled Water with AgI and Particulate Matter Groups

	Method	Mean	Standard Deviation
1	distilled water with AgI and particulate	35	0.866025
2	distilled water with INP and particulate	33	1.118034

null hypothesis: $H_0: \sigma_1 = \sigma_2$ alternate hypothesis: $H_a: \sigma_1 < \sigma_2$ lower one tailed test

Since s2 > s1, Fcalc = s2²/s1² = (1.118034)²/(0.866025)² = 1.667

Degree of freedom: v1=(n1-1)=1 between groups, v2=(n2-2)=6 within groups

95% confidence level: $F_{1,6}$ =5.987

Since 1.667<5.987 $F_{1,6}$ > Fcalc; therefore, the null hypothesis is true

Method 1 (distilled water with AgI and particulate matter) has the same amount of standard deviation and precision as method 2 (distilled water with INP and particulate matter), with 95% confidence.

DISCUSSION

The focus of this project was to investigate whether INP has less effect on the nitrogen intake of ryegrass than AgI and to compare the two ice nuclei's abilities in catalyzing ice nucleation.

A. Effect on Nitrogen Intake

The results indicate that INP had no influence on the growth rate of ryegrass, suggesting that INP did not affect the nitrogen-intake of ryegrass.

During the 2-week exposure to AgI, the growth rate of the ryegrass decreased by 31% in height and 15% in mass. This indicates that AgI affected the metabolism of the nitrogen-fixing bacteria, resulting in a decreased nitrogen-intake by the ryegrass. The T-test results show a strong correlation (95% confidence level, p<0.05) between the exposure to AgI and the decrease in growth rate.

The results of this project corroborate with the findings of Y. Tsuchiya *et al.* The research conducted by Y. Tsuchiya *et al.* in 2003 suggests that regions cloud-seeded with AgI have excessive levels of silver ions. Whereas Y. Tsuchiya *et al.* focused on the correlation between decreased oxygen uptake by microorganisms in the soil, this project focused on the effects of both AgI and INP on the nitrogen intake of ryegrass.

The influence of AgI and INP on the nitrogen in take of ryegrass is of primary importance because ryegrass is one of most plants that depend on the nitrogen-fixing bacteria to fixate nitrogen. Although some research has been conducted to determine the effects of AgI on microorganisms in the soil, little research has been done to find a suitable alternative for AgI that could decrease the ecological ramifications of cloud-seeding.

The findings of this project has major implications because the results indicate that INP would have little influence on the nitrogen intake of plants if it replaces AgI as an ice nuclei in seeding clouds.

T-Test: Comparing µ of Ryegrass mass with INP and without INP

	Method	Mean	Standard Deviation
1	without INP	30.20	0.834965
2	with INP	26.20	0.653025

null hypothesis: $H_o: \mu = \mu_o$ alternate hypothesis: $H_A: \mu > \mu_o$ upper one tailed test

95% confidence level: $t_{6,95\%}$ = 2.447 T-value < $t_{6,95\%}$

Method 2 does not have more influence on the mass of ryegrass than method 1, with 95% confidence.

B. Catalyzing Ice Nucleation

Droplet-Freezing Assay

The results of the droplet-freezing assay indicate that the presence of PM in the water has a significant influence on the abilities of INP and AgI in catalyzing ice nucleation.

When PM was absent, the group containing AgI had 13% more frozen water than the group containing INP. Most importantly, the abilities of INP and AgI in catalyzing ice nucleation are the same when PM is present in the water. The T-test was used to determine that the 6% difference between the amount of frozen water in the AgI and INP groups was not statistically significant.

The findings of this project corroborate with the research conducted by *Muryoi et al.*. Whereas *Muryoi et al.* focused on determining the ice-nucleating temperature of AgI, this project compared AgI with INP in attempt to determine whether INP has sufficient capability to replace AgI in cloud-seeding.

The abilities of AgI and INP in catalyzing ice nucleation when PM is present simulate their performance as ice nuclei used to seed clouds in the atmosphere, where PM is constantly present.

The results indicate that INP would be as efficient as AgI in catalyzing ice nucleation and inducing precipitation if it replaces AgI as an ice nucleator used in cloud-seeding. mass.

Since the results from part A of this project indicate that INP has less ecological ramifications than AgI, the findings from part B suggest that INP may be a suitable alternative for AgI due to its high capability in catalyzing ice nucleation.

The replacement of AgI with INP would have a great impact on the global water distribution because cloud-seeding would be able to be more broadly used. In addition to the current usage of making artificial snow, cloud–seeding could be used to induce precipitation in regions suffering from drought or to clear smog. Since INP is insoluble in water, it could be easily filtered from the precipitation to increase the quality of the water and to improve the lives of people living in dry regions of the Earth.

Cooling Curves and Heat of Crystallization

The results reveal that the test groups containing AgI consistently had a larger amount of heat of crystallization than the groups containing INP.

When PM was present, the AgI group had 50% more heat of crystallization than the INP group. The heat of crystallization that was released by the test groups during ice nucleation decreased the overall rate of cooling.

Since the groups containing AgI had a larger amount of heat of crystallization than the groups containing INP when PM was present, the AgI groups had a larger decreased rate of cooling. Hence, the large amount of heat of crystallization released by the AgI group when PM was present acted as a hindrance to the catalyzing of ice nucleation.

CONCLUSIONS

The results of this project indicate that INP, due to its high capability of catalyzing ice nucleation and low ecological impact compared to AgI, may be a viable alternative for AgI in cloud-seeding.

1. INP had no statistically significant effect on the nitrogen-intake of ryegrass (p<0.05); groups that were exposed to AgI had a 30% decrease in growth rate in height and 15% decrease in growth rate in mass.
2. When PM was absent, the test group containing AgI had 13% more frozen water than the group containing INP. When PM was present, there was only a 6% difference between the amount of frozen water in the groups containing INP and AgI. There was no statistically significant difference in the abilities of INP and AgI in catalyzing ice nucleation when PM was present.
3. The cooling curves indicate that a larger amount of heat of crystallization was released during the ice nucleation of the group containing PM with AgI than the group containing INP and PM. The cooling curves suggest that the large amount of heat of crystallization released by the AgI group when PM was present decreased the overall rate of cooling and hindered AgI's ability in catalyzing ice nucleation.

The findings of this project indicate that INP has less ecological ramifications than AgI. In addition, the commercial cost for INP was 40 dollars less than that of AgI. Hence, INP may be an environmentally-friendly and commercially-viable alternative for AgI in weather modification methods, such as cloud-seeding.