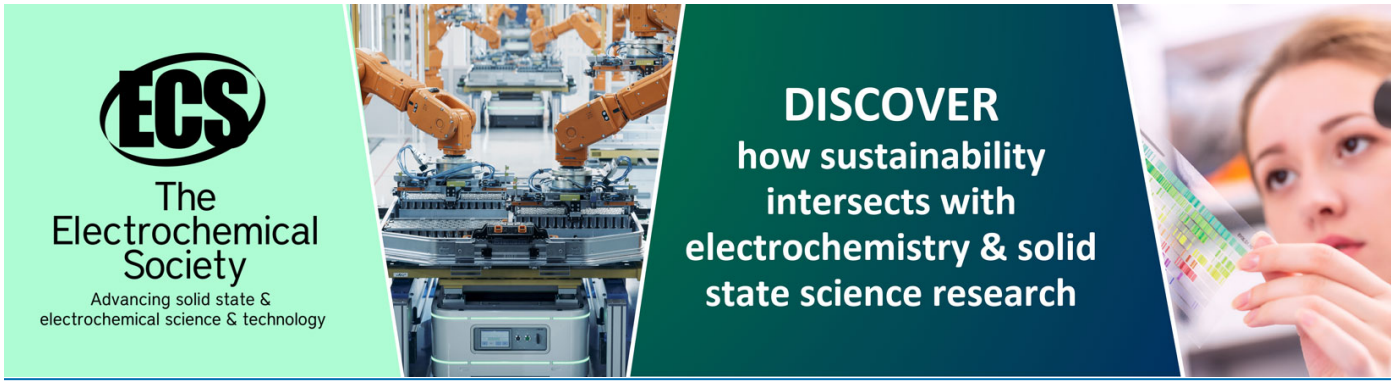
Enumeration of ice nucleation active bacteria and severity of frost injury (*embun upas*) on potato in Wonosobo, Dieng Plateau



IOP Conf. Series: Earth and Environmental Science **185** (2018) 012032 doi:10.1088/1755-1315/185/1/012032

Enumeration of ice nucleation active bacteria and severity of frost injury (*embun upas*) on potato in Wonosobo, Dieng Plateau

A Susilowati[1], L Y Oktiningtiyas and R Setyaningsih

Study Program of Bioscience, Graduate Program, University of Sebelas Maret, Jl. Ir. Sutami 36A Kentingan Jebres Surakarta 57126, Indonesia

E-mail: arisusilowati@staff.uns.ac.id

Abstract. The altitude of the Dieng plateau is between 1,500-2,500 meters above sea level (m ASL), with an average temperature of 14ºC. During the dry season, the temperature in this area becomes very cold that reaches -2ºC, therefore frost injury may occur in potato, which is often referred to as *embun upas* (frozen dew). Frost injury causes the production of potatoes in Dieng to decline. Frost injury in plants is characterized by spots symptom, which can be brown, yellow, or black on leaves surface with the chlorotic ring that surrounds them. The phenomenon of frost injury is thought to be related to the activity of ice formation by the Ice Nucleation Active (INA) bacteria and triggered by cold temperatures (-2°C). This study aimed to determine the number of INA bacteria and its correlation with frost injury spots on potato leaves and to discover the class of INA bacteria based on ice formation temperature. The sampling of the potato leaves used purposive sampling method. Leaves showed frost injury symptom with different severity were taken from a different altitude of ± 2,000 m ASL (Dieng Wetan village) and ± 2,500 m ASL (Sembungan village). Bacterial isolation was done by spread plate method on Nutrient Agar supplemented with 2.5% glycerol. The activity of ice nucleation was determined using tube nucleation method. Estimation of INA bacteria number was done by multiple-tube nucleation method 3.3.3. Correlation between the number of INA bacteria and percentages of frost injury spots was analyzed using SPSS bivariate. The results showed that the highest INA bacteria number i.e. >2.75 x 10^4 was found on scale 3 of leave spots while the lowest value of 7.25 x 10^3 on scale 1. Correlation between the populations of INA bacteria with the scale of frost injury spots showed 0.844. The correlation values ranging from 0.80 to 1.00 indicating a strong association between the INA bacterial number and the scale of frost injury spots of potato leaves. Based on the ice forming temperatures, INA bacteria were classified into three classes, i.e., class A that can freeze water at temperatures of -2 to -4°C, class B -5 to -7°C and freezing point of class C below -8°C.

1. Introduction

Dieng Plateau is located in Central of Java Province and has three regencies, which are Banjarnegara, and Wonosobo and Temanggung. The altitude of the Dieng plateau is between 1,500-2,500 meters

above the sea level (m ASL). *Embun upas* is the locally Indonesian name of ice nucleation diseases on potato leaves on Dieng plateau. *Embun upas* generally appears in the Dieng plateau on July or August of each year. During that time, the temperature is at the critical point reaching zero and even minus causing the appearance of *embun upas*. The lowest temperature that occurs at a critical point can reach -8^{o}C. The emergence of *embun upas* results in frost injury on the potato that ultimately leads to the death of the potato and the risk of crop harvesting failure [1]. *Embun upas* becomes of the limiting factors in potato production in the Dieng Plateau, causing loss to the potato farmers reaching hundreds million rupiah, after more than 35 ha of potato plantation xperienced frost damage in 1994 [2]. The research location of Dieng Plateau (Central Java) and potato plantation in Sembungan village can be seen in Figure 1A and 1B.

Figure 1A. Location of Dieng plateau which includes Banjarnegara, Wonosobo and Temanggung (Central Java).

Figure 1B. Sampling Area: Sembungan Village (7°14'24.94"S-109°55'0.97"T) is dominated by potato plantations in terraces.

The frost injury on potato has not been well understood by most farmers and that the numbers of INA bacteria are responsible for its formation. INA bacteria have been considered as the main causative factor of frost injuries on potato because of their ice nucleation property that can initiate theformation of ice crystalin plant tissue. Bacteria that cause frost injury are called INA bacteria [3]. The INA bacteria are bacteria that live on the leaves surface (phyllosphere) and able to catalyze the formation of frost injury on the leaves surface.

Reports of frost injury mostly come from subtropical countries, like in Japan, which occurred on tea plants [4], cabbage, broccoli and mulberry [5] and in the United States, which occurred on corns' plants [6], oranges, wheat, and tomatoes [7]. Hirano and Christen later proved that frost injury that occurs on some species of plants are caused by the activity of bacteria in the leaves surface and is harmful because it causes plants death [8]. Research on frost injury bacteria from the tropics has not been widely reported or published. The data of INA bacteria studied on potato in Wonosobo, Dieng Plateau will provide information about the novelty of this bacterium in the tropics.

Further research concerning bacteria in the tropics needs to be conducted, considering its negative impact on agriculture due to the *embun* phenomenon caused by ice nucleation activity. Basic knowledge revealed about INA bacteria on potato plant can later be used to arrange strategies to overcome and prevent the occurrence of damage due to frost injury on potato plants in Dieng plateau. In this study, we covered about the estimation of INA bacteria number in potato leaves, determination of disease severity scale caused by INA bacteria, and the identification of INA class based on its freezing temperature.

2. Material and methods

2.1. Estimation of INA bacterial number

Estimation of INA bacteria number can be done using multiple tube nucleation method. Samples of potato leaves weighed, 2 grams were homogenized in 50 mL phosphate buffers in 0.1% protease-peptone. Taken as much as 1 mL and inserted into a test tube containing 9 mL sterile phosphate buffers. Afterwards, 3 series of dilutions i.e., 10^{-1}, 10^{-2}, 10^{-3} were performed into a tube containing 9 mL of phosphate buffers. In each series of dilutions, duplicate replications were performed so that the dilution of the 3-tube series for each dilution series was obtained. The bacterial suspension as the result of dilution was introduced into the circulating alcohol bath at the temperature of -10ºC for 10 minutes. umber of bacteria per gram of fresh leaves weight was estimated based on the Most Probable Number (MPN) method. The number of total INA bacteria was determined by counting the number of tubes giving positive frozen reaction and then comparing the pattern of positive results (the number of tubes showing frozen at each dilution) with standard MPN statistical tables [9-10].

2.2. Disease severity of frost injury on potato

The sampling of the potato leaves used purposive sampling method. Leaves showed frost injury symptom with different severity were taken from a different altitude of ± 2,000 m ASL (Dieng Wetan village) and ± 2,500 m ASL (Sembungan village). Disease severity was expressed in the spotting scale (Table 1).

The association of INA bacterial population with disease severity was analyzed using SPSS with correlation bivariate test.

Table 1. Diseases severity of frost injury on potato leaves expressed in the spotting scale.

Spot Scale	Description
0	No symptoms on the leaves
1	Little spots, not very real, about 10% of the leaves area infected

2	Little spots spread on the leaves and easily observed, but do not cause any apparent damage, 11-30% of the leaves area infected
3	Symptoms spread on the leaves area, damage and chlorosis are limited, 31-50% of the leaves area infected
4	Widespread symptoms occur, leaves necrosis, 51-71% of the leaves area infected
5	Spotting almost or spreading throughout the leaves area, causing necrosis to death, 71% or of the leaves area infected

2.3. Isolation of INA bacteria

For each leave, a sample of 2 grams was cut with a size of 0.5 cm^2 and the sample was put into a 250 mL Erlenmeyer flask containing 50 mL of 0.1 M phosphate buffer pH 7 and 0.1 M protease peptone. Erlenmeyer flask was shaken on a rotary shaker at a speed of 150 rpm for 2 hours. One mL of the samples were taken and put into a test tube containing 9 mL of sterile distilled water. Three series of dilution i.e., 10^{-1}, $10^{-2,}$ 10^{-3} were performed, and from each dilution 100 µL of the sample was taken then plated on NA medium containing 2.5% glycerol (NAG) using spread technique into the petri dish. Glycerol-containing media is a common medium used to isolate ice-forming bacteria because it can optimize the growth of cultures [11]. Glycerol as a major carbon source is capable of enhancing nucleation activity, according to [12] studies suggesting that some bacteria with weak or lost nucleation activity are able to restore their activity after growth in nutrients agar added glycerol at 22ºC. Each dilution series was spread on two Petri dishes. After the surface of the media dried sufficiently was then wrapped in paper and incubated at 18-24ºC with the reversed position for 2 days [13].

2.4. Collection of pure isolates

Colonies of bacteria that have grown in NA media + 2.5% glycerol that had different morphologies were transferred into an agar slant by a scratch method using inoculating needles in an aseptically Laminar Air Flow Cabinet. Single bacterial cells formed a single colony was then grown for 4-6 days in the incubator at a temperature of 22ºC, then stored in the refrigerator at 5ºC [14].

2.5. Activity Test for Ice nuclei formation

The activity of ice nucleus formation was conducted using multiple nucleation tube tests. Four to six days old bacterial colonies on an agar slant medium were transferred using inoculation needle and re-suspended in 400 µl sterile phosphate buffers and tested for the ice nucleation activity at temperature -4°C, -7°C and -10°C in a circulating alcohol bath for 10 minutes. Positive test results indicated that phosphate buffers when dropped by bacterial suspension freeze within 30 seconds at a temperature of -4°C or less, it was considered that the colony contains active ice nuclei or contains INA bacteria [15-16].

3. Results

Based on the positive results of Most Probable Number (MPN) with three series of dilutions at a different altitude of Dieng Wetan Village (± 2000 m ASL) and Sembungan Village (± 2500 m ASL) respectively, obtained a freezing tube showing the number of INA bacteria (Figure 2).

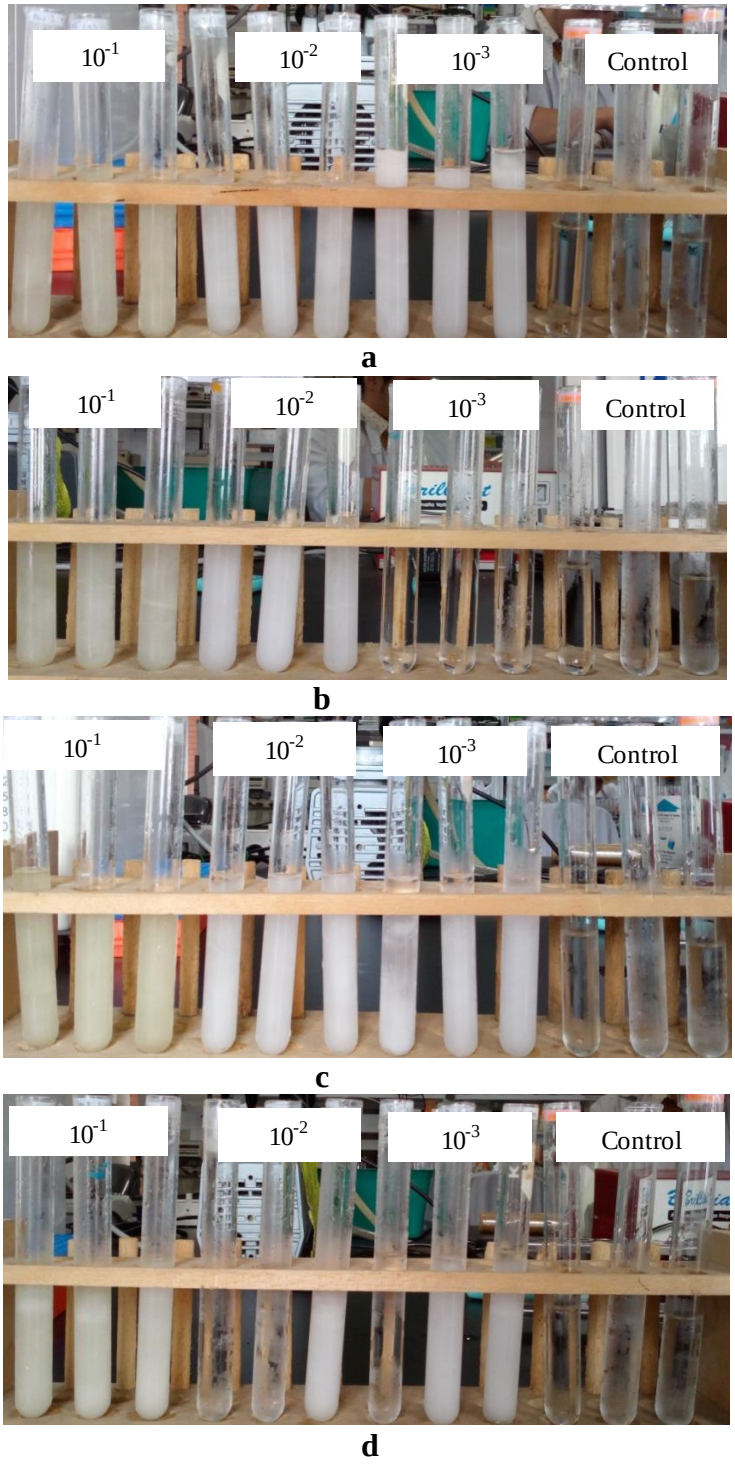


Figure 2. Positive frozen tube on MPN test. Dieng Wetan station I a). Tube combination 3-3-3; Dieng Wetan station II b) Tube combination 3-3-2; Sembungan stasiun I c) tube combination 3-3-3; Sembungan stasiun II d) tube combination 3-1-2.

MPN values in replication one and two at each at sampling area of Dieng Wetan and Sembungan (Table 2 and 3). MPN value indicated the highest INA bacterial population in potato plant in Dieng

plateau with tube combination 3.3.3 i.e >2.75 x 10^4. Average number of bacteria based on MPN on the spotting scale of 3, 2 and 1 (Tabel 4). The average number of bacteria on a scale of 3 is > 2.75 x 10^4, the number of bacteria on scale 2 is >1.67 x 10^4, scale 1 in Dieng Wetan > 1.52 x 10^4, and scala 1 in Sembungan 7.25 x 10^3.

Table 2. Estimation of bacterial number based on MPN table, replication 1.

Sampling Station	Scale of Spots	Replication 1			
		10^{-1}	10^{-2}	10^{-3}	Estimation of Bacteria number MPN/g leaves
Dieng Wetan Station I	3	3	3	3	>2.75 x 10^4
Dieng Wetan Station II	1	3	3	0	6 x 10^3
Sembungan Station I	2	3	3	3	>2.75 x 10^4
Sembungan Station II	1	3	1	2	3 x 10^3

Table 3. Estimation of bacterial number based on MPN table, replication 2.

Sampling Station	Scale of Spots	Replication 2			
		10^{-1}	10^{-2}	10^{-3}	Estimation of Bacteria number MPN/g leaves
Dieng Wetan Station I	3	3	3	3	>2.75 x 10^4
Dieng Wetan Station II	1	3	3	3	>2.75 x 10^4
Sembungan Station I	2	3	1	2	3 x 10^3
Sembungan Station II	1	3	3	1	1.15 x 10^4

Table 4. The average of the estimated number of INA bacteria based on the table MPN, Thomas formula series 3.3.3.

Sampling Station	Spotted Scale	Estimation of Bacteria number MPN/g leaves
Dieng Wetan Stasiun I	3	>2.75 x 10^4
Dieng Wetan Stasiun II	1	>1.67 x 10^4
Sembungan Stasiun I	2	>1.52 x 10^4
Sembungan Stasiun II	1	7.25 x 10^3

Potato leaf samples from different altitude have the varying severity of disease based on spotting scale scaling, i.e. 3 and 1 scale at ± 2,000 m ASL, 2 and 1 at an altitude of ± 2,500 m ASL (Figure 3)

Figure 3. Spot scales on the leaves of potato plants based on the scoring, a) scale 3: 31-50% infected leaf area; b) scale 2: 11-30% infected leaf area; c) scale 2: 11-30% infected leaf area; d) scale 1: 10% infected leaf area.

Based on the correlation analysis showed the correlation value of 0.844 and significance 0.156 (Table 5).

Table 5. Analysis of correlation of bacterial estimation with spot scale using SPSS Bivariate Correlations.

		Bacterial Population	Spot Scale
Bacterial Population	Pearson Correlation	1	.844 (*)
	Sig. (1-tailed)		.156
	N	4	4
Spot Scale	Pearson Correlation	.844 (*)	1
	Sig. (1-tailed)	.156	
	N	4	4

The bacteria isolated from potato leaves were 84 pure cultures, but based on the nucleation activity test only 20 isolates were clotted. From the results of ice nucleation test in this study, We found three classes of INA bacteria based on freezing temperature, i.e. class A that freezes at temperature -4°C, class B at temperature -7°C and class C at -10°C (Table 6).

Table 6. Classification of class INA based on a temperature difference of ice nucleation activity from potato plant leaves.

Bacterial isolate code	Number of bacterial isolate	FreezingTemperature	Class of INA
SM2-a1a, SM2-b1a	2	-4	A
DW1-a1a, SM1-b2b, SM2-b2a, SM2-c1a	4	-7	B
DW1-a2b, DW1-b1b, DW2-b1a, SM1-c1c, SM1-c1d, SM1-c2b, SM2-c1b, SM2-a1c, SM2-a2c, SM2-b1d, SM2-c2e, SM2-c2c, SM2-c2a, SM2-a2c	14	-10	C
Total number of INA bacteria	20		

Note: DW1 = Dieng Wetan station 1, DW2 = Dieng Wetan station 2, SM2 = Sembungan station 1, SM2 = Sembungan station 2

4. Discussion

4.1. Estimation on the number of ina bacteria dieng plateau potato plant leaves

The calculation of INA bacteria was done using Multiple Tube Nucleation (MPN) method, which is a method used to estimate the amount of ice from bacterial suspension and bacterial number on plant [13]. A test was conducted using the tubes to determine the number of ice nucleation based on the number of frozen tubes at each dilution. This method assumes that one of the frozen tubes contains at least one ice nucleus [17-18]. The optimum temperature for the estimation test was -5°C for 10 minutes in a circulating alcohol bath, which was sensitive enough to detect the formation of ice nuclei by INA bacteria [19-21].

Based on the calculation of MPN value, the highest INA bacteria population in potato plant in Dieng plateau from eight samples of $>2.75 \times 10^4$ were found on the scale 3 of leaf spots while the lowest value of 7.25×10^3 on scale 1. Lindow states that the population of INA bacteria can reach 10^2-10^7 cell/g plant tissue [3]. In this study, the population of INA bacteria from potato plants ranged from 10^3 to 10^4. Gross *et al*, has estimated that the population of INA bacteria in fruit plants ranges from 10^2 to 10^4 [22]. In addition to the availability of bacterial number in the sample, determination of the magnitude of the INA bacteria number was also affected by the dilution series at the time of estimation. According to the result of positive tubes MPN 3-3-3 that corresponding with an uncertain amount of the bacteria (more than 1100 MPN/g), it seems to need analysis more than 10^{-4} to get a more precise estimate number. Regrettably, we did not do further dilution in this study.

4.2. Correlation of INA bacterial population with disease severity of potato plant frost injury

Analysis of disease severity was done by calculating the correlation between the scale of the leaf spot and the bacterial population obtained from the estimation of the bacterial population using the scale or disease severity scoring method [13]. Based on the scoring method on potato plant samples with spot scales varied on the four stations.

Based on scoring towards the severity of potato disease, the highest spot scale was scale 3 with 31-50% spot width, while the lowest scale was scale 1 with 10% spot width. The correlation results between INA bacteria number and spot scale on potato plant was analyzed using SPSS with Bivariate Correlations test. The Pearson Bivariate Correlations test assumes that if the r-value of correlation is > 0.05 then there is a correlation between the two variables, and if the sig (1-tailed) <0.05 then both variables have significant correlation [23]. The results of this study showed that the value of r = 0.844 means there was a correlation between the two variables. Sugiyono states that if the correlation value ranges from 0.80 to 1.00 then there is a strong relationship between the two variables [24]. For the significance value of 0.156>0.05 means the value of significance is lower than 95%, the value is about 84.4%.

4.3. *Classification of INA bacteria class*

The nucleation activity can be discovered when the bacterial suspension in the microtube freezes after its insertion into the circulation alcohol bath. The test was performed on three different temperatures namely -4°C, -7°C and -10°C for 10 minutes. The difference in temperature was due to differences in the activity of each bacterial suspension in accordance with its class group [25]. The results of the nucleation activity test by INA bacteria were obtained from several classes of INA bacteria based on the freezing temperature.

Based on the test of nucleation activity, not all isolates were found positive for INA bacteria. There were several classes of INA bacteria from the existing isolates, 2 isolates were classified into class A with freezing temperature of -4°C, 4 isolates were classified as class B with freezing temperature -7 °C and 14 isolates were classified as class C with freezing temperature -10 °C. Four bacteria isolates were

found at an altitude of ± 2000 m ASL while 16 bacteria isolates were obtained at an altitude of ± 2500 m ASL. INA bacteria were classed based on freezing temperature as class A which can freeze water at the temperature of -2 to -4°C. While the freezing point of class B is -5°C to -7°C and below -8°C for class C [26]. It is also supported by Stephanie report of isolated INA bacteria from rainwater which was classified as class B INA bacteria that freeze at -7°C [27].

5. Conclusion

Highest of INA bacteria population in potato leaves i.e. 2.7 x 10^4 was found on scale 3 of leave spots while the lowest value of 7.25 x 10^3 on scale 1. There is strong correlation of INA bacteria population and the scale of frost injury spots even the significance is lower than 95%. Based on the ice forming temperatures, INA bacteria are classified into three classes, i.e., Class A can freeze at temperatures of -2°C to -4°C, class B -5°C to -7°C and class C below -8°C.

Reference

[1] Turasih 2016 *Adaptation strategy to climate change on farmer's household in Dieng Plateau* (Bogor: Bogor Agricultural Institute)
[2] Arwiyanto T 1996 Isolation of ice core bacteria on potatoes *J. Perlindungan Tanaman Indonesia* **2**:12-4
[3] Lindow S E 1993. Novel method for identifying bacterial mutant with reduced epiphytic fitness *J. Appl. Environ Microbiol* **36** 831-8
[4] Goto K, Inaba T and Goto M 1988 Factors affecting frost damage of vegetable crops by artificial: spraying of ice nucleation-active bacteria in a freezing chamber *J. Proc Assoc PI Protec Shikoku* **23** 47-55
[5] Goto M, Gotto T and Inaba T 1989 Identification of ice nucleation sites measured in situ low radiation inactivation analysis *J. Proc Natl Phytopath Soc Japan* **55** 330-5
[6] Lindow S E, Amy D C, and Upper C 1978. Erwinia herbicola: A bacterial ice nucleus active in increasing frost injury to corn *J. Phytopathology* **68** 523-7
[7] Lindow S E 1983 b The role of bacterial ice nucleation in frost injury to plants *J. Annu Rev Phytathol* **21** 363-84
[8] Christner B C, Cai R, Morris C E, McCarter K S, Foreman C M, Skidmore M L, Montross S N and Sands D C 2008 Geographic, seasonal, and precipitation chemistry influence on the abundance and activity of biological of ice nucleators in rain and snow *J. Proc Natl Acad Sci USA* **58** 1334-8
[9] Fardias S 1993 *Analysis of Food Microbiology* (Jakarta: PT. Raja Grafindo)
[10] Blodgett R 2006. Appendix 2, Most Probable Number from Serial Dilution. BAM (Bcteriological Analytical Manual), Chapter 4. FDA (Food and Drug Administration), California.
[11] Lindow S.E. 1990. Bacterial ice nucleation in frost injury to plants *J. Ann. Rev. Phytopathol.* **21** 363-84
[12] Mahdieh R, Hasanzadeh N, Khodaygan P and Riahi- Madvar A 2018 Ice nucleation active bacteria from pistachio in Kerman Province, *Iran J. Plant Pathol.* **100** 51-58
[13] Manandhar H K, Ram D T, Sarala S, Sharada,J., Shrinkhala M, Suk B G, Sajal S, Epsha P, Anju P, Balkrishna J, Gyanu Manandhar, Devendra G, Devra I J and Bhuwon R S 2016 *A field guide for identification and scoring methods of diseases in the mountain crops of Nepal* (Nepal : Biodiversity International) 77-81
[14] Lindow S E, Arny D C and Upper C D 1978 Distribution of ice nucleation-active bacteria on plants in nature *J. Appl Environ Microbiol* **36** 831-6
[15] Nejad P, Ramstedt M, Granhall U, Roos S, Mclvor I 2006 Biochemical characterization and identification of Ice-Nucleation-Active (INA) Willow pathogens by means of BIOLOG®MicroPlate, INA gene primers and PCR-based 16S rRNA-gene analyses *J. Plant Diseases and Protection* **113** 97-106

[16] Cazorla F M, Olalla L, Toresl J A, Perez-Garcia A, Codina J C and de Vicente A 1995 A method for estimation of population densities of Ice-Nucleating Active Pseudomonas syringae in buds and leaves of mango *J. Applied Bacteriology* **79** 341-6
[17] Muriel J, Attard E, Sancelme M, Deguillaume L, Guilbaud C, Morris C E, Amato P and Delort A M 2013 Ice nucleation activity of bacteria isolated from cloud water *J. Atmospheric Envir.***70** 392-400
[18] Govindarajan A G and Steven E L 1988 Size of bacterial ice-nucleation sites measured in situ by radiation inactivation analysis *J. Biochemistry* **85** 1334-8
[19] Montesions E and Viraldell P 1991 Relationships among population levels of Pseudomonas syringae, amount of the nuclei, and incidence of blast dormant flower in Commercial Pear Orchards in Catalunya, *Spain J. Phytopathology* **81** 113-9
[20] Baertlein D A, Lindow S E, Panapoulos N J M, Lee S P, Min-drinos M N and Chen T H H 1992 Expression of bacterial ice nucleation gene in plants *J. Plant Physiology* **100** 1730-6
[21] Hirano S S, L Stuart B and Christen D U 1985 Ice nucleation temperature of individual leaves in relation to population sizes of ice nucleation active bacteria and frost injury *J. Plant Physiol* **77** 259-65
[22] Gross D C, Cody Y S, Proebsting E L, Radamaker J K and Spotts R A 1983 Distribution, population dynamics, and characteristics of ice nucleation-active bacteria in Deciduous Fruit Tree Orchards *J. Applied Envir.Microb.***46** 1370-9
[23] Singgih S 2016 SPSS Complete Guide Version 23 (Jakarta: PT Elex Media Komputindo) Chapter 14 335-43
[24] Sugiyono 2007 Qualitative-quantitative research methods and R & D " (Bandung: Alfabeta)
[25] Keift T L and Ruscceti T 1990 Characterization of biological ice nuclei from A Lichenes *J. Bacteriol* **172** 3519-23
[26] Yankofsky S A, Levin Z, Bertold T and Sandlermand N 1981 Some basic characteristics of bacterial freezing nuclei *J. Applied Meteorol.* **20** 1013-9
[27] Stephanie and Waturangi D E 2011 Distribution of ice nucleation-active (INA) bacteria from rain-water and air *J. Biosciences* **18** 108-12