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Influence of water activity on the ice-nucleating activity of *Pseudomonas syringae*

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Pseudomonas syringae **is known as a biological ice-nucleating agent. The bacterium has the unusual property of increasing the temperature at which water freezes by a few degrees. However, the ice-nucleating activity (INA) always remains lower for *in vitro* cultivated cells, than for cells grown *in planta*. We examined the effects of the hydrophobic environment and of water availability, on the *in vitro* growth and INA of *P. syringae*. The hydrophobic environment was modified by addition of fatty acids, vegetable oils or silicone oil to the culture medium. Addition of olive oil (1%), or traces of silicone oil in the culture medium had a positive effect upon the expression of INA. Variations in water activity from 0.990 to 0.988 by addition of sugar beet fibres or sodium chloride in the culture medium were followed by an increase in INA. This study suggested that control of the medium's water activity must be considered as an important parameter for optimization of INA in *P. syringae*.**

Keywords: water activity; ice-nucleating activity; *Pseudomonas syringae*; membrane fluidity

Introduction

Phytopathogenic strains such as *Pseudomonas syringae* are able to catalyze ice formation at warm subfreezing temperatures (–2 to –4°C). The ice-nucleating activity (INA) depends on an outer membrane protein of *P. syringae*, Ina Z, which could be linked to the membrane by a glycosylphosphatidylinositol (GPI) anchor [19]. In a given population, a small proportion of the bacterial cells (10^{-6} threshold cell frequency) possess a higher INA than the others [21]. The most active cells, termed type I, present ice-nucleating temperatures between –2 and –5°C. The types II and III correspond to ice-nucleating temperatures between –5 and –7°C and –7 to 10°C respectively [25].

These differences in INA expression are related to a differential maturation of the structure of the nucleation site, to the anchorage of the Ina Z protein to the outer membrane and to the aggregation of the Ina Z protein into patches [17]. While ice-nucleating sites active at –10°C are approximately the size of three ice-nucleating proteins (*ca* 360 kDa), sites active at –5 and –2°C have masses of about 2500 and 19 000 kDa, corresponding to about 20 and 120 ice-nucleating proteins respectively. Such large sizes may be sensitive to changes in the physical properties of the membrane [8,12]. These studies further address the hypothesis that the likehood of formation of ice protein aggregates and their stability would be influenced by membrane structure, specifically membrane fluidity.

The fact that *P. syringae* can increase freezing temperature by a few degrees may provide important benefits to industry with applications in snow making [24] or in the production of frozen foods [22,23]. These applications need the development of a large biomass production process of such biological nucleating agents. The culture conditions and the medium composition must be optimized [1,11] in order to obtain a high biomass concentration characterized by a high INA.

In this context, it is interesting to compare the INA of the phytopathogenic strain *P. syringae* when it is cultivated *in vitro*, and when the INA is measured *in planta*. Generally, the INA of bacteria grown *in vitro* remains lower than those after growth on plants [14]. In order to optimize the culture conditions of these bacteria, the *in planta* nutritive and environmental parameters have to be considered. Since *P. syringae* is a natural inhabitant of plants, it is reasonable to assume that INA is influenced by environmental factors such as atmospheric hygrometry. Dickinson [5] showed the importance of a high relative humidity for the growth of *P. syringae in planta*, while Hirano and Hupper [9] found a higher growth and INA when the atmospheric hygrometry was low. Pooley and Brown [15] studied the effect of culture mode on expression of the ice nucleation phenotype, and noted that cells grown on agar plates were considerably more active than those from broth culture. Nevertheless, no one was concerned by the effect of water availability on the expression of INA *in vitro*.

The amount of available water in the environment of microorganisms affects growth, respiration, enzyme synthesis, sporulation and other physiological functions [18]. The aim of our study was to point out the importance of water activity (a_w) on the *in vitro* growth and the INA of *P. syringae*, and to determine if this parameter could be related to a variation of membrane fluidity leading to a modification of aggregation of Ina Z proteins. For that purpose, INA and growth extent were studied in relation to the addition of hydrophobic environment-modifying agents and a_w-controlling agents in the culture medium.

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Materials and methods

Bacterial strain and culture conditions

P. syringae No. 7420 was obtained from the Institut Pasteur collection. It was maintained on nutrient agar or deep frozen with 15% glycerol in the culture medium.

The standard culture medium was a semi-defined medium which was complemented with additives depending on the assays. As previously described [3], its composition was: glucose 90 g L^{-1}; meat peptone 28 g L^{-1}; K_2HPO_4 2.87 mmol L^{-1}; KH_2PO_4 3.67 mmol L^{-1}; trisodium citrate 56 mmol L^{-1}; and was supplemented with a salt solution leading to the following final concentrations: $CaCO_3$ 10 μmol L^{-1}; $ZnSO_4 \cdot 6H_2O$ 12.1 μmol L^{-1}; $FeCl_3 \cdot 6H_2O$ 100 μmol L^{-1}; $MnCl_2 \cdot 4H_2O$ 25 μmol L^{-1}; $CuCl_2 \cdot 2H_2O$ 4.98 μmol L^{-1}; $CaCl_2 \cdot 6H_2O$ 8.1 μmol L^{-1}; H_3PO_2 3.16 μmol L^{-1}; $NaMoO_4 \cdot 2H_2O$ 10 μmol L^{-1}; MgO 1.24 mmol L^{-1}.

Sugar beet fibres were obtained from AgroIndustrie Recherche et Développement (ARD, Pomacle, France). Palmitic and myristic acids were from Sigma, vegetable oils from Lesieur (France) and silicone oil Rhodorsyl 47V20 from Rhone Poulenc (France).

Precultures and cultures were carried out in 500-ml Erlenmeyer flasks and incubated at 24°C on an oscillary shaker (110 rpm).

Analytical techniques

Biomass evaluation: Growth was evaluated at the end of the exponential phase by turbidity measurements at 600 nm (1 optical density unit at 600 nm [OD 600] = 1.1×10^8 cells ml^{-1}).

Ice-nucleating activity determination: Two milliliter-samples of cell culture in late exponential phase were withdrawn. Cells were harvested by centrifugation (10 min; $7000 \times g$), then resuspended in sterile distilled water in order to obtain 7.5×10^9 cells ml^{-1}. The ice-nucleating activity was determined by using the standard drop freezing method [20]. Drops from the cell suspension (10 μl) were deposited on aluminium sheets and cooled at the rate of 1°C min^{-1}. The number of frozen drops was counted at each temperature step. Freezing temperature spectra were obtained for every culture condition as a function of temperature in percent of frozen drops. The qualitative estimation of the INA was expressed as the temperature at which 90% of the drops were frozen (T_{90}). The T_{90} values reported here were mean values of three measurements which were determined with an experimental error of ± 0.1°C.

Serial dilutions were used in order to obtain the entire ice nucleus spectrum of bacterial suspensions. The ice nucleus concentration in each dilution was normalized to the concentration in the original suspension. The quantitative estimation of the INA was given by the nucleation frequency which is the fraction of cells active at a given temperature, determined as freezing nuclei units per cell (FNU $cell^{-1}$) by Vali's equation [20].

The maximal discrepancy calculated for the log concentration of ice nuclei values was 0.25, leading to an error coefficient of 1.8 FNU $cell^{-1}$.

Determination and modification of water activity: Before sterilization, the water activity (a_w) of the media was modified by addition of various amounts of sodium chloride, sugar beet fibres or oils. Water activity was measured at the beginning of the culture, using an hygrometer (AWX 3001, Bioblock Scientific, Illkirch, France) with an experimental error of ± 0.002.

Results

Influence of the addition of fatty acids or oils to the culture medium

The aim of this study was to point out the effect of the qualitative and quantitative modification of the hydrophobic environment during growth on the INA. Govindarajan and Lindow [8] and Lindow [12] indicated that INA decreases when the membrane fluidity increases. Their results suggested that a hydrophobic environment provided by lipids is required for structural organization of the ice nucleus, and the expression of the phenotype. Other authors confirmed [17] that the ice-nucleating sites can be disaggregated when the membrane fluidity increases.

In the present study, the hydrophobic environment was modified by addition of fatty acids [palmitic acid (C18:0) and myristic acid (C14:0)] to the culture medium. The choice of these fatty acids was made because myristic acid is found in the GPI anchor of the variant surface glycoprotein of the trypanosoma [6] and because palmitic acid is a metabolic precursor of myristic acid.

The INA measured when bacteria were grown in the standard medium without lipid addition, were $T_{90} = -3.5$°C and 3×10^{-8} FNU $cell^{-1}$ at –3.5°C, and considered as the control values. When the concentration of added pure fatty acids was increased from 1% to 5% (v/v), T_{90} decreased (by 0.2°C to 0.5°C) while the nucleation frequencies remained about 10^{-8} FNU $cell^{-1}$ at –3.5°C (Table 1a).

When vegetable oils were used at 1% (v/v), an increase of INA was observed. T_{90} increased by 0.5°C to 1.4°C, while nucleation frequency reached 3×10^{-6} FNU $cell^{-1}$ at –3.5°C (Table 1b). At concentrations from 2.5–5% (v/v), the T_{90} decreased by 0.1°C to 1.6°C and the nucleation frequency decreased to about 10^{-10} FNU $cell^{-1}$ at –3.5°C. Concurrently, control tests were performed in the standard medium without bacteria and without oils. In these controls, INA was not detected.

From the quantitative point of view, our results showed that when the concentration of oils reached 2.5% (v/v), an inhibitory effect was observed on INA, characterized by a low production of ice nuclei per cell.

Vegetable oils contain various proportions of saturated and unsaturated fatty acids. The noted variations between the different oils might be due to variation of the nature of the fatty acids possibly metabolized and integrated in the bacterial membrane leading to a modification of the aggregation of the Ina Z proteins. Poly-unsaturated fatty acids are highly fluid, but mono-unsaturated and saturated fatty acids are characterized by their low fluidity.

Our results showed that the highest INA ($T_{90} = -2.1$°C and 3.10^{-6} FNU $cell^{-1}$ at –3.5°C) was obtained with olive oil at 1% (v/v) (Table 1b). Olive oil is characterized by its high content of mono-unsaturated fatty acids (76% of the

Table 1 Influence of the addition of various proportions of fatty acids and oils to the culture medium, on the ice nucleation activity of *Pseudomonas syringae*, expressed as T_{90} (°C) and FNU cell^{-1} at –3.5°C

		Proportion of oil added (v/v)				
		0%	0.01%	1%	2.5%	5%
(**a**)	Palmitic acid (C18:0)	–3.5 3×10^{-8}	–3.5 3×10^{-8}	–3.7 1.7×10^{-8}	–4.0 8.6×10^{-9}	–3.8 1.1×10^{-8}
	Myristic acid (C14:0)	–3.5 3×10^{-8}	–3.5 3×10^{-8}	–3.8 1.1×10^{-8}	–3.7 2.1×10^{-8}	–4.0 1.5×10^{-8}
(**b**)	Olive oil	–3.5 3×10^{-8}	–3.5 3×10^{-8}	–2.1 3×10^{-6}	–5.0 5×10^{-10}	–5.0 2.3×10^{-9}
	Rape oil	–3.5 3×10^{-8}	–3.5 3×10^{-8}	–3.1 3×10^{-6}	–4.0 1.1×10^{-9}	–3.7 2.9×10^{-9}
	Maize oil	–3.5 3×10^{-8}	–3.5 3×10^{-8}	–3.0 3×10^{-6}	–4.0 1.1×10^{-9}	–4.5 5.5×10^{-10}
	Peanut oil	–3.5 3×10^{-8}	–3.5 3×10^{-8}	–3.0 3×10^{-6}	–3.5 2.8×10^{-8}	–4.9 1.7×10^{-9}
	Sunflower oil	–3.5 3×10^{-8}	–3.5 3×10^{-8}	–3.0 3×10^{-6}	–5.0 5×10^{-10}	–5.1 5×10^{-10}

total amount of fatty acids). These results suggested that the addition of 1% oil with a high level of low fluidity fatty acids in the culture medium may provide good conditions for the proper assembly and structural organization of ice nucleation sites.

Influence of addition of silicone oil

The presence of lipids in the culture medium due to addition of vegetable oils, even at low concentrations, may modify metabolism and growth of *P. syringae* [10]. In order to determine the influence of modification of the hydrophobic environment on INA, we needed to suppress the possible nutritional effect of oils on the bacteria. For that purpose, we used an amorphous mineral oil, silicone oil in a range of 0.01–20% (v/v). In a preliminary study, we carried out assays with silicone oil as the only carbon source and no growth of *P. syringae* was observed (data not shown).

The water activity of these media and the INA obtained with *P. syringae* cells withdrawn at the end of the culture period were evaluated (Table 2).

Addition of traces of silicone oil (0.01% v/v) to the standard medium led to a slight decrease of a_w value (0.993 *vs* 0.998 in the standard medium). The INA obtained with traces of silicone oil was very high ($T_{90} = -1.5$°C). In contrast, traces of vegetable oils have been shown to have no effect on the INA (Table 1b). Moreover, with higher proportions of silicone oil (1–20% v/v), a_w decreased from 0.985 to 0.961 while the T_{90} values remained very high. Although we observed inhibition of INA with increasing concentrations of vegetable oils, we detected no inhibition with increasing concentrations of silicone oil.

Table 2 Influence of the addition of various proportions of silicone oil on the water activity (a_w) of the culture medium and on the INA of *P. syringae*, expressed as T_{90} (°C) and FNU cell^{-1} at –2.5°C

Silicone oil (%) (v/v)	a_w	T_{90} (°C)	FNU cell^{-1} (–2.5°C)
0	0.998	–3.5	2.3×10^{-9}
0.01	0.993	–1.5	3×10^{-6}
1	0.985	–2.9	7×10^{-9}
5	0.977	–2.6	5.5×10^{-9}
10	0.970	–2.5	7.6×10^{-9}
20	0.961	–2.5	1×10^{-9}

From the quantitative point of view, the nucleation frequency reached 3×10^{-6} FNU cell^{-1} at –2.5°C when a trace of silicone oil was added to the culture medium. With higher proportions, the control value of nucleation frequency was restored (about 10^{-9} FNU cell^{-1}). These results suggested that the addition of traces of silicone to the standard medium led to the production of numerous and highly structured ice nucleation sites.

Influence of the modification of the medium's water activity

The influence of the medium's water activity on cell growth extent and on INA was studied. We chose sodium chloride and sugar beet fibres as a_w-depressors, and tested concentrations of 0.1–1.0 mol L^{-1} and 0.5–2.5% (w/v) respectively. The a_w values measured in these media decreased from 0.990 to 0.957 with sodium chloride and to 0.942 with fibres.

A decrease in a_w led to a decrease in growth extent as illustrated in Figure 1. At an a_w of 0.957 (1.0 mol L^{-1} NaCl), the optical density dropped to 5% of the control value obtained at an a_w of 0.990. However, the sodium chloride salt might present limited interest as an a_w-controlling agent and NaCl may be toxic to cells when its concentration reaches 1.0 mol L^{-1}. Moreover, this salt has an antifreeze effect and may interfere with INA measurements, even if the cells were carefully washed to eliminate traces of culture medium.

Sugar beet fibres, which were thought to be neither toxic nor available as a carbon source to *P. syringae*, were added to the medium as an a_w-depressing agent from 0.5 to 2.5% (w/v). As the sugar beet fibre concentration increased, the medium's a_w decreased and it led to a limitation of growth. A rapid decrease in optical density was observed below an

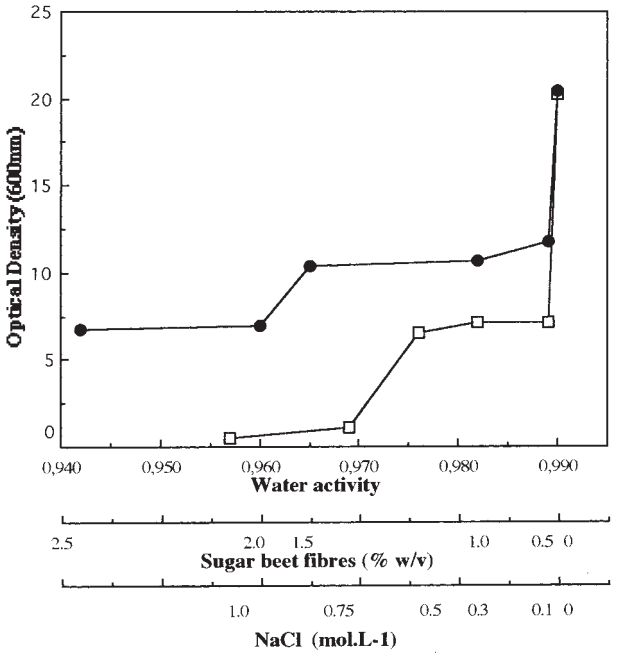

Figure 1 Growth of *Pseudomonas syringae* expressed as optical density (OD), as a function of the water activity of the medium (a_w) with NaCl (–□–) and sugar beet fibers (–●–) as a_w depressors.

a_w of 0.990, and it reached 33% of the initial value when the a_w was 0.942 (Figure 1).

The effect of decreasing a_w on INA was studied with sodium chloride and sugar beet fibres as a_w-depressors (Figure 2).

With sodium chloride, the INA strongly increased up to $T_{90} = -2.0°C$ for a slight decrease of a_w (0.990–0.988). An a_w of 0.988–0.982, obtained by addition of 0.1–0.3 mol L^{-1} of sodium chloride in the medium, led to the highest INA ($T_{90} = -2.0°C$). Below an a_w of 0.982, INA expressed as T_{90} seemed affected and decreased with a_w decreasing.

From the quantitative point of view, as the a_w decreased from 0.990 to 0.982, the nucleation frequency increased from $<10^{-9}$ to 1.5×10^{-8} FNU $cell^{-1}$ at –2°C. Therefore, the addition of 0.3 mol L^{-1} of sodium chloride in the medium led to the production of numerous and highly structured ice nucleation nucleus.

With the addition of sugar beet fibres, the INA reached the optimal value ($T_{90} = -2.0°C$) when a_w decreased from 0.990 to 0.988. When the a_w decreased below 0.988 (0.5% sugar beet fibres w/v), the INA remained at its optimal value, and the threshold ice-nucleating temperature was still –2.0°C at an a_w of 0.942.

The nucleation frequency increased from 10^{-9} to about 10^{-8} FNU $cell^{-1}$ at –2°C when the a_w decreased from 0.990 to 0.982. Then, as the a_w decreased below 0.982 (1–2.5% sugar beet fibres), the nucleation frequency remained at about 10^{-8} FNU $cell^{-1}$. These results showed that the INA obtained with *P. syringae* grown on standard medium supplemented with 1–2.5% sugar beet fibres expressed a high concentration of very active nuclei.

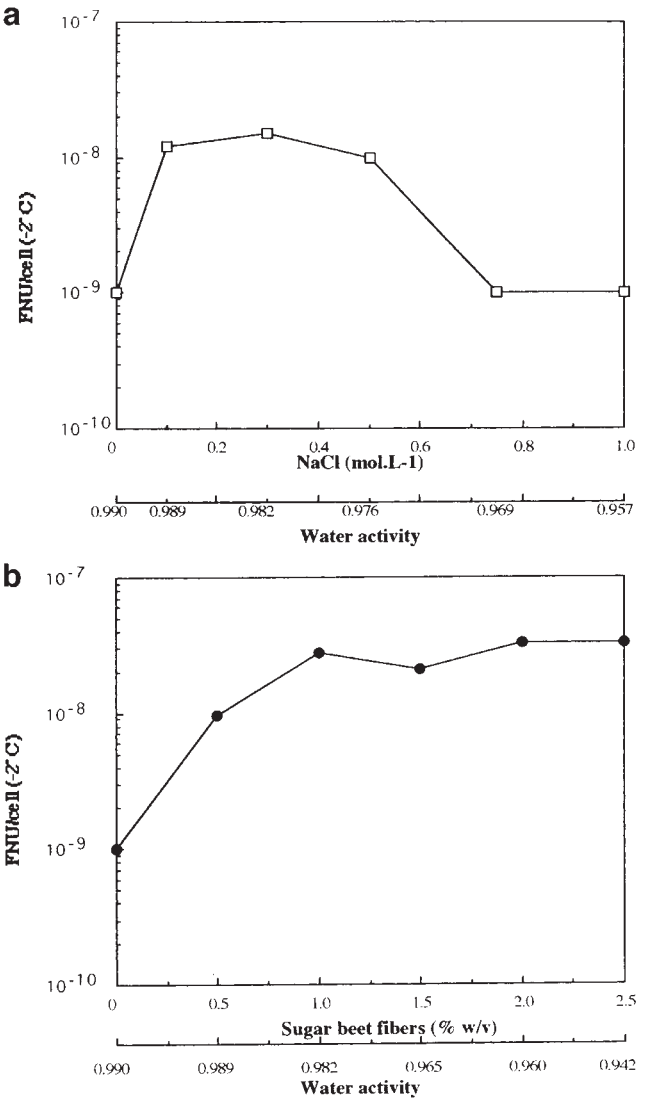

Figure 2 (a) Ice nucleation activty of *Pseudomonas syringae* expressed as nucleation frequency (FNU $cell^{-1}$ at –2°C), as a function of water activity of the medium (a_w) with NaCl as a_w depressor. (b) Ice nucleation activity of *Pseudomonas syringae* expressed as nucleation frequency (FNU $cell^{-1}$ at –2°C), as a function of water activity of the medium (a_w) with sugar beet fibres as a_w depressor.

Discussion

The INA is higher when *P. syringae* is established on a plant ($T_{90} = -0.6°C$) than when it is grown *in vitro* [14]. The influence of *in vivo* environmental parameters on INA is partly known. Physicochemical parameters such as temperature or pH [11,13], and the composition of the *in vitro* culture medium [3] influence the evolution of the INA. However, little is known about the mechanism involved in the control of INA.

In other microbiological systems, numerous studies have shown the importance of the control of the medium's water activity upon growth and metabolism [2]. Since *P. syringae* is a nucleating agent of water, the impact of water availability (expressed as water activity) on growth and INA must be considered.

The relationship between the amount of available water

in the micro-environment and membrane fluidity was already pointed out [7], and Curtain *et al* [4] showed that membrane fluidity of *Dunaliella* sp decreased when sodium chloride concentration increased in the medium. In this work, the protein mobility in the membrane varied as a function of the osmotic stress. From the structural point of view, a lipid phase separation model was suggested in which, for low water availability, the phospholipid bilayer would turn into a hexagonal micellar structure [16]. Work with *P. syringae* [8] showed that highly fluid lipids were less effective in reconstituting ice nucleation activity in delipidated bacterial membranes, than lipids having a lower fluidity. These results were confirmed by Lindow [12], who directly established a link between stability of ice nuclei and the concentration of 2-phenethyl alcohol (a membrane-fluidizing agent) in which the cells were suspended. A large decrease in the stability of bacterial ice nuclei occurred when cells were exposed to increasing amounts of the membrane-fluidizing agent.

Our study focused on the relationships between physico-chemical conditions during the culture of *P. syringae* (hydrophobic environment and water activity), and INA. Results presented here showed that INA was modified by addition of fatty acids or vegetable oils to the culture medium. Inhibition of INA was pointed out when fatty acids and vegetable oils were added at 1% and 2.5% respectively. It is interesting to point out that the olive oil added at 1% (v/v), characterized by its high content of low fluidity fatty acids, led to the highest INA, expressed as T_{90} and nucleation frequency. In agreement with Lindow [12], these results showed that membrane fluidity may be controlled during the growth of *P. syringae*.

Silicone oil addition to the medium led to a larger increase of INA. Whatever the concentrations used, no inhibition of INA was detected. With only traces of silicone oil in the standard culture medium, a very high INA was obtained ($T_{90} = -1.5$°C and 3.10^{-6} FNU cell^{-1} at −2.5°C). This was unexpected and three hypotheses are put forward to explain the action of the silicone oil: (1) it was integrated in the cell membrane leading to a modification of membrane fluidity; (2) it stabilized the proteinaceous structure of the outer membrane; or (3) it induced a modification of the culture medium's a_w.

In this study, silicone oil was shown to act as an a_w-depressor and as an INA-improving agent. Since modification of the culture medium's a_w was known to be involved in modification of membrane fluidity, silicone oil might be considered a membrane-fluidity-modifying agent.

Neither toxic to cells, nor used for growth, sugar beet fibres appeared as an interesting a_w-depressor. Under water stress conditions, we observed a different effect upon growth and upon INA. When a_w was under 0.990, growth began to decrease sharply, while INA was enhanced. These results are consistent with previous studies [1] which reported that INA was enhanced under growth limitation conditions.

From the present study, we can suggest that the control of the medium's water activity is an interesting parameter for optimization of the INA. It must be considered for the development of an optimized medium aimed at producing *P. syringae* with high ice-nucleating activity. The use of sugar beet fibres (0.5% w/v) or traces of silicone oil as a_w-depressing agents in the culture medium, led to an enhancement of *in vitro* INA expressed as a high concentration of very active nucleus.

Further studies on the modification of membrane fluidity would be useful to show the involved molecular mechanism under water stress conditions and to establish its relationships with high efficiency ice nucleation sites in *P. syringae*.

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