ECOLOGY OF *PANTOEA AGGLOMERANS* 2066-7 STRAIN: A BIOLOGICAL CONTROL OF BACTERIA ONION DISEASES

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ABSTRACT

The growth response of the biocontrol agent *Pantoea agglomerans* 2066-7 to change in water activity (a_w), temperature, and pH was determined *in vitro* in basic medium. The minimum temperature at which 2066-7 was able to grow was 7°C, and the growth of 2066-7 did not change at varying pH levels (4–10.34). The best growth was obtained at a water activity of 0.98 in all media modified with the four solutes (glucose, glycerol, NaCl and polyethylene glycol). The solute used to reduce water activity had a great influence on bacterial growth, especially at unfavorable conditions (low temperature). This study has defined the range of environmental conditions (a_w, pH, and temperature) over which the bacteria may be developed for biological control of plant diseases.

Keywords: *Pantoea agglomerans*, ecology, water activity and plant diseases

INTRODUCTION

The control of diseases in fruits and vegetables is still mainly based on the use of synthetic pesticides, although the demand for produce free from chemical residues and the emergence of pesticides -resistant pathogen strains is constantly increasing.

Biological control using microbial antagonists has attracted much interest as an alternative to chemical products (**Droby and Chalutz, 1992**; **Vinas *et al.,* 1998**). However, very few of the biocontrol agents studied have been successfully commercialized.

Following application on fruits and vegetables, biocontrol agents are exposed to a range of variable biotic and abiotic stress factors, such as competition, predation, changes in temperature, osmolarity, low pH, availability of nutrients and water. Therefore, biocontrol in the field has often been limited by fluctuating environment and the narrow range of conditions over which successful establishment and effective pest and/or disease control is possible (**Hallsworth and Magan, 1994a,b**).

Dehydration of the product and maintenance in a dry environment is one of the best way to formulate microbial agents, so that they can be handled using the normal distribution and storage channels (**Rhodes, 1993**).

Unfortunately, not all microorganisms are amenable to drying and many tend to lose viability during both the drying process and storage. Biological systems are currently preserved by reducing their water content and thereby the water activity (a_w) of the medium. Thus, water activity is a measure of the availability of water for biological functions and relates to water present in a food in "free" form. In food system, total water of moisture is present in "free" and "bound" forms. Bound water is necessary to hydrate the hydrophilic molecules and to dissolve the solutes and not available for biological functions; as well, it does not contribute to water activity. The free water in a food is necessary for microbial growth. It is necessary for the transport of nutrients and the removal of waste materials, to carry out enzymatic reactions, to synthesize cellular materials, and to take part in other biochemical reactions. Each microbial species (or group) has an optimum, maximum, minimum water activity level for growth. When the water activity is reduced below the minimal level for growth of a microorganism, the cells remain viable for a while. Nevertheless, if the water activity is reduced drastically, microbial cells will lose viability, generally rapidly at first, and then more slowly (**Magan and Lacey, 1988; Plaza *et al.,* 2003; mathlouthi, 2001**).

Temperatures and pH of environment are also limited factors to the development of microorganisms. However, to assure a good application of biological control agent in the field and during storage a study of the optimal water activity,

temperatures and pH of development of this agent is very important (**Cañamás *et al.,* 2009**).

In this work and at the first time in Morocco we studied the ecology of a biological control agent of onion bacteria disease. The experiments were conducted in the laboratory of Plant Protection URPP- INRA-Meknes in collaboration with the Laboratory of Plant Biotechnology and Molecular Biology, Faculty of Sciences-Meknes. The main objective of this work is to study the improvement of a_w, temperature and pH tolerance observed in *Pantoea agglomerans* 2066-7.

MATERIALS AND METHODS

Effect of temperature on growth of *P. agglomerans*

To test the ability of *P. agglomerans* 2066-7 to grow at different temperatures onto YPGA medium (5 g.L^{-1} yeast, 5 g.L^{-1} peptone and 10 g.L^{-1} glucose, 18 g.L^{-1} agar), the bacterium was incubated during 24 to 48h under 7°C, 25°C, 30°C, 36°C, 38°C and 40°C.

Effect of pH on growth of *P. agglomerans*

In order to perform the survival study, 9mL of non-acidified (control pH 6.9 ± 0.1) and liquid media at pHs 2, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 10 or 13 ,were inoculated with *Pantoea agglomerans* 2066-7. From the pre-culture, the liquid medium was inoculated by 20µL of 10^7 UFC.mL^{-1} of *P. agglomerans* suspension. The medium employed was "YPG", consisted of 5 g.L^{-1} yeast, 5 g.L^{-1} peptone and 10 g.L^{-1} glucose, for the culture of *P. agglomerans*. All pH determinations were made with the Beckman pH meter, and the pH was adjusted with normal HCl or normal NaOH. The growth was tested by measure of optical density by spectrophomometre (600nm).

Non- acidified basal liquid medium was used as a control.

Population dynamics of *P. agglomerans*

Population dynamics of 2066-7 *P. agglomerans* bacterium were determined on the surfaces of onion bulbs, with or without wounds. Two sets of bulbs were rinsed in fresh water after harvest. One of these sets was wounded (1 mm long and 2 mm deep). All bulbs were dipped in a 2066-7 suspension 10^8 CFU mL^{-1} for 30 s. Once dried, bulbs were placed on tray packs in plastic boxes and incubated at moist chamber at 7°C and 25°C. Populations of 2066-7 were monitored at 0 (just prior to storage), 24, 48, 288 and 312 h on bulbs stored at 25°C, and 0, 1, 2,

18, 20, and 21 days on cold stored bulbs. Four bulbs constituted a single replicate and each treatment was replicated four times. The experiment was carried out twice.

The pieces of peel surface of 1g were removed (including the wounded areas in the case of wounded bulbs) with a knife. Pieces of peel were shaken in 9 ml sterile water on agitator for 30 mins with 125 t/min. Serial dilutions of the washings were made and plated on YPGA Medium (yeast extract, 5 g .L^{-1}; peptone, 5 g. L^{-1}; glucose, 10g .L^{-1} and agar, 15 g .L^{-1}). Colonies were counted after incubation at 25°C in the dark for 48 h. Population sizes were expressed as CFU ml^{-1} of bulb surface.

Effect of water activity on growth of *P. agglomerans*

Basic medium

The basic medium used in this research consisted of sucrose (10 g .L^{-1}) plus yeast extract (5 g .L^{-1}) with a pH of 6-7 and a$_w$ of 0.995 (**Costa *et al.*, 2001**). This medium demonstrated good and cost-effective production while maintaining biological control efficacy.

Osmotic stress characterization with different solutes

To obtain information about the a$_w$ solute profile of *P. agglomerans* bacterium, was grown in basic liquid medium and a known amount of CFU were inoculated in Petri dishes that contained the basal agar medium modified with the ionic solute; NaCl and with the nonionic solutes; glycerol, glucose and polyethylene glycol (PEG 600), to 0.98, 0.97, 0.96 and 0.95 a$_w$ (**Teixido *et al.*, 2006**). The solid agar media were inoculated by spread plating a 20µL aliquot of a 10^5 CFU mL^{-1} bacterial suspension of *P. agglomerans* incubated at 7°C, 25°C and 30°C and then visually examined them every 24 h to determine the presence or absence of growth of colonies. This was performed for each a$_w$ and solute condition. All treatments were carried out with four replicates, and the experiment was repeated twice.

Media from the same a$_w$ were always sealed in plastic polyethylene bags to maintain the equilibrium relative humidity conditions and prevent water loss. Percentage of cultivability was calculated comparing CFU on treatments in relation with the control (basal agar medium).

Statistical analysis

Growth rates were subjected to the variance analysis (SAS Institute, INRA). Statistical significance was judged at the $P < 0.05$ level. When analysis revealed statistically significant differences, Duncan's multiple range test for separation of means was performed.

Response surface methodology (RSM) with a generalized linear model design was applied with the SPSS 20. Temperature (7, 25 and 30°C) and a$_w$ (0.98, 0.97, 0.96 and 0.95) were investigated.

RESULTS

Effect of temperature on growth of *P. agglomerans*

Table 1, show the ability of *P. agglomerans* strain to growth at a temperature from 7°C to 38°C.

Table 1 Temperature influence on growth of *P. agglomerans* 2066-7 strain

Temperature	7°C	25°C	30°C	36°C	38°C	40°C
Growth rate	(+)	(+)	(+)	(+)	(+)	(-)

Legend: (+) Growth, (-) Absence of growth

Effect of pH on growth of *P. agglomerans*

Figure 1, show the adaptation of *P.agglomerans* at large range of pH conditions. The initial of colony growth was observed after 24h of incubation on the basic and acidified medium (from 4 to 10.34). The growth was absent in a pH values less than 4 and high than 10.34.
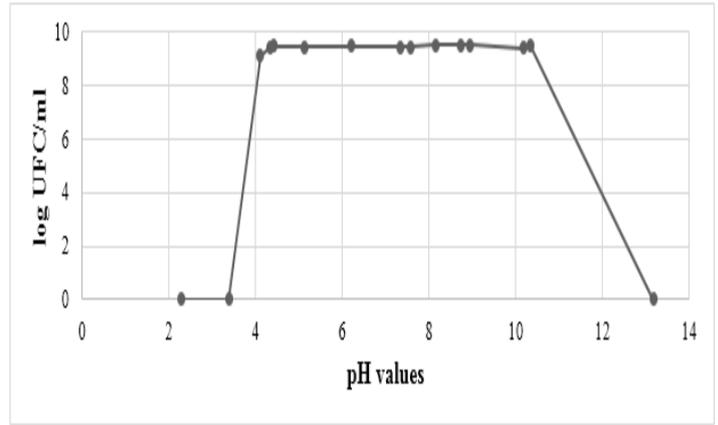
Figure 1 Effect of pH on *P. agglomerans* 2066-7 growth

Population dynamics of *P. agglomerans* bacterium

Population of *P. agglomerans* on bulb surfaces showed the same pattern under the two tested temperature conditions (Fig.2). During the first 24h, the population degreased to return to the initial concentration after 12 and 20 days of incubation under 25°C and cold temperature respectively.
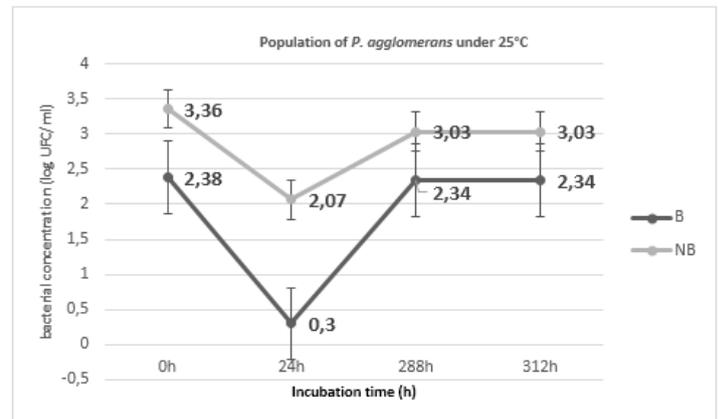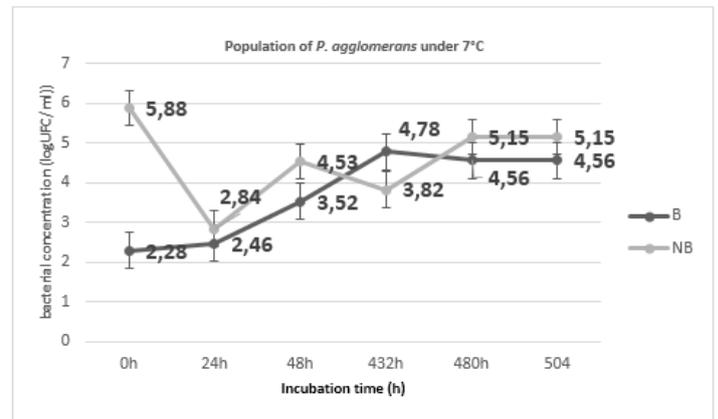
Figure 2 Population dynamics of *P. agglomerans* 2066-7 on surfaces of unwounded (NB). and wounded (B). Onion bulbs incubated at 7°C for 21 days and at 25°C for 13 days. Points represent the means of four replicates and the vertical bars are standard errors.

Osmotic stress characterization with different solutes

The cultivability of *P. agglomerans* at suboptimum a$_w$ media modified with different solutes is shown in figure 3. The growth of the bacterium was very limited in the presence of glucose under 7°C and 30°C and in the presence of glycerol under 7°C. However, it was completely inhibited at values of a$_w$ ≤ 0.97 and at 0.95 under 25°C on the presence of glycerol and glucose respectively. Generally, under of greater water stress and low temperature there was increased in lag times prior to growth initiation. All solutes exhibited 100% cultivability or near than at ≥ 0.96 under 25°C. These results reflect a good adaptation of *P.agglomerans* on modified medium under 25°C.
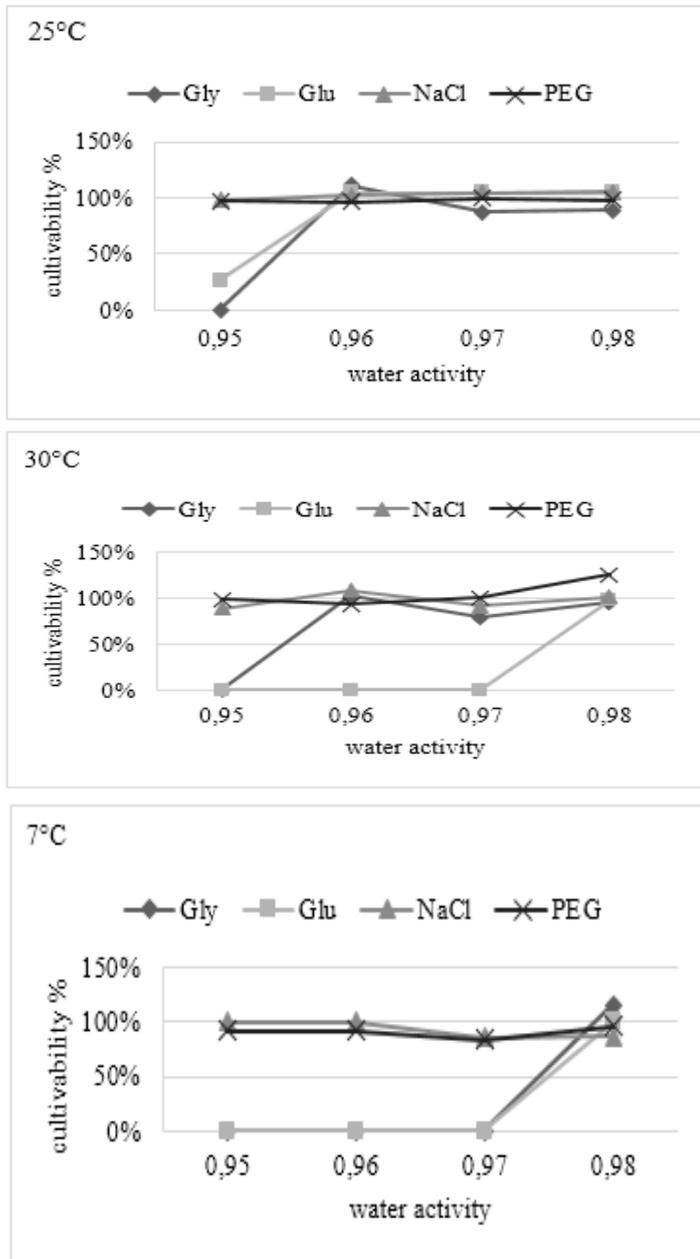
Figure 3 Percentage of cultivability (CFU on treatments in relation with CFU on control basic agar medium) of *P. agglomerans* on low water activity (a_w) modified media using different solutes: glycerol (Gly), polyethylene glycol (PEG), glucose (Glu) and NaCl. Tested a_w were 0.98, 0.97, 0.96 and 0.95. Values are mean of two experiments of four replicates each.

Figure 4, show the growth rate (μ ((LogUFC*10exp2/mL)/j)) of *P. agglomerans* at 7°C on the basic medium supplemented with various solutes us to adjust the water activity. In general, the growth rate degreased as the water activity of the medium degreased. The growth rate was highest at water activity of 0.98 whatever the solute. In the presence of polyethylene glycol or NaCl, *P. agglomerans* was able to grow at all values of a_w. However, the growth stopped at a_w of 0.95 in the presence of glucose or glycerol.

At 25°C, initial of colony growth advanced one day as compared to initiation at 7°C, moreover, the growth rate was higher at 25°C than at 7°C. At a_w of 0.96 and 0.95 the growth rate was highest on basic medium supplemented with glycerol or glucose and NaCl or polyethylene glycol respectively. Growth was stopped at 0.95 a_w in the presence of glucose or glycerol. On basic medium supplemented with polyethylene glycol or NaCl, *P. agglomerans* was able to grow at all a_w values tested at 30°C, the growth stopped at 0.97 and 0.95 on modified medium by glucose and NaCl respectively. Growth rates were much lower as at 30°C than 25°C and at 7°C than 30°C.

Statistical analysis of data, based on variance analysis with three criteria, provided evidence of significant effect ($P < 0.05$) of solutes and incubation temperature and interactions thereof on the growth rate of *P. agglomerans*. Duncan's multiple range analysis confirmed a significantly higher growth rate at 0.98 that any other conditions tested, whatever the solute used to adjust the a_w of the medium (Fig.5-A). Apropos of incubation temperature, the higher growth rate

was noted under 25°C with a value of 1.7718 (LogUFC*10exp2/mL)/j), under 7°C the value was 0.94 (LogUFC*10exp2/mL)/j) (Fig.5-C).

Finally, the test revealed two distinct groups for the influence of solutes; the growth rate was slightly high on medium   modified with polyethylene glycol (1.69) or glycerol (1.52) that unmodified medium (1.516), and reduced when NaCl (1.50) added and reduced more strongly in the presence of glucose (0.59) (Fig.5-B).
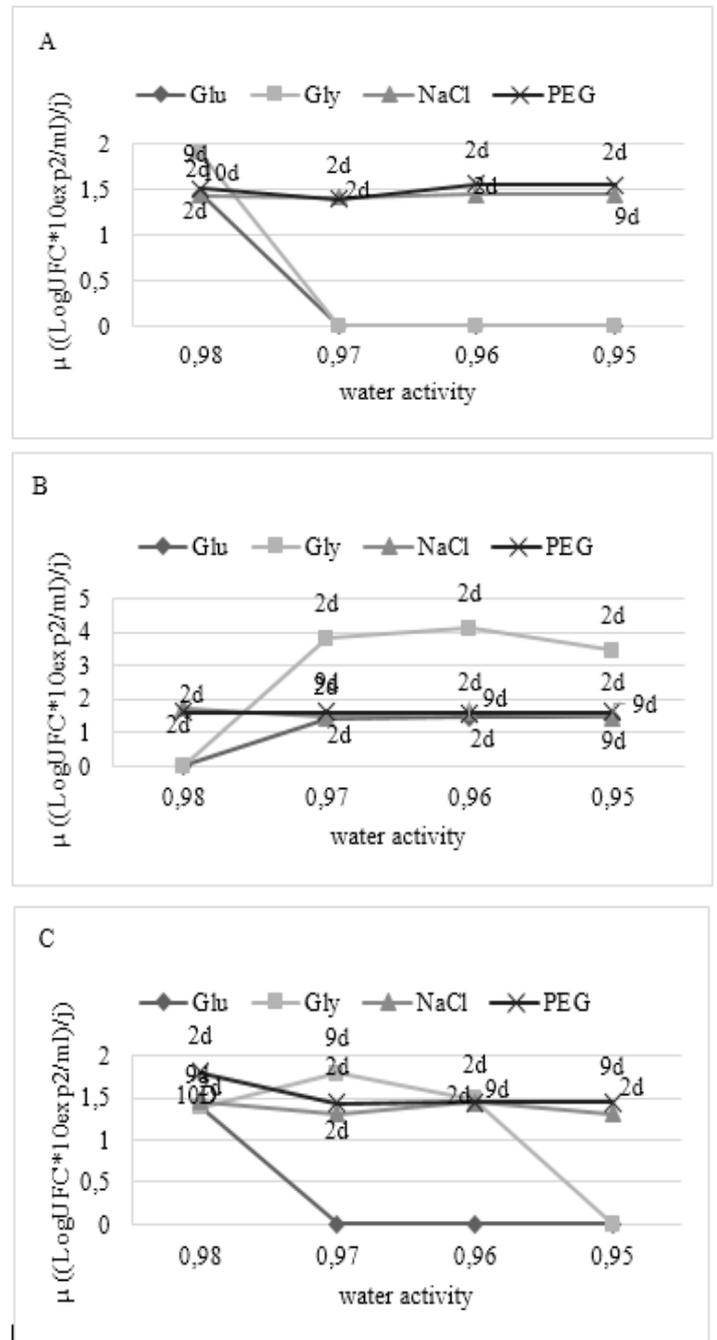
Figure 4 Effect of water activity on growth rate (μ) of *P. agglomerans* in modified medium with, glycerol (Gly), polyethylene glycol (PEG), glucose (Glu) and NaCl at 7°C (A), 25°C (B) and 30°C (C).The number of days for initiation of growth is shown.
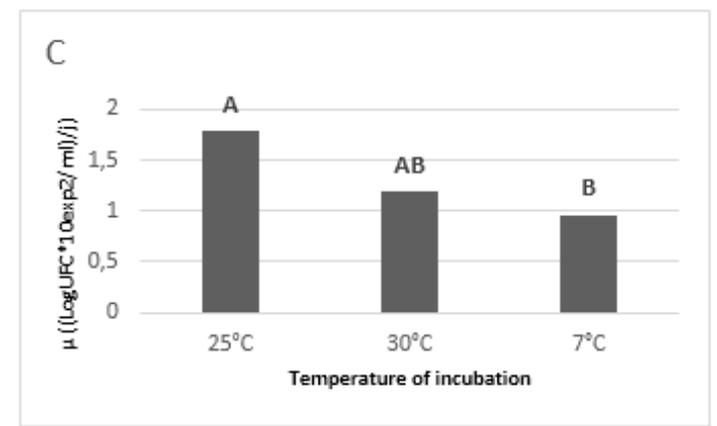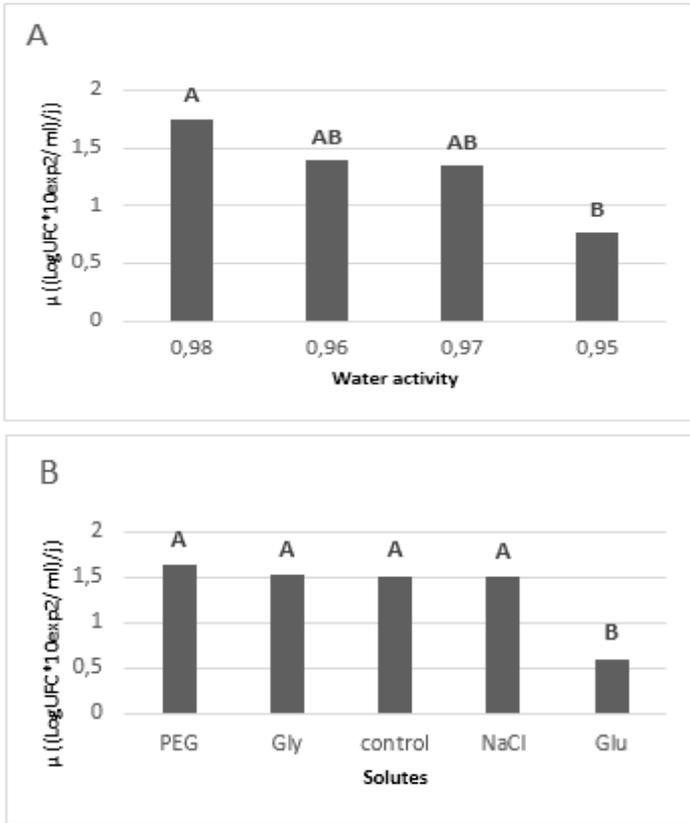
Figure 5 Comparison of *P. agglomerans* growth rate (μ) averages per treatment [water activity (A), solutes (B) and temperature of incubation (C)] performed by the Duncan's range multiple test. The treatments having same letters are not significantly different (P <0.05).

Modelling

The average growth rates obtained with the model under the various conditions are reported in the table 2. No difference was observed between the observed values and those predicted by the all solutes model. To determine the conditions for growth of *P. agglomerans*, responses showing the predicted effect of a_w and temperature were down from the generalized linear model. For the four models (Fig.6), the response surface showed a growth rate sensitive to the incubations temperature and water activity.

Growth was predicted to be higher at an a_w of 0.98, whatever the temperature tested, the highest growth rate was observed, and the optimum of growth rate was noted at 0.96 under 25°C.

Table 2 Experimental and predicted values of growth rate of *P. agglomerans* obtained by applying generalized linear model for temperature and a_w with glycerol, polyethylene glycol and NaCl models

Environment factor		Extension growth rate					
		Glycerol		polyethylene glycol		NaCl	
Temperature	a_w	Observed	Predicted	Observed	Predicted	Observed	Predicted
7°C	0.95	0	0	1.6	1.6	1.44	1.44
25°C	0.95	0	0	1.644	1.644	1.72	1.72
30°C	0.95	0	0	1.45	1.45	1.37	1.37
7°C	0.96	0	0	1.66	1.66	1.44	1.44
25°C	0.96	4.2	4.2	1.62	1.62	1.62	1.62
30°C	0.96	1.5	1.5	1.53	1.53	1.71	1.71
7°C	0.97	0	0	1.42	1.42	1.41	1.41
25°C	0.97	3.85	3.85	1.62	1.62	1.457	1.457
30°C	0.97	1.96	1.96	1.55	1.55	1.44	1.44
7°C	0.98	1.89	1.89	1.65	1.65	1.43	1.43
25°C	0.98	3.45	3.45	1.62	1.62	1.45	1.45
30°C	0.98	1.44	1.44	2.23	2.23	1.54	1.54
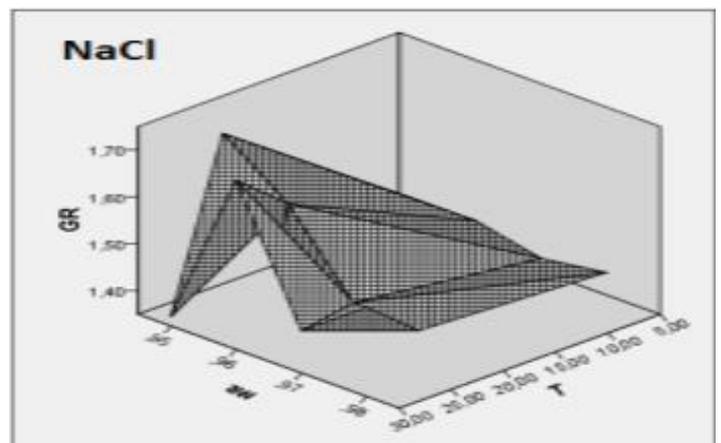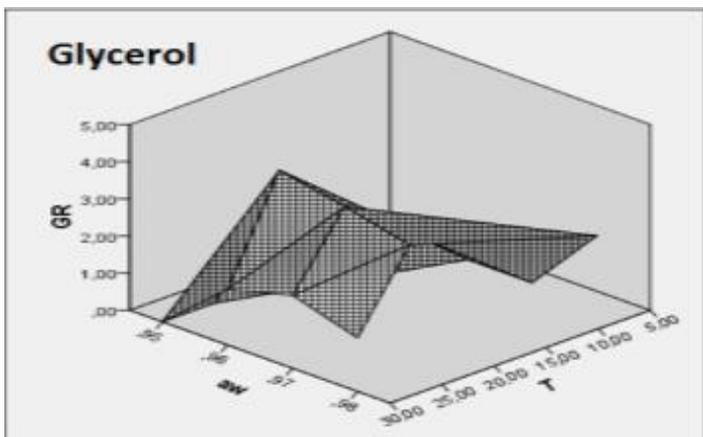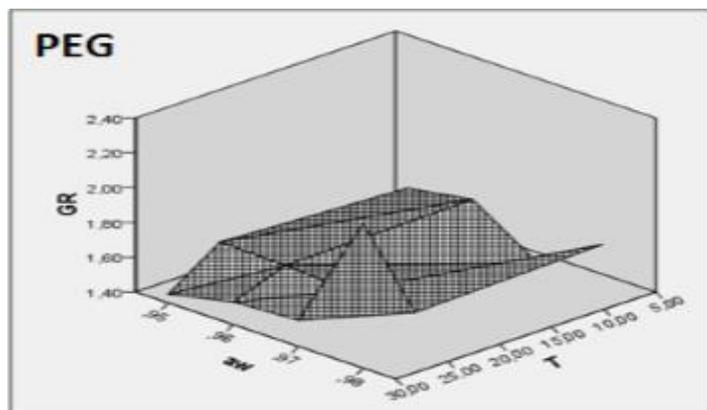
Figure 6 Response surface representing the predicted effect of water activity (aw) and temperature (T) by the three models glycerol, NaCl and Polyethylene glycol (PEG) on growth rate of *P. agglomerans* (GR).

DISCUSSION

Biological control under laboratory or controlled conditions has been demonstrated to be very effective in controlling bacteria disease in large number of studies. However, few biological control products have been commercialized and effectively used under practical conditions. The main reason for this relative lack of success is probably the fact that biological control agents are living organisms and thus limited by fluctuating environmental conditions both during application (with field conditions being the most restrictive) and the formulation process and shelf life. In this work, we tried to improve tolerance of *P. agglomerans* to low a_w and survival during the drying process with osmotic stress, survival on onion bulbs under 25°C and cold temperature (7°C), tolerance on different pH conditions and survival under different temperatures. Moreover, in this study we have modelled the growth rate of *P. agglomerans* according to temperature and a_w, two major factors affecting bacteria growth.

P. agglomerans growth was higher than on unmodified medium when polyethylene glycol was used to modify a_w. **Teixido et al. (2006)** found that *P. agglomerans* was less tolerant of low a_w in the presence of polyethylene glycol on medium, this difference of results may be due to the strain nature and isolation environment. In the presence of the glucose the growth was very limited under the three temperatures. Also the results show that, on basic medium supplemented or not with various solutes used to after the water activity of the medium, *P. agglomerans* grows best at temperature of 25°C and at an a_w ranging from 0.96 to 9.98. At low temperature (7°C), *P. agglomerans* can grow at a low a_w (0.95), depending on the solute used to reach this a_w. Similar studies have been carried out reported that other *P. agglomerans* strain and fugal species can grow at low a_w under 25°C (**Teixido et al., 2006 ; Lahlali et al., 2005**). The choice of solute used to modify the water activity of a medium has a significant impact on the growth rate of *P.agglomerans* (**Teixido et al., 2006**).

Our study likewise show that polyethylene glycol, glycerol and NaCl have a lesser effect on the growth rate of *P. agglomerans* than glucose, and it was higher on the presence of polyethylene glycol than unmodified medium.

Most predictive models have been developed for pathogenic bacteria. The main problem was that the difficulty of acquiring sufficient reproducible data, suitable for modelling (**Buchanan, 1993; Gibson and Hocking, 1997**). In the presence study we have modelled the combined effects of temperature and a_w on the *P. agglomerans* growth rate. The data obtained with polyethylene glycol, glycerol and NaCl were modelled by means of linear model.

The model provide better understanding of the development of antagonist *P. agglomerans*. It give a better idea of antagonist respecting the temperature and a_w. Concerning the pH conditions, we improve the good adaptation of *P. agglomerans* under a large range of pH conditions. Moreover, the growth of *P. agglomerans* 2066-7 strain was very effective inside onion bulb wounds at 7°C and 25°C. A similar colonization of *P. agglomerans* strains was reported on apple and pear wounds under cold temperature at different atmospheric conditions (**Bennik et al., 1998; Nunes et al., 2001**).

This indicates an excellent adaptation of strain 2066-7 to cold storage and 25°C temperature, which is an important feature for biological control agents of onion diseases. The antagonist could survive in the microenvironment of the wound and prevent disease.

REFERENCES

BENNIK, M.H.J., VORSTMAN, W., SMID, E.J., GORRIS, L.G.M. 1998. The influence of oxygen and carbon dioxide on the growth of prevalent *Enterobacteriaceae* and *Pseudomonas* species isolated from fresh and controlled-atmosphere-stored vegetables. *Food Microbiology*, 15, 459–469. http://dx.doi.org/10.1006/fmic.1998.0187 .

BUCHANAN, L.R. 1993. Predictive food microbiology. *Trends in Food Science & Technology*, 4, 6 – 11. http://dx.doi.org/10.1016/S0924-2244(05)80004-4 .

CAÑAMAS T.P., VIÑAS I., ABADIAS M., USALL J., TORRES R., TEIXIDO N. 2009. Acid tolerance response induced in the biocontrol agent *Pantoea agglomerans* CPA-2 and effect on its survival ability in acidic environments. *MicrobioLogicaL Research*, 164, 438—450. http://dx.doi.org/10.1016/j.micres.2007.02.007 .

COSTA E., USALL J., TEIXIDÓ N., DELGADO J., VIÑAS I. 2002. Water activity, temperature, and pH effects on growth of the biocontrol agent *Pantoea agglomerans* CPA-2. *Can. J. Microbiol*, 48, 1082–1088. http://dx.doi.org/10.1139/w03-001 .

DROBY, S., CHALUTZ, E. 1992. Biological control of postharvest diseases: a promising alternative to the use of synthetic fungicides. *Phytoparasitica Suppl,* 20, 149–153. http://dx.doi.org/10.1007/BF02980427 .

GIBSON, A.M., HOCKING, A.D., 1997. Advances in the predictive modelling of fungal growth in food. *Trends in Food Science &Technology*, 8, 353– 358. http://dx.doi:10.1016/S0924-2244(97)01065-0

HALLSWORTH, J.E., MAGAN, N. 1994a. Effects of KCl concentration on accumulation of acyclic sugar alcohols and trehalose in conidia of three entomopathogenic fungi. *Lett Appl Microbiol*, 18, 8–11. http://dx.doi.org/10.1111/j.1472-765X.1994.tb00785.x .

HALLSWORTH, J.E., MAGAN, N. 1994b. Effect of carbohydrate type and concentration on polyhydroxy alcohol and trehalose content of conidia of three entomopathogenic fungi. *Microbiology*, 140, 2705–2713. http://dx.doi.org/10.1099/00221287-140-10-2705 . .

LAHLALI, R., SERRHINIB, M.N., JIJAKLI, M.H. 2005. Studying and modelling the combined effect of temperature and water activity on the growth rate of *P. expansum*. *International Journal of Food Microbiology*. 103, 315—322. http://dx.doi.org/10.1016/j.ijfoodmicro.2005.02.002.

MAGAN, N., LACEY, J., 1988. Ecological determinants of mould growth in stored grain. *International Journal of Food Microbiology,* 7, 245–256. http://dx.doi.org/10.1016/0168-1605(88)90043-8 .

MATHIOUTHI, M. 2001. Water content, water activity, water structure and the stability of foodstuffs. *Food Control*, 12, 409—417. http://dx.doi.org/10.1016/S0956-7135(01)00032-9 .

NUNES, C., USALL, J., TEIXIDO, N., VINAS, I. 2001. Biological control of postharvest pear diseases using a bacterium *Pantoea agglomerans* (CPA-2). *Int J Food Microbiol*, 70, 53–61. http://dx.doi.org/10.1016/S0168-1605(01)00523-2.

PLAZA, P., USALL, J., TEIXIDO, N., VINAS, I. 2003. Effect of water activity and temperature on germination and growth of *Penicillium digitatum*, *P. italicum* and *Geotrichum candidum*. *Journal of Applied Microbiology*, 94, 549– 554. http://dx.doi.org/10.1046/j.1365-2672.2003.01909.x.

RHODES, D.J. 1993. Formulation of biological control agents. In Exploitation of Microorganisms ed. Jones, D.G. pp. 411–439. London: Chapman & Hall. http://dx.doi.org/10.1007/978-94-011-1532-2_16

TEIXIDO, N., CANAMAS, T.P., ABADIAS, M., USALL, J., SOLSONA, C., CASALS, C., VINÂS, I.2006. Improving low water activity and desiccation tolerance of the biocontrol agent *Pantoea agglomerans* CPA-2 by osmotic treatments. *Journal of Applied Microbiology*, ISSN, 1364-5072. http://dx.doi.org/10.1111/j.1365-2672.2006.02948.x

VINAS, I., USALL, J., TEIXIDO´ N., SANCHIS, V. 1998. Biological control of major postharvest pathogens on apple with Candida sake. *Int J Food Microbiol*, 40, 9–16. http://dx.doi.org/10.1016/S0168-1605(98)00009-9 .