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

Soumia Sadik1,2, Hamid Mzaouaz2, Abdellatif Benbouazza1, and El Hassan Achbani* 1

Address(es): PhD student, Miss Soumia Sadik, 1Laboratory of Plant Protection URPP, INRA, 50000-Meknes, Morocco, phone number: +212679897059/+212650198107, 2Laboratory of Plant Biotechnology and Molecular Biology, Faculty of Sciences, 50000 Meknes, Morocco.

*Corresponding author: achbani105@gmail.com, sadik.soumia@hotmail.com

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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 ways to formulize 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, 2004).

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 20g.L of 107 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 spectrophotometer (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 10^7 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; 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 an aₚ 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 aₚ solute profile of *P. agglomerans* bacterium, was grown in basic liquid medium and a known amount of CPU 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 aₚ (Teixido et al., 2006). The solid agar media were inoculated by spread plating a 20µL aliquot of a 10⁷ 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 aₚ and solute condition. All treatments were carried out with four replicates, and the experiment was repeated twice.

Media from the same aₚ 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 aₚ (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 grow at a temperature from 7°C to 38°C.

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

<table>
<thead>
<tr>
<th>Temperature</th>
<th>7°C</th>
<th>25°C</th>
<th>30°C</th>
<th>36°C</th>
<th>38°C</th>
<th>40°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(-)</td>
</tr>
</tbody>
</table>

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 decreased 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 aₚ 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 aₚ ≤ 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 increase in lag times prior to growth initiation. All solutes exhibited 100% cultivability or near than at least 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<sub>w</sub>) modified media using different solutes: glycerol (Gly), polyethylene glycol (PEG), glucose (Glu) and NaCl. Tested a<sub>w</sub> 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 (Log(UFC*10<exp>2/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 decreased as the water activity of the medium decreased. 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<sub>w</sub>. However, the growth stopped at a<sub>w</sub> 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<sub>w</sub> 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 a<sub>w</sub> of 0.95 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<sub>w</sub> 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<sub>w</sub> 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 (Log(UFC*10<exp>2/ml))/j), under 7°C the value was 0.94 (Log(UFC*10<exp>2/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).
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 aw 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 aw 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 aw with glycerol, polyethylene glycol and NaCl models

<table>
<thead>
<tr>
<th>Environment factor</th>
<th>Extension growth rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>aw</td>
</tr>
<tr>
<td></td>
<td>Observed</td>
</tr>
<tr>
<td>7°C</td>
<td>0.95</td>
</tr>
<tr>
<td>25°C</td>
<td>0.95</td>
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<tr>
<td>30°C</td>
<td>0.95</td>
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<tr>
<td>7°C</td>
<td>0.96</td>
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<tr>
<td>25°C</td>
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<td>30°C</td>
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<tr>
<td>7°C</td>
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<tr>
<td>25°C</td>
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<td>30°C</td>
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<tr>
<td>7°C</td>
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<tr>
<td>25°C</td>
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<tr>
<td>30°C</td>
<td>0.98</td>
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</table>
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 and survival during the drying process with osmotic stress, survival on onion bulbs under 25°C and cold temperature (7°C), tolerance of the biocontrol agent P. 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

CONCLUSIONS

Several studies have been carried out reported that other P. agglomerans strain and fugal species can grow at low a_s 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).

The model provide better understanding of the development of antagonist P. agglomerans. It give a better idea of antagonist respecting the temperature and a_s. 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.

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REFERENCES


