Environmentally Friendly and Safe Technologies for Quality of Fruits and Vegetables

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SECTION 4. ENVIRONMENTALLY FRIENDLY AND SAFE METHODS TO CONTROL POSTHARVEST LOSSES

24. IS IT POSSIBLE TO IMPROVE BIOCONTROL AGENTS TO PRACTICAL APPLICATIONS? THE *PANTOEA AGGLOMERANS* CPA-2 EXAMPLE

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Abstract
A major hurdle in exploitation of biocontrol agents is the limited tolerance of fluctuating environmental conditions practically and the difficulties in developing a shelf-stable formulated product as effective as fresh cells. Most of microorganisms are very sensitive to drying processes involved in formulation and biological control is usually limited by the narrow range of conditions below microorganisms are able to survive, establish and effectively control pests and diseases. *P. agglomerans* cells grown at low water activities using NaCl exhibited osmotic adaptation and also demonstrated thermotolerance and desiccation tolerance after spray drying, freeze drying and fluidized bed drying. Different formulation strategies of *P. agglomerans* cells were tested in order to improve survival under field conditions and efficacy in controlling postharvest rots, including lyophilised cells, osmotic adaptation by NaCl treatments and additives. In general, osmotic adapted and lyophilised *P. agglomerans* cells showed greater survival rates than non-osmotic adapted or fresh cells when these bacterial treatments were sprayed at field conditions. However, this superiority was only found when additive Fungicover was added to suspension treatments. The improved formulation of *P. agglomeran*s provided an effective control for oranges against natural postharvest pathogens infections and *P. digitatum* artificial infections. These results allowed us to conclude that it is possible to improve environmental stress tolerance and ecological competence of *P. agglomerans* by integrating certain formulation strategies. Enhancing stress tolerance and formulation strategies could be appropriate approaches to obtain consistency and broaden the spectrum of use of biocontrol agents.
Keywords: biological control, citrus, fluidized bed drying, improving environmental stress resistance, pre-harvest treatments, spray drying

Introduction
Biological control of postharvest diseases of fruits has advanced greatly during the past decade. Concerns regarding human health and environmental risks associated with chemical residues in foods have been the main driving force of the search for new and safer control methods. Among the proposed alternatives, the use of naturally occurring antagonistic microorganisms has been the most extensively studied. Several microbial antagonists, based on either yeast or bacteria were developed and commercially tested. However the success of these products remains limited and just a few microorganisms are commercial available to control postharvest decay of citrus and pome fruits such as Bio-Save 10 (*Pseudomonas syringae* ESC-10, Jet harvest solutions, USA), Shemer (*Metschnikowia fructicola*, Bayer Crop Science, AG), Boniprotect (*Aureobasidium pullulans*, Bio-protect, Germany) and Candifruit (*Candida sake* CPA-1, Sipcam Inagra, Spain).

There are several reasons of the limited number of commercial available biocontrol agents, such as the limited tolerance of fluctuating environmental conditions practically and the difficulties in developing a shelf-stable formulated product that is as effective as fresh cells. This is because of the simultaneous exposure of the microorganism to environmental stress conditions such as low a_w (dehydration) and high temperatures. Thus, improvement in physiological quality of the biocontrol agents during production which can enable survival and activity under such environmental conditions are an important challenge for exploitation and potential suitability in commercial conditions.

It has been demonstrated with both food-borne pathogens (O'driscoll *et al.* 1996; Mattick *et al.* 2001; Greenacre *et al.* 2003) and probiotics (Ananta & Knorr 2003; Prasad *et al.* 2003) that adaptation to hostile environmental conditions also has the potential to alter cellular physiology such that the organism becomes more resistant to further stress. In the case of osmotic stress, the significant changes reported in bacteria include the accumulation of compatible solutes such as amino acids and sugars (Csonka 1989).

Subjection to a mild stress makes cells resistant to a lethal challenge with the same stress condition. Preadaptation to one particular stress condition can also render cells resistant to other stress imposing conditions: this phenomenon is known as cross protection (Sanders *et al.* 1999).

Detailed studies have shown that the strain CPA-2 of *Pantoea agglomerans* - previously classified as *Erwinia herbicola* - is an effective antagonist to the major postharvest fungal pathogens of pome and citrus fruits (Teixidó *et al.* 2001; Nunes *et al.* 2001, 2002; Usall *et al.* 2008) and it is in commercialization process in Spain as a solid formulation named Pantovital by BIODURCAL S.L. Commercial and technical formulations of these two biocontrol agents are been developed in the Postharvest Pathology, IRTA, Lleida, Catalonia.

Before our studies, there were very few reports describing the physiological osmotic stress responses in biological control agents, and all of them have been on filamentous fungi (Hallsworth & Magan 1996; Pascual *et al.* 2000) or yeasts (Teixidó *et al.* 1998a,b; Abadias *et al.* 2001). During 12 years our group has focussed part of the research in improving biocontrol agents (*C. sake* and *P. agglomerans*) behaviour in front stress conditions achieving interesting results that allow enhance biocontrol treatments at field conditions, improve biocontrol agents behaviour during formulation process and broaden their spectrum of action. The main results with osmotic adaptation achieved with *P. agglomerans* are summarized in this chapter.

Improving Low Water Activity Tolerance by Osmotic Treatments

The improvement of tolerance to low water activity (a_w) and desiccation in *P. agglomerans* cells subjected to mild osmotic stress during growth was studied using different solutes to change a_w of growth media. It was shown that cells grown in media at low a_w using NaCl exhibited osmotic adaptation in solid media at low a_w obtaining high production level and maintaining biocontrol efficacy (Teixidó *et al.* 2006). Osmotic-adapted cells also demonstrated thermotolerance (Teixidó *et al.* 2005).

The role of different compatible solutes in adaptation of the bacterium to osmotic stress was determined and this study suggested that glycine-betaine and ectoine play a critical role in environmental stress tolerance improvement (Teixidó *et al.* 2005; Cañamás *et al.* 2007).

Formulation Process Enhancement

P. agglomerans cells grown at low water activities using NaCl not only exhibited osmotic adaptation but also demonstrated better desiccation tolerance after spray drying, freeze drying and fluidized bed drying than non-modified cells.

Spray Drying Process

Cells grown in NaCl modified media which showed the best low a_w adaptation were tested in spray-drying trials to check desiccation tolerance. *P. agglomerans* was grown on unmodified, and NaCl 0.98, 0.97 and 0.96 a_w modified basic media for 24 and 48 h at 30 ºC and 150 rpm agitation on a rotary shaker. Harvested cells were resuspended in $MgSO_4$ 10% suspension to obtain an initial concentration of $1x10^{10}$ cfu mL^{-1}. These suspensions were then incubated for 30 min at room temperature, constantly shaken to allow cell adaptation and then spray-dried at an inlet temperature of 140 ºC and a delivery rate of 500 mL h^{-1}. The powder obtained was rehydrated with reconstituted nonfat skimmed milk (10%), which acted as a rehydration medium. Used methodology was optimized by Costa *et al.* (2002).

Significant differences between growth treatments were found with respect to the survival of spray-dried cells (Data not shown). The best survival was achieved with cells grown for 48 h in NaCl 0.97 medium

(29%), followed by cells grown for 48 h in NaCl 0.98 (23%). The survival rate of cells grown in unmodified medium was always less than 7%. In the case of NaCl, the survival of pre-stressed cultures improved when cells were incubated for 48 h instead of 24 h. The opposite tendency was observed with control cells. Our research demonstrated that the treatments that showed the best adaptation to low a_w also presented better survival during the spray-drying process than control cells. These results confirm others obtained by Prasad *et al.* (2003), who found that when pre-stressed with either heat (50 ºC) or salt (0.6 M NaCl), *Lactobacillus rhamnosus* HN001 showed a significant improvement in viability compared with a non-stressed control culture after storage at 30 ºC in its dried form. The drying technique applied in this case was fluidised bed drying.

Although desiccation improvement of modified NaCl cells is clear, it is not sufficiently good in practical terms to consider spray-drying as an appropriate strategy for dehydrating this biocontrol agent.

Fluidized Bed Drying Process

The effect of osmotic treatments on the viability of cells after fluidized bed drying process was studied. Assayed treatments were the same described above in the previous section, using NaCl to adjust a_w (0 g L^{-1} NaCl (0.99 a_w)- P, 35 g L^{-1} NaCl (0.98 a_w)-P980 or 53 g L^{-1} NaCl (0.970 a_w)-P970) place another treatment with 25 g L^{-1} NaCl (0.988 a_w)- P988. For an optimal level of growth in P, P988, P980 and P970 treatments, incubation times were 20, 20, 22 and 30 h, respectively. For P980 and P970 cultures were also obtained after 48 h of incubation, corresponding this time to an optimal level of osmoresistance of *P. agglomerans* cells (P980+48 h and P970+48 h treatments) as it had been demonstrated by Teixidó *et al.* (2006).

Results pointed out those treatments which were effective to improve survival of *P. agglomerans* cells during drying process. The best survival values of *P. agglomerans* cells were achieved with osmotic treatments when cells grown at a_w of 0.98 and 0.97 for 48 h using NaCl to adjust a_w (Fig 1). These treatments also shown the highest viabilities following spray-drying process in studies carried out by Teixidó *et al.* (2006). However, P988 osmotic treatment was chosen for further experiments because it was a cheaper and optimized production treatment. Although P980+48h and P970+48h treatments gave higher viabilities than P988 osmotic treatment, they showed lower levels of biomass production. Other authors such as Prasad *et al.* (2003) obtained also higher viabilities in osmotic shocked cells of *Lactobacillus rhamnosus* HN001 (DR20) than non-adapted after drying in fluidized bed dryer.

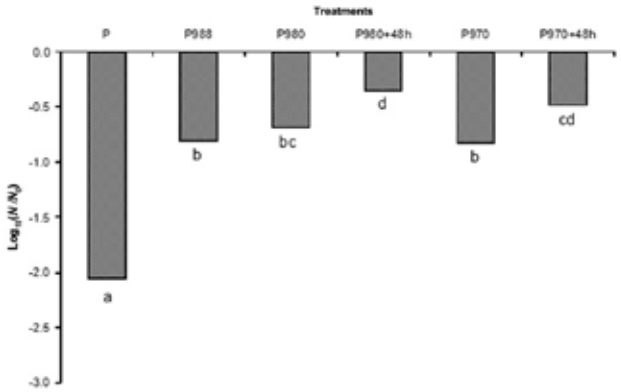

Fig 1. Survival of non-osmotically (P treatment) and osmotically adapted cells of *P. agglomerans* after dehydration in a fluidized bed dryer for 20 min at 40 ºC. Levels of viability after drying process were expressed as logarithmic value of survival fraction $\log_{10}(N\,N_0^{-1})$. Results are the means of at least two independent fluidized bed drying trials with two independent replications per trial. Columns with different letter are statistically different according to Duncan's multiple range test at $P<0.05$.

Freeze Drying Process

Significant differences in the viability of *P. agglomerans* cells after freeze-drying and shelf life period were observed depending on the presence or absence of NaCl (25 g L^{-1} P988) on growth medium (Fig 2).

Just after drying process, survival of *P. agglomerans* cells grown in unmodified or NaCl-amended medium was 90.2 and 100% respectively. Differences among both treatments increased along the storage or shelf life of formulated products and after 6 months of storage at 4 °C, cells grown in NaCl medium maintained 100% of viability and the ones grown in basic medium showed less than 25% of cell survival. It is also remarkable that cells grown in 25 g L^{-1} NaCl-medium showed viabilities higher than 80% after one year of storage. Survival and shelf life achieved are reasonably good for commercial application.

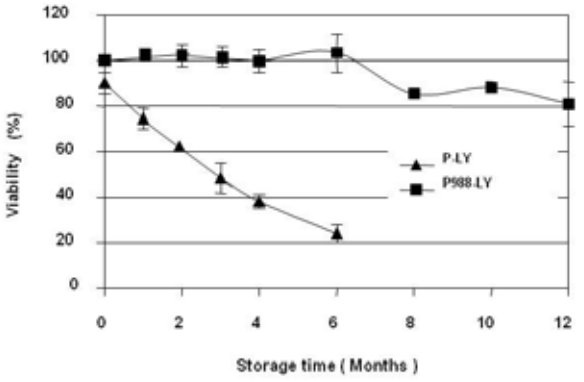

Fig 2. Survival of non-osmotically P-LY and osmotically P988-LY adapted cells of *P. agglomerans* after freeze-drying and shelf life of dehydrated product stored at 4 °C during one year. Results are means of four independent samples and vertical bars indicate standard errors.

Preharvest Treatments Enhancement

Osmotic adapted cells described above were used in preharvest treatments in order to control the main postharvest diseases on citrus fruit.

In the first experiment (Cañamás *et al.* 2008a), it was observed that *P. agglomerans* cells (osmotic-adapted and non-adapted) were unable to maintain a stable population on the fruit surface and consequently, preharvest applications resulted ineffective against both naturally and artificially inoculated *P. digitatum*. This low survival rates revealed the sensibility of *P. agglomerans* cells to environmental field conditions. These results also led us to conclude that it is necessary a minimal antagonist population levels on fruit surfaces in order to guarantee competition with pathogens for sites and nutrients, and to subsequently obtain an efficient control. The establishment of bacterial populations on plant surfaces is a critical phase in disease control. Microbes can be inactivated by several environmental factors, including sunlight, temperature, humidity, leaf surface exudates and competitors.

The effect of main environmental factors on *P. agglomerans* cells, such as relative humidity and solar radiation was studied and different formulation strategies were used in order to enhance survival on fruit surface under field conditions and subsequently enhance biocontrol efficacy (Cañamás *et al.* 2008a).

Osmotic adapted *P. agglomerans* cells, especially when these cells grew at 0.98 a_w were more resistant than non-adapted cells when both were applied in oranges and stored in chambers at a low Relative Humidity (43%). However, the minimum values of relative humidity registered during the first field assay were between 15 and 50%. These extreme values may restrict the survival and growth of *P. agglomerans* cells. The relative importance of each of these factors depends on why and where a particular product is used. In applications in foliar environments (which were our case), solar radiation, and especially the ultraviolet (UV) portion of the spectrum, is probably the main important factor affecting the persistence of microbial insecticides (Rhodes 1993; Filho *et al.* 2001). This detrimental effect of UV radiation was also

checked in a laboratory assay in which a major decrease in *P. agglomerans* population was observed during exposure to sunlight.

In laboratory studies, different additives, such as summer oils, alginate, glycerol and food additives at different concentrations were tested mixed with *P. agglomerans* in order to study its compatibility. The non-toxic ones (Citroline, Summer oil, Alginate, Sunspray, Glycerol, Siapton and Fungicover) were added to the biocontrol agent, sprayed on detached oranges and left outdoors. Population dynamics of the antagonist on fruit surface was determined along the time. Fungicover (FC) was the most effective additive for improving the adherence and persistence of *P. agglomerans* cells on oranges and it was also compatible with the antagonist. Firstly, adherence was improved by Fungicover since the *P. agglomerans* cell population just after application and drying (0 h), was greater than when cells were only sprayed with water. It was also visually observed that spreading, wetting and dispersion were also clearly improved. This could have been due to the fact that the additive Fungicover contains fatty acid derivatives in an alcohol solution. These components could have reduced the surface tension of the cell suspension and thereby improved the spread and wetness of the spray over the plant surface (Burges 1998). On the other hand, the persistence of *P. agglomerans* cells was also improved outdoors under springtime environmental conditions in the presence of Fungicover at 5%. It has not been possible to exactly elucidate the mechanism(s) by which this additive was able to protect the antagonist population. The additive Fungicover could also have protected *P. agglomerans* cells from solar radiation as sunscreen, physically reflecting and scattering, or selectively absorbing radiation, converting short wavelengths to harmless longer ones (Jones & Burges 1998).

Fungicover is an edible film-forming compound for fruits and vegetables to reduce weight loss, delay senescence, improve natural brightness and reduce physiological disorders. It also reduces droplet size and improves uniformity of distribution on the surface to be protected. This additive did not show any fungicidal effect on *P. digitatum* (Cañamás *et al.* 2008a).

In this study it has also been demonstrated that inoculum formulation can influence the persistence of *P. agglomerans* cells. Bacterial treatments prepared with lyophilised (LY) *P. agglomerans* cells become more resistant to environmental conditions than fresh cells (SH), as Stockwell *et al.* (1998) observed when the bacterial antagonists *Pseudomonas fluorescens* A506 and *Erwinia herbicola* C9-1R were applied under field conditions.

Different strategies could be used to improve *P. agglomerans* cell survival on oranges under non-controlled environmental conditions and all them were applied in two representative field trials on 'Lane late' and 'Valencia' oranges, in order to evaluate the effectiveness of different bacterial formulations of *P. agglomerans* applied at preharvest for controlling postharvest decays caused by natural infection and also by artificial infection (*Penicillium digitatum*) (Cañamás *et al.* 2008b).

Population dynamics of *P. agglomerans* during trials (under field conditions and at postharvest) are shown in Fig 3. In both experiments greater adherence and persistence of populations were observed when the biocontrol agent cells were sprayed using the additive Fungicover, being the population level similar to that treatment applied at postharvest.

Results for the efficacy of preharvest treatments for artificial infection by *P. digitatum* are shown in Fig 4. In experiment 1 (Fig 4A) the P988-LY+FC and P988-SH+POST treatments were significantly more effective than the other preharvest treatments and non differences were found between both after 15 d. In experiment 2 (Fig 4B) and after 15 d of storage, all the preharvest treatments with the additive Fungicover showed effective control against *P. digitatum* with decay values of below 12.5%. Treatment P988-LY also showed effective control with 12.8% decay. However, only treatments P988-LY+FC, P98-LY+FC and P97-LY+FC with decay values of between 7 and 5.3%, exhibited levels of control on *P. digitatum* no statistically different to the antagonist postharvest treatment P988-SH+POST.

The protective effect of the additive Fungicover was again confirmed by results and this effect varied according to whether or not the *P. agglomerans* cells had been osmotic adapted and if the cells had been

lyophilised or not. It is therefore likely that adding Fungicover to formulations could protect cells against field conditions. At the same time, it was possible to observe that populations of osmotic adapted cells showed a higher level of survival than non-adapted cells when the additive FC was used. Furthermore, the positive effect of applying lyophilised *P. agglomerans* cells instead of fresh cells was also evident when treatments were combined with Fungicover.

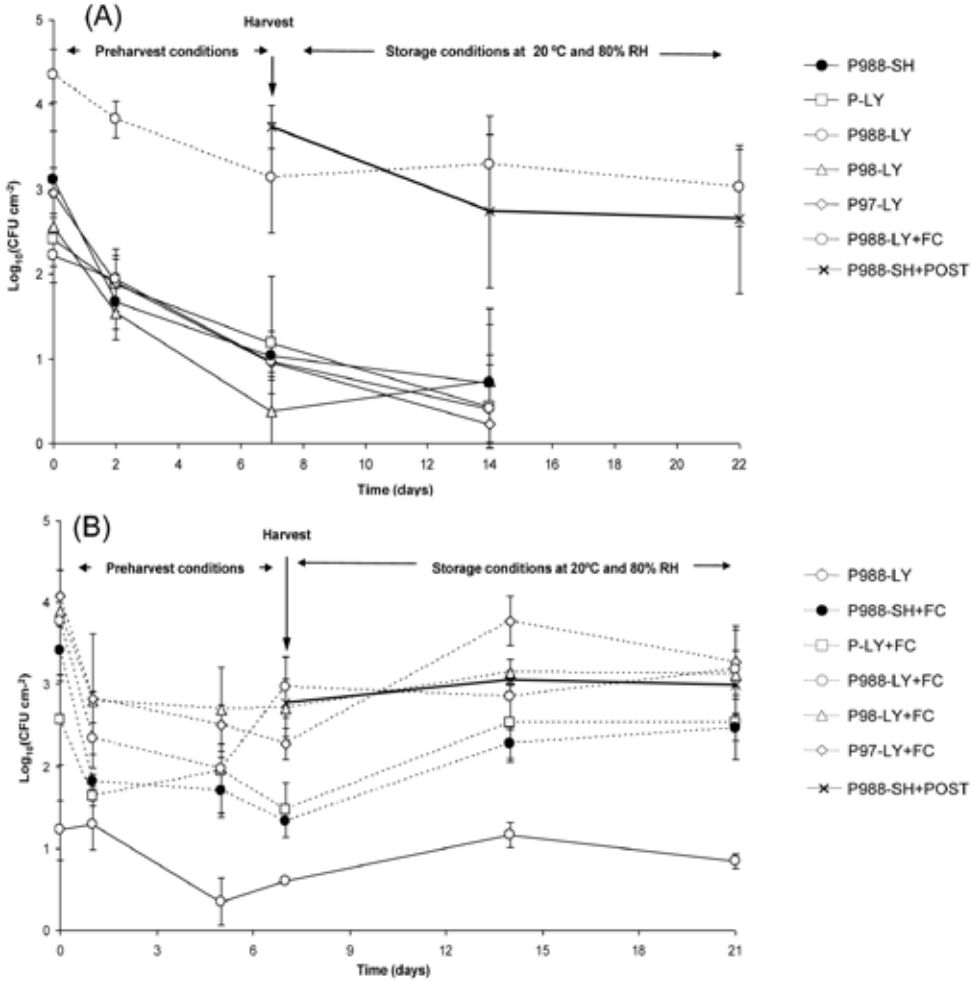

Fig 3. Population dynamics of *P. agglomerans* treatments during field and storage conditions. Bacterial treatments were prepared from lyophilized (LY) or fresh (SH) and from non-adapted (P) or osmotic adapted *P. agglomerans* inocula in presence of 25 g L^{-1} 0.988 a_w (P988), 35 g L^{-1} 0.98 a_w (P98) or 53 g L^{-1} 0.97 a_w (P97) of NaCl in the medium, respectively). The additive Fungicover (+FC) was used in some treatments at a concentration of 5% in order to check its adherence and persistence effect on the populations of *P. agglomerans* cells. An adequate volume of non-adapted or osmotic adapted *P. agglomerans* inocula for each bacterial treatment was mixed into 30 litres of water in a plastic recipient to obtain a final concentration of 2×10^8 cfu mL^{-1}. Bacterial treatments were sprayed onto orange fruits cv Lane Late (Experiment-1A) or Valencia late (Experiment-2B) one week before harvest. Treatment P988-SH+POST was applied at postharvest dipping oranges in a solution at 1×10^8 cfu mL^{-1} before the storage period. Results are means of four independent samples and vertical bars indicate standard deviations.

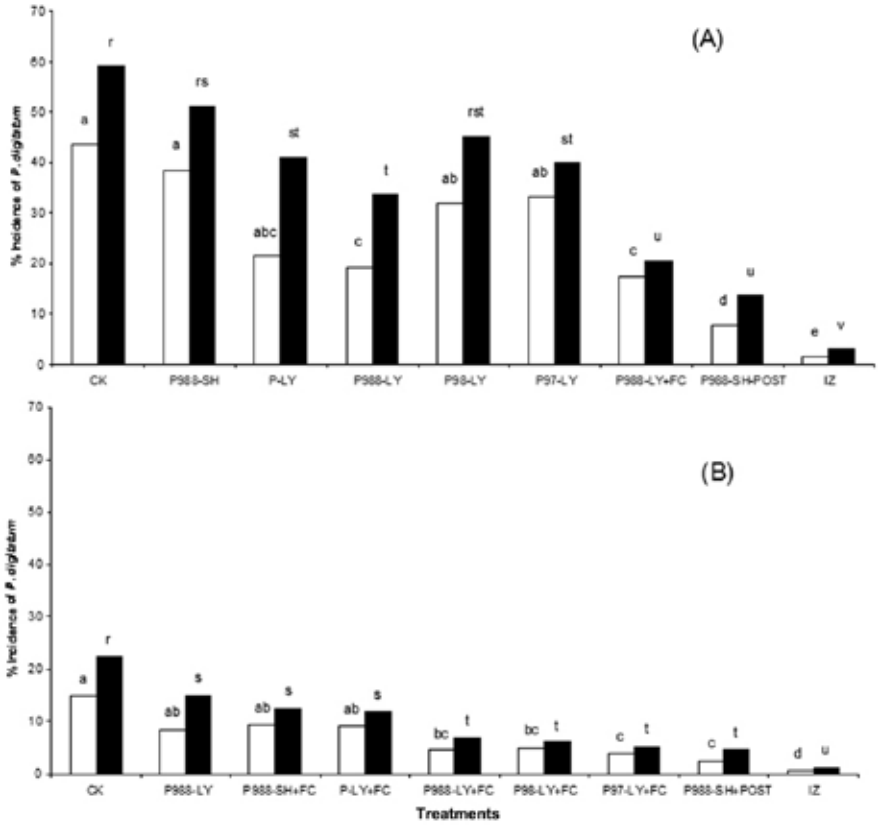

Fig 4. Effectiveness of preharvest treatments against artificial infection of the fungal pathogen *P. digitatum* compared with postharvest treatments: "*P. agglomerans* and commercial fungicide Imazalil (IZ) at 1.125 g/L". Fruits were stored for 15 d at 20 °C and 85% RH. The incidence of decayed fruits was scored after 7 (white bars) and 15 d (black bars) of storage and expressed as % decay produced by *P. digitatum* on orange cultivars, Lane late and Valencia late in Experiments 1(A) and 2 (B), respectively. Different letters in the bars indicate significant differences between means according to a Duncan's Multiple Range Test ($P<0.05$).

Results indicated that there was a close relationship between the population level associated with a given treatment under field conditions and the level of control achieved by this treatment during storage. Moreover, only bacterial treatments, which were prepared from lyophilised and osmotic adapted cells, showed a level of control comparable to postharvest treatments with the biological control *P. agglomerans* when they were applied with additive Fungicover. These preharvest treatments were also associated with population levels that were higher under field conditions. These findings were in concordance with those of Tian *et al.* (2004) who found that only *Rhodotorula glutinis* and *Cryptococcus laurentii*, whose populations remained at high and stable levels, significantly reduced fruit decay during storage at 25 °C.

We conclude that survival and stability of *P. agglomerans* populations could be maintained under field condition by integrating certain formulation strategies: adding additives, ecophysiological osmotic adaptation and lyophilisation. Thus, it has highlighted that it is very important to optimize both, distribution of the biological control agent on the host surface and survival under field conditions.

The results presented in this chapter are an example that the induction of stress adaptation responses is a

useful and practical tool, that could broaden new possibilities for improving performance of biocontrol agents to other hosts and diseases and it could improve their antagonistic activity in a wide range of conditions.

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