Chapter 10
Pichia anomala and *Candida oleophila* in Biocontrol of Postharvest Diseases of Fruits: 20 Years of Fundamental and Practical Research

Massart Sebastien and Mohamed Haissam Jijakli

Abstract The economic losses caused by post-harvest pathogens of apple and pear can still reach 25 %. There is currently an increasing demand to develop sustainable methods to control these post-harvest pathogens. Biocontrol agents are interesting candidates to answer this demand. Nevertheless, their commercial development is sometimes hampered by a low or non-reliable efficacy comparing to fungicide treatments. Fundamental research on the mode of action of the BCA and of its ecological fitness could help to overcome that phenomenon. This chapter reviews the progresses made during two decades to understand the mode of action and the ecological niche of two BCA, *Pichia anomala* strain K and *Candida oleophila* strain O. These advances required the combination of various methodologies (in vitro and in situ) and techniques (microbiology, microscopy, genome characterization, transcriptome, proteome, gene disruption…) which are summarized here. Importantly, the practical impact of these discoveries to improve the efficacy of the biopesticide is also highlighted.

Keywords Sustainable control • BCA • Ecological fitness • Fungicides • Biocontrol

Introduction

Post-harvest diseases of apple and pears, such as *Botrytis cinerea*, *Penicillium expansum* and *Gloeosporioides* group, are still provoking important economic losses which can reach 25 % of the harvested fruits. To date, these pathogens are mainly controlled by pre- and post-harvest fungicide treatments. However, the consumers are becoming more reluctant to accept chemical residues in food and

M. Sebastien • M.H. Jijakli (✉)
Laboratory of Phytopathology, University of Liège, Gembloux Agro-Bio Tech, Passage des déportés, 2, 5030 Gembloux, Belgium
e-mail: mh.jijakli@ulg.ac.be; jijakli.h@fsagx.ac.be

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there is an increasing concern about the environmental and ecological impacts of fungicide treatment. Additionally, the withdrawal of fungicides and the development of resistant strains are limiting the number of available and efficient fungicides. There is therefore an increasing demand to develop alternative and sustainable methods to control post-harvest pathogens of apple and pears.

Biological control is generating a great enthusiasm as a sustainable and "eco-friendly" control method. The annual turnover of biopesticide is growing at a pace of 20 % per year to reach 1.7 billion $ in 2011, representing 5 % of the total pesticide market.

Post-harvest biological control is one of the most promising markets because the application sites are limited to the harvested commodities, the environmental conditions are defined and stable in storage rooms and the harvested commodities are of high value. So far, numerous biological control agents (BCAs) have been isolated for their biocontrol properties against post-harvest apple and pear pathogens. Nevertheless, the development of a BCA in an efficient commercial product is complex and there are currently only two bacteria and four yeasts currently registered as biopesticide (Jijakli 2011).

This bottleneck is mainly due to a lower or non-reproducible efficacy of the biopesticide comparing to fungicide treatment. This represents a major drawback for the development of biopesticides and needs to be addressed.

The efficacy of a biopesticide can be improved and stabilized through a better understanding of the mode of action of the BCA and of its ecological niche. This information will allow the development of the most appropriate formulation and timing of application and, ultimately, it will facilitate the registration of the product.

This chapter presents a study case focusing on two BCA: *Pichia anomala* strain K and *Candida oleophila* strain O, isolated in our laboratory more than 20 years ago (Jijakli and Lepoivre 1993). We review the fundamental and applied researches carried out to understand the mode of action and the ecological niches of these BCA. Importantly, we underline also the impact of these researches on the efficacy improvement of the biopesticide.

Results, Fundamental Research

BCA On-Site: A Complex Interplay

Once applied on the fruit surface, the BCA will face a complex microenvironment (see Fig. 10.1) which is influenced by the host genotype and its physiology, by the presence and concentration of pathogens and by the microflora composition of the commensal microorganisms. Multiple interactions between them occur at the site of action and may influence the BCA efficacy. Moreover, this site is also under the influence of environmental parameters like humidity, temperature, UV light...

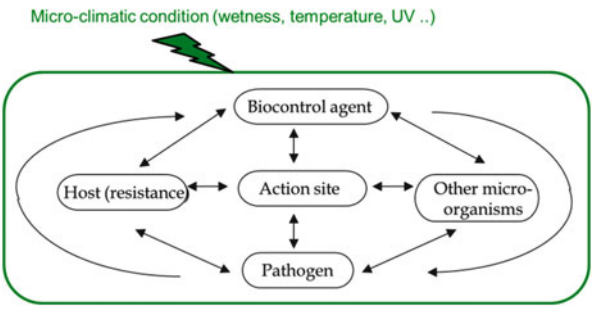

Fig. 10.1 Complex interplay at the site of action of a BCA on fruit

Understanding the mode of action and deciphering the ecological niche of a BCA may represent therefore a complex task. Moreover, even if molecular biology tools have been developed and used with BCA (Massart and Jijakli 2007), this research is still hampered by the general scarcity of molecular tools and genome knowledge compared to conventional yeasts like *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*.

So far, the experimental strategies have relied mainly on the simplification of the micro-ecosystem by *in vitro* studies, providing indirect evidences on the mode of action or on the ecological niche of the BCA. As *in vitro* studies do not necessarily reflect the *in situ* reality, these findings had to be confirmed later on by *in situ* studies.

Understanding the Mode of Action of Pichia anomala *Strain K*

Knowledge of the modes of action of a BCA is crucial for developing successful post-harvest biocontrol strategies. This knowledge allows (i) a rational optimization of the method and timing of application, (ii) a more efficient formulation design to enhance and stabilize the BCA efficacy, (ii) a targeted selection of more effective BCAs and (iv) is mandatory to register a BCA for commercial use (Jijakli 2011).

The modes of action of a BCA are generally classified in four main groups: nutrient or site competition, antibiosis, direct interaction between the pathogen and the BCA and induction of host resistance (Wilson and Wisnieswski 1994). It is important to keep in mind that the biocontrol properties of most BCA do not rely on a major mechanism but rather on multiple modes of action acting together or sequentially.

The complexity of interactions at the action site and the multiplicity of the modes of action make mandatory the development of complementary approaches to understand the modes of action and the ecological niche of the BCA. The approaches undertaken for *Pichia anomala* strain K are summarized in Fig. 10.2.

Microbiological and biochemical approaches are traditionally the first approaches applied to understand the modes of action of a BCA. Over the past

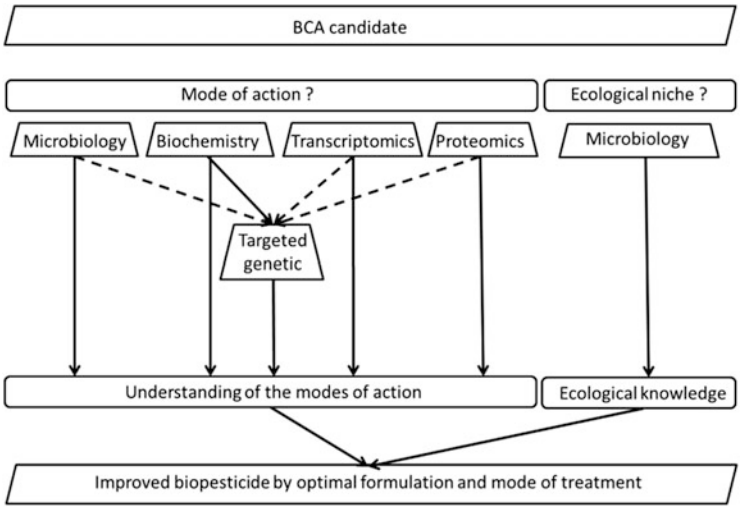

Fig. 10.2 Scheme of experiment necessary to understand the mode of action and the ecological niche of a BCA. With → experiments done on strain K and -→ other possible experiments

decade, the development of molecular techniques has brought innovative tools to complete these approaches. The molecular tools allowed a better understanding and even demonstrated the mechanisms underlying biocontrol properties of BCAs. Briefly, they correspond to targeted or non-targeted gene identification, high throughput gene expression profiling through mRNA or protein analysis, and gene inactivation and/or overexpression. There have been reviewed in detail elsewhere (Massart and Jijakli 2007).

Microbiological and Biochemical Approaches

Microbial approaches are classically the first experiments carried on to understand the mode of action of a BCA. These approaches can be developed *in situ* or *in vivo*. Antibiosis of *P. anomala* strain K against post-harvest pathogens has never been detected whatever the tested *in vitro* assay (unpublished results). The nutrient and site competition has been studied *in situ* on wounded sites (Jijakli and Lepoivre 1993). At 25 °C, population of strain K in wounds grew to reach a maximum density 12 h after application. The protective level of strain K against *B. cinerea* also reached a maximum when the pathogen was applied 12 h after strain K application. Interestingly, microscopic observation showed that the germination of *B. cinerea* was significantly reduced in presence of strain K at the wounding site, even when the pathogen and the BCA were applied simultaneously with no subsequent protection. This indicated that inhibition of spore germination was not the only mechanism of pathogen biocontrol.

Biochemical studies were carried out on the hydrolytic enzymes produced by strain K. The enzymatic activity of culture filtrate of strain K was analyzed and it revealed endo- and exo-β-1,3-glucanase activities but no chitinolytic activity. A marked increase in glucanase activity was observed when using cell wall preparation from *B. cinerea* as the sole carbon source compared to glucose (Jijakli and Lepoivre 1998). An exo-β-1,3-glucanase (PaExg2), presenting the highest specific activity, was purified from culture filtrates of strain K. PaExg2 showed inhibitory effect on germ tube growth and conidial germination (up to 29 % inhibition) of *B. cinerea*, causing morphological changes in germ tubes. Exo-β-1,3-glucanase activity was also detected in apple wounds treated with strain K. Overall results suggested that exo-β-1,3-glucanase activity might be involved in the protective effect of *P. anomala* strain K against *B. cinerea* (Jijakli 2011).

Genome Organization

The genome of *P. anomala* strain K was characterized in order to better understand its organization and to design the most appropriate disrupting strategies (Friel et al. 2005). Through Pulse-Field Ge Elecrophoresis (PFGE), the number of chromosomes of strain K was estimated at 6, ranging in size between 1.1 and 3.2 Mb and representing a genome of 11.7 Mb. The comparison of several isogenic strains through PFGE suggested a significant genomic instability as the number or the length of chromosomes varied between strains. These observations were confirmed by molecular hybridization using four probes corresponding to URA3, LEU2, PAEXG1 and PAEXG2 genes. The strain K can therefore be considered as an aneuploidy strain. Haploid strains, called Kh(n) with n as number of the strain, were also obtained from strain K by ascus microdissection.

Targeted Genetic Approach

A targeted genetic approach relies on the selection, the cloning, the disruption and/or the over-expression of one of several genes potentially involved in biocontrol properties. These genes are selected based on the results obtained through biochemical, microbiological, transcriptomic and proteomic approaches. This approach requires therefore *a priori* knowledge in order to select the most appropriate gene candidates.

For *P. anomala* (strain K), the biochemical studies suggested an involvement of exo-β-1,3-glucanase in the biocontrol properties. Grevesse et al. (2003) designed degenerated primers to amplify exo-β-1,3-glucanase genes in the genome of strain K. Two genes, called PAEXG1 and PAEXG2 were cloned and sequenced. The PAEXG2 gene was further inactivated by disruption in a uracile-auxotroph strain derived from strain K. Surprisingly, there was no difference in biological control

properties against *B. cinerea* between strain K and PAEXG2-mutated strain. This represented a contradiction between biochemical and genetic approach.

Given the complexity of interactions between strain K, *B. cinerea*, and apple wounds, multiple genes are likely to contribute to biocontrol (Friel et al. 2007). In order to solve the observed contradiction, another disruption approach was undertaken to sequentially disrupt PAEXG1 and PAEXG2 genes by adapting the URA3-blaster technique previously developed for *Saccharomyces cerevisiae* (Alani et al. 1987). The results showed that the biocontrol properties of the strain were affected by single inactivation of PAEXG1 or PAEXG2 gene and by the double inactivation of both genes compared to the parental strain. These results were not in accordance to those published previously for PAEXG2 disruption (Grevesse et al. 2003). The explanation was brought through biocontrol assays carried on *in situ*. Friel et al. (2007) showed that the relative contribution of exo-β-1,3-glucanase was strongly depend on the quantity of applied strain in the wound and the maturity of apples. Indeed the mutated strains exerted no protective effect when low concentrations were applied to fresh apple fruit, but their protective effect was similar to that of the parental strain when they were applied to mature apple fruit at medium or high concentration or to fresh apple fruit at high concentration. This demonstrated that the importance of glucanase in biocontrol properties was depending on the experimental conditions studied, underlining the complexity of the interplay between the mechanisms of action of the strain and their dependence to the density of the biocontrol strain at the site of action.

Open Transcriptomic Approach

As mentioned above, the biocontrol properties of a BCA often depend on numerous genes interacting with each other sequentially or in parallel. For example, the mycoparasitic properties of the biocontrol agent *Trichoderma virens* rely on at least 18 genes (Steyaert et al. 2003). An "open" strategy complementary to the targeted approach holds great interest to identify other genes involved in biocontrol properties.

This "open" approach relies on the identification of genes differentially expressed by the BCA in several environmental conditions. Typically, the gene expression is compared between biocontrol-inducing conditions and control conditions. Amongst the protocols developed, the cDNA Amplified Fragment Length Polymorphism protocol was applied to identify genes potentially involved in biocontrol properties of strain Kh5, a haploid strain derived from strain K and presenting the same biocontrol properties. Strain Kh5 was grown *in vitro* on a medium containing glucose or cell wall preparation of *B. cinerea* as the sole carbon source. Eleven candidate genes were identified. Their differential expression was confirmed independently by real-time PCR. These genes corresponded to β-glucosidase, transmembrane transport, citrate synthase, and external amino acid sensing and transport. Some of these functions could be related to cell wall

metabolism and potentially involved in mycoparasitic properties (Massart and Jijakli 2006).

After this identification step, the molecular tools developed to disrupt candidate genes could be applied to further characterize their implication in biocontrol properties.

Open Proteomic Approach

The proteomic approach also corresponds to an open strategy studying the cell protein contents and highlighting the variations in the proteome according to the different conditions tested. It is complementary and often combined with transcriptomic approach as it targets the gene product instead of the transcribed mRNA. Kwasiborski et al. (2012) developed an *in situ* model and an extraction protocol both compatible with a 2-D gel electrophoresis protocol (see Fig. 10.3). The developed *in situ* model allowed exchanges between organisms, maintained the inhibitory effect of the antagonist while obtaining yeast quantity compatible with the downstream proteomic study and limiting the apple constituents' contaminations.

Proteins from strain Kh6, a haploid strain derived from strain K and presenting the same biocontrol properties, were extracted in exponential and stationary phases in the presence or absence of *B. cinerea* (Kwasiborski et al. 2014).

Exponential and stationary phase proteomic profiles differed, suggesting different physiological states of the yeast. In the exponential phase, results showed that most of the proteins influenced by the presence of the pathogen were involved in the energetic metabolism and in the protein synthesis. In the absence of the pathogen,

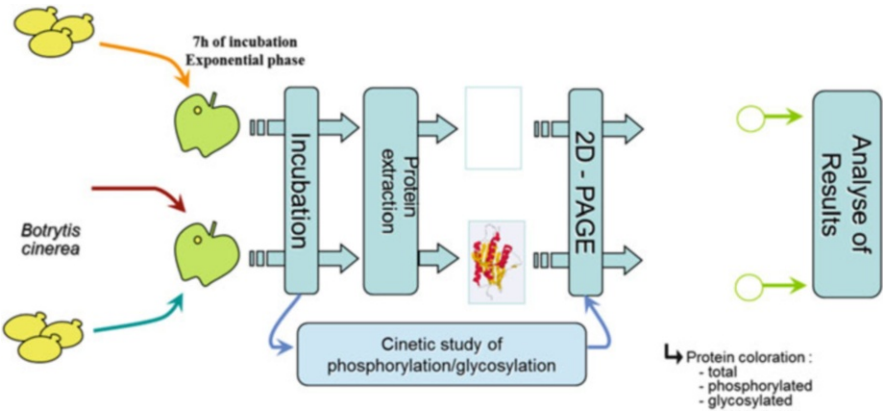

Fig. 10.3 *In situ* proteomic experiment to identify proteins specifically produced by *P. anomala* strain K in presence of *B. cinerea* in apple wounds

strain Kh6 produces energy through the glycolysis pathway while the presence of the pathogen oriented the energetic metabolism to the oxidative phosphorylation. More specifically, the BCA activates the pentose phosphate pathway. In addition, the presence of the pathogen led to an overexpression of proteins involved in nucleotides synthesis and transcription. These adaptations suggested that strain Kh6 modified its metabolism to optimize energy and nucleic acids production in order to colonize the wound as fast as in the absence of the pathogen.

During the stationary phase, strain Kh6 orientated its metabolism to the alcoholic fermentation in order to face the nutrients impoverishment of the wound, whatever the presence or absence of the pathogen. However, the overexpression of proteins implicated in the protein synthesis seemed to indicate a metabolic delay of strain Kh6 in presence of the pathogen.

These results showed that, in the presence of *Botrytis cinerea*, strain Kh6 is able to colonize efficiently the wound and to adapt its metabolism for limiting the growth and nutrient use of *B. cinerea*. This explained and confirmed the previous observations based on microbiological studies and suggesting a role of colonization in the mode of action of the strain.

Ecological Studies on Strain K and Strain O

Ecological studies are focused on the influence of environmental parameters on the growth and biocontrol properties of a BCA. These ecological studies on the BCA can also be completed by similar studies on the targeted pathogens to allow a better understanding of the complex relationship between both microorganisms.

Ecological studies will address key questions for the BCA. For a post-harvest application, the ecological studies will evaluate if the BCA is well adapted to the existing storage conditions. Moreover, the comparison with pathogen's niche will allow the selection of the most appropriate storage condition to control the pathogen, favoring BCA growth and limiting pathogen's growth. For a pre-harvest application, ecological studies will highlight the adverse environmental parameters hampering the establishment and the growth of the BCA. This knowledge will allow the development of a formulation limiting this negative influence.

Ecological studies can be done on artificial media *in vitro* as a pre-screening experiment or *in situ* on fruit surface as a confirmation experiment. In any case, *in vitro* studies must be completed by *in situ* experiments. The environmental parameters most often evaluated are the UV radiation, the relative humidity (or water activity *in vitro*) and the temperature.

UV Radiation

The influence of artificial UV-B radiation on strain K was evaluated in Petri dishes and *in situ* on apple. Clear differences in LD_{90} values were observed between both conditions: 1.6 kJ/m^2 *in vitro* (0.69 h of natural sunlight) and 5.76 kJ/m^2 *in situ* (2.46 h of natural sunlight). In order to protect strain K against the adverse effect of sunlight, eight UV-protectants were tested *in vitro* and *in situ* alone or in combination (Lahlali et al. 2011a). Five of the selected UV-protectants reduced yeast mortality caused by UV-B radiation on apple fruit surfaces. Amongst them, lignin and folic acid, increased significantly the ability of strain K to control *in situ* the post-harvest pathogen *Penicillium expansum* while the three other ones decreased the biocontrol efficacy. For strain O, the LD_{90} values were 1.45 kJ/m^2 *in vitro* and 5.5 kJ/m^2 *in situ*. Amongst the tested UV-protectants, riboflavin and uric acid were effective *in situ* to protect strain O against UV treatment. The addition of uric acid to strain O in biocontrol assays against *P. expansum* did not modify significantly the biocontrol efficacy (49.2 % vs. 47.7 %) (Lahlali et al. 2011b).

These *in situ* results must be further confirmed by real pre-harvest application with the selected UV-protectants.

Relative Humidity, Temperature and Initial Concentration

The environmental parameters influencing the biocontrol properties can be studied alone, like for UV light, or in combination. This latter option is particularly interesting to investigate the effects of parameters which might have synergic or antagonist actions on biocontrol efficacy. Nevertheless, studying the combined effects of several parameters might complicate the experimental design as the number of conditions to be compared growths exponentially.

For example, the effects of relative humidity (RH) and temperature should preferably studied in combination as they are often closely linked to each other. Lahlali and coworkers (2008) developed models to predict the combined effects of RH (75–98 %), temperature (5–25 °C) and applied concentration (10^4–10^8 CFU/ml) on the *in situ* growth of strain K and of strain O. Importantly, a Box and Behnken (1960) experimental design was applied to optimize the number of experiments. Multiple regression analyses showed that the model yielded a good prediction of yeast density. The RH had a greater effect than temperature. The number of yeast cfu per square centimeter of apple fruit surface increased with increasing RH, temperature, and initial applied yeast concentration. The optimal growth conditions corresponded to 25 °C and 98 % HR for both strains (Lahlali et al. 2008). These *in situ* results confirmed previous *in vitro* experiments showing a higher effect of water activity compared to temperature (Lalhali, unpublished results).

Results, Practical Advances Gained from Fundamental Research

Optimizing the Concentration of the BCA for Effective Protection

Modelling the growth of strain K and strain O, was particularly useful in order to select the most appropriate quantity of cells to be applied to reach a yeast density on apple surface which ensure an efficient protection against post-harvest pathogens. The models predicted that an initial application of 2.10^7 (strain O) or 10^7 CFU/ml (strain K) allowed to reach the threshold density of 10^4 CFU/cm^2.

Predicting the BCA Population Density After Pre-harvest Application

The developed model is able to predict the yeast population densities on the apple surface 48 h after field spraying of biocontrol agents (Lahlali et al. 2009). Depending on weather conditions, it might therefore be useful to evaluate the success of BCA colonization after pre-harvest application and to decide if an additional post-harvest treatment should be recommended.

Adapting the Storage Conditions

The effect of environmental parameters on the growth of pathogens can also be modeled (Lahlali et al. 2006). The models built for the BCA and for the pathogen can be compared in order to identify ecological conditions favorable to the BCA and adverse for the pathogen. For strain K and strain O, it might be recommended to maintain saturated HR at the beginning of post-harvest conservation as it has a negative influence only on *P. expansum* growth and not the development of both antagonistic strains.

Optimizing the BCA Formulation

The ecological studies highlighted the sensitivity of strain K against UVB and the development of a protecting formulation has been mentioned above. The results highlighted the positive effect of UV-protectant against UVB radiations.

The model developed for strain K and O suggested also an important effect on low RH on the *in situ* growth of the strain. Thanks to theses observation, the rational development of a formulation targeting a better tolerance to low RH can be prioritized for the practical pre-harvest application (Lahlali and Jijakli 2009).

The knowledge of the mode of action can also led to the targeted development of formulations which will enhance the BCA efficiency. An example might be the addition of cell wall preparation to BCA controlling the pathogens through mycoparasitisms or induction of plant defense.

Filling the Regulatory "Dossier"

Knowledge of the mode of action of a BCA will be an asset to get official registration for commercialization. Indeed, understanding precisely the mode of action, together with a genome sequencing of the strain, can rule out the antibiotic production by the BCA. In addition ecological studies can demonstrate the absence of growth or the death of the BCA at 37 °C, which will minimize the risk of opportunistic development in human body, more specifically for people with immune-deficiencies.

Summary

The journey from the isolation of a BCA to its commercialization is particularly challenging. This chapter highlights the complexity of the interactions and the need of applying a comprehensive panel of methodologies to get a better insight in the mode of action and in the ecology of a BCA.

Scientific evidences and strong demonstration of the BCA properties are brought by a smart combination of *in vitro* and *in situ* models, open and targeted strategies, traditional (microbiology and biochemistry) and innovative (genetic, transcriptomic and proteomic) technologies. In this chapter, we have also shown that fundamental researches can led to practical progress in the large scale application of a BCA.

More specifically, 20 years of researches have led to a better characterization of antagonist activity of *P. anomala* strain K and *C. oleophila* strain O against post-harvest pathogens of apple. The increasing knowledge gained on strain O throughout these years has been crucial in the successful registration of this strain at EU level in 2013 (Product name: Nexy from Lesaffre company – France). Fortunately, the experience gained from this long development will most probably shorten considerably the time between isolation and commercialization for a new BCA in the future.

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