Discovery, development and technology transfer of biocontrol agents for postharvest disease control.

Discovery, development and technology transfer of biocontrol agents for postharvest disease control

D. Spadaro[1,2], A. Garibaldi[1], M.L. Gullino[1,2]
[1]AGROINNOVA, University of Torino, Via L. da Vinci 44, 10095 Grugliasco (TO), Italy
[2]Dept. Agricultural, Forestry and Food Sciences (DISAFA), University of Torino, Via L. da Vinci 44, 10095 Grugliasco (TO), Italy

Keywords: biofungicide, efficacy, fruit, mechanism of action, molecular characterization, yeast.

Abstract

Several biocontrol agents (BCAs) have been discovered and tested for their efficacy in controlled and semi-commercial conditions. After the efficacy evaluation and preliminary studies on the mode of action, patenting is an essential step before contacting potential private companies interested in developing biofungicides. Several small enterprise are interested in developing BCAs, but the long and expensive registration process in Europe often discourages them. Further steps undertaken by the research sector include a deep characterization of the mechanism of action, and the development of molecular tools to track the microorganisms in the environment. The biomass production process and the development of appropriate stabilization and formulation are key issues to extend the shelf life of the biocontrol product and to develop a commercial biofungicide. These steps need the close collaboration between the industry and the research sector. Other possible ways of introducing the BCA on the market could include the creation of university spin off companies. To introduce biofungicides in the postharvest sector, appropriate technology transfer and demonstration activities are very important to involve the producers and the packinghouses, often reluctant to introduce BCAs in the food chain. The current manuscript offers an overview of the results achieved at the University of Torino, during the last years.

INTRODUCTION

Biological control using microbial antagonists (Janisiewicz and Korsten, 2002; Spadaro and Gullino, 2004) has emerged as one of the most promising alternatives, either alone or as part of an integrated pest management, to reduce pesticide use. During the past 30 years, several bacterial, yeast and fungal biocontrol agents have been exploited and widely investigated against different postharvest fungal pathogens. The high number of researches carried out in the field of postharvest biological control, testified by the number of publications, but the limited number of microorganisms registered represent the main difficulty of the research field, where most of the results acquired remain confined in the laboratory (Droby et al., 2009). The selection and development of a biocontrol product is a costly and long process, that will be explained in the present review.

ISOLATION

The first step in developing biocontrol agents (BCAs) is the isolation and screening process which will largely influence its efficacy and ultimately its success under commercial conditions. The isolation procedure of potential antagonists depends on

the characteristics of the pathogen infection.

To control postharvest diseases, investigators usually isolated naturally occurring microorganisms from fruits just before harvesting or during storage. The fruit surface is an excellent source of naturally occurring antagonists against postharvest fruit decay. A shortcoming of this strategy is that it favours the selection of antagonists that are generally fast growers with the ability to colonize a specific niche rich in nutrients, that mainly exhibit preventive or protective activity, and appear to have little effect on latent infections. Present screening methods also favour the selection of organisms whose primary mechanism of action is nutrient competition. A direct consequence of the type of screening procedures currently in use is the observation that several research programs in postharvest biocontrol (BC) worldwide have independently identified and selected antagonists from a narrow range of species (Table 1 and Table 2).

The fructoplane has provided the most abundant and most desirable source for isolating antagonists against postharvest fruit pathogens. However, the antagonists may also come from other closely related or unrelated sources. The phylloplane has also been a good source of antagonists, as it may share part of the resident microflora of fruits as well as contain other microorganisms dislodged from the fruit. Screening collections of yeast or starter cultures used in the food industry may also yield effective antagonists. Soil also may be an abundant and diverse source of antagonists.

Since the method of screening will have a major impact on the type and properties of the antagonist that are identified, it is important to evaluate the consequence of the methods for screening that are presently being utilized and appraise whether or not they can be improved.

SELECTION

The selection and development of a BC product is a costly and long process. For this reason, the initial steps in the research programme should involve the accurate selection of potential antagonists. For BC to be successful, the conditions that favour a potential antagonist should be the same or similar to those favouring the pathogen. The best antagonists perform well over the full spectrum of conditions conducive to pathogen development.

Most of the investigators evaluated the efficacy of microorganism strains on artificially wounded fruits since *in vitro* antagonistic properties of a strain does not always lead to *in vivo* protection activity. Most BCAs currently registered or under development for postharvest are able to control fruit decays originating from wound infections made during or after harvest.

Many postharvest decays of stone fruits and subtropical fruits develop in storage from latent infections occurring in the orchard. These infections are difficult to control because the intimate relationship of the pathogen with the host has been already established, and melanised appressoria often formed by these fungi on fruit surface are very resistant to environmental factors and penetration by fungicides. Screening against latent infections could benefit from focusing on organisms exhibiting mechanisms of BC which could most likely succeed against the melanised structures of the pathogen (Janisiewicz, 2010).

Strains of an antagonist species can be compared for effectiveness in controlling fruit decay, and for phenotypic characteristics that are useful in determining their commercial potential. For example, differentiation criteria for decay control on apple can include the BC efficacy of the strains, spectrum of activity (pathogens to be tested,

cultivar range, fruit maturity stages), ability to colonize wounded and sound fruit surfaces under various conditions, utilization of substrates occurring in fruits, or growth at cold storage temperatures and at 37°C (human body temperature). BCAs are more acceptable if they can be applied together with current practices, and information on the compatibility of the BCAs with chemicals used in the postharvest system, should be developed. Additional criteria may include resistance to environmental stress in the orchard application of BCAs, and pathogenicity of the antagonists to fruits, since strains of some antagonists with good BC potential. Since the antagonists are applied to consumable products (produce), they must meet strict requirements for human safety. The initial determination of the antagonist safety, based on the available literature, should be made early in the program. The final determination is made during commercial development of the product, and is based on an elaborate and costly toxicological profile. Other requirements for the commercial development of a BCA, involving mass production, formulation, application, and distribution, require a more intimate knowledge of commercialization of microbial pesticides.

Among the different BCAs, yeasts deserve particular attention, as their activity does not generally depend on the production of toxic metabolites, which could have a negative environmental or toxicological impact. The high number of yeasts among the postharvest antagonistic agents could be related to the fact that yeasts are tolerant to extreme environmental conditions of storage (temperature close to 0°C, high relative humidity, low oxygen level) and adapted to fruit characteristics (high sugar concentration, high osmotic pressure and low pH).

EXTENSION OF USE

Potential BCAs often have some significant limitations: they have a reduced action range, because they partially act on specific hosts against well-defined pathogens on particular environmental conditions. A method to select antagonists with a broader spectrum of action, preferably for commercial development, is the carrying out of initial efficacy tests on various pathogens and fruit and vegetable species. Other strategies to extend the use of BCAs include the use of antagonist mixtures, the integration of BCAs with other physical and chemical protection means, and the preharvest use of BCAs.

ANTAGONIST MIXTURES

It has been difficult to select individual strains with a broad spectrum of activity against major and minor pathogens that are effective when used on fruits at various maturity levels. Mixtures of compatible strains may be needed to provide the necessary spectrum of activity (Guetsky et al. 2002). Application of mixtures of microbial antagonists has certain advantages: i) widening the spectrum of microbial activity resulting in the control of two or more postharvest diseases; ii) increasing the effectiveness under different situations such as species, cultivars, maturity stages, and locations; iii) enhancing the efficiency and reliability of BC (permitting a reduction in the application times and treatment costs) as the components of the mixtures act through different mechanisms like antagonism, parasitism, and induction of resistance in the host; iv) combining different BC traits without the transfer of alien genes through genetic transformation.

The enhancement of the efficacy of microbial antagonists may be due to: better utilization of substrate, resulting in acceleration of the growth rate; removal of substances inhibitory to one organism by the other microbial agent; production of nutrients by one

microbe that may be used by another; and formation of a more stable microbial community that may exclude other microbes, including pathogens.

The benefits of using an antagonist mixture are clear, but implementation of this approach requires approval from the industry producing BCAs, because it entails a doubling of the cost to commercialize the antagonist mixture as compared to a single antagonist. The economic viability of this approach is favoured if mixtures include at least one antagonist that has already been commercialized.

PREHARVEST USE

One of the major obstacles to the development of postharvest BCAs is their inability to control previously established infections, such as latent infections. Therefore, preharvest application of microbial antagonistic culture are often effective to control postharvest decay of fruits (Ippolito and Nigro, 2000). There are two distinct approaches in applying BCAs in the field to control postharvest decays of fruits. In one, antagonists developed for postharvest application are applied just before harvest. The intent is to precolonize the fruit surface with an antagonist immediately before harvest, so that wounds inflicted during harvest can be colonized by the antagonist prior to colonization by a pathogen. In the other approach, antagonists, selected for field application, are applied throughout the fruit development, to reduce latent infections that can originate as early as bloom time and cause fruit decay after harvest when the natural mechanisms of resistance have broken down.

An antagonist applied in the field prior to harvest will have longer to interact with the pathogen as compared to an antagonist applied after harvest. However, applying and maintaining an active ingredient on the foliar surface presents a number of difficulties because BCAs would have to withstand exposure to variable and frequently hostile conditions on the vegetation canopy for long periods of time. To be successful in preharvest application, potential antagonists should be able to tolerate low-nutrient availability, UV rays, high temperature and dry conditions. Additives have often been used to try to meet these objectives.

INTEGRATED USE

During the past few decades, many attempts have been made to develop alternatives to fungicides to control postharvest decays on various commodities (Spadaro et al., 2004; Yu et al., 2008). They include environmental modification, such as storing commodities at temperatures suppressive to pathogen development, modifying relative humidity and the atmosphere; treating with hot air or water; inducing resistance by applying elicitors or UV irradiation; applying substances generally regarded as safe (GRAS: calcium infiltrations, sodium carbonates or ethanol); by applying animal and plant natural products; and by sterilizing fruits and handling water with UV irradiation or ozone. However, none of these methods, when used alone, provided satisfactory levels of decay control, although some appeared to be very useful when applied in combination with BC, resulting in additive or even synergistic levels of decay control.

BC product performance can be enhanced by applying the antagonist in a cascade similar to the hurdle technology strategy used in the food industry. By incorporating various control steps along the packing line from receiving to packing, different combinations of products can be tested that more specifically suit individual packinghouses. For instance, by first drenching or fine-spraying fruit with a disinfectant such as chlorine, followed by hot water dip or ethanol spray, hot air drying, and finally

BC treatment incorporated into wax, effective control of postharvest diseases can be provided. Creating a vacuum on the fruit surface and subsequently filling it with natural antagonists will not only reduce competition with other epiphytes, but will also stimulate the plant natural defence system.

MECHANISMS OF ACTION

A good understanding of the mode of action of BCAs towards plant pathogens is essential to develop appropriate selection strategies, production, formulation and methods of application, and to facilitate registration procedures.

1. Competition for nutrients and/or space

Competition is defined as niche overlap, where there is simultaneous demand for the same resource by two or more microbial populations. Competition for nutrients (e.g. carbohydrates, nitrogen, oxygen) and space is considered to be a primary mode of action against postharvest fungal pathogens. In this case, both the pathogen and the antagonist should have the same requirement for a specific nutrient or resource. Competition can be an effective biocontrol mechanism when the antagonist is sufficiently concentrated and present at the correct time and location and if it can utilize limited nutrients more efficiently than the pathogen. Yeasts and some bacteria can successfully compete with the pathogen, inhibiting its growth but often leaving it alive (Janisiewicz *et al.* 2001; Zhang *et al.* 2010). In particular, competition for iron is believed to play a significant role in biocontrol interactions. Iron is essential for fungal growth and pathogenesis, and iron sequestration by non-pathogenic microbes could be exploited in novel systems for biocontrol of postharvest pathogens (Saravanakumar *et al.* 2008).

2. The role of biofilm formation

Biofilm formation is a development process of microorganisms leading to formation of morphologically distinct multicellular structures, altered gene expression patterns, and enhanced resistance to stresses. Recently, the ability to form biofilms on the inner surface of wounds was indicated as a possible mechanism of biocontrol. The biocontrol activity of biofilm-forming yeast strain is tightly related to the morphological phase of cell harvesting after growing in liquid culture. Only yeast cells collected from the biofilm phase are effective in limiting pathogen growth, apparently being able to colonize the inner surface of artificial wounds with more efficiency (Giobbe *et al.* 2007).

3. Production of antimicrobial compounds

Antibiosis is defined as the inhibition or destruction of a microorganism by substances produced by another microorganism. Some of the most active bacteria are producers of antibiotics, whose action, at least partially, determines their effectiveness. Bacteriocins, antibacterial proteins, produced by bacteria, are able to form pores in the membrane of target cells and deplete the trans-membrane potential, resulting in a leakage of cellular materials. Strains of *Bacillus subtilis* have extensively been studied as BCAs of plant pathogens, and they often produce a range of antimicrobial cyclic lipopeptides, including iturins, fengycins and surfactins (Touré *et al.* 2004; Chen *et al.* 2008; Arrebola et al. 2010). The main concern, related to the use of antibiotics in food products, is the development of human pathogens resistant to these compounds and the possible development of resistance in fruit pathogens. Even if antibiotic producers appear to be

able to control wound infections established before antagonist application, at the moment, there are not such BCAs registered for use on fruit.

4. **Parasitism and release of hydrolases**

Parasitism occurs when the antagonist feeds on or within the pathogen, resulting in a direct destruction or lysis of fungal propagules and structures. Most phytopathogenic fungi have cell walls composed of complex polymers of β-1,3- and β-1,6-glucans, mannoproteins, as well as some chitin, which play an important role in maintaining the cell integrity and protecting against biotic and abiotic stresses. Breakdown of fungal cell wall requires the participations of the different enzymes, especially β-1,3-glucanase and chitinase. Glucanases and chitinases can be directly or indirectly involved in the mechanism of antagonistic yeasts against pathogens. Mycoparasites utilize fungal cell-wall-degrading enzymes, such as glucanases, chitinases and proteases to dissolve the fungal cell walls and penetrate the cells (Friel et al., 2007; Saravanakumar et al., 2009; Zhang *et al.* 2012).

5. **Induction of resistance**

Indirect antagonism implies mechanisms which are not a direct result of the activity of the BCA, but are the consequence of the response of the fruit tissue to the presence of beneficial microorganisms. Some BCAs can interact with the host tissue, particularly the wounds, increasing the cicatrisation processes. Several antagonistic yeasts are as much effective as applied before pathogen inoculation. Plant defence mechanisms include the hypersensitive response, formation of structural barriers, synthesis of phytoalexins, lignification of plant cell walls, synthesis of lytic enzymes, as well as expression of a wide range of pathogenesis-related proteins (PRPs) (Chan *et al.* 2007; Nantawanit et al. 2010). New omic tools can be used to evaluate the global effect of the application of biocontrol agents on the transcriptome and/or proteome of fruit (Jiang *et al.* 2009). A recent paper about a microarray analysis on the transcriptome of grapefruit after treatment with *Metschnikowia fructicola* showed that 1007 putative unigenes showed significant expression changes following yeast application (Hershkovitz *et al.* 2012). The most important induced genes included respiratory burst oxidase, mitogen-activated protein kinase cascades, G-proteins, chitinase, PAL, chalcone synthase and 4-coumarate-CoA ligase.

6. **The role of oxidative stress**

Oxidative stress plays a crucial role also in biological control (Castoria et al., 2003). The ability to survive and proliferate in wounded host tissues is pivotal for postharvest BCAs, and wounding of fruit tissue is associated with the accumulation of ROS, such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (OH^-), that can effect host response, pathogen virulence and yeast efficacy (Macarisin *et al.* 2010).

On the peel of grapefruit treated with *Metschnikowia fructicola*, peroxidase, SOD, and CAT were down-regulated. Moreover, suppression was correlated with significantly higher levels of H_2O_2, O_2^- and OH^- production in yeast-treated surface wounds (Hershkovitz *et al.* 2012). In plant defence against pathogens H_2O_2 can be involved in hypersensitive response mediated by a mitogen-activated protein cascade, lignin biosynthesis, induction of specific heat shock transcription factors, accumulation of PR genes, and antimicrobial compounds such as phytoalexins.

MICROBIAL ECOLOGY AND ENVIRONMENTAL TRACKING

Besides knowledge of the mechanisms of action, monitoring tools need to be developed to study the ecological fitness of antagonistic microorganisms after fruit treatment. The study of the population dynamics can help to interpret and predict the BC efficacy in relation to formulation and modality of application. Moreover, the registration of a specific strain of an antagonist requires its identity and stability be ascertained with precision, to ensure efficacy and genetic integrity of the product and to protect intellectual property. One of the major characteristics for an antagonist to be used in BC is its precise identification and its traceability, to permit to follow its environmental fate in space (dispersion) and in time (survival), after release. Moreover the effectiveness depends to a large extent upon the survival of the antagonists in competition with other microorganisms on the surface of fruits. A study of the ecology of the BCA is therefore necessary but often complicated by the existence of other microorganisms on the fruit that are morphologically similar to the antagonistic strain. Several monitoring methods have been developed to identify and to quantify microorganisms, the choice of the method being dependent on the required level of specificity. In the case of BCAs, a strain-specific detection level is needed as they are released into the environment where other strains of the same species of the BCA may be present.

The lack of morphological distinction among similar BCAs on Petri dishes has led to the development of more specific molecular methods. When DNA sequences are not available in databases for the design of PCR primers, the search for anonymous target DNA sequences has generally proven to be successful. Random markers as products of the PCR-based random amplified polymorphic DNA (RAPD) and arbitrarily primed-PCR (AP-PCR) have been widely used because of the simplicity of the method and its ability to discriminate closely related microorganisms without prior knowledge of the target sequences. However, reproducibility can be a problem for the applicability of the RAPD method, especially for weakly amplified bands. Therefore, the RAPD markers are converted into sequence-characterized amplified regions (SCAR) markers which are reproducible, can be organism-specific, and may represent a single locus in the genome, advantages that RAPD markers lack (De Clercq et al., 2003). More robust and reliable techniques, such as amplified fragment length polymorphism, based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA can be applied on potential BCAs. Specific fingerprints using amplified fragment length polymorphism (AFLP) technique have been applied to monitor the population of *Metschnikowia pulcherrima* (Spadaro et al., 2008). A more complex monitoring system, based on a quantitative-competitive PCR with an enzyme-linked oligosorbent assay, was developed to monitor *P. anomala* on apples (Pujol et al., 2004). Quantitative real time PCR has also been used to quantify BCAs (Schena et al., 2002; Spotts et al., 2009).

PRODUCTION

Often, during the research phase, several promising and useful microorganisms are found, but the subsequent step of production for commercialization – the so-called industrial phase – can represent the bottleneck of the whole development process. The scarcity on the market of microbiological formulates can be attributed to the high production costs and to the inadequate profit margins, especially when referred to the initial investment. When scaling up the production of an antagonist yeast strain, it is important to select candidates that can be mass-produced despite the detachment from the

natural habitat of growth. Mass production has to be cost-effective, because the use of the product has to be promoted in the postharvest disease control industry, which is investing only a small proportion of production costs into postharvest treatments. Growth media may therefore have to comprise industrial by-products or waste material. Although BCAs are more environmentally friendly, the sales price of the product must compete with that of currently used fungicides. The art of growing a BCA successfully is greatly dependent on the amount of technical information available on the specific microorganism strain. A rapid, efficient and cheap mass production of yeast or bacterial antagonists, generally by liquid fermentation, is one of the key issues to achieve the commercial use of the biofungicide (Wraight et al. 2001). Filamentous fungi on the opposite are generally produced by solid state fermentation. Liquid fermentation involves two additional steps at the end of a run: the separation of the inoculum through filtration and the inoculum concentration through centrifugation and dehydration. This last step may alter the functionality and BC capability of the microorganism, especially when it is characterized by rapid heating. To increase the biomass production of an antagonistic yeast on a laboratory scale, the optimization of the growth conditions, using different complex nutrient sources, is essential. The culture media can greatly influence the efficacy of the BCAs. To scale-up a laboratory fermentation process to an industrial level, it is fundamental to find nitrogen and carbon sources that provide maximum biomass production and minimum cost of media, whilst maintaining BC efficacy (Spadaro et al., 2010a). The use of commercial by-products with the same nutritional qualities can result in a cheap alternative for the yeast biomass production. Anyway, often by-products are not standardized as purified products and they may contain impurities that need to be removed before fermentation. Moreover, their composition may vary according to season and origin. For these reasons, appropriate procedures should be employed to standardize the industrial growth media. Harvesting stationary phase cells is desirable to enhance cell survival under stress conditions such as low water potential or drying.

STABILIZATION AND FORMULATION

The microbial agent should be formulated as a product having a storage stability of at least 6 months. Formulation is necessary in order to present the product in a usable form and to optimize the efficacy, stability, safety and ease of application of the product. The formulation must therefore provide conditions which retain viability during preparation, storage and application, and favour survival of the agent, in the environment. The choice of the type of formulation depends primarily on the delivery target, as well as on the biology and ecology of the BCA, pathogen and host, and cropping system. The need of storing and preserving the microbial pesticide requires an increase in the shelf-life, which is one of the main limiting factors. The treatment consists of stabilizing the viability of the microorganism. This can be achieved i) in liquid state maintained by refrigeration; ii) by freezing in presence of cryoprotectant substances; iii) by dehydrating the product. The methods based on dehydration – fluidized-bed drying, spray drying and freeze drying – allow optimum conditions for storage, handling, distribution and formulation of the microorganism (Abadias et al., 2001; 2005; Spadaro et al., 2010b). The shelf life of a biological product refers to the period of time during which the antagonistic cells remain viable and effective. A correct formulation can be decisive in the improvement of the efficacy and extension of the product shelf-life, facilitating the storage for periods of time commercially acceptable (Burges, 1998). Formulations have a profound effect on BCAs and products, including shelf life, ability to grow and survive

after application, effectiveness in disease control, ease of operation and application, and cost. Additionally, formulated preparations of the antagonist should also be evaluated as quickly as possible to determine if commercial methods of antagonist production have a major impact on BC activity. The antagonistic microorganisms so far developed have been formulated as liquid suspensions or as wettable powders, because they should be applied through dipping, drenching or spraying. Formulations can influence the survival and activity of BCAs on fruit surfaces and in wounds. The application of adjuvants can protect and stimulate the establishing of the antagonist on the host surface (Usall et al., 2010). Formulations that include wetters to facilitate reabsorption of moisture from air may reduce this problem. Wetters not only make water spray stay on fruit surfaces but, like oil carriers, they also enable organisms to reach otherwise inaccessible places such as stomata and lenticels, thereby improving the chances of establishing antagonists for disease control. Reducing the oxygen level in the formulation by adding oxygen adsorbents or by using vacuum-packing is also important. Vacuum-packing of yeast granules or powders will further prolong the shelf-life of the product. Research is needed to determine the value of each additive alone and also in the presence of the other ingredients. Sodium alginate, carboxymethylcellulose and chitosan, are adhesivants and can be added to yeast cell suspension, to increase the activity of the formulate. Chitosan has also a fungistatic activity demonstrated against the main postharvest pathogens of strawberry.

PRACTICAL APPLICATIONS

At present there are few commercial products available in the market for postharvest use (Table 3). Biosave™ (*Pseudomonas syringae*) was originally registered in the USA for postharvest application to pome and citrus fruits, and this was later extended to cherries, potatoes and more recently to sweet potatoes. Recently, Shemer™ (*Metschnikowia fructicola*) was registered in Israel for both pre- and postharvest application on various fruit and vegetables including apricots, citrus, grapes, peach, pepper, strawberry and sweet potato. In addition, a commercial formulation of *Candida sake* was recently developed and registered for use on pome fruit in Spain under the name Candifruit™. BoniProtect™, developed in Germany, is based on antagonistic strains of the yeast-like fungus *Aureobasidium pullulans* and is used in preharvest to control wound pathogens developing in postharvest on apples. Avogreen™ has been used for control of Cercospora spot, a postharvest disease of avocado in South Africa, but its use has been limited, due to inconsistent results. A product based on *Candida oleophila* (Aspire™) and another product based on *Cryptococcus albidus* (Yieldplus™) were commercialized for some years but they were not successful. Aspire™ was registered in the United States for postharvest application to citrus and pome fruits, but the product was taken off the market 3 years after its large scale commercial introduction. Moreover, Nexy, based on another strain of *C. oleophila*, has been recently approved for use first in Belgium and then in the whole European market.

CONCLUSIONS

The most critical criterion for the success of a BC product is whether or not it performs effectively under commercial conditions, providing an acceptable and consistent level of control of the target diseases (Fravel et al. 1999). In most cases, as a part of the last phase of the commercial development process, BC preparations are usually tested on their targeted crops at different locations using specific application methods. In order to

conduct meaningful tests, large scale production of a formulated BCA is required. BC of plant diseases in general, and on fruit after harvest in particular, is a niche market, with a relatively small profit potential. Thus, finding an industrial partner has been the first challenge to public-sector researchers seeking to commercialize a BCA. Companies (including chemical companies) are always looking for new opportunities in the same or related markets as their existing products. If a biological product fits a market segment not occupied by a company existing product line, then a biologically based product can be a desirable new product. An example is in the area of postharvest fruit disease control. Existing fungicides for postharvest disease control have been reduced in number over the last decade because of regulatory restrictions and the development of pathogen resistance. On the other hand, an effective BCA that does not have toxicity problems is relatively easier and much less expensive to register. So it seems that the market would favour the development of new BC products. Large-scale feasibility tests should be conducted in cooperation with the company. The company should investigate the potential for registration and formulation of the antagonist before making the commitment. Developing different formulations is required to address the different needs of growers in terms of mixing, integrating with existing chemicals, and application methods. It is essential that a formulated product, despite mass production of large quantities, retains the properties of the initial lab-grown cultures. The formulation must retain its species purity (not be contaminated) and the microbial cells must retain their genetic stability, cell viability, attributes as colonizers on fruit surfaces, as well as other aspects of their mechanism of action. Industrial fermentation is accomplished under conditions quite different from those in shake culture. The process must be cost-effective, rely on industrial by-products as nutrients and fermentation must be completed within 24–30 h. Downstream processing involves various steps, such as drying, addition of volume materials (inert ingredients), adhesives, emulsifiers and adjuvants. All these actions may adversely affect the properties of the selected BCA. The effect of commercial conditions on the physiological state of the BCA and its activity following rehydration is also critical. Information on the effect of industrial production practices as well as formulation technologies themselves should be investigated early in the development process before the product reaches costly commercial tests. Results of tests performed under commercial or semi-commercial conditions with formulated BC preparations indicate that inconsistency and variability in the level of disease control are among the most significant barriers preventing widespread implementation of BC technology. In order to improve reliability and efficacy, efforts have been made to enhance efficacy and reliability by various means. To build confidence in the product within the fruit industry, pilot tests should be conducted in commercial packinghouses. Extensive technical support and quality control are essential in the success of the product. Marketing BC products requires specialized knowledge of the target plant disease, the BCA, integrated disease control practices, production and storage systems, and microbial ecosystems. The success of implementing BC or integrated disease management systems will depend largely on product knowledge and a thorough understanding of the complexity of the disease and postharvest environment. Distributing both the product and the knowledge necessary for its successful use will be the only effective way to ensure long-term market acceptance. The use of BCAs as an alternative to the synthetic, chemical fungicides that are presently used to control postharvest pathogens has many constraints and obstacles that make it difficult to implement their use as a practical control strategy. The advances made and commercial products so far developed, although limited, nevertheless represent promising possibilities. In most cases,

however, even commercially available products still need to be fine-tuned and enhanced. A probable scenario is that the use of postharvest BC in general will continue to increase slowly.

Scientists, growers and consumers alike must accept the fact that BCAs are usually not as effective as pesticides. The success of BC greatly depends on influencing the consumer to prefer inner quality to outward appearance. BC should be viewed more and more as an important component of an integrated disease management scheme for a significant and permanent reduction of pesticide use. The science and practice of postharvest BC is still in its infancy compared to the fungicidal treatment, but the progress made in this area during the past twenty years has been remarkable. Gradual removal of the major regulatory barriers to registration of antagonists for postharvest disease control in different countries is encouraging. New biofungicides under development further testify the increasing interest in BC of postharvest diseases.

ACKNOWLEDGEMENTS

This research was funded by the projects "SafeFoodControl – Development of innovative systems and technologies for the production, storage, processing and valorization of Piedmontese fruit and vegetables" and by the project "PROACTIN - Innovative technologies of production, storage and processing of kiwifruit in the presence of *Pseudomonas syringae* pv *actinidiae"* granted by the Piedmont Region.

Literature cited

Abadias M., Benabarre A., Teixidò N., Usall J., Viñas I. (2001). Effect of freeze drying and protectants on viability of the biocontrol yeast *Candida sake. International Journal of Food Microbiology* 65, 173-182.

Abadias M., Teixidó N., Usall J., Solsona C., Viñas I. (2005). Survival of the postharvest biocontrol yeast *Candida sake* CPA-1 after dehydration by spray-drying. *Biocontrol Science and Technology* 15, 835-846.

Arrebola E., Jacobs R., Korsten L. (2010). Iturin A is the principal inhibitor in the biocontrol activity of *Bacillus amyloliquefaciens* PPCB004 against postharvest fungal pathogens. *Journal of Applied Microbiology* 108, 386-395.

Burges H.D. (1998). *Formulation of Microbial Biopesticides: Beneficial microorganism, nematodes and seed treatments*, pp. 396. Dordrecht: Kluwer Academic Publishers.

Castoria R., Caputo L., De Curtis F., De Cicco V. (2003). Resistance of postharvest biocontrol yeasts to oxidative stress: A possible new mechanism of action. *Phytopathology* 93, 564-572.

Chan, Z., Qin, G., Xu, X., Li, B. & Tian, S. (2007) Proteome approach to characterize proteins induced by antagonist yeast and salicylic acid in peach fruit. *Journal of Proteome Research* **6,** 1677-1678.

Chen H., Wang L., Su C.X., Gong G.H., Wang P., Yu Z.L. (2008). Isolation and characterization of lipopeptide antibiotics produced by *Bacillus subtilis*. *Letters in Applied Microbiology* 47, 180-186.

De Clercq D., Cognet S., Pujol P., Lepoivre P., Jijakli M.H. (2003). Development of SCAR marker and a semi-selective medium for specific quantification of *Pichia anomala* strain K on apple fruit surface.

Droby S., Wisniewski M., Macarisin D., Wilson C. (2009). Twenty years of postharvest biocontrol research: Is it time for a new paradigm? *Postharvest Biology and Technology* 52, 137-145.

Fravel D.R., Rhodes D.J., Larkin R.P. (1999). Production and commercialization of biocontrol products, in *Integrated pest and disease management in greenhouse crops*, (ed. R. Albajes, M.L. Gullino, J. van Lenteren, Y. Elad), pp. 365-376. Dordrecht: Kluwer Academic Publishers.

Friel D., Pessoa N.M.G, Vandenbol M., Jijakli M.J. (2007). Separate and combined disruptions of two exo-β-1,3-glucanase genes decrease the efficiency of *Pichia anomala* (strain K) biocontrol against *Botrytis cinerea* on apple. *Molecular Plant-Microbe Interactions* **20**, 371-379.

Giobbe S., Marceddu S., Scherm B., Zara G., Mazzarello V.L., Budroni M., Migheli Q. (2007). The strange case of a biofilm-forming strain of *Pichia fermentans*, which controls *Monilinia* brown rot on apple but is pathogenic on peach fruit. *FEMS Yeast Research* **7,** 1389-1398.

Guetsky R., Shtienberg D., Elad Y., Fischer E., Dinoor A. (2002). Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. *Phytopathology* **92**, 976-985.

Hershkovitz, V., Ben-Dayan, C., Raphael, G., Pasmanik-Chor, M., Liu, J., Belausov, E., Aly, R., Wisniewski, M. & Droby, S. (2012) Global changes in gene expression of grapefruit peel tissue in response to the yeast biocontrol agent *Metschnikowia fructicola. Molecular Plant Pathology* **13**, 338–349.

Ippolito A., Nigro F. (2000). Impact of preharvest application of biological control agents on postharvest diseases of fresh fruits and vegetables. *Crop Protection* **19**, 715-723.

Janisiewicz W.J., Tworkoski T.J., Kurtzman C.P. (2001). Biocontrol potential of *Metschnikowia pulcherrima* strains against blue mold of apple. *Phytopathology* **91,** 1098-1108.

Janisiewicz W.J., Korsten L. (2002). Biological control of postharvest diseases of fruits. *Annual Review of Phytopathology* **40,** 411-441.

Janisiewicz W.J. (2010). Quo vadis of biological control of postharvest diseases, in *Post-harvest Pathology. Plant Pathology in the 21st Century, Vol. 2.*, (ed. D. Prusky, M.L. Gullino), pp. 137-148. Dordrecht: Springer.

Jiang, F., Zheng X., Chen J. (2009). Microarray analysis of gene expression profile induced by the biocontrol yeast *Cryptococcus laurentii* in cherry tomato fruit. *Gene* **430**, 12-16.

Macarisin D., Droby S., Bauchan G., Wisniewski M. (2010). Superoxide anion and hydrogen peroxide in the yeast antagonist-fruit interaction: A new role for reactive oxygen species in postharvest biocontrol? *Postharvest Biology and Technology* **58**, 194-202.

Nantawanit N., Chanchaichaovivat A., Pinijpan B., Ruenwongsa P. (2010). Induction of defense response against *Colletotrichum capsici* in chili fruit by the yeast *Pichia guilliermondii* strain R13. *Biological Control* **52,** 145-152.

Pujol M., De Clercq D., Cognet S., Lepoivre P., Jijakli M.H. (2004). Monitoring system for the biocontrol agent *Pichia anomala* strain K using quantitative competitive PCR-ELOSA.

Saravanakumar D., Ciavorella A., Spadaro D., Garibaldi A., Gullino M.L. (2008). *Metschnikowia pulcherrima* strain MACH1 outcompetes *Botrytis cinerea*, *Alternaria alternata* and *Penicillium expansum* in apples through iron depletion. *Postharvest Biology and Technology* **49**, 121-128.

Saravanakumar D., Spadaro D., Garibaldi A., Gullino M.L. (2009). Detection of enzymatic activity and partial sequence of a chitinase in *Metschnikowia pulcherrima*

strain MACH1 used as postharvest biocontrol agent. *European Journal of Plant Pathology* **123**, 183-193.

Schena L., Finetti Sialer M., Gallitelli D. (2002). Molecular detection of strain L47 of *Aureobasidium pullulans*, a biocontrol agent of postharvest diseases. *Plant Disease* **86,** 54-60.

Spadaro D., Gullino M.L. (2004) – State of art and future perspectives of biological control of postharvest fruit diseases. International Journal of Food Microbiology, 91 (2), 185-194.

Spadaro D., Ciavorella A., Lopez G., Garibaldi A., Gullino M.L. (2010b). Effect of protectants and initial cell concentration on viability of freeze-dried cells of *Metschnikowia pulcherrima*. *Canadian Journal of Microbiology* **56**, 809-815..

Spadaro D., Ciavorella A., Zhang D., Garibaldi A., Gullino M.L. (2010a). Effect of culture media and pH on the biomass production and biocontrol efficacy of a *Metschnikowia pulcherrima* strain to be used as a biofungicide for postharvest disease control. *Canadian Journal of Microbiology* **56**, 128-137.

Spadaro D., Garibaldi A., Gullino M.L. (2004). Control of *Botrytis cinerea* and *Penicillium expansum* on apple combining a biocontrol agent with hot water dipping and acibenzolar-S-methyl, baking soda, or ethanol application. *Postharvest Biology and Technology* **33**, 141-151.

Spadaro D., Sabetta W., Acquadro A., Portis E., Garibaldi A., Gullino M.L. (2008). Efficacy and genetic diversity of *Metschnikowia pulcherrima* strains isolated from different food matrices against postharvest diseases in apple. *Microbiological Research* **163**, 523-530.

Spotts R.A., Wallis K.M., Serdani M., O'Gorman D.T., Sholberg P.L. (2009). Real time polymerase chain reaction for rapid and quantitative determination of *Cystofilobasidium infirmominiatum* on the surfaces of apple, pear, and sweet cherry fruit. *Postharvest Biology and Technology* **51**, 227-231.

Touré Y., Ongena M., Jacques P., Guiro A., Thonart P. (2004). Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botryitis cinerea* on apple. *Journal of Applied Microbiology* **96,** 1151-1160.

Usall J., Teixidó N., Abadias M., Torres R., Cañamas T., Viñas I. (2010). Improving formulation of biocontrol agents manipulating production process, in *Post-harvest Pathology. Plant Pathology in the 21st Century, Vol. 2*, (ed. D. Prusky, M.L. Gullino), pp. 149-169. Dordrecht: Springer.

Wraight S.P., Jackson M.A., de Kock S.L. (2001). Production, stabilization and formulation of fungal biocontrol agents, in *Fungi as Biocontrol Agents,* (ed. T.M. Butt, C. Jackson, N. Magan), pp. 253-287.

Yu T., Zhang H., Li X., Zheng X. (2008). Biocontrol of *Botrytis cinerea* in apple fruit by *Cryptococcus laurentii* and indole-3-acetic acid. *Biological Control* **46**, 171-177.

Zhang D., Spadaro D., Garibaldi A., Gullino M.L. (2010). Screening and efficacy evaluation of three antagonists against postharvest brown rot of peaches. *Postharvest Biology and Technology* **55**, 174-181.

Zhang, D. Spadaro, D., Valente, S., Garibaldi, A. & Gullino, M.L. (2012) Cloning, characterization, expression and antifungal activity of an alkaline serine protease of *Aureobasidium pullulans* PL5 involved in the biological control of postharvest pathogens. *International Journal of Food Microbiology* **153,** 453-464.

Table 1: Main yeast or fungal species with antagonistic properties against postharvest pathogens of fruits and vegetables studied in the last 20 years

Acremonium breve	*Metschnikowia andauensis*
Aureobasidium pullulans	*Metschnikowia fructicola*
Candida ciferrii	*Metschnikowia gruessii*
Candida ernobii	*Metschnikowia pulcherrima*
Candida membranifaciens	*Muscodor albus*
Candida oleophila	*Oxyporus latemarginatus*
Candida saitoana	*Pichia anomala*
Candida sake	*Pichia angusta*
Cryptococcus albidus	*Pichia guilliermondii*
Cryptococcus flavus	*Pichia membranaefaciens*
Cryptococcus humiculus	*Pseudozyma fusiformata*
Cryptococcus infirmominiatus	*Rhodosporidium paludigenum*
Cryptococcuslaurentii	*Rhodotorula glutinis*
Cryptococcus magnus	*Rhodotorula mucilaginosa*
Debaryomyces hansenii	*Sporidiobolus pararoseus*
Filobasidium floriforme	*Sporobolomyces roseus*
Hanseniaspora uvarum	*Trichoderma harzianum*
Kloeckera apiculata	*Trichoderma viride*
Leucosporidium scotti	*Trichosporon pullulans*

Table 2: Main bacterial species with antagonistic properties against postharvest pathogens of fruits and vegetables studied in the last 20 years

Bacillus amyloliquefaciens
Bacillus subtilis
Bacillus licheniformis
Bacillus pumilus
Burkholderia cepacia
Burkholderia gladioli
Burkholderia glathei
Brevundimunas diminuta
Enterobacter aerogenes
Enterobacter cloacae
Pantoea agglomerans
Pseudomonas aeruginosa
Pseudomonas cepacia
Pseudomonas corrugata
Pseudomonas fluorescens
Pseudomonas putida
Pseudomonas syringae
Rahnella aquatilis
Stenotrophomonas maltophilia

Table 3: Biofungicides for the control of postharvest diseases commercialized since 1995.

Commercial product	**Antagonist**	**Pathogens controlled**	**Host fruits**	**Availability**
Biosave 100	*Pseudomonas syringae*	*Botrytis cinerea, Penicillium* spp., *Mucur piriformis, G. candidum*	Pome, citrus fruit, cherry, potato, sweet potato	USA
Biosave 110	*Pseudomonas syringae*	*B. cinerea, Penicillium spp., M. piriformis, G. candidum*	Pome fruit, potato, sweet potato	USA
Aspire	*Candida oleophila*	*B. cinerea, Penicillium spp.*	Citrus fruit, pome fruit	USA, no more
YieldPlus	*Cryptococcus albidus*	*B. cinerea, Penicillium spp.*	Pome fruit	S. Africa, no more
Avogreen	*Bacillus subtilis*	*Cercospora purpurea, Colletotrichum gloeosporioides*	Avocado	S. Africa, no more (preharvest)
Shemer	*Metschnikowia fructicola*	*B. cinerea*	Table grape, strawberry, sweet potato	Israel
Candifruit	*Candida sake*	*B. cinerea, P. expansum*	Pome fruit	Spain
BoniProtect	*Aureobasidium pullulans*	*B. cinerea, P. expansum*	Pome fruit	Austria (preharvest)
Nexy	*Candida oleophila*	*B. cinerea, P. expansum*	Pome fruit	Belgium, UE