State of art and future perspectives of biological control of postharvest fruit diseases

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.
DOI: 10.1016/S0168-1605(03)00380-5

STATE OF ART AND FUTURE PERSPECTIVES OF BIOLOGICAL CONTROL OF POSTHARVEST FRUIT DISEASES

Davide Spadaro and Maria Lodovica Gullino*

Di.Va.P.R.A. – Plant Pathology, University of Torino, Via L. da Vinci 44, 10095 Grugliasco (TO), Italy.

Abstract

Synthetic fungicides, when admitted, are the primary means to control postharvest diseases of fruits. Biological control using antagonists has emerged as one of the most promising alternatives to chemicals. During the last twenty years, several biocontrol agents have been widely investigated against different pathogens and fruit crops. Many biocontrol mechanisms have been suggested to operate on fruit including antibiosis, parasitism, induced resistance and competition. Trying to extend the use of the biofungicides, there have been deep studies on application of antagonists mixes, preharvest use, and integration with chemical and physical means of protection. The formulation and application methods are key issues for the efficacy and final success of the commercial product. The new molecular techniques are useful tools in the characterisation of the microorganisms and enhancement of their biocontrol capabilities, through genetic engineering. Although a huge number of scientific papers published on biological control, this method at the moment should be viewed as an important component of an integrated disease management scheme if a significant and permanent reduction of pesticide use is our goal. Anyway scientists, growers and consumers alike must accept the fact that BCA's are usually not as effective as pesticides.

* Corresponding author: Davide Spadaro, Di.Va.P.R.A. – Plant Pathology, University of Torino, Via L. da Vinci 44, 10095 Grugliasco (TO), Italy; phone: +39-6708696; fax: +39-6708541, email: spadaro@agraria.unito.it

Keywords: antagonist, fruit, mechanism of action, postharvest disease.

1. Introduction

Fruits are an important part of the human diet, because they supply essential nutrients such as vitamins, minerals, and they are important to human health and well-being, for their contents in antioxidants and anticancer substances. An increasing awareness by consumers that diet and health are linked has resulted in a greater consumption of fruits. At the same time, consumers are also more concerned about the safety of the fruits they eat, and they ask for food free from pesticide residues, toxins and pathogens.

Losses due to pests and diseases attacks on fruit in field and during storage, transit, and commercialisation steps, before reaching the consumer, are not easily assessed, but can result in 25% of the total production in industrialised countries (Harvey, 1978). In developing countries damages are often higher, exceeding 50%, because of the lack of adequate storage structures (Eckert and Ogawa, 1985). The high water content of plant products, such as fruit, is one of the features that makes them more susceptible to pathogens attack, since they are in orchard (Harvey, 1978). Another factor favourable to pathogenic fungi, particularly to the necrotrophic ones, is the presence during storage on the plant organs of wounds, often produced during harvest and transport of fruit, which represent an ideal way of access for microorganisms.

Synthetic fungicides, when admitted, are the primary means to control postharvest diseases. However, several reasons, such as the growing public concern over the human health conditions and the environmental pollution associated with pesticide usage in orchards (Wilson and Wisniewski, 1994), the development of fungicide resistant strains of postharvest

pathogens (Romano et al., 1983; Spotts and Cervantes, 1986) and the lack of reregistration of some of the most effective fungicides (Gullino and Kuijpers, 1994) have encouraged the search of alternative approaches.

Biological control well fits with the concept of sustainable agriculture, because it mostly exploits natural cycles with zero or reduced environmental impact. Among the biological strategies adoptable in postharvest, the induction of resistance in the fruit, the use of plant or animal products with a fungicidal activity, and, above all, the application of antagonistic microorganisms can be considered. Biological control using antagonists (Wilson and Wisniewski, 1994) has emerged as one of the most promising alternatives, either alone or as part of an integrated pest management to reduce pesticide use.

The postharvest environment represents a particular sector for the development of biological measures. Peculiar difficulties are present in the control of postharvest diseases (Chalutz and Droby, 1998): the disease control level required is extremely high (also 95-98%); the nutritional safety imposes special care to the direct use of living microorganisms on food products; the potential market to employ a biofungicide expressly developed for postharvest use is relatively small. On the other side, the possibilities of success for postharvest biological means can be numerous (Chalutz and Droby, 1998): the storage conditions partially controlled, such as temperature and humidity, can switch the host-pathogen-antagonist equilibrium towards the antagonist and the laboratory trials and results have a higher possibility to be transferred into practice; the application site of the antagonist, which is the fruit, is limited, permitting an increase of the biocontrol agent (BCA) efficacy and avoiding the presence of some interfering factors; finally the high value of fruit can justify a treatment with a product relatively expensive.

During the last 20 years, several biocontrol agents have been exploited and widely investigated against different postharvest fungal pathogens (*Alternaria*, *Botrytis*,

Colletotrichum, Monilia, Penicillium, Rhizopus spp.) on different host species. Many of the first studies were aimed at the study of the mode of action and the evaluation of the efficacy of some potential biocontrol bacteria, such as *Brevibacillus subtilis*, producer of antibiotics (Pusey et al., 1986). The consideration that the application of bacterial antagonists on the fruit was not commercially acceptable, at least in the short period, brought to switch the interest on antagonists using modes of action different from antibiosis. Wilson and Wisniewski (1994) indicated the characteristics of an ideal antagonist: genetic stability, efficacy at low concentrations and against a wide range of pathogens on various fruit products, simple nutritional requests, survival in adverse environmental conditions, growth on cheap substrates in fermenters, lack of pathogenicity for the host plant and lack of production of metabolites potentially toxic for humans, resistance to the most frequently used pesticides, compatibility with other chemical and physical treatments. Yeasts, between the potential BCAs, possess a good number of the mentioned features and, during the last years, research focused on the selection and study of these microorganisms.

At present, three products containing *Pseudomonas syringae* Van Hall, active against *Botrytis, Penicillium, Mucor* and *Geotrichum* spp., named Bio-Save 100, Bio-Save 110 and Bio-Save 1000 and commercialised by EcoScience Corp. (Janisiewicz and Jeffers, 1997), and a product containing *Candida oleophila* Montrocher, effective against *Botrytis* and *Penicillium* spp., named Aspire and commercialised by Ecogen Inc. (Hofstein et al., 1994), are available on the market for postharvest protection. Other yeast species have been extensively tested and could be registered in relatively short times. Among the microorganisms under development, there are antagonistic strains belonging to the species *Aureobasidium pullulans* (Ippolito et al., 2000), *Candida saitoana* (El-Ghaouth et al., 1998), *Candida sake* (Teixido et al., 1998) and *Metschnikowia pulcherrima* (Spadaro et al., 2001a).

2. Mechanisms of action

Information on the mechanisms of action for most of the antagonists investigated is still incomplete, because of the difficulties encountered during the study of the complex interactions between host, pathogen, antagonist and others microorganisms present in the site of interaction. However a good understanding of the mode of action is essential to develop appropriate formulation and methods of application, and to obtain registration.

Several possible biocontrol mechanisms have been suggested to operate against post-harvest rots on fruit including antibiosis, parasitism or direct interaction with the pathogen (extracellular hydrolases), induced resistance and competition for nutrients and space (Droby and Chalutz, 1994).

2.1. Antibiosis

Some of the most active bacteria are producer of antibiotics, whose action, at least partially, influences these BCA's effectiveness. *B. subtilis* was able to produce iturin, a powerful antifungal peptide (Gueldner et al., 1988) but also gramicidin S (Edwards and Seddon, 2001), *Pseudomonas cepacia* showed to synthesise pyrrolnitrin, that used alone can control *Botrytis cinerea* and *Penicillium expansum* attacks on pome fruit (Janisiewicz et al., 1991). Also from these observation, it was possible to develop phenylpyrroles fungicides (Gehmann et al., 1990). Often, over antibiosis, other mechanisms are present: *Penicillium digitatum* strains resistant to pyrrolnitrin are still inhibited by *P. cepacia* (Smilanick and Denis-Arrue, 1992). The main concerns, related to the use of antibiotics in food products, regard the selection of human pathogens resistant to these chemicals and the likely rapid development of resistance in fruit pathogens. Even if antibiotic producers are able to control wound infections occurred before antagonist application or latent infections, at the moment there are not such antagonists

146 registered for use on food.

147

148 *2.2 Competition*

149 Others selected microorganisms, particularly yeasts, act mainly establishing with the pathogen
150 a competition for space and/or utilisation of some nutrients (Piano et al., 1997; Filonow,
151 1998; Spadaro et al., 2001a). Yeasts can successfully compete with the pathogen, inhibiting
152 its growth but often leaving it alive. Also some bacteria act for competition, for example
153 *Enterobacter cloacae* against *Rhizopus stolonifer* on peaches (Wisniewski et al., 1989). In the
154 competition for space yeasts are helped by the formation of an extracellular polysaccharide
155 capsule that can promote adhesion to fruit surface (Andrews et al., 1994). Competition for
156 nutrients was demonstrated for *Pichia guilliermondii* against *P. digitatum* cocultivated on
157 synthetic means (Droby et al., 1989): the addition of exogenous nutrients resulted in a reduced
158 efficacy, because antagonists offered better results when nutrients were scarce. A rapid
159 multiplication and colonisation by antagonist cells in the wound was elucidated in various
160 interactions (Droby et al., 1989; Smilanick and Denis-Arrue, 1992; Piano et al., 1997). Recent
161 studies on the repartition of radiolabelled glucose between the antagonist yeasts
162 *Sporobolomyces roseus* or *Cryptococcus laurentii* and the pathogen *B. cinerea* point out a
163 strong sugar assumption by the BCAs, that blocks fungus conidial germination for nutrients
164 deprivation (Filonow, 1998). In fruit wounds, nutrients competition is likely extended to other
165 nutrients, such as nitrogen compounds low concentred. Janisiewicz et al. (2000) recently have
166 developed a non-destructive method using tissue culture plates with cylinder inserts
167 containing defusing membrane at one end to study competition for nutrients without
168 competition for space.

169

170 *2.3 Parasitism*

Antagonist and pathogen can interact also through a direct parasitism. Wisniewski et al. (1991) observed a strong adhesion *in vitro* of *P. guilliermondii* antagonist cells to *B. cinerea* mycelium, perhaps due to a lectin link. Such adhesion is blocked by exposure to compounds able to alter the protein integrity and the respiration process. Moreover *P. guilliermondii* shows an high activity of β-1,3-glucanase enzyme, that could be associated to pathogen link and result in the degradation of the fungal cell walls (Jijakli and Lepoivre, 1998). Also *Aureobasidium pullulans* in apple wounds produces extracellular exochitinase and β-1,3-glucanase, that could play a role in the biocontrol activity (Castoria et al., 2001). El-Ghaouth et al. (1998) found, through ultrastructural and cytochemical studies, that *Candida saitoana* yeast cells, when cultivated together with *B. cinerea* mycelium, link fungal hyphae that show cytological damages, such as papillae and other protuberances in the cell wall, and degeneration of the cytoplasm. Finally some yeasts, such as *S. roseus* and *C. laurentii*, when applied, are able to reduce, on apple carposphere, the conidial adhesion and germination of *B. cinerea*, that is favoured by butyl acetate, volatile aroma produced by the fruit (Filonow, 2001).

2.4 Induced resistance

Some BCAs can interact with the host tissue, particularly the wounds, increasing the cicatrisation processes (Droby and Chalutz, 1994). Several antagonistic yeasts are as much effective as applied before pathogen inoculation. This observation has brought to suppose that yeast cell application could induce resistance processes in the fruit skin. Some *Candida* strains are able to cause chemical and osmotic changes in apple tissues, favouring antagonist settlement (McLaughlin et al., 1990). A *P. guilliermondii* strain stimulates the production of ethylene, an hormone able in grapefruit to activate the phenylalaninammonium-lyase (Wisniewski et al., 1991), enzyme involved in the phenols, phytoalexins and lignins synthesis.

A phytoalexins accumulation (scoparon and scopoletin) was noted in citrus fruit treated with yeast cell (Rodov et al., 1994). In addition to control decays, *A. pullulans* can cause on apple fruit a transient increase in β-1,3-glucanase, chitinase, and peroxidase activities starting 24 h after treatment (Ippolito et al., 2000).

3. Extension of use

Potential biocontrol agents 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 antagonist with a broader spectrum of action, preferably for commercial development, is the carrying out of initial efficacy tests on various pathogens and fruit species (Wilson et al., 1993; Lima et al., 1999).

3.1 Antagonist mixtures

In the enhancement of a biocontrol system, work could focuses on a promising approach, which is the development of antagonist mixtures. There is a superior probability to have an effective biological control with a mixture of several antagonists, complementary and non competitive, that with only one. Such mixture has some peculiar advantages (Janisiewicz, 1998): over the broadening of the spectrum of action (different fruits, cultivars and ripening stages), it can increase the efficacy (less biomass necessary) and offer a higher reliability, permitting a reduction in the application times and treatment costs. Moreover, it permits the combination of different genetic traits, avoiding genetic engineering. The evaluation of casual combinations of antagonists isolated from apple fruits and leaves resulted in the selection of a mixture of *P. syringae* and *S. roseus*, superior to both BCAs applied alone in the control of the Penicillium rot (Janisiewicz and Bors, 1995).

3.2 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. Field application of the BCAs may enable early colonisation of the fruit surfaces, protecting from these infections (Ippolito and Nigro, 2000). To be successful in preharvest application, potential antagonists should be able to tolerate low-nutrient availability, UV rays, high temperature and dry conditions. Leibinger et al. (1997) applied in preharvest a mixture of a yeast *A. pullulans* and a bacterium (*B. subtilis*) obtaining a level of control of *P. expansum* and *B. cinerea* on apple similar to fungicides. Teixido et al. (1998) applied unmodified and low water activity tolerant cells of *Candida sake* before harvest to control blue mould of apples, obtaining similar disease control.

3.3 Integrated use

Biological means of control can not at the moment solve all the problems of postharvest rots during fruit storage and they must be considered instruments to be used in combination with other methods of control in an integrated vision of disease management. For example, biocontrol agents can be combined with waxes and fungicides applied not only in post but also in pre-harvest (Pusey, 1994). In some laboratory and semi-commercial trials the efficacy of the BCA *P. guilliermondii* was consistently increased by the addition of small concentrations of imazalil or thiabendazole, reaching the levels of control of the fungicide alone (Droby et al., 1993). Antagonists, if employed together with fungicides, have to be tolerant or they request the development of resistant strains. Yeasts generally are tolerant to many of the fungicides used in postharvest: *Metschnikowia pulcherrima* (Spadaro et al., 2001a) is tolerant to relative high concentrations of benzimidazoles (benomyl and

246 thiabendazole) and dicarboximides (vinchlozolin and procymidone). Moreover
247 microorganisms need to survive to thermal, hygrometry and atmospheric storage conditions.
248 The efficacy of *M. pulcherrima* against *B.cinerea* and *P. expansum* is increased when fruits
249 are stored at low temperatures optimal for host, respect to storage at 23°C, because the
250 antagonist is more adaptable than the pathogens to cold temperatures (Spadaro et al., 2001a).
251 Among the strategies evaluated during the last years, it is to remember the combination of
252 biological treatments with other techniques alternative to chemicals: thermotherapy (Barkai-
253 Golan and Phillips, 1991), ultraviolet rays (Chalutz et al., 1992), animal and plant natural
254 products (Aharoni et al., 1993), calcium infiltrations (Janisiewicz et al.,1998), sodium
255 bicarbonate (Teixido et al., 2001) or ethanol (Spadaro et al., 2001b).
256 Calcium chloride infiltrations combined to antagonist application on apples increases control
257 of *P. expansum* after 3 and 6 months of storage at 1°C, compared to simple biological
258 treatment (Janisiewicz et al., 1998). A even more integrated approach, experienced on “Gala”
259 apples (Conway et al., 1999) consists of a heat treatment (4 days at 38°C), followed by a
260 calcium chloride (2%) infiltration and a *Pseudomonas syringae* cell suspension application.
261 The result of the three treatments carried out together was a reduction of the Penicillium rots
262 (91%) higher respect to calcium, antagonist, or heat treatments applied alone.
263 The efficacy of *Pantoea agglomerans* for the control of green mould was improved when
264 combined with sodium bicarbonate, or baking soda (Teixido et al., 2001), a common food
265 additive, allowed with no restrictions for many application by European and North American
266 regulations, and listed as approved ingredients on products labelled as “organic”. The
267 combination of sodium bicarbonate, or ethanol, or acybenzolar-S-methyl with *M. pulcherrima*
268 cell suspension and heat treatment was also studied by Spadaro et al. (2001b). Ethanol occurs
269 naturally in fruit and many other food products, and its toxic effects on spores of fungal
270 pathogens have been reported: the flesh treated with the alcohol was significantly firmer and

injury to the fruit did not occur.

It is possible to conclude that the possibilities to apply antagonists in the context of integrated control are very extensive.

4. Improvement of biocontrol agents

4.1 Formulation and application

Differently from soilborne or open field pathogens, where a disease control of 70-80% can be considered satisfying, postharvest disease control requests a higher level of efficacy and more consistent results. Even considering the most effective BCA's studied until now, they rarely reach the levels of efficacy of the fungicides. It is therefore necessary an increase in their antagonistic activity to achieve a practical use.

A rapid, efficient and cheap mass production of the antagonist, generally by fermentation, is a key issue. The efficacy of many antagonists of wound pathogens is directly related to the number of antagonist propagules inoculated (Hofstein et al., 1994), so that a really simple way to increase the effectiveness is the application of a higher number of cells. To scale-up a laboratory fermentation process to an industrial level, it is fundamental to find the nitrogen and carbon sources that provide maximum biomass production and minimum cost of media, whilst maintaining biocontrol efficacy. Costa et al. (2001) have studied yeast extract, dry beer yeast, sucrose, and molasses as possible substrate for the production of the biocontrol agent *P. agglomerans.*

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 (Janisiewicz and Jeffers, 1997). The application of adjuvants can protect and stimulate the establishing of the antagonist on the host surface. The addition of calcium salts increases the

296 activity of several antagonistic yeasts (Janisiewicz et al., 1998). The addition of glycerol and
297 trealose to the culture means augmented the osmotic tolerance and control capability of *C.*
298 *sake* against *P. expansum* on apple (Janisiewicz, 1998). Sodium alginate,
299 carboxymethylcellulose and chitosan, are adhesivants and can be added to yeast cell
300 suspension, to increase the activity of the formulate. The three adhesivants have been added to
301 a strain of *M. pulcherrima* (Piano et al., 1998) significantly increasing the efficacy against
302 grey rot on apple. Chitosan has also a fungistatic activity demonstrated against the main
303 postharvest pathogens of strawberry (El-Ghaouth et al., 1992). Recently, El-Ghaouth et al.
304 (2000a) developed a biocontrol product called "bioactive coating" consisting of a unique
305 combination of an antagonist with glycolchitosan, a chemically-modified chitosan. The
306 bioactive coating made it possible to exploit the antifungal property of glycolchitosan and the
307 biological activity of the antagonist. Moreover, when applied as a pretreatment, sodium
308 carbonate enhanced the efficacy of the bioactive coating (El-Ghaouth et al., 2000b).
309 Also application systems have a fundamental role in the determination of the final results. The
310 coatings most often applied to citrus fruit contain shellac, which is a purified product of the
311 hardened resinous secretion of the scale insect *Kerria lacca*. Having developed coating
312 formulations before biological control, it is important to test the suitability of such products
313 for antagonistic applications. McGuire (2000) found that, after 3 or 4 months storage, *C.*
314 *oleophila* survival was more efficacious when applied in the shellac than when applied by
315 preliminary immersion and subsequent drying of fruit prior to shellacking. On the contrary,
316 Chalutz and Droby (1998) had noted that the application of antagonistic yeasts in aqueous
317 suspension before waxing results in an efficacy much more consistent respect to a unique
318 application of antagonists and wax.
319 Another issue to face in the commercial production of biocontrol agents is the storage, that
320 should be as long as possible. The BCA’s should have a storability of at least 6 months and

preferably of 2 years (Pusey, 1994). Abadias et al. (2001a) found that freezing at –20°C was the best method to preserve the viability of *C. sake* cells after freeze drying. Survival of the cells was higher using 10% skim milk as a protectant, and increased by using other appropriate protectants, such as lactose, glucose, fructose or sucrose. Skimmed milk was also the best rehydratation medium with 1% peptone (Abadias et al., 2001b). However the efficacy of freeze-dried cells was significantly lower than that of fresh cells.

The transition from fungicide use to employment of biological means will be as easier as more flexible are the biofungicides.

4. 2 The nutritional environment

Temperature, humidity and gas composition in warehouse are optimised to guarantee a high fruit quality during storage and should not be changed but it is possible to manipulate the chemical environment to favour the antagonist. The addition of nutrients preferably metabolised by the antagonist and difficultly by the pathogen was suggested in several antagonist-pathogen interactions (Janisiewicz, 1998). The application of two nitrogen compounds, L-asparagine and L-proline (Janisiewicz et al., 1992), and an analogue of sugar, 2-deoxy-D-glucose (Janisiewicz, 1994) showed a consistent increase of the control of *P. expansum* on apple. Both aminoacids stimulated *P. expansum* germination but slowed mycelial growth. Also L-glutamine added to *M. pulcherrima* showed a direct influence on the yeast, because its application with the antagonist contributed to reduce Botrytis rot, while without yeast, it was ineffective (Piano et al., 1998). The sugar analogue 2-deoxy-D-glucose could be a useful additive to antagonistic microorganisms, provided that it has a fungicidal action on the major postharvest pathogens of apple and peach fruit (El-Ghaouth et al., 1997) and that the antagonist is resistant to its toxic effects. Recently the sugar analogue was found to be compatible with the antagonistic yeast *C. saitoana* and effective against apple and citrus

346 fruit decay (El-Ghaouth et al., 2000c).

347 Nutritional composition can influence not only density and competitiveness of the population,
348 but also the production of metabolites crucial in many control systems, such as antibiotics
349 (Gueldner et al., 1988) and cell wall degrading enzymes (Wisniewski et al., 1991). The form
350 and concentration of nitrogen and carbon sources can therefore be important factors in the
351 synthesis and secretion of key compounds in the biocontrol mechanism.

352

353 *4.3 Manipulation of antagonists*

354 It is possible to increase the ecological competence of microorganisms manipulating
355 antagonists with techniques of mutagenesis (physical or chemical) or of sexual recombination,
356 through protoplast fusion or continued culture.

357 Because nitrogen seems to be a limiting substance, it was noted that biocontrol increases
358 adding some aminoacids. Therefore biocontrol strains with a superior capability of
359 exploitation of nitrogen compounds present, or with a higher transport or metabolism rate of
360 the limiting factor, could be developed (Janisiewicz, 1998). Some phenolic compounds,
361 among them benzoic or chlorogenic acid, present at the wound sites, influence negatively the
362 colonisation by yeasts; it is therefore conceivable trying to obtain strains resistant to these
363 phenolic compounds (Bizeau et al., 1989). Mutants that use new substrates, not metabolised
364 by the pathogen, to provide a nutritional advantage, could also be induced (Janisiewicz,
365 1998).

366 With genetic engineering techniques, features for the exploitation of plant products or
367 additives applied together with the antagonist, for fungicide resistance, for carposphere
368 colonisation in storage conditions, fro synthesis of compounds favouring antagonism
369 (antibiotics or siderophores), could be transferred into the potential antagonistic
370 microorganisms (Pusey, 1994). Because one mechanism involved in the biocontrol of

postharvest fruit pathogens is mycoparasitism, lytic enzymes such as chitinases (Chernin et al., 1997), proteases and glucanases (De la Cruz et al., 1995) produced by bacterial and fungal microorganisms, could be inserted in the potential antagonists to improve the degradation of the pathogen cell walls, resulting in death or inhibition of growth of the attacked fungus. For biocontrol of soilborne pathogens, for example, a chitinase gene, isolated from *Serratia marcescens*, was introduced into the endophytic bacterium *Pseudomonas fluorescens* to improve the control of *Rhizoctonia solani* on bean (Downing and Thomson, 2000).

Another idea could be the insertion of the gene for amylase under constitutive promoter in some BCA's, because many fruits are rich in amid and antagonists could use effectively this carbon source having a consistent advantage. First experiments of transformation have been successful: *M. pulcherrima* was transformed with the green fluorescent protein gene (Nigro et al., 1999), *C. oleophila* was transformed with β-glucuronidase gene (Chand-Goyal et al., 1998), and histidine auxotrophs of *C. oleophila* were transformed with *HIS3, HIS4* and *HIS5* genes (Chand-Goyal et al., 1999). All these studies were accomplished only to obtain variants of the antagonistic strains with a genetically stable marker to expedite studies on the ecology of the yeasts on the fruit surface, but are really useful to put successive insertions of useful genes right. In all cases the transformed antagonists maintained the biocontrol capability.

5. Molecular characterisation

New molecular technologies improving the preliminary selection of biocontrol agents and the monitoring of field applied antagonists may greatly facilitate the selection and screening of yeast isolates having a superior antagonistic activity. Morphological and cultural characteristics alone are not sufficient to distinguish between strains of the same species and do not allow the exploitation of any possible naturally available genetic variability. Moreover

the effectiveness of the preharvest strategy 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's is therefore necessary but often complicated by the existence of other microorganisms on the fruit that are morphologically similar to the antagonistic strain. Molecular approaches can help to differentiate a biocontrol agent from an epiphytic microorganism. Between the technologies already used to obtain DNA fingerprintings the random amplified polymorphic DNA (RAPD) and the arbitrarily primed PCR (AP-PCR) are to remember (Schena et al., 1999; 2000). Both these methods are based on the amplification of random genomic DNA fragments by arbitrarily selected PCR primers and have the major disadvantage that they are very sensitive to the reaction conditions, DNA quality and PCR temperature profiles, which limit their applications.

New techniques more robust and reliable, such as amplified fragment length polymorphism (Vos et al., 1995; De Barros et al., 1999), based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA, and PCR amplification of the ITS regions of the ribosomal DNA (Masih et al., 2001) can be applied on potential biocontrol agents.

6. Conclusions

From this review, it appears that some significant progress has been made toward biological and integrated control of postharvest diseases on fruits. Some biofungicides are already on the market in a few countries, and will probably become more widely available as they are registered in more areas. Other BCA should reach the market soon. Postharvest conditions provide an ideal niche for BCAs since they are less subject to sudden climate changes, and are often equipped with a sophisticated system of climate control.

It is unrealistic to assume that perfect conditions for the development of BCA's will always prevail in the warehouse, and as a result, biofungicides will rarely stand alone as a complete measure of disease control under all conditions. For this reason, scientists, growers and consumers alike must accept the fact that BCA's are usually not as effective as pesticides. The success of biological control greatly depends in a change of mentality of the consumer, not anymore willing to have fruit with satisfying exterior aspects, but with an inner quality. Biological control should be viewed at the moment as an important if not essential component of an integrated disease management scheme if a significant and permanent reduction of pesticide use is our goal.

Acknowledgements

The manuscript has been written by the first author and revised by the second one. Work supported by a grant from Italian Ministry for Environment and Territory.

References

Abadias, M., Benabarre, A., Teixido, N., Usall, J., Vinas, I., 2001a. Effect of freeze drying and protectants on viability of the biocontrol yeast *Candida sake*. Int. J. Food Microbiol. 65, 3, 173-182.

Abadias, M., Teixido, N., Usall, J., Benabarre, A., Vinas, I., 2001b. Viability, efficacy, and storage stability of freeze-dried biocontrol agent *Candida sake* using different protective and rehydration media. J. Food Prot. 64, 6, 856-861.

Aharoni, Y., Copel, A., Fallik, E., 1993. Hinokitiol (b-thujaplicin), for postharvest decay control on "Galia" melons. N. Z. J. Crop Hortic. Sci. 21, 165-169.

446 Andrews, J.H., Harris, R.F., Spear, R.N., Lau, G.W., Nordheim, E.V., 1994. Morphogenesis
447 and adhesion of *Aureobasidium pullulans*. Can. J. Microbiol. 40, 6-17.
448 Barkai-Golan, R., Phillips, D. J., 1991. Postharvest heat treatment of fresh fruits and vege-
449 tables for decay control. Plant Dis. 75, 1085-1089.
450 Bizeau, C., Ponchant, F., Moreau, M., 1989. Evolution of yeast flora on cider apples of
451 Britanny. Proc. 7th Int. Symp. on Yeasts, Perugia, Italia. Yeast, vol. 5. John Wiley &
452 Sons, Ltd., Chichester, UK, 491-494.
453 Castoria, R., De Curtis, F., Lima, G., Caputo, L., Pacifico, S., De Cicco, V., 2001.
454 *Aureobasidium pullulans* (LS-30) an antagonist of postharvest pathogens of fruits: study
455 on its modes of action. Postharvest Biol. Technol. 22, 7-17.
456 Chalutz, E., Droby, S., 1998. Biological control of postharvest disease. In: Boland, G.J.,
457 Kuykendall, L.D. (Eds.), Plant-Microbe Interactions and Biological Control Marcel
458 Dekker, New York, USA, pp. 157-170.
459 Chalutz, E., Droby, S., Wilson, C.L., Wisniewski, M.E., 1992. UV-induced resistance to
460 postharvest diseases of citrus fruit. Phytochem. Phytobiol. B.: Biol. 15, 367.
461 Chand-Goyal T., Eckert J.W., Droby, S., Atkinson, K., 1998. A method for studying the
462 population dynamics of *Candida oleophila* on oranges in the grove, using a selective
463 isolation medium and PCR technique. J. Microbiol. Res. 153, 3, 265-270.
464 Chand-Goyal, T., Eckert, J.W., Droby, S., Glickmann, E., Atkinson, K., 1999. Transformation
465 of *Candida oleophila* and survival of a transformant on orange fruit under field
466 conditions. Curr. Genet. 35, 51-57.
467 Chernin, L.S., De La Fuente, L., Sobolev, V., Haran, S., Vorgias, C.E., Oppenheim, A.B.,
468 Chet, I., 1997. Molecular cloning, structural analysis, and expression in *Escherichia coli*
469 of a chitinase gene from *Enterobacter agglomerans*. Appl. Environm. Microbiol. 63, 834-
470 839.

471 Conway, W.S., Janisiewicz, W.J., Klein, J.D., Sams, C.E., 1999. Strategy for combining heat
472 treatment, calcium infiltration, and biological control to reduce postharvest decay of
473 “Gala” apples. HortScience 34, 4, 700-704.
474 Costa, E., Teixido, N., Usall, J., Atares, E., Vinas I., 2001. Production of the biocontrol agent
475 Pantoea agglomerans strain CPA-2 using commercial products and by-products. Appl.
476 Microbiol. Biotechnol. 56, 367-371.
477 De Barros, L., Rainieri, S., Henschke, P.A., Langridge, P., 1999. AFLP fingerprinting for
478 analysis of yeast genetic variation. Int. J. Syst. Bacteriol. 49, 915-924.
479 De La Cruz, J., Pintor-Toro, J.A., Benitez, T., Llobell, A., Romero, L.C., 1995. A novel endo-
480 1,3-glucanase, BGN 13.1, involved in the mycoparasitism of *Trichoderma harzianum*. J.
481 Bacteriology 177, 6937-6945.
482 Downing, K.J., Thomson, J.A, 2000. Introduction of the *Serratia marcescens* chiA gene into
483 an endophytic *Pseudomonas fluorescens* for the biocontrol of phytopathogenic fungi.
484 Can. J. Microbiol. 46, 4, 363-369.
485 Droby, S., Chalutz, E., 1994. Mode of action of biocontrol agents of postharvest diseases. In:
486 Wilson, C.L., Wisniewski, M.E. (Eds.), Biological Control of Postharvest Diseases.
487 Theory and Practice. CRC Press, Boca Raton, USA, pp. 63-75.
488 Droby, S., Chalutz, E., Wilson, C.L., Wisniewski, M.E., 1989. Characterization of the
489 biocontrol activity of *Debaryomices hansenii* in the control of *Penicillium digitatum* on
490 grapefruit. Can. J. Microbiol. 35, 794-800.
491 Droby, S., Hofstein, R., Wilson, C.L., Wisniewski, M.E., Fridlender, B., Cohen, L., Weiss, B.,
492 Timar, D., Chalutz, E., 1993. Pilot testing of *Pichia guilliermondii*: a biocontrol agent of
493 postharvest diseases of citrus fruit. Biol. Control 3, 47-52.
494 Eckert, J.W., Ogawa, J.M., 1985. The chemical control of postharvest diseases: subtropical
495 and tropical fruits. Annu. Rev. Phytopathol. 23, 421-454.

Edwards, S.G., Seddon, B., 2001. Mode of antagonism of *Brevibacillus brevis* against *Botrytis cinerea in vitro*. J. Appl. Microbiol. 91, 4, 652-659.

El-Ghaouth, A., Arul, J., Grenier, J., Asselin, A., 1992. Antifungal activity of chitosan on two postharvest pathogens of strawberry fruits. Phytopathology 82, 398-402.

El-Ghaouth, A., Smilanick, J.L., Brown, G.E., Ippolito, A., Wisniewski, M., Wilson, C.L., 2000a. Application of *Candida saitoana* and glycolchitosan for the control of postharvest diseases of apple and citrus fruit under semi-commercial conditions. Plant Dis. 84, 243-248.

El-Ghaouth, A., Smilanick, J.L., Wilson, C.L., 2000b. Enhancement of the performance of *Candida saitoana* by the addition of glycolchitosan for the control of postharvest decay of apple and citrus fruit. Postharvest Biol. Technol. 19, 103-110.

El-Ghaouth, A., Smilanick, J.L., Wisniewski, M., Wilson, C.L., 2000c. Improved control of apple and citrus fruit decay with a combination of *Candida saitoana* and 2-deoxy-D-glucose. Plant Dis. 84, 249-253.

El-Ghaouth, A., Wilson, C.L., Wisniewski, M., 1998. Ultrastructural and cytochemical aspects of the biological control of *Botrytis cinerea* by *Candida saitoana* in apple fruit. Phytopathology 88, 282-291.

El-Ghaouth A., Wilson C.L., Wisniewski M., 1997. Antifungal activiy of 2-deoxy-D-glucose on *Botrytis cinerea*, *Penicillium expansum*, and *Rhizopus stolonifer*. Ultrastructural and cytochemical aspect. Phytopathology 88, 772-779.

Filonow, A.B., 2001. Butyl acetate and yeasts interact in adhesion and germination of *Botrytis cinerea* conidia in vitro and in fungal decay of golden delicious apple. J. Chem. Ecol. 27, 831-844.

Filonow, A.B., 1998. Role of competition for sugars by yeasts in the biocontrol of gray mould of apple. Biocontrol Sci. Technol. 8, 243-256.

521 Gehmann, K., Nyfeler, R., Leadbeater, A.J., Nevill, D., Sozzi, D., 1990. CGA 173506: a new
522 phenylpirrole fungicide for broad spectrum disease control. Proc. Brighton Crop Prot.
523 Conf., Pests and Diseases, 399-406.
524 Gueldner, R.C., Reilly, C.C., Pusey, R.L., Costello, C.E., Arrendale, R.F., Cox, R.H.,
525 Himmelsbach, D.S., Crumley, F.G., Cutler, H.G., 1988. Isolation and identification of
526 iturins as antifungal peptides in biological control of peach brown rot with *Bacillus*
527 *subtilis*. J. Agric. Food Chem. 36, 366-370
528 Gullino, M.L., Kuijpers, L.A.M., 1994. Social and political implications of managing plant
529 diseases with restricted fungicides in Europe. Annu. Rev. Phytopathol. 32, 559-579.
530 Harvey, J.M., 1978. Reduction of losses in fresh market fruits and vegetables. Annu. Rev.
531 Phytopathol. 16, 321-341.
532 Hofstein, R., Friedlender, B., Chalutz, E., Droby, S., 1994. Large scale production and pilot
533 testing of biocontrol agents of postharvest diseases. In: Wilson, C.L., Wisniewski, M.
534 (Eds.), Biological Control of Postharvest Diseases – Theory and Practice. CRC Press
535 Inc., Boca Raton, FL, USA, pp. 89-100.
536 Ippolito, A., El-Ghaouth, A., Wilson, C.L., Wisniewski, M., 2000. Control of postharvest
537 decay of apple fruit by *Aureobasidium pullulans* and induction of defense responses.
538 Postharvest Biol. Technol. 19, 265-272.
539 Ippolito, A., Nigro, F., 2000. Impact of preharvest application of biological control agents on
540 postharvest diseases of fresh fruits and vegetables. Crop Prot. 19, 610-619.
541 Janisiewicz, W.J., 1994. Enhancement of biocontrol of blue mold with nutrient analog 2-
542 deoxy-D-glucose on apples and pears. Appl. Environ. Microbiol. 60, 2671-2676.
543 Janisiewicz W.J., 1998 – Biocontrol of postharvest diseases of temperate fruits – Challenges
544 and opportunities. In: Boland, G.J., Kuykendall, L.D. (Eds.), Plant-Microbe Interactions
545 and Biological Control. Marcel Dekker, New York, USA, pp. 171-198.

546 Janisiewicz, W. J., Bors, R., 1995. Development of a microbial community of bacterial and
547 yeast antagonists to control wound-invading postharvest pathogens. Appl. Environ.
548 Microbiol. 61, 3261-3267.
549 Janisiewicz, W.J., Conway, W.S., Glenn, D.M., Sams, C.E., 1998. Integrating biological
550 control and calcium treatment for controlling postharvest decay of apples. HortScience
551 33, 1, 105-109.
552 Janisiewicz, W.J., Jeffers, S.N., 1997. Efficacy of commercial formulation of two
553 biofungicides for control of blue mold and gray mold of apples in cold storage. Crop Prot.
554 16, 629-633.
555 Janisiewicz, W.J., Tworkoski, T.J., Sharer, C., 2000. Characterizing the mechanism of
556 biological control of postharvest diseases on fruits with a simple method to study
557 competition for nutrients. Phytopathology 90, 1196-1200.
558 Janisiewicz, W.J., Usall, J., Bors, B., 1992. Nutritional enhancement of biocontrol of blue
559 mold on apples. Phytopathology 82, 1364-1370.
560 Janisiewicz, W.J., Yourman, L., Roitman, J., Mahoney, N., 1991. Postharvest control of blue
561 mold and gray mold of apples and pears by dip treatments with pyrrolnitrin, a metabolite
562 of *Pseudomonas cepacia.* Plant Dis. 75, 490-494.
563 Jijakli, M.H., Lepoivre, P., 1998. Characterization of an exo-b-1,3-glucanase produced by
564 *Pichia anomala* strain K, antagonist of *Botrytis cinerea* on apples. Phytopathology 88, 4,
565 335-343.
566 Leibinger, W., Breuker, B., Hahn, M., Mengden, K., 1997. Control of postharvest pathogens
567 and colonization of the apple surface by antagonistic microorganisms in the field.
568 Phytopathology 87, 1103-1110.
569 Lima, G., Arru, S., De Curtis, F., Arras, G., 1999. Influence of antagonist, host fruit and
570 pathogen on the biological control of postharvest fungal diseases by yeasts. J. Ind.

571 Microbiol. Biot. 23, 223-229.
572 Masih, E.I., Slezack-Deschaumes, S., Marmaras, I., Barka, E.A., Vernet, G., Charpentier, C.,
573 Adholeya, A., Paul, B., 2001. Characterisation of the yeast *Pichia membranifaciens* and
574 its possible use in the biological control of *Botrytis cinerea*, causing the grey mould
575 disease of graopevine. FEMS Microbiol. Lett. 202, 2, 227-232.
576 Mcguire, R.G., 2000. Population dynamics of postharvest decay antagonists growing
577 epiphytically and within wounds on grapefruit. Phytopathology 90, 1217-1223.
578 Mclaughlin, R.J., Wisniewski, M.E., Wilson, C.L., Chalutz, E., 1990. Effects of inoculum
579 concentration and salt solutions on biological control of postharvest diseases of apple
580 with *Candida* sp.. Phytopathology 80, 456-461.
581 Nigro, F., Finetti Sialer, M.M., Gallitelli, D., 1999. Transformation of *Metschnikowia*
582 *pulcherrima* 320, biocontrol agent of storage rot, with the green fluorescent protein gene.
583 J. Plant Pathol. 81, 3, 205-208.
584 Piano, S., Cerchio, F., Migheli, Q., Gullino, M.L., 1998. Effetto di diverse sostanze
585 sull'attività dell'antagonista *Metschnikowia pulcherrima* 4.4 contro *Botrytis cinerea* e
586 sulla sua sopravvivenza sulle mele. Atti Giornate Fitopatologiche, 3-7 maggio 1998, 495-
587 500.
588 Piano, S., Neyrotti, V., Migheli, Q., Gullino, M.L., 1997. Characterization of the biocontrol
589 capability of *Metschnikowia pulcherrima* against Botrytis postharvest rot of apple.
590 Postharvest Biol. Technol. 11, 131-140.
591 Pusey, P.L., 1994. Enhancement of biocontrol agents for postharvest diseases and their
592 integration with other control strategies. In: Wilson, C.L., Wisniewski, M.E. (Eds.),
593 Biological Control of Postharvest Diseases. Theory and Practice. CRC Press, Boca
594 Raton, WV, USA, pp. 77-88.
595 Pusey, P.L., Wilson, C.L., Hotchkiss, M.W., Franklin, J.D., 1986. Compatibility of *Bacillus*

596 *subtilis* for postharvest control of peach brown with commercial fruit waxes, dicloran and
597 cold-storage conditions. Plant Dis. 70, 622-626.
598 Rodov, V., Ben-Yehoshua, S., D'hallewin, G., Castina, T., Farg, D., 1994. Accumulation of
599 the phytoalexins, scoparone and scopoletine in citrus fruit subjected to various
600 postharvest treatments. Int. Symp. Natural Phenols in Plant Resistance, Acta Hortic. 381,
601 517.
602 Romano, M.L., Gullino, M.L., Garibaldi, A., 1983. Evaluation of the sensitivity to several
603 fungicides of post-harvest pathogens in North-western Italy. Mede. Fac. Landoubouww.
604 Gent 48, 591-602.
605 Schena, L., Ippolito, A., Zahavi, T., Cohen, L., Droby, S., 2000. Molecular approaches to
606 assist the screening and monitoring of postharvest biocontrol yeasts. Eur. J. Plant Pathol.
607 106, 681-691.
608 Schena, L., Ippolito, A., Zahavi, T., Cohen, L., Nigro, F., Droby, S., 1999. Genetic diversity
609 and biocontrol activity of *Aureobasidium pullulans* isolates against postharvest rots.
610 Postharvest Biol. Technol. 17, 189-199.
611 Smilanick, J.L., Denis-Arrue, R., 1992. Control of green mold of lemon with *Pseudomonas*
612 species. Plant Dis. 76, 481-485.
613 Spadaro, D., Piano, S., Duverney, C., Gullino, M.L., 2001b. Use of microorganisms, heat
614 treatment, and natural compounds against Botrytis rot on apple. Proc. 2nd International
615 Conference on the alternative control methods against plant pests and diseases, Lille,
616 France, 4-7 marzo 2002, in press.
617 Spadaro, D., Vola, R., Piano, S., Gullino, M.L., 2001a. Mechanisms of action and efficacy of
618 four isolates of the yeast *Metschnikowia pulcherrima* active against postharvest
619 pathogens on apples. Postharvest Biol. Technol. 15, in press.
620 Spotts, R.A., Cervantes, L.A., 1986. Population, pathogenicity, and benomyl resistance of

Botrytis spp., *Penicillium* spp., and *Mucor piriformis* in packinghouses. Plant Dis. 70, 106-108.

Teixido, N., Usall, J., Palou, L., Asensio, A., Nunes, C., Vinas, I., 2001. Improving control of green and blue molds of oranges by combining *Pantoea agglomerans* (CPA-2) and sodium bicarbonate. Eur. J. Plant Pathol. 107, 7, 685-694.

Teixido, N., Vinas, I., Usall, J., Magan, N., 1998. Control of blue mold of apples by preharvest application of *Candida sake* grown in media with different water activity. Phytopathology 88, 960-964.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van De Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M., 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23, 21, 4407-4414.

Wilson, C.L., Wisniewski, M.E., Droby S., Chalutz E., 1993. A selection strategy for microbial antagonists to control postharvest disease of fruits and vegetables. Scientia Hort. 53, 183-189.

Wilson, C.L., Wisniewski, M.E., 1994. Biological Control of Postharvest Diseases. Theory and Practice. CRC Press, Boca Raton, USA.

Wisniewski, M.E., Biles, C., Droby, S., Mclaughlin, R., Wilson, C.L., Chalutz, E., 1991. Mode of action of the postharvest biocontrol yeast, *Pichia guilliermondii*. I. Characterization of attachment to *Botrytis cinerea*. Physiol. Mol. Plant P. 39, 245-258.

Wisniewski, M.E., Wilson, C.L., Hershherger, W., 1989. Characterization of inhibition of *Rhizopus stolonifer* germination and growth by *Enterobacter cloacae*. Can. J. Bot. 67, 2317-2323.