YEAST SPECIES FOR BIOCONTROL OF APPLE POSTHARVEST DISEASES : AN ENCOURAGING CASE OF STUDY FOR PRACTICAL USE

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1. INTRODUCTION

Since early 1970's, postharvest diseases of apple annually cause losses of 15-25 % despite modern storage facilities including controlled atmosphere (CA) or Ultra Low Oxygen (ULO) facilities (Bondoux, 1992). Factors that favour microbial growth, such as physiological senescence of fruits, mechanical injuries, as well as physiological disorders due to undesirable storage conditions can promote and explain these postharvest decays.

In Belgium and France (Bondoux, 1992) most losses are attributable to *Penicillium expansum* LK, *Botrytis cinerea* Pers. and *Gloeosporides* sp. (including *Cryptosoporiopsis malicorticis* (Cordl.) Nannf., *Colletotrichum gloeosporioides* (Penz.) and *Trichoseptoria fructigena* Maubl.). In USA and UK, *Botrytis* and *Penicillium* are the most important agents of postharvest diseases (Rosenberger, 1991). During storage, wounds are the first sites of fection for the initiation of diseases caused by the grey mould (*B. cinerea*) or the blue mould (*P. expansum*) agents. Both fungal agents infect fruits by deposit of airborne or waterborne conidia on wounds during harvesting and handling before storage. Infections of the *Gloeosporides* sp. occur in the field but quiescent latent and escape notice at harvest (Bondoux, 1992).

Control measures are principally based on the protection of fruits from pre- and post-harvest infection with pre- and postharvest fungicide treatments. However, in the context of consumer reluctance to chemical residues in food and public concern for environmental safety, there is an increasing demand to develop alternative methods to control diseases. That demand becomes a critical need with respect to the possible deregistragion of effective and widely used fungicides (Wellings, 1996) and the development of fungicide-resistant strains of postharvest pathogens (Franclet, 1994). Several novel approaches are emerging as possible

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alternatives to synthetic fungicides, including induction of natural defence mechanisms of harvested products, application of natural biocides, genetic resistance, and biological control agents (BCA's) (Wilson *et al.*, 1994).

The first phases of development of a biological control product can be considered as being very similar to the development of a chemical pesticide: early screening of a reliable activity, high efficacy in realistic conditions and adequate formulation to meet the expectation of the growers. However, because biological control agents (BCA) used for the control of plant diseases are primarily living organisms, their mass production, the study of their mode of action and their safety requirements are likely to be somewhat different than those for chemical pesticides. Public policy must translate that specificity in homologation procedures based on a case by case study.

Although the relevance of BCA appears limited until now, biological control of postharvest diseases are often presented as a specially promising area as testifying by already commercialised agents. In this respect, the paper exemplifies the potential and limits of such biocontrol strategy by presenting the case study of two yeast's, *Pichia anomala* (strain K) and *Debaryomyces hansenii* (strain O) exhibiting high and reliable protective activity agair *Penicillium* sp. and *Botrytis cinerea* in postharvested apples.

2. POSTHARVEST DISEASES OF FRUITS : A PREDILECTION FIELD OF BIOLOGICAL CONTROL

Biological control is generating a great enthusiasm to play a role in sustainable agriculture although the relevance of BCA's in plant pathology appears limited until now. If everybody recognises the existence of natural phenomenon's of microbial antagonism, the question is to know how to manipulate naturally occurring antagonistic micro-organisms to achieve a reliable and effective strategy of disease control meeting the requirements of the market. In this respect, postharvest biological control could be considered as specially promising on a practical point of view because (1) the application sites are limited to the fruits, (2) the environmental conditions are defined and stable in storage rooms, and (3) the harvested commodities are of high value.

Literature presents numerous examples of biocontrol of fruit diseases (Table 1). Furthermore, biocontrol products such as Biosave™ (*Pseudomonas syringae* van Hall, Esc-11) and Aspire™ (*Candida oleophila* Montrocher, I-182) are already commercialised by Ecogen Inc. (Longhorn, PA) and Ecoscience Corp. (Worcester, MA), respectively and are used among other on postharvest apples against wound diseases.

Before becoming an economically feasible alternative to chemical control, BCA's have to satisfy different requirements related to biological, technological and toxicological properties. We will review the requirements that an "ideal antagonist" must meet through our research dealing with the biological protection of postharvest apples. An "ideal antagonist" should have the following characteristics: effective at low concentrations in several postharvest host pathogen combinations; able to survive under adverse environmental conditions such as low temperatures and controlled atmospheres adopted prevailing in storage facilities ; amenable to inexpensive production and formulation with a long shelf life; easy to dispense; compatible with commercial handling practices; genetically stable ; non pathogenic for the consumer and for the host commodity.

Table 1. Examples of BCA's used to control diseases on apples.

Disease	Antagonist	Nature of BCA and application period	Mode of action	Reference
B. cinerea	*Trichoderma pseudokoningii*	Fungus, blossoming to harvest	Parasitism and antibiosis	Tronsmo and Ystaas (1980)
	Trichoderma harzianum	Fungus, blossoming to harvest	Parasitism	Tronsmo and Ystaas (1980)
	Metschnikowia pulcherrima	Yeast, storage	Competition	Piano *et al.*, (1997)
	Cryptococcus humicola, Filobasidium floriforme, Rhodosporidium toruloides	Yeast's, storage	Competition	Filonow *et al.*, (1996)
B. cinerea and *P. expansum*	*Pseudomonas cepacia*	Bacteria, storage (pyrrolnitrine) and competition	Antibiosis	Janisiewicz and Roitman (1988); Chalutz and Droby (1993)
	Acremonium breve	Yeast, storage	Competition and induction of host resistance	Janisiewicz (1987, 1988)
	Cryptococcus laurentii	Yeast, storage	Competition	Roberts (1991); Wilson *et al.*, (1993); Chalutz and Droby (1993)
	Pichia guilliermondii	Yeast, storage	Competition, parasitism	Wilson and Wisniewski (1989); Chalutz and Droby (1993)
	Trichosporon sp. and *Candida* sp.	Yeast, storage	Competition	Aloi *et al.*, (1991)
B. cinerea, P. expansum; and *Mucor* sp.	*Pseudomonas syringae* (Biosave ã)*	Bacteria, storage	Induction of host resistance	Ecogen Inc. (Longhorn, PA)
	Candida oleophila (Aspire ã)*	Yeast, storage	Competition	Ecoscience Corp. (Worcester, MA)
Venturia inaequalis	*Athelia bombacina*	Fungus, blossoming to harvest	Competition and antibiosis	Janisiewicz (1991)

* : commercial name of the BCA

3. ISOLATION AND SELECTION OF BIOCONTROL AGENTS

3.1. Isolation and Characterisation of Epiphytic Micro-organisms

Theoretically, the isolation procedure of potential antagonists depends on the characteristics of the infection by the pathogen. To control post-harvest diseases, investigators usually isolated naturally occurring micro-organisms from fruits just before harvesting or during storage (Aloi *et al.*, 1991; Gullino *et al.*, 1991b; Janisiewicz, 1991). Nevertheless, an absolute relationship between efficacy and origin of isolation doesn't exist. Actually, micro-organisms exhibiting antagonistic properties against *B. cinerea* and *P. expansum* have been isolated from soil, leaves of apple tree or from fruits or leaves of other plants (Janisiewicz, 1988; Janisiewicz and Roitman, 1988; Wilson and Wisniewski, 1989). In this respect, an elegant and fast method of antagonist isolation has been adopted by Wilson *et al.*, (1993). They applied rinsing waters from tomatoes and apples directly on wounds inoculated with the pathogen (*B. cinerea*) and isolated antagonistic micro-organisms from wounds which did not exhibit any symptom.

We isolated micro-organisms from rinsing waters of Golden Delicious at harvest and after different periods of storage (Jijakli, 1996). We pointed up the coexistence of filamentous fungi, yeast's and bacteria on surface of apples whatever the storage period. Fungi belonging to the genera *Cladosporium*, *Penicillium*, *Aureobasidium*, *Alternaria*, *Mucor*, *Fusarium*, *Cephlosporium*, *Stemphylium*, *Trichoderma*, *Epicoccum*, *Pyrenochaeta*, *Pithomyces* and

Stigmella were principally observed. After two months of storage, epiphytic populations of bacteria and yeast's reached a maximum density of 5 x 10^7 cfu/ml and 3 x 10^6 cfu/ml respectively. In our study, the dominant population in term of density level was attributed to bacteria for each isolation period, while Janiciewicz (1996) observed that yeast's were the dominant population on Golden Delicious in term of different number of species.

Until now, microbial populations and their ecology on fruit surface are poorly known (Leibinger *et al.*, 1997). A better knowledge of characteristics of epiphytic micro-organism should lead to a more rational isolation scheme of antagonists well fit to their habitat and competitive with regard to other naturally occurring micro-organisms. This gap could be explained by the lack of reliable methods to correctly take a census of micro-organism populations on the plant surface. For example, the plating method under-estimated yeast populations from rinsing tissues of plants (Fokkema, 1991), although it is still the sole technique used in the scope of postharvested fruits.

3.2. Selection of Efficient BCA's

Most of the investigators (Aloi *et al.*, 1991; Gullino *et al.*, 1991a; Janisiewicz, 1987; 1988; Janisiewicz and Roitman, 1988; Wilson and Wisniewski, 1989) evaluated the efficacy of micro-organism strains on artificially wounded apples since *in vitro* antagonistic properties of a strain does not always lead to *in vivo* protection activity (Elsheriff and Grossmann, 1994).

Among 329 epiphytic micro-organisms (yeast's and bacteria), we selected two yeast's, *Pichia anomala* (strain K) (Hansen) Kurtzman and *Debaryomyces hansenii* (strain O) (Zopf) Lodder and Kreger-van Rij for their high and reliable biocontrol activity against infection by *B. cinerea* or *Penicillium* sp. on wounded Golden Delicious (Jijakli and Lepoivre, 1993). Treatment of wounded sites with 50 µl of yeast suspension (107 cfu/ml) was sufficient to inhibit rot development induced by 50 µl (106 spores/ml) of either *B. cinerea* or *Pencillium* sp. at 5°C and 25°C.

Numerous yeast strains exhibiting antagonism against *Botrytis* and/or *Penicillium* have been reported in the literature : *Acremonium breve* W. Gams (Janisiewicz, 1988), *Candida sake* (Saito and Ota) van Uden and Buckley, *C. tenuis* Diddens and Lodder (Wilson and Wisniewski; 1989), *C. oleophila* (Mercier and Wilson, 1994), *Candida* sp. (McLaughlin *et al.*, 1990), *C. guilliermondii* (Castellani) Langeron and Guerra , and *Kloechera apiculata* Janke (McLaughlin and Wilson, 1992), *Candida* sp. and *Trichosporon* sp. (Aloi *et al.*, 1991, Gullino *et al.*, 1991a), *Sporobolomyces roseus* Kluicer et van Niel (Janisiewicz *et al.*, 1994). The high frequency of yeast among the antagonistic agents reported could be related to the fact that yeast's are tolerant to extreme environmental conditions of storage rooms (temperature close to 0 °C, high relative humidity) and adapted to apple characteristics (high sugar concentration, high osmotic pressure and low pH) (Janicisiewicz, 1991). However, bacteria such as *Bacillus subtilis* (Ehrenberg) Cohn (Pusey and Wilson, 1984; Sholberg *et al.*, 1995), *Pseudomonas cepacia* Palleroni et Holmes (Janisiewicz and Roitman, 1988) or *P. syringae* van Hall (Janisiewicz and Bors, 1995) were also reported as effective BCA's on apples against both pathogens.

3.3. Major Parameters Controlling the Level of Protection

The study of parameters affecting the level of protective activity under sub-optimal conditions contributes to the selection of the most efficient BCA's. This study determines also the conditions giving a high and reproducible protective activity against postharvest diseases.

Our work (Jijakli *et al.*, 1993b) demonstrated that antagonistic activity of different strains of yeast's, isolated from apple surface were firstly dependent on the incubation time before inoculation of *B. cinerea* or *Penicillium* sp. (Table 2). Protection level increased with time between application of the antagonist and inoculation of the pathogen. The most efficient strains, *P. anomala* (strain K) and *D. hansenii* (strain O) reduced significantly the diameter of decay lesion, even when inoculation of the pathogen and application of the yeast were performed simultaneously.

In other respect, there was a quantitative relationship between spore concentration of *B. cinerea* and the amount of antagonist required for disease control (Table 3). The application of 108 cfu/ml of *P. anomala* (strain K) was necessary to completely inhibit lesions caused by *B. cinerea* upon inoculation with 50 µl of 106 spores/ml whereas 108 cfu/ml of *D. hansenii* (strain O) protected fruits against inoculation with 50 µl of 105 spores/ml of *B. cinerea*. The same parameters influencing the level of protection were already identified by other authors (Gullino *et al.*, 1991b ; Janisiewicz *et al.*, 1994 ; McLaughlin *et al.* ,1990 ; Mercier and Wilson, 1995 ; Roberts, 1991).

The temperature of fruit incubation and the humidity at the wound site were also identified as factors controlling the protective level (Gullino *et al.*, 1991a ; Mercier and Wilson, 1995). When apple wounds were artificially dried, Mercier and Wilson (1995) observed a decreased of both population level of *C. oleophila* and protection level against *B. cinerea*.

Table 2. Lesion development (mm) on wounded Golden Delicious apples treated with 50 µl of antagonistic yeast suspension (about 107 cfu/ml); and then inoculated with 50 µl of pathogen suspension (106 spores/ml) after different incubation times of the antagonist (Jijakli *et al.*, 1993a).

	B. cinerea[b]				*Penicillium* sp.[b]			
Incubation times	0 h	12 h	24 h	48 h	0 h	12 h	24 h	48 h
2.13[c]	21.6[a]	9.4 [d]	6.9 [d]	0.5 [d]	20.2	15.6 [d]	6.9 [d]	5.4 [d]
1.58	24.9	12.2 [d]	11.2 [d]	9.1 [d]	16.4 [d]	15.6 [d]	14.2	9.0
9C5	24.7	19.5	7.0 [d]	3.1 [d]	20.2	22.6	19.1	10.4
5F2	27.0	13.9	7.1 [d]	19.7 [d]	18.5	16.7 [d]	15.5	4.1 [d]
K	10.7[d]	8.6 [d]	0.0 [d]	0.1 [d]	10.6 [d]	12.2 [d]	3.7 [d]	0.0 [d]
O	15.2 [d]	5.7 [d]	4.7 [d]	3.0 [d]	19.2	18.1	3.1 [d]	2.0 [d]
9A4	22.0	19.5	14.1	5.4 [d]	25.4	22.6	19.2	14.2
Control[e]	33.8	25.1	31.9	29.4	23.6	23.4	22.6	19.0

a: Data represent average lesion diameter (mm) measured 5 days after pathogen inoculation.

b: Pathogen

c: Antagonistic strains

d: Means of lesion diameters of the antagonist-treated apples are significantly different ($p=0.001$) from the control mean (in the same column) according to Dunnett's procedure.

e : Untreated apples inoculated with the pathogen only.

Data shown for 1 of the 2 trials (Data of separate trials were not pooled because variances differed significantly)

Table 3. Lesion development (mm) on wounded Golden Delicious apples inoculated with various spores concentrations of *B. cinerea* or *Penicillium* sp. 24 h after treatment of different concentration of *P. anomala* (strain K) or *D. hansenii* (strain O) (Jijakli *et al.*, 1993a).

Yeast concentration (cfu/ml)	*B. cinerea* spores concentration (spores/ml)				*Penicillium* sp. spores concentration (spores/ml)			
	10^7	10^6	10^5	10^4	10^7	10^6	10^5	10^4
P. anomala								
10^8	3.5[ad]	0.0[d]	0.0[d]	0.0[d]	4.6[d]	0.0[d]	1.1[d]	0.0[d]
10^7	4.7	0.7[d]	1.4[d]	1.6[d]	9.4[d]	2.6[d]	0.0[d]	3.7[d]
10^6	17.0	5.2[d]	10.2[d]	0.0[d]	17.2[d]	18.0	14.1	20.2
10^5	24.4	11.2[d]	5.9[d]	5.0[d]	18.1[d]	12.9[d]	15.4	4.9[d]
control[b]	24.5	28.1	25.0	22.1	21.0	21.4	21.6	19.1
D. hansenii								
10^8	9.5[ad]	2.9[d]	0.0[d]	0.0[d]	4.7[d]	6.2[d]	8.5[d]	4.6[d]
10^7	14.2	2.6[d]	3.4[d]	0.0[d]	15.5	4.0[d]	2.1[d]	0.0[d]
10^6	17.1	18.6[d]	9.1[d]	6.7[d]	11.0	15.2	10.1[d]	4.7[d]
10^5	13.2	7.9[d]	2.6[d]	0.9[d]	14.9	16.1	8.4[d]	9.2[d]
control[b]	24.5	28.1	25.6	22.1	21.0	21.4	21.6	19.1

a: Data are the average lesion diameter (mm) measured 5 days after pathogen inoculation.

b: Untreated apples inoculated with the pathogen only.

c: Means of lesion diameters of the antagonist-treated apples are significantly different to the control mean (in the same column) according to Dunnett's procedures (p=0.001)

Data shown for 1 of the 2 trials (Data of separate trials were not pooled because variances differed significantly)

4. MASS PRODUCTION OF BCA's

A screening of the potential efficient antagonists based on their capacity to be produced and dried in mass must follow the study of the protective properties. The capacity to produce in mass and to dry the micro-organisms has to be evaluated. The cost production of a micro-organism includes the culture media and the energy needed for production and drying process. Appropriate adjuvants relative to the drying process (protectants, carriers,...) must be added to increase the viability of BCA's during the different processes.

P. anomala (strain K) and *D. hansenii* (strain O) were tested for their technological properties. They were produced in fermentor (media 863) by CWBI (Centre Wallon de Bio-Industrie, Gembloux, Belgium) and fermentation products were dried by lyophilisation. Both antagonistic strains can be produced in fermentor and dried while maintaining their antagonistic activity (Jijakli *et al.*, 1993b).

The technologies of mass production are too often neglected and published data in this area remains spare because of industrial secrets. A closer collaboration between plant pathologists and teams working on mass production and formulation of micro-organisms would greatly stimulate the crossing of BCA's from laboratory to practical use. In this respect, useful insights could be expected from research's on BCA's against insect pests and weeds (Guillon, 1993).

5. FORMULATION OF BCA's

A formulation has to be developed to ensure a reasonably long shelf life of the BCA's and to facilitate their preparation and their application with the standard equipment of the producer. A good formulation will protect the antagonist from adverse conditions, increase the survey and/or enhance the efficiency of micro-organisms. A formulation which reduced antagonist concentration without affecting the protective level, will improve the economical feasibility of the product.

Nutrients are frequently reported as adjuvants which stimulate or stabilise the protective level of antagonistic strains in postharvest diseases (Janisiewizc, 1994; Janisiewizc *et al.*, 1992). We selected 15 carbohydrates and 16 nitrogenous compounds as potential adjuvants of yeast formulation (Jijakli *et al.*, 1993a). Only one sugar analogous (2-deoxy-D-glucose or 2-gluc) showed a protective effect against *B. cinerea* when applied alone and increased the level of protection from about 60 % to 90 % when added to *P. anomala* (strain K) or *D. hansenii* (strain O) suspension (105 cfu/ml). The sole application of 2-gluc inhibited also the development of *B. cinerea* on bean (Jejelowo *et al.*, 1988). This analogous of glucose reduced *in vitro* spore germination and hyphal growth of *B. cinerea* and *P. expansum* (Janisiewizc 1994; Jejelowo *et al.*, 1988; Jijakli *et al.*, 1993a) and could act as a competitive inhibitor of glucose metabolism (Janisiewizc, 1994). 2-gluc could be a proper additive if toxicological requirements of any additive of formulation are fulfilled.

On the other hand, none of the other nutrients (L-asparagin, L-proline, galactose, mannitol, riblose and sorbitol), selected for their *in vitro* and/or *in vivo* antagonist stimulation or pathogen inhibition either by Janisiewicz *et al.*, (1992) or Harper *et al.*, (1981) enhanced the protective activity of *P. anomala* (strain K) or *D. hansenii* (strain O) in our experiments. These different results show that an effect observed in a specific plant-antagonist-pathogen combination is not automatically transposable to an other system when the nutrient affects specifically the antagonistic agents. In opposite, when the nutrient has a specific effect on basic metabolism of the pathogen with no subsequent inhibition of the antagonist, we can expect an easier transposition in different systems.

The application of calcium chloride (2 % w/v) in mixture with *P. anomala* (strain K) or *D. hansenii* (strain O) enhanced the protective level against *B. cinerea* and *Penicillium* sp. McLaughin *et al.*, (1990) and Gullino *et al.*, (1991b) observed similar results when calcium is applied together with an antagonistic strain. The influence of calcium on increasing the resistance of host tissues is often reported (Conway, 1991 ; Messiaen, 1994), while its action on micro-organisms is still poorly studied. Nevertheless, the synergistic action of a combined treatment calcium-antagonist leads McLaughlin *et al.*, (1990) to suggest the secretion of new antifungal metabolites by the BCA's.

6. MODE OF ACTION

6.1. Difficulties and Importance of the Study of Modes of Action

As we move towards the application of biological control, new research problems are emerging among which the mechanisms of action of BCA's were too often considered only as an academic concern. The understanding of the modes of action of biocontrol agents is a prerequisite to (1) developing rational selection procedures yielding a second generation of more effective antagonistic microbial strains, (2) carrying out appropriate production and formulation enhancing antagonistic efficacy (3) providing a quality control procedure, and (4) fulfilling some requirements of the toxicological and registration procedure for commercial use.

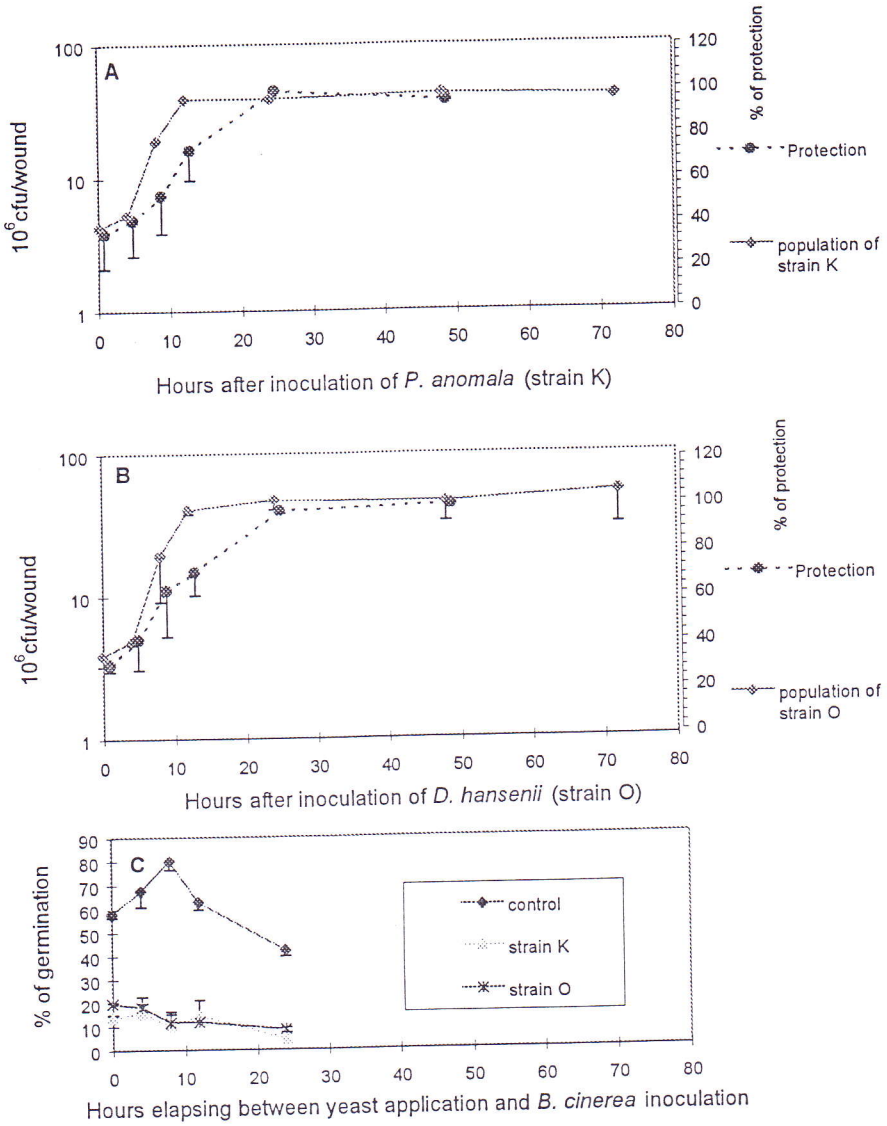
Unfortunately, knowledge's on the mode of action of many antagonists of postharvest diseases are still limited. The comprehension of the mechanisms of action are hampered by the complex interactions between host-pathogen-antagonist. Moreover, the mechanisms studied *in vitro* in order to simplify these complex interactions, do not necessarily reflect *in situ* reality. Nevertheless, in the absence of antibiotics production by most antagonistic yeast's, it appears that the mode of action of yeast's could comprise one or several of the following processes : nutrient or site competition, direct interactions between the biocontrol agent and the pathogen, and induced host resistance (Wilson and Wisnieswski, 1994).

6.2. Nutrient Competition as Part of Mode of Action of *P. anomala* (strain K) and *D. hansenii* (strain O) Against *B. cinerea*

Experimental evidence of the implication of nutrient competition in the antagonistic relationship is still missing although several studies suggested that competition for nutrients might play a role in the antagonistic activity. Some authors (Chalutz *et al.*, 1991; Roberts, 1991; Wisniewski *et al.*, 1989) highlighted the ability of antagonists to rapidly multiply at the wounded sites of fruits but without relating this aptitude to their protective activity. Results of other worker (Droby *et al.*, 1989; Wisniewski *et al.*, 1991) showed that the addition of nutrients can restore both the germination of the pathogen and its development on fruits in presence of the antagonist.

We also investigated the ability of *P. anomala* (strain K) and *D. hansenii* (strain O) to colonise the wounds in relation with their protective activity against *B. cinerea* on apples and with regard to the *in situ* conidial germination of *B. cinerea* (Jijakli *et al.*, 1993a). Populations of *P. anomala* (strains K) and *D. hansenii* (strain O) in wounds increased at 25°C to reach a maximum density (approximately 1 log unit over the initial density) after 12 hours of incubation (Figure 1A and B) similarly to the protection level against *B. cinerea* which also reached a maximum after 12 hours of yeast incubation.

On the other hand, *in situ* spore germination of *B. cinerea* was markedly reduced on wounded sites treated with strain K or strain O, even when pathogen and yeast were applied simultaneously with no subsequent protection (Figure 1C). This suggested that other factor(s) than inhibition of spore germination may be involved in biocontrol effectiveness.



Hours elapsing between yeast application and *B. cinerea* inoculation

Figure 1. Effect of population densities of *P. anomala* (A) and *D. hansenii* (B) on level of protective activity against *B. cinerea*. Data from population densities represent mean colony from three trials (one wound site/trial). Each wound was triplicate-plated. Data from protection level represent the mean % of protection (as compared to the control which was not treated with yeast before inoculation of *B. cinerea*) from two trials (6 wounds/trial). Vertical bars represent standard error of the mean. (Jijakli *et al.*, 1993a).

Effect of *P. anomala* and *D. hansenii* on spore germination (C). Data represent the mean % and associated standard error (vertical bars) of spore germination from one of the two trials (1 replicate per trial). Twenty fields were observed per replicate and a spore was considered as germinated when the germinating tube was longer than the spore.

6.3. ß-1,3-glucanases as Part of Mechanism of Action *P. anomala* (strain K) Against *B. cinerea*

Production of hydrolytic enzymes which degrade cell walls of phytopathogens has been regularly reported as a mechanism of suppression of soil borne pathogens by some biocontrol agent such as *Trichoderma* genus (Bélanger *et al.*, 1995; Benhamou and Chet, 1996; Elad, 1996; Lorito *et al.*, 1994) but lytic enzymes have rarely been reported in biological control of postharvest diseases (Wisnieswski *et al.*, 1991).

We investigated the possible role of ß-1,3-glucanases and chitinases in the antagonistic properties of *P. anomala* (strain K) against *B. cinerea* on apples (Jijakli and Lepoivre, 1998). While chitinase activities were not detected from culture filtrates of strain K after various growth conditions (incubation period, source of carbon), endo- (EC 3.2.1-39) and exo-ß-1,3-glucanase (EC 3.2.1-58) activities were detected in the culture filtrates of strain K (Figure 2). Higher specific activities for both enzymes were obtained in media containing 2g/l of *B. cinerea* cell wall preparation (CWP) as sole carbon source as compared to media with glucose or laminarin (2 g/l). Endo- and/or exo-ß-1,3-glucanases activity from *T. harziamum* Rifai, *P. guilliermondii* Wickerham or *Serratia marescens* was also higher in media supplemented with fungal cell walls than in media containing laminarin (Elad *et al.*, 1982, Ordentlich *et al.*, 1988, Wisnieswski *et al.*, 1991). Exoglc1, an exo-ß-1,3-glucanase purified until homogeneity from *P. anomala* (strain K) culture filtrates, showed a stronger inhibitory effect on germinative tube growth than on conidial germination of *B. cinerea* (Figure 3). Moreover, the enzyme caused morphological changes such as leakage of cytoplasm and cell swelling on *B. cinerea*. Hydrolytic enzymes produced by other antagonists such as *Stachybotrys elegans* Barron or *Schizophyllum commune* Fries : Fries (Chiu and Tzean, 1995; Tweddell *et al.*, 1995) caused similar effects on hyphal growth of *Rhizoctonia solani* (Künh) and *Fusarium monoliforme* Wollenweber.

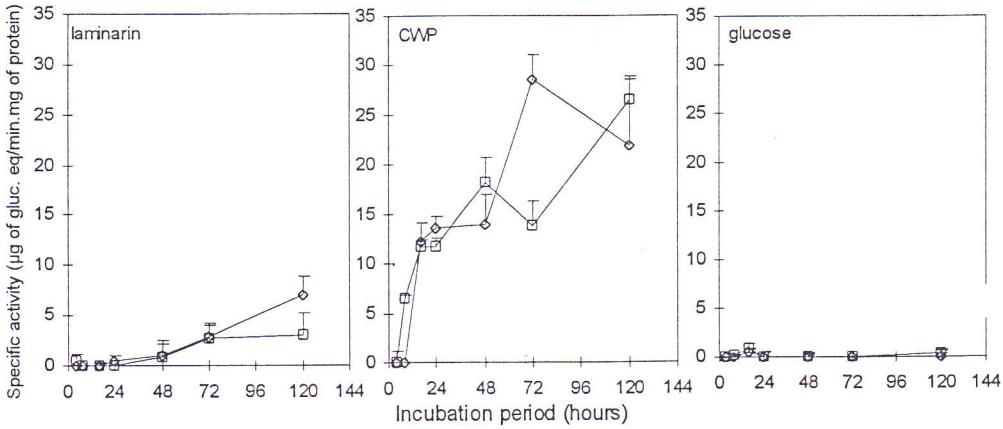


Figure 2. Time courses of endo-ß-1,3-glucanase(◇) and exo-ß-1,3-glucanase(□) activity from strain K supernatant of YNB medium containing *B. cinerea* cell wall fragments (CWP), laminarin or glucose as sole carbon source (2mg/ml). One specific unit of ß-1,3-glucanase (endo-or exo-) is defined as the amount of enzyme causing the release of 1µg of glucose equivalent per miligram of protein per min. Assays were performed in triplicate and the entire experiment was repeated three times. Values presented are averages of the 3 experiments. The error bars represent standard deviations of the means of the 3 experiments (Jijakli and Lepoivre, 1998).

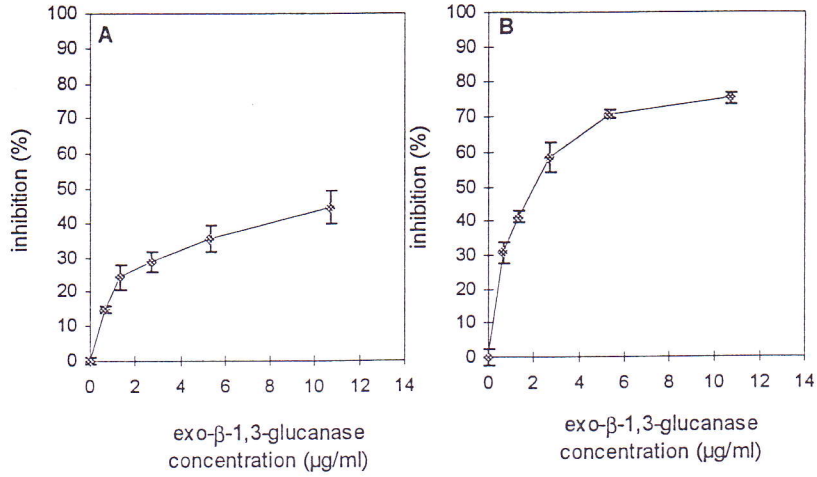


Figure 3. Effect of purified exo-ß-1,3-glucanase from *P. anomala* on spore germination (A) and germ tube elongation (B) of *B. cinerea*.
The percentage of conidia germinating was determined as the percentage of the first 100 spores found on a microscope slide. Length of 20 germ tubes was measured and averaged. For each enzyme concentration, the mean percent of inhibition and the standard deviation (error bars) were calculated. The data collected from two separate experiments with four replicates per treatment were pooled (Jijakli and Lepoivre, 1998).

In other respect, water used to rinse apple wounds treated with strain K and with CWP were assayed for exo-glucanase activity (Table 4). Exo-ß-1,3-glucanase activity was detected on apples treated with strain K. That activity appears to be related to exoglc1 on the basis of its electrophoretic mobility in native gel. Moreover, the addition of CWP to suspension of *P. anomala* stimulated both *in situ* exo-ß-1,3-glucanase activity and protection level against the pathogen (Table 4). To our knowledge, this is the first demonstration of *in vivo* glucanases production by yeast's.

Table 4. *In vivo* detection of exo-ß-1,3-glucanase activity from *P. anomala* strain K and protection from this strain against *B. cinerea* on apples (Jijakli and Lepoivre, 1998)

Treatment[x]	exo-ß-1,3-glucanase activity (µg eq. gluc./wound)[y]	Decay lesion (mm) after *B. cinerea* inoculation[z]
Control	3.4 ± 0.47 a	35.5 ± 8.43 a
CWP	2.1 ± 1.58 a	40.3 ± 4.65 a
Strain K	10.8 ± 0.52 b	15.6 ± 4.31 b
Strain K + CWP	32.7 ± 5.51 c	6.9 ± 3.65 c

x :Wounded sites of apples were treated with 50 µl of distilled water (Control and CWP) or 50 µl of strain K suspension (105cfu/ml) (Strain K). After 24 hours, 50 µl of CWP were applied at the wound sites (CWP and strain K + CWP). Treatments were arranged in a total randomized design.
y : 24 h after treaments, exo-ß-1,3-glucanase activities were measured. Means of enzymatic activity (± standard deviation) were expressed as µg equivalent of glucose/wound. Treatments were arranged in a total randomized design (three replicates per treatment). The experiment was conducted three separate times. After analysis of variance (ANOVA), data from the three experiments were pooled. Resulting means with a common letter do not differ significantly at P = 0.01(Fisher's LSD).
z : 24 h after treaments, wounded sites were inoculated with *B. cinerea* suspension (106 spores/ml). Treatments were arranged in a total randomized design. The experiment was conduted in two replicates searately. After analysis of variance (ANOVA), data from the two experiments were pooled. Resulting means were separated by the Ficher's LSD at P = 0.01.

The possible production of exoglc1 on apples and its effect on *B. cinerea in vitro* strengthen the hypothesis that exo-ß-1,3-glucanase contributes to the biocontrol of *B.cinerea* by *P.anomala* strain K without demonstrating its action. The following criteria may be used to determine if a particular compound is directly involved in biological control of fungal pathogens : (1) the purified compound shows fungicidal or antimicrobial properties, (2) the compound may be detected *in situ*, when producing strains are present, (3) the biocontrol ability of mutants defective in compound of interest is reduced in the laboratory and in practical conditions, (4) the complementation of the mutant with DNA sequence restoring the synthesis of the compound of wild strain restores also biocontrol ability. Because of an easier way to engineer prokaryotes, all of these criteria have been already used to determine the involvement of metabolites produced by antagonistic bacteria (Glick and Bashan, 1997; Wilson and Wisnieswski, 1994). In opposite, the criteria involving molecular tools were not yet reported to support the implication of a compound produced by antagonistic yeast's. Moreover, their possible polyploidy complicates the genetic investigations. However, in contrast to most other organisms, integrative recombination of transforming DNA in yeast's proceeds exclusively via homologous recombination and that property constitutes the basis of our further investigations on the mechanisms of biocontrol of *P. anomala* (strain K).

We isolated two exo-ß-1,3-glucanases encoding genes from a *P. anomala* (strain K) genomic library (Grevesse *et al.*, 1998). These genes named *PAEXG*1 and *PAEXG*2 were found to share significant similarities at the deduced amino acid level with exo-ß-1,3-glucanases from other fungi. Evidence that *PAEXG*2 coded for the purified exoglc1 was given by the sequencing of the N-terminal region of exoglc1. Because possible allelic effects render genetic studies more complicated, *P. anomala* (strain K) isolated from an apple in its diploid form, was induced to sporulate and ascospores were isolated by micromanipulation. Segregation of *PAEXG*1 and *PAEXG*2 in 10 haploid segregants was studied by Southern blots in relation with their *in vitro* exo-ß-1,3-glucanase activity production and their *in vivo* protective activity against *B. cinerea*. All segregants showed an exo-ß-1,3-glucanase activity production equivalent to the production of the diploid strain (or even higher) and retained some significant biocontrol activity at a lower or equivalent level in comparison with the diploid strain. No relation was found between these properties and the segregation of *PAEXG*1 or *PAEXG*2 showing that either *P. anomala* (strain K) is homozygous at both loci and/or other genetic factors (genes or regulatory elements) are active in the protective effect. The implication of *PAEXG*1 and *PAEXG*2 in the antagonism will be studied *in vivo* by their disruption through integrative transformation in the genome of the haploid material. This disruption strategy will give the experimental evidence for the hypothetical action of exo-ß-1,3-glucanase in the protective properties of *P. anomala* (strain K).

7. INTEGRATION OF BIOLOGICAL CONTROL TO OTHER TREATMENTS AGAINST DISEASES ON APPLES

The complete substitution of pesticides by biological control does not constitute a realistic goal. Biological control must be considered like a new potential strategy to be integrated to a panel of other methods. In this respect, the compatibility of BCA's treatments must be firstly evaluated with regard to their integration to the succession of fruit conditioning operations before storage.

A first strategy to widen the spectrum of antagonistic action consists in mixing several BCA's (Falconi and Megden, 1994; Janisiewicz, 1996; Leibinger *et al.*, 1997). A higher protection of postharvest apples against *P. expansum* was observed with the application of a

mixture of *S. roseus* and *P. syringae* in comparison with the effects of their separate application (Janisiewicz and Bors, 1995). The development of five different wound diseases on pears was totally controlled by the treatment of yeast combination [*Cryptoccocus laurentii* (Kufferath) Skinner, *C. infirmo-miniatus* (Okunuki) Phaff and Fell and *Rhodotorula glutinis* (Fresenius) Harrison] (Chand-Goyal and Spotts, 1996)

The combination of chemical treatments with BCA's was more efficient than the application of the sole antagonistic micro-organsism. The success of biocontrol integration to chemical treatments will depends on the selection of the best combinations of fungicide-wax-antagonist (Pusey, 1986). The compatibility of a strain of *P. guilliermondii* with a commercial wax and a fungicide (thiabendazole) was evaluated against *P. digitatum* on postharvest citrus (Droby *et al.*, 1993). Other investigators observed the same phenomenon with other bio-chemical treatments (McGuire, 1994 ; Chand-Goyal and Spotts, 1996). In some cases, the combination of biological and chemical applications allowed the reduction of chemical concentration (Droby *et al.*, 1993).

The integration of the biological agents to other physical measures (thermotherapy, gamma and UV irradiation, film-forming polymers treatments) could be an other solution to widen the spectrum of activity of BCA's (Wilson *et al.*, 1994).

The grower attitude will depend on the capacity of non chemical strategy to inhibit both latent and wound infections usually targeted by traditional fungicide treatments. The control of *Gloeosporium* infections developing postharvest apple rots from orchard latent infections, has to be assumed on postharvest apples in addition to the protection of wound pathogens (*Botrytis* and *Penicillium*) (Jijakli and Lepoivre, 1995). In this context, heat treatments with water (45° C for 10 min) appeared to be efficient against *Gloeosporides* lenticel infections (*C. gloeosporioides*, *C. malicorticis* and *T. fructigena*) by inactivation of spores or hyphae located on skin and on external part of fruit flesh (Bondoux, 1992 ; Eckert, 1975). However, thermotherapy treatment with water bath can enhance the sensitivity of apples to wound pathogens such as *Alternaria* sp., *B cinerea* or *P. exansum* (Edney and Burchill, 1967).

In order to integrate biological control to thermotherapy, we applied three different treatments separately or in combination on postharvest Golden Delicious : (1) dipping the apples in water at 45°C for 10 min, (2) dipping the fruits for 2 min in a water suspension of two antagonistic yeast's, *Pichia anomala* (strain K) and *Candida sake* (strain O) (107 cfu/ml each), or (3) dipping the fruits in an emulsion of a 2% film-forming antitranspirant (2 % Nu-film-P or NFP, 96 % of poly-1-p-Menthen, Miller laboratory) for 2 min. Thermotherapy alone reduced the incidence of *Gloeosporides* lenticel infections from 54.4 % (untreated apples) to 4.6 %, but enhanced sensitivity of the apples to *Penicillium* spp (Table-5). The higher sensitivity to *Penicillium* infections could be explained by lenticel damage (Edney and Burchill, 1967) or partial sterilization of fruit surface enhancing the subsequent *Penicillium* contamination. Control of this pathogen was restored by dipping the fruits in yeast suspension in NFP emulsion after the heat treatment. The quality parameters (weight, size, skin color, firmness, acidity and refractometric indice) of the fruits were not affected by any of the treatments (Jijakli *et al.*, 1993a). During a second year of trials, combination of heat treatment and yeast application could entirely control infections caused by *B. cinerea* and *Gloeosporium* rot and the percentage of apples rotted by *Penicillium* spp. was reduced from 18.2 % (untreated fruits) to 3.8 % without affecting any quality parameters (Table-6), thus confirming the efficacy of such integrated treatments against major fungal diseases on postharvest apples. Nevertheless, the evaluation of the practical feasibility of an integrated approach combining biological control and thermotherapy must be further studied with a less intensive scheme of chemical application.

Table 5. Separate and combined effects of thermotherapy, biological control and antitranspirant application on post-harvest diseases of apples in 1993 (Jijakli *et al.*, 1993b)

	Control	45°C[b]	45°C +NFP[c]	45°C K+O[d]	45°C+ K+O+ NFP	K+O	NFP	NFP+ K+O
Total infection	72.4a	55.2	61.3	50.0	40.2	56.2	57.5	62.5
Gloeosporides	54.4	4.6	4.6	2.3	1.5	37.7	33.8	41.5
Penicillium spp.	8.2	20.0	32.3	19.2	11.5	3.1	10.0	6.1
Alternaria spp.	4.8	13.1	10.0	10.8	11.5	4.6	5.4	7.7
Fusarium spp.	3.4	7.7	5.4	3.1	6.9	0.8	0.8	2.3
Cylindrocarpon spp.	3.4	3.8	3.8	5.4	6.9	3.8	1.5	3.8
Rhizopus spp.	0.0	1.5	0.0	0.0	0.0	0.0	0.0	0.0
Non determined	4.1	8.5	11.5	9.2	9.2	10.0	10.0	6.9

a = mean percentage of infection from 130 apples, b = apples were dipped in heat water (45°C) for 10 min,
c = apples were dipped in Nu-Film-P (2%), d = apples were dipped in strain K et O suspensio (107 cfu of each strain/ml).

Table 6. Separate and combined effects of thermotherapy, biological control and antitranspirant application on post-harvest diseases of apples in 1994

	Control	45°C+K+O +NFP[b]
Total infection	24.2 a	4.6
Gloeosporides	1.5	0.0
Penicillium spp.	18.2	3.8
Botrytis cinerea	6.1	0.0
Fusarium spp.	0.0	0.0
Alternaria spp.	0.0	0.0
Non determined	0.8	0.8

a = mean percentage of infection from 130 apples, b = apples were successively dipped in heat water (45°C) for 10 min and in a suspension of yeast's in a 2 % NFP emulsion, c = dry heat treatment carried out at 38° C for 24 h, d = apples were successively heated in a steam room and dipped in a suspension of yeast's in a 2 % NFP emulsior

8. HOMOLOGATION

Biological agents used for the control of plant diseases are primarily living organisms. The impact on the environment and therefore, safety requirements are likely to be somewhat different than those for chemical pesticides.

OECD, with particular inputs from the Directorate of Sciences and Technology and Environment, published a booklet which concerned the release of micro-organisms in the environment. Most countries, including the European Union, have incorporated the main conclusions of the OECD reports (OECD, 1995) in their national regulation.

In this respect, the European Union Council Directive of 15 July, 1991 (91/414/EEC) concerns the marketing of plant protection products. According to this directive, any products used in the biological control of plant diseases must be considered as plant protection products

and have therefore to fulfil different specific requirements. The technical dossier of registration of the BCA must supply the necessary information's for evaluating the foreseeable risks, whether immediate or delayed, which the substance may entail for humans and the environment. These requirements mainly concern the identity of the organism, its biological properties, the description of the analytical methods available for its identification, its toxicological impact (pathogenicity and infectivity), and the ecotoxicological properties. The associated costs for the registration of plant protection products containing biological agents are directly related to the level of requirements and rapidly become so high that the development of products for the biological control of plant disease could be discouraging for companies and leads into temptation of circumventing these regulations. Public policy could thus promote biological alternative to chemical control by authorising realistic registration procedures based on a case by case study. If the active substance belongs to a group of micro-organisms well known for their non pathogenicity, different costly studies such as long term toxicity should be not required for the evaluation of the dossier. While the responsibility of the registration usually falls upon the company developing a commercial product, some requirements also concern the criteria of the screening tests set up for the selection of the agents. In this respect, we must ıphasise possible problems encountered in the registration of BCA's whose mode of action ınclude antibiosis or that belong to groups of micro-organisms comprising some potential pathogenic species or strains.

Large scale introduction of biocontrol agents also requires specific evaluation of their environmental impact. A requisite to any study related to the persistence of artificially introduced micro-organisms in the environment is the ability to track them. In this purpose, molecular fingerprinting of *P.anomala* (strain K) was attempted by means of random amplification of DNA (RAPD). Polymorphism was observed in the RAPD profiles of different strains of *P.anomala* and between different species of yeast. The nucleotide sequence of a strain K-specific amplified product was used to design specific primers which allow the unequivocal recognition of the antagonist. That method primarily used for identification of the BCA and monitoring its population on the fruit surface, could also be used to perform studies of ecophysiology of the BCA, with the aim of understanding and predicting its antagonistic activity as affected by environmental conditions of storage of the fruits.

9. CONCLUSIONS

Many challenges must be met before biocontrol of postharvest diseases can be sucssfully used on a commercial basis (Figure 4). Biological control is often generating a great enthusiasm although the still limited relevance of BCA today. The postharvest environment may be one in which the best chance to develop successful biological control exists because many aspects of it can be controlled. Moreover, consumer demand for alternatives to postharvest chemical treatments constitutes a major and special impetus in the search for biocontrol agents. Already commercially available products (such Aspire™ and Biosave™) demonstrate the realism of the approach.

However, encouragement's from environmental agencies and central government as well as grower education remain crucial in determining the economic climate within biological control will operate.

It remains doubtful that the first generation of biocontrol agents will easily provide by themselves the efficacy and the consistency associated with conventional fungicides. However, biocontrol agents already selected in different laboratories could constitute a significant part of integrated systems including physical treatments or lower doses of chemicals to provide adequate control of postharvest diseases.

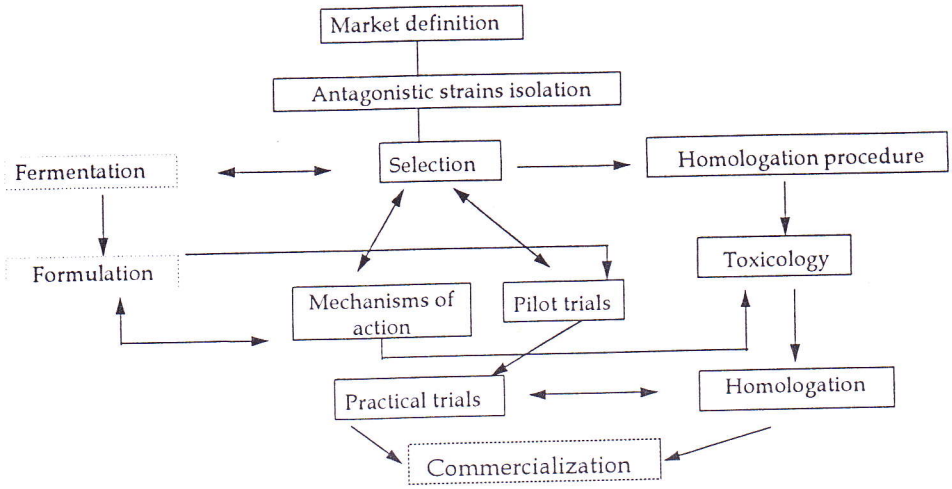


Figure 4. Steps leading to the practical use of BCA's

In the long term, basic information's on the genetically determined factors that control survival, colonisation, effectiveness in the field and storage and properties of mass production are required to overcome the random process of selection.

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