United States
Department of
Agriculture

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Research
Service

ARS-92

June 1991

Biological Control of Postharvest Diseases of Fruits and Vegetables, Workshop Proceedings

Shepherdstown, West Virginia
September 12-14, 1990

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From September 12-14, 1990, a workshop sponsored by the United States/Israeli Agricultural Research and Development Fund was held at the Bavarian Inn in Shepherdstown, West Virginia on the *Biological Control of Postharvest Diseases of Fruits and Vegetables*. The purpose of the workshop was to bring together government and university researchers with industry representatives who are interested in developing alternatives to synthetic fungicides for the control of postharvest diseases. It is hoped that this meeting and its proceedings will accelerate the commercialization of innovative alternatives to synthetic fungicides for the control of postharvest diseases of fruits and vegetables.

C. Wilson
E. Chalutz

Contents

I. BACKGROUND LECTURES

We have only recently been considering the biological control of postharvest diseases of fruits and vegetables. Research in this area is based on our understanding and manipulation of epiphytic microflora.

Managing Epiphytic Microflora for Biocontrol

Harvey W. Spurr, Jr.[1], Vern J. Elliott[1], and Wayne M. Thal[2]

Abstract

Epiphytic microflora are ubiquitous. Much effort is devoted to finding antagonists among the microflora to use as biological control agents for plant disease. Although research results are encouraging, most attempts to obtain satisfactory control under field conditions are variable. Levels of control are usually low. We suggest reorienting research and putting emphasis on microbial ecology. We need to do biological control research without promising biological control.

Introduction

Is biological control of postharvest disease a reality? Looking at some recent titles you might assume it is. For example: "Biological control of Rhizopus rot of peach with *Enterobacter cloacae*" (Wilson, Franklin, Pusey, 1987) or "Postharvest control of stone fruit brown rot by *Bacillus subtilis*" (Pusey, Wilson, 1984). Although these titles along with many others in the phytopathology literature may suggest that biological control is a reality, we know that it *is not* and that's one reason for this review.

The "Other" Microorganisms

Why is biological control of plant disease – preharvest or postharvest, so elusive? Again if the answer were available we would not be here, however, we would like to speculate that it has to do with the numbers. In this case it has to do with the numbers that make up populations of microflora. Microflora is what we are going to discuss.

A recent editorial by R. J. Sullivan entitled: "The Other Microorganisms" (1989) discussed some numbers. The average human contains about 10 trillion cells and 10 times as many or about 100 trillion microbes which are components of the normal flora. "Some of these organisms are nonpathogenic; most are opportunists, who if given the opportunity of increasing growth or invading new territory, will cause infection." Some of these organisms also provide services such as synthesizing essential vitamins, while others protect us from invasion by pathogenic microbes. They protect us by competing for space, food, and the production of a variety of antibiotic chemicals. Does this sound familiar?

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Sullivan points out that the natural inhibitors are potent. They maintain a balance among the microbes normally present and inhibit the growth of newer arrivals. When the normal flora is disturbed, as in antibiotic therapy or acute diarrhea, this inhibition can break down causing one of the normal residents to overgrow the others. An example of this is the overgrowth of *Candida albicans* in both the mouth and vagina when the normal microbial balance is upset.

Sullivan briefly describes "normal flora" of the skin, mouth and other organs and various environmental factors controlling which organisms grow and where such as pH, temperature, water availability, specific nutrients, secretary antibodies. In conclusion, Sullivan states these microflora are our companions. They vary in variety and number, but are always there. "They need us to survive, as their host, but we very much need them for what they produce for us and the competition they provide with other, more potentially harmful microorganisms."

The analogy seems clear. Plants and animals both host microflora. Normal plant flora consists of both nonpathogenic and pathogenic microorganisms. The number of bacteria ranges from 10^3 to 10^7 per sq.cm. on leaves or per g of root depending on host and environmental parameters. Depending on the size and distribution of bacterial cells, less than 1 percent to 37 percent of this sq.cm. will be physically covered. Numerous yeast and fungi are also present and the interactive dynamics vary with seasonal growth and development of the host (Blakeman, 1985). The enumeration and function of nonpathogenic bacterial species, in particular, is less well defined than for animal flora. This is an important limitation to managing the plant flora which are known to reduce disease, reduce frost injury and fix nitrogen for plant nutrition (Spurr, 1990).

Thus, medical researchers have developed considerable basic knowledge of "normal flora." It is understood that normal flora includes some pathogenic species. The location, description quantification and function of human species of flora is more complete than for plants, as might be expected based on the larger amount of resources available for such investigations. The consequences of upsetting the flora are better documented in humans can be managed in ways to prevent disease. However, few examples of biological control are available which depend upon the introduction of microflora.

Plant Epiphytic Microflora   Back to the question, how can we manage epiphytic microflora to control disease? More specifically, how can we manage microflora to control postharvest disease and rots of fruits and vegetables? First, we should define what we mean by "manage". In regard to this discussion "manage" means those actions or activities a person can take which will alter epiphytic microflora control of disease. Based on current experience and knowledge we can hypothesize certain management activities which may have an impact on microflora. It has not been demonstrated that all of these have an impact. Therefore, we need to do additional research and for this have prepared a list of research activities (Table 2).

It has been stated that preharvest, harvest (transition) and postharvest activities impact epiphytic microflora. Figure 1 shows how environmental conditions in the field (preharvest) are different from conditions in storage (postharvest). Primarily, in the field, the environment is less controllable and cultural practices vary. Microflora develop in response to these factors over the growing season and are in some "state" by harvest time. The impact of this state to storage disease development is usually unknown. This state includes both nonpathogenic and pathogenic microbes. It may include latent infections. At harvest, the transition period, fruit (vegetables) are subjected to handling and processing. Thus, there may be: bruising and wounding, washing and dipping, heating and cooling during the transition to storage. This harvesting and processing transition has considerable impact on epiphytic microflora and their physical-chemical environment. Also, it provides an opportunity to intentionally modify the microflora and their environment in numerous ways prior to storage (Wilson and Wisniewski, 1989). Once the commodity moves to storage (postharvest), environmental conditions which drive the growth or development of microflora are regulated and are often fairly constant. This provides additional opportunities to manage.

Management Activities

We recognize that the epiphytic microflora live on surfaces. We want to manage this microflora to control postharvest disease. Some management activities which can impact or alter the microflora are listed in Table 1. The activities were selected because they are broad and inclusive, covering a range of sub-activities and listed according to the time when they apply. These activities are readily recognized as usable inputs or actions. They can be selected and implemented in many instances without a thorough knowledge of their total impact on future occurrences such as the development of postharvest disease. Our primary interest usually focuses on: "select and apply biological and/or chemical agents." This management activity, perhaps more than the others listed, requires a considerable research effort. It should be emphasized that successful disease control will depend on understanding all the listed management activities and using them in concert. We also recognize the need for research to support and develop these management activities.

Research Activities

Most research on biological control of disease has been directed to obtaining a quick success. Scientists readily grasp that microflora suppress pathogen growth *in vitro*. Thus, research has centered on rapid procedures (bioassays) for selecting and then introducing potential biological control agents. This is usually a selection of one antagonist to control one pathogen referred to as the "silver bullet" approach (Spurr and Knudsen, 1985). The result is a growing list of antagonists which partially control disease depending on conditions. Also, the result is a growing recognition that biological control is dependent upon a thorough knowledge of ecosystems and their management (Baker and Dunn, 1990; NAS Committee, 1989).

To be more specific about research needs, Table 2 was constructed to list research activities which are required to generate the knowledge upon which to develop management activities. The activities are listed somewhat in the order by which they should be addressed. What is being stressed here is a need to place our emphasis on long-term research with an ecological basis. This knowledge will be used to develop management activities. We also stress the need to integrate data and research activities to develop computer based models. These models will likely need to be developed by working cooperatively with one or more scientists.

It may seem strange that selection of commodity and disease targets are listed. This was done to emphasize that success in achieving biological control, especially in the near term, probably depends to a degree on making careful selections in these areas. This goes along with the stated idea that postharvest disease should be easier to control than preharvest disease because conditions encountered in storage are less variable.

Current Research

In pointing out the pitfalls of using silver bullets and stressing an overall need for studies of microbial ecology, it is necessary to present some suggestions as to what specific research approaches may be employed. To develop suggestions we have examined the reports presented at the 5th International Symposium on the Microbiology of the Phyllosphere and the joint annual meeting of The American and Canadian Phytopathological Societies. Both meetings were held during the summer of 1990. It should be noted that 932 "Abstracts of Presentations" were published from the 1990 APS/CPS meeting and 100, 11 percent, were on biological control. The majority of the biological control abstracts, 63 percent, were silver bullet reports resulting in partial control. The following paragraphs present some non silver bullet research which we think is important to making progress toward achieving biological control.

Bacteria often occur in biofilms in aquatic environments. All natural and industrial ecosystems examined, support the contention that bacteria adhere to surfaces and cluster together in biofilms (Casterton, 1990). These biofilms consist primarily (95%) of polysacharrides. The films protect the enclosed bacteria from drying, ultraviolet irradiation, mechanical abrasion, antibacterial viruses, predators, surfactants, antibodies and antibiotics. Single or monoculture, films tend to occur under low nutrient conditions. The concept of biofilms needs to be studied and perhaps applied by researchers interested in biological control. Bacteria in biofilms are physiologically different than those growing in liquid fermentation without protective biofilms.

Research on the characterization of bacterial microflora associated with peanut foliage documents the diversity of genera present (Elliott and Spurr, 1990). Seventy percent of 300 isolates identified showed the following genera by percent of occurrence: *Pseudomonas* 29, *Micrococcus* 22, *Methylobacterium* 20, *Corynebacterium* 14, *Xanthomonas* 13, *Flavimonas* 11, *Clavibacter* 10, *Enterobacter* 9, *Bacillus* 8, *Microbacterium* 7, *Agrobacterium* 6, *Yersinia* 5, *Comamonas* 2, *Klebsiella* 2, misc.

genera 9, unknown 16. Looking at this diversity in genera and realizing that they are found in associations on peanut leaf surfaces, emphasizes the need for research on microbial ecology. We need to learn how these associations of bacteria function and survive.

It has often been said that determining survival or epiphytic fitness of bacteria should lead to developing strains for biological control. Lindow (1990) used Tn5 mutagenesis to identify traits which were important to the survival of *Pseudomonas syringae* on bean leaf surfaces under fluctuating conditions of leaf wetness. One hundred twenty six of 5300 random mutants exhibited reduced fitness. Of the phenotypic traits which could be identified, osmotolerance and motility and some catabolic capabilities seemed important to survival. Capsular polysaccharide, tobacco hypersensitivity, *in vitro* growth rate and fluorescence did not appear to be important to survival. This approach will eventually define traits related to fitness and provide a genetic basis for developing new strains.

Pseudomonas fluorescence   Mixing genetically engineered strains of *Pseudomonas fluorescence* proved a valuable tool for determining survival in soil and the rhizosphere of wheat in relation to the production of phenazines by the strains (Mazzola, Cook, Thomashaw and Weller, 1990). The phenazines are toxic to the fungal pathogen causing "take-all" disease of wheat. These strains could be identified when extracted from soil or roots. Genetic engineering techniques were used to make related strains that produced phenazines and those which did not. The strains were introduced into soil and survivors quantified after planting five successive cycles of wheat. It was concluded that phenazine production contributes to the survival in soil habitats of strains. Genetic engineering techniques for marking stains provided a means for locating and enumerating bacterial strains in complex environments. This is essential to develop knowledge for management activities.

Studies of the multiple interactions among saprophytes, pathogens, nutrients and fungicides in the phyllosphere of cereals were important for the development of a simulation model to guide the introduction of biocontrol agents (Dik, 1990). Competition for nutrients results in antagonism between yeasts and pathogens. This is not sufficient for control, so fungicide must be applied. Broad-spectrum fungicides also reduce yeasts and thus there is more nutrient which stimulates the pathogens even in the presence of fungicides. The presence of yeasts enhanced both fungicide performance and decreased disease and aphid damage by limiting the detrimental effect of honeydew on leaf physiology. Thus, yeasts on the leaf surface could decrease the need for both fungicides and insecticides. A simulation model for yeast population growth was developed to guide the application of chemicals and to further evaluate the impact of yeasts in this example of integrated control. This work provides an example of how models can assist with making decisions concerning the complex interactions involved in management for biological control.

Conclusions

We began by discussing the ubiquitous microflora and their impact on living hosts - animal or plant. It was emphasized also that microflora exist as diverse populations and are subjected to various environmental fluctuations and stresses. Our desire to harness these microflora for benefits such as disease control depends on using management activities which include physical, chemical and biological inputs. We cannot move quickly to implement these based on simplistic theories, as experience has already proven. We must do the basic research to learn about the complexities such as how a mixed population multiplies and survives. We have to undertake studies of survival and competition among microorganisms using the advantages provided by techniques based on genetic engineering. We must develop data sets to manage the complexities with the help of computer models. To achieve biological control it is necessary to do biological control research without promising biological control!

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Table 1. Management activities targeted for biological
control of postharvest disease.

Preharvest Activities

    Select:
        Varieties
        Cultural practices - seed treatment, tillage, pruning
        Chemicals - pesticides, fertilizers
        Biological agents

Harvesting/Processing Activities

    Select:
        Harvest time, environmental conditions
        Harvesting, handling, culling methods
        Processing methods
        Biological and/or chemical agents

Postharvest Activities

    Select:
        Environment - temperature, humidity, atmosphere
        composition
        Time in storage
        Biological and/or chemical agents

Table 2. Research activities required to develop management
activities for biological control of postharvest disease

    Select commodity

    Select disease target(s) on commodity site

    Study microbial ecology of site
        Determine makeup of microfloral population
        Determine impact of environment on microflora
        Determine impact of preharvest, harvest and
        postharvest activities on disease development

    Study impact of physical, chemical and biological inputs
on disease development

Construct models to guide management activity

Field
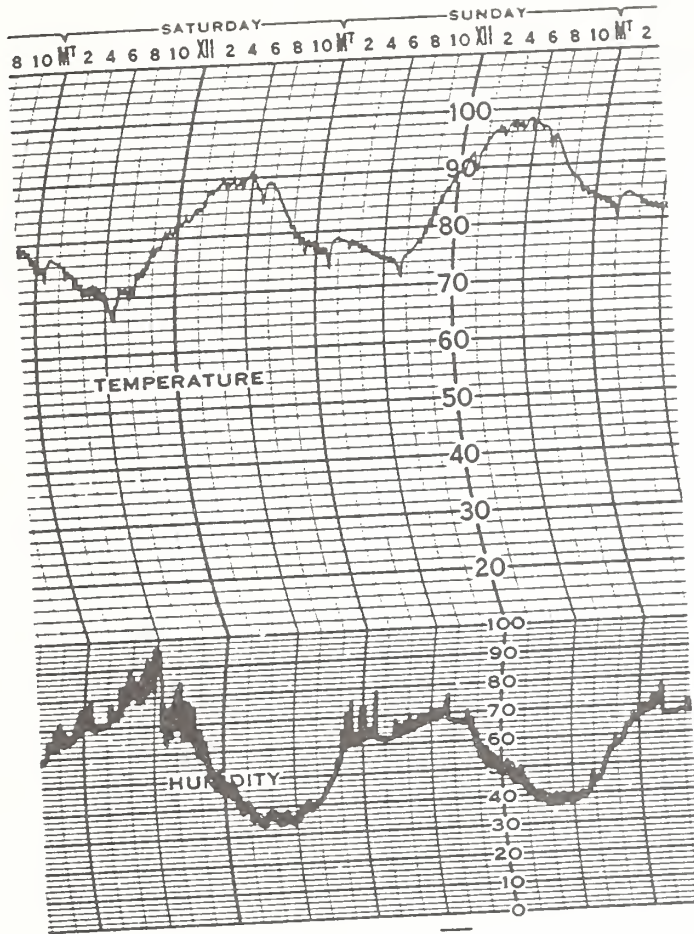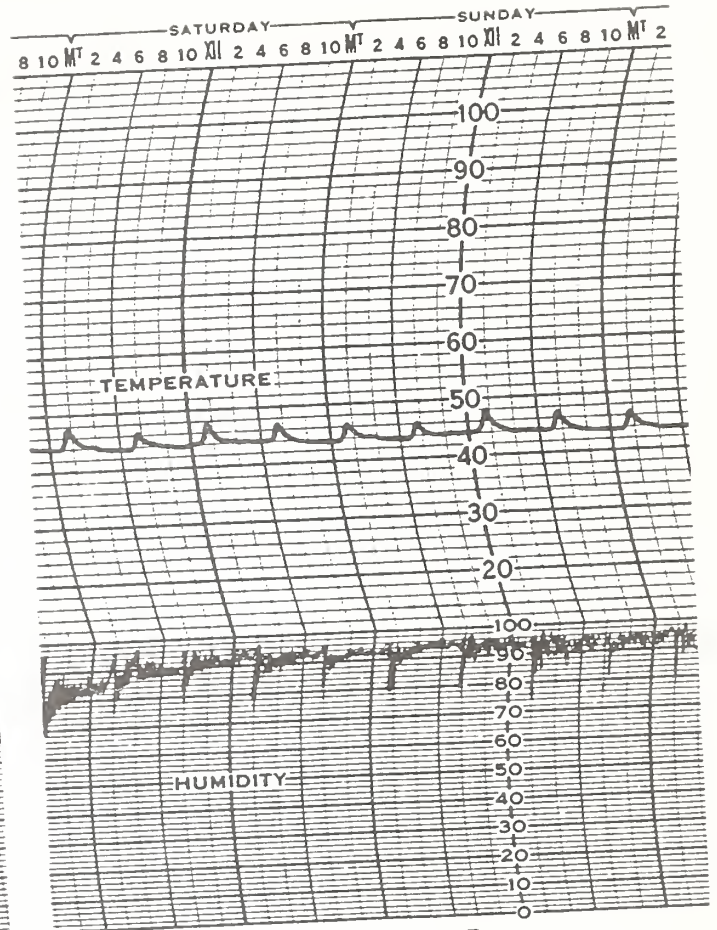
Figure 1. Comparison of temperature and humidity in a field to a storage chamber. Microflora, nonpathogens and pathogens, on plant products must survive the transition from an uncontrolled, variable field environment through harvesting and processing to a controlled, less variable storage chamber.

Role of Chemical Fungicides and Biological Agents in
Postharvest Disease Control

Joseph W. Eckert[1]

Abstract

Treatments to control postharvest diseases of fresh fruits and
vegetables must prevent development of latent infections on
immature fruit, late-season infections preceding harvest, and
infections initiated in injuries associated with harvesting
and handling the crop. Postharvest treatments developed
before 1960 were mostly effective against wound-invading
pathogens: *Penicillium, Rhizopus, Monilinia, Ceratocystis,*
and *Botrytis,* but were not very useful against preharvest
infections. Systemic fungicides developed since 1965, notably
benzimidazoles (thiabendazole, benomyl, and carbendazin),
sterol inhibitors (imazalil, prochloraz, and propiconazole),
and phenylamides (metalaxyl) have given excellent control of
wound infections as well as quiescent field infections:
anthracnose and stem-end rots of tropical fruits, banana crown
rot, apple lenticel rots, and late season infections of
Monilinia and *Phytophthora.* The intensive use of these
fungicides has resulted in pathogen-resistance in citrus,
apples, and stone fruits. The problem can be managed by
alternating non-selective fungicides and by inoculum
reduction. Biological control agents have provided control of
some field infections and wound-invading pathogens, both by
protective action. Curative action seems weak except when
bio-control formulations contained pre-formed antibiotics or
chemical fungicides.

Introduction

Fresh fruits and vegetables are susceptible to attack by
several pathogenic fungi and bacteria after harvest because
they are high in water and nutrients and have lost most of the
intrinsic resistance that protected them during their
development while attached to the plant. Disease results in
not only a direct loss of food, but may also accelerate
ripening of the entire shipment and contaminate sound fruits
with toxic products of the pathogen and diseased plant
material. Postharvest losses in well-managed shipments
typically run 5-10%; total loss may occur when heavily
infected or damaged produce are stored in an unfavorable
environment.

Strategies for Postharvest Disease Control

Observations and research over many years have identified
several major factors, involving the host, pathogen, and
environment, that greatly influence the development of
postharvest disease in fruits and vegetables. Depending upon
the specific crop and marketing conditions, one or more of

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these factors can be manipulated to reduce the incidence or severity of disease. The prudent ship per/marketer will use at least several of these factors because they interact for most effective disease suppression. In addition, the failure of a single component of the program will not result in a total loss in disease control.

Enhance Host Resistance

Fruits, as they ripen, invariably become more susceptible to invasion by postharvest pathogens. Hence, treatments such as low temperature, a low O_2/high CO_2 atmosphere, ethylene removal, and growth regulators (e.g., 2,4-D and gibberellin) that delay senescence, significantly suppress the development of postharvest pathogens in infected fruit (Alvarez, 1980). An environment favorable to wound healing (high humidity-moderate temperature) prevents infection of root/tuber crops, citrus, and other fruit where defense mechanisms can be elicited. These aspects of postharvest disease control have been emphasized in other reviews (Brown, 1989; Sommer, 1982; 1985).

Interfere with Pathogen Development

The opportunities for fruit infection may be limited since it requires, synchronously: the presence of the pathogen, physiological susceptibility of the host, an entry route, and a favorable environment. The incidence of infection can be reduced by a control measure (e.g., sanitation, inoculum exclusion) that reduces the availability or infectiousness of the pathogen or by one that reduces host susceptibility (e.g., careful handling, wound curing, fungicides etc.). The incidence of stem-end rots of citrus fruits caused by *Diploidia, Phomopsis*, and *Alternaria* which enter through the calyx of young fruitlets, is dependent upon rainfall early in the growing season (Eckert and Brown, 1986). These diseases have been experimentally reduced by removal of the inoculum source (e.g., by pruning infected dead wood from the trees and by debuttoning the fruit after harvest). Similarly, the infection of the cut stem of bananas, mangos, and papayas and superficial injuries on the surface of citrus fruits must take place before the wound-healing process prevents access of the pathogen to the internal tissue of the fruit.

Disease Control Strategies

In planning disease-control strategies, it is useful to recognize three types of infections that may lead to postharvest disease: 1. Latent (quiescent) infections which are initiated on immature fruit in the field. Further development of the pathogen at the infection site is prevented by resistance of the immature fruit until it begins to ripen (Brown and Swinburne, 1980; Dickman and Alvarez, 1983). Anthracnose of tropical fruits (*Colletotrichum* spp.) and stem-end rots of citrus (*Diplodia, Phomopsis,* and *Alternaria*) are well-known examples of quiescent infection. 2. Infection of unripe fruit at an advanced stage of development in the field. Progressive development of the disease is suppressed by resistance of the fruit until it ripens several days or weeks after infection. Botrytis rot of grapes and strawberries, brown rot (*Monilinia*) of stone fruits (Kable, 1971), and brown rot (*Phytophthora*) of citrus fruits are diseases which arise from late-season infection of developed

fruit. 3. Infection through wounds such as the site of severance from the plant and those caused by mechanical and environmental stress to the surface of the fruit (Harvey, 1978; Sommer, 1982). Generally, the pathogen begins to develop immediately in the wounded tissue, the rate dependent upon the inoculum level, degree of fruit ripeness, and the environment. Banana crown rot and pineapple black rot are typical diseases initiated by infection through the severed attachment site, whereas Penicillium mold of citrus and Rhizopus rot of stone fruits are initiated by infection at mechanical wounds on the surface of the fruit. Certain diseases (e.g., brown rot of stone fruits) may be initiated by both preharvest infection through the fruit epidermis and by postharvest infection through mechanical wounds. Decay control treatments must address both aspects of the disease.

Chemical Treatments for Control of Postharvest Diseases

Selecting an Effective Fungicide

About 20 organic compounds have been used extensively over the past 30 years to control postharvest diseases (Fig. 1). The chemistry and history of these compounds are summarized in *The Pesticide Manual* (Worthing, 1987) and their applications for specific fruits and vegetables are discussed in several reviews (Eckert and Ogawa, 1985, 1988; Eckert and Brown, 1986; Eckert, 1990). The selection of an effective fungicide to control a postharvest disease depends upon: 1) the requirements of a specific disease problem (e.g., the sensitivity of the pathogen species and its location); 2) the chemical, physical, and biological properties of the fungicide; 3) temperature and humidity; 4) the tolerance of the host to the treatment.

Quiescent Infections

Infections that are quiescent at the time of harvest are much more difficult to eradicate than germinating spores of the same pathogen. Appressoria possess a thick cell wall and are notoriously difficult to kill with most contact fungicides (Greene, 1966). Alternately, some appressoria may germinate on the fruit before harvest and give rise to a slender germ tube that becomes quiescent between the epidermal cells of the host. Postharvest decay arising from latent infections has been controlled by several strategies. Fungicides sprayed on the developing fruit in the field prevent development of spores and appressoria of pathogens. Protective sprays applied on a 7-14-day schedule have been extensively used to control anthracnose on mangos (Prusky et al., 1983), papayas (Alvarez and Nishijima, 1987), bananas (Slabaugh and Grove, 1982) and avocados (Muirhead et al., 1982).

Late-season Field Infections

Late-season field infections, favored by late summer rainfall, give rise to several postharvest diseases: e.g., lenticel rot of apples, brown rot of peaches, and gray mold of grapes (Kable, 1971). These diseases have been controlled by protective or curative fungicide treatments applied to the fruit before harvest. Preharvest fungicides to control late-season quiescent infections, are often broad-spectrum protective fungicides such as fixed coppers, mancozeb, and chlorothalonil (Eckert and Ogawa, 1988). Iprodione should be considered for diseases caused by *Alternaria, Botrytis,* and

Monilinia since this fungicide is highly active against these pathogens (Prusky et al., 1983; Alvarez and Nishijima, 1987). Postharvest treatments to eradicate latent or incipient infections generally require a systemic fungicide and 50°C water for effective disease control. Metalaxyl and fosetyl-Al are selectively active against incipient infections of *Phytophthora* (Edney and Chambers, 1981; Cohen, 1981). Stem-end rots of citrus have been controlled for over three decades by treating the fruit after harvest with 2,4-D to retard senescence of the fruit button (calyx) which usually harbors a quiescent infection of one of the stem-end rot pathogens (Eckert and Brown, 1986). Papayas, mangos, and avocados can be treated with a systemic fungicide (benomyl, thiabendazole, imazalil or prochloraz) and hot water to eradicate appressoria or latent infections in the surface tissues of the fruit or its stem-end (Muirhead et al., 1982; Alvarez and Nishijima, 1987; Spalding and Reeder, 1978, 1986).

Wound-invading Pathogens

Wound-invading pathogens may be controlled by several postharvest treatments: 1) disinfest the surface of the fruit and its environment; 2) eradicate or suppress germinating spores in wounds on the surface of the fruit; 3) reduce wound susceptibility with protective fungicides, or treatments that stimulate wound defense mechanisms (Brown, 1989; Ben-Yehoshua et al., 1988). The probability of infection at a superficial wound on citrus fruits is related to the number of spores of *Penicillium* or *Geotrichum* deposited into the wound (Baudoin and Eckert, 1985; Wild and Eckert, 1982). Therefore, a substantial reduction in the spore population in the fruit environment will reduce the incidence of decay. A standard commercial practice in fruit packinghouses is to disinfest the atmosphere with formaldehyde, boxes and other equipment with quaternary ammonium compounds, and the surface of the fruit with an active chlorine solution.

Fungicides After Infection

Fungicides applied after harvest to control wound-invading pathogens must reach the site of infection--a wound in the peel of the fruit or the exposed vascular bundles of a cut stem, and be inhibitory to the pathogen in that environment. Many compounds that are highly fungitoxic *in vitro* fail to control the same pathogen when it is inoculated into a wound. The performance of fungicides against wound pathogens can be influenced by the pH of the plant tissue and by accumulation or detoxification of the fungicide by constituents of the wounded tissue. Detoxification can be a major factor in the effectiveness of chemically-reactive compounds such as hypochlorous acid, captan, and amines.

Wound Infection

Postharvest fungicides prevent wound infection by direct inhibition of the pathogen, although the elicitation of fungitoxic plant metabolites (phytoalexins) and wound barriers may be involved also (Barak, Edgington and Ripley, 1984; Brown, 1989). Although nonselective water-soluble salts of fungicides such as o-phenylphenol, sodium carbonate, and salicylanilide have been used to control wound pathogens of citrus fruits (*Penicillium*) and pineapples (*Ceratocystis*), the degree of control was greatly improved by development of systemic fungicides in the late 1960s. A major breakthrough in the control of wound pathogens, especially those

responsible for crown rot of bananas and stem-end rots of citrus, papaya, mango and pineapple, was made possible by the introduction of thiabendazole, benomyl, imazalil and procholoraz (Brown, 1984; Slabaugh and Grove, 1982; Eckert, 1990; Eckert and Ogawa, 1986). Apparently, these systemic fungicides diffuse into the tissue surrounding the wound to inhibit growth of the pathogen at sites that are not accessible to the traditional water-soluble fungicides.

Spectrum of Fungicidal Activity

Most postharvest fungicides have a spectrum of antifungal activity that is sufficiently broad to control most postharvest pathogens on a specific crop. However, many fungicides have poor activity against *Rhizopous, Mucor, Alternaria, Phytophthora,* and *Geotrichum*. In these cases, it may be necessary to combine or alternate two fungicide treatments. For example, dicloran is combined with benomyl or thiabendazole to control brown rot and *Rhizopus* rots of stone fruits. Iprodione and metalaxyl can be added to primary fungicide treatments to improve control of *Alternaria* and *Phytophthora*, respectively.

Most fruits and vegetables are packaged without individual wraps, risking the spread of disease and debris from infected to healthy produce during marketing. Some postharvest fungicide treatments are highly valued for suppressing disease spread: biphenyl/citrus fruits; imazalil/citrus fruits; dicloran/stone fruits; SO_2/grapes. This property is not common amongst postharvest fungicides; it appears to be due to the accumulation of the fungicide in the epidermis and exocarp of the fruit (Brown, 1984, Brown and Dezman, 1990; Eckert et al., 1979).

Progress, Problems, and Prognosis for Chemical Treatments

Fungicides and Application Techniques

Fungicides and application techniques are available today to provide practical control of the major postharvest diseases of fruit and vegetable crops. This statement is based, to a certain degree, upon experimental successes rather than commercial accomplishments. The development of the benzimidazole fungicides in the late 1960s provided a conceptual breakthrough in postharvest disease control. Prior to that time, the postharvest treatment of fruit crops was limited almost entirely to the application of nonselective fungicides soon after harvest to inactivate wound pathogens. The benzimidazoles demonstrated the potential of high-potency selective fungicides that could penetrate into fruit tissues to prevent the development of quiescent infections and other subsurface inocula without injuring host cells or interfering with the induction of defense mechanisms. The benzimidazoles and other highly active fungicides (e.g., dicloran, imazalil, prochloraz, etaconazole, and guazatine) could enter injured tissue and, in some cases, penetrate through the intact cuticle, protecting the fruit against subsequent infection, suppressing the expansion of visible lesions, and inhibiting fungus sporulation and disease spread. Some of these compounds are remarkably effective in controlling crown rots and stem-end rots, apparently because they can penetrate into the stem tissues or are drawn into the vascular elements of the cut stem. The development of systemic fungicides during

this period was fortunate since these treatments supported major changes in the packaging, postharvest handling, and marketing of citrus fruits, bananas, and to a lesser extend other tropical fruits.

Benzimidazole Fungicides

The successful application of the benzimidazole fungicides for the control of several major postharvest diseases has magnified the importance of pathogens insensitive to this class of fungicides: *Alternaria, Stemphylium, Acremonium, Geotrichum, Phytophthora*, mucors, and benzimidazole-resistant variants of *Penicillium, Botrytis, Fusarium,* and *Diplodia* that were selected by the intensive use of these fungicides. In fact, some investigators have reported that postharvest treatment with benzimidazole fungicides may increase the severity of diseases caused by benzimidazole-insensitive pathogens (Slabaugh and Grove, 1982; Sugar and Powers, 1986).

Ergosterol Inhibitor

Since the mid 1970s, several compounds that inhibit ergosterol biosynthesis ("EBIs") in sensitive fungi have been investigated for many of the same postharvest applications as the benzimidazoles--i.e., for eradication of quiescent infections and for disease suppression. Imazalil, prochloraz, and etaconazole/propiconazole have provided good control of pathogens that are not inhibited by the benzimidazole fungicides. Imazalil is applied commercially to citrus fruit and bananas after harvest to control pathogen species and variants that are resistant to the benzimidazoles. The EBI fungicides vary in antifungal spectrum, and certain members of the group exhibit surprising activities: Prochloraz and imazalil have strong activity against *Alternaria*; etaconazole/propiconazole show unusual activity against *Geotrichum*. In addition to the EBIs, other fungicides have unique activity against certain postharvest pathogens difficult to control. Iprodione has controlled Alternaria rot in situations where strong systemic action is not essential. This fungicide also controls postharvest diseases caused by *Penicillium, Botrytis, Sclerotinia,* and *Monilinia*. Guazatine is uniquely effective against *Geotrichum* sour rot as well as benzimidazole-resistant *Penicillium* spp., and this fungicide is being developed commercially as a postharvest treatment for citrus fruits (Brown, 1988). Finally, metalaxyl and fosetyl-AL have been successful in eradicating *Phytophthora* (pre-harvest infections) on citrus fruits (Gaulliard, J. M. and Pelossier, 1983; Cohen, 1981).

While virtually all of the important diseases have been controlled under simulated-commercial conditions, the effective chemicals and techniques may not be permitted in all countries, and may not be available, affordable, or practical under all circumstances. Nonetheless, research over the past two decades has revealed the potential of postharvest chemical treatments to control, and in many cases, eradicate, incipient infections of diverse pathogens on harvested fruit crops.

Antimicrobial Chemicals

Antimicrobial chemicals seem destined to play a major role in postharvest disease control for the foreseeable future. They are effective, reliable, convenient to apply and cost effect. Furthermore, residues on treated produce can be accurately measured and the safety of the treatment can be assessed by

accepted toxicological criteria. Despite their fundamental role in postharvest disease control, chemical treatments are confronted by several problems that threaten their future potential.

Hazard From Synthetic Pesticides

The fear of cancer resulting from foreign substances (synthetic chemicals) in foods has strongly influenced the public to demand regulatory action affecting pesticides that have been determined to cause cancer in experimental animals (National Research Council, 1987). The superficial logic of this issue notwithstanding, there is a sizeable body of evidence indicating that possible hazards from synthetic pesticide residues are very small compared to those from naturally-occurring carcinogens in foods (Ames, 1989). Over 50% of all chemicals that have been tested in rats and mice have been found to be carcinogens at the highest dosages administered. Of 392 chemicals tested, 58% of the synthetic chemicals and 45% of the natural chemicals were carcinogenic in at least one animal species. Given the tiny residues of synthetic pesticides in foods, the natural carcinogens in fresh fruit and vegetables would clearly seem to pose a greater health hazard than the synthetic pesticides. These data certainly do not support the supposition that "natural" foods are intrinsically more healthful than those that have been treated with pesticides. Nonetheless, the carcinogen "label" (Natural Research Council, 1987) resulted in the loss of benomyl as a postharvest fungicide. In 1989, the DuPont Co. decided not to further support any of the U.S. postharvest registrations for benomyl, despite the unique importance of this fungicide for control of stem-end rots and other diseases, especially on Florida citrus fruits (Brown et al. 1988).

Pesticidal Regulation

The registration of postharvest fungicides is a problem which affects all pesticides that are used in small amounts and, therefore, are only marginally profitable to their manufacturers. Additional data related to toxicology, metabolism or environmental fate which may be required for reregistration of a pesticide cannot be economically justified by anticipated future sales of these minor-use pesticides. The postharvest fungicides, sec-butylamine and biphenyl, are no longer registered in the U.S. as a consequence of this problem.

Fungicide Resistant

Loss in effectiveness of several postharvest fungicides has been attributed to the selection and proliferation of fungicide-resistant biotypes of the pathogen in the population (National Research Council, 1986). The intensive and continuous use of biphenyl, SOPP, thiabendazole, benomyl, sec-butylamine, and imazalil to prevent citrus fruit decay after harvest has resulted in the build-up of resistance to these fungicides in *Penicillium digitatum* and *P. italicum*. Serious losses of the crop have been encountered in all citrus producing areas of the world (Eckert, 1990). Multiple resistance to fungicides has also developed in *Penicillium expansum* on apples (Rosenberger and Meyer, 1981). In several tropical fruit crops, the hazard of pathogen resistance is clear since one fungicide, or related compounds, are applied to the fruit in the plantation and, several weeks or months

later, to the harvested fruit in the packinghouse. The benzimidazole fungicides, benomyl, carbendazim, and thiophanate-methyl have been widely used in banana plantations to control Sigatoka disease, a foliar pathogen. Since the pathogens responsible for postharvest decay of bananas are ubiquitous colonizers of plant debris in the plantation, it is not surprising that the fungicide sprays aimed at Sigatoka disease also select benzimidazole-resistant strains of post-harvest pathogens. Slabaugh and Grove (1982) observed a dramatic decline in crown rot control on bananas harvested from Central American plantations which had been intensively sprayed with benomyl for Sigatoka control. The pathogens responsible for postharvest disease were not well controlled by thiabendazole, another benzimidazole fungicide, applied to banana hands in the packinghouse to control crown rot during shipment. The immediate solution to this problem is to change the postharvest treatment to an unrelated fungicide, e.g., imazalil, which is highly active against benzimidazole-resistant banana pathogens. Recently, however, several fungicides that are cross-resistant to imazalil are being extensively applied in the plantation to control Sigatoka disease, leading to concern about the long-term effectiveness of registered postharvest fungicides on bananas.

Spalding (1982) isolated benzimidazole-resistant strains of *Colletotrichum gloeosporioides, Diplodia natalensis,* and *Phomopsis citri* from decaying mangos in Florida packinghouses. Presumably, the fungicide-resistant strains were selected by the intensive use of benomyl to control anthracnose on the fruit in the plantation. Postharvest treatments of imazalil and etaconazole gave good control of anthracnose and stem-end rots during storage and ripening the fruit decay, but benomyl, thiabendazole and thiophanate-methyl were ineffective, presumably because benzimidazole-resistant strains of the pathogen were present on the fruit at the time of harvest.

Alternatives to Chemical Fungicides

The best strategies for postharvest disease control combine treatments that reduce disease pressure and suppress the emergence of fungicide-resistant pathogens. The most important of these are: 1) Inoculum reduction through sanitation or exclusion (Bancroft et al., 1984). 2) Careful harvesting and handling to minimize the number of susceptible wounds. 3) Curing of tuber crops and fruits to heal wounds when appropriate (Brown, 1989; Baudoin and Eckert, 1985; Ben-Yehoshua et al., 1988). 4) Storage in a environment optimum for maintaining host resistance and suppressing pathogen development (Fitzell and Muirhead, 1983; Sommer, 1985). 5) Nonselective fungicide treatments such as sodium carbonate, sodium bicarbonate, active chlorine, and sorbic acid, heated to 45°-50°C when appropriate. Brushing oranges in soap reduced anthracnose, apparently by reducing the number of appressoria on the fruit surface (Smoot et al., 1971). 6) Individual wraps and container-dividers to isolate diseased fruit. Polyethylene wraps have improved curing and prevented disease spread (Ben-Yehoshua, 1985; Miller and Risse, 1988).

Biological Control

A considerable effort has been made over the past decade to determine the potential of biological control as an alternate to chemical treatments for control of postharvest diseases (Wilson and Pusey, 1985; Wilson and Wisniewski, 1989). Only a brief comparison of the intrinsic value of chemical treatments and biological control agents in relation to postharvest disease control is in order here. The details of the biological agents and their effi ciency will be dealt with in other chapter of this volume.

Although some successes have been reported in the biological control of postharvest diseases initiated by field infection (i.e., strawberries and grapes, Tronsmo and Dennis, 1977; Janisiewicz, 1988), most of the research on biological control of postharvest diseases has focused on the control of wound pathogens on stone fruits, pome fruits, and citrus fruits (Wilson and Wisniewski, 1989). The concept of colonizing wounds with an antagonistic bacterium is clear in the early report of Gutter and Littauer (1953), but little interest developed in this area until 1980 when the problems of chemical fungicides became apparent. Earliest efforts to control postharvest diseases involved the use of *B. subtilis* (Pusey and Wilson, 1984; Singh and Deverall, 1983, Utkhede and Sholberg, 1986), *Trichoderma* and *Rhodotorula* (DeMatos, 1983) and *Pseudomonas cepecia* (Janisiewicz and Roitman, 1988). All of these microorganisms colonize wound sites and elaborate fungitoxic substances which prevent development of pathogens such as *Monilinia, Alternaria, Penicillium digitatum, Penicillium italicum, Geotrichum* and *Penicillium expansum*. Pusey (1989) comprehensively reviewed research and development work on *Bacillus subtilis* which he characterizes as a "biological fungicide", recognizing that the effectiveness of this bacterium is due to a family of antifungal polypeptides (*Iturins*) that it releases into fruit wounds (Gueldner et al., 1988).

Antibiosis

Biological control agents that owe their effectiveness to the elaboration of fungicidal substances in wound sites are subject to exactly the same problems as synthetic chemical fungicides, and possibly a few more that are unique to living microorganisms. Firstly, U.S. regulatory agencies should require detailed information on the toxicology and metabolism of the antifungal antibiotics (*Iturins*) produced by *B. subtilis*, pyrollnitrins produced by *Pseudomonas cepacia* (Janisiewicz and Roitman, 1988; Wilson and Chalutz, 1989) and trichothecenes produced by *Myrothecium roridium* (Appel, 1989; Gees and Coffey, 1989). Published properties of these compounds (Merck, 1989) should give rise to concern for their use on foods, even if the biocontrol formulations contained only washed microbial cells which generate the antibiotics only in colonized wounds (Smilanick et al., 1991). The application of living cells of *P. cepacia* and related pseudomonads to fresh fruits and vegetables would require a detailed evaluation of their pathogenicity to immunosuppressed humans. Finally, biocontrol agents that generate antifungal antibiotics should select resistant biotypes of the plant pathogen in the population which could reduce the effective ness of the biocontrol agent against postharvest disease. Janisiewicz (1988) has discussed the analagous problem with *Agrobacterium radiobacter*.

Competition

Recent investigations on biological control of postharvest diseases have emphasized the use of microorganisms that are not antagonistic to the pathogen *in vitro*, but which appear to control disease by heavily colonizing the wound and competing with the pathogen for nutrients and space. Some evidence indicates that these microorganisms may elicit defense mechanisms of the host as well. The yeasts, *Aureobasidium* and *Debaryomycetes* (Pichia) have been investigated in this connection (Droby et al., 1989; Wilson and Chalutz, 1989; Stretch, 1989). The application of *Debaryomycetes* and related yeasts is encouraged by the fact that these microorganisms are frequently associated with foods and might be more acceptable to consumers than exotic bacteria that could contaminate treated produce with xenobiotics. In a recent study, Chalutz and Wilson (1990) found that treatment of grapefruit with *Debaryomycetes* provided only limited eradicative action against *Penicillium digitatum*, probably reflecting the time lag required for *Debaryomycetes* to become effective (i.e., colonize the wound). *Debaryomycetes* provided effective disease control if applied simultaneous with the plant pathogen; however, the treatment failed when the yeast was applied seven hours after inoculation of the fruit with *P. digitatum* spores. In comparison, effective chemical fungicide treatments will eradicate wound pathogens 30 hours after inoculation. Smilanick et al., 1991, reported that *P. cepacia* and *P. corrugata* treatments controlled *Monilinia fructicola* 12 hours after inoculation, but the culture filtrate showed little antifungal activity. The effectiveness of *P. corrugata* was similar to that of two systemic fungicides tested, but the *Pseudomonads* could not control quiescent (late season) infec tions of *M. fructicola*.

Wound Site

Although many questions remain to be answered concerning the usefulness of biological control against postharvest diseases, the picture is emerging that these agents will be most useful against wound pathogens, since these sites are most readily colonized by selected bacterial and fungal antagonists. Since these biological agents must increase significantly in cell mass before they inhibit the pathogen, it is unlikely that washed cell formulations can be depended upon for eradication of established wound infections or quiescent infections on peaches, tropical fruits or citrus. Several investigators have suggested the formulation of a biocontrol agent with a chemical fungicide in order to broaden the spectrum of activity (*B. subtilis* + dicloran; Pusey, 1989; Pusey et al., 1988). Chalutz and Wilson (1990) noted that *Debaryomycetes* was more resistant to thiabendazole and imazalil than *P. digitatum* suggesting that a chemical fungicide might be added to the biocontrol formulation to increase its eradicative action and to reduce the selection pressure for the emergence of pathogens biotypes resistant to either the biocontrol agent or the chemical fungicide.

Acknowledgment

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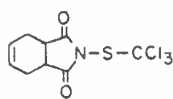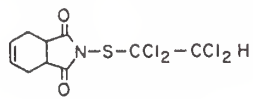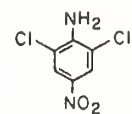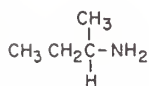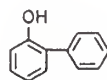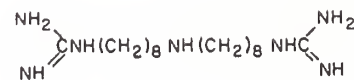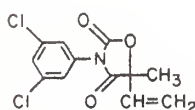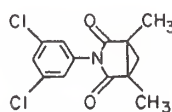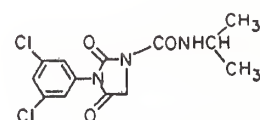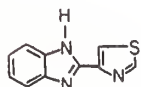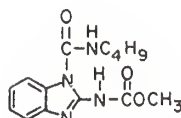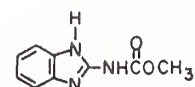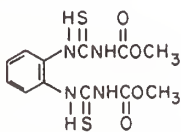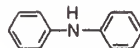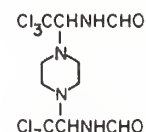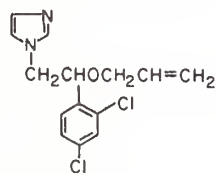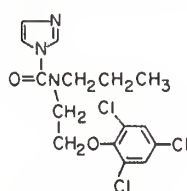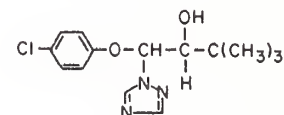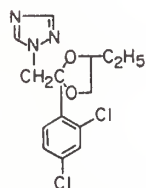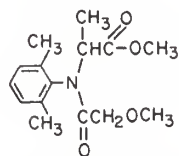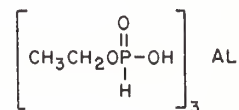
Figure 1. Postharvest fungicides.

Our Workshop as a Bridge Between Research and Commercialization

Edo Chalutz[1]

Abstract

In my short talk today, I wish to briefly highlight the research we have been doing on biological control of postharvest diseases, while touching on the main points of our current thinking on how biocontrol agents would best be applied. The title of my talk is intended to relate the main lines of our research and thinking to the program of the workshop. The title also reflects our belief that time is ripe for discussions to take place between researchers and industry representatives on the large-scale testing and application of laboratory findings on biological control of postharvest diseases. Such findings are now being obtained by a very rapidly growing number of scientists. This is because of:

-- the increased environmental concerns regarding the use of chemical fungicides, particularly on food products.

-- withdrawal from the market of important fungicides used to control postharvest diseases and

-- the development of resistance against important fungicides.

Advantages of Postharvest Biocontrol

The potential advantage that postharvest application of biocontrol agents might have (over field or soil applications) also suggest that alternatives to chemical fungicides are not only needed, but that they should now be evaluated (both biologically and economically) under commercial conditions. We hope that participants of the workshop will express their findings and thoughts on this subject, so that by the end of the meeting, the potential applicability of this new technology could be better evaluated.

As researchers, we have many options to proceed with our work. This meeting should also help direct the research, or part of it, toward the applicability of biocontrol agents. With the help of BARD, the United States-Israel Binational Agricultural Research and Development Fund, and some support by the industry, we can now conduct these discussions.

As some of you know, BARD has also supported our research on biological control of postharvest diseases. This collaborative research (USDA - NS - ARO), which has recently been expanded to also include large-scale operations, with the involvement of a private firms, has been conducted by several scientists, both in the U.S. and ISRAEL. Most of them are here today, and will present our findings during the meeting.

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[1]ARO, The Volcani Center, Bet Dagan, Israel

Naturally Occurring Microflora	Our approach in the initial phase of the work has been to examine the naturally-occurring microflora on the surfaces of fruits, in order to isolate antagonists, preferably non-antibiotic-producing ones. By washing the surfaces of citrus fruits (picked after a long dry period) and culturing the microorganisms in the washings, a dense population - consisting mostly of bacteria and yeasts - developed on the plates. However, only after dilution of these washings, did also filamentous fungi, of the type that cause postharvest diseases, develop on the plates. Thus, if the findings on the plates reflect the natural situation on the surface of the fruit, then it could be assumed that some of the bacteria and yeast, naturally present on the fruit surface, inhibit the growth of other microorganisms, including plant pathogenic filamentous fungi.

If these microorganisms indeed protect the fruit from infection, then, removing them, should increase incidence of diseases. We tried to test this assumption: in controlled experiments, gentle washing and drying of grapefruit prior to placing the fruit in prolonged cold storage, increased the incidence of natural infection. Grapefruit prolonged storage is shown in Figure 2. This is really a very well known phenomenon.

Upon individual culturing of many of the microorganisms isolated from fruit surfaces and then, reapplying them to surface wounds of citrus (or apple) fruits, we were able to screen for and isolate several bacteria and yeasts that protected the wounds.

This bioassay for isolating and testing efficacy of microbial antagonists was relatively easy to use and provided clear data.

Bioassay for Antagonists

Bioassay is shown in Figure 3. One of these antagonists, the yeast US-7, which was isolated from the surface of lemon fruits, turned out to be one of the most effective isolates evaluated. It was initially identified as *Debaryomyces hansenii,* and recently reidentified as *Pichia guilliermondii.* US-7 on lemon fruit is shown on Figure 4.

In the next session of our workshop, we will hear of the experience of several workers in isolating and testing several biocontrol agents (including this yeast) effective against a number of fruit, vegetable, and flower diseases.

Mechanism of Action

How does the yeast US-7, or other antagonists, inhibit disease? One, most-obvious way would be by the production of antibiotics by the antagonist.

The US-7 yeast antagonist did not seem to exert its effect through the production of antibiotics: It did not inhibit the growth in culture of any of the postharvest pathogens (as did the bacterium antagonist *Bacillus subtilis*), nor did its culture filtrate exhibit any activity in the bioassay.

The following is shown in Figure 5.I. - US-7 in culture - lack of inhibition, and in II, *B. subtilis* inhibition.

Other possible modes of action are:

Competition for nutrients.
Induction of host resistance mechanisms.
Direct interaction between the antagonist and the pathogen.
Other mechanism?

We will consider these different modes of action in our morning session tomorrow, along with ways to enhance efficacy of biocontrol agents.

But even when a highly effective antagonist is found, will it exhibit high activity in large-scale and commercial tests? We have tried to answer this question by conducting larger scale experiments and examining such factors as:

> survival of the antagonist on the fruit during prolonged cold storage.

> its compatibility with waxes, fungicides, and other factors likely to be encountered under commercial conditions.

Resistance to Fungicides
The US-7 antagonist actually performed better at low storage temperatures than it did at room temperature, and when it was applied to citrus fruit prior to waxing then when applied to unwaxed fruit. But, perhaps the most interesting observation was its relatively high resistance to fungicides commonly used to control postharvest diseases (compared with the fungal pathogens).

US-7 resistance to TBZ and imazalil is shown in Figure 6. This enabled us to develop and test an integrated control approach in which we combined the biocontrol procedure with very low concentrations of the fungicide.

Larger-scale Tests
US-7, wax, and TBZ on grapefruit in storage is shown in Figure 7. After obtaining encouraging results in these tests (which consisted of several cartons per treatment of non-inoculated, injured grapefruit), the larger-scale tests were initiated.

Tomorrow afternoon, the large-scale production and application of biological control agents will be discussed, along with questions concerning their registration and patenting.

We think that the US-7 yeast antagonist has several important features that make it particularly suitable as a biocontrol agent. These are summarized in Figure 8.

In our last session tomorrow night, we will consider other non-fungicidal methods for the control of postharvest diseases of fruits and vegetables. The innovative approaches we will hear about, could perhaps be applied together with biological control procedures, thus offering applicable alternatives to chemical fungicides in the not-too-distant future.

II. PRESENT STATUS OF THE BIOLOGICAL CONTROL OF POSTHARVEST DISEASES OF FRUITS AND VEGETABLES.

Recent research indicates that biological control in the postharvest environment may have a greater chance for success than biological control in the field.

Characterization of Postharvest Biological Control of Deciduous Fruit Diseases by *Cryptococcus* spp.

R. G. Roberts[1]

Abstract

Recent postharvest pathology research at the USDA Tree Fruit Research Laboratory in Wenatchee, WA, has established the suitability of using certain naturally-occurring epiphytic yeasts in the genus *Cryptococcus* for biocontrol of postharvest disease. *Cryptococcus laurentii, C. flavus*, and *C. albidus* var *aerius,* have been isolated from the surfaces of apple and pear fruit and leaves which are effective in preventing or reducing postharvest disease development in apples, pears, and cherries. These yeasts are able to rapidly colonize, prosper, and survive for long periods in wounds on apple and pear fruit under both cold and warm temperatures (0-20°C) and in regular (ambient) or controlled atmospheres (1.5% CO_2, 2.0% CO_2). Efficacy of these yeasts for biocontrol is dependent upon yeast concentration and storage temperature, but is not affected by fruit tissue calcium levels. In filter paper disk assays on agar media, growth of these yeasts was not inhibited by benomyl, DPA, SOPP, Mertect 340-F, or Rovral, but was slightly inhibited by 500 ppm Captan.

Introduction

Resistance to Benomyl

The increased interest and research effort in the biological control of plant disease seen during recent years has been a response to both biological and regulatory events. On the East coast of the US, the use of benomyl as a postharvest fungicide was compromised by the widespread development of resistance (Rosenberger and Meyer, 1979), probably exacerbated by preharvest use. While benomyl resistance was also reported on the West coast (Spotts and Cervantes, 1986), benomyl remained an effective postharvest tool in Washington state until the postharvest label was pulled in 1989 because its use was reserved for postharvest applications. While other benzimidazoles are still available for postharvest use, they do not overcome the problem of resistance, and tend to be less effective than was benomyl. With the Alar controversy of 1988-89 and the more general discussion of pesticide residues on food products that ensured, fresh impetus was given to the search for alternative means of crop protection.

Development of alternative postharvest disease control systems that employ microbials is dependent not only upon finding antagonists that are effective, but also ones that have ecological, biochemical, and physiological traits that make them suitable for use within the constraints imposed by postharvest environments. The ability of a microbial pesticide to colonize and persist on and in fruit at effective levels, to be compatible with other postharvest practices, processes and

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chemicals, and to be effective under cold and modified atmosphere conditions are among the constraints that will determine the suitability of a given microbial for commercial use. Recent postharvest biocontrol studies using yeasts in the genus *Cryptococcus* indicate that this group of epiphytes are well suited for use in the postharvest environments encountered in the fruit and vegetable industries.

Isolation from Plant Surfaces

Members of the genus *Cryptococcus* are easily isolated from the surfaces of leaves, stems, fruit, and buds of apple, pear and other economically important crop plants (Andrews and Kennerly, 1980; Clark, Wallace, and David 1954; Fokkema, den Houter, Kosterman, and Nelis, 1979; Fokkema, van de Laar, Nelis-Blomberg, and Schippers, 1975; Roberts, 1990; and Williamson and Fokkema, 1985). Our own experience has been that fruit orchards in which insect control has been marginal, especially of white files (apples) and pear psylla, have been rich sources of epiphytic yeasts effective in postharvest biocontrol, both in quantity and in diversity. Such trees have been the source of the most effective biocontrol yeasts encountered in recent studies in Washington, including *C. flavus* (Saito) Phaff et Fell, *C. laurentii* (Kufferath) Skinner, and *C. albidus* (Saito) Skinner var *aerius*. The honey dew from these insects coats the leaves and fruits and being rich in sugars, are presumed responsible for the often high populations of yeasts found on these leaves and fruits. In addition to the "white" yeasts, which include *Cryptococcus* spp., "pink" yeasts have also been isolated from these trees which have demonstrated efficacy against postharvest diseases of tree fruits. Our knowledge of the diversity and population structure of epiphytic yeasts on these plants with possible value as postharvest biocontrol agents is limited, and deserves additional study.

Identification

As with other yeasts, *Cryptococcus* strains epiphytic on fruit trees are classified according to their morphology, mode of reproduction, and physiological tests (Rodriguez de Miranda, L. Genus 5). Cell morphology is variable, but all strains reproduce by multilateral budding, and ascospores, ballistospores, chlamydospores and teliospores are not produced. *Cryptococcus* strains can utilize inositol as the sole source of carbon, and do not ferment any sugar. Agar cultures tend to be slimy in appearance and whitish to cream-colored, especially when young, and are thus among those yeasts referred to a "white yeast." Pigment production can occur in older cultures, but is always pale in color. Physiological characteristics and patterns of carbohydrate utilization for selected biocontrol strains of *Cryptococcus* are given in Tables 1 and 3. Morphological characters of these strains are given in Table 3. *Cryptococcus laurentii* differs from *C. flavus* primarily in its ability to synthesize starch and in the morphology of its vegetative cells. Patterns of carbohydrate assimilation, nitrate assimilation, and lack of growth at 37°C are otherwise similar. *Cryptococcus albidus* var. *aerius* differs from both *C. laurentii* and *C. flavus* by its lack of acid production on glucose, positive nitrate assimilation, and lack of erythritol assimilation. All strains shown here can utilize a wide range of carbohydrates as their sole carbon source, which presumably enhances their epiphytic fitness and may account for their relatively high frequency of isolation from sugar-rich sources.

Colonization and Persistence

Wound competence under different environmental conditions may be an important character for evaluation of microbial agents with commercial potential. Rapid colonization of apple fruit wounds by *C. laurentii* at temperatures ranging from 5-20°C has been previously reported (Roberts), and additional studies (unpublished data) have shown wound colonization to be significant even at 0°C. Recent yeast population dynamics studies with Golden Delicious apple fruit and d'Anjou pear fruit (Shefelbine) have shown that both *C. laurentii* and *C. flavus* rapidly colonize wounds in apple and pear fruit under cold storage conditions (1-2°C) and under both ambient and controlled atmospheric conditions (1.5% CO_2, 2.0% CO_2). The shape of the growth curves in RA and CA are very similar, and establish that these yeasts are able to colonize, proper, and survive in fruit wounds under conditions present during long term storage of deciduous tree fruits. Yeasts in this group are therefore both psychrophilic and microaerophilic. Both of these traits are very desirable for microbials that are to be used on agricultural commodities that are to be stored under cold temperatures and modified atmospheres.

Effect of fruit maturity and storage temperature on biocontrol efficacy.

A direct relationship between storage temperature and biocontrol efficacy has been established in studies using *C. laurentii* for control of gray mold in Golden Delicious apple fruit (Roberts, 1990; Roberts and Raese, 1990) and with *C. laurentii*, *C. flavus*, and *C. albidus* var. *aerius* for control of Mucor rot of d'Anjou pear fruit (Roberts, 1990). With gray mold of apple, disease incidence decreased significantly as incubation temperature decreased when wounds were protected by preinoculation treatment with varying concentrations of C. laurentii. With Mucor rot of pear, a similar relationship between decay incidence and storage temperature has been observed in wounds treated with *C. laurentii*, *C. flavus*, and *C. albidus* var. *aerius*.

Fruit maturity has a pronounced effect on biocontrol efficacy with both apple and pears and with all *Cryptococcus* strains tested. In studies using *C. laurentii* and ripe and green Golden Delicious apple fruit, treatment of ripe fruit with strain 87-108 of *C. laurentii* before inoculation with *B. cinerea* gave little or no control of gray mold upon subsequent incubation at 20°C. Similar treatment of green fruit, however, gave excellent levels of biocontrol similar to those observed in numerous other studies. The ripe and green apple fruit were characterized by differing average YID values (79.2 vs. 72.8), pressures (9.8 lbs. vs. 15.1 lbs.) pH (3.65 vs. 3.39), and soluble solids (10.2% vs. 11.0%), respectively. Increased biocontrol efficacy against Mucor rot was also observed when unripe d'Anjou pears were treated with a variety of *Cryptococcus* strains and then inoculated with Mucor spores compared to similar treatment of ripe pears (Roberts, 1990).

From these studies it can be inferred that there are certain conditions of use for these yeasts which will optimize their effectiveness. Generally speaking, these conditions are

entirely consistent with what are currently accepted as good postharvest handling and production practices (Willett, et al., 1989). For maximum benefit from biocontrol strains of *Cryptococcus,* fruit should be harvested at optimal maturity levels. Production practices that enhance inherent resistance to postharvest disease such as cover sprays of calcium chloride, while not directly affecting the efficacy of *Cryptococcus,* can increase resistance to some postharvest diseases of apples (Raese, et al., 1989; Roberts and Raese, 1990). *Cryptococcus* suspensions should be applied to fruit as soon after harvest as possible and then cooled as rapidly as possible to allow *Cryptococcus* populations to become established while slowing down pathogen development. The most effective formulations and delivery systems for these yeasts have yet to be developed, but the environmental conditions to optimize biocontrol with *Cryptococcus* spp. are clear.

Sensitivity to Agrichemicals

Compatibility of biocontrol agents with the postharvest environment, including agrichemicals with which fruit comes in contact, is an important aspect of commercial suitability of any postharvest biocontrol agent. To determine the sensitivity of *Cryptococcus* strains to the postharvest chemicals currently in or proposed for use in commercial packinghouse operations, 500 mg/L suspensions of Benlate, Mertect 340-F, Captan, sodium orthophenylphenate (SOPP), Rovral, and a 2,000 mg/L suspension of DPA were prepared, then spotted onto filter paper disks on agar plates which had been seeded with suspensions of different *Cryptococcus* strains. The widths of inhibition zones after three days growth at 25°C were measured in millimeters from the edge of the filter paper to the edge of the yeast lawn, and are presented in Table 4. Of the materials tested, only Captan was slightly inhibitory to *Cryptococcus* strains, with no zones of inhibition present with any of the other chemicals. This implies that the use of *Cryptococcus* will be compatible with other materials currently being used, and suggests the use of *Cryptococcus* in combination with reduced levels of one or more of these fungicides to control decay. Such an approach could potentially reduce fungicide residues present on fruit, and might prevent or delay the onset of fungicide resistance.

Conclusions

Cryptococcus laurentii, C. flavus, and *C. albidus* var *aerius* have been shown to give effective levels of biological control of several postharvest diseases of apple and pear fruit, including gray mold, blue mold, and Mucor rot. These psychrophilic, microaerophilic, medically-unimportant yeasts rapidly colonize and survive in wounds in apple and pear fruit under a variety of temperature and atmospheric conditions similar to those found in commercial fruit storages in the US, but are most effective when applied to freshly harvested fruit stored at cold temperatures. All strains tested are able to utilize a wide range of carbohydrates, which probably contributes to their demonstrated epiphytic fitness and wound competence. Although slightly inhibited by Captan, all strains are able to grow in the presence of other commonly used agrichemicals, which suggests they might be used in

conjunction with reduced rates of fungicides to decrease
residue levels and prevent or delay development of resistance.
The sum of their physiological and ecological characteristics,
as well as their demonstrated efficacy against postharvest
fruit diseases, characterize this group of epiphytic yeasts as
being very suitable for use in the modern postharvest
environment.

Acknowledgments

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TABLE 1. Physiological characters of *Cryptococcus albidus* var *aerius* (**C.a.**), *C. laurentii* (**C.l.**), and *C. flavus* (**C.f.**) used in part for species identification. (+) = growth or production, (-) = no growth or production, (vw) = very weak growth or production.

ISOLATE NUMBER	GROWTH ON INOSITOL	GLUCOSE ACID	GLUCOSE GAS	NITRATE ASSIMILATION	GROWTH AT 37 C	STARCH PRODUCTION
C.a.89-212	+	-	-	+	-	-
C.l.89-129	+	+	-	-	-	+
C.l.89-138	+	+	-	-	-	+
C.f.89-154	+	+	-	-	vw	-
C.f.89-156	+	+	-	-	-	-
C.f.89-160	+	+	-	-	-	-
C.f.89-211	+	+	-	-	-	-

TABLE 2. Patterns of carbohydrate utilization of biocontrol strains of *Cryptococcus albidus* var *aerius* (C.a.), *C. laurentii* (C.l.), and *C. flavus* (C.f.). (+) = utilization as sole carbon source, (-) = not utilized as sole carbon source, LAC = lactose, CEL = cellobiose, RHA = rhamnose, ERY = erythritol, SUC = sucrose, MAL = maltose, MELI = melibiose, MELE = melezitose.

ISOLATE NUMBER	CARBOHYDRATE ASSIMILATION							
	LAC	CEL	RHA	ERY	SUC	MAL	MELI	MELE
C.a.89-212	+	+	+	-	+	+	+	+
C.l.89-129	+	+	+	+	+	+	+	+
C.l.89-138	+	+	+	+	+	+	+	+
C.f.89-154	+	+	+	+	+	+	+	+
C.f.89-156	+	+	+	+	+	+	+	+
C.f.89-160	+	+	+	+	+	+	+	+
C.f.89-211	+	+	+	+	+	+	+	+

TABLE 3. Lengths, widths, and length/width ratios of vegetative cells of biocontrol strains of *Cryptococcus albidus* var *aerius* (C.a.), *C. laurentii* (C.l.), and *C. flavus* (C.f.), taken after growth in 2% glucose-yeast extract-peptone broth.

STRAIN	LENGTH (μm)	WIDTH (μm)	L/W RATIO
C.l.87-108	4.2 - (5.6) - 6.6	2.7 - (3.7) - 4.6	1.2 - (1.5) - 2.1
C.l.89-129	4.2 - (5.1) - 5.9	3.2 - (3.7) - 4.2	1.2 - (1.4) - 1.5
C.f.89-154	3.8 - (5.0) - 6.0	3.5 - (4.5) - 5.6	1.0 - (1.1) - 1.4
C.f.89-156	4.3 - (5.1) - 5.8	3.9 - (4.6) - 5.4	1.0 - (1.1) - 1.3
C.f.89-160	3.7 - (5.2) - 5.8	3.6 - (4.7) - 5.7	1.0 - (1.1) - 1.3
C.f.89-211	4.2 - (5.1) - 6.1	4.0 - (4.7) - 5.9	1.0 - (1.1) - 1.3
C.a.89-212	4.1 - (5.8) - 7.0	3.6 - (4.7) - 6.4	1.0 - (1.2) - 1.6

TABLE 4. Effect of agricultural chemicals on growth of biocontrol strains of *Cryptococcus albidus* var *aerius* (C.a.), *C. laurentii* (C.l.), and *C. flavus* (C.f.). Suspensions of agrichemicals were spotted into 1 cm diameter filter paper disks on nutrient agar plates previously seeded with different strains of *Cryptococcus* spp., then incubated 3 days at 25 C. Numbers represent the radius of the inhibition zone measured from the edge of the disk to the edge of yeast lawn. All chemicals were used at 500 ppm active ingredient, except for DPA, which was used at 2,000 pm.

STRAIN	MERTECT 340-F	SOPP	DPA	ROVRAL	BENLATE	CAPTAN
C.l.87-108	0	0	0	0	0	2.0
C.l.89-129	0	0	0	0	0	2.0
C.f.89-154	0	0	0	0	0	2.0
C.f.89-156	0	0	0	0	0	1.3
C.f.89-160	0	0	0	0	0	1.3
C.f.89-211	0	0	0	0	0	3.4
C.a.89-212	0	0	0	0	0	3.3

Biological Control of Postharvest Diseases of Pome Fruits

W. Janisiewicz[1], J. Roitman[2], and N. Machoney[2]

Abstract

The justification for developing biological control of postharvest diseases has been discussed earlier on numerous occasions and has already been mentioned at this meeting. I would like to emphasize the fact that problems related to the effectiveness and registration of fungicides used in pome fruit production and storage are severe. The pome fruit industry relies heavily on pesticides and any restriction on their use can have severe economic consequences. Recently, Benlate was withdrawn from registration for postharvest use and other fungicides are being reviewed. The problems with fungicides and the progress in developing biological control of gray-mold, caused by *Botrytis cinerea,* on grapes and brown-rot, caused by *Monilinia fructicola,* on stone fruits prompted initiation of the program on biological control of postharvest diseases of pome fruits.

From the beginning, the project has focused on exploring the resident microflora of apple and pear fruit and leaves for antagonistic activity against major postharvest pathogens such as *B. cinerea* and *P. expansum.* It was surprising to find that this microflora harbors many microorganisms from various taxonomic groups (bacteria, yeast, yeast-like fungi) with antagonistic capabilities (Chalutz, et al., 1988, Janisiewicz, 1987; and Janisiewicz, 1991 (in press)). However, it quickly became apparent that only a small portion of these antagonists are potential prospects for further practical development. Since we are to limit our presentation to recent research, I will briefly outline the approach that led to the discovery of two antagonists and describe recently conducted larger scale tests and work related to improving their effectiveness.

Basic approach

The main objective of the program has been to develop biological control of *P. expansum* and *B. cinerea* on apple and pear. These fungi were targeted first because they are major postharvest pathogens of apple and pear, and they infect primarily through wounds where biocontrol has the best chance of success. Development of iatrogenic diseases on various fruits suggested that natural antagonists might occur among saprophytic microorganisms of fruit and might significantly affect disease development. A critical factor in developing biocontrol has been in the selection of fruit and leaves of apple and pear as the isolation site for microorganisms. This has proven to be an effective approach which resulted in the isolation of many antagonists from pome fruits (Janisiewicz, 1985, 1987, and Roberts, 1990) and other fruits (Chalutz, 1988,). The isolated microorganisms have been subjected to primary screening for antagonistic activity

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against *P. expansum* and *B. cinerea* on fruit and *in vitro* tests. After secondary screening where the minimum effective concentrations were determined (Janisiewicz, 1987), the antagonists were tested for compatibility with postharvest treatments, additives, and storage conditions (Janisiewciz, 1987). For the most promising antagonists, population dynamics at the wound site were determined under various storage conditions (Janisiewicz, 1991 (in press)). Larger scale tests are being conducted under semi-commercial and commercial conditions on various fruit cultivars. At this point we also initiated work to develop antagonist formulations. A pilot test using commercial conditions has been planned.

The mechanisms of biocontrol have been studied for both antibiotic-producing and non-producing antagonists. Metabolites of an antagonist, *Pseudomonas cepacia*, were isolated and analyzed for antagonistic activity against *B. cinerea*, *P. expansum*, and *Mucor piriformis* (Janisiewicz and Roitman, 1987, 1988; Roitman, et al., 1991 (in press)). The effective compound was purified and identified as pyrrolnitrin. When applied to fruit, it gives total protection against blue-mold, gray-mold and mucor rot of pome fruits, and various diseases of other fruits (Janisiewicz et al., 1991 (in press); 1989, Takeda et al., 1989). The next step involved enhancing antagonist effectiveness through manipulation of its biocontrol mechanism. In the case of *P. cepacia*, it was stimulation of pyrrolnitrin production.

Larger Scale Test After laboratory experimentation and prior to conducting an expensive pilot test, it is important to conduct larger scale tests where fruit are treated with antagonists and stored to simulate, as closly as possible, commercial application. Recently, a large scale test using the antagonist *Pseudomonas syringae* to control *B. cinerea* and *P. expansum* on various pear cultivars was conducted. The effectiveness of the biocontrol agent was compared on Bartlett, Red Bartlett, Bosc, and Anjou cultivars. Fruit were wounded with a sharp instrument resulting in the removal of a 3^3 mm block of tissue, or with a 6 penny common nail pertruding about 4 mm from a wooden block. The wounded fruit were placed in a basket and dipped in a tank containing 15 L of a conidia suspension (1×10^4 conidia/ml) of the pathogen conidia mixed with various concentrations of the antagonist. The treated fruit were placed on fruit trays in one bushel fruit boxes with polyethylene liners and stored at 18°C for 7 days or at 1°C for 30 days. The antagonist gave excellent control of *B. cinerea* on Anjou, Bartlett, and Red Bartlett on both types of wounds at 18°C. Control was less effective on Bosc pears (Fig. 1). Similar control was achieved at 1°C; however, in the case of Anjou, Bartlett and Red Bartlett, more decay developed from nail wounds (Fig. 2). Control of *P. expansum* was excellent on fruit with cut wounds but weaker on fruit wounded with the nail. In most cases more decay developed on Bosc pears than on the other three cultivars, and on fruit with nail wounds than on fruit with sharp cut wounds. Bosc has been shown to be more susceptible than other pear cultivars to side rot. Bosc might also be more susceptible

to other pathogens which would explain the reduced effectiveness of the biocontrol agent on this cultivar. In laboratory tests, Bosc fruit were completely protected against *B. cinerea* and *P. expansum* when the concentration of the antagonist was increased. Whether reduced effectiveness of biocontrol on Bosc pears was due to higher susceptibility of this cultivar or lower effectiveness of the antagonist is unknown. However, results from population study of the antagonist at the wound site showed similar increases of the antagonist population on all cultivars during the experiment. This suggests that higher susceptibility of the fruit is the more likely possibility. Since Bosc pears are harvested at different times from other pears, it might be practical to develop an antagonist specific for this cultivar.

Enhancing Biocontrol

In studying mechanisms of biocontrol and improving the effectiveness of antagonists, efforts were focused on the antagonist *P. cepacia*. Pyrrolnitrin, a powerful antifungal compound was isolated from the bacterium and culture medium. This compound inhibits growth and germination of many pathogens of pome, stone, and citrus fruits. Although the mechanism of biocontrol of *P. cepacia* has not been fully explained, pyrrolnitrin probably plays a major role in the antagonism. Therefore, to improve antagonist effectiveness, it would be advantageous to increase the production of pyrrolnitrin. Experiments were conducted to determine production of pyrrolnitrin under various growth conditions (Roitman, et al., 1991 (in press)). *Pseudomonas cepacia* was grown in minimum salts, nutrient broth, and King's B media with pH adjusted to 5.8 or 7.0 prior to inoculation with the bacterium. Production of pyrrolnitrin after 96 hr was much greater in minimum salts medium than in nutrient broth or King's B media. More pyrrolnitrin was produced at pH 5.8 than at pH 7.0 (Table 1). The pH of the salt medium changed little over time but increased greatly in the other two media during the 168 hr growth period (Fig. 3A). Production of total phenylpyrrole metabolites was also higher in the minimum salts medium (Fig. 3B). An interesting relation was observed in the composition of the phenylpyrrole metabolites in the media during 168 hr growth. Concentration of pyrrolnitrin and 2-chloropyrrolnitrin increased but other aminophenyl metabolites [TriChloroAmino (TCA) and DiChloroAmino (DCA)], after an initial increase, declined rapidly and were barely detectable after 168 hr (Fig. 4). This type of change would be expected from a precursor-product relationship. Therefore, it is proposed that DCA and TCA are the precursors of pyrrolnitrin and 2- chloropyrollnitrin, respectively.

```
TRYPTOPHAN   ->   ->   ->   MONOCHLOROAMINO
                                    l
PYRROLNITRIN      <-       DICHLOROAMINO
                                    l
2-CHLOROPYRROLNITRIN <- TRICHLOROAMINO
```

By changing growth conditions, the yield of desireable metabolites of the antagonist increased. Tests are underway to determine if the creation of favorable conditions for production of pyrrolnitrin will increase the antagonist's effectiveness. Elucidation of the synthetic pathway would be helpful in cloning genes responsible for producing pyrrolnitrin.

Concluding remarks

To make significant progress in biocontrol of postharvest diseases of fruits, research must be conducted on various aspects of biocontrol simultaneously. Research should not be limited to a single biocontrol agent, especially when control of many diseases on various fruits is considered. The possibility of using antagonist mixtures for control of multiple diseases was demonstrated earlier. This might be a good approach for solving the problems related to antagonist survival and effectiveness on fruit at various stages of maturity and storage conditions.

It is encouraging to see increased work on biological control of postharvest diseases of fruits. Currently biocontrol research of postharvest diseases of pome fruits is conducted in six countries and at several laboratories in the United States. This creates a need for increased communication and the development of standard research methods for comparing results from various laboratories. Many scientists working in this field, in this country and abroad are associated in the Postharvest Biocontrol of Fruit and Vegetables subgroup of the NE-87 project on Control of Postharvest Decay of Fruit and Vegetables. This group attempts to develop standards for the type of wounds, antagonist and pathogen inoculum concentrations, timing of inoculation, evaluation of fruit decay development, and fruit storage conditions. Standards will benefit both scientists and those in the private sector interested in commercial development of biocontrol.

It is important that scientists working with biocontrol agents make antagonists available to other scientists. Restricting avaibility of antagonists to the individuals involved in a project diminishes confidence in the results of biocontrol research. It is imperative that prior to commercial release, an antagonist be evaluated in several laboratories. Failing to do this can impede research and development in the biocontrol industry. There is currently great potential for biocontrol of postharvest diseases of fruit and vegetables, either as a sole treatment or integrated with other control methods; if we did not believe that, most of us would not be in this workshop. However, we should proceed with caution and not repeat an earlier mistake of promising more of biocontrol than is actually possible.

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Takeda, F., W. Janisiewicz, J. Roitman, N. Mahoney, and
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Table 1. Effect of broth composition, time and initial pH on the production
of pyrrolnitrin (mg/l broth) in shake fermentation of *Pseudomonas cepacia*.

Media	24 hr pH		48 hr pH		96 hr pH		168 hr pH	
	5.8	7.0	5.8	7.0	5.8	7.0	5.8	7.0
Minimum salts	3.6	3.2	11.5	9.3	51.5	39.5	60.5	49.9
Nutrient broth	2.4	2.2	6.0	5.6	7.2	6.6	7.2	6.8
King's B	1.7	1.5	1.9	2.1	4.7	5.5	5.0	5.6
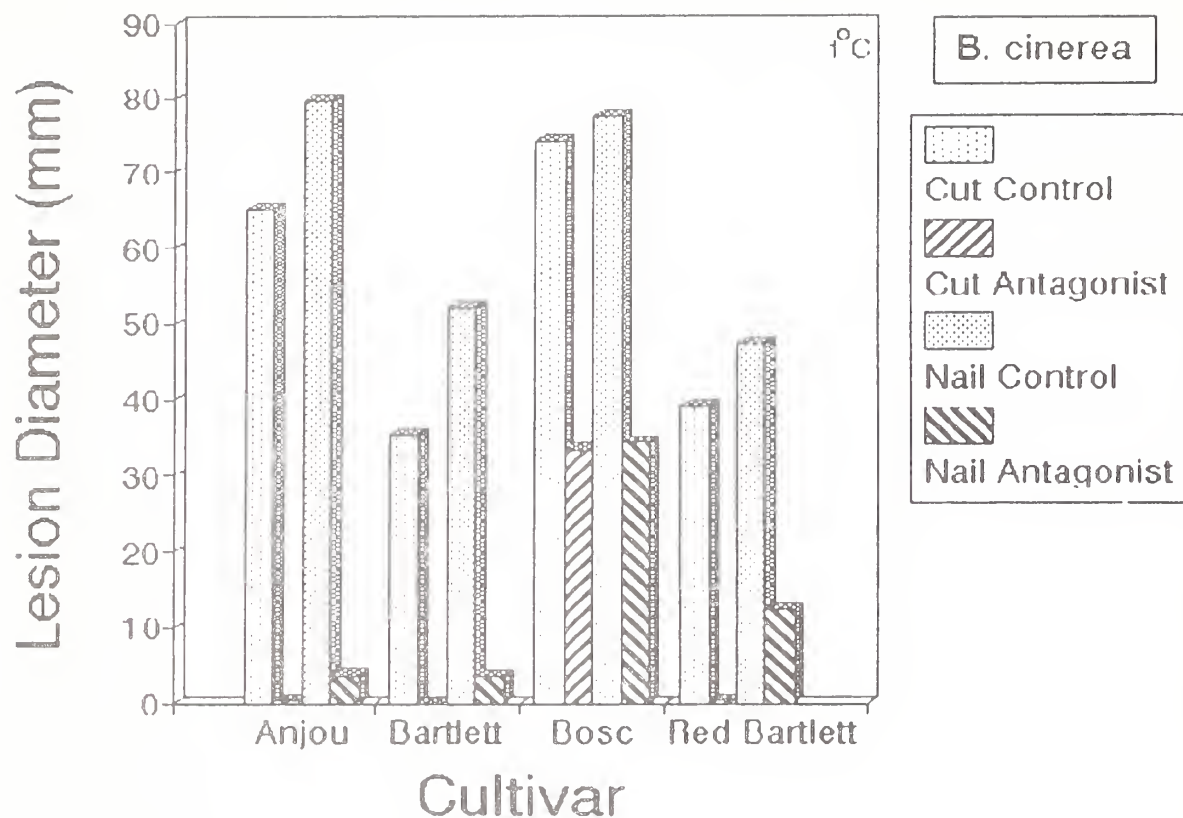
Figure 1. Diameter of lesions originating from two types of wounds on pear fruit of different cultivars. The fruit were dipped in a suspension of *B. cinerea* conidia (1×10^4 conidia/ml) or in a mixture of conidia and the antagonist *P. syringae* isolate L-59-66. The fruit were evaluated after 30 days storage at 1°C.
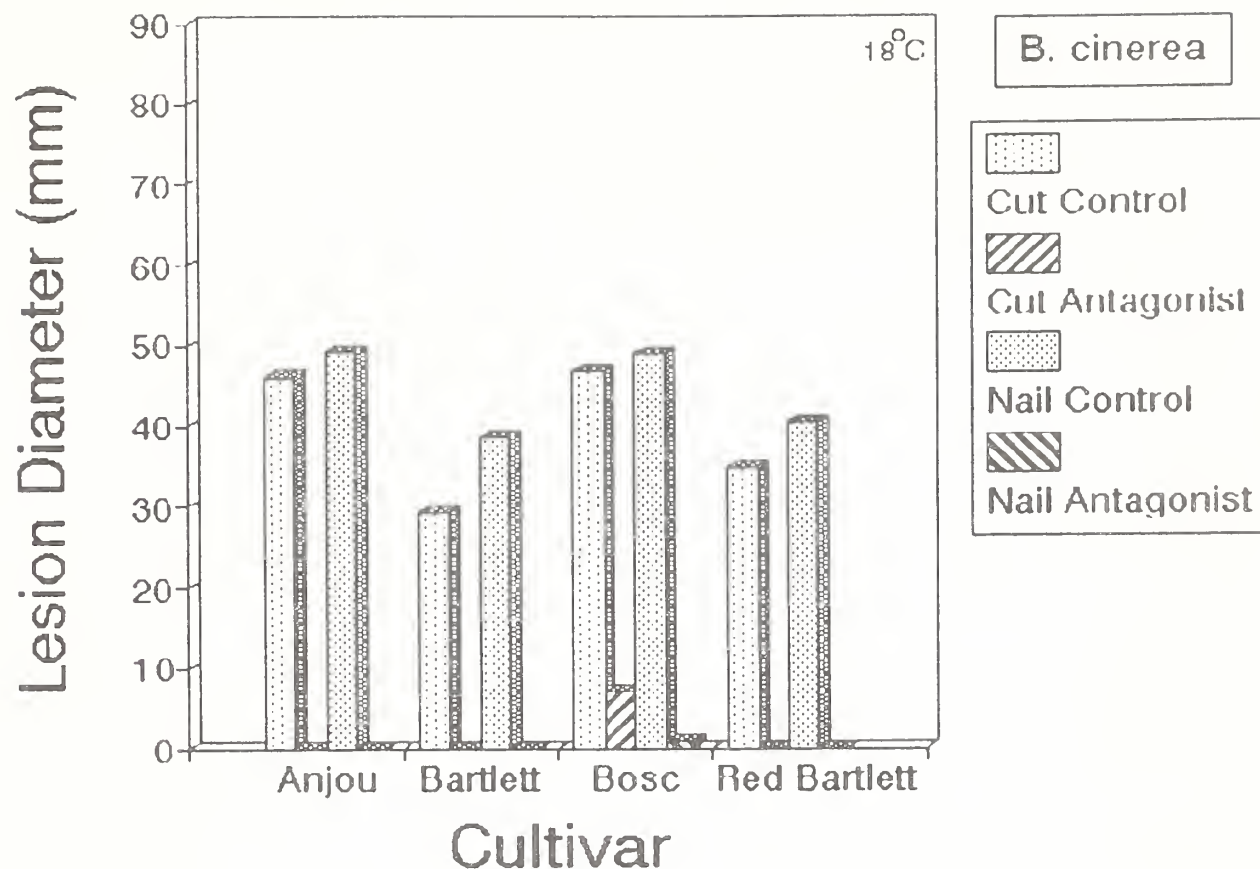
Figure 2. Diameter of lesions originating from two types of wounds on pear fruit of different cultivars. The fruit were dipped in a suspension of *B. cinerea* conidia (1×10^4 conidia/ml) or in a mixture of conidia and the antagonist *P. syringae* isolate L-59-66. The fruit were evaluated after 7 days storage at 18°C.
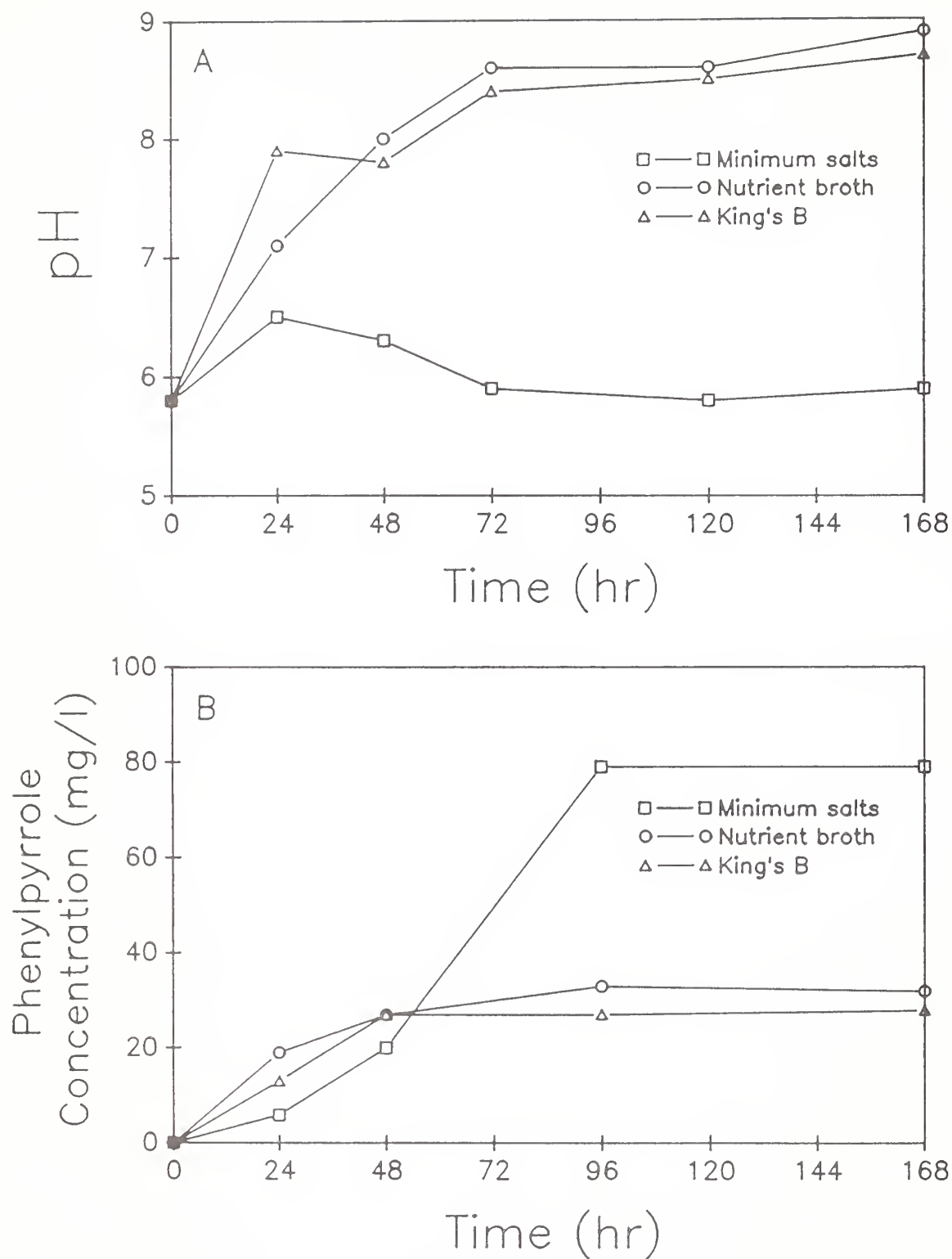
Figure 3. a) PH changes during growth of *Pseudomonas cepacia* in various culture media.
b) Production of phenylpyrrole metabolites by *Pseudomonas cepacia* in various culture media. (Adopted from Roitman et al., 1991)
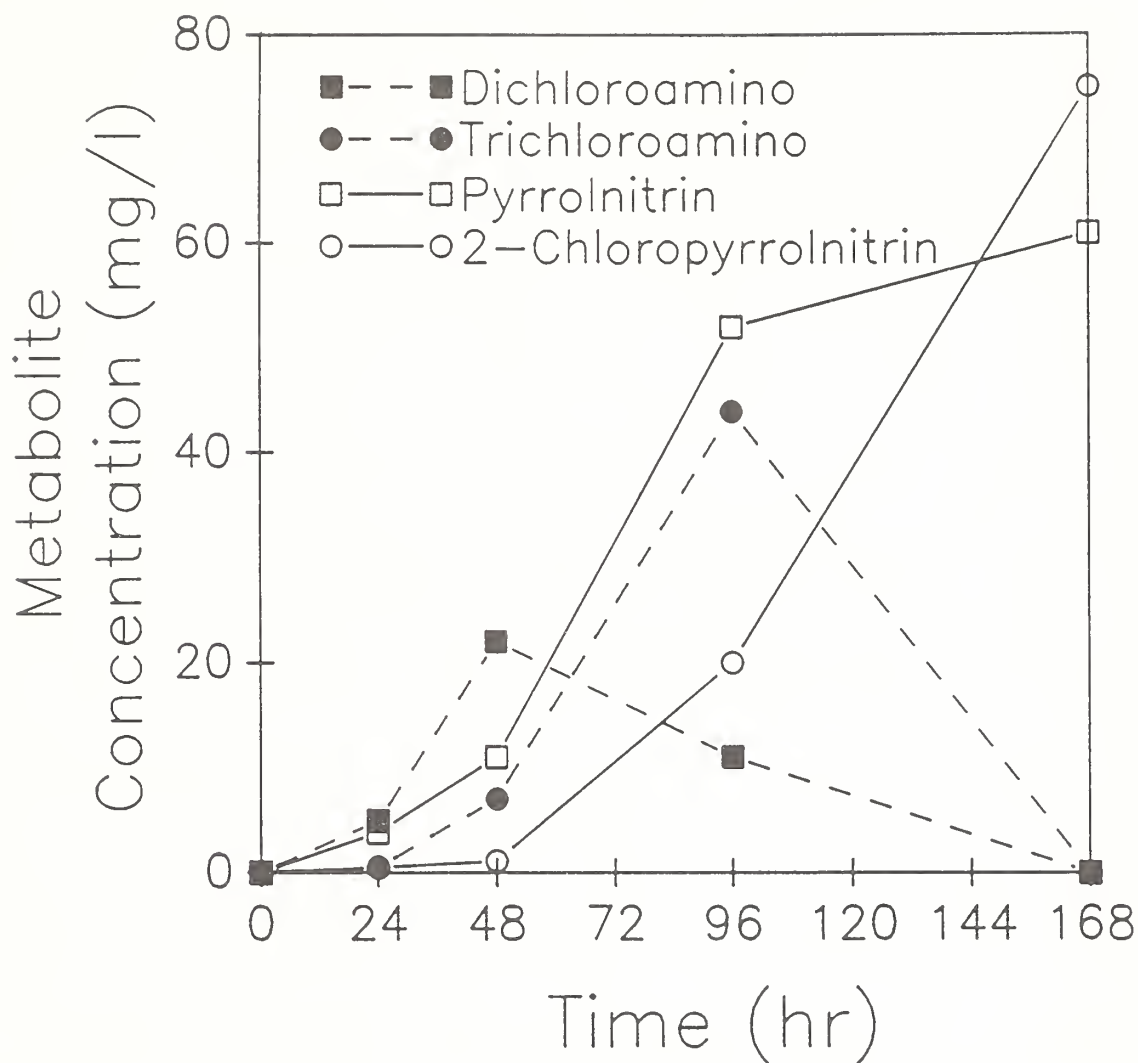
Figure 4. Time related changes in concentrations of nitrophenylpyrrol metabolites of *Pseudomonas cepacia* grown in minimum salt medium. (Adopted from Roitman et al., 1991)

Biological Control of Postharvest Diseases of Citrus Fruit

Samir Droby[1], Edo Chalutz[1], Lea Cohen[1], Bathia Weiss[1], and Charles Wilson[2]

Abstract

The yeast *Pichia guilliermondii*, isolated from the surface of lemon fruit, inhibited the development of green and blue mold and sour rot of several citrus fruit cultivars. It was more effective against green mold than blue mold or sour rot. Control of green mold of grapefruit was maintained for 21 days at 11 or 22°C. The antagonist also reduced the incidence of green mold decay of naturally infected injured, grapefruit stored at 11°C for 21 days. *P. guilliermondii* was also found to be compatible with low fungicide concentrations. Effective control of the green mold of grapefruit was demonstrated in semi-commercial tests when the yeast was applied with low concentration of TBZ followed by wax treatment.

Introduction

Green and blue mold caused by *Penicillium digitatum* and *P. italicum*, respectively, and sour rot caused by *Geotrichum candidum* account for most postharvest losses of citrus fruit worldwide (Bancroft et al., 1984). These diseases have been traditionally controlled by synthetic chemical fungicides, often in combination with physical means such as refrigeration. Fungicide efficacy is frequently decreased by the development of resistant strains of the pathogens (Brown, 1977; Delp, 1980/ Eckert and Ogawa, 1985). In addition, public awareness and concern regarding pesticide residues in our foods has greatly increased in recent years. A recently published National Academy of Sciences report states that "for certain regions the loss of all oncogenic compounds – particularly fungicides – would cause sever short-term adjustments in pest control practices because of the lack of economically viable alternatives" (Anon., 1987).

These findings, and public pressure to reduce the use of pesticides, emphasize the need to find alternative means for controlling postharvest diseases of citrus. One alternative could be biological control.

An early observation of a potential microbial biocontrol agent of postharvest diseases of citrus was reported by Gutter and Littauer (1953) who isolated from citrus fruit a strain of *Bacillus subtilis* that inhibited the growth in culture of several citrus fruit pathogens. More recently, the efficacy of this bacterium as an antagonist of postharvest diseases was studied with citrus (Singh and Deverall, 1984).

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Our study was undertaken to find new biological control antagonists of citrus pathogens. We were particularly interested in finding antagonists that did not produce antibiotic substances as a part of their mode of action. Such antagonists would gain better public acceptance since no exotic antibiotics would be introduced into the food chain during their application.

Experimental

Selecting Natural Antagonists

Selecting naturally occurring antagonists to treat surfaces of fruits for the control of postharvest diseases, requires special considerations. Since such antagonist will be consumed, their toxicity and allergenic properties to man will have to be assessed. To avoid such potential difficulties, in our search for antagonists to control postharvest diseases of citrus fruit we have attempted to select antagonists that are likely to be safe when used on the surfaces of fruits. The isolation procedure of the non-antibiotic producing antagonists was already reported in several previous publication. The antagonistic activity of potential biocontrol agents was tested initially on grapefruits and then on other citrus cultivars. In all experiments, surface-sterilized fruits were wounded around the stem end, with three or four wounds per fruit. Forty microlitre of cell suspension of the antagonist were placed in each wound, allowed to dry and then inoculated with spore suspension of the pathogen. The treated fruits were incubated under moist conditions at 24°C. The efficacy of the antagonist in reducing green mold infections of naturally infested grapefruit was evaluated on wounded fruit. Each fruit was wounded at four locations equally separated from each other. The fruit was then dipped momentarily in a cell suspension (10^9 cells per milliliter) of *Pichia guilliermondii*. Control fruits were dipped in water. All fruits were then waxed and placed in cold storage.

Colonizing Wound Site

To determine the ability of *P. guilliermondii* to colonize the wound site of the treated fruit at different times during storage at 11°C, wounds were cut and shaken vigorously in sterile water for 1 h. After serial dilutions, washing liquid was plated on PDA containing 250 ug/ml of chloramphenocol. The number of colonies were counted after 48 h of incubation at 25°C.

Results and Discussion

By the above procedure we have selected three yeast antagonists (Table 1). Two, designated US-7 and B-2, demonstrated effective activity against postharvest diseases of citrus fruit. The antagonistic activity of the G-4 isolate against citrus pathogens was very limited as compared to the above isolate. In addition, the G-4 and US-7 isolated exhibited broad spectrum of activity against other postharvest diseases of fruits and vegetables such as gray mold of apples, Rhizopus rot of peaches, Botrytis and Rhizopus decay of table grapes and tomato and other postharvest wound pathogens (Chalutz et al., 1988; Chalutz and Wilson, 1990; Wilson and Chalutz, 1989).

| *Pichia guilliermondii* | The US-7 isolate (*P. guilliermondii*) inhibited green mold, blue mold, and sour rot of several citrus fruit cultivars (Table 2). Although the antagonist reduced decay on all cultivars tested, its efficacy varied between cultivars; it was more effective on pummelo and grapefruit and less effective on the other cultivars. The antagonist was also more effective in reducing green mold than in reducing blue or sour rot. In kumquat, which were injured during picking and then dipped in the antagonist cell suspension, natural infections were reduced from 27% in the control of 12% in the treated fruit after 6 days of incubation. |

The persistence of the antagonistic activity of *P. guilliermondii* against green mold of grapefruit in inoculated fruit during incubation periods of up to 21 days. When fruit treated with the antagonist was incubated at 22°C, the incidence of infection normally did not increase after the first 7 days; incidence remained at 9-11% during the remainder of the incubation period (Table 3). At lower incubation temperatures (11°C), infection incidence gradually increased during the 3-week incubation period. Under these conditions, infection incidence among treated fruit was 1% after 14 days and less then 7% after 21 days (Table 3).

Efficacy of *P. guilliermondii* was also maintained in naturally infected fruit stored at 11°C for 21 days (Table 4). While, in the control, infection incidence increased to 22% after 14 days and to 33% after 21 days of storage, dipping the injured fruit in the antagonist cell suspension reduced the incidence of natural infections by 90%. Under these conditions, the TBZ-treated fruit maintained a low incidence of infection throughout the storage period.

| **Growth Rate of** *Pichia guilliermondii* | The growth rate of *P. guilliermondii* on NYDA plates was determined also in the presence of various concentrations of TBZ and imazalil, two fungicides used to control postharvest rots of citrus fruit (Eckert and Ogawa, 1985). The concentrations of these chemicals that inhibited 50% of the growth of the yeast antagonist were 3000 ppm for TBZ and 5 ppm for imazalil. The corresponding concentrations for P. digitatum inhibition were 0.2 ppm for TBZ and 0.06 ppm for imazalil. |

| **Compatibility with Fungicides** | To test the compatibility of the yeast antagonist applied to the fruit with the fungicide TBZ, large-scale experiments were carried out with injured grapefruits which were not inoculated artificially. The results of these tests(Fig. 1) demonstrated the validity of the integrated control approach for effective control of postharvest decay of grapefruit under extended cold storage conditions. These tests further indicated that the yeast cell concentration at the wound sites was maintained high and constant throughout the storage period (Fig. 2). |

| **Desirable Characteristics** | Several features of the US-7 yeast isolate suggest that it may be particularly suitable as a biocontrol agent of postharvest diseases of citrus fruit: The antagonist is (a) effective against a wide range of postharvest pathogens; (b) not a produced of antibiotics; (c) indigenous to the fruit environment; (d) persistent on the fruit or wound surface under a wide range of temperatures; (e) relatively resistant to postharvest fungicides; (f) commonly found in food products; and (g) easy to grow and handle. |

Acknowledgments

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Table 1. Inhibition of green mold decay of grapefruit by
three yeast antagonists.

Yeast	Source	% Infection
Pichia guilliermondii (US-7)	Lemon	3
Rhodotorula mucilaginosa (B-2)	Grapefruit	11
Hanseniaspora uvarum (G-4)	Grape	45
Water control		96

Table 2. Inhibition of green mold, blue mold and sour rot of several citrus fruit cultivars by *Pichia guilliermondii*.

Citrus cultivar	Green mold*	Blue mold*	Sour rot*
Grapefruit	6	6	9
Shamouti orange	10	15	--
Valencia orange	8	19	--
Lemon	10	--	18
Temple	10	--	--
Pommelo	2	4	--

*Percent infection after 6 days of incubation at 24°C. Infection of control fruit was 96% or higher for green and blue mold and 77% or higher for sour rot.

Table 3. Persistence of inhibition of green mold of grapefruit by
Pichia guilliermondii (US-7) at two incubation temperatures.

Treatment	% Infection after storage Period (days)		
	7	14	21
Incubation at 22°C			
Control	90.0 a*	100.0 a	100.0 a
P. guilliermondii	8.8 c	8.8 b	11.1 b
Incubation at 11°C			
Control	62.4 b	95.5 a	100.0 a
P. guilliermondii	0.0 1.1 c	6.7 b	.

* Within colums, values followed by different letters are
significantly different (P=0.05) according to Duncan's multiple test.

Table 4. Inhibition of green mold of grapefruit by *Pichia guilliermondii* in naturally infected fruit stored at 11°C.

Treatment	% Infection after storage Period (days)		
	7	14	21
Control	4.7 a	21.8 a	33.3a
P. guilliermondii	1.0 ab	2/6 b	2.6 b
TBZ (2000 ppm)	0.5 b	0.5 b	0.5 b

Within columns, values followed by different letters are significantly differ (P=0.05) according to Duncan's multiple range test.
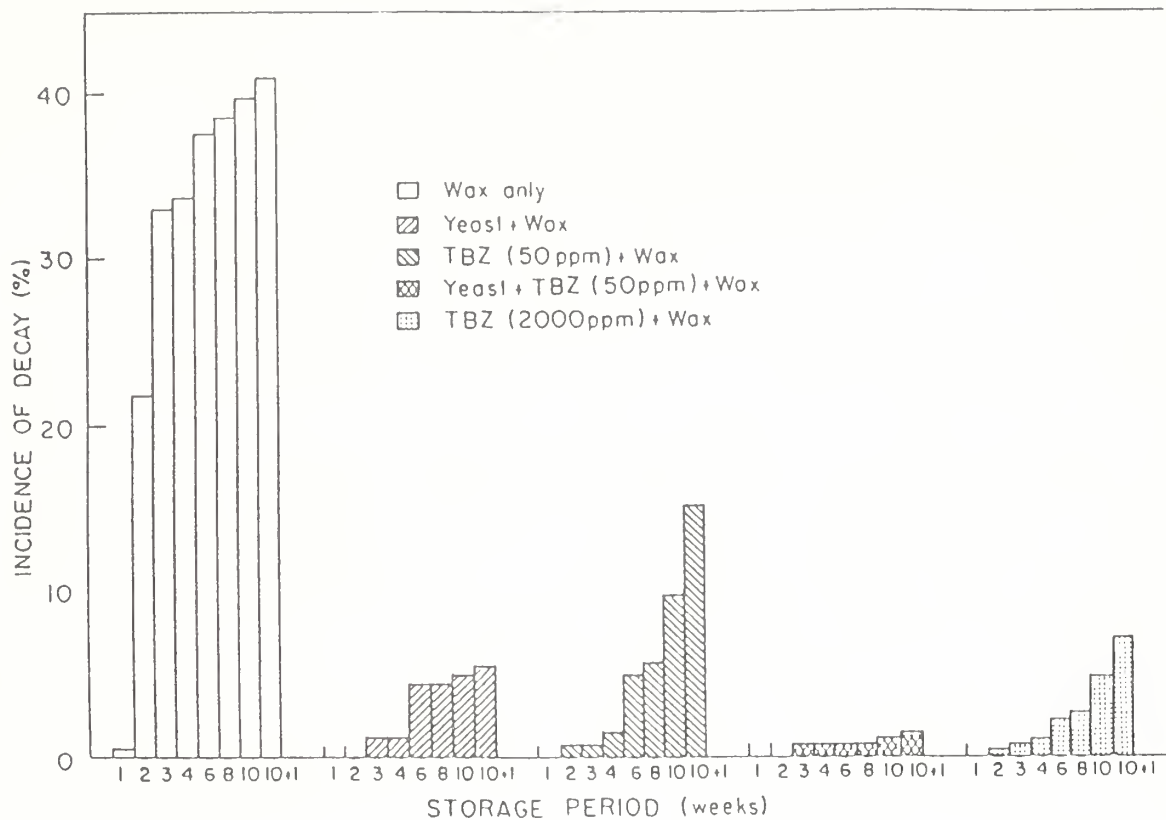
Figure 1. Compatibility of *P. guilliermondii* with TBZ and wax for the control of postharvest mold rots. Each fruit was injured prior to dipping it in the appropriate aqueous solution. It was then dried, packed in commerical cartons ad placed in cold storage at 11°C.
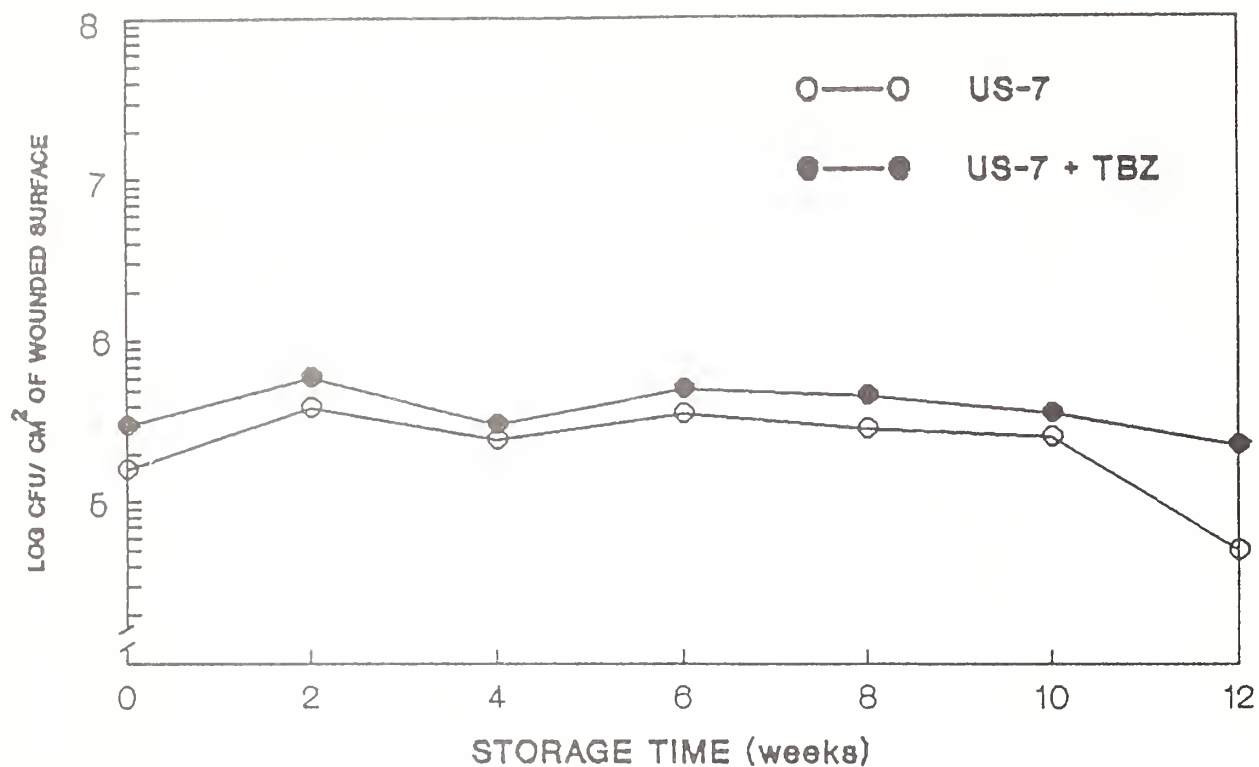
Figure 2. Growth of *P. guilliermondii*, with and without the addition
of TBZ (50 ppm), on grapefruit stored at 11°C.

Biological Control of Botrytis, Rhizopus and Alternaria Rots of Tomato Fruit by *Pichia guilliermondii*

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Abstract

The yeast antagonist *Pichia guilliermondii* was effective in reducing incidence of *Botrytis cinerea*, *Rhizopus stolonifer* and *Alternaria alternata* decay of tomato fruit. A water suspension of the yeast cells applied to wounds on the surface of the fruit prior to inoculation with spore suspensions of the pathogens, reduced disease by 90%. The efficacy of the antagonist in reduction of the gray mold disease of tomato was affected by the concentration of both the yeast cells and the spore suspension. No reduction of disease was exhibited by an autoclaved preparation of yeast cells, or by a yeast culture filtrate. *P. guilliermondii* also did not inhibit the growth of the pathogens on potato dextrose agar while under similar conditions the antibiotic-producing bacterium *Bacillus subtilis* clearly inhibited the growth of the pathogens. The antagonist grew readily on injured tomato fruit leachate and multiplied rapidly at the wound site. Under limiting nutrient conditions the presence of the yeast cells inhibited the growth in culture of the pathogens but this inhibition could be reversed by the addition of nutrients to the medium. Dipping tomato fruit in the yeast cell suspension, right after harvest, did not reduce the decay development on the fruit. We concluded that nutrient competition between the yeast and the pathogen is involved in the mode of action of *P. guilliermondii* in reducing gray mold of tomato fruit.

Introduction

In recent years, biological control of postharvest diseases of fruits has received considerable attention. Increased public concern about the presence of fungicide residues in foods and the development of resistance by pathogens to major fungicides are two of the reasons. Although successful application of effective antagonists under commercial conditions has, so far, been demonstrated with only a few systems (Dobus, 1984; Pusey et al., 1988; Pusey et al., 1986), numerous laboratory studies revealed the potential of this approach for the control of postharvest diseases (Janisiewicz, 1988; Wilson and Pusey,

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1985). We have recently investigated biological control of postharvest diseases of citrus fruit (Chalutz et al., 1988; Chalutz and Wilson, 1989). Among the antagonists isolated from the surface of lemon fruit, a yeast, *Pichia guilliermondii* (which by mistake was regarded as *Debaryomyces hansenii* (Zopf) van Rij.), exhibited characteristics which made it a good candidate as a biocontrol agent for postharvest diseases. It was highly antagonistic under laboratory conditions against several citrus wound pathogens, did not produce antibiotics in culture, it was persistent on the fruit and wound surface, was relatively resistant against postharvest fungicides and is commonly found in food products (Chalutz and Wilson, 1989). Furthermore, this yeast was also effective against postharvest diseases of other commodities, such as table grapes and apple (Chalutz et al., 1988; Wisniewski et al., 1988). The present study was aimed at evaluating the efficacy of *P. guilliermondii* as a biocontrol agent of postharvest pathogens of tomato fruit and characterizing some features of this interaction.

Materials and Methods

The yeast *Pichia guilliermondii* was cultured for 48 hr in 50 ml nutrient yeast dextrose broth (NYDB) in 100 ml Erlenmeyer flasks on a rotary shaker (100 RPM) at 26°C. Cells were than separated by centrifugation (9000 RPM), rinsed twice by resuspension in sterile deionized water, and adjusted to a final concentration of 10^9 colony forming units (CFU) per ml.

The antagonist was tested against the following tomato fruit pathogens (Barkai-Golan, 1981; Ramsey et al., 1952): *Alternaria alternata* (Fr.) Keissler, *Botrytis cinerea* Pers., and *Rhizopus stolonifer* (Ehr.: Fr.) Lind.

Freshly harvested ripe tomato fruits, cv FA F1-121, were washed in water, wiped dry, and placed, stem-end down, in plastic trays (50x100x15 cm) on a moist paper, 40-50 fruits per tray. Two conical wounds, 1-2 mm deep; and 1-2 mm in diameter, were cut in the top part of each fruit using the tip of a dissecting needle protruding from a rubber stopper. Aliquots of 30 µl of the yeast cell water suspension were then applied to each wound, with sterile water used as the control, and the droplets were allowed to dry for 1-2 hr at room temperature. The fruits were then inoculated by placing 20 µl of a pathogen spore suspension on each wound. Unless otherwise indicated, the concentration of antagonist cells was 10^9 cells per ml. The concentration of spores used for inoculations was that concentration which resulted in at least 80% infection of control wounds after 7 days of incubation. The concentration varied from 10^3 to 10^6 spores per ml, depending on the pathogen and the degree of maturity of the fruit. For most tests with *B. cinerea*, a concentration of 10^5 spores per ml was used. Spore suspension was prepared from 7-day-old, potato dextrose agar (PDA)-grown cultures. Following inoculation, the trays were covered with a high density polyethylene sleeve and kept at 22 - 24°C for several days. Percent infection was calculated by counting the number of infected sites after an incubation period of up to 7 days. In each experiment, 40 to 50 inoculation sites (20 to 25 fruits) per treatment were evaluated and the experiment was repeated at least 3 times.

For evaluation of the possibility to use *P. guilliermondii* under commercial, conditions, cherry tomatoes of the cv. 'EY-12' and regular tomatoes of the cv. 'Dikla' were harvested in the field and were dipped (for 25 seconds) in the yeast suspension (10^9 CFU per ml deionized water) within 45 min of harvest. Other fruits were treated with only deionized water, the third treatment was with 0.05% of the fungicide 'Alisan' and nontreated fruits served as controls. There were 2 experiments with the cherry tomatoes and only 1 with the 'Dikla' cv. With the cherry there were 5 replications of 40 fruits each and with the 'Dikla' 4 replications of 20 fruits each. The fruit was stored for 7 days at 12°C and 90% RH and then was transferred to storage at 22°C and about 85% RH. The number of rotten fruits was determined after 7, 9 and 11 days.

Interaction Between Antagonist and Pathogen

For evaluation of the possible interaction between the antagonist and the pathogens in culture, 15-mm discs of 5-day-old, nutrient yeast dextrose agar (NYDA) cultures of the antagonist were placed on PDA plates in which the pathogen spore suspension was seeded. The effect of the yeast antagonist on the growth of the pathogen was compared with that of a known bacterial antagonist, *Bacillus subtilis* (Cohn) Prazmowski. In addition, the interactions between the antagonist and the pathogens were also tested by placing 4 mm agar discs of a 7-day-old culture of the pathogen in petri dishes containing 15 ml of a synthetic medium (Lilly and Barnett 1951), with and without the antagonist cells, according to the method of Wisniewski et al. (1989). The effect of the antagonist on growth of the pathogens in the synthetic medium was evaluated by determination of the dry weight of the cultures after one week of incubation at 24°C (Wisniewski et al., 1989).

For evaluating the effect of water-soluble nutrients present in tomato fruit on the efficacy of *P. guilliermondii* in the inhibition of gray mold, fresh tomato fruits were autoclaved, homogenized for 1 min and then centrifuged at 9000 RPM for 10 min. The supernatant was diluted with water and filter-sterilized for the preparation of the pathogen spore suspensions used for inoculation.

Population changes of *P. guilliermondii* on intact or wounded, inoculated fruit surfaces were evaluated by cutting the inoculated sites (5x5 cm) and vigorously shaking the tissue in 10 ml of sterile water for 1 hr. After appropriate dilutions, 10 ml of the washing liquid were plated in triplicates on NYDA and the number of colonies counted after 48 hr of incubation. Data are presented as CFU of *P. guilliermondii* per cm^2 of wound surface.

Results

Pichia guilliermondii effectively reduced the incidence of all three diseases studied. A cell suspension of the antagonist, applied to wounds on the surface of the fruit prior to inoculation, reduced, by approximately 90%, the number of inoculated sites on which the fungi developed (Table 1). Efficacy of the antagonist was affected by the yeast concentration in the wound as well as by the number of spores of the pathogen used for inoculation (Figure. 1). At a high

spore concentration (10^6 spores/ml), low concentrations of the antagonist did not reduce percent infection and only the highest antagonist concentration used (10^9 cells/ml) reduced infection to low values. On the other hand, when wounds were inoculated with low concentrations of spores, percent infection was effectively reduced by all the concentrations of the antagonist cells tested (Figure 1).

No Antibiosis

Both the autoclaved cell suspension and the culture filtrate, sterilized by filtration, failed to provide any protection (Table 2). Similarly, agar discs of *P. guilliermondii* placed on PDA in which spores of the pathogen were seeded, failed to inhibit the growth of any of the three pathogens tested, while a similar disc of *B. subtilis* inhibited the growth of all three pathogens, as was evident by the formation of an inhibition zone around the discs (data not shown).

The yeast antagonist grew readily on tomato fruit wound leachate (Figure 2A) and rapidly multiplied at the wound sites of injured tomato fruit (Figure 2B).

Overcoming Inhibition

The inhibition of *B. cinerea* by *P. guilliermondii* could be partially overcome by the addition of the soluble nutrients present in tomato juice to the spore suspension used for inoculation. The higher the concentration of the juice extract, the lower was the protection of inoculation sites by the yeast antagonist (Table 3).

The presence of the yeast with the pathogens in a synthetic minimal medium inhibited mycelial growth of all three pathogens (Table 4). This inhibition was more pronounced when the pathogen was incubated on a used medium, but when such a medium was sterilized and replenished with nutrients before inoculation, normal mycelial growth was restored. In an enriched medium, the presence of the yeast cells did not inhibit the growth of *B. cinerea, A. alternata* or *R. stolonifer*.

In both field experiments there was no advantage to the treatment with *P. guilliermondii* with regards to decay development (Figure 3). Both *Alternaria* and *Botrytis* were the decay causing fungi of these field grown fruits.

Discussion

Our results indicate that *P. guilliermondii* inhibited three major postharvest wound pathogens of tomato fruit under conditions that favor disease development (Table 1). While the efficacy of the yeast antagonist depended on the relationship between the number of antagonist cells and the concentration of the pathogen spores (Figure 1), the rapid multiplication of the yeast cells at the wound site (Figure 2) and the fact that one or a small number of spores usually suffice to cause decay, suggest that the antagonist may retain its efficacy also under natural conditions.

Pichia guilliermondii lost its antagonistic activity when the cells were killed, and its culture filtrate did not exhibit any activity (Table 2). In addition, the yeast antagonist showed no inhibitory activity against the fungal pathogens on

PDA plates. Under similar conditions, *B. subtilis,* an antagonist known for its ability to inhibit pathogens by secretion of antibiotic materials (Gueldner et al., 1988; Korzybski et al. 1978), resulted in the formation of clear mycelial inhibition zones around its colonies. Thus, as in the inhibition of postharvest diseases of citrus fruit by *P. guilliermondii* (Chalutz and Wilson, 1990), the mode of action of this yeast in antagonizing the tomato fruit pathogens is not through the production of antibiotics.

Competition for Nutrients

Several lines of evidence suggest that successful competition for nutrients at the wound site could be the main mechanism by which *P. guilliermondii* inhibits *B. cinerea* and other postharvest pathogens: a) the antagonism could be partially reversed by the addition of nutrients to the wound during inoculation – the degree of reversal depending on the concentration of nutrients added (Table 3); and b) the culturing of the antagonist cells with the pathogens on a minimal synthetic medium resulted in marked reduction in the growth rate of the pathogen only under limited nutritional conditions (Table 4). The growth of *P. guilliermondii* in the medium did not result in any residual inhibitory effect on the pathogen which could not be restored by the mere addition of nutrients to the used medium (Table 4). These data, when considered together with the observations on the lack of inhibition on the nutrient-rich PDA medium, the rapid growth of the antagonist at the wound site during the critical first 24 hrs of incubation (Figure 2), and the non-specific nature of *P. guilliermondii* antagonism inhibiting several wound postharvest pathogens of such diverse commodities as citrus (Chalutz and Wilson, 1990), apple (Wisniewski et al. 1988), grape (Chalutz et al. 1988) and tomato, strongly suggest that competition for nutrients is one major mode of action of *P. guilliermondii* in the inhibition of the tomato pathogens. The fact that the yeast treatment did not reduce the postharvest decay development in field grown tomatoes, which were not inoculated artificially, (Figure 3) might imply that on time of harvest the pathogenic fungi were already established in the fruit tissue and therefore the yeast was not effective in controlling the decay.

Competition for nutrients as the mode of action of biocontrol agents has been suggested in the past (Blakeman and Fokkema, 1982). Recently it was shown to play a major role in the biological control of the damping-off disease (Elad and Chet, 1987) and fruit postharvest diseases, including Rhizopus rot of peach fruit (Wisniewski et al. 1989). Thus, with the lack of evidence of antibiotic production or direct interactions between *P. guilliermondii* and the pathogens it antagonizes, the very rapid depletion of nutrients at the wound site, resulting in the inhibition of spore germination and germ-tube elongation of the pathogens, is likely the major mode of action by which the yeast antagonizes artificially inoculated tomato and other postharvest pathogens of fruits.

Acknowledgments

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Table 1. Inhibition of postharvest rots of tomato by *Pichia guilliermondii* (isolate US-7).

Pathogen	Antagonist	Incubation time (days)			
		2	3	4	7
		Percent infection			
Rhizopus stolonifer	US-7	5.2	8.5	13.0	--
	Control	90.8	97.1	100.0	--
Botrytis cinerea	US-7	--	0	5.3	7.0
	Control	--	42.4	57.5	80.1
Alternaria alternata	US-7	--	0	6.1	8.4
	Control	--	85.5	97.9	100.0

Table 2. Inhibition of *Botrytis cinerea* decay of tomato by the antagonist *Pichia guilliermondii* (isolate US-7), as affected by pretreatments of the antagonist cells.

Pretreatment	Percent infection[z]
None	3.1 a
Autoclaved	47.2 b
Culture filtrate	48.1 b
Sterile medium	51.4 b
Water control	48.4 b

[z]Values followed by different letters are significantly different (P=0.05) according to Duncan's multiple range test.

Table 3. Inhibition of *Botrytis cinerea* decay of tomato by *Pichia guilliermondii* (isolate US-7), as affected by tomato juice nutrients added to the spore suspension.

Treatment	Percent infection[z]
Water control	59.7 a
US-7	4.6 c
US-7 + 10% juice prep.[v]	6.8 c
US-7 + 50% juice prep.	16.4 c
US-7 + 100% juice prep.	38.0 b

[z]Values followed by different letters are significantly different (P=0.05) according to Duncan's multiple range test.

[v]Tomato fruit was autoclaved, homogenized and centrifuged, and the filter-sterilized liquid was added to the spores prior to inoculation.

Table 4. Growth rate in culture of several tomato postharvest pathogens in the presence of the antagonist *Pichia guilliermondii* (isolate US-7).

Treatment	*Alternaria alternata*	*Botrytis cinerea*	*Rhizopus stolonifer*
	Dry weight of mycelium (mg)[z]		
Control	58.3 a	48.9 a	41.3 a
US-7	17.4 b	18.8 b	18.3 b
Control used[v]	0.9 c	1.5 c	--
Replenished[x]	58.6 a	50.1 a	--
Enriched[w]	78.6 d	49.6 a	125.4 c
US-7	68.2 d	48.4 a	112.3 c

[z]Values in the same column (same fungus) followed by different letters are significantly different (P=0.05) according to Duncan's multiple range test.

[v]*P. guilliermondii* was cultured on the synthetic medium for 48 hr prior to the use of the medium.

[x]The used medium was replenished with original nutrients.

[w]The medium was enriched with yeast extract and nutrient broth.
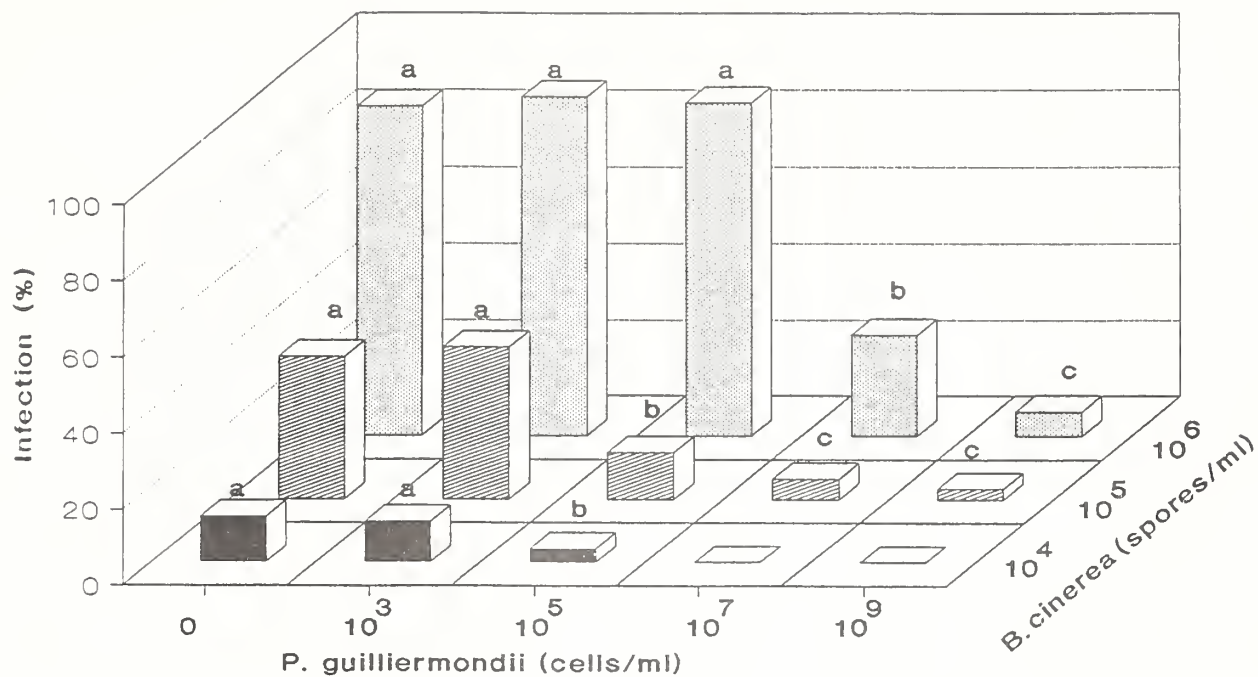
FIG. 1. The relationship between concentration of the pathogen spore suspension and the antagonist cells in the inhibition of *Botrytis cinerea* decay of tomato by *Pichia guilliermondii* (isolate US-7). Values in the same column (spore concentration) followed by different letters are significantly different (P=0.05) according to Duncan's multiple range test.
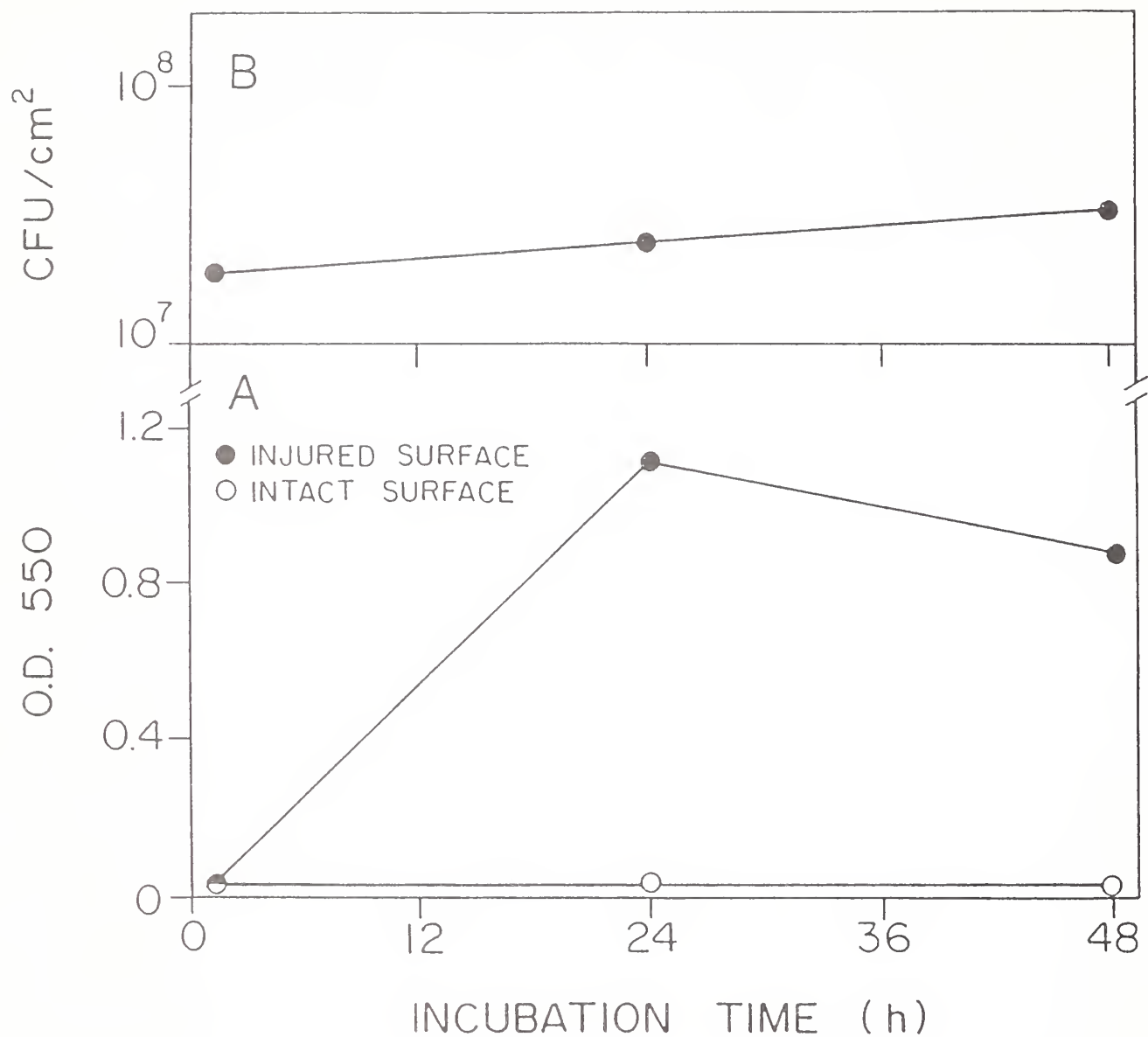
FIG. 2. Growth of *Pichia guilliermondii* on fruit leachate (A) or on the tomato fruit surface (B). Absorbance at 550 nm was used to evaluate the turbidity of the cultures.
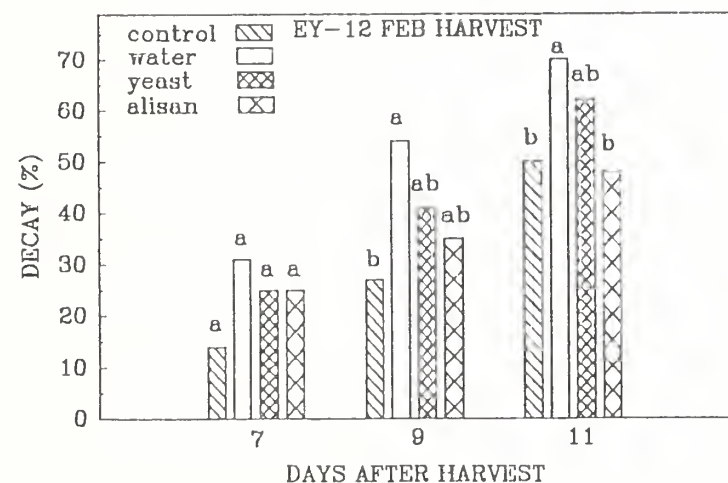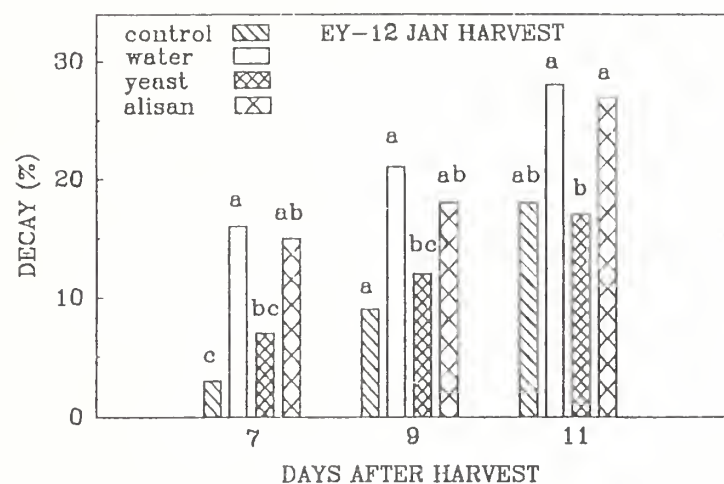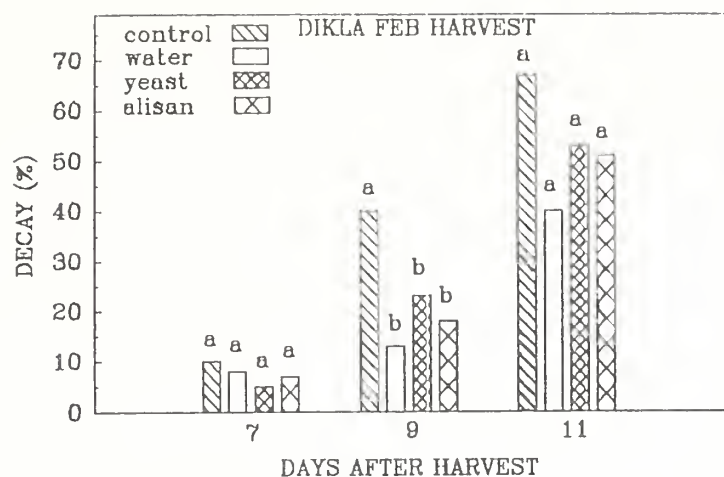
FIG. 3. Decayed tomato fruits in postharvest field experiments with *P. guilliermondii*. Fruit observations were made after 7 days of storage at 12°C and after additional 2 and 4 days of storage at 22°C. At each observation day, values followed by different letters are significantly different (P=0.05) according to Duncan's multiple range test.

Control of Powdery Mildews in Cucumber and Rose by *Stephanoascus* spp.

W. R. Jarvis[1] and R. R. Belanger[2]

Abstract

Powdery mildews contribute to poor quality in marketed cucumber fruit and render roses, begonias, african violets, chrysanthemums and other florist crops unmarketable. In the greenhouse environment outstanding control of *Sphaerotheca fuliginea* on cucumber and *S. pannosa* var. *rosae* on rose has been obtained by *Stephanoascus flocculosus* and, to a lesser degree, by *S. rugulosus*. The optimum temperature for biological control is about 26°C and water vapor deficits of less than about 0.65 kPa are required. Both environmental requirements are provided by a greenhouse fogging system.

Introduction

Conventional pesticide control of the powdery mildew diseases of greenhouse vegetable and ornamental crops is in a most unsatisfactory state. *Sphaerotheca fuliginea* (Schlect.) Poll., the common cause of powdery mildew of cucurbits in N. America, is resistant to benzimidazoles (Schepers, 1984; Schroeder and Provvidenti, 1969); dimethirimol (Schepers, 1984); biteranol, fenarimol, imazalil, and triforine (Schepers, 1983). Dinocap, though effective, tends to be phytotoxic at high temperatures, and has a very long legislated period (30d in Canada) between application and harvest. Microfine sulfur is effective but if repeated applications are made it builds up to phytotoxic levels. In florist crops, all fungicides leave unsightly residues that detract from market value, while dodemorph acetate used against rose powdery mildew (*S. pannosa* (Wallr.) Lev. var. *rosae* Wor.), costs about US \$11,800/ha/year (J. Lessard, personal communication). The position is further complicated in that pesticides are not weathered in the greenhouse environment but are a constant environmental presence. The time is therefore ripe for biological control.

Early attempts to control cucumber powdery mildew biologically centered on exploiting the common hyperparasite *Ampelomyces quisqualis* Ces. (Jarvis and Slingsby, 1977). It was quite effective when sprays of spore suspensions were interspersed with water sprays but problems with registration protocol shelved this approach. Attempts were then made to enhance the indigenous populations of *A. quisqualis* by sprays of microbial nutrients. These did not enhance populations of *A. quisqualis* but did reveal outstanding control by a *Sporothrix* sp. (W.R. Jarvis, unpublished results). Subsequently, two new *Sporothrix* species were isolated from the wild and shown to have

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Stephanoascus teleomorphs (Traquair, Shaw and Jarvis 1988), namely *Stephanoascus flocculosus* Traquair, Shaw & Jarvis (anam. *Sporothrix flocculosa* Traquair et al.) and *Stephanoascus rugulosus* Traquair et al. (anam. *Sporothrix rugulosa* Traquair et al).

Greenhouse trials on cucumber at Harrow, Ontario, and on roses at Ste. Foy, Quebec demonstrated the outstanding promise of particularly one species, *St. flocculosus*, and patents are pending on both species and their mode of action.

Many other antagonistic fungi have been reported from powdery mildews (Table 1) including *Tilletiopsis* spp. but in our experience (unpublished results) none approaches the two *Stephanoascus* spp. in disease control.

Experimental

Cucumbers

Cucumbers: Long English seedless cucumbers *Cucumis sativa* L., cv. Corona were raised in pots and transplanted into sterilized soil groundbed at the 4-true-leaf stage, and supported on strings in vertical cordons. Fruit thinning, deleafing, fertilization and irrigation followed normal commercial practice for Ontario (Smith, 1988).

Roses

Roses: Minature roses (*Rosa multiflora* L.), cv. Ruiredro were purchased from a local grower and grown in a greenhouse until development of the disease.

Fungal Cultures

Fungal cultures: Cultures of *Sporothrix flocculosa* Traquair, Shaw & Jarvis and *Sp. rugulosa* Traquair, Shaw & Jarvis were maintained on malt extract-yeast extract agar (Jarvis et al. 1988). Conidial suspensions were prepared in sterile water and diluted to 10^6 conidia mL^{-1}. *Tilletiopsis washingtonensis* was similarly maintained and cultures were harvested for conidial suspensions.

Powdery Mildew

Powdery mildew: On both cucumbers and roses, *Sphaerotheca fuliginea* and *S. pannosa* var. *rosae*, respectively, appeared in the greenhouse without inoculation.

Disease Assessment

Disease assessment: The degree of development of each powdery mildew was assessed on an arbitrary scale described for *S. fuliginea* by Spencer (1979).

Antagonist Assessment

Antagonist assessment: The degree of colonization of each fungal antagonist on the powdery mildew was assessed on the arbitrary scale described by Jarvis et al (1988).

Effect of Temperature

Effect of temperature: In the case of roses, mildew-infested plants were transferred to growth chambers maintained at 18, 22, 26, 30 or 34°C for a week under saturated humidity. Four plants were placed in each of the five chambers and received one of the following treatments: application of a conidial suspension of *Sp. flocculosa*, or *Sp. rugulosa*, or *T. washingtonensis* or distilled water. The chambers were compartmentalized to prevent cross-contamination. Each day, five leaves from each plant were sampled and colonization was scored as previously described (Belanger and Haljaoui, 1990). This experiment was repeated three times.

In the case of cucumbers, leaf discs bearing incipient colonies of powdery mildew were cut with a cork borer, and floated on water in wells mounted in petri dishes (Jarvis et al. 1988). Powdery mildew colonies were inoculated by spraying them in their wells with a spore suspension of either *Sporothrix* sp. until lightly misted. The wells were surrounded by water, and replicated dishes were incubated in illuminated growth chambers at selected temperatures (Jarvis et al. 1988).

Effect of Humidity

Effect of humidity: Relative humidity in petri dishes was controlled by a graded series of glycerolwater mixtures (Jarvis et al., 1988).

Greenhouse Studies

Greenhouse studies: Mature cucumber plants were sprayed to incipient run-off with conidial suspensions of the antagonists. Relative humidities of >70% (vpd <0.95kPa) were maintained by means of a microfine water-fogging system (Micro-Cool Inc.), which also maintained a greenhouse temperature between 25 and 30°C, close to the optimum for the development of *S. fuliginea*. Biological control was compared with a standard microfine sulfur spray treatment ($1gL^{-1}$) in a fully-randomized complete block design. Treatments were applied at 2-week intervals. These studies were frequently interrupted by breakdown of the fogging equipment which proved quite unreliable.

At intervals, leaf discs 1.6 cm diam., were removed with a cork borer, washed in sterile water, and the washings serially-diluted on yeast-malt agar for enumeration of the antagonists. Similar discs were also removed from the skin of mature cucumber fruits for enumeration.

To determine the effect of relative humidity (r.h.) on rose powdery mildew, *Sporothrix*-inoculated, mildew-infested rose plants were placed in growth chambers maintained at r.h. of 70, 80 or 90% under a temperature of 25 + 1°C for one week. The experimental design was similar to the one described above. The effect of r.h. on the infestation of *S. fuliginea* on cucumber leaf discs was determined by substituting glycerol-water mixtures for water in the experimental design described for temperature studies as described above.

Results and Discussion

Effect of Temperature

Effect of temperature: On both *S. fuliginea* and *S. pannosa* var. *rosae* the greatest colonization occurred at 26°C with *Sp. flocculosa* achieving complete overgrowth in approximately 48 hr and *Sp. rugulosa* and *T. washingtonensis* in 5 days (Figures 1 and 2). For all three antagonists tested, temperature below 22°C and above 30°C generated little antagonistic activity against *Sphaerotheca pannosa* f. sp. *rosae*. However, at any given temperature, *Sp. flocculosa* always achieved a higher rate of colonization than the other two antagonists.

Effect of Humidity

Effect of humidity: Only r.h. of 90% permitted complete colonization of *S. pannosa* f. sp. *rosae* by all three antagonists (Figures 3). Once again *Sp. flocculosa* exhibited a faster activity than the other two antagonists tested. *Sp. rugulosa* superior activity was also noticeable at r.h. 80 and

70%. By contrast, *Sp. rugulosa* and *T. washingtonensis* never attained maximum colonization below r.h. 90% with the latter being the least active especially at r.h. 70%. The effect of relative humidity on the colonization of *S. fuliginea* on cucumber was remarkably similar (Figures 4 and 5) to that of *S. pannosa* f. sp *rosae*. At r.h. approaching 100% colonization took less than 12 h.

In trials with cucumbers on a commercial scale, for which representative results are summarized in Tables 2, 3 and 4, *S. flocculosa* again proved superior to *S. rugulosa*, and provided that an adequate relative humidity was maintained, gave good control of powdery mildew. Both *S. flocculosa* and *S. rugulosa* maintained effective populations, and have been isolated from leaf discs beforfe transplanting. The numbers of colony-forming units on fruit are low and are unlikely to present a hazard to consumers.

Provided that the relative humidity of the greenhouse does not fall below 70% (vpd>0.95 kPa), and is preferably maintained at about 80%, (>0.6 kPa) control of *Sphaerotheca fuligines* and S. *pannosa* var. *rosae* by *Sporothrix flocculosa* is excellent. Control by *S. rugulosa* is good. The high relative humidity also helps to control *S. fuliginea* but this observation by Abiko and Kishi (1979) may be attributed, in part, to the presence of unsuspected antagonists. The provision of a fogging system in the greenhouse not only cools the air by evaporative cooling to between 26 and 30°C, near the optimum for *Sporothrix* spp., but also provides the necessary high humidity. This form of cooling and humidifying does not invite water-dependent pathogens such as *Botrytis cinerea* because the plants never get wet; the fine water droplets evaporate in the air (Jarvis 1989).

The two *Sporothrix* species described here are taxonomically distinct from the human pathogen *S. schenckii*, which has an *Ophiostoma*, not a *Stephanoascus*, teleomorph. Preliminary toxicology (Belanger and Jarvis, unpublished) shows that *S. flocculosa* is not harmful to laboratory animals on ingestion or intraperitoneal infection.

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Table 1. Some examples of fungi antagonistic or hyperparasitic to powdery mildews

Powdery mildew	Antagonist *Hyperparasite	Reference
Cystotheca wrightii	*Ampelomyces quisqualis*	Yakawa et al. 1971a
Erysiphe graminis f. sp. *hordei*	*Tilletia pallescens*	Klecan et al. 1990
Erysiphe polygoni	*Cladosporium cladosporioides* *Curvularia lunata*	Srivastava & Bisht 1983
Erysiphe polygoni	*Ampelomyces quisqualis*	Yarwood 1932
Microsphaera euonymi-laponicae	*Ampelomyces quisqualis*	Yukawa et al. 1971b
Sphaerotheca fuliginea	*Ampelomyces quisqualis*	Jarvis & Slingsby 1977
Sphaerotheca fuliginea	*Tilletiopsis* sp.	Hoch & Provvidenti 1979
Sphaerotheca fuliginea	*Tilletiopsis albescens* *Aphanocladium album* *Ampelomyces quisqualis* + 13 other fungi	Hijwegen 1988
Sphaerotheca fuliginea	*Tilletiopsis minor*	Hijwegen 1986
Sphaerotheca fuliginea	*Acremonium alternatum*	Malathrakis 1987

Table 2. Mean severity rating[1] of powdery mildew of cucumber in a greenhouse following treatment with *Sporothix flocculosa* and microfine sulfur.

	Days after transplanting										
Treatment	13	19	26	33	40	47	54	61	68	75	82
S. flocculosa	0	0	0	0	0	0.02	0.05	0.36	1.31	2.93	5.00
Sulfur	0	0	0	0	0	0	0	0	0.14	0.54	–

[1]Rating on 0-5 scale: 0= no powdery mildew, 5= leaves completely covered.

Table 3. Mean severity rating[1] of powdery mildew of cucumber in three greenhouses at ambient relativehumidity and two controlledrelative humidities (RH%) and following treatment with *Sporothrix flocculosa,* and *S. rugulosa.*

Treatment	RH%	Days after transplanting					
		0	7	14	21	28	35
S. flocculosa	ambient	1.00	1.06	1.56	1.88	3.50	5.00
	60–65	1.00	1.00	0.93	0.94	1.40	2.10
	80–85	0.75	0.75	0.81	0.77	0.72	1.40
S. rugulosa	ambient	1.00	1.38	1.25	2.02	3.40	5.00
	60–65	0.93	1.06	1.06	1.67	2.34	3.10
	80–85	0.93	1.00	1.00	0.81	1.09	1.90
Check	ambient	0.93	1.12	1.56	2.41	3.70	5.00
	60–65	1.00	1.00	1.13	1.40	2.19	2.70
	80–85	0.93	1.00	0.93	0.96	0.96	2.00

[1]Rating on 0.5 scale; 0 = powdery mildew, 5 = leaves completely covered.

Table 4. Colony-forming units (cfu) of *Sporothrix flocculosa* on discs from leaves and fruit skin of cucumber sprayed at 2-week intervals.

	Days after transplanting				
	0	14	28	42	56
Leaf discs	1.04	116.6	289.6	786.3	1306.3
Fruit skin discs	–	–	–	–	1333.7
Check, leaf discs	0	3.1	0	3.1	3.1
Check, fruit skin discs	–	–	–	–	0
Sulfur-treated leaf discs	0	2.1	5.0	0	7.25
Sulfur-treated fruit skin discs	–	–	–	–	4.13
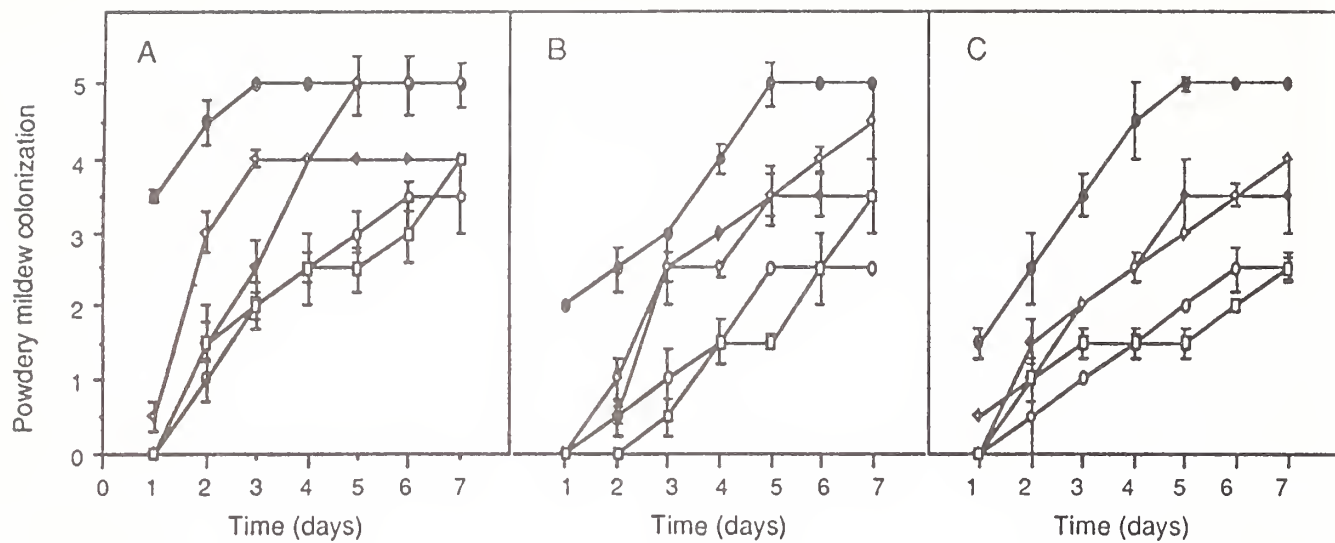
Fig. 1. Effect of temperature on colonization of <u>Sphaerotheca pannosa</u> f. sp. <u>rosae</u> by **A**. <u>Stephanoascus flocculosus</u>, **B**. <u>St</u>. <u>rugulosus</u>, and **C**. <u>Tilletiopsis washingtonensis</u>. O-O = 18°C, ◆-◆ = 22°C, ●-● = 26°C, ◇-◇ = 30°C, and ☐-☐ = 34°C. Vertical bars represent standard error of the mean.
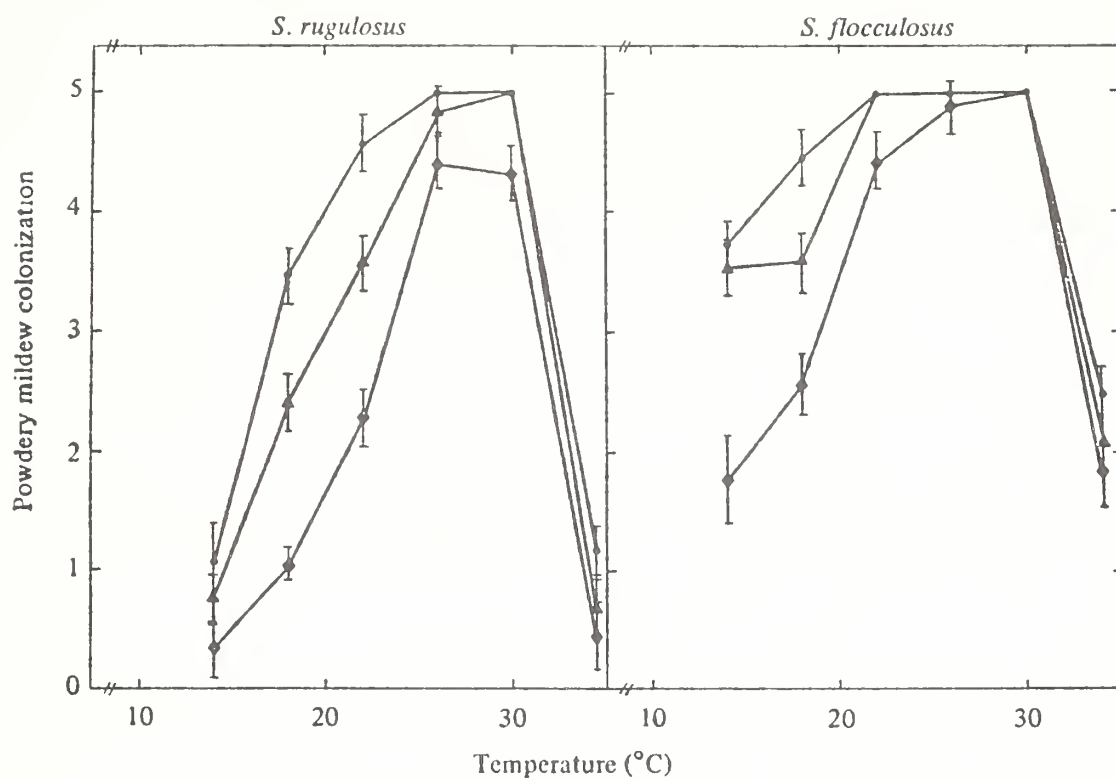
Fig. 2. Influence of temperature on colonization of *Sphaerotheca fuliginea* by *S. flocculosus* and *S. rugulosus*. Points represent the mean colonization rating with standard deviation bars. ◆—◆ = 24 h, ▲—▲ = 48 h, ●—● = 72 h.
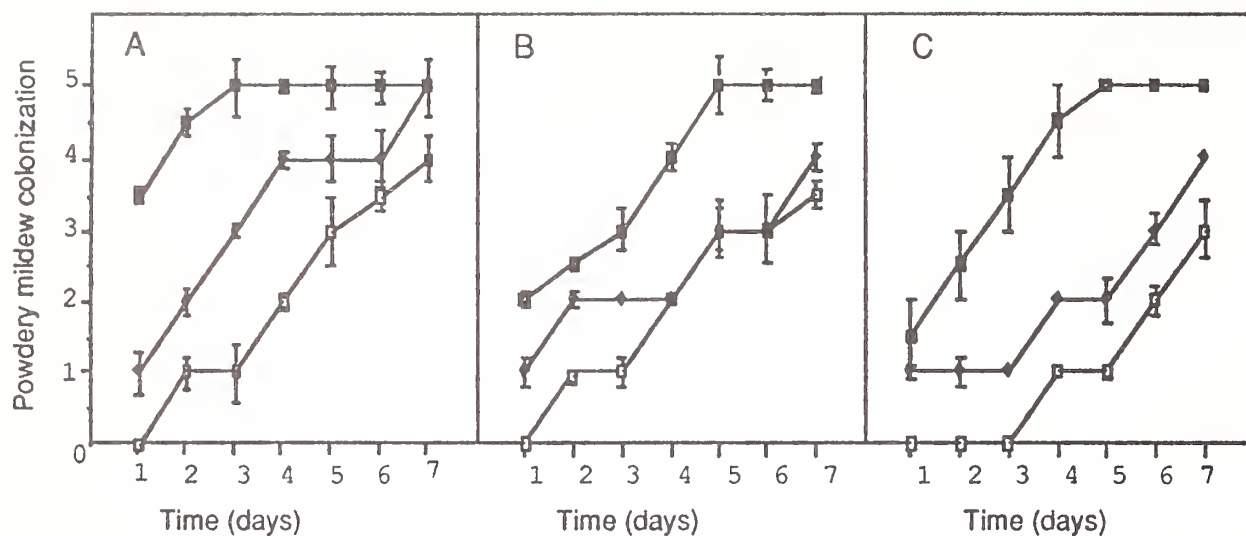
Fig. 3. Effect of relative humidity on colonization of Sphaerotheca pannosa f. sp. rosae by A. Stephanoascus flocculosus, B. St. rugulosus, and C. Tilletiopsis washingtonensis. ■—■ = 90%, ◆—◆ = 80%, and □—□ = 70%. Vertical bars represent standard error of the mean.
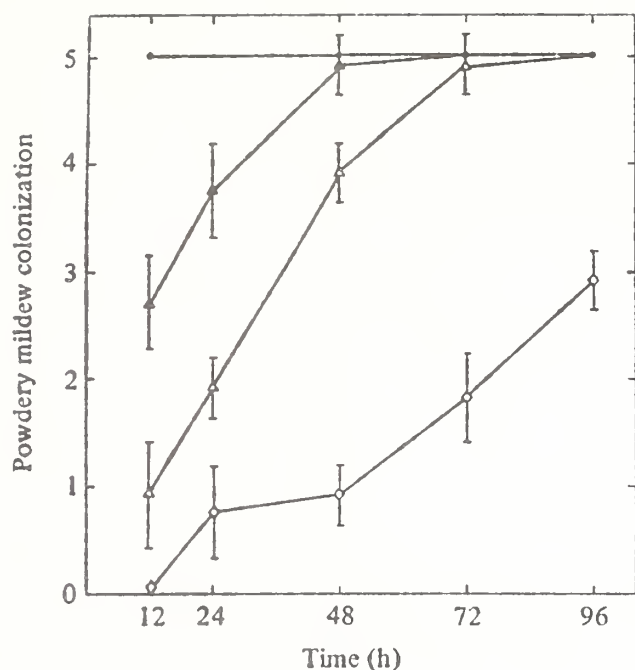
Fig. 4. Influence of relative humidity on colonization of *Sphaerotheca fuliginea* by *S. flocculosus*. Points represent the mean colonization rating with standard deviation bars. ●—● = 100% r.h., ▲—▲ = 90% r.h., △—△ = 80%., ◇—◇ = 70% r.h.
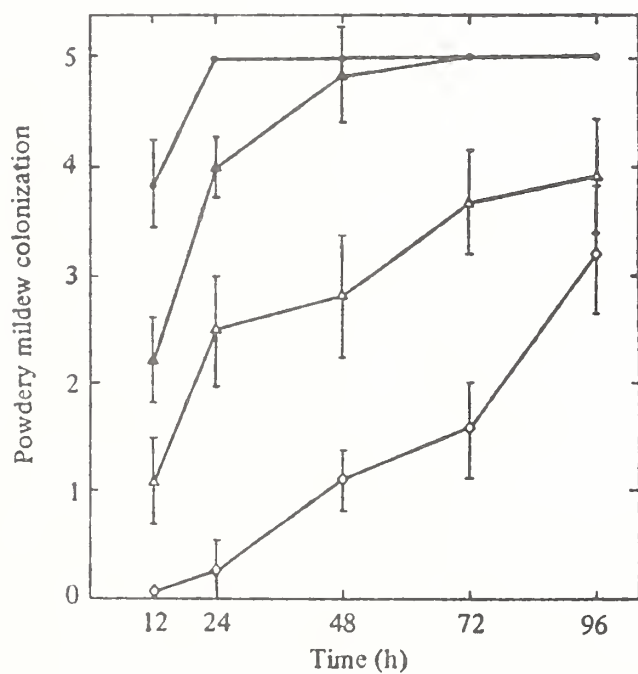
Fig. 5. Influence of relative humidity on colonization of *Sphaerotheca fuliginea* by *S. rugulosus*. Points represent the mean colonization rating with standard deviation bars. ●—● = 100% r.h. ▲—▲ = 90% r.h., △—△ = 80%., ◇—◇ = 70% r.h.

Preharvest and Postharvest Biological Control of *Rhizopus* and *Botrytis* Bunch Rots of Table Grapes with Antagonistic Yeasts

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Abstract

The yeasts *Pichia guilliermondii* and *Hanseniaspora uvarum* were effective in the control of *Rhizopus stolonifer* and *Botrytis cinerea* rots and of other postharvest diseases of grapes. Dipping injured or non-injured berries in a 48-hour culture of the antagonists protected the berries against subsequent inoculation with the pathogens. The antagonists were particularly effective in protecting naturally infected whole clusters of grapes against decay by both pathogens, either alone or in combination with each other or other rot-causing fungi. The effectiveness of the antagonists when applied as a postharvest dip, was not substantially reduced during 1 to 2 weeks of storage of 0°C after treatment. *P. guilliermondii* was also effective in reducing postharvest decay of grapes when applied in the vineyard, as a preharvest spray, 3 days before harvest. A more premature application was less effective and prolonged storage at 0°C enabled *B. cinerea* to overcome the effect of the antagonist. Neither yeast antagonist appeared to inhibit the *Rhizopus* fungus in culture by secreting antibiotics into the growth medium and their mode of action is yet to be fully elucidated.

Introduction

A bunch rot of grapes, *Vitis vinifera* L., caused by *Rhizopus stolonifer* (Ehr. ex Fr.) Lind has become prevalent in Israel on most table grape cultivars (Barkai-Golan, 1981). Whereas most grape bunch rot fungi, among which *Botrytis cinerea* Pers. predominates (Nelson, 1979), can be controlled by fumigation with sulfur dioxide (Nelson, et al., 1967), the effective dose for the control of *R. stolonifer* is often phytotoxic and causes bleaching of the berries. Moreover, the application of this chemical in foods has recently been banned for most commodities. Grapes have not so far been included in the ban, but it is likely that they will be in the near future, particularly in light of increased public concern regarding pesticide contamination of food. While the storage of grapes at 0°C inhibits *Rhizopus* development (Matsumoto, et al., 1967), any interruption in the cold storage chain and the transfer of fruit from storage to the shelf is accompanied by rapid development of *Rhizopus* rot. *B. cinerea* continues to develop at 0°C and may therefore predominate over *R. stolonifer* after storage, in spite of the more rapid development of the latter at temperatures above 0°C (Nelson, 1979). It is obvious, therefore, that innovative control measures of postharvest diseases of grapes are needed.

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Biological control of postharvest diseases of fruits has met with success with *Monilinia* and *Rhizopus* rots on peaches (Pusey, et al., 1986; Wilson, et al., 1985) and with *Penicillium* blue-mold on apples (Janisiewicz, 1987). We have recently investigated biological control of postharvest diseases of citrus fruits. Among the antagonists studied, a yeast *Pichia guilliermondii*, previously known as *Debaryomyces hansenii* (Zopf) van Rij. isolated from the surface of lemon fruit, had characteristics that made it a good candidate as a biological control agent for postharvest diseases, since it was highly effective, under laboratory conditions, against different citrus wound pathogens - *Penicillium digitatum* Sacc., *P. italicum* Wehmer and *Geotrichum candidum* LR. ex. Pers. In addition, it did not seem to produce antibiotics in culture against the pathogens and, as far as is known, it does not have related species that produce plant diseases. Another yeast species, which has been isolated from the fruit surface of grapes, is *Hanseniaspora uvarum*. This investigation was undertaken to test the effectiveness of both yeasts as biocontrol agents of the major postharvest diseases of grapes, *R. stolonifer* and *B. cinerea*.

Materials and Methods

Postharvest treatments: To test the effectiveness of *P. guilliermondii* (designated US-7) and *H. uvarum* (designated G-4) in inhibiting rot development in grapes, the following procedure was used. The antagonists were incubated in 100 ml nutrient yeast dextrose broth (NYDB) in 250 ml Erlenmeyer flasks on a rotary shaker (100 rpm) at 28°C for 48 hr. Freshly harvested grapes, of the Perlette and Thompson Seedless cultivars, were dipped momentarily in the yeast-NYDB mixture, either as whole clusters with non-injured berries, or as individual berries which had been removed from the stems by pulling, thereby causing a wound. In initial tests with single berries, other types of artificial wounds were tested, such as piercing non-injured berries with a needle. One to two h after the berries had been dipped in the antagonist preparation and dried, they were inoculated by dipping in the pathogen spore suspension (10^4 spores per ml), or by placing a single decayed berry in the center of a group of non-injured berries ("nesting"). In some experiments, however, artificial inoculation was not used since the control, non-treated grapes developed a sufficiently high incidence of *Rhizopus* or *Botrytis* decay by natural infections originating in the vineyard. The treated berries were placed in small plastic baskets, held in polyethylene-covered cartons. Whole treated clusters were placed directly in commercial shipping cartons, lined with perforated polyethylene bags, which were folded over the fruit. Incubation was carried out at room temperature or in cold storage, as indicated. Decay incidence was determined by counting the number of infected berries. Each treatment in each experiment consisted of at least three replicates of 20 berries or four replicates of five intact clusters, placed in half of a shipping carton.

Results

Results of initial trials indicated that *P. guilliermondii* was effective in reducing the incidence of *R. stolonifer* and *B. cinerea* rots both in injured and in non-injured grape berries (Table 1). Reduction of decay by the yeast antagonist was most pronounced in berries that were not injured prior to inoculation and were inoculated by placing a decayed berry amongst non-injured berries.

Hanseniaspora uvarum

Following extensive screening tests a second effective yeast antagonist, *H. uvarum*, was isolated from the surface of grape berries. The effectiveness of this antagonist was similar to that of *P. guilliermondii* in reducing *Rhizopus* rot in single injured berries, infected naturally or by nesting (Table 2). Their effectiveness in decay reduction was particularly high in tests carried out with whole cluster. In this non-injured fruit, a dip of intact clusters in either suspension prevented increase in the incidence of decay beyond 3% irrespective of the natural causal organism (Table 3). Storing the treated fruit for 1 or 2 weeks at 0°C did not reduce substantially the effectiveness of the antagonists in decay reduction.

P. guilliermondii was also effective in inhibiting postharvest decay, when applied to grapes as a preharvest treatment (Table 4). In non-injured fruit of whole clusters, such a treatment reduced both natural infections arising from the vineyard, and *R. stolonifer* incidence in berries which were inoculated by "nesting" after harvest.

Longevity of Effectiveness

The longevity of the effectiveness of the preharvest spray was examined by spraying "Shami" vines 10, 7 and 3 days prior to harvest with a suspension of *P. guilliermondii*. The most prevalent decay organism was *B. cinerea*, the incidence of which was reduced significantly, on fruit stored at 20°C for 7 days, by all three spray treatments (Figure 1A). Treatment 3 days prior to harvest was somewhat more effective than earlier sprays. However, during 4 weeks' storage at 0°C, the incidence of *Botrytis* rot increased considerably and none of the treatments retained their effectiveness (Figure 1B),. On the other hand, further development of bunch rot in the vineyard was arrested, during 2 weeks following harvest (Figure 2).

Enhancing Efficacy

An attempt was made to increase the efficacy of the preharvest spray by multiple applications in a *Rhizopus* infected vineyard. A single application 3 days prior to harvest was shown to be as effective as three sprays, and the incidence of *Rhizopus* decay in both treatments did not differ significantly from that of a fungicide treatment (Table 5). However, when the fruit was harvested 2 weeks after the last spray application, the effectiveness of decay control declined considerably.

No Antibiosis

Neither yeast antagonist appeared to inhibit *Rhizopus* rot by antibiotic action. When tested in culture by placing plugs of a 48-h-old yeast-NYDA culture on culture plates on which the pathogen was grown, no inhibition zone was observed. However, when the same procedure was used to test the antibiotic action of another bacterial antagonist, designated I-16, such a zone

was clearly evident. In addition, direct microscopic examinations revealed that *P. guilliermondii* did not affect spore germination of mycelial growth of the pathogens in culture.

Discussion

The results show that *P. guilliermondii*, a yeast antagonist of postharvest diseases of citrus fruit, was also effective in inhibiting *R. stolonifer* and *B. cinerea* rots of grapes and other minor grape postharvest fungi. In addition, another yeast antagonist, *H. uvarum*, which was isolated from grapes, was found to be equally effective.

Postharvest Dip The antagonists protected injured grape berries but were particularly effective in reducing incidence of decay of naturally infected, non-injured grapes when applied as a postharvest dip. The results of most tests indicated an increased incidence of decay in the NYDB-treated, control fruit. This phenomenon resulted from the rich nutritional environment provided by this treatment to the surface of the berry, thus enhancing the development of rot-causing grape pathogens. Nonetheless, the antagonist-NYDB preparation reduced decay effectively, even when compared with the water-treated control.

Commercial Handling Of particular interest are the results presented in Table 3, which demonstrate the effect of the antagonists under conditions that may be encountered during commercial handling of table grapes. Under such conditions, the antagonists were highly effective in reducing decay. However, one of the important quality parameters of table grapes is the presence of a bloom on the surface of the berry. The postharvest dip in the antagonist preparation removes some of the bloom, thereby adversely affecting the perceived quality of the treated fruit. For this reason, we tested the possibility of treating the fruit with an antagonist before harvest.

Vineyard Application A spray in the vineyard 3 days prior to harvest was found to have no adverse effect on the appearance of the fruit, but was effective in controlling the postharvest development of decay caused by both *R. stolonifer* and *B. cinerea*, under certain conditions. The treatment became less effective if the time between application and harvest was extended, or if the fruit was stored for more than 2 weeks at a temperature which allowed pathogen growth. Although *R. stolonifer* does not develop at 0°C (Matsumoto, et al., 1967), *B. cinerea* continues to do so (Nelson, et al., 1967). Thus, with a heavy natural vineyard infection by the latter pathogen, the preharvest yeast spray, which was effective for a short postharvest storage period at 20°C, lost its effectiveness after 4 weeks' storage at 0°C.

The continued effect of *P. guilliermondii* observed in the vineyard after harvest, suggests that this antagonist might also be an effective control agent for vineyard diseases, and this aspect will be investigated further.

Mode of Action The mode of action of the yeast antagonists in the inhibition of postharvest diseases of grapes or citrus fruit is being

investigated, but has not been fully elucidated. While antibiotic action is not likely to play a role, since no inhibition of *Rhizopus* or *Botrytis* by *P. guilliermondii* could be observed in culture, other possibilities – such as direct effects of the antagonist on the pathogen, induced host resistance, and competition between the antagonist and the pathogen for nutrients or space – all appear to be plausible. The mode of action of the yeast antagonists will have to be understood before the full potential of this biocontrol procedure can be assessed.

Acknowledgment

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Table 1. Inhibition of *Rhizopus stolonifer* and Botrytis cinerea rots of grapes by the yeast antagonist *Pichia guilliermondii*.

Treatment		Mode of inoculation	Incidence of decay (%)	
			Rhizopus stolonifer	*Botrytis cinerea*
Non-injured	control	None	16	39
	protected	None	14	0x
Non-injured	control	Nesting	60	79
	protected	Nesting	6x	27x
Injured(pierced)	control	Spore suspension	98	–
	protected	Spore suspension	40x	–
Injured(pulled)	control	Spore suspension	58	88
	protected	Spore suspension	11x	35x

Freshly harvested single berries were dipped in sterile NYDB (control) or in a 48-h culture of the yeast antagonist (protected) and then placed in plastic trays at room temperature for 5 days. Incidence of decay was determined by counting single berries.

*Differs significantly from the paired control at P=0.05.

Table 2. Inhibition of Rhizopus rot of grapes by the yeast antagonists *Pichia guilliermondii* (US-7) and *Hanseniaspora uvarum* (G-4).

Treatment	Single berries (Injured)	
	Natural infection	Inoculation by "nesting"
	Incidence of decay (%)	
Water	23a	78a
Sterile medium (NYDB)	68a	92a
US-7	8b	13b
G-4	13b	15b

Single berries were pulled from freshly harvested cultures and dipped in a 48-h-old yeast antagonist culture. Some of the berries were then inoculated by placing a single decayed berry inside a small plastic basket which contained 20 healthy berries ("nesting"). Incidence of decay was evaluated after 5 days at room temperature.

Within columns, values followed by different letters are significantly different (P-0.05) according to Duncan's multiple range test.

Table 3. Inhibition of postharvest rots of grapes by the yeast antagonists *Pichia guilliermondii* (US-7) and *Hanseniaspora uvarum* (G-4).

Treatment	Incidence of decay (%)[x]	
	Experiment #1	Experiment #2
Non-treated control	17b	6bc
Water	22b	11b
Sterile medium (NYDB)	48a	25a
US-7	2c	1c
G-4	3c	2c

Freshly harvested whole grape clusters were dipped in a 48-h-old antagonist culture or in the appropriate controls. The clusters were then placed, without artificial inoculation, in commercial shipping cartons and kept at 17°C for 8 days. Decay in Experiment #1 was caused mostly by *Rhizopus*, while that in Experiment #2 was caused mostly by *Botrytis* and, to a lesser extent, by *Aspergillus* and *Rhizopus*.

[x]Within columns, values followed by different letters are significantly different (P=0.05) according to Duncan's multiple range test.

Table 4. Inhibition of postharvest decay of "Perlette" grapes by preharvest treatments with the yeast antagonist *Pichia guilliermondii.*

Treatment	Whole clusters (natural infection)	Single berries (inoculated with *Rhizopus* by nesting)
	Incidence of decay (%)[x]	
Control-untreated	6.6a	23a
Water	8.4a	–
P. guilliermondii	3.2b	10b

Grape clusters were sprayed in the vineyard with a 48-h-old culture of the yeast antagonist. Control fruit was not treated or sprayed with water. Three days later, the clusters were picked and placed in storage at 20°C for 7 days and then evaluated for decay development. Berries of other vine-treated clusters were pulled and placed, immediately after harvest, in plastic baskets with one Rhizopus-infected berry placed near every 20 berries. Incidence of decay was evaluated in single berries after 5 days at room temperature.

[x]Within columns, values followed by different letters are significantly different (P=0.05) according to Duncan's multiple range test.

Table 5. Inhibition of *Rhizopus stolonifer* postharvest decay of
'Thompson Seedless' grapes by preharvest treatments with *Pichia
guilliermondii* compared with a fungicide spray.

| Treatment[w] | No. of applications[y] | Incidence of decay (berries/kg)[x] | |
		Harvest I[z]	Harvest II
Control – H_2O	1	31.2a	27.0a
P. guilliermondii	1	13b	15.0ab
	3	9.6b	10.5b
Anvil 0.05%	1	6.1b	3.1b

[w] – 0.02% Tween-20 added to water and yeast sprays.

[y] – Spray dates: 10/VII/90, 12/VII/90, 15/VII/90. Single yeast spray
– 15/VII/90, Anvil – 10/VII/90.

[z] – Harvest dates: I – 17/VII/90 (artificially infected). II –
24/VII/90 (not infected).

[x] – Eight replicaters of 2 kg of fruit were packed in polyethylene
lined cartons at each harvest. The incidence of decay was
evaluated by counting the number of rotten berries after 2 days
at 0°C and 3 days at 20°C. More than 90% of the decay was caused
by *R. stolonifer*.

a-b Within columns, values followed by a common letter do not differ
significantly (P=0.05) according to Duncan's multiple range test.
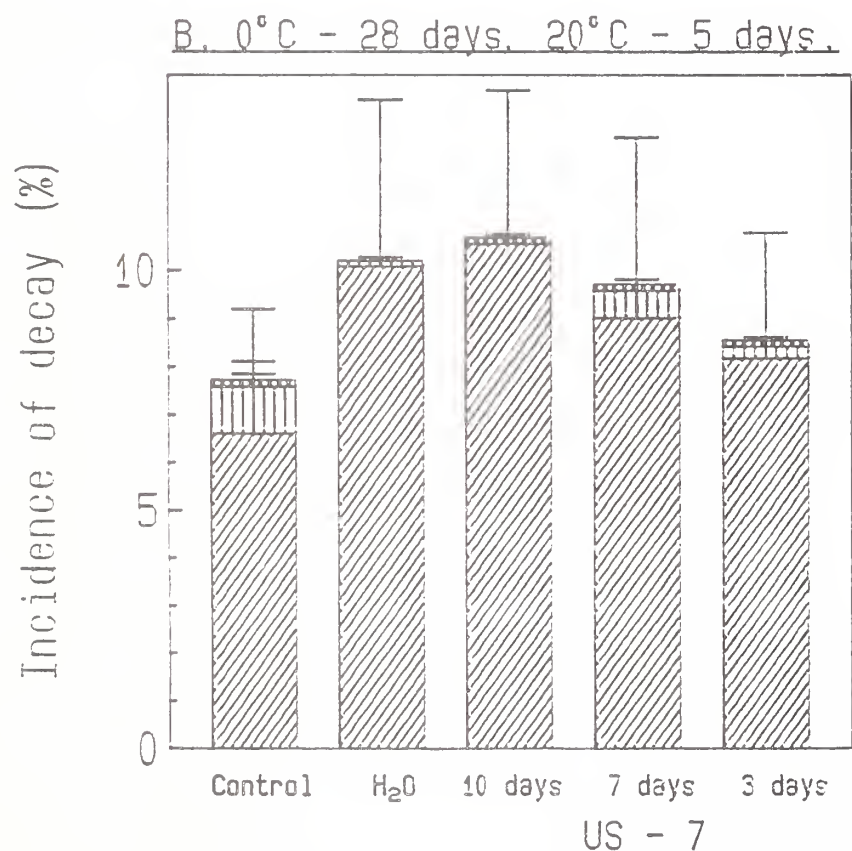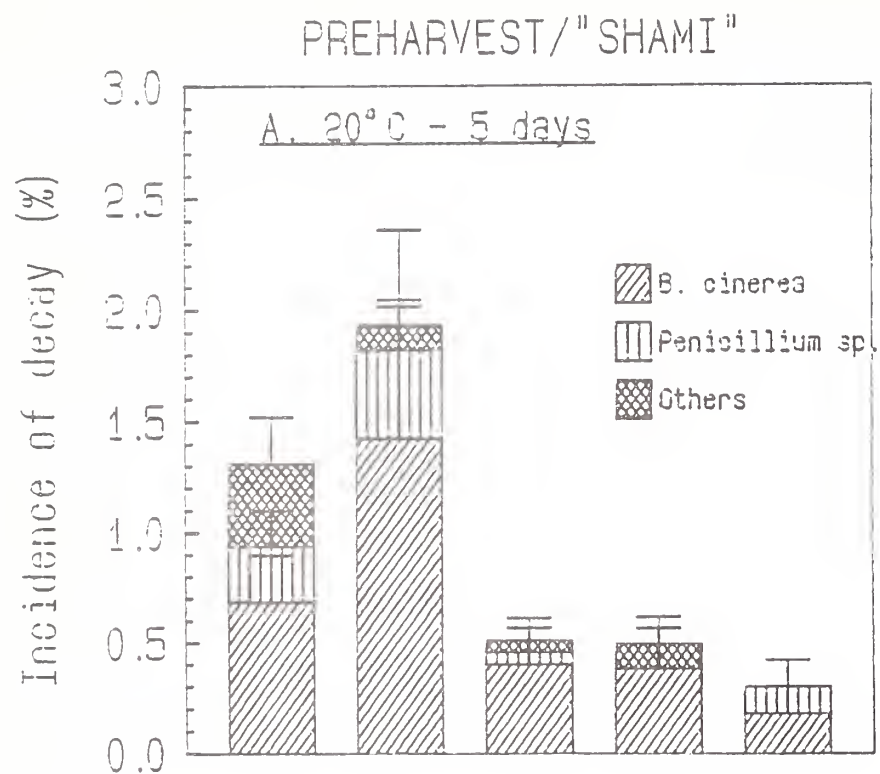
FIG.1. Preharvest application of *Pichia guilliermondii* to control natural
postharvest decay of "Shami" grapes.
   A. After 5 days of 20°C.
   B. After 4 weeks at 0°C plus 5 days at 20°C.
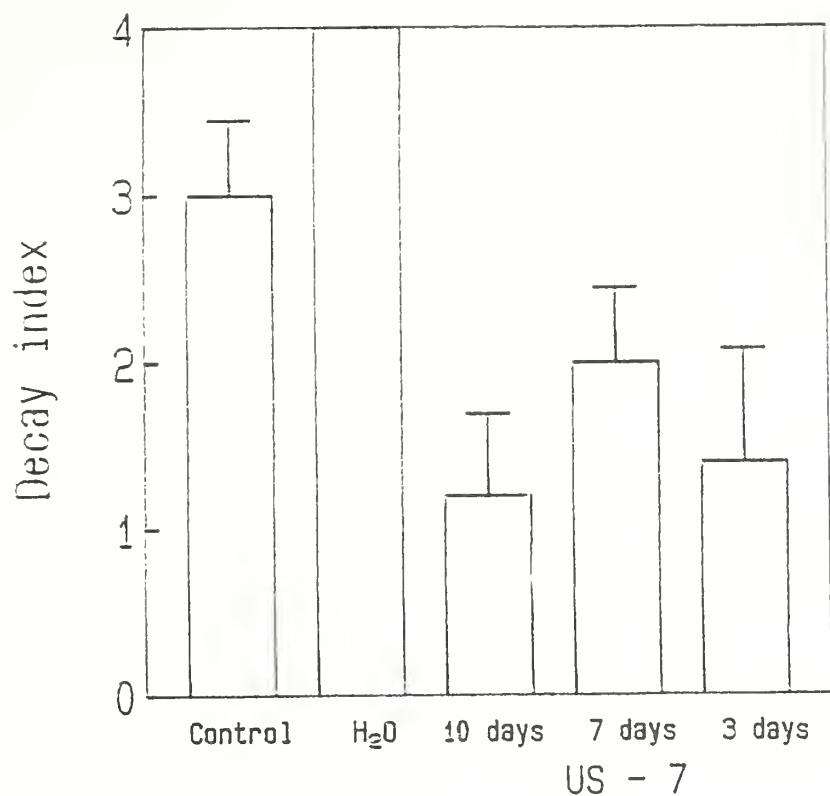
FIG.2.   Decay index^z on "Shami" grape clusters in the vineyard.   2 weeks after
         spray application of *Pichia guilliermondii*.

            ^{z} Index: 1 – one decayed berry/cluster.
                              2 – 2–10 decayed berries/cluster.
                              3 – 11–20 decayed berries/cluster.
                              4 – >20 decayed berries/cluster.

Biocontrol of Postharvest Bacterial Diseases of Fruits and Vegetables

H. E. Moline[1]

Abstract

Biocontrol agents discovered with activity against postharvest bacterial diseases to date have been limited to two genera of bacteria, *Erwinia* and *Pseudomonas*. *Pseudomonas fluorescens* and *P. putida* strains have been identified that are antagonistic to *Erwinia carotovora*, causing bacterial soft rot (BSR) of potato. *Erwinia cypripedii* was also shown to be antagonistic to *E. carotovora* using carrots, tomatoes, and peppers as test hosts. *Erwinia herbicola* has been shown to be antagonistic to *E. amylovora*, which causes fire blight of apples and pears, but this antagonism has not been shown to be sufficient to be of economic value.

Introduction

Smith (1919) is generally credited with being the first to use the term biological control to describe the introduction of exotic natural enemies for the suppression of insect pests. However, the first well publicized natural predator introduction was accomplished in 1888, the use of the vedalia beetle to control the cottony cushion scale (Doutt, 1958). General biological control has been operational as long as pests and antagonists have. It has helped to maintain biological balance in the ecosystem. In the absence of conscious recognition of this natural antagonism many cultural practices may have served to reduce or eliminate this component of crop disease management (Baker and Cook, 1974).

The term biocontrol has been applied widely to include the spectrum of pest control measures other than the application of chemicals. In spite of more than 60 years of research on biocontrol of plant pathogens, few commercial applications of biocontrol agents have been marketed to date. This is not meant to imply that there is not a market for potential biocontrol agents, but development of new agents will continue to be painfully slow.

The best example of the effective exploitation of the profusion of interactions and mutual antagonisms that occur among populations of microorganisms in the soil environment is the biological control of crown gall (Garrett, 1988). Biocontrol is effected by an antagonist, *Agrobacterium radiobacter*, which actively competes with the pathogen *A. tumefaciens*, in the infection court, in addition to producing an unusual type of antibiotic that selectively inhibits most pathogenic agrobacteria (Kerr, 1980).

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Among bacteria isolated from the rhizosphere a wide variety of genera have been shown to have the potential to suppress diseases caused by a number of soil-borne plant pathogens (Schippers, 1988). Some of these, especially *Pseudomonas* spp. and *Bacillus* spp, have been shown to significantly suppress disease and increase yield of crops in field trials (Schippers et al., 1987). The role of fluorescent *Pseudomonas* spp. in soils naturally suppressive to diseases has been well documented (Baker et al., 1986).

A number of mechanisms appear to be responsible for the interaction between biocontrol agents and target pathogens. Antibiotics produced by the biocontrol agent actively inhibit the growth of some pathogens. Competition for substrate at the wound site has been shown to favor biocontrol agents in a number of cases. In some cases this substrate competition includes the ability to tie up iron (Fe^{3+}), making it difficult for the pathogen to survive in the microenvironment (Leong, 1986; Neilands, 1981). Induction of host resistance (induced resistance) may function in combination with other modes of resistance, especially in the case of postharvest disease responses, where wound pathogens are a major cause of decay and the senescent nature of substrate reduces its potential to respond. In many instances the antagonism may be the result of a complex of the above interactions (Wilson et al., 1989).

The potential market for antibacterial microbials may be stronger than that for antifungal agents, due to the scarcity of suitable and effective bactericides for use against many bacterial pathogens (Jutsum, 1988).

Bacterial Soft Rot   Bacterial soft rot, caused by *Erwinia carotovora*, is one of the most serious postharvest diseases of fruits and vegetables. It is responsible for most transit and storage losses of potatoes, tomatoes, and leafy vegetables.

Potatoes   Potatoes: Bacterial soft rot is the major cause of postharvest losses of potatoes. The causal bacteria (*Erwinia carotovora*) is always present in high numbers on the surface and within lenticels of healthy tubers and can rapidly decay bruised tissue. The search for biocontrol gents to reduce the incidence of bacterial soft rot (BSR) of potatoes has been underway for a number of years (Table 1). Burr et al., (1978) and Kloepper (1983) demonstrated that potato yield can be increased significantly by treatment of seed pieces with *Pseudomonas fluorescens* or *P. putida* after harvest significantly reduced postharvest tuber decay. They also observed increased yield after treatment of seed pieces prior to planting, which they attributed to reduced incidence of pre- and postharvest BSR. Postharvest tuber treatment resulted in a greater reduction of soft rot, although *P. putida* was able to colonize tubers as a result of seed piece inoculation. They recovered 10^4 colony forming units (CFU) per gram of peel 5 days after postharvest treatments, compared with populations of 50 cfu per gram of peel on daughter tubers grown from seed pieces treated prior to planting. Since Pseudomonads are recognized as the largest group of microorganisms that produce antibiotics, these investigators postulated that the reduction in bacterial soft rot severity may be a result of abiosis and

competition. McGuire and Kelman (1984) demonstrated that there was a relationship between calcium content of potato tubers and BSR. Their studies indicate that tubers containing higher calcium content had significantly less decay than untreated tubers.

Tomatoes & Peppers

Tomatoes & Peppers: Our survey of microorganisms found on the surface of tomato and bell pepper fruit has resulted in the isolation of two bacterial isolates having antagonistic action against a number of *Erwinia carotovora* isolates causing BSR. Microbes used in the study were obtained by taking washings from tomatoes and peppers. Dilutions of wash water were plated onto potato dextrose agar (PDA), acid PDA, and nutrient agar. Initial screening of microflora was accomplished using 13 mm diameter carrot slices that were aseptically removed from carrots with a cork borer. Bacterial and yeast isolates screened for antagonistic behavior were cultured on nutrient agar and 24-48 hr cultures were suspended in sterile distilled water at a concentration of 10^8 colony forming units (cfu) per ml. Carrot discs were dipped in this suspension and placed on water agar in plastic petri dishes. Positive results were indicated by failure of disks to become macerated when subsequently inoculated with two drops of a water suspension containing 10^7 cfu per ml of soft rotting isolates of *E. carotovora*. Carrot disks dipped in sterile distilled water and challenged with *E. carotovora* were macerated within 2 days at 20°C. Decay ratings were made on a 0 - 5 scale with 0 indicating no decay and 5 indicating maceration of the disks. The two isolates that were antagonistic to *E. carotovora* were tested for antibiotic activity by dipping carrot slices in heat killed spore suspensions of the antagonists. Samples treated in this manner were not antagonistic to any *E. carotovora* isolates.

Subsequent tests were carried out to further evaluate those cultures demonstrating antagonistic activity against *E. carotovora* in carrot disk bioassay using whole tomato and pepper fruits. Fruits were puncture wounded on four sides and antagonists were placed in the wounds with a pasteur pipette at the same concentration used for carrot disk assays. After allowing the fruit to dry for two hours 10^7 cfu per ml *E. carotovora* cells were placed in the wounds with a pasteur pipette. Fruit not treated with antagonists were pretreated with sterile distilled water, incubated two hours, and challenged with *E. carotovora*. Treated fruits were incubated at 20°C for 2 - 3 days and decay was rated on a 0 - 5 scale similar to the carrot disks.

Preliminary results indicate that while the antagonists do not completely eliminate bacterial soft rot they do significantly reduce decay (Table 2). Studies are being conducted testing additional strains of *E. cypripedii* as well as strains of *E. herbicola* for possible and antagonistic action against BSR.

In addition to testing tomato and pepper microflora for biocontrol activity, a number of other previously reported biocontrol agents were screened for activity against the soft rot pathogens using the carrot disk assay. Those tested

included *Bacillus subtilis* (Pusey & Wilson, 1984), *Pichia guilliermondii* (Chalutz et al., 1988), *Pseudomonas cepacia* (Janisiewicz, 1987), and Pseudomonads isolated from suppressive soils (Cook & Rovira, 1976). None of these antagonists demonstrated any antibacterial activity in our screening tests.

Fire Blight–
Apples

Apples: Although fire blight is generally considered to be a disease affecting apple and pear trees, because of import restrictions on fruit coming from areas where it occurs it has serious postharvest implications (Dueck, 1974; Van der Zwet et al., 1990). Therefore, a brief discussion of the problem is included.

In 1928 Rosen reported that a yellow bacterium isolated from a fire blight canker was inhibitory to *Erwinia amylovora*. The bacterium was subsequently identified as a strain of *E. herbicola*. Riggle and Klos (1972) studied an *E. herbicola* strain that they isolated from fire blight cankers in association with *E. amylovora* and found it offered partial control of fire blight. Since *E. herbicola* consumed all organic nitrogen in a synthetic medium and reduced the pH to a level inhibitory to *E. amylovora* they postulated that it may exclude *E. amylovora* from infection corridors by competition for nutrients. Subsequent studies have been unable to demonstrate that inoculation with *E. herbicola* could provide a method to reduce the level of fire blight in orchards sufficient for commercial application. Two US research centers are currently investigating other antagonists as possible biocontrol agents against fire blight. They are the USDA, ARS, Appalachian Fruit Research Station at Kearneysville, W.V. and the USDA, ARS, Tree Fruit Research Laboratory at Wenatchee, WA.

There are a number of other postharvest bacterial diseases that may lend themselves to biocontrol, although most of them do not cause the magnitude of losses realized as a result of BSR

Conclusions

Researchers have investigated the use of natural antagonistic microflora for the control of only a very few bacterial postharvest diseases. However, biocontrol of other postharvest bacterial diseases may also merit investigation (Table 2). In all cases studied to date microbes identified as being antagonistic against bacterial pathogens have been bacteria. This does not preclude the possibility that other types of antagonistic microbes might be identified. A number of yeasts and fungi produce potent antibiotics which may be active against bacterial postharvest pathogens. Some of these agents have been discussed during these proceedings.

To date the most promising bacterial biocontrol discoveries have been with the use of Pseudomonads to increase yield and reduce bacterial soft rot of potatoes. Colyer and Mount (1984) postulate that the reduction of bacterial soft rot is effected by competition for substrate and abiosis. Kempe and Sequeira (1953) attributed the reduced BSR severity in potatoes in the presence of an antagonistic Pseudomonas strains to substrate competition. I found no evidence of abiosis in my evaluations of *E. cypripedii* strains and feel that their action against BSR may be due to substrate competition also. There is evidence

that *E. herbicola* strains antagonistic against plant pathogenic Erwinias may have abiosis and substrate competition as a basis for their antagonism (Gibbins, 1978).

Competition for substrate may produce very complex interactions between microflora of the soil. The ability of some bacteria to bind iron and remove iron from the soil, thereby limiting the growth of other bacteria has been intensively studied (Neilands, 1981). Some bacteria are also able to remove nitrogen from the soil and by doing so may limit the growth of pathogenic soil-borne bacteria (Veverka et al. 1988). These and other complex interactions between microflora in the soil may be responsible for limiting disease.

Application of bacterial biocontrol agents to fruits and vegetables, either pre- or postharvest, should not present an insurmountable problem. Techniques have been developed for foliar applications of bacterial biocontrol agents on a large scale that can serve as models for pre- or postharvest application to fruits and vegetables (Knudson & Spurr, 1988; Schroth et al., 1984).

Our limited laboratory screening tests may not be able to detect many potential antagonists that function in the environment. As we continue to search for biocontrol agents we will certainly learn a great deal more about the ecology of the soil-borne and foliar-fruit microflora. It is doubtful that any one biological will become the panacea of biocontrol, since the activity of those agents tested date appears to be quite specific. Its more likely that integrated control practices employing biocontrol agents and beneficial chemicals like calcium (Conway, 1989) will result from our studies of plant senescence, host-pathogen interactions, and biological control agents.

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Table 1. Potential biocontrol agents tested against postharvest
bacterial diseases.

Crop	Disease	Antagonist	Reference
Apple	Fire blight	*Erwinia herbicola*	Riggle & Kos, 1972
Mushroom	Brown blotch	*Pseudomonas fluorescens*	Nair & Fahy, 1972
Potatoes	Bact. soft rot	*P. putida*	Colyer & Mount,1984
"	"	" *P. putida*	Burr et al., 1978
"	"	" *P. fluorescens*	Burr et al., 1978
"	"	" " "	Kloepper, 1983
Tomatoes & Peppers	"	" *Erwinia cypripedii*	Moline, unpubl.

Table 2. Protection of carrot disks, tomatoes, and peppers from bacterial soft rot by preinoculation with *Erwinia cypripedii*.

Expt.	Preinoculated with *E. cypripedii*			Inoculated with *E. carotovora* only		
	No. of inoc.	% infected	sev.[z] index	No. of inoc.	% infected	sev index
Carrot disks	200	1.2	0.6	200	93	4.2
tomatoes	160[y]	9.3	1.1	160	96	4.4
peppers	80[y]	6.7	0.9	40	87	4.6

[z] The severity index was based on a 0-5 scale, 0 indicating no decay and 5 indicating completely decayed.

[y] four inoculations were made on each fruit.

Table 3. Postharvest bacterial disease candidates for biological control.

Crop	Disease	Pathogen
Celery	Bacterial Blight	*Pseudomonas appi*
Lima beans	Bacterial spot	*P. syringae*
Peas	Bacterial blight	*P. pisi*
Plums	Bacterial spot	*Xanthomonas pruni*
Potatoes	Brown rot	*P. solanacearum*
	Red xylem & Pink eye	*P. fluorescens*
	Ring rot	*Corynebacterium sepedonicum*
Sweet potatoes	Bacterial soft rot	*Erwinia chrysanthemi*

III. MODE OF ACTION OF BIOCONTROL AGENTS.

Through an understanding of the mode of action of biocontrol agents we can more intelligently select and utilize them.

Antibiosis as Mode of Action in Postharvest Biological Control

P. L. Pusey[1]

Abstract

The B-3 strain of *Bacillus subtilis* or its antifungal peptides, identified as iturin compounds, may have use in plant disease control. In response to a concern over the safety of using B. *subtilis* on harvested food, consideration is given to the possibility of using as biocontrol agents, or as a source of antifungal compounds, microorganisms that are involved in the production of fermented foods. These organisms or their products have been a part of man's diet for thousands of years. Also reviewed are antibiotics used in food preservation and plant disease control.

Iturin Produced by Bacillus subtilis

The B-3 strain of *Bacillus subtilis* (Ehrenberg) Cohn has been demonstrated to control postharvest brown rot of stone fruit under laboratory and simulated commercial conditions (Pusey and Wilson, 1984; Pusey et al., 1988). In initial screening tests which led to the discovery of B-3, a number of bacteria produced zones of inhibition when cultured on solid media with *Monilinia fructicola* (Wint.) Honey but had little or no effect against the fungus when tested on fruit. However, the B-3 bacterium proved effective both in culture and on fruit. Cell-free filtrates from culture of B-3 protected fruit from the pathogen and the filtrates retained activity even when autoclaved (Pusey and Wilson, 1984). Cells of B-3 that were separated from the culture broth were effective against fruit decay but autoclaved cells offered no protection. This indicated that living cells, applied to the fruit produce antifungal metabolites or are antagonistic toward the pathogen in some other way (e.g. compete for nutrients).

The biologically active substance produced by B-3 could be extracted from cultures by lowering the pH of the cell-free filtrate or supernatant to 2.5 and collecting the precipitate by centrifugation (McKeen et al., 1986). Purification was achieved by extraction in diethyl ether, HPLC with a C-18 reversed-phase absorbent, and by droplet countercurrent chromatography with chloroform+methanol+water (7+13+8 by volume) (Gueldner, 1988). Analytical procedures used to determine the structure of the peptides were mass spectrometry, nuclear magnetic resonance spectrometry, and IR spectrometry. The D or L forms of individual amino acids was determined by capillary gas chromatography.

Based on the analyses, it was concluded that the major antifungal peptides produced by B-3 are the same as the iturin antibiotics reported in France (Delcambe et al., 1976) and Japan (Isogai et al., 1982). Iturins are cyclic peptides made up of seven alpha-amino acids (two D-asparagine residues, L-asparagine, L-glutamine, L-proline, L-serine and D-tyrosine) and one beta- amino acid. The various beta-amino acids are referred to as iturinic acids.

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The iturin antibiotics are active against very few bacteria but have a wide antifungal spectrum. In clinical trials with animals and humans these peptides were shown to have potential value in the topical treatment of fungal skin diseases (Delcambe et al., 1976). They were of particular interest because of their low toxicity and lack of allergenic properties. According to a Japanese patent (Rikagaku, 1984), iturin A was effective against the plant diseases cucumber grey mold, cucumber anthracnose, rice blast and rice sheath blight.

Culture extracts containing the iturin antibiotics of B-3 were active against a wide range of plant pathogenic fungi (McKeen et al., 1986) (Table 1). Although the extracts were fungistatic rather than fungicidal against spores of M. *fructicola* (McKeen et al., 1986), scanning electron micrographs indicated that the cell walls and membranes of fungal hyphae on the fruit surface were degraded in the presence of B-3 (Hazen, unpublished).

Antibiotic-producing Microorganisms in Fermented Food

Food safety considerations: When research on the use of *B. subtilis* for postharvest control was presented to representatives of a produce marketing company and a large food chain in the United Kingdom, at their request, it was pointed out by a company microbiologist that *B. subtilis* had been associated with food poisoning. Forty-nine cases were reported in the U.K. and 15 cases in Australia and New Zealand (Kramer and Gilbert, 1989). The bacterium was implicated in these cases but was never proven to be the causal agent using Koch's postulates. At the time of the author's visit in the U.K., food safety and the problem of poisoning from organisms such as *Listeria* and *Salmonella* in meat products was a subject of national attention. Needless to say, the idea of using a bacterium such as *B. subtilis* on harvested food was not met with great enthusiasm. However, biocontrol as a general approach to combating postharvest spoilage was viewed as a valid alternative to the use of chemicals and one that should be explored.

It can be argued that *B. subtilis* as a species is quite diverse, with a wide variety of strains that produce many different metabolites (Ganesan and Hoch, 1984; Katz and Demain, 1977). Even though some strains might produce toxic substances, this does not mean that B-3 or other strains do. It should be noted, too, that in Japan a strain of *B. subtilis* is used in the food "natto," which is fermented soybeans. Regardless of whether the B-3 strain of *B. subtilis* is proven safe for human consumption, it was felt by company representatives in the U.K. that B-3 would not be acceptable because it has the same species name as bacteria that have been associated with food poisoning. Their concerns related primarily to protecting the company image and avoiding disapproval by a safety-conscious society.

The above experience led the author to seriously think about what microorganisms would be considered safe for human consumption, and thus, serve as acceptable candidates for postharvest biocontrol of plant diseases. Possibly the

organisms considered to be the safest are those that are already used in food. Familiar examples of food prepared by a fermentation process involving microorganisms include: cheese, yogurt, summer sausage, leavened bread, vinegar, soy sauce, sauerkraut and pickled cucumbers and olives. Food such as these consist of microorganisms or products of microorganisms that have been part of man's diet for thousands of years (Daeschel, 1989; Gilliland, 1985; Pederson, 1979; Rose, 1982). If we can find food fermentative organisms that are shown to have potential use in postharvest disease control, presumably safety would be much less of an issue and the probability of gaining approval with minimal or no toxicity testing should be relatively high.

Antibiotics Produced by Fermentative Bacteria

Throughout history fermentation has been one of the most important methods of preserving foods (Gilliland, 1985; Pederson, 1979; Rose, 1982). Organisms involved in the fermentation process produce substances that are inhibitory to spoilage organisms. These substances include organic acids, alcohols, peroxides, diacetyl, secondary reaction products and antibiotics. Most of the known antibiotics produced the lactic acid bacteria (Table 3.) inhibit only other bacteria. However, reuterin has a broad spectrum of activity which includes fungi (Daeschel, 1989).

For more than 40 years, research has been conducted on the use of lactic acid bacteria and/or their products to enhance the preservation of foods that normally do not contain such organisms. Hirsch et al. (1951) were the first to evaluate the application of nisin to Swiss cheese, where it effectively prevented blowing (gassing) attributable to the growth of clostridia. Nisin is now used as a food preservative on a variety of fresh and processed foods in many countries (Jay, 1983); Daeschel, 1989; Wagner and Moberg, 1989). Another example of success concerns a product called Microgard (Wesman Foods, Inc., Beaverton, Ore.) which is grade A skim milk that has been fermented by *Propionibacterium shermanii* and then pasteurized (Weber and Broich, 1986). Initial studies (Salih, 1985; Weber and Broich, 1986) demonstrated that this product prolonged the shelf life of cottage cheese by inhibiting psychotropic spoilage bacteria. Microgard is approved for use by the Food and Drug Administration and is currently added to about 30% of the cottage cheese produced in the United States (Daeschel, 1989). It is inhibitory to Gram-negative bacteria, some yeasts, and some molds but not Gram-positive bacteria (Al-Zoreky, 1988). The inhibitory component of Microgard was found to be a proteinaceous substance with a molecular weight of 700.

Use of microorganisms in fermented foods has long been considered a safe process because of the lack of any evidence to the contrary. Hence, it is reasonable to assume that the antimicrobial substances produced by such organisms at the levels found in fermented foods are also safe. However, some of these substances are subject to regulatory restrictions if they are intentionally added to a food in a semipurified or purified form (Daeschel, 1989).

Testing of fermentative bacteria on fruits: Twenty-five isolates of fermentative bacteria representing genera shown in Table 1 were received by the author from the ARS, Northern Regional Research Center in Peoria, IL, and subjected to preliminary testing for biocontrol potential on peach fruit. The bacteria were grown in nutrient yeast dextrose broth at 30°C for 48 hr and whole cultures were brushed onto the fruit. After incubation at 25°C for 48 hr, one strain of *Pediococcus cerevisiae* Balcke was shown to reduce the development of brown rot but not *Rhizopus* rot (Table 3). In plates coinoculated with *P. cerevisisae* and *M. fructicola*, inhibition zones developed, indicating that the bacterium may produce an antibiotic. *P. cerevisiae* is involved in the commercial fermentation of vegetables, including cabbage, olives and cucumbers (Fleming, 1982). It's potential in biocontrol is being further investigated.

Antibiotics Used in Food preservation

In considering the use of antibiotic-producing organisms in postharvest biocontrol, it may be useful to briefly review the general subject of antibiotics as food preservatives. Shortly after antibiotics were proven to be effective in the treatment of infectious human diseases, studies were begun to extend their use to foods as preservatives (Curran and Evans, 1945). By around 1955, two categories of antibiotics for food preservation had emerged, those that were effective for use in fresh foods and those effective primarily in certain processed food (Jay, 1983). For the former category, the tetracycline antibiotics were most effective with chlortetracycline being more effective than oxytetracycline. For use in processed foods such as cheeses and as adjuncts to heat in canning, the most effective antibiotics tested were subtilin, tylosin and nisin. A short time later, a third category to appear was made up of antimycotic agents to control food-borne fungi. Natamycin was shown to be the most effective of those investigated in this category.

Of the above agents, only nisin is approved for use as a food preservative in the United States (Wagner and Moberg, 1989). The only other antibiotic used widely in food is natamycin, which was accepted in 1976 by the Joint Food and Agriculture Organization/World Health Organization Expert Committee (Jay, 1983). The committee accepted natamycin because it does not affect bacteria, produces unusually little resistance among fungi, is infrequently involved in cross-resistance among other antifungal polyenes, and DNA transfer does not occur with fungi to the extent that it does with bacteria. Although tetracyclines are effective in controlling spoilage of fresh meats, their use on food conflicts with their use in human medicine. High levels of acquired resistance in intestinal bacteria rapidly appear when tetracyclines are ingested. Subtilin has never been officially allowed for use in any country, but could yet prove to be of value in heat-processed foods since it is not used medically or in animal feeds (Jay, 1983). The economic feasibility of toxicological studies, however, may be prohibitive unless subtilin can be shown to be more effective than nisin. Tylosin, a non-polyene macrolide antibiotic, was shown effective as a heat adjunct in canned foods. Currently, there is a lack of interest in tylosin as an antimicrobial in foods because of the use of macrolides clinically and the cross-resistance that develops between them.

Antibiotics Tested in Postharvest Disease Control

In addition to the intense interest in antibiotics for food preservation during the 1950's, antibiotics were also tested for use in controlling plant diseases. Goodman (1959) listed 45 fungal diseases and 27 bacterial diseases of plants against which antibiotics had been tested. Postharvest diseases included in the list are presented in Table 6. These compounds seemed like the long sought miracle cure for many diseases of plants, including serious postharvest diseases as bacterial soft rot (U.S. Dept. Agr., 1956). None is currently used to control postharvest diseases. Primary reasons for this are that the high cost of the compounds prevented their widespread use and, secondly, their application to fresh fruits and vegetables would endanger the medicinal effectiveness of the antibiotics (Ryall and Lipton, 1979).

Relatively few antibiotics are used widely for the preharvest control of plant diseases (Table 7). Of the three used in the United States, cycloheximide is the only antibiotic that is effective against fungal diseases. Other antibiotics with activity against fungi are produced in Japan and used mainly in the Orient. Cycloheximide has a high toxicity, in terms of the oral LD_{50} possibly the highest among fungicides used in agriculture, but some antibiotics used outside the U.S.A. appear to have a very low toxicity.

Concluding remarks

Antifungal peptides from the B-3 of *B. subtilis* were identified as iturin. B-3 and/or its antibiotic may be of value in the control of plant diseases.

Concern by a segment of the fresh food industry over the safety of using *B. subtilis* on food led the author to consider the possibility of using as biocontrol agents or as source of antifungal products, microorganisms that are involved in the production of fermented foods. Since these organisms or their metabolites have long been a part of man's diet, safety should be less of an issue. Researchers in the food preservation area have for many years taken this approach. It is perhaps significant that the only antibiotic approved in the U.S. as a food preservative is nisin, which comes from a bacterium important in the production of fermented milk products. In a preliminary screening of fermentative bacteria, one isolate of *P. cerevisiae*, which is involved in the fermentation of vegetables produced inhibition zones against *M. fructicola* in culture and significantly reduced brown rot development on fruit.

A review of past research on antibiotics tested for use as food preservatives or agents in plant disease control may be relevant to current considerations on the use of antibiotic-producing antagonists or antibiotics alone in the control of postharvest diseases. What can we learn from the research of the 1950's when tests were conducted with hundreds of different antibiotics most of which have been forgotten? What criteria were used in determining their acceptability for particular uses? In evaluating antibiotics for food or agricultural use,

were compounds rejected because of low efficacy, high toxicity, low economic feasibility, development of pathogen resistance, conflict with medicinal use, or for other reasons? We should consider whether antibiotics presently being investigated have advantages over those already tested. If they do not, it may be unwise for us to continue investing time and resources on control strategies involving these compounds. On the other hand, because of changes in economics or social attitudes, it may be that what was unacceptable in the past may be acceptable today. For instance, a relatively nontoxic antibiotic that was previously discarded because of low efficacy as compared to synthetic fungicides, might now be the more preferable agent to use on harvested crops.

Many of the antibiotics considered for use against spoilage bacteria in food were rejected because of a conflict with their medicinal use. Fortunately for the plant pathologist working with fungal diseases, this appears to be a lesser concern with antibiotics that inhibit fungi but do not affect bacteria.

The development of pathogen resistance is a major reason for the rejection of many antibiotics for use on plants. Natamycin, used widely as an antifungal food preservative, is an example of an antibiotic to which very little resistance is known to develop. It seems likely that other antibiotics with this attribute exist.

A brief look at the list of antibiotics currently used for treating fungal plant diseases reveals that the major producers and users of these compounds are in the Orient. Some of these antibiotics have a lower LD_{50} than sulfur and are applied to plant crops at lower ppm rates than those of fungicides now used in the U.S. on harvested fruits and vegetables. This further supports the idea that biocontrol involving antibiotic production as the mode of action could, from a safety standpoint, be a plausible approach to postharvest disease control.

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Table 1. Antifungal spectrum of antibiotic
extract derived from *Bacillus subtilis* (B-3)

Fungi	Width of inhibition zone (mm)
Armillarea mellia	0
Botryosphaeria dothidea	34
B. obtusa	35
Botrytis cinerea	26
Ceratocystis ulmi	26
Coniothyrium olivaceum	22
Cytospora sp.	21
Endothia parasitica	37
Epicoccum purpurascens	27
Fusarium sp.	18
Geotrichum cingulata	13
Glomerella cingulata	27
Monilinia fructicola	31
Penicillium cactorum	23
Phytophthora cactorum	12
Pythium phanidermatum	0
Pythium irregulare	0
Rhizopus sp.	16
Scelerotium rolfsii	0

Table 2. Major genera of microorganisms used in fermented
food (Pederson, 1979).

Bacteria	Yeasts	Molds
Acetobacter	*Candida*	*Aspergillus*
Bactobacillus	*Kloeckera*	*Cladosporium*
Leuconostoc	*Pichia*	*Penicillium*
Propionibacteria	*Saccharomyces*	
Pediococcus	*Saccharomyces*	
Streptococcus	*Schizosaccharomyces*	
	Torulopsis	

Table 3. Examples of lactic acid bacteria that produce
antibiotics.

Bacterium	Antibiotic	Organisms inhibited
Streptococcus lactis	nisin	G+
S. cremoris	diplococcin	G+
Lactobacillus plantarum	lactolin	G+
L. acidophilus	lactocidin	G+
	acidophilin	G+, G−
	acidolin	G+, G−
L. reuterii	reuterin	G+, G−, Y, F

G+ and G− = Gram-positive and Gram-negative bacteria,
respectively; Y = yeasts; F = fungi.

Table 4. Representative bacteria involved in food fermentation that were screened for potential use in postharvest control of peach fungal decay.

	Lesion diameter (mm)	
Treatment	Brown rot	Rhizopus rot
Control	22	10
Bacillus subtilis (B-3)*	0	14
Pediococcus cerevisiae (B1153)	4	10
Propionibacterium freudenreichii (B3523)	17	23
Leuconostoc citrovorum (B1232)	17	26
Streptococcus lactis (B633)	17	27
Lactobacillus bulgaricus (B734)	21	26

*Included as standard

Table 5. Summary comparison of major antibiotics considered
for use in food (Jay, 1983)

Property	Tetra-cyclines	Subtilin	Tylosin	Nisin	Natamycin
Widely used in food?	No	No	No	Yes	Yes
First food use, year	1950	1950	1961	1951	1956
Microbial spectrum	G+, G-	G+	G+	G+	Fungi

G+ and G- = Gram-positive and Gram-negative bacteria,
respectively.

Table 6. Tests during the 1950's with antibiotics to control postharvest diseases (Goodman, 1959).

Disease	Pathogen	Antibiotic	No. of references
Bacterial soft rot	*Erwinia caratovora*	ch,ox,st	2
Storage rot	*Botrytis cinerea*	cy,gr	4
Peach brown rot	*Monilinia fructicola*	cy,fu,my,ol,pa	8

ch = chlortetracycline; cy = cycloheximide; fu = fungicidin; gr = griseofulvin; my = mycostatin; ol = oligomycin; ox = oxytetracycline; pa = patulin; st = streptomycin.

Table 7. Most widely used antibiotics for preharvest
 control of plant diseases (Thomson, 1988).

Antibiotic	Pathogens controlled	Toxicity (LD$_{50}$ in mg/kg)	Used in U.S.
Streptomycin	bacteria	9000	Yes
Terramycin	bacteria	Not specified	Yes
Cycloheximide	fungi	2	Yes
Kasugamcin	fungi	22,000	no
Blasticidin	fungi	53	no
Polyoxin	fungi	21,200	no
Validamycin	fungi	20,000	no
Mildiomycin	fungi	4,120	no

Nutrient Competition as a Mode of Action of Postharvest
Biocontrol Agents

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Abstract

The interaction between the antagonistic yeast *Pichia
guilliermondii* and the green mold fungus *Penicillium digitatum*
on grapefruit were studied on culture and on the fruit to
characterize the mechanism of action by which the yeast
exhibits its biocontrol activity. The antagonist did not
produce antibiotics in culture and was ineffective in
protecting against the diseases when killed by heat or
chemicals. Incidence of green mold was dependent upon the
pathogen spores and the antagonist yeast cells. Control of
green mold was most effective at 10^9 cfu/ml of *P.
guilliermondii*. The role of available nutrients in the
biological control activity of the yeast antagonist was
assessed. Significant inhibition of spore germination and
hyphal growth of *P. digitatum* in culture was achieved by the
addition of the yeast cells to a minimal synthetic medium.
Inhibition of *P. digitatum* by the antagonists in culture and on
the fruit peel could be overcome by the addition of exogenous
nutrients. Thus, competition for nutrients at the site of
infection plays a role in the mode of action of *P.
guilliermondii* against *P. digitatum*.

Introduction

Most postharvest pathogens of fruits and vegetables are wound
parasites which depend mainly on exogenous nutrients for the
germination and initiation of the pathogenic process.
Therefore, reduction in the nutrient base appears to be a
suitable method to reduce chances for successful interaction.
This is particularly true for surface wounds which are rich in
nutrients and moisture. We thus assume that one of the
important features of a biocontrol agent of postharvest
diseases could be its ability to be adapted to the wound site
environment and compete successfully with the pathogen for
nutrients and space.

**Requirements of an
Antagonist**

The following features are required for an antagonist to be
successful competitor at the wound site: (a) better adapted
than the pathogen to various environmental and nutritional
conditions; (b) rapid growth at the wound site; (c) effective
utilizer of nutrients at low concentrations; (d) survive and
develop on the surface of the commodity and the infection site
under extreme temperature, pH and osmotic conditions that are
unfavorable for the pathogen.

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| Pichia guilliermondii | The yeast *Pichia guilliermondii* has been demonstrated as an effective biological control agent of postharvest diseases of several fruits and vegetables (Chalutz et al., 1988a; Chalutz et al., 1988b; Wilson and Chalutz, 1989). Several modes of action have been suggested for the biocontrol activity of *P. guilliermondii* (Chalutz et al., 1988c). Elucidation of the mechanisms by which antagonists inhibit postharvest pathogens is important for successful application and effective use of biocontrol agents under commercial conditions.

In this paper, the role of competition for nutrient in the mode of action by which *P. guilliermondii* inhibits postharvest pathogens, is characterized.

Experimental

Cultures of *Pichia guilliermondii* were grown on nutrient yeast dextrose agar (NYDA) containing 8 g nutrient broth, 5 g yeast extract, 10 g D-glucose, and 20 g agar in 1 L of water. A loop of the culture was transferred to a 100-ml Erlenmeyer flask containing 50 ml of NYDB and incubated on a rotary shaker at 26 C for 48 h. Cultures were washed twice with distilled sterilized water, following the removal of the growth medium by centrifugation at 7000 rpm for 10 min. Cell pellets were resuspended in distilled sterilized water and brought to the initial concentration.

In all experiments, surface-sterilized grapefruits were wounded around the stem end, with three or four wounds per fruit. The wounds were treated with 40 ul of a water cell suspension of the yeast antagonist (10^8 cells/ml). After the drops were dried, 20 ul of a spore suspension of *Penicillium digitatum* (10^5 spores/ml) were applied to each wound. Percent infection was scored following one week of incubation at 24°C.

Killed Cells

Killed cells of *P. guilliermondii*, obtained either by exposing a cell suspension to chloroform vapors or by autoclaving it, were also used to evaluate their efficacy in inhibiting *P. digitatum*. The antagonist-pathogen relationship was assessed by varying the concentration of the pathogen spores (from 10^4 to 10^7 spores/ml) while keeping the yeast cell concentration at 10^9 cfu/ml, or by varying the yeast concentration (from 10^5 to 10^9 cfu/ml) while holding the pathogen spore concentration at 10^5 spores/ml.

Population Densities

The population densities of the yeast antagonist at the wound site were compared with those on unwounded fruit surface at intervals of 1, 4, 24 and 48 h. Wounds or unwounded fruit tissue were cut and shaken vigorously in 10 ml of sterilized distilled water for 1 h. After one-tenth serial dilutions, 100 ul of the washing liquid was plated on NYDA and the number of colonies were determined after 48 h of incubation at 25°C.

Peel leachate was prepared from wounded grapefruit (50 wounds/fruit). Wounded fruit was shaken in 200 ml of distilled water and then the washing liquid was filtered through Whatman no. 1 filter paper and filter-sterilized before use. Washing liquid from unwounded fruit was used as a control. |

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Competition Tests	Competition tests between the yeast antagonist and *P. digitatum* were performed in a synthetic medium (Lilly and Barnett, 1951). Two PDA discs, 0.5 mm in diameter, containing hyphae of *P. digitatum* were placed in 15 ml of synthetic medium in 90-mm diameter petri dished. Aliquots of 0.1 ml of a washed cell suspension of *P. guilliermondii* (1.6 x 10^6 cfu/ml) were added to the medium in each plate. Dry weights of the fungus, after growth in the presence and absence of the antagonist, were determined following 7 days of incubation at 24°C. The effect of *P. guilliermondii* on spore germination and germ tube elongation of *P. digitatum* was also assessed. Spores of *P. digitatum* were suspended in peel leachate, and aliquots of 1 ml were then transferred to the wells of tissue culture plate. Fifty ul of washed cell suspension of *P. guilliermondii* (10^6 cfu/ml) were added to each well. Percent germination and germ tube elongation were determined after 24 h of incubation at 24°C.
	Peel macerate was prepared from water-soaked peel at the margins of *P. digitatum* lesions on grapefruit. Aliquots of 100 ul of concentrated spore suspension of *P. digitatum* were added to different dilutions of the peel macerate to give a final spore concentration of 10^5 spores/ml, which was used to inoculate fruit wounds pretreated with the yeast antagonist.
Forming Mutants	Cells of *P. guilliermondii* was mutagenized by harvesting the exponentially growing cells in NYDB and by washing in sterile water twice with an equal volume of citrate buffer (pH 5.5). The cells were mutagenized with ethylmethan sulfonate (EMS) for 140 min. After treatment with EMS, cells were washed twice with sterile water and then plated in NYDA at dilutions which gave 200 colonies per plate after growing at 30°C for 3 days. Colonies were screened for auxotrophs by replicating them onto minimal medium (Lilly and Barnett, 1951) and scoring the lack of growth after 2 days of incubation.

Results and Discussion

The ability of *P. guilliermondii* to prevent infection of *P. digitatum* was lost when the antagonist cells were killed either by autoclaving or by exposure to chloroform vapors (Table 1). Only live cells of the yeast antagonist were highly effective in protecting grapefruit surface wounds from infection o *P. digitatum*. Cell-free culture filtrate of *P. guilliermondii* was also ineffective in reducing the incidence of infection. Saccharomyces cervisia failed to inhibit infection of *P. digitatum* (Table 1). According to these findings, it may be assumed that *P. guilliermondii* did not inhibit the disease by production of antibiotic substances.

Inhibiting *P. digitatum*	A concentration of 10^9 cfu/ml of the antagonist cells was the most effective in inhibiting the infection of *P. digitatum* (Fig. 1). Decreasing concentrations of the antagonist cells resulted in an increased rate of infection. Increasing the concentration of spores of *P. digitatum* from 10^4 to 10^6 spores/ml resulted in reduction of biocontrol activity of the yeast antagonist. Percent infection increased to about 50% at the highest spore concentration (Fig. 2). This result demonstrates that the activity of *P. guilliermondii* depended on the concentration of both the pathogen and the antagonist.

The antagonist grew rapidly in wound leachate and maintained a high cell concentration for 48 h of incubation, while leachate of the intact fruit did not support any growth of *P. guilliermondii* cells (Fig. 3). At the wound site, the population of *P. guilliermondii* increased almost 100-fold within 24 h, while on the intact fruit peel surface the population remained at the initial level, 48 h after incubation (Fig. 3).

Inhibiting Spore Germination

Spore germination of *P. digitatum* was markedly inhibited when cells of *P. guilliermondii* were added to the wounded fruit leachate (Table 2). Reducing the concentration of the wounded fruit leachate by 50% or increasing the initial concentration of *P. guilliermondii* two-fold further reduced percent germination of 8 and 6%, respectively. The inhibition was partially overcome by adding 10% grapefruit juice to the germination medium, resulting in an increase from 18 to 40% in spore germination (Table 3). Addition of cells of *P. guilliermondii* to a synthetic minimal medium reduced the growth of *P. digitatum* (Table 3). Dry weight of hyphae after 7 days incubation was only about one-half of the dry weight of the fungus grown without the yeast cells or in the presence of bakers' yeast. Although the growth of *P. digitatum* was very low in minimal medium n which the yeast antagonist had been cultured for 48 h, replenishment of this "used" medium with its original ingredients resulted in normal growth. When similar competition experiments were performed on medium enriched n nutrients, the presence of *P. guilliermondii* did not affect the growth of *P. digitatum*.

Overcoming Inhibition

Protection of grapefruit wounds by *P. guilliermondii* was partially overcome by the addition of nutrients obtained from macerated peel of grapefruit to spore suspension of *P. digitatum* used for inoculation. The high the concentration of he added nutrients, the more pronounced was the reversal of the yeast inhibition of *P. digitatum* infection (Fig. 4). At concentration of 40% of the nutrient solution, infection was 80% after 7 days of incubation.

Mechanism of Action

These results suggest that a possible mechanism by which *P. guilliermondii* might achieve biological control is through effective competition with the competition for nutrients between *P. guilliermondii* and *P. digitatum* takes place under restrictive nutrient conditions, while no such competition is evident when a surplus of nutrients is available. Therefore, the nutritional environment available at the wound site may create a favorable microenvironment for *P. guilliermondii* to colonize rapidly, multiply, and compete effectively with the pathogen for nutrients.

More direct evidence for the role of competition for nutrients in *P. guilliermondii* mode of action was obtained from results of the assays performed with a mutant isolate of *P. guilliermondii* which totally lost its biocontrol activity. This mutant (M-7) was ineffective against *P. digitatum* on grapefruit and *Botrytis cinerea* on apples (Table 4). In order to characterize some of the features of the mutant, its growth rate on different media was compared to that of the biologically active wild type (US-7). Both the wild type and

the mutant grew normally and reached high population levels already after 24 h of incubation at 24°C (Fig. 5). However, the growth of the mutant on minimal medium or on wound leachate was negligible (Fig. 6,7). At the wound site, the population of the mutant remained at the initial level during the incubation period while population of the wild type increased almost 100-fold with 24 h (Fig. 8). *In vitro* tests revealed that the ineffective isolate did not inhibit spore germination (Table 5) Considered together, these results indicate that the mutant lost its ability to utilize and grow on a low concentration of nutrients and subsequently the pathogen was not inhibited due to the lack of nutrients.

Several reports on the interaction between epiphytic microorganisms showed that bacteria and yeasts are able to take up nutrients from dilute solutions more rapidly an in greater quantity an efficacy than are the germ tubes of fungal pathogens. This may result in a marked reduction in the amounts of exogenous nutrients available for the pathogen (Blakeman and Brodie, 1977; Brodie and Blakeman; 1976, Fokkema, 1981). Under these conditions, pathogen spore germination and hyphal development are greatly restricted. Nutrient competition has been identified as a factor in several biological control systems. Elad and Chet (1987) indicated that nutrient competition played a major role in the biocontrol of Pythium damping off by bacterial antagonists. Wisniewski et al. (1989) showed that *Enterbacter cloacae* inhibited germination of *Rhizopus stolonifer* through nutrient competition. Our data indicate that nutrient competition also plays a role in the antagonism of *P. guilliermondii* against *P. digitatum.*

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Table 1. Inhibition of *Penicillium digitatum* decay of
grapefruit by the antagonist *Pichia guilliermondii* (US-7) as
affected by various treatments of the antagonis cells.

Treatment	Infection
Water control	100 a
US-7	5 b
US-7, autoclaved	100 a
US-7, exposed to chloroform vapors	100 a
US-7, culture filtrate	95 a
Saccharomyces cerevisiae	95 a

Table 2. Inhibition of spore germination and germ tube elongation of *Penicillium digitatum* by *Pichia guilliermondii* (US-7) on low nutreint medium.

Treatment	Spore germination (%)	Relative germ tube elongation
Water	0	—
NIPL, control[a]	0	—
IPL, control[a]	72 a	—
IPL + US-7	18 a	+++
IPL (0.5) + US-7[a]	8 c	+
IPL + US-7 (2x)[a]	6 c	+
IPL + US-7 + 10% grapefruit	40 b	++

[a] NIPL, noninjured peel leachate; IPL, injured peel leachate; IPL (0.5), half-strength IPL; US-7 (x2), doubling of the initial concentration of US-7 cells.

+, less or equal spore length; ++ twice or threefold the spore length; +++, more than threefold the spore length. Values following by different letters are significantly different (P=0.05) according to Duncan's multiple range test.

Table 3. Growth of *Penicillium digitatum* in a synthetic medium as affected by the presence of the antagonist *Pichia guilliermondii* (US-7).

Treatment	*Penicillium digitatum* (dry weight of mycelium, mg)
Control	46.4 a
US-7	22.0 b
Saccharomyces cervisiae	46.0 a
cell-free filtrate from US-7 culture[a]	3.0 c
Replenished cell-free filtrate[b]	46.2 a
Enriched[c]	55.1 a
Enriched + US-7	36.7 a

[a]*Pichia guilliermondii* (US-7) was cultured on a synthetic medium for 48 h prior to use of the cell-tree filtrate.

[b]The used medium was replenished with original ingredients.

[c]The synthetic medium was enriched with yeast extract and nutrient broth at their original concentration.

Table 4. Biocontrol activity of *Pichia guilliermondii* (US-7) and the auxotrophic mutant (M-7) against *Penicillium digitatum* on grapefruit and *Botrytis cinerea* on apples.

Treatment	Infection (%)
	Apple
Water control	80.0
US-7	0.0
M-7	100.0
	Grapefruit
Water control	88.0
US-7	0.0
M-7	89.0

Table 5. Effect of *Pichia guilliermondii* (US-7) and the
auxotrophic mutant (M-7) on spore germination of *Botrytis cinerea*
and *Penicillium expansum*.

Treatment	Germination	
	B. Cinerea	*P. expansum*
Control	97.7	88.8
US-7	0.0	0.0
M-7	97.0	89.8
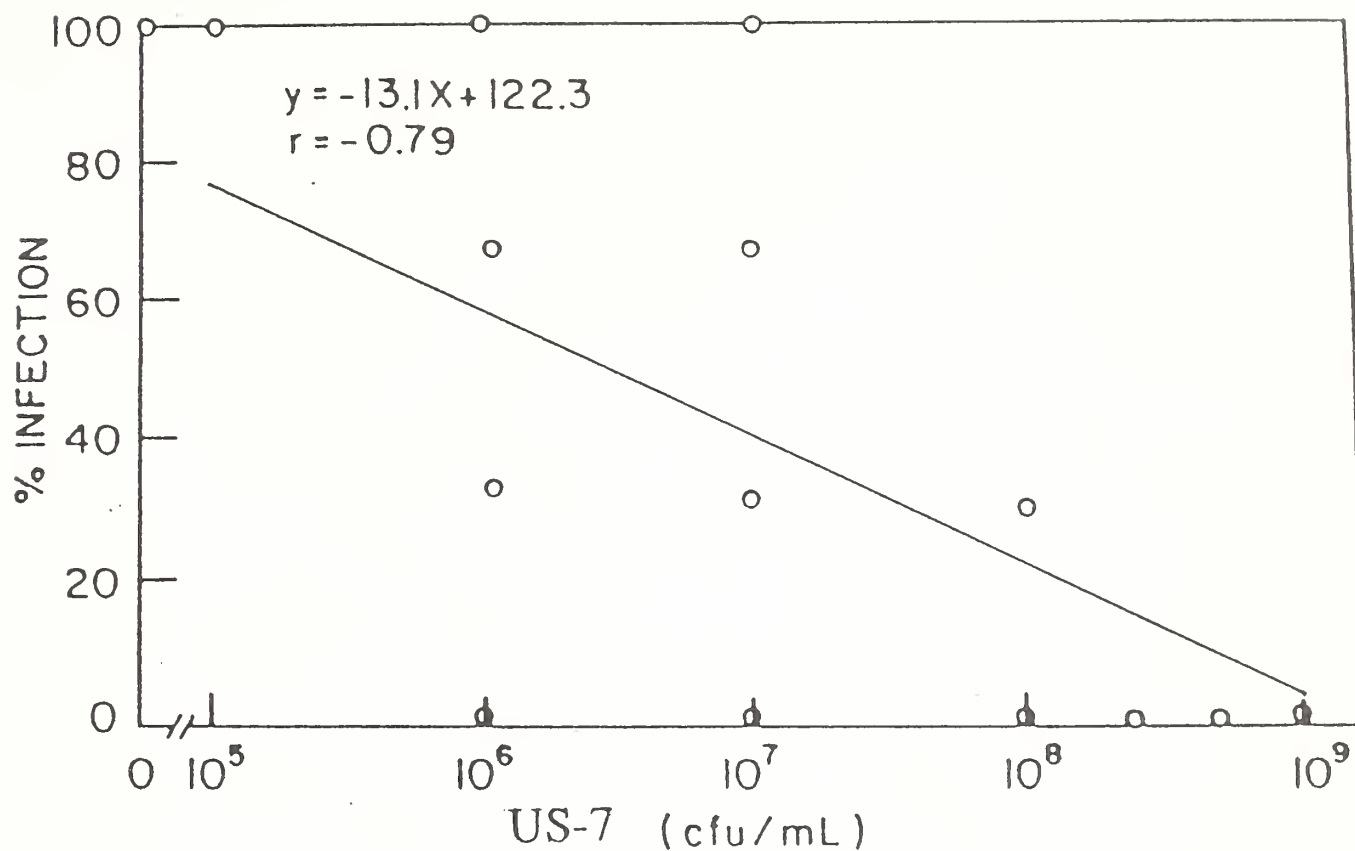
Figure 1. Relationship between *Pichia guilliermondii* cell
concentration and development of green mold on grapefruit.
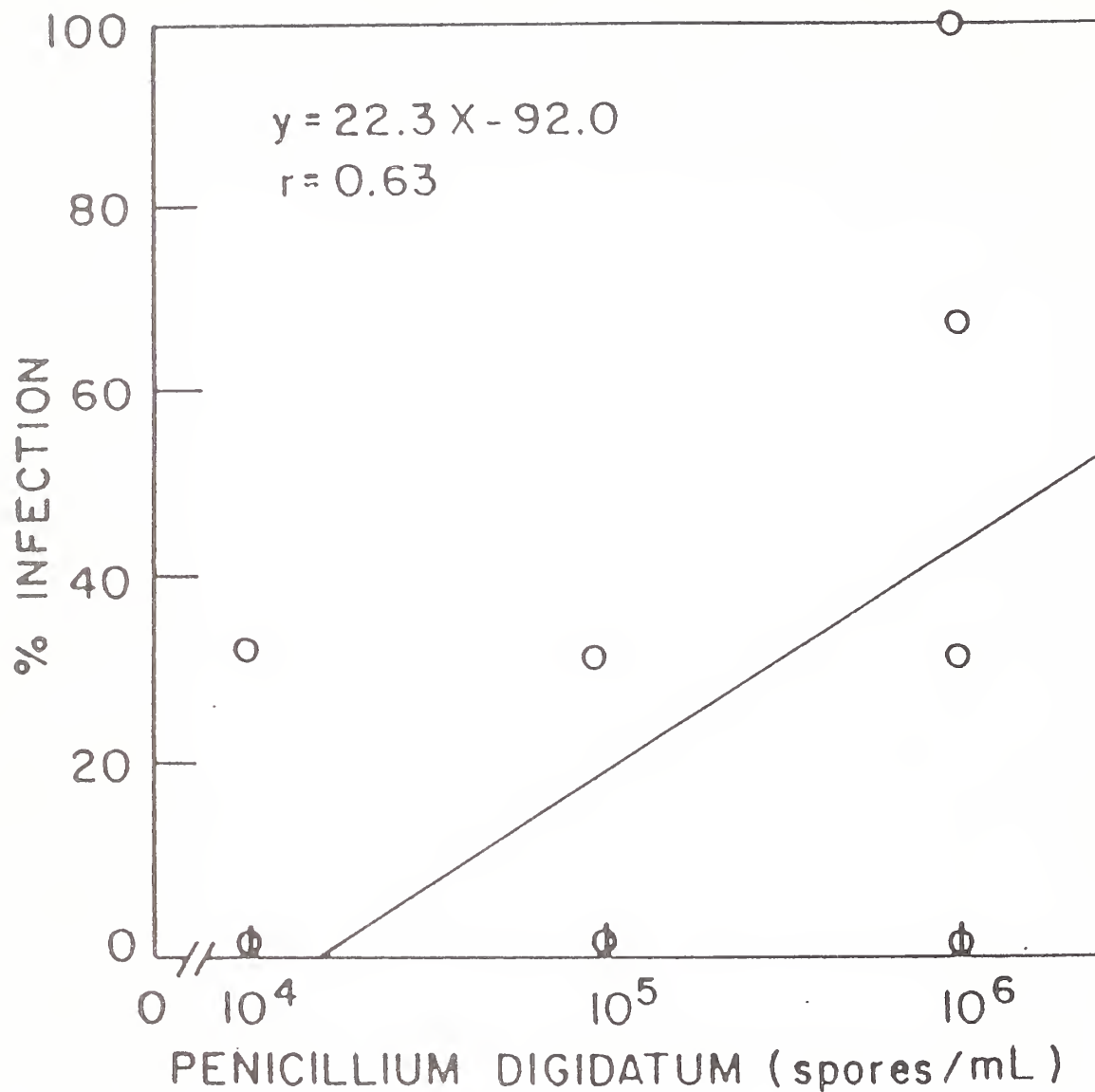
Figure 2. Relationship between *Penicillium digitatum* spore
concentration and the development of the green mold on grapefruit
treated with *Pichia guilliermondii* (US-7).
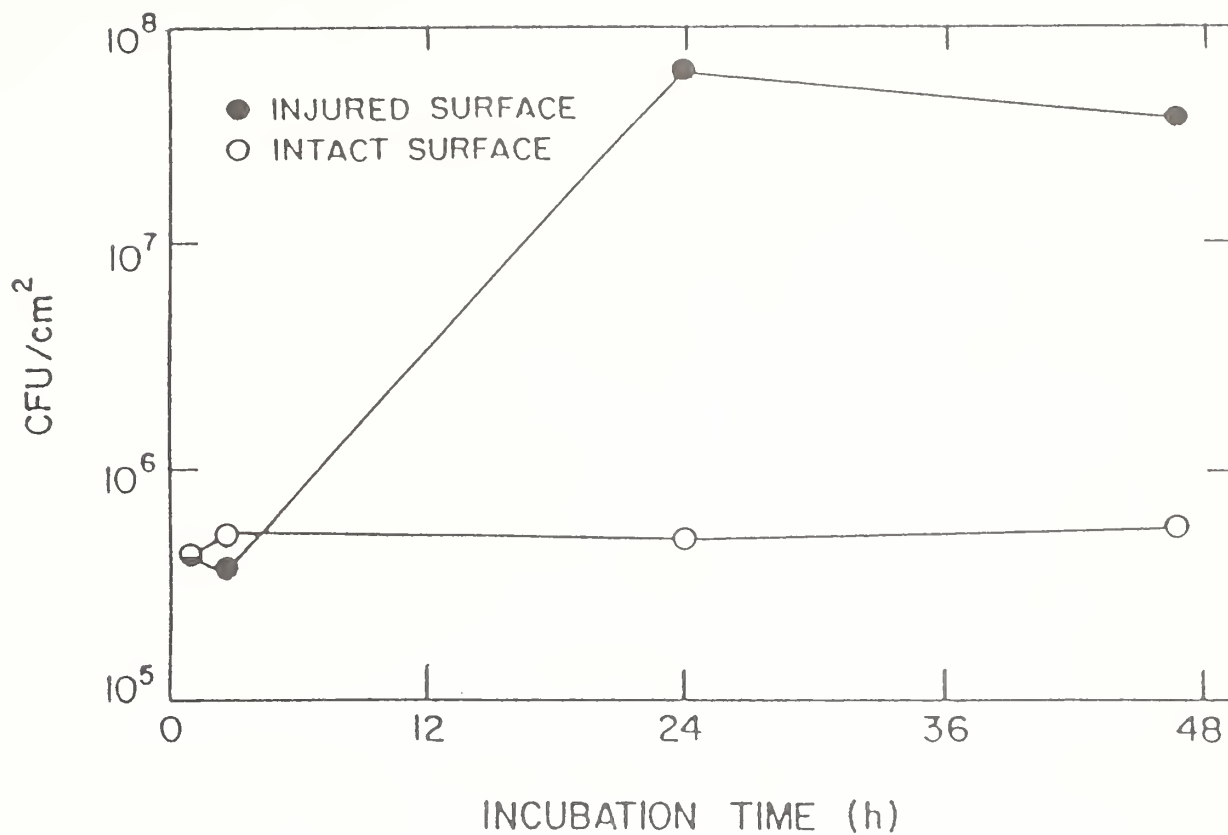
Figure 3. Growth of *Pichia guilliermondii* (US-7) on leachate from grapefruit.
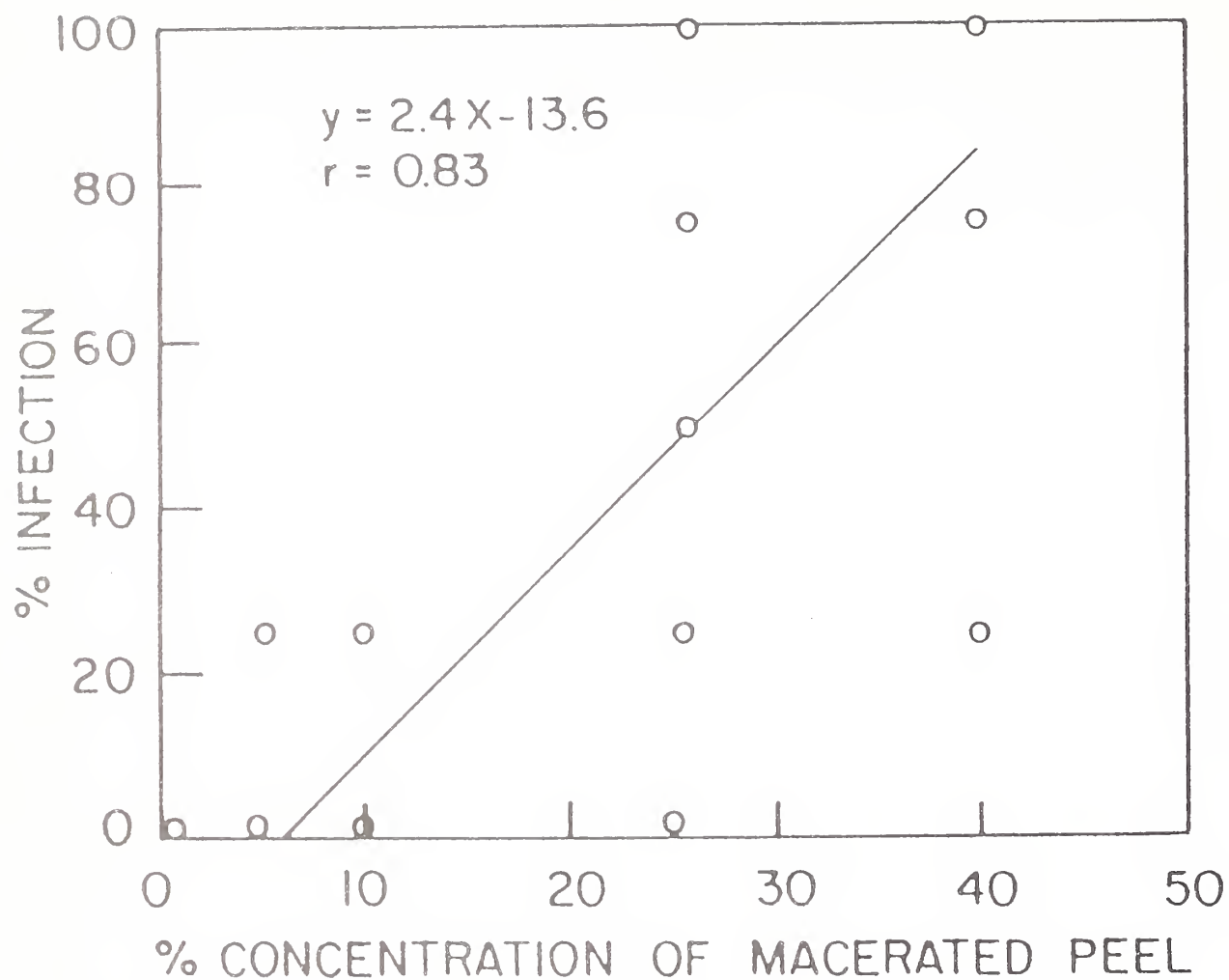
Figure 4. Protection of *Pichia guilliermondii* (US-7) against
Penicillium digitatum infection on grapefruit as affected by the
additoin of nutrients from wounded grapefruit peel to the inoculum
of the pathogen.
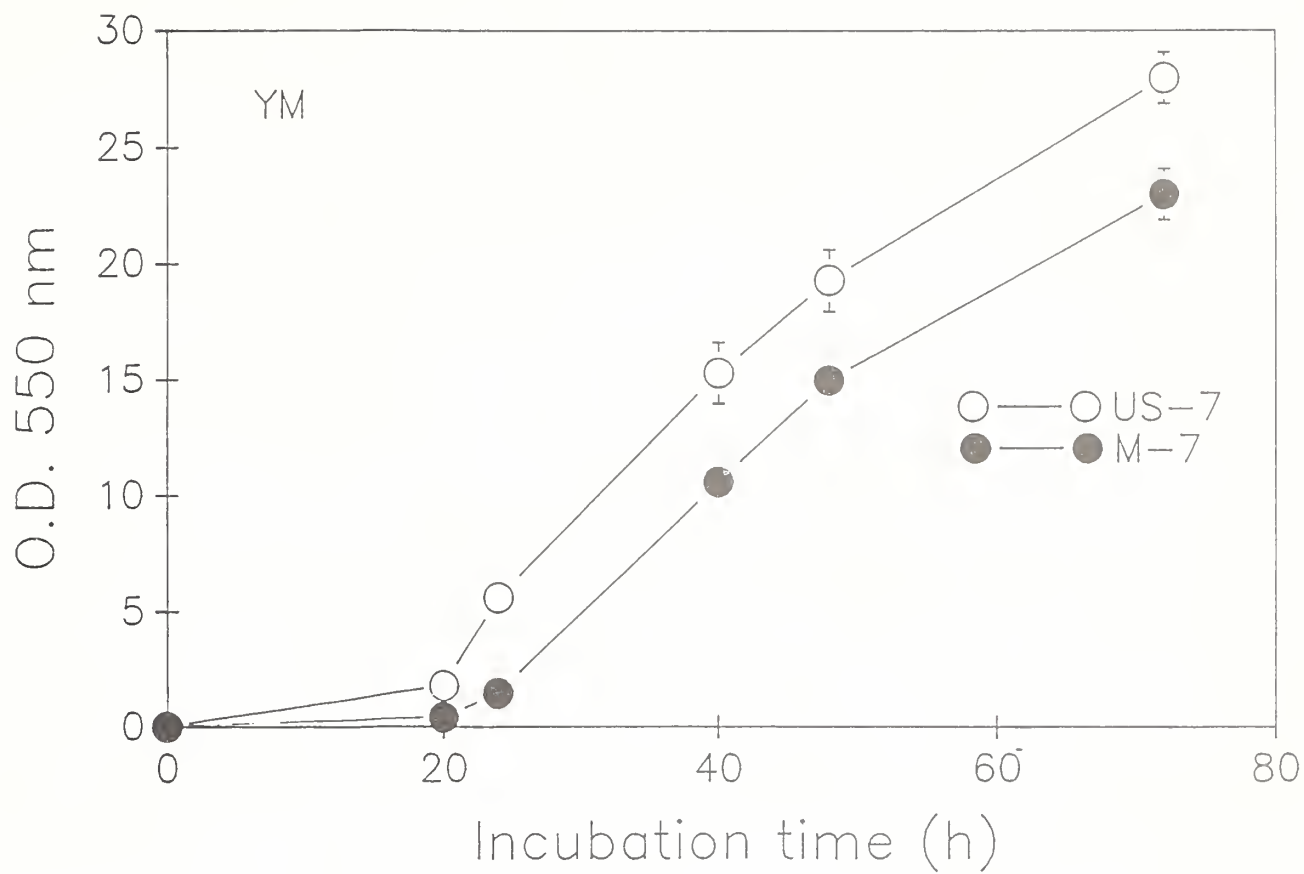
Figure 5. Growth of *Pichia guilliermondii* (US-7) and the auxotrophic mutant (M-7) in a rich yeast and malt extracts medium.
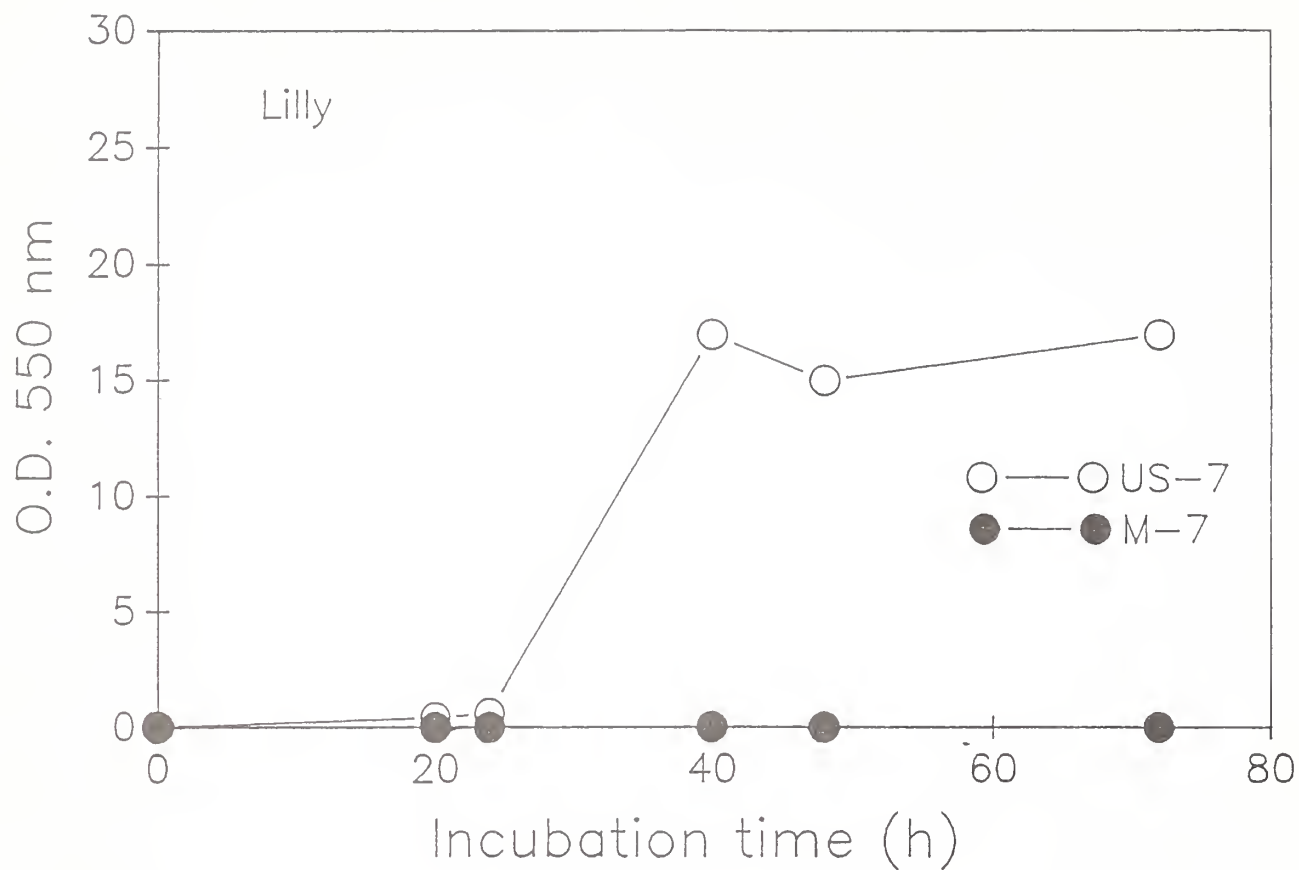
Figure 6. Growth of *Pichia guilliermondii* (US-7) and the auxotrophic mutant (M-7) in minimal medium (Lilly and Barnett).
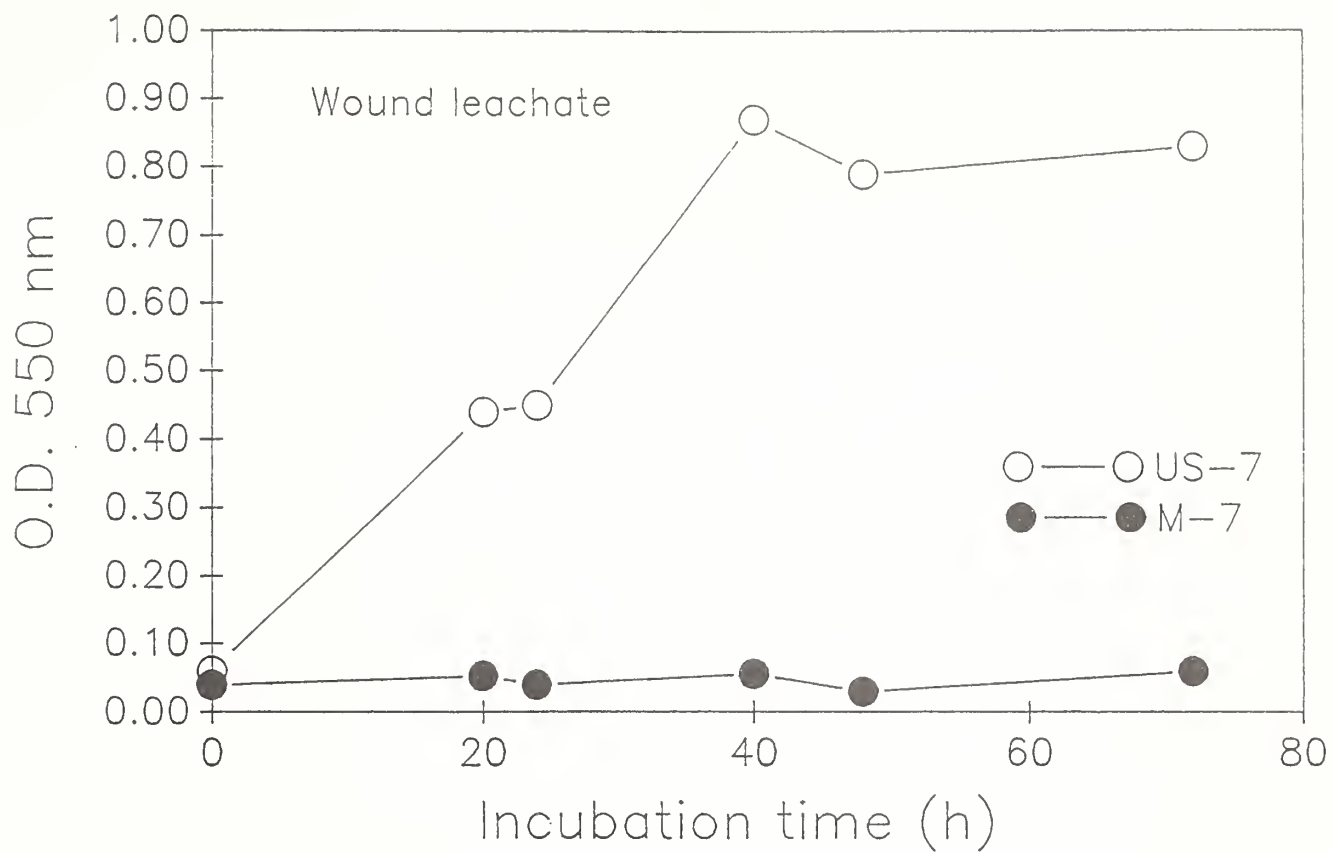
Figure 7. Growth of *Pichia guilliermondii* (US-7) and the auxotrophic mutant (M-7) in wound leachate from wounded apple.
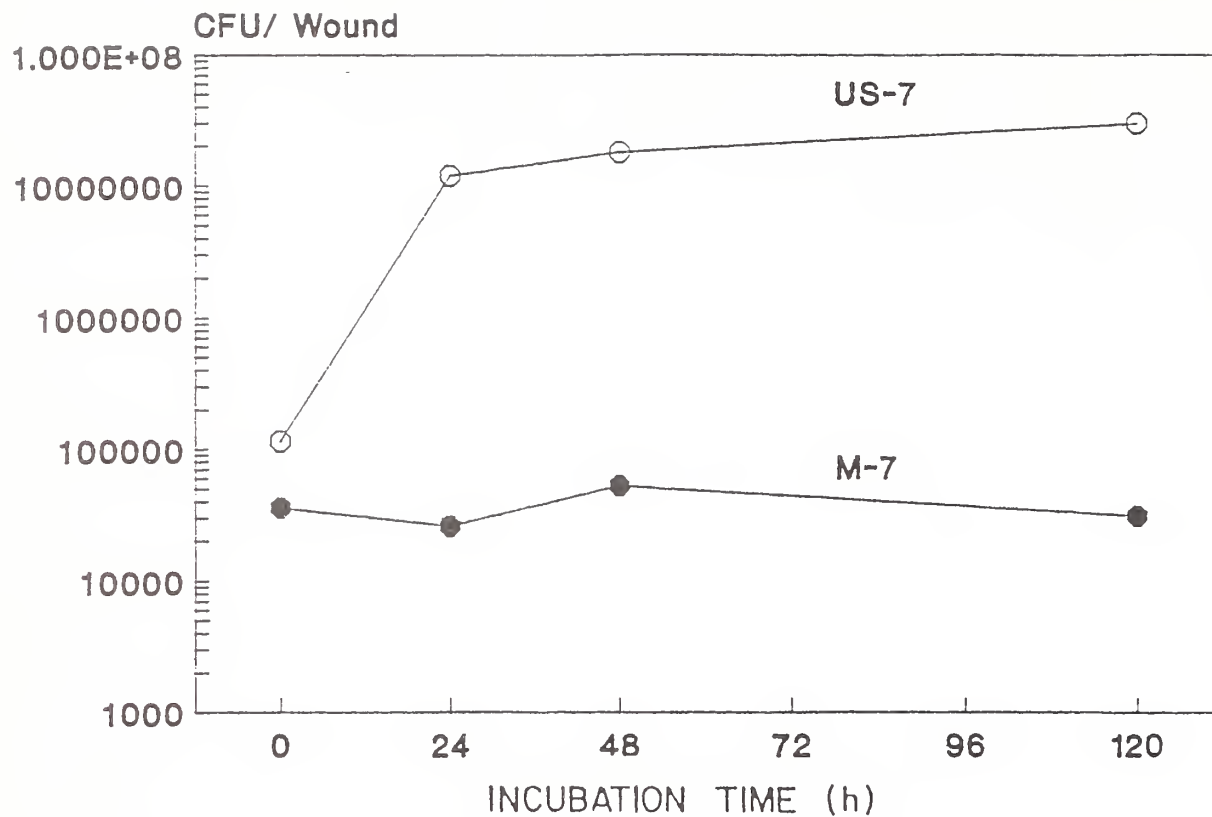
Figure 8. Growth of *Pichia guilliermondii* (US-7) and the
auxotrophic mutant (M-7) in wound sites on the surface apple.

Induced Resistance in Relation to Fruit and Vegetables

C. L. Biles[1]

Abstract

Resistance to plant pathogen attack is a multi-faceted event. When discussing resistance it is important to remember that resistance is the rule and susceptibility is the exception. That is, a plant is resistant to almost all organisms that it encounters. Michele Heath (1981) emphasizes two kinds of resistance expressed by plants; general (non-host) and specific cultivar (host) resistance. General resistance is expressed by the plant against most pathogens. Although general resistance is observed, little is known about the factors that contribute to the phenomenon. Non-host resistance is usually considered preformed and non-specific. Examples that are present in fruit are the cuticle, natural volatiles (Wilson, 1986), antifungal phenolics (Swinburne, 1978), and polygalacturonase inhibitors (Fielding, 1981). Cultivar resistance occurs when the specific metabolic 'accomodation' of the pathogen to the host species renders the nonhost defense ineffective. In reaction to this basic compatibility, the resistant host reestablishes a successful defense mechanism. The defense mechanism is often secondary metabolites of the phenylpropanoid pathway, such as, phytoalexins, phenolics, hydrolytic enzymes, and lignification. Nonhost resistance is usually considered a multigene (horizontal) effect while cultivar resistance is one gene.

Induced Resistance

Induced resistance is a third type of resistance observed in plant-pathogen interactions. Induced resistance, according to Ouchi (1983), is defined as a dynamic resistance based on physical and chemical barriers induced by a preliminary or concomitant inoculation with an incompatible pathogen or nonpathogen, or by treatment with their products.

Localized induced resistance has been reported for several crops (Ku, 1982; Ouchi, 1983). Induced resistance of watermelon to *Fusarium oxysporum* f. sp. *niveum* is an example (Biles and Martyn, 1989). *F. oxysporum* f. sp. *niveum* is a soil-borne vascular wilt pathogen that invades the root hairs, colonizes the xylem tissue and then causes wilting due to water blockage (Beckman, 1987). There are three known races of F. o. f. sp *niveum*; race 0, 1, and 2. There are resistant varieties to races 0 and 1, but no resistant varieties to race 2. Avirulent and the closely related cucumber wilt pathogen were tested as inducers of resistance to the virulent race 2. Plants were grown in a greenhouse and dipped in an avirulent or nonpathogen conidial suspension after 24 hr they were inoculated with the virulent race 2. The avirulent race 0 and 1 induced resistance to the virulent race 2 as well as the cucumber wilt pathogen.

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Induced resistance has also been found to be systemic. Kuc' et al. (1982) have shown that by inoculating a lower cucumber leaf with *C. lagenarium* and then inoculating an upper leaf a few days later, resistance is established. Induced resistance has been transferred from the induced leaves to the fruit (Kuc, personal communication; Caruso and Kuc, 1977) and from induced tobacco plants to progeny (Roberts, 1983). Systemic resistance was also shown in the watermelon-Fusarium wilt system described previously. When the roots of the watermelon seedling was preinoculated with a nonpathogen *F. o.* f. sp. *cucumerinum* isolate and the leaves later inoculated with *C. lagenarium* conidia, the lesion size was reduced (Biles and Martyn, 1989).

Induced Proteins

Several workers have found that when resistance is induced a number of proteins are induced (Collinge and Slusarenko, 1987). These include several prominent enzymes of the phenylpropanoid pathway, flavanoid synthesis, hydrolytic enzymes, and enzymes involved in lignin synthesis. These enzymes are elicited by fungi, bacteria, viruses, heat stress, UV irradiation, and salicylic acid.

Signal molecules involved in plant defense responses include -glucans, glycoproteins, lipids, oligosaccharides, and ethylene. These molecules elicit phytoalexins, proteinase inhibitors, and hydroxyproline rich glycoproteins (Halverson and Stacey, 1986).

Ethylene

Ethylene is a plant hormone that has been implicated in induction of a resistant response in plant-pathogen interactions (Esquerre-Tugaye et al., 1979). It is of interest to note that ethylene has been found to promote, inhibit, and have no effect on the resistance status of the host (Biles et al., 1990). The role of ethylene and ethylene-induced proteins appears to be dependent on the host and pathogen involved. To illustrate this, our laboratory has recently found that ethylene stimulated susceptibility of cucumbers inoculated with *Colletotrichum lagenarium*.

Mauch and Staehelin Model

In beans (*Phaseolus vulgaris*) ethylene stimulates chitinases and B-glucanases which hydrolyse fungal cell walls in vitro (Abeles et al., 1971; Mauch et al., 1988). An interesting model has been advanced by Mauch and Staehelin (1989) in relation to induced resistance. They found that ethylene-induced chitinases and B-glucanases were predominately located in the vacuole. These enzymes are capable of degrading fungal cell walls and have been proposed to be a defense mechanism of plants to fungal pathogens (Abeles et al., 1971; Mauch et al., 1988). Using immunocytochemical localization techniques, they showed that minor amounts of B-glucanase occurs in the cell wall middle lamella. Upon pathogen attack, B-glucanase located in the cell wall middle lamella cleaves off fungal glucoside residues which act as elicitors. These elicitors in turn stimulate the nuclear DNA and the result is an increase in phytoalexins, and fungal degrading enzymes. The majority of the hydrolytic enzymes are deposited in the vacuole. Upon disruption of the cell wall by the fungus or a stimulated hypersensitive response by the plant cell, the hydrolytic enzymes are released. This action floods the hyphal tips and causes lysis.

This model involves the induction of pathogenesis related proteins. In contrast, Kopp et al., (1989) recently found that the glucan elicitor derived from *Phytophthora megasperma* f. sp *glycinea* elicited phytoalexins, but not PR-proteins. The glucan fungal elicitor did not directly affect the virus, and did not interfere with virus disassembly. The induced resistance in this case did not depend on the induction of PR-proteins, the phenylpropanoid pathway, lignin-like substances, or callose-like material.

Phytoalexins

In the case of fruits and vegetables, resistant responses of the potato tuber have been studied in great detail in regard to phytoalexin production (Muller and Borger, 1940, cited in Kuc and Rush, 1985). In a similar way, phytoalexins and secondary products have been investigated in other fruits and vegetables. In regard, to the antagonist yeast, *Pichia guilliermondii* (87), the level of control exhibited is very good in a variety of crops (Wilson and Wisniewski, 1989). What is the mechanism of control and does the yeast stimulate secondary metabolites?

Yeast Induced Ethylene

Preliminary data conducted by Droby and Chalutz suggest that the yeast does stimulate ethylene production and enhanced levels of phenylalanine ammonia lyase in citrus (unpublished data). Enhanced ethylene in citrus has been shown to increase resistance (Brown and Barmore, 1977) to *Colletotrichum lagenarium*. However, Barmore et al., (1976) has also found that ethephon treatment used to degreen citrus increases susceptibility to stem end rot caused by *Diplodia natalensis*. In our laboratory, preliminary results also suggests that the yeast isolate enhances ethylene synthesis, but the abundance of wound ethylene produced often confounded interpretation of the results (Wisniewski, personnal communication). Application of fungal cell walls, dead yeast cells and cell walls, and degradative enzymes did not induce resistance in apple fruit (unpublished data). The conclusion of this author is that *P. guilliermondii* (87) does stimulate the fruit metabolism. There is, however, no evidence to conclude that induced resistance plays a major role in the control exhibited by *P. guilliermondii*.

UV-light

Another physical induction has been observed in the laboratories of C. L. Wilson and Clauzell Stevens. Using UV-C irradiation, they have observed a reduction of fungal infection on onions, sweet potatoes, apples, and peaches (Lu et al., 1987); Stevens, in press; C. L. Wilson and C. Stevens, unpublished data). This affect appears to be more than just germicidal. The UV appears to induce a resistant response when the appropriate levels are used. Other workers have observed that UV stimulates phytoalexin production in plants (Bridge and Klarman, 1973; Hadwiger and Schwochau, 1971). Christ and Mosinger (1988) showed that local resistance of tomato leaves was induced by repeated short periods of irradiation. Reduction of *Phytophthora infestans* lesions corresponded to a buildup of PR-proteins.

Heat Treatment

Heat treatment has also been observed to induce resistance in fruit. Spotts and Chen (1987) found that heat induced pears were more resistant to *Mucor* and *Phialophora* in storage. Couey and Nishijima (unpublished data) also found that papaya heated and then inoculated had less disease than treatments heated after inoculation.

Conclusion

In conclusion, fruits and vegetables do have several general (nonhost) defense mechanisms and appear they have the ability to respond to physical stimuli with disease resistance. It would behoove us to investigate the general defense mechanisms of fruit and try to enhance and manipulate them. Physical stimulation that elicits a defense response is also a possible alternative to chemical treatment.

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The Use of the Yeast *Pichia guilliermondii* as a Biocontrol Agent: Characterization of Attachment to *Botrytis cinerea*

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Abstract

An isolate (87) of the yeast *Pichia guilliermondii,* protects apples from postharvest fruit rotting fungi *Botrytis cinerea* and *Penicillium expansum.* To examine the yeast-pathogen interaction, *B. cinerea* was grown on agar plates overlayed with cellophane. Effective and non-effective yeast isolates were applied near the young hyphal growth. Samples were taken 24 hr later from the area where the fungi and yeast had intersected. Light microscopy revealed a general attachment of the effective biocontrol agent and a non-effective isolate (117) of *Debaryomyces hansenii.* Low temperature scanning electron microscopy (LTSEM) indicated that both species of yeast attached to the fungal hyphae but the 87 isolate attached fastidiously. Twenty-four hours after applying the 87 isolate to *B. cinerea,* pitting and collapse of the hyphae were observed. These features were not observed with the ineffective isolate of *D. hansenii.* Culture supernatants from *P. guilliermondii* yielded 2-3 fold more +-(1-3) glucanase activity than *D. hansenii.* The data indicate that tenacious attachment, along with the secretion of cell wall degrading enzymes, may play a role in the biocontrol activity of this yeast antagonist.

Introduction

Pichia guilliermondii

Biological control of postharvest diseases of fruit is an area of great potential (Wilson and Wisniewski, 1989). The use of an isolate (87) of *Pichia guilliermondii* has shown broad spectrum activity in the control of a number of postharvest diseases of citrus (Chalutz and Wilson, 1990) Droby, et al., (1989) and temperate fruit (McLaughlin, et al., 1990 and Wisniewski, et al., 1988). In several previous publications this isolate had been referred to as *Debaryomyces hansenii,* however, more detailed characterization of this isolate has changed the classification to *P. guilliermondii* (McLaughlin, et al., 1990).

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Although nutrient competition has been suggested as the principle mode of antagonism (Droby, et al., 1989), attachment of the yeast to pathogen hyphae and extensive production of an extracellular matrix have been observed (Wisniewski, 1988). These factors may play a key role by either enhancing nutrient competition or by some other undetermined mechanism. The importance of attachment in antagonism has been proposed for other biological control systems utilizing *Trichoderma harzianum* (Elad, et al., 1983) and *Enterobacter cloacae* (Nelson, et al., 1986; Wisniewski, et al., 1989).

The principle objective of the present study was to examine the nature of the attachment of the yeast to hyphae of the postharvest pathogen of apple, *Botrytis cinerea*. It was of interest to determine if the attachment was due to a specific lectin-type binding or was of a more general, non-specific nature involving the extracellular matrix. Furthermore, we wanted to investigate whether or not attachment played a direct role in antagonism, by facilitating enzymatic hydrolysis of hyphal cell walls by *P. guilliermondii*.

Experimental

Botrytis cinerea cultures were obtained from apples and maintained on potato dextrose agar (PDA). *P. guilliermondii* (87) was isolated from a lemon surface as previously described (Wilson and Chalutz, 1989). *Debaryomyces hansenii* (117, ATCC #36239) was obtained from the American Type Culture Collection, Rockville, MD, USA. The yeast isolates were maintained on silica gel and grown in yeast media culture flasks for 24 – 48 hr before experimental use, as previously described (McLaughlin, et al., 1990).

Fungal and yeast interaction

The pathogen (1 X 10^5 conidia/ml) was plated on either acidic PDA, apple juice agar or apple slices (cv Golden Delicious) overlayed with cellophane (Spectra/Por, Spectrum Medical Industries, Inc., Los Angeles, CA). After 24 hr, 40ml of the yeast isolate (1 X 10^8 colony forming units per ml) was placed at the margin of fungal growth. At 4, 24 or 48 hr intervals, the cellophane portion where fungal and yeast interaction could be observed was removed from the agar plate, washed with a stream of distilled water for 30-60 seconds and viewed with light or scanning electron microscopy. Similar results were obtained from all media sources used.

In order to further characterize the attachment mechanism, several compounds were applied to the yeast and pathogen to test if a disruption of attachment would occur. Cellophane with the pathogen alone was removed and placed in a 45 mm petri dish. The yeast with the specified compound was applied to the fungus and allowed to incubate. The compounds used are listed in Table 1. After 4 hr, the fungal hyphae was observed under light microscopy for any attachment. Attachment was rated as + or –, where – indicates no attachment and increasing degrees of attachment were indicated by + or ++.

| Enzyme Activity | To test whether or not isolate 117 or 87 exhibited +-(1-3) glucanase activity, the isolates were grown in several substrates (Table 2) consisting of 1% (w/v) carbon source in a minimal salt media. They were grown in shake culture for 48 hr (stationary phase). The individual cultures were then centrifuged (1,0000 xg) and the supernatant dialyzed for 18 hr against deionized H_2O with 3 changes of H_2O in a 4L container. The samples were then lyophilized and reconstituted with one ml of 0.05 M acetate buffer, pH 5.0. A 200 ml aliquot was added to 800 ml of 1% laminarin (No. L-9634, Sigma, St. Louis, MO) which was used as a substrate for the enzyme, and 0.02% sodium azide. The enzyme-substrate mixture was incubated for 2 or 24 hr at 37°C and tested for total reducing sugars using dinitrosalicylic acid reagent (Miller, 1959). Glucose was used as a standard. The amount of reducing groups was measured spectrophotometrically at 500 nm. Background levels of reducing sugars were determined with a time O supernatant extract from each treatment. The time O extract was added to the laminarin enzyme substrate just prior to boiling at 100°C for 5 minutes. Boiling the supernatant prior to incubation with laminarin gave similar results. |

LTSEM

Low Temperature Scanning Electron Microscopy (LTSEM): The majority of experiments were conducted with hyphae of *B. cinerea* incubated 24 or 48 hr with the yeasts as described above. In some experiments, attachment of *P. guilliermondii* to hyphae of *Penicillium expansum* was also examined. *P. expansum* cultures were maintained as previously described (McLaughlin, et al., 1990) and handled in a manner identical to *B. cinerea*. Attachment of yeast to cotton or glass fibers was also examined. Small sections of cellophane (6 mm^2) in areas of fungal/yeast interaction were removed from the surface of the PDA, rinsed thoroughly with a stream of distilled water and mounted on an aluminum stub using silver paint as an adhesive. In order to enhance the removal of the yeast, some samples were rinsed with either 1% Tween (v/v) or 2% $CaCl_2$ (w/v). Additionally, some samples were briefly sonicated. An Oxford CT-1500 Cryo Preparation System and Cold Stage (Oxford, England) mounted on a Cambridge S-120 scanning electron microscope (Cambridge, England) were used to prepare and examine the specimens. Samples were quenched in LN_2-slush, transferred to the cold stage, and etched at -80°C for 3-5 minutes. The samples were then removed from the specimen chamber, coated with gold/palladium at -160°C and subsequently transferred back to the cold stage and kept at -165 to -175°C. Specimens were viewed at 5-10 kV.

TEM

Transmission Electron Microscopy (TEM): Samples of *Botrytis* hyphae incubated with the yeast for 48 hr were examined. Small samples of material (1 mm^2) were placed in 3% (v:v) gluteraldehyde in 25 mM sodium phosphate buffer (pH 6.8) at 4°C and postfixed in 2% osmium tetroxide (w/v) at 4°C. Samples were dehydrated in a graded ethanol series, followed by propylene oxide and embedded in epoxy resin. The material was sectioned with a diamond knife, mounted on copper grids and stained with uranyl acetate and lead citrate. Grids were examined at 75kV, using a Hitachi H-600 transmission electron microscope.

Results

Ultrastructure

LTSEM observations of 24 hour cocultures of *P. guilliermondii* *B. cinerea* indicated that the yeast had become tenaciously attached to the hyphae and to each other despite extensive rinsing of samples with distilled water or 1% Tween from a wash bottle during sample preparation (Fig. 1). In contrast, coculturing of *B. cinerea* with an isolate of *D. hansenii* (117), selected for use as a comparison because of its inability to exhibit biological control of *B. cinerea*, indicated only a loose association of the yeast with the fungus (Fig. 2).

In many instances, individual yeast cells of *P. guilliermondii* gave the appearance of having sunk into the hyphal cell wall (Fig. 3). To enhance dislodgement of *P. guilliermondii*, some samples were rinsed with 2% $CaCl_2$ or sonicated prior to sample preparation. These treatments were mildly effective in dislodging the yeast from some areas of the hyphae. When areas of hyphae were observed where yeast cells presumably had either become detached on their own or dislodged during sample preparation, irregularities in wall confirmation were observed. Discrete areas of the hyphae cell wall appeared concave, giving the hyphal strand a general appearance of being pitted (Fig. 4). In some areas the pitting was quite extensive. Similar observations were made on hyphal/yeast cell interactions in cocultures of *Penicillium expansum/P. guilliermondii* (Figs. 5-6).

Further observations were made on sectioned material. In cocultures of *D. hansenii/B. cinerea*, hyphal ultrastructure was normal in appearance and did not exhibit evidence of stress, despite the presence of numerous yeast cells (Figs. 7). In contrast, hyphae that had been cocultured with *P. guilliermondii* were often convoluted in appearance and appeared moribund (Fig. 8). It also appeared that in many hyphae the wall had undergone some swelling and partial degradation (Figs. 9-10). Although many areas of the wall appeared to have a concave appearance, it was not clear if this related to the pitting observed with LTSEM or was a more general phenomenon of cell wall degradation.

In areas where *P. guilliermondii* appeared to be in direct contact with hyphae of *B. cinerea*, yeast cells appeared to be lying within a depression of the hyphal cell wall (Fig. 11). At times, the yeast cells appeared to be firmly embedded within these depressions and surrounded by an extracellular matrix. In contrast, yeast cells of *D. hansenii* were only superficially attached to the hyphal cell wall via an extracellular matrix (Fig. 12).

Enzyme Activity

After 2 hr at 37°C, activity was 4 fold greater or more in supernatant extracts of *P. guilliermondii* grown in fructose, mannose, glucosamine, sucrose, and galactose than in supernatant extracts of *D. hansenii* grown on the same carbon sources (Table 1). +-(1-3) glucanase activity of *D. hansenii* extract was only detected in fructose and D-glucosamine cultures.

Attachment

The results, as reported in Table 2, indicated that all the salt solutions tested ($CaCl_2$, $MgCl_2$, and $MnCl_2$) and the protein degrading enzymes (protease and trypsin) blocked attachment when the yeast and the fungus were cocultured in the presence of the test compound. The use of sugars or laminarase, which have been shown to block lectin binding, were unsuccessful in preventing attachment of *P. guilliermondii* to hyphae of *B. cinerea*. The exception of this result was with the use of 2-deoxyglucose. Soaps (SDS and Tween), B-mercaptoethanol, and low temperature (4°C) were mildly effective in blocking attachment. Finally, blocking of respiration with sodium azide also blocked attachment. It was also observed that yeast cells were unable to attach to either cotton or glass fibers.

Discussion

Attachment of yeast cells to other organisms has been shown to play a major role in their biological activity (Douglas, 1987; Jones and Epstein, 1989; Hamer, et al., 1988). The isolate of *P. guilliermondii* investigated in this study appears to a have a strong attachment mechanism that is blocked when respiration is inhibited or when cells are exposed to compounds that effect protein integrity. When yeast cells were washed, the ability to attach was partially restored. This indicates that the protein(s) that is integral to attachment is most likely located on the yeast cell surface and/or within the surrounding extracellular matrix. Nelson, et al., (1986) showed that *E. cloacae* attachment could be negated if sugars were applied. They concluded that a lectin-type binding was functioning in attachment. A lectin was also concluded to play a major role in the agglutination of spores of *Trichoderma harzianum* by culture supernatants of *Sclerotium rolfsii* and *Rhizoctonia solani* (Elad, et al., 1983). This agglutination was blocked both by sugars and trypsin. In our study, 5% 2-deoxyglucose was the only sugar that blocked attachment. This does not rule out the possibility of blocking attachment with the use of other sugars. The yeast was also unable to attach to cotton or glass fibers. Collectively, the data indicate that a lectin or other type of agglutin may be involved in selective binding of *P. guilliermondii* to other fungi. Qualitative observations have demonstrated a differential ability of *P. guilliermondii* to attach to various genera of fungi (data not shown).

Further experiments showed that both the isolates investigated produce glucanase. The effective *P. guilliermondii* isolate (US-7) produced higher levels of +-(1-3) glucanase. The close association of *P. guilliermondii* to the fungal cell wall would enhance the effectiveness of any hydrolase excreted by the yeast to the extracellular matrix. Chitinases and B-glucanases have been observed in yeast (Douglas, 1987) and are instrumental in the budding process. Filamentous fungal hydrolases have also been shown to degrade pathogen cell walls (Elad, et al., 1983).

Other workers (Droby, et al., 1989) have suggested that
nutrient competition plays a major role in the biocontrol
activity of *P. guilliermondii*. Our results indicate that the
yeast is firmly attached to hyphae of *B. cinerea* and that this
attachment in conjunction with the production of +-(1-3)
glucanase results in a partial degradation of the hyphal cell
wall. The cell wall degradation was evidenced by a pitted
appearance, visible with both LTSEM and transmission electron
microscopy. In contrast, the ineffective isolate of *D.
hansenii* (117) showed only a superficial ability to attach to
hyphae of *B. cinerea* and only a small amount of +-(1-3)
glucanase activity when grown on several carbon substrates.
Therefore, the efficacy of *P. guilliermondii* appears to be
dependent not only on it's ability to colonize a wound site
(Droby, et al., 1989), but may also depend on it's ability to
both firmly attach to hyphae of the pathogen and exhibit high
levels of +-(1-3) glucanase activity.

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Table 1. The effect of various carbon sources on the activity of
extracellular +-(1-3) glucanase produced by *Pichia guilliermondii* (87)
and *Debaryomyces hansenii* (117) grown on a mineral medium.

Substrate	umol reducing sugar/L/hr isolate	
	87	117
Pectin	nd[a]	nd
Chitin	nd	nd
Fructose	0.16±0.03	0.04±0.03
Mannose	0.17±0.04	nd
D-Glucosamine	0.08±0.01	0.02±0.01
Apple juice	nd	nd
Sucrose	0.08±0.03	nd
D-Galactose	0.12±0.02	nd

[a]nd = below detectable level.

Table 2. Attachment of *Pichia guilliermondii* (87) to *Botrytis cinerea* after treatment with various salts, sugars and chemical agents.

Treatment[a]	Attachment after 4 hr[b]
Yeast isolate 87	++
Tween (1%) v/v	+
SDS (0.01 %)	+-
B-mercaptoethanol (0.01%)	+-
$CaCl_2$ (1%)	-
$MnCl_2$ (1%)	-
$MgCl_2$ (1%)	-
Protease (2 mg/ml)	-
Trypsin (2 mg/ml)	-
Laminarase (2 mg/ml)	++
D-glucose (5%)	++
2-deoxyglucose (5%)	-
Sodium Azide (1%)	-
D-galactose (5%)	+
D-mannose (5%)	+

[a] all reagent were prepared w/v, except where indicated.
[b] ++ = high level of attachment, + = attachment, +- = attachment but sparse, - = no attachment

Figure 1. Coculture of *P. guilliermondii* and
           B. cinerea. Tenacious attachment of
           yeast to fungal hyphae is visible.
           X1,510.

Figure 2. Coculture of *D. hansenii* and *B. cinerea*. Only superficial attachment of yeast to pathogen was visible. X2,450.

Figure 3 - 4. Coculture of *P. guilliermondii* and *B. cinerea.* Embedding of yeast into hyphal wall and pitting of the surface of hyphal cell wall (arrow) is visible. X3,320 and X 3,550, respectively.
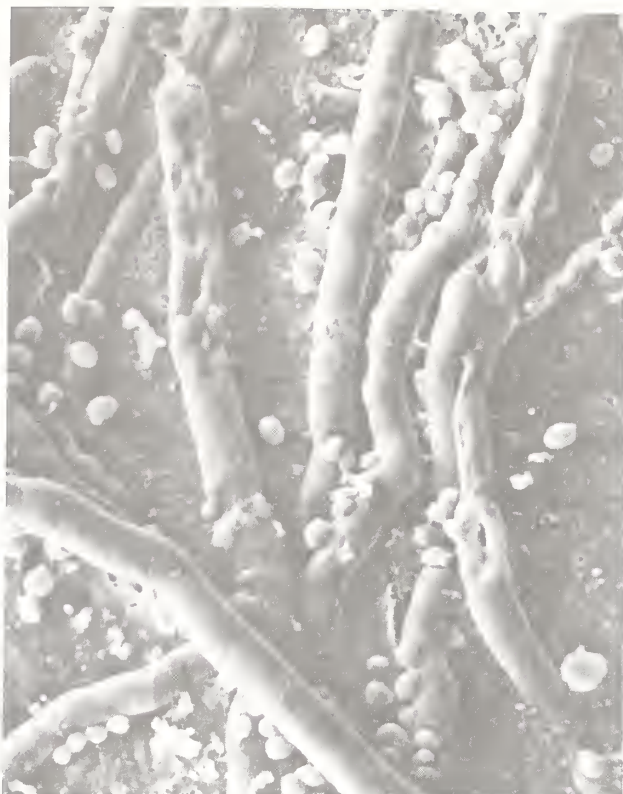
Figure 5 - 6. Coculture of *P. guilliermondii* and *P. expansum*. Note extensive pitting in the hyphae cell wall. In some instances, holes in the hyphal cell wall are visible (arrow). X1,110 and X2,350, respectively.
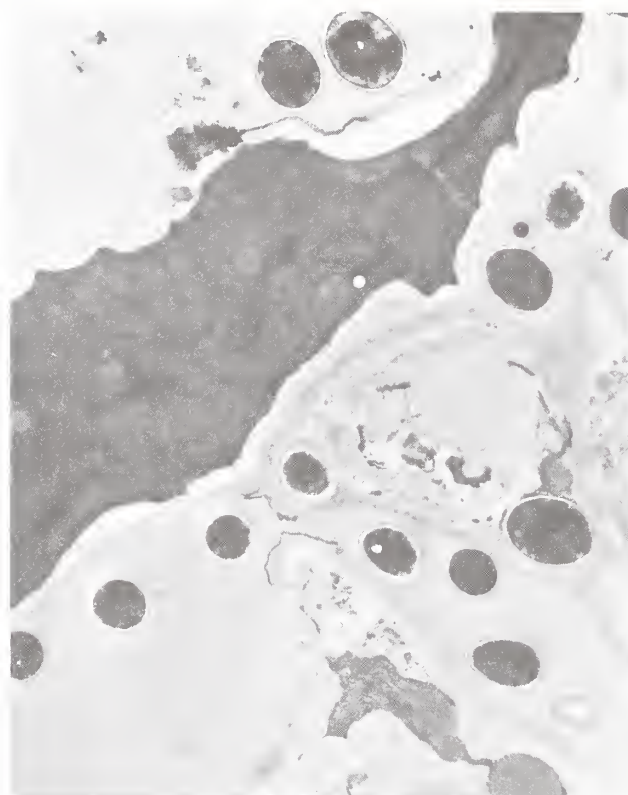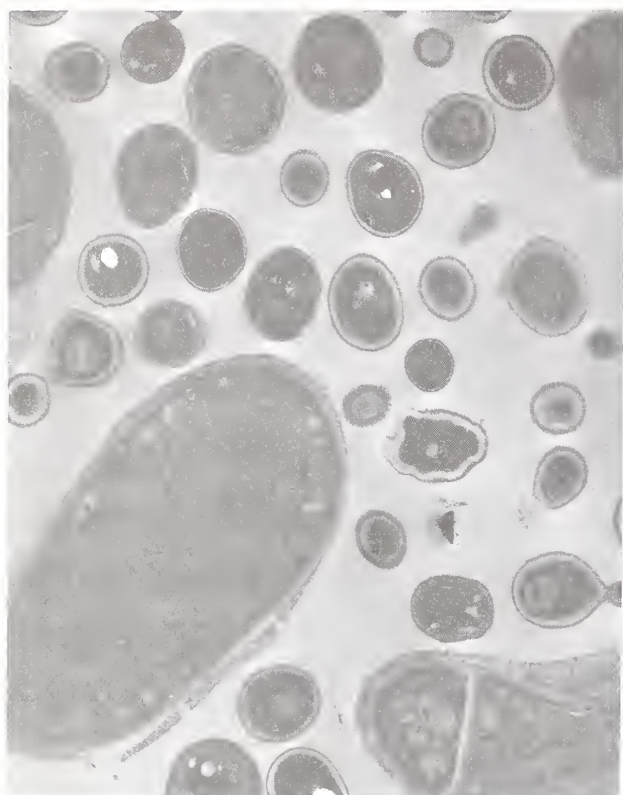
Figure 7 - 8. Ultrastructure of *D. hansenii/B. cinerea* and *P. guilliermondii/B. cinerea*, respectively. Note moribund hyphae in *P. guilliermondii/B. cinerea*. X3,570 and X5,625, respectively.
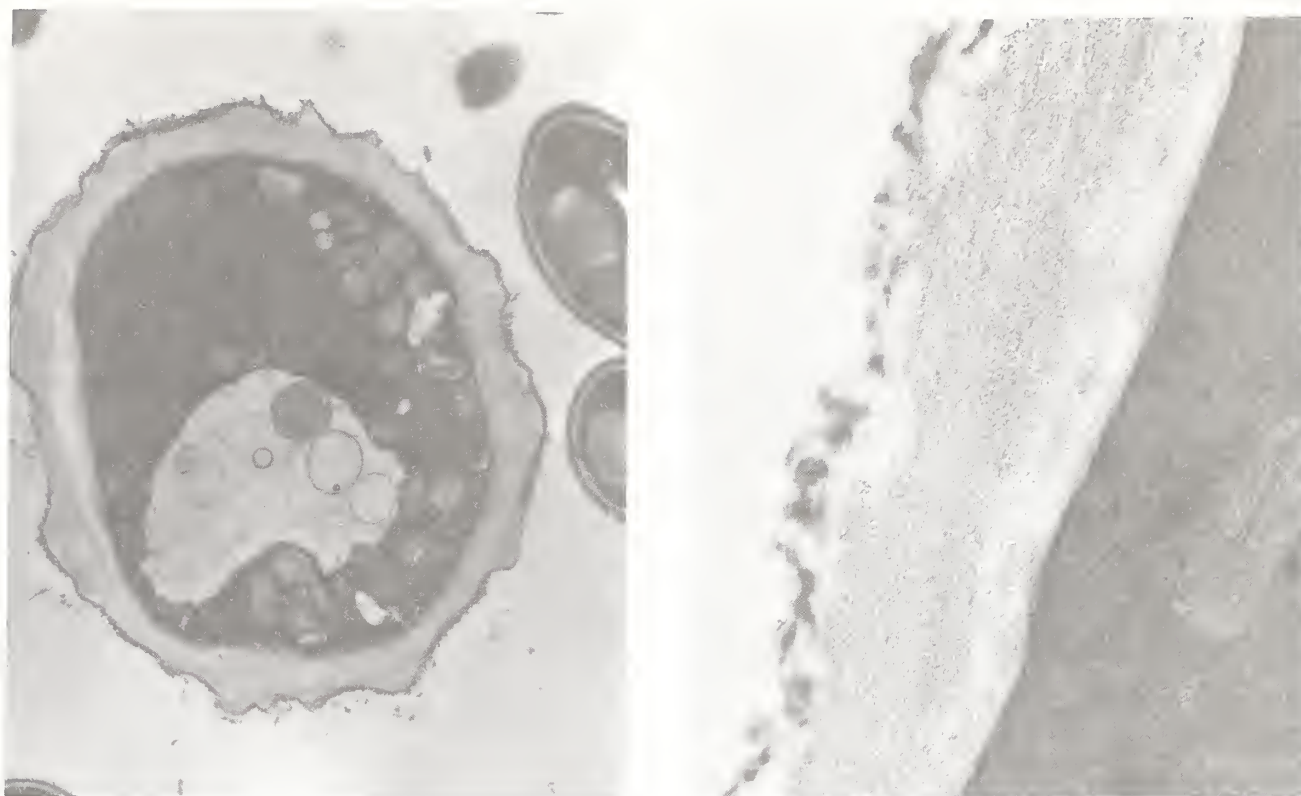
Figure 9 - 10. Partially degraded *B. cinerea* hyphal cell walls observed in cocultures
of *P. guilliermondii/B. cinerea*. X11, 250 and X46,950, respectively.
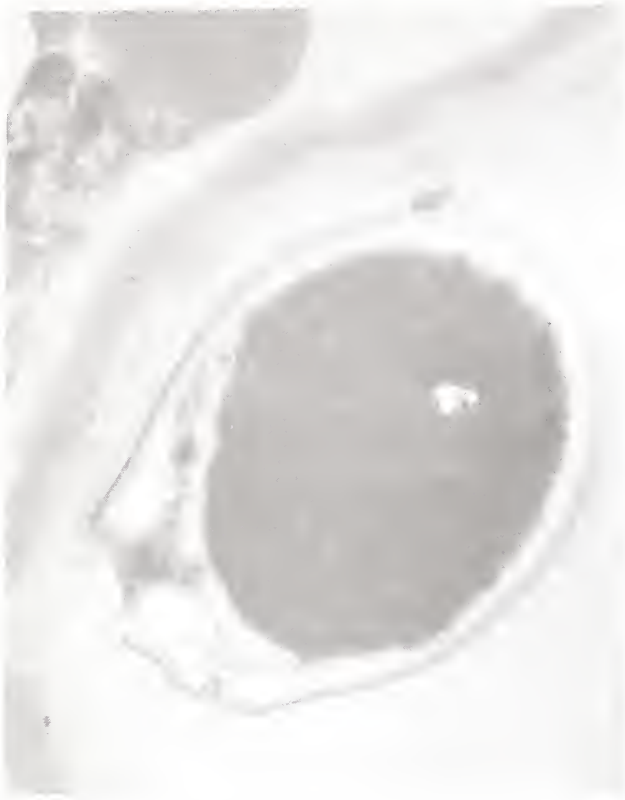
Figure 11. Yeast cell of *P. guilliermondii* embedded in depression of hyphal cell wall of *B. cinerea*. X33,300.
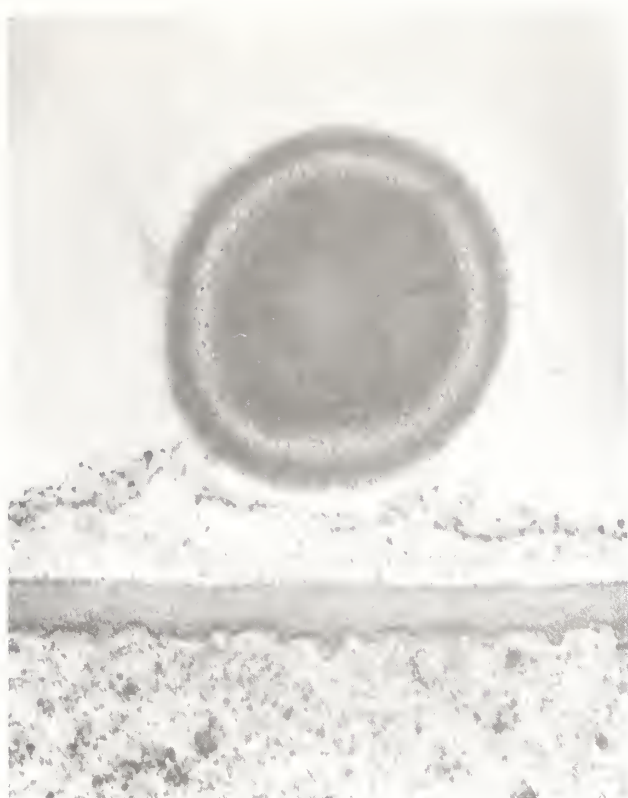
Figure 12. Superficial attachment of
         D. hansenii yeast cell to hyphal
         cell wall of *B. cinerea*. X34,200.

A Review and Current Status of Research on Enhancement of Biological Control of Postharvest Diseases of Fruit by Use of Calcium Salts with Yeasts

R. J. McLaughlin[1]

Abstract

Previous research has shown that *Candida guilliermondii* and other species of yeast, including *Kloeckera apiculata*, are effective for reducing postharvest losses in various fruits due to *Botrytis cinerea* and *Penicillium* spp. The amount of control conferred by these yeasts primarily depends on the inoculum level of the yeasts used to treat the fruit and the challenge inoculum level of the pathogen. Control of apple decay due to *B. cinerea* with these yeasts is demonstrated by a substantial reduction in disease incidence and severity, while that obtained in assays for *Penicillium* rot control is significant but low. Addition of various salt solutions to these yeast suspensions has shown that $CaCl_2$ can dramatically increase the amount of control obtained. Calcium chloride, in the absence of yeasts, does not facilitate significant reduction of disease. Control of *Botrytis* rot can be obtained with lower amounts of yeast and *Penicillium* rot control is much improved. Experimental results reported in this paper indicate that the mode of action of $CaCl_2$ occurs by causing a reduction of conidial germination and germ tube elongation of the postharvest decay pathogens.

Introduction

Postharvest losses in pome fruit due to fungal decay can be significant. Losses can be prevented or minimized by treatment of fruit after harvest with microbial antagonists or fungicides. Fungal and bacterial antagonists, investigated as an alternative to fungicides, have been found effective for the control of various postharvest diseases in peach (Pusey and Wilson, 1984; Pusey, et al., 1988; Wisniewski, et al., 1989), apple (Janisiewicz, 1987; Janisiewicz, 1988; McLaughlin, et al., 1990b; Roberts, 1990), pear (Janisiewicz and Roitman, 1988), citrus (Chalutz, et al., 1988; Chou and Preece, 1968; Gutter and Littauer, 1953; Wilson and Chalutz, 1989), cherry (Utkhede and Sholberg, 1986), and grape (Dubos, 1984). Specifically, the biocontrol agents that have been studied for the control postharvest diseases of peach have included *Bacillius subtilis* for the control of Brown Rot, caused by *Monilinia fructicola* (Pusey, et al., 1988), and *Enterobacter cloacae* for the control of *Rhizopus* rot, caused by *Rhizopus stolonifer* (Wisniewski, et al., 1989). For the control, of apple decays due to *Penicillium expansum* and *Botrytis cinerea*, the antagonistic microorganisms have included the filamentous fungus *Acremonium brevae* (Janisiewicz, 1987), the bacterium *Pseudomonas cepacia* (Janisiewicz and Roitman, 1988) and several species of yeasts (McLaughlin, et al., 1989; McLaughlin, et al., 1990b; Roberts, 1990).

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Yeast strains of *Cryptococcus laurentii* and *Candida guilliermondii* (previously described as *Debaryomyces hansenii* (McLaughlin, et al., 1990a)), have been studied for the biological control of *Botrytis* and *Penicillium* rot of apple (McLaughlin, et al., 1990b; Roberts, 1990; Wisniewski, et al., 1988). The proposed mechanism by which strains of *C. guilliermondii* elicit control is by nutrient competition (Droby, et al., 1989). Preliminary data from our lab indicates that *C. guilliermondii* strains may also effect partial degradation of the mycelium of *B. cinerea* (McLaughlin et al., unpublished).

Biological control of postharvest decays can be enhanced by use of calcium chloride in the yeast suspensions that are applied to fruit (McLaughlin, et al., 1990b). This report reviews previous work in this area and reports research directed towards determining the means by which $CaCl_2$ enhances the biological control ability of yeasts.

Experimental

Source of Fruit

Apples (cv. Golden Delicious) were harvested at commercial maturity and stored at 2°C for 3 mo or less before use.

Yeast and Pathogen Strains

Candida guilliermondii strain 87 (=US-7) was isolated from the surface of lemon fruit as previously reported (Wilson and Chalutz, 1989). *Kloeckera apiculata* strain 138 (=G-4) was isolated by Samir Droby (Agricultural Research Organization, The Volcani Institute, Bet Dagan, Israel) from the surface of Thompson Seedless grapes.

Isolates of the postharvest pathogens *B. cinerea* and *P. expansum* were obtained from diseased fruit at the Appalachian Fruit Research Station, Kearneysville, WV, and routinely stored as lyophilized cultures.

Culture conditions and preparation of yeast and pathogen spore suspensions. Yeast strains were cultured and prepared for application to fruit as previously described (McLaughlin, et al., 1990b). Conidia of *B. cinerea* were obtained and prepared as previously described (McLaughlin, et al., 1990b). Fruit were challenged with 20 μl aqueous suspensions of 10^5 spores/ml of *B. cinerea*.

Biocontrol Assays on Apple Fruit

Fruit were surface disinfested and air-dried 4 hr prior to wounding (McLaughlin, et al., 1990b). Single wounds (5 mm deep by 3 mm wide) were made in each fruit as previously described (Wilson and Chalutz, 1989). Immediately after wounding, 50 μl of an aqueous yeast suspension was pipetted into each wound site. Calcium chloride (2% w/v) was included in cell suspensions of strain 87 and G-4 in some treatments. The yeast suspensions were left to dry in the wound site for 2 hr at ambient temperature (24–26°C) before challenge with 20 μl of pathogen spore suspension. The challenge inoculum was 10^5 conidia/ml of *B. cinerea*.

Spore suspensions were prepared in sterile distilled water amended with 0.01% Tween 20 (McLaughlin, et al., 1990b). Treatments were completely randomized in each test. Fruit were

incubated at 24°C and observed for percent infection at 7 days after challenge inoculation. There were three to six trials consisting of ten to twenty replicates per treatment.

Growth of the yeast *in vivo* in the presence and absence of $CaCl_2$. Fruit were treated with yeast cells suspended in aqueous or 2% $CaCl_2$ suspensions as in the previous assay. Fruit were incubated at 24°C and wounds were assayed for populations of yeasts by dilution plating onto nutrient-yeast dextrose agar (McLaughlin, et al., 1990b). Wounds were removed from fruit by use of a sterilized no. 6 cork borer and comminuted in sterile distilled water for 60 sec in a Virtis homogenizer set at 60 power prior to dilution plating.

Growth of *B. cinerea*

Growth of *B. cinerea* and *P. expansum in vitro* as affected by $CaCl_2$. Apple juice agar was prepared using 200 ml of freshly-prepared, filter-sterilized juice from "Golden Delicious" apples, plus 800 ml of sterile distilled water and 15 g of agar. Calcium chloride was added to half of the media to a final concentration of 2% (w/v). Spore suspensions (10 µl at 10^4 conidia/ml) of *B. cinerea* or *P. expansum* were added to the center of each plate for each type of agar. Plates were incubated at 26°C and radial growth was measured over 7-10 days. There were three replicates per treatment.

Germ Tube Elongation of *B. cinerea*

Germination and germ tube elongation of *B. cinerea* conidia *in vitro* as affected by $CaCl_2$ and nutrient levels. Germination and germ tube elongation of *B. cinerea* conidia was measured in microtiter plate wells containing various concentrations dilutions of apple juice (0 - 5 mM reducing sugars final concentration) land calcium chloride (0 - 175 mM final concentration). Conidia from 7 day-old cultures were added to obtain a final concentration of 5×10^3 conidia per well. After addition of conidia, total liquid volume per well was 150 µl. Plates were covered, wrapped in parafilm to minimize moisture loss and placed in a rotary shaker-incubator set at 26°C and 250 rpm. After 24 hr of incubation the plates were taken from the incubator and germination and germ tube elongation were measured. Fifty microliters of 10% glutaraldehyde was added to each well to arrest further germination and germ tube elongation during the measuring process. The experiment was repeated three times.

Results and Discussion

Previously reported work has shown that aqueous suspensions of *C. guilliermondii* strains 87 and 101 can dramatically reduce the severity and incidence of *Botrytis* rot of apple (McLaughlin, et al., 1990). Control of Penicillium rot is significant; but only lesion size is reduced and not lesion frequency. Tests with various salt solutions (2% w/v) aqueous unbuffered solutions of $CaCl_2$, $CaCO_3$, $FeSO_4$, KCl, $MgCl_2$, $MnCl_2$, and NaCl) were designed to determine if salt solutions, having varying osmotic potentials, would facilitate control of the disease. These tests demonstrated that significant enhancement of control could be achieved with certain salts ($CaCl_2$, $CaCO_3$, and KCl). However, the ability of these salts to facilitate improved control was not related to the osmotic potential of these solutions. The two most effective salt solutions, $CaCl_2$ and $CaCO_3$, suggested that the Ca^{+2} cation may play an important role.

Enhancement of control with $CaCl_2$ is unlikely to occur as a result of physiological changes in the host tissue that result in increased resistance to decay of pectic substances, as reported by Conway (1982) and Conway and Sams (1983). In these studies, fruit vacuum-infiltrated with 8 (w/v) solutions of $CaCl_2$ had a decay reduction of only 10%. Dipping of fruit in $CaCl_2$ solutions having concentrations as high as 12% (w/v) did not reduce decay. The observation was confirmed by McLaughlin et al., (1990b), in that topical treatment with $CaCl_2$ did not reduce Penicillium or Botrytis decay when applied in the presence of yeast cells.

The ability of $CaCl_2$ to facilitate control can be dependent on the yeast strain and the relative concentration of $CaCl_2$ that is used. For instance, strain 101 of *C. guilliermondii*, when applied at 10^7 cfu/ml, requires concentrations of 2% $CaCl_2$ to facilitate control of Botrytis rot, while strain 87 will give a similar level of control at a $CaCl_2$ concentration of 1% (McLaughlin, et al., 1990b).

Calcium chloride can result in the enhancement of control with other yeast species. As shown in Figure 1, disease incidence of Botrytis rot on Golden Delicious apples was significantly lowered, compared to treatments with yeast alone and the water control, when yeast cells of strain 87 and strain 138 of *Kloeckera apiculata* were applied in the presence of 2% $CaCl_2$.

One of the hypothesis on the mode of action of $CaCl_2$ was that control was facilitated by improving the colonization ability of the yeast in the wound site. This does not seem to be the case since populations of yeast, followed over a period of 96 hr after application, do not significantly differ (Figure 2). Population levels are asymptotic at 72 hr after application to the wound site. Populations reached ca. 7.2 (+ 0.1) log cfu/wound site in each treatment.

Another possibility was that $CaCl_2$ could inhibit the growth of the pathogen in the wound site. Growth of *B. cinerea* and *P. expansum* in apple juice agar, with and without 2% $CaCl_2$, was measured to determine if this was the case (Figure 3) This study indicated that $CaCl_2$ does not inhibit the growth of B. cinerea, but the growth of *P. expansum* was significantly affected. Average colony diameters of treatments in the presence of $CaCl_2$ were significantly lower at 4 and 7 days after plating them in agar plates that were not amended with $CaCl_2$.

The effect of $CaCl_2$ was found to be dependent on the concentration of reducing sugars in the microtiter plate studies that measured germination and germ tube elongation. Germination of *B. cinerea* was reduced significantly in wells where 0.10 mM reducing sugars were present, a significant negative correlation was observed with increasing $CaCl_2$ concentration ($r^2 = 0.54$, probit $(y) = -0.007(x) + 1.4$). There was 94.0% (+3.6) germination at 0% $CaCl_2$ and 63.7% (+5.0) at 175 mM $CaCl_2$. Significant effects increasing $CaCl_2$ concentration were not observed in treatments at higher levels of reducing sugars. Germ tube elongation was most dramatically reduced when $CaCl_2$ concentrations were higher than 100 mM, regardless of reducing sugar concentration (Figure 4). At 75

mM CaCl$_2$, there was little evidence of an effect on germ tube elongation at the 5.0 mM reducing sugar level. Calcium chloride at 75 mM or greater consistently reduced germ tube length at lower reducing sugar concentrations.

These data demonstrate that the effect of CaCl$_2$ *in vitro* is related to both its concentration and the reducing sugar concentration of apple juice. If these data can be extrapolated to explain the ability of calcium chloride to facilitate control in the wound site, then this phenomenon must be strongly linked to the yeast's ability to reduce sugar levels in the wound site and thus reduce germination and growth of the pathogen. Preliminary data indicate that this relationship can also be observed with *P. expansum* and that other salts, such as MgCl$_2$, do not elicit a similar effect (data not shown).

Data that indicate nutrient competition as a mode of action for strain 87 is consistent with this hypothesis (Droby, et al., 1989). Other reports in the literature have shown decreased infection or germ tube elongation by *B. cinerea* can result from depletion of nutrients, which in some cases is attributed to phylloplane-associated bacteria and fungi (Barash, et al., 1964; Brodie and Blakeman, 1975; Clark and Lorbeer, 1976, 1977; Kosuge and Hewitt, 1964). The data that shows the dependence of the CaCl$_2$ is not strain or species-specific in control enhancement, strongly implicate nutrient competition or site exclusion as mechanisms that characterize the biological control ability of these yeasts. The competitive ability of the yeasts, as measured by the ability to grow and occupy the wound site and thus reduce available nutrients to the pathogen's conidia, seems intuitively necessary in order for them to facilitate control. This phenomenon may also work in concert with undetermined mechanisms that result in mycelial collapse and degradation in vivo.

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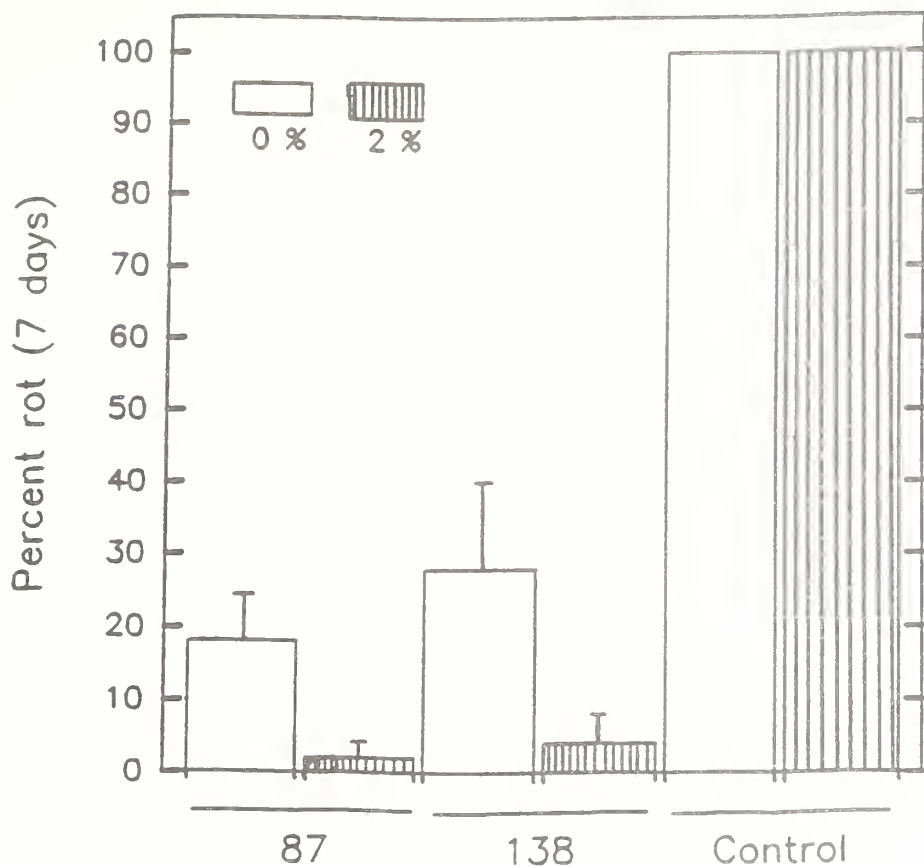
FIG. 1. Effect of calcium chloride on biological control of *Botrytis* rot of 'Golden Delicious' apple with strain 87 of *Candida guilliermondii* and strain 138 of *Kloeckera apiculata*. Fruit were treated with aqueous or 2% (w/v) $CaCl_2$ suspensions containing no yeast or yeast cells at 10^8 cfu/ml and challenged 2 hr later with a 10^5 conidia/ml suspension of *Botrytis cinerea*. Bars are standard errors of the means.
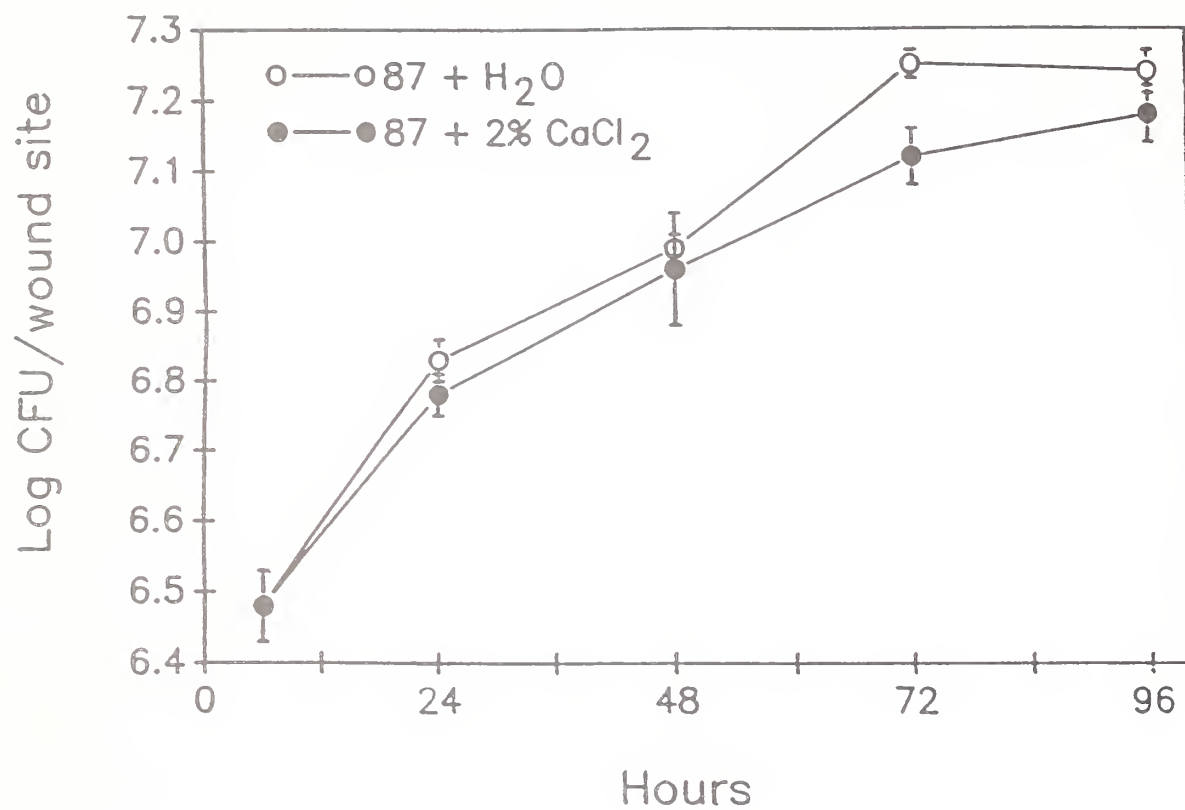
FIG. 2. Population recovery of strain 87 of *C. guilliermondii* in apple wounds as affected by application with or without 2% CaCl$_2$.
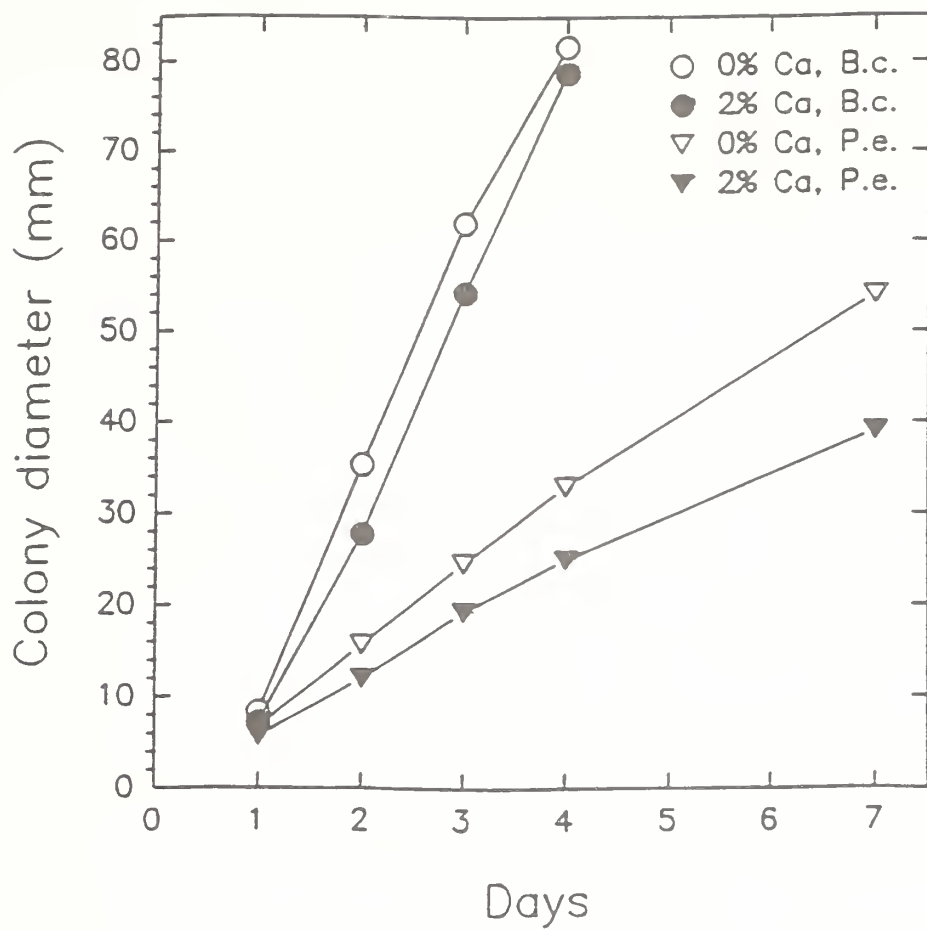
FIG. 3. Effect of calcium chloride on growth of *Botrytis cinerea* (B.c.) and *Penicillium expansum* (P.e.) *in vitro* on apple juice agar at 26°C.
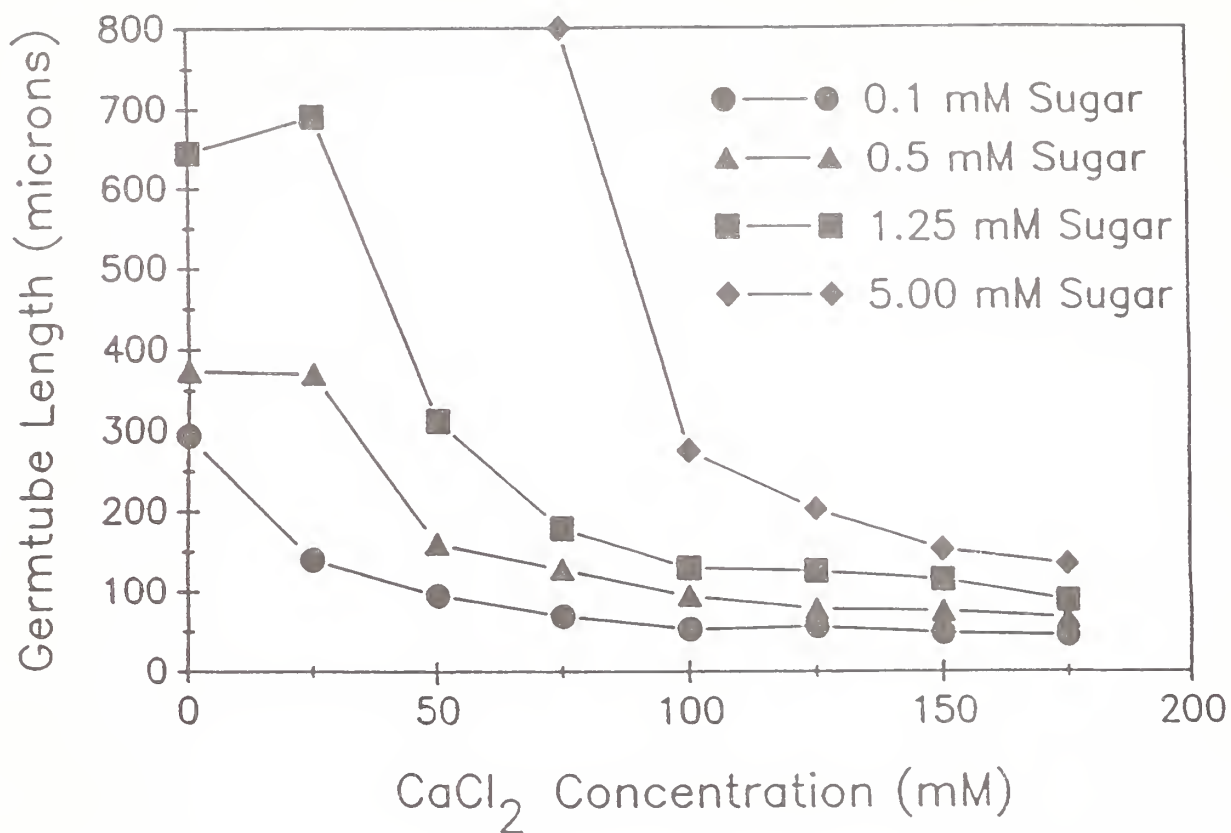
FIG. 4. Effect of calcium chloride and reducing sugar concentration in apple juice on germ tube elongation of *Botrytis cinerea* after 24 hr of incubation at 26°C.

IV. LARGE-SCALE PRODUCTION OF BIOLOGICAL CONTROL AGENTS.

Producing and applying antagonists on a large scale presents new problems and challenges.

Scaling-up the Production for Application of an Antagonist - from Basic Research to R & D

R. Hofstein[1], S. Droby[2], E. Chalutz[2], C. Wilson[3], and B. Fridlender[1]

Abstract

The conceptual spirit of scaling-up the production of biopesticides stems from the general theme of the biological sciences. Basic research focuses on the fundamental phenomenon of a biological process and biotechnological processes are the reflection of it in terms of a functional microorganism(s) which acts as the executing machinery of that process in the development of biopesticides. During the phase of basic research, towards the development of a biopesticide, an antagonistic microorganism is being isolated and characterized according to its potential to serve as the active component of a biopesticide. The yeast-like strain US-7 (*Pichia guilliermondii*) which was isolated from citrus fruit and has been proposed as an antagonist of *Penicillium* fruit-rot has been subjected to the developmental process of a biofungicide for the control of post-harvest diseases. The development involves an industrial process of fermentation, product formulation and semi-industrial 'field-trials' in packing houses. The design and execution of such an R & D program is discussed here in details. The rationale behind it is exemplified by a series of experiments which examine the potential to control post-harvest diseases of citrus crops.

Introduction

Biological control which relies on microbial antagonists has been implicated in insect control (Dulmage, 1981) and in plant-disease control (Baker and Scher, 1987). A number of post-harvest diseases have also been controlled by introduced antagonists (Wilson, 1989). A yeast-like microorganism which has lately been characterized as a naturally occurring species of *Pichia* (US-7 = *Pichia guilliermondii*) effectively antagonized the development of molds and rots of citrus varieties during storage (Wilson and Chalutz, 1989).

The laboratory results on *in vitro* as well as *in vivo* efficacy of US-7 justified the efforts to promote the natural antagonist into a commercial product. The mode of action of the microbial isolate indicated that the post-harvest environment is advantageous for its antagonistic function.

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Steps in Commercialization

There are several key steps which have to be undertaken as the immediate responsibility of any organization who is in charge of developing a biopesticide. First and most importantly, the microorganism ought to be easily harnessed and respond properly to the artificial conditions of mass-production. Next, the fermentation process has to be cost-effective and therefore, the industrial fermentation should be adjusted to optimal utilization of waste products as source of nutrients for the multiplying cells (Trilli, 1986). Thirdly, the biomass should consist mostly of those cells which preserve the antagonistic features together with recovery of stability during storage (shelf-life). These two can be achieved with an adequate formulation that is usually tailored specifically to the purpose of a biological agent in general (Connick, 1988) and for fungal agents in particular (Papavizas, 1986). Last but not least, the antagonistic activity of the microbial pesticide ought to be expressed in a simulating fashion when *in vitro* and *in vivo* tests are used as a preempt to the full-scale field trials. These tests then become the quality assurance analyses.

Pichia guilliermondii

The yeast isolate *Pichia guilliermondii* (US-7) was selected for the development of a biofungicide of post-harvest diseases for reasons that are derived from the basic criteria of product development. The isolate responds positively to the artificial conditions of biomass production (i.e. semi-industrial fermentation on waste products as raw material). Its mode of action was proposed to be via competition with pathogenic fungi for space and nutrients on wounded fruits and such a mechanism is more acceptable simply because it does not involve biosynthesis and secretion of potentially hazardous antibiotic compounds as shown by Droby et al. (1989). The isolate was found to be compatible with major non-toxic ingredients of the storage and application formulation. Toxicological tests have assigned to it a safety clearance which is generally of utmost importance and is particularly crucial for market acceptability of a biofungicide (Consumer Product Testing - official document, 1990).

Postharvest Diseases of Citrus

We have targeted our efforts primarily towards the control of post-harvest diseases in citrus crops among which are included blue mold, green mold, gray mold (appears also on grapes) and several other rots which can cause a substantial loss during shipment and storage and therefore represent a significant segment of the world market for post-harvest disease control. In the future we will extend the same line of scaling-up activities, production of biocontrol agents against brown-rot in peaches which was reported by Pusey et al. (1986), to be suppressed by microbial agents, development of products for the control of gray mold in apples and grapes. The potential of biofungicides as an alternative approach or as part of integrated pest management (IPM) will be discussed in view of product efficacy and its contribution to the urgently required reduction in the use of chemical fungicides immediately prior to harvest or post harvest.

Experimental

Microbial Cultures and Inoculi

The yeast-like isolate US-7 was characterized as a strain of *Pichia guilliermondii* by standard characterization techniques (Microbial Properties Research - official document, 1990). This strain was subjected to key Tier I toxicological tests (acute oral toxicity in rats and acute dermal toxicity in rabbits) and was determined to be a non-toxic article (Consumer Product Testing, 1990).

Cultures of US-7 were prepared on nutrient yeast dextrose agar (NYDA) and inoculi for fermentation were prepared in Erlenmeyer flasks containing (Nutrient Broth, Yeast extract, Dextrose, NYDB) according to the procedure which was previously reported by Droby et al. (1989).

Scale-up Fermentation Processes

In order to fulfill the requirements of 'field-trials' in citrus packing-houses we have adjusted the fermentation conditions which were previously described by Droby et al. (1989). There the biomass was produced in nutrient yeast dextrose broth (NYDB) mixed together in Erlenmeyer flasks. The scale-up and process optimization were performed in a 15L bench top fermentor (Microgen, New-Brunswick). The growth medium for production of US-7 comprised of NYDB (8.0 gr nutrient broth, 5.0 gr yeast extract and 10.0 gr D-glucose, all mixed in 1000 ml deionized water). It was important in this particular project and is also recommended for any other scaling-up processes, to start by linear extrapolation of the laboratory results namely, the first few batches which are produced as a supply for field-trials have to be fermented on standard laboratory type growth media. Therefore during the first period of the program all batches of US-7 were prepared in a bench-top fermentor on the NYDB growth medium.

Later on, with progress in the scaling-up program, NYDB was replaced by much cheaper industrial waste materials such as cotton seed meal, corn-steep liquor or partially digested peptones. Each of these raw materials was used either as a particulate preparation following an extensive cycle of sterilization or as a 10-20% extract in plain water. At the end of the fermentation process, the biomass was harvested by centrifugation or by microfiltration (dewatering through a selective filtration membrane). The recovered paste was washed and resuspended in tapwater up to the original volume or to a ten-fold concentrated suspension which was conveniently stored as such at 4°C.

Quality Assurance Tests

In vitro tests in agar plates measured the extent of *Penicillium* spore germination when sporal suspensions were seeded in potato/dextrose agar (PDA) in the presence of variable amounts of the antagonist. The rate of germination was compared with that of sporal suspensions growing in the absence of antagonist.

In vivo efficacy of cellular samples was determined by a method which was developed by Wilson & Chalutz (1989). Basically, grapefruit were punched to form wounds and these were loaded with drops of cellular suspensions of the antagonist followed by spray of *Penicillium digitatum* sporal suspensions.

Inhibition of fruit rot at or next to the wounds indicated quantitatively the efficacy of the product.

Post-harvest Mangaement in Packing Houses of Citrus Crops:

Three methods of post-harvest disease management were assessed in several packing-houses of citrus crops. The three methods were: 1) dipping of fruit in antagonist suspensions; 2) spraying the antagonist through a standard nozzle and 3) drenching of fruit while it is running on the sorting conveyer. The citrus varieties which were subjected to the efficacy trials were Mineola and Topaz peelable mandarins, Jaffa oranges and grapefruit. In order to evaluate the optimal conditions for fruit treatment, the experiment was integrated into the daily routine of the packing-house except for the chemical fungicides which were replaced by the biopesticide. We had to confirm that no chemical residues in the sorting line would interfere with the experiments and therefore prior to the actual exposure of 'experimental fruit' to the treatment, all lines and nozzles were extensively washed with water followed by a pre-run of antagonist suspension in a few cycles.

The level of chemical residues was determined by means of mass-spectrometry which can detect levels of less than one ppm of the commonly used chemical fungicides. Whenever the experiment included an IPM approach, the cellular suspension of US-7 was premised with 200 ppm of tiabendazol (TBZ). The biological or IPM treatments were always compared with the standard packing- house treatment which included 2000 ppm TBZ, 2000 ppm Imazalil and 4000 ppm Ridomil.

The standard lay-out of a processing line in citrus packing-houses comprise of several working stations. The fruit receives an extensive wash with plain water and then a mild soap wash as it enters the sorting line. It is then air-dried before reaching the station of fungicide treatment. The most commonly used systems are the drenching or the spray units but theoretically, dipping can also be considered if necessary. The sprayed fruit then rolls under blowers for dehydration followed by sorting, waxing and packing.

Evaluation of Disease Development

A standard method was used for evaluation of biological control of fungal pathogens. Fruit was packed in cardboard boxes (such as those commonly used for shipment to European markets) and stored at 17°C for at least 17 days. Following storage, boxes were withdrawn from the storage and infested fruits were counted so as to obtain a figure on percent decay. The inspectors had to point out the distribution of different pathogens, namely, whether it was molds or other rots (sour rot etc.) which might have caused the decay.

Before placement of the fruit into incubation under storage conditions, samples were taken for laboratory assessment of the degree of fruit coverage with the antagonist, detection of residual chemical fungicides and for *in vivo* testing of quality assurance according to a protocol which was described above.

Results and Discussion

The scale-up of any process from small scale in the laboratory to a semi-industrial level is not always reached by a linear extrapolation. The bridging experiment between the basic research and the R & D stage was an efficacy test which determined the concentration of the biological agent required for the control of a pathogen. Grapefruit was wounded and treated with varying concentrations of cells (cfu/ml) and groups of 5-10 fruits were then sprayed with varying density of *Penicillium digitatum*. The experimental design was similar to the one which was developed for *in vivo* quality assurance tests. The most effective control was within the range of $1.0-2.0 \times 10^8$ cfu/ml of *P. guilliermondii* cell suspensions for a pathogen in the range of 10^5 spores/ml (Figure 1). The dose-response curve served as the base-line for all the experiments in the packing-house. The fermentation of US-7 in the bench-top fermentor required minor modifications from the conditions which were adapted for production in Erlenmeyer flasks. After several cycles of controlled fermentations, the process has reached an optimal growth on NYDB (see 'Experimental' for composition) for 48 hours at 28°C. The aeration levels requirements were at 1.0 VVM and the rate of mixing was 350-400 rpm. The mixing and aeration inside the fermentor created foams which had to be suppressed with 1% polypropylene glycol (p-2000, anti-foam). The fermentation on NYDB was very efficient and the yield obtained exceeded 10^9 cfu/ml (up to $2-3 \times 10^9$ cfu/ml). However, the high cost of that growth medium required replacement by industrial waste material. Preliminary tests have indicated that US-7 can utilize nitrogen and carbohydrates from many different sources. It can use meals such as cotton seed meal and soy meal; complex carbohydrates such as corn-steep liquor and molasses, as well as citrus pulp extracts and partially digested peptones. The rates of biomass production and quality assurance by *in vitro* tests were summarized and are presented in Table 1.

Biomass Production

The results clearly indicate that biomass production which preserves the antagonistic activity can be attained following fermentation on cheap waste material. This is an extremely useful factor in the development of a biopesticides which ought to be attractive in efficacy as well as low price. The batches of US-7 which were routinely prepared within pilot scales were harvested either on a continuous (sharpless) or a regular centrifuge (Sorvall RT 5000). Alternatively, the fermentation broth was successfully concentrated in a microfiltration system using a Sigma type membrane (Filtron Inc.). The paste was then collected and resuspended as a Concentrated Microbial Suspension (CMS: $2-3 \times 10^{10}$ cfu/ml) and stored at 4°C. During the 'field-trials' in several packing-houses we have tested three different methods of antagonist application namely, dipping of fruit in a pre-mixed solution, drenching of rolling fruit or spray application through high pressure (low volume nozzles).

Application Methods   Dipping of fruit in a tank was preceded by dilution of CSM to 5 $\times$ 10^8 cfu/ml in tap water at ambient temperature and the actual dipping lasted for 30 seconds which fruit was air dried and packed in cardboard boxes. The rate of decay was compared with that of control treatment which was the dipping of fruit in plain tap water. The results for Temple and Topax, peelable mandarins is presented in Figure 2a, b, similar results were also obtained for Jaffa oranges and grapefruit (data not shown). Even though the dipping process reduced the rate of decay very effectively, there are two major technical problems one of which is the absence of dipping tanks as part of inline systems in packing houses. The other obstacle is that presumably due to recycle of the solution in the same tank for large quantities of fruit without replacements there is accumulation of pathogen inoculum.

Spray application or drenching were also suggested for antagonist application. In spray application the cellular suspension was used as a relatively concentrated formulation ($>10^9$ cfu/ml) which was added into a 10L tank that was attached to the spray-nozzle system. Drenching was conducted with large volumes of more dilute solutions ($\sim 10^8$ cfu/ml) which effectively washed the rolling fruit along the conveyer on the sorting line. Laboratory tests indicated that the antagonist covered the fruit evenly and at appropriate rates when either method was used. Topax mandarin variety was treated by spray application or by drenching in two different packing-houses. The results obtained following drenching are presented in Figure 3a and Figure 3b, for total decay and that attributed to molds, respectively. Analysis of the results following 17 days of incubation at 17°C indicated that the total decay due to rots and molds was suppressed by 25 – 30% when the microbial antagonist was added to the Drencher without any supplements. However, the disease was controlled to a much better extent when the IPM approach was undertaken by mixing microbial antagonist and low rates of a chemical fungicide. The most effective antagonistic composition for suppression of total decay and for mold control was that of US-7 mixed with very low rates of TBZ (200 ppm). The efficacy was comparable to that which was reached by the standard packing- house procedure even though the concentration of TBZ was about 10% of the standard rate and in the absence of all the other chemicals. Prior to the IPM testing, the microbial product was tested in a compatibility test and was found to be unharmed by either TBZ or standard brands of wax. It should be emphasized that the latter involved high rates of chemical fungicides (2000 ppm TBZ, 2000 ppm Imazalil, 4000 ppm Ridomil). Similar results were observed following spray application of the antagonist through a nozzle at rates of 3-5 gallon/hour. The drencher and the sprayer alike were found useful for treatment of peelable varieties as well as Jaffa orange.

A fundamental conclusion from these results is that the antagonist can effectively control the pathogens following application by several non-related methods. Such a flexibility is crucial since for successful market penetration, the product has to be adjusted to many different existing agritechnologies and not the reverse.

Registration Requirements	For a pesticide to be accepted as a registered product, it has to consist of a proven performance in a commercial environment and to be environmentally and medically safe for use. The first prerequisite can be fulfilled by quality assurance tests as those which were conducted for each and every batch that was produced for field trials and then by subjecting the potential product to the natural (sometimes stressful) conditions of the packing-house. A series of experiments were directed to that goal and in a particular packing-house, several "sets" of grapefruit and Topax deliveries were accepted for sorting and packing. These sets were picked in a number of different orchards so as to randomize the conditions. Fruit was divided into two groups so that one half was subjected to the standard procedure (PH = Packing-House) whereas the other received the biological treatment.
Semi-industrial Scale Operation	In this semi-industrial scale of operation, a packing-house was hired for a whole day and the entire array of instruments was thoroughly washed to discard possible chemical residues. Half of each set of harvested grapefruit was rolled into the sorting-line. The microbial antagonist was applied through two parallel nozzles whose capacity was 3.5 gallon per hour. A peristaltic pump delivered the MCS from a 10L reservoir with microbial density of 8×10^8 cfu/ml. Following treatments of the three 'biological sets' the array was rearranged and the 'chemical sets' were fed into the line to obtain a standard pH treatment. At the end of the experiment, all samples were packed in cardboard boxes and incubated under the standard conditions (17 days at 17°C). The sets of the Topaz variety were tested during the mid-season and rates of decay due to molds (Figure 4a) as well as the total decay (Figure 4b) was subjected to comparisons of US-7 as part of IPM versus standard packing-house procedures. There was no significant difference between the two treatments and the biological agent was somewhat better. The grapefruit experiment was conducted as an exact replicate of the conditions in the packing house. Larger fruit quantities were taken for storage and only the total decay was assessed. This experiment was conducted twice, once at mid-winter (Figure 5b) when grapefruit is less sensitive to rots and molds and the second was done towards the end of the season when the rate of decay is usually increasing (Figure 5a). In the first round the rate of decay was indeed low as shown at least for sets 1 and 3 and there is no significant difference between the biological and the standard treatment. Later in the season there the rate of decay in control non-treated samples increased drastically but was suppressed evenly by the PH and the US-7/TBZ200 fungicidal treatments.

The scale-up of production processes which were accompanied by an extensive program of 'field-trials' in packing-houses enabled us within a relatively short period of time to accelerate the efforts towards registration of a product. It is event that the antagonistic efficacy of the microorganism was successfully restored and therefore it could compete with the standard chemical treatment in prevention of fruit decay due to post- harvest diseases. Most of the criteria for the acceptance of a microbial pesticide were fulfilled with respect to US-7 and particularly as part of IPM programs.

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Table 1. Growth rate of US-7 on industrial waste material and antagonistic activity – *in vitro* tests.

Growth Medium	Cell counts (cfu/ml)		%inhibition of
	24 hr ferm.	48 hr ferm.	Penicillium germinat.
NYDB	8×10^8	2×10^9	94.2
cotton seed meal + corn steep liquor	2×10^8	3×10^8	80.0
cotton seed meal + glucose syrup (starch industry)	6×10^8	3×10^9	81.2
soymeal + corn steep liquor	6×10^8	9×10^8	75.0
industrial peptone+ corn steep liquor	3×10^8	7×10^8	92.0
industrial peptone+ glucose syrup	1×10^8	2×10^9	93.6
orange peel extract	3.6×10^8	1×10^9	89.3
molasses	2.7×10^7	3.6×10^7	80.0
orange peel extract + molasses	1.3×10^8	9.2×10^8	92.0
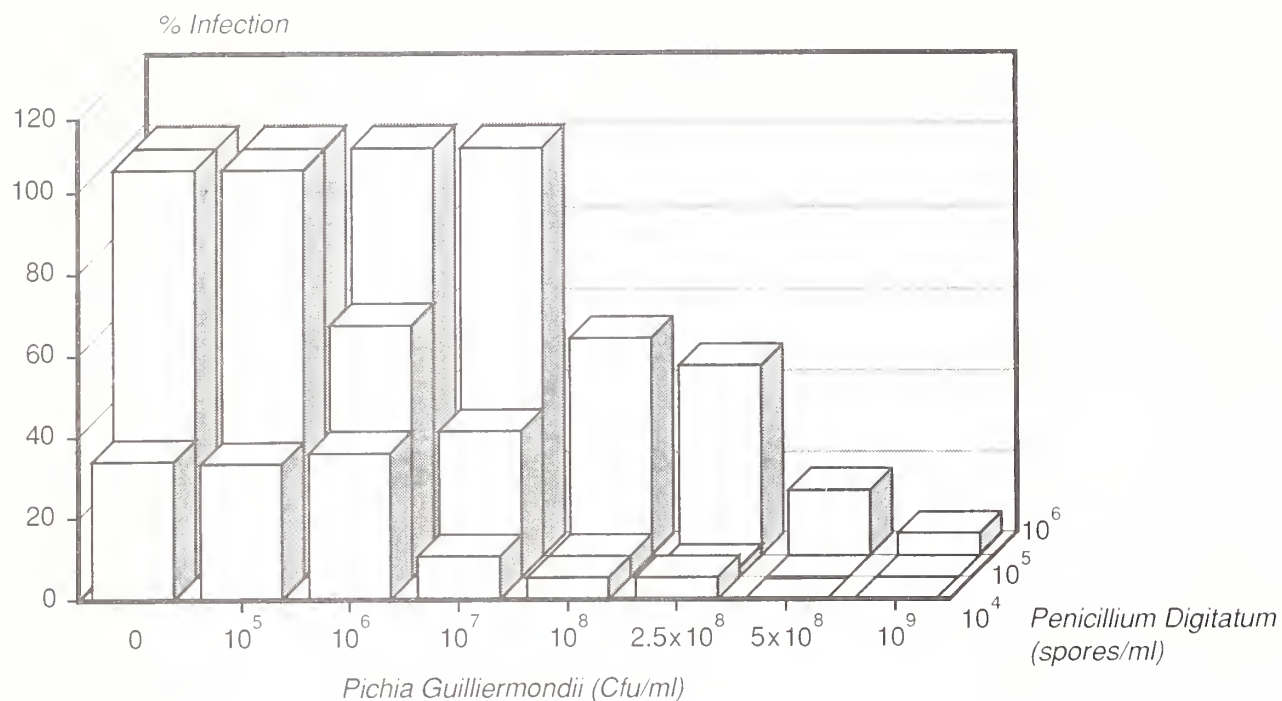
FIG. 1. Dose response for the biological control of *Penicillium digitatum* (10^4, 10^5, 10^6 spores/ml) on by increasing concentrations of the yeast-like agent *Pichia guilliermondii*. The biological control is the reverse of the percent infection of artificially inoculated grapefruit in the laboratory.

DIPPINGS

TEMPLE VARIETY
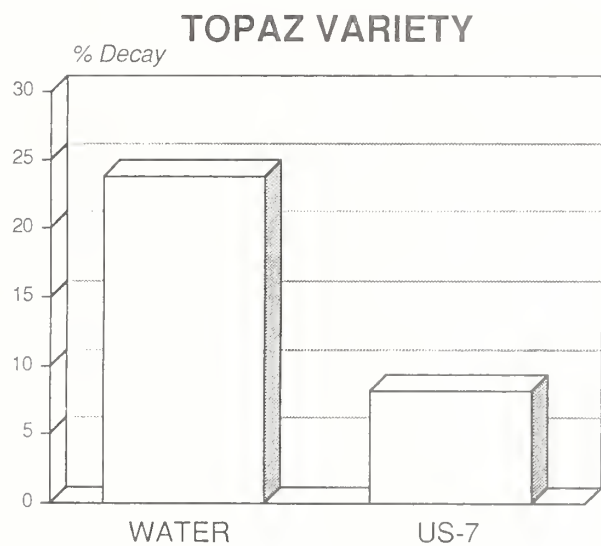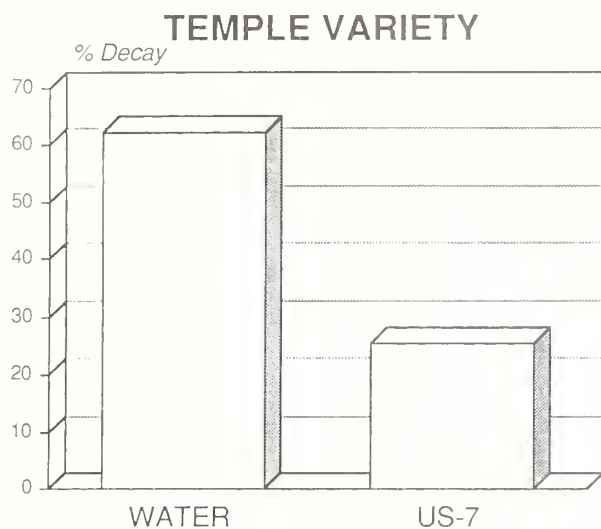
TOPAZ VARIETY

FIG. 2. Percent total decay of fruit after 17 days of storage at 17°C
following dipping in plain water or US-7 suspension (10^9 cfu/ml).
A. dipping of the Temple variety;
B. dipping of the Topaz variety.
Each treatment included about a hundred fruits.

TOPAZ - Total decay
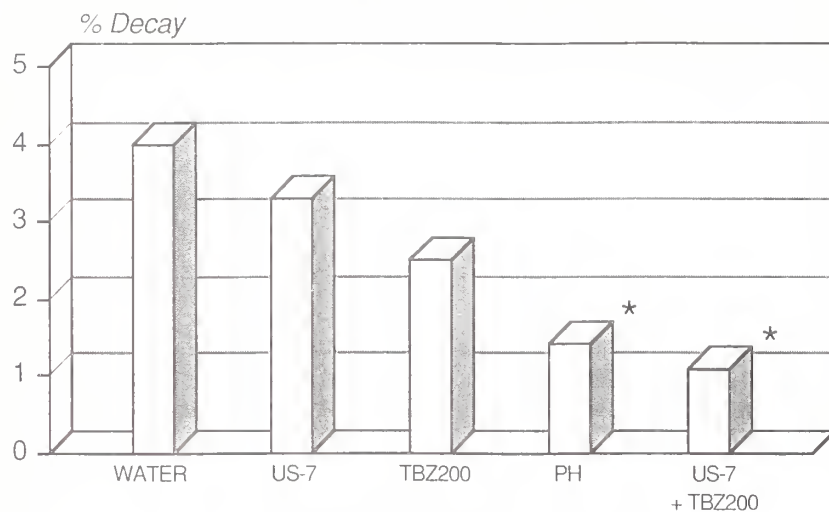
Statistical Significance (p < 0.05)

TOPAZ Molds
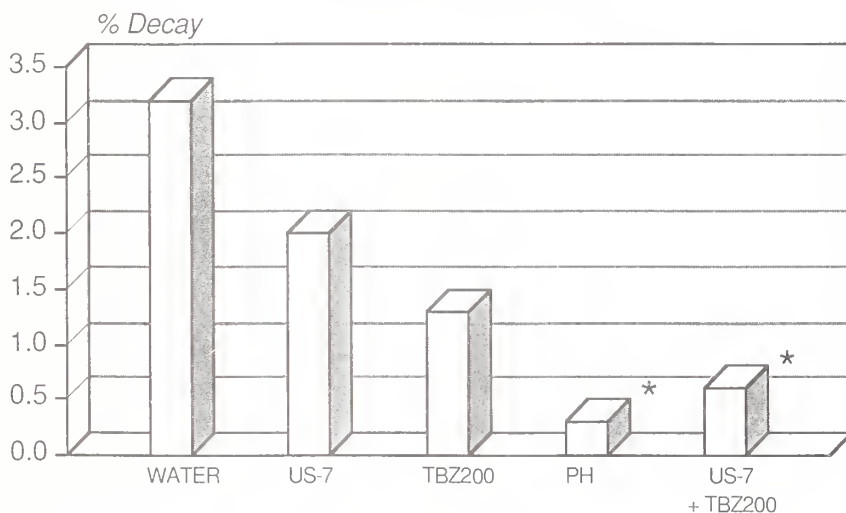
Statistical Significance (p < 0.05)

FIG. 3. Decay of fruit during storage following drenching of the Topaz
variety with US-7 (10^8 cfu/ml), 200 ppm TBZ, the standard chemical
treatment (PH) and an IPM system of US-7 plus 200 ppm, TBZ.
   A. percent of total decay due to lack of the above treatment in
      comparison to water wash.
   B. percent of decay due to molds only.

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TOPAZ Molds

% Molds

Bar chart with vertical axis from 0.0 to 1.6. Three sets each with PH and US-7 bars:
- SET 1: PH ≈ 1.0, US-7 ≈ 1.35
- SET 2: PH ≈ 1.0, US-7 ≈ 0.55
- SET 3: PH ≈ 1.5, US-7 ≈ 0.9

PH –   Standard packing-house treatment
US-7 – Biocontrol with P. Guilliermondii

TOPAZ - Total decay

% Decay

Bar chart with vertical axis from 0.0 to 2.5. Three sets each with PH and US-7 bars:
- SET 1: PH ≈ 1.65, US-7 ≈ 1.25
- SET 2: PH ≈ 1.95, US-7 ≈ 1.95
- SET 3: PH ≈ 2.1, US-7 ≈ 2.25
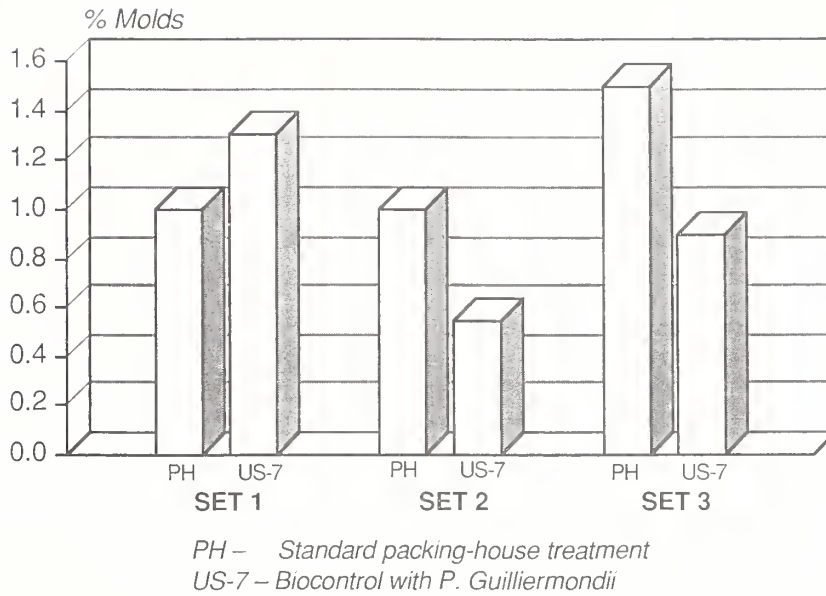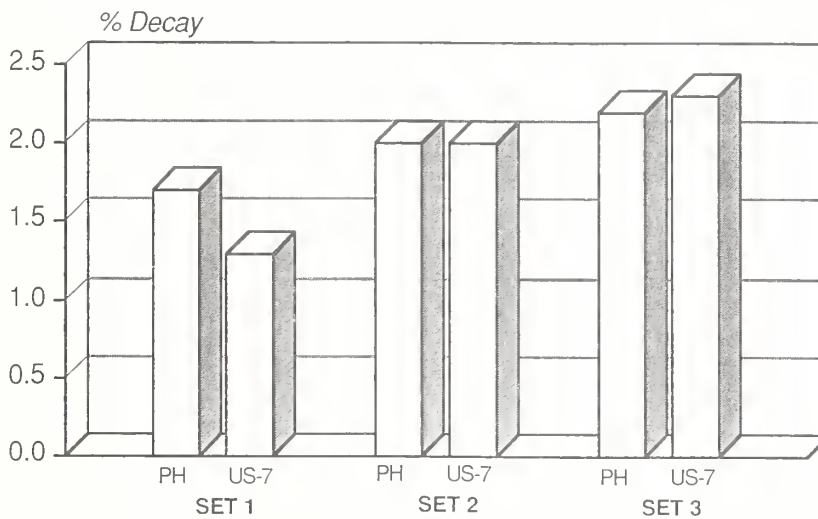
Packing-house (PH) treatment includes 2000 TBZ, 2000 Imaz.,
4000 ppm Ridomil, US-7 Treatment includes 200 ppm TBZ

FIG. 4.   Semi-industrial scale of biological control (US-7) compared with the
          standard packing-house (PH) system. Each set represents a delivery
          from a different orchard as is the common routine of the
          packing-house. Note that the US-7 treatment is part of IPM with 200
          ppm TBZ.
     A.   the decay of fruit as expressed by percent molds that develop during
          the storage (17 days) at 17°C.
     B.   the total decay which was induced by various fungal pathogens during
          storage.

GRAPEFRUIT - END OF SEASON
Total Decay
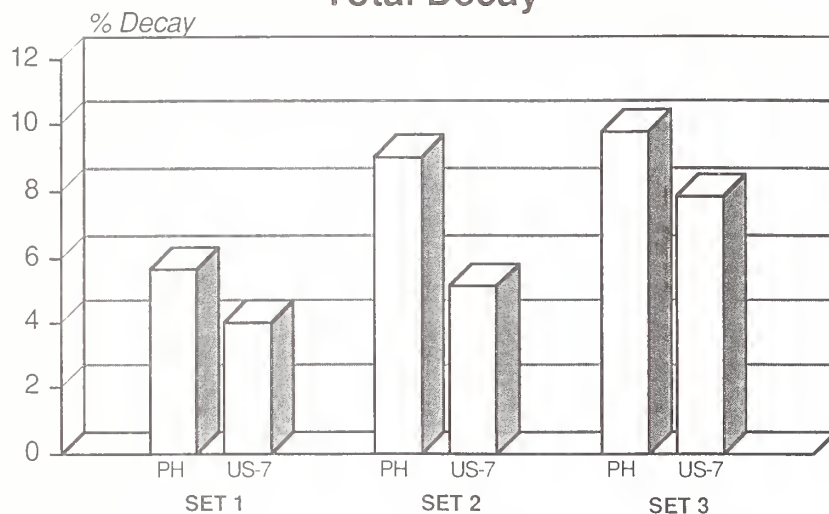
GRAPEFRUIT - MID WINTER
Total decay
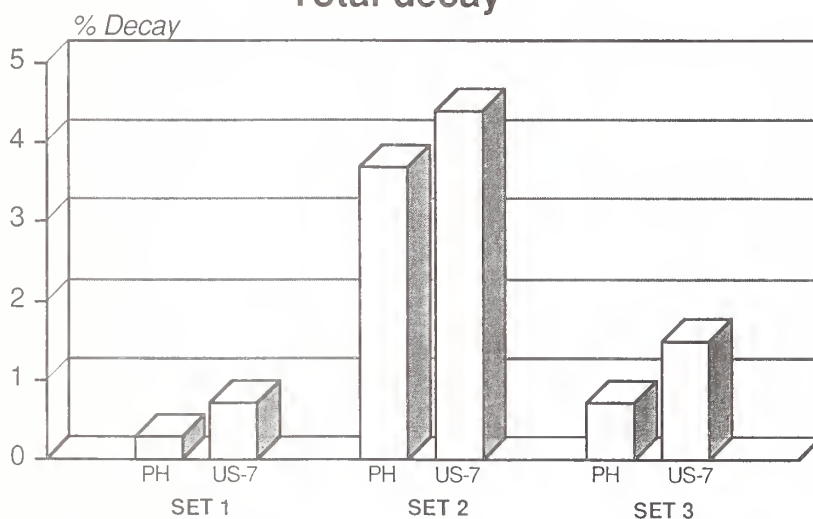
FIG. 5. Semi-industrial scale of biological vs. chemical control of post-harvest disease grapefruit (total decay). Sets of fruit were tested in the mid-winter (B) as opposed to the end of the season (A).

Integration of Biocontrol Agents with Postharvest Systems

P.L. Pusey[1]

Abstract

Implementation of *Bacillus subtilis* (B-3) in postharvest control of peach brown rot was investigated. The basic approach was to use the biocontrol agent in place of a chemical fungicide and to do this with minimal or no changes in present commercial systems. In laboratory tests, the antagonist was compatible with waxes used commercially on fruit and with dicloran, a fungicide used for the control of *Rhizopus* rot. Disease control was successfully demonstrated in pilot tests in which bacterial preparations were mixed with wax and applied to fruit as a spray on simulated packing lines.

Laboratory Simulation of Commercial Conditions

In laboratory tests, the B-3 strain of *Bacillus subtilis* (Ehrenberg) Cohn controlled brown rot caused by *Monilinia fructicola* (Wint.) Honey when the bacterium was applied to peaches, nectarines, apricots, plums and cherries (Pusey and Wilson, 1984). Subsequent tests with peaches were conducted to determine the feasibility of using B-3 in a commercial operation. The antagonist was shown to be effective within the full range (>10C) at which *M. fructicola* grew and caused fruit decay. Control was achieved on both wounded and nonwounded fruit by either dipping or spraying with cell suspensions of the bacterium.

In considering implementation of B-3 in peach packinghouses, the approach taken was to try incorporating B-3 without making changes in current practices, except for the substitution of fungicide with B-3. The most widely used method of applying fungicide to harvested peaches is by adding the chemicals to liquid wax that is sprayed or brushed onto the fruit. The B-3 organism was tested in combination with commercial fruit waxes commonly applied to harvested stone fruit (Pusey et al., 1986). Two waxes tested consisting of a mineral oil and paraffin base, and two others consist of a water base. Application of the bacterium was shown to be compatible with these waxes on both wounded and non-wounded fruit.

Like the chemical fungicides used for control of brown rot, B-3 has little or no effect against *Rhizopus* rot, another important postharvest disease of stone fruit caused by *Rhizopus stolonifer* (Ehr. ex Fr.) Vuill. Generally, two fungicides are needed after harvest to control both brown rot and *Rhizopus* rot. Tests conducted to determine whether B-3 could be combined with dichloran the fungicide commonly used for *Rhizopus* control (Pusey et al., 1986) showed that B-3 and dicloran were not only compatible, but that the two had an additive effect against brown rot. Some efforts have been made to find an effective biological agent against *Rhizopus* (Wilson et al., 1987). Conceivably, this would make possible the total biological control of post-harvest decay of stone fruit.

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It was also important to know whether the antifungal activity of B-3 could be retained during low-temperature storage of fruit (Pusey et al., 1986). Fruit were treated with B-3 and then held at 2-4° C for various periods before being inoculated with the fungus and incubated at 20°C. Even after the maximum period tested (21 days), B-3 controlled brown rot.

Pilot Tests on Peach-packing Lines

Production of B-3 was scaled up from laboratory flasks to a 250-litre fermenter, to provide sufficient quantities of B-3 for pilot tests and also to simulate commercial production of the bacterium. A low-cost medium was selected based on its support of B-3 growth and antibiotic production (Pusey et al., 1988). The primary nitrogen sources were from defatted cottonseed flour and soybean meal, materials previously used with success in the fermentation of *B. thuringiensis* (Dulmage, 1971). Preparations of B-3 from flask and fermenter cultures were stored at 4°C, either as a paste or powder.

Tests were performed in 1986 using simulated peach-packing lines at the U.S. Department of Agriculture, Byron, GA, and at Clemson University, Clemson, SC (Pusey et al., 1988). The B-3 organism was applied in combination with water-based wax and dicloran using a spray nozzle system. In these experiments, B-3 (2×10^8 to 7×10^8 CFU/Kg of fruit) was equal to benomyl (1-2 mg/Kg of fruit) in controlling brown rot. Also, B-3 stored as a paste or powder was as effective as B-3 from fresh cultures. A final test was conducted in a commercial packing house in Musella, GA. The test was complicated by an inability to remove fungicide residues completely from the packing line equipment. Consequently, it was not possible in this test to demonstrate either B-3 or the fungicide standard provided control in the packing house.

Concluding Remarks

The B-3 strain of *B. subtilis* is compatible with commercial procedures used in handling peaches and, thus, could be incorporated as a biocontrol agent in a postharvest operation. Commercialization of B-3 will possibly depend on whether the market potential is great enough to invest in toxicity testing. The antagonist has been shown to be effective against *Botryis cinerea*, *Glomerella candidum* and *M. fructicola* on apples and against *B. cinerea* on grapes (Pusey, 1989). Fermenta ASC, which has a license for a U.S. patent (Pusey and Wilson, 1988) on the use of B-3 for stone fruit, continues to test B-3 in screening tests and has so far found that it controls several other diseases, including powdery mildew of apple, bean rust, coffee rust, wheat leaf rust and black sigatoka of banana (personal communications).

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Compatibility of Biocontrol Agents with Present Processing Technology

R. A. Spotts[1]

Abstract

The tremendous diversity of crop-pathogen-postharvest environment combinations presents a challenge to the commercialization of biological control. Biocontrol agents must be resistant to chemicals used for control of fungal, bacterial, and physiological disorders and diseases. Biocontrol agents must be compatible with commercial handling systems, including dump tanks and flumes, drenches, line spray applicators, and heat tunnels. In addition, biocontrol must be effective in a wide range of temperatures and storage atmospheres. With proper planning and innovative research, these challenges can be met.

Introduction

Many challenges must be met before biocontrol can be used successfully on a commercial basis for control of decay of fruits and vegetables. The following discussion focuses primarily on systems used in the tree fruit industry in the Pacific Northwest, but the principles can be applied to a wide range of crops and production areas.

Crop Diversity and Decay Resistance

Many large fruit packinghouses handle several crops such as apples, pears, and sweet cherries. In the United States, Red Delicious is the leading apple cultivar with a yield of over 97 million bushels (20 Kg), but production of nine other cultivars exceeds 4 million bushels (USDA and IAI estimate, 1989). Similarly, several pear and sweet cherry cultivars are grown in significant quantities. Cultivars vary in resistance to decay and each may require a different biocontrol strategy. For example, the pear cultivars Bosc and Comice are highly susceptible to side rot caused by *Phialophora malorum* Kidd & Beaum, but d'Anjou is relatively resistant (Sugar, 1989).

Pathogen Diversity

Perhaps even more striking than crop diversity is the complex group of pathogenic bacteria and fungi that cause postharvest decay of fruits and vegetables. *Pezicula malicorticis* (H. Jacks.) Nannf. causes bullseye rot of apple and pear and can infect fruit from petal fall to harvest (Spotts, 1985). *Coprinus psychromorbidus* Redhead & Traquair, a low temperature basidiomycete, appears to infect fruit just before harvest (Spotts, Traquair, and Peters, 1981). The most common postharvest pathogens of pome fruit, *Penicillium expansum* Link and *Botrytis cinerea* Pers. infect fruit during and after harvest through wounds or stem ends (Pierson, Ceponis, and McColloch, 1971). It is unlikely that any single biocontrol agent will control such a diverse group of pathogens. For example, we found that *Clavibacter michiganensis* subsp. *sepedonicus* gave good control of blue mold, moderate control of gray mold, and had no effect on *Mucor* rot. In addition, it is

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uncertain that postharvest application of biocontrol agents will eradicate latent infections caused by *P. malicorticis* and *C. psychromorbidus*.

Compatibility with Prebarvest and Postharvest Chemicals

Several fungicides may be applied for decay control within the last 4 days before harvest. Thus, biocontrol agents applied postharvest would contact fungicide residues on fruit surfaces. In addition, several fungicides and antiscald chemicals (diphenylamine for apples, ethoxyquin for pears) are applied to fruit in postharvest drenches or line sprays (Willett et al. 1989). Although effective biocontrol may reduce or eliminate the need for some chemical applications, biocontrol agents must be selected with resistance to the chemicals commonly used in fruit and vegetable production.

Fruit Handling Systems

Fruit are harvested into wooden bins that contain about 450 Kg. Fruit bins are drenched with fungicide and an antiscald chemical and stored in air or controlled atmosphere storage. However, in a presize system, fruit are floated from bins, sorted, treated with a fungicide in water or wax, sized, then placed back into bins for storage. Alternately, fruit may be packed and stored in boxes containing about 20 kg rather than in bins.

Recycled drench solutions accumulate dirt and debris containing fungal spores. These solutions are often changed daily, with drencher volumes commonly exceeding 4000 liters. Application of biocontrol agents in this system would require considerable quantities of material.

Immersion dump tank and flume systems are used to remove fruit from bins and move it through the packinghouse. Solution volume often exceeds 12,000 liters. A flotation salt such as sodium sulfate or sodium ligninsulfonate is necessary to float pears but no apples. Water temperature usually is under 10°C but may be heated to 25°C to clean apples for waxing. Some packinghouses use a heat sterilization method for dump tank water. Water is heated to 55°C for 25 minutes, then allowed to cool before fruit are added (Spotts and Cervantes, 1985). The number of decay spores also is reduced by chlorination or use of sodium-o-phenylphenate (Spotts and Cervantes, 1986). Other research methods to reduce decay spores include ozonation and filtration. The application of biocontrol agents in these types of systems has not been tested.

Fungicides often are applied to fruit in a line spray in water or wax. These systems are nonrecirculating but require only small volumes of solution. One liter of wax solution will cover over 1300 kg of fruit. Waxed fruit are dried in a heat tunnel at 50 to 60°C. Biocontrol agents could be incorporated easily into a line spray system.

Fruit Storage Systems

Apples are stored at − 1 to 4°C and pears at −1.1 to −0.5°C (Hardenburg, Watada, and Wang, 1986). In the Pacific Northwest, fruit are stored up to 9 months. Fruit are shipped to market at transit temperatures of 0 to 2°C. Winter pears require ripening at 20°C for 5 to 10 days. Thus, biocontrol agents must function in a wide temperature range to give control of decay from storage to the consumer.

Controlled atmospheres (CA) are required for long-term cold storage of apples and pears. d'Anjou pears can be stored 7 months in air if they are pretreated with 12% CO_2, 8 months in 2% O_2 + 0.1% CO_2, or 9 months in 1% O_2 + 0.1% CO_2. Atmospheres of less than 2% O_2 often have adverse effects on microorganisms (Spotts, 1984). These effects can be used advantageously to reduce decay (Chen, Spotts, and Mellenthin, 1981). However, biocontrol agents must tolerate low O_2 - high CO_2 environments if they are to provide decay control in commercial CA storages.

Conclusion

Many biological and physical factors must be considered when selecting or developing biocontrol agents for commercial use. Packinghouse personnel are accustomed to broad spectrum fungicides that function in a wide range of postharvest handling and storage systems. Mistakes are likely to occur in making the switch to living biocontrol agents that are more sensitive to environmental conditions than are fungicides or bactericides. If biocontrol agents are too narrow in their pathogen-crop-environment action spectrum, companies may hesitate to make the financial commitments necessary for registration. These challenges must be met if biocontrol is to succeed on a commercial scale for reduction of postharvest decay of fruits and vegetables.

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Postharvest Shelf Life of Organically Grown Produce: Current Perceptions and Need for Further Study in the USA

Terry Schettini[1]

Introduction

OrgGP

ConvGP

Following a number of media reports in 1989 regarding food safety in the USA there was an explosion of interest in providing organically grown fruits and vegetables in the fresh produce sections of supermarkets. By the spring of 1990, however, much of the interest in providing this source of fresh produce had waned. The reasons reported in the popular press and trade journals were varied and complex. Two factors that were commonly cited were that organically grown produce (OrgGP) had a shorter shelf life, or that it resulted in greater shrinkage, than conventionally grown produce (ConvGP) in the market place (Anderson, 1988; Fabricant, 1990; Felgner, 1990; Food Chemical News, 1990; Mejia, 1990). Following discussions between the author and representatives from the public and private sectors concerning these factors, it became apparent that further clarification of these terms (shelf life and shrinkage) and related issues was indicated for several reasons (Schettini, 1990).

Three Reasons Why Clarification of Terms and Identification of Actual Problems is Needed:

1. The terminology used to describe the post harvest quality of OrgGP in various reports, or the interpretation of these reports, has made it difficult to determine what problems actually exist; and continued ambiguity will only hinder the proper identification and characterization needed to resolve them.

2. In addition, the following issue may be significant to the fresh produce industry in the immediate future: if the decreased use of conventional pesticides on OrgGP does result in poorer postharvest quality then the increasing volume of produce grown under reduced pesticide use programs may also be at risk for postharvest problems. The reason is that these products will lack some of the same protectants as OrgGP during postharvest handling and distribution.

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3. Finally, and of specific relevance to the participants of this workshop, successful implementation of practices for biological control of postharvest diseases requires the development of integrated pest management strategies that consider both the interaction between biological control agents and the pre- and postharvest application of pesticides, as well as their combined effect on postharvest quality of fresh fruits and vegetables.

Therefore, reports on the effects of production practices on postharvest quality require further scrutiny in order to minimize disruptions to the industry during the development of, and transition to, alternative production systems. Since produce grown with reduced pesticide use programs is not routinely distinguished from ConvGP in the marketing system, and OrgGP is, this paper reports perceptions and studies specific to the shelf life and shrinkage aspects of postharvest quality of organically grown produce.

Shelf Life

Physiological Shelf Life Versus Economic Shelf Life

At least two interpretations of the term 'shelf life' can be inferred from various articles and discussions.

Physiological Shelf Life

(1) physiological shelf life- the biological capability of a product to maintain its postharvest quality when handled properly (for example, produce free of disease and injury may store longer than diseased or injured produce).

Economic Shelf Life

(2) economic shelf life- the useful life of a product in the produce section in the market as affected by marketing practices (for example, high prices may result in slow sales of a product; the remaining inventory may deteriorate and must be discarded).

The distinction between these two usages of the term shelf life is important. For example, differences in physiological shelf life between OrgGP and ConvGP infers that produce grown under the two crop production systems but handled properly in the distribution chain will differ in postharvest quality over time. In contrast, differences in economic shelf life can include factors independent of the crop production system. In addition, conclusions about differences in physiological shelf life require controlled comparisons of crop production practices while studies of economic shelf life require surveys of experienced industry representatives.

Studies on Shelf Life

A search of the scientific and trade literature found no evidence that supports the contention that OrgGP, in general, has a shorter physiological shelf life than ConvGP (see also p. 12 in Fishman). However, several studies reported that produce (usually root crops) grown with organic, or biodynamic, methods stored as well or better as produce grown with conventional methods (Hansen, 1981; Knorr and Vogtmann, 1983; Linder, 1985; Nilsson, 1979).

Two recent studies included data from surveys of fresh produce merchandisers (Cook, 1990; Jolly, 1989). In one study on the attitudes of merchandisers toward OrgGP, the majority of respondents believed that the shelf life of OrgGP was "worse"

than ConvGP (Table 1, Jolly, 1989). This report concurs with the press reports mentioned earlier but was not designed to determine the reasons behind these attitudes.

One Report on Shrinkage

Shrinkage

Shrinkage is the portion of fresh produce inventory that is unsellable or remains unsold. Shrinkage influences the profitability of fresh produce and is affected by both physiological and economic factors. In an ongoing study of the profitability of OrgGP a survey of retailers and grower/shippers was conducted. The respondents reported that OrgGP had a shrinkage of up to 25% versus 5-6% for ConvGP (Cook, 1990). This higher shrinkage is a major factor in the decreased profitability of OrgGP and therefore the decreased interest by industry in OrgGP. It is worthwhile to discuss shrinkage further since its relation to shelf life, as mentioned earlier, is unclear and the terms may, incorrectly, be used interchangeably.

More on Shrinkage: Why may there be more shrinkage for OrgGP? None of the reasons listed below are unique to OrgGP but one or more of these may be occurring in this relatively new and rapidly developing sector of the fresh produce industry.

Improper Harvest Date

1. Improper harvest date- especially if the produce is too ripe for subsequent handling;

Improper Post-harvest Handling

2. Improper postharvest handling - for example, lack of cooling facilities to remove field heat;

Distribution Inefficiences

3. Distribution inefficiencies - apparently the need exists to improve the marketing networks with brokers and shippers;

Ineffective Marketing

4. Ineffective marketing - for example, inappropriate displays;

Slow Turnover

5. Slow turnover - inventory remains on the shelf longer than anticipated for a number of reasons;

Shorter Shelf Life

6. Shorter shelf life - true physiological deficiencies. Let's discuss the last three areas a bit further.

Ineffective Marketing

Ineffective Marketing: This reason was discounted in the study by Cook (1990). The respondents reported that the OrgGP was advertised regularly, placed into bulk displays and integrated into the main produce sections. The same study had no questions directed specifically at determining the physiological shelf life of the produce. One conclusion from the survey was that the higher shrink of OrgGP was due to slow turnover.

In a report in a trade journal, a merchandiser observed that "Although many [OrgGP] items arrived at the warehouse and store levels in good shape ... quality was not sound enough to withstand organic's slow turnover" (Glynn, 1990). So, slow turnover seems to be a common theme for organically grown commodities.

Slow Turnover

Slow Turnover: Why does OrgGP have a turnover that is slower than ConvGP? The two most stated reasons are high price and poor appearance.

High Price

High Price: The price premium that is usually charged for OrgGP is reportedly due primarily to higher production costs for these products AND the high demand for these products relative to the supply. However, this "demand" may be a perceived, or public relations generated, demand rather than actual market place demand. This perceived/PR demand is apparently generated by grocery markets that either are trying to support this fledgling industry or feel they must have organic produce on their shelves as a public relations practice. The actual demand, that is the number of customers willing to pay the price premium on OrgGP, evidently is lower than the supply of produce offered at the higher price. This results in slower turnover and consequently higher shrinkage. Once production costs decrease and the perceived/PR demand readjusts to the true demand, the price of organically grown produce should decrease. This should result in faster turnover and reduced shrinkage.

Poor Appearance

Poor Appearance: Another observation made by some produce merchandisers is that the appearance of organically grown produce is "worse" than conventionally grown produce (Table 1, Jolly, 1989). This may be due to insufficient culling by growers/packers/shippers as well as improper pre- and postharvest handling. There are a number of reasons for poor appearance as well as differences in opinion on the importance of appearance in consumer decision making.

So what about appearance? What is poor versus acceptable appearance to the consumer? Many discussions are based on the assumption that consumer attitudes toward the cosmetic attributes of fresh produce can not be changed. A survey of consumers conducted in the summer of 1989 indicates that such changes may be possible (Bunn, 1990).

In the above survey, it was reported that oranges can tolerate a visible degree of superficial thrip damage without affecting the taste, nutritive value, or storage ability of the fruit, nor the yield or health of the tree. Shoppers were asked if they would be more willing to purchase an unblemished orange versus an orange with 10% or 20% damage (Table 2). At first, only 5 to 6% of the shoppers were more willing to buy the blemished orange; however, after they were informed that the cosmetically damaged fruit were produced using half as many pesticides, 58 to 63% of the shoppers were more willing to buy the oranges with 20% or 10% thrip damage, respectively. Therefore it may be possible to reeducate the public as to what is acceptable cosmetic quality.

Physiological Shelf Life

Studies on Physiological Shelf Life: Finally, what about true physiological shelf life, or storage ability? In the storage studies mentioned earlier it was reported that OrgGP stored as well or better than ConvGP. However, two items should be noted about these studies: first, the crops studied were all storage or root crops (beets, carrots, cabbage, potatoes and turnips); secondly, the treatments were limited to different fertility practices (NPK versus organic fertilizers) rather

than comparing different crop production systems that are comprised of integrated fertility AND pest management strategies.

Conclusions

Additional controlled comparisons of produce grown in contrasting crop production systems is needed. Steps in this direction are being made by postharvest physiologists participating in two ongoing studies of which the author is aware: a whole-farm comparison of established organic and conventional tomato farms in California; and small experimental plots of tomatoes grown using different production techniques in Pennsylvania. More studies are needed with more commodities grown in established production plots using integrated production strategies rather than just differences in fertility practices. Postharvest diseases are a major factor in the storage ability of fresh produce and the effects of crop production practices on postharvest disease need to be more fully explored. What is needed are more collaborative studies involving postharvest physiologists and horticulturists to determine if the physiological attributes of organically grown produce affect its physiological shelf life.

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TABLE 1. MERCHANDISER ATTITUDES TOWARD ORGANIC CONVENTIONALLY GROWN FOODS

ORGANIC IS:	BETTER	WORSE	SAME	UNSURE
APPEARANCE	0	11	1	0
SHELF LIFE	0	7	3	2

AFTER JOLLY, 1989.

TABLE 2. PERCENT OF RESPONDENTS WILLING TO BUY
COSMETICALLY IMPERFECT ORANGES PRIOR TO AND FOLLOWING
INFORMATION ABOUT REDUCED PESTICIDE USE (N=229).

WILLINGNESS TO BUY "IMPERFECT"	LEVEL 1 DAMAGE		LEVEL 2 DAMAGE	
	BEFORE	AFTER	BEFORE	AFTER
LESS	78	25	87	34
SAME	16	12	9	9
MORE	6	63	5	58

AFTER BUNN, et al., 1990.

V. PATENTING AND REGISTRATION PROCEDURES.

It is good to consider possible patenting and registration hurdles at the early stages of developing a biocontrol agent.

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Patent Protection for Microorganisms and Their Use in Biocontrol

S. C. Wieder[1]

Introduction

Although the concept of biocontrol has been known for some time, *Bacillus thuringiensis* has been used for years as a biocontrol agent, it has only been recently, as a result of the Diamond v. Chakrabarty Supreme Court Decision (Diamond, 1980) and the ever tightening regulatory controls of the Environmental Protection Agency (EPA) that microorganisms are considered attractive alternatives to chemical pesticides. However, to add the impetus necessary for industry to change its course toward biocontrol, patent protection is vital.

Patenting of Microorganism Overview

Louis Pasteur was granted U.S. Patent No. 141,072 in 1873 having a claim directed to "yeast, free from organic germs of disease, as an article of manufacture." This patent is the first of many subsequent patents to microorganisms. However, in view of a number of Supreme Court decisions (American Fruit Growers, 1931 and Funk Bros., 1940) it became the policy of the U.S. Patent Office to exclude microorganisms for patent protection as being "living matter" or "products of nature" until 1980.

"The Hand of Man"

The Supreme Court in a landmark five to four decision (Diamond, 1980) held that the microorganism claimed by Ananda Chakrabarty was indeed a "manufacture" or "composition of matter" within the meaning of 35 U.S.C. 101. Subsequent to this decision the U.S. Patent Office now allows patents with claims directed to microorganisms that can be shown to be made by "the hand of man". While opening the door to patent protection for biocontrol agents, this decision precipitated a rush to obtain patents that actually inhibited industry from becoming active participants in biocontrol technology. Many companies even now are reluctant to invest resources to develop biological control agents because the issuing of a patent from the date of application may take years (GAO, 1989). While this is one consideration, many companies already in biocontrol are nervous about getting patent protection at all, since the biotechnology case law is still evolving and thus property rights to the naturally occurring biocontrol agents, particularly microorganisms, are not well defined. In spite of these concerns patent applications for biocontrol technology are increasing due in part to regulatory agencies, such as EPA, taking a hard look at chemical pesticides.

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Enablement Concerns and Microorganism Patent Applications

While patent practice varies from country to country, the concept of enablement can be considered a common denominator. Under U.S. law, 35 U.S.C. 112 requires that the "specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is the best mode contemplated by the inventor of carrying out his invention."

Additionally, the specification under patent rule 37 C.F.R. 1.71(b) "must describe completely a specific embodiment of the process, machine, manufacture, composition of matter or improvement invented, and must explain the mode of operation or principle whenever applicable."

These requirements provide unique problems to the inventor of biocontrol inventions. What is enablement in the context of a biocontrol agent? To the genetic engineer the simple recitation of a gene sequence oriented in the proper position in a plasmid and inserted into a host by conventional methods may be sufficient disclosure for those in the field to duplicate the effort. However, in many cases inventions directed to microorganisms cannot simply be described in a manor that would readily enable the ordinary skilled artisan to make and use the invention. In these cases, the U.S. Patent Office requires a deposit of the microorganism before the issuance of a patent in an International Deposit Authority (IDA) recognized under the Budapest Treaty. The U.S. as well as other contracting states under the Budapest Treaty recognize the following IDA's for receiving deposits of microorganisms for the purpose of patent procedure.

Deposits of Microorganisms

Agricultural Research Culture Collection (NRRL) - USA
American Type Culture Collection (ATCC) - USA
Australian Government Analytical Laboratories (AGAL) - Australia
Centraalbureau Voor Schimmelcultures (CBS) - Netherlands
Collection Nationale De Culture De Micro-organismes (CNCM) - France
Commonweath Agricultural Bureau (CAB), International Mycological Institute - United Kingdom
Culture Collection of Algae and Protozoa (CCAP) - United Kingdom
Deutsche Sammlung Von Mikroorganismen (DSM) - Federal Republic of Germany
European Collection of Animal Cell Cultures (ECACC) - United Kingdom
Fermentation Research Institute (FRL) - Japan
Institute of Micro-organism Biochemistry and Physiology of the USSR Academy of Slcience (IBRM) - Soviet Union

In Vitro International, Inc. (IVI) - USA
Mezogazdasagi Es Ipari
Mikroorganizmusok Magyar Nemzeti
Gyujtemenye (MIMNG) - Hungary
National Bank for Industrial
Microorganisms and Cell Cultures
(NBIMOC) - Bulgaria
National Collection of Industrial
Bacteria (NCIB) - United Kingdom
National Collection of Type Cultures
(NCTC) - United Kingdom
National Collection of Yeast Cultures
(NCYC) - United Kingdom
USSR Research Institute for Antibiotics
of the USSR Ministry of the Medical
and Microbiological Industry
(VNIIAA) - Soviet Union
USSR Research Institute for Genetics
and Industrial Microorganism
Breeding of the USSR Ministry of the
Medical and Microbiological Industry
(VNII) Genetika) - Soviet Union

The deposit of microorganisms necessary to practice a biocontrol invention in one of these IDA's, before or at the time of filing a patent application, assures the inventor that he or she has fulfilled the enablement requirement in most countries for the availability of the microorganism. In U.S. practice, the deposit of a microorganism may be made at any time prior to the issuance of a patent (Lundak, 1985).

Patent applications having claims directed to one or more novel microorganisms *per se*, in which a duplication of the inventors' methods would not produce the exact microorganism as claimed, usually requires a deposit in an IDA for enablement. Likewise method claims that require the use of novel microorganisms would necessitate a deposit to practice the invention.

Failure to deposit a microorganism can result in an enablement rejection by the Patent Office that cannot be overcome.

Scope of a Biocontrol Invention

The scope of the invention is an important concern to investigators in the biocontrol field. Often, species of microorganisms are screened for a desired utility. Many times these species are known to possess this utility, but at a level not worth commercializing. If a strain is found that possesses the desired utility at a greatly elevated level, one now worth pursuing commercially, a patent may be pursued for the isolate. Claims to the specific isolate, usually deposited as required by the Patent Office, are narrow. Protection is limited to one individual isolate. Patents having such narrow scope are little incentive for industry to venture into biocontrol production without careful consideration as to the ease with which a competitor can compete in the market without infringing. This is not necessarily the case with genetically modified microorganisms, or for strains of microorganisms producing novel products i.e. antibiotics, (Bergy, 1979). As biocontrol technology unfolds the burden falls equally on the scientist and the patent practitioner to ascertain the appropriate scope of a biocontrol invention.

Discussion

There are numerous other considerations the Patent Office both
here and abroad use for determining patentability such as
obviousness, however these considerations depend on the art and
literature published in the field. Often these references vary
widely from discipline to discipline. It is sufficient to say
that the U.S. Patent Office in the consideration and
determination of what is obvious use these three factual
inquires:

1. Determination of the scope and contents of the prior art.

2. Ascertaining the differences between the prior art and
   the claims in issue; and

3. Resolving the level of ordinary skill in the pertinent
   art (Graham, 1966).

Additional definitions of obviousness will not be attempted
here since there are volumes written on the subject and it is
heatedly discussed constantly before the courts. With the
regulatory agencies activity pursuing, licensing and
registering biocontrol agents private industry is looking with
interest at pursuing microorganisms for use in biocontrol as
effective alternatives to chemical pesticides, herbicides and
fungicides (Richter, 1988). In view of these facts it is
important for the scientist to consider patenting of a
microbiological invention while concurrently giving careful
consideration to enablement and scope of that invention through
a deposit.

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Environmental Protection Agency Oversight of Microbial Pesticides

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Abstract

The purpose of this paper is to provide an overview of the Environmental Protection Agency's oversight of microbial pest control agents. The application of the Federal Insecticide, Fungicide, and Rodenticide Act and Sections 408 and 409 of the Federal Food, Drug, and Cosmetic are discussed. General data requirements for EPA registration of microbial pest control agents are discussed along with the specific requirements for nontarget aquatic organism testing.

Background

This report presents an overview of the Environmental Protection Agency's (EPA) oversight of microbial pest control agents (MPCAs). These agents are regulated under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and Sections 408 and 409 of the Federal Food, Drug, and Cosmetic Act (FFDCA). MPCAs include living microorganisms such as bacteria, fungi, protozoa, algae, and viruses that are introduced into the environment to prevent, repel, destroy or mitigate the population or biological activities of another life form considered to be a pest under Section 2(t) of FIFRA.

The first of these microbial pest control agents (*Bacillus popilliae*) was registered in 1948. At this writing, there are 21 microbial pesticide active ingredients used in several hundred products registered for use in agriculture, forestry, mosquito/blackfly control, and homeowner situation. (See Figure 1). The Office of Pesticide Programs (OPP) formally recognized in 1979 that MPCAs are distinct from conventional chemical pesticides and made the commitment to develop appropriate testing guidelines for microbial pesticides. The guidelines for microbial and biochemical pesticides were published in 1982 as the Pesticide Assessment Guidelines, Subdivision M. (The microbial section of Subdivision M was updated and revised in July of 1989.) In 1984, data requirements for MPCAs were codified in the Code of Federal Regulations, Title 40 Part 158.170 (40 CFR Part 158.170).

FIFRA and FFDCA Requirements for Microbial Pesticides

The main aspects of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) as they relate to the pesticide registration process are summarized below. EPA's oversight extends from premarket testing, small-scale field test notifications and Experimental Use Permits (EUPs), through full commercial use of a pesticide product, Section 3 Registrations. Any pesticide product that is to be used on a food crop to be distributed in commerce must have a tolerance

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(maximum legal residue level) or temporary tolerance under Section 408 or 409 of the FFDCA or must be granted an exemption from the requirement of a tolerance. All MPCAs registered to date for use on food crops are currently exempt from the requirement of a tolerance.

In the process of pesticide development, field testing is often necessary to evaluate the environmental fate and efficacy of a pesticide. Title 40 of the Code of Federal Regulations Part 172 (40 CFR Part 172) describes when it is necessary to obtain an Experimental Use Permit under Section 5 of FIFRA. Briefly, if the pesticide is to be used on a food crop that will be distributed in commerce or the size of the test acreage is greater than 10 acres on land or 1 surface acre of water, an EUP is required. For nonfood uses, it is generally presumed that an EUP is not required for field tests under 10 acres on land or 1 surface acre of water. Other criteria used to determine when an EUP must be obtained are set forth in 40 CFR Part 172.3. An EUP is of limited duration and requires that the test be carried out under controlled conditions.

In 1984, EPA recognized that there may be potentially significant impacts from the use of genetically altered and nonindigenous MPCAs in the environment, even at the small-scale testing stage. To address this concern, EPA issued an interim policy statement that announced that the presumption in the EUP regulation (40 CFR 172.3) that an EUP would not be required for small-scale testing would not be applicable to such tests involving genetically altered and nonindigenous microbial pesticides. This policy requires notification of EPA prior to small-scale field testing of genetically altered and nonindigenous MPCAs so that the Agency can determine whether an EUP is required. The 1984 interim policy was subsequently incorporated into a 1986 policy statement that retains the notification requirement. The Agency is currently working on revising the existing EUP regulation to codify the notification requirement.

As noted earlier, before a company can market a pesticide product, it generally must obtain a Section 3 registration; however, there are two additional means under FIFRA whereby a company may distribute a pesticide product in the absence of an experimental use permit. The first of these is pursuant to an emergency exemption under Section 18 of FIFRA. Under this section, federal or state agencies may request an unregistered use of a currently registered pesticide product or the use of an unregistered pesticide product. Such a request can only be granted when there is a potentially severe economic or human health impact and no other alternatives are available for pest control. A Section 18 exemption usually allows use of the particular pesticide product for a year, however the time for use allowed may be more or less. In addition to emergency exemptions under Section 18, cases exist where a particular

pesticide product may be registered for one or more uses, but not for a particular use which is determined by the state as being a special local need. In these cases, the state may register that use or formulation needed for the special local need under Section 24(c) of FIFRA. The EPA has 90 days to disapprove of such state registrations. If the Agency does not respond, then that use and or formulation, heretofore not part of a federal registration, becomes part of either an existing or a new federal registration. (Refer to Section 24(c) (2) of FIFRA for specific details.)

Data Requirements

The recommended test methods provided in the 1989 revised Pesticide Assessment Guidelines Subdivision M and corresponding data requirements in 40 CFR Part 158 are set forth in four basic areas: product and residue analysis, environmental fate, nontarget organism testing, and human health effects. The testing schemes for human health and nontarget organism effects are tiered, i.e. certain testing is not required unless triggered by initial testing. Residue analysis and environmental fate requirements are usually triggered by human health effects data and nontarget organism data respectively. An example of this is the nontarget organism/environmental fate tier testing scheme. (See Figure 2). At the first tier, short term testing utilizes maximum hazard dosing. If no adverse results are observed in Tier I, then further testing is not warranted nor is environmental fate data required. In the first tier of nontarget organism testing, avian oral, freshwater fish, freshwater aquatic invertebrate, and honeybee testing are required. In addition, tests to evaluate MPCA effects on wild mammals, plants, and beneficial insects are required depending on the proposed use site, target organism and degree of anticipated exposure.

Like the nontarget organism testing, the toxicology testing is also tiered. (See Figure 3). Tier I consists of studies including oral toxicity/pathogenicity, dermal toxicity, pulmonary toxicity/pathogenicity, intravenous toxicity/pathogenicity, primary eye irritation, reporting of an observed hypersensitivity incidents, and cell culture tests with viral pest control agents.

Pursuant to FIFRA Section 3 and within the parameters of 40 CFR Part 158 and Subdivision M, EPA has the authority to ask for data to address any additional questions regarding any of the aforementioned data areas. EPA also has the authority to waive data requirements if based on information provided by the applicant, Agency scientists determine that they are not applicable to the risk assessment or inappropriate for the MPCA in question.

Nontarget Aquatic Organism Data Requirements

How does EPA assess the risk of a MPCA to nontarget aquatic organisms? In Tier I, several studies are required for all end-use products intended for outdoor application and all manufacturing use products that legally may be used to formulate such end-use products. The first of these is the freshwater fish toxicity/pathogenicity study. If direct application to water is not expected from the use pattern of the product, then only one species of fish need be tested, preferably the rainbow trout. If direct application to water is expected, then bluegill sunfish is to be tested as well as the rainbow trout. As with all Tier I tests, maximum hazard dosing is required.

Besides freshwater fish studies, freshwater aquatic invertebrate toxicity and pathogenicity testing is required. Unless the pesticide product is to be applied directly to water, one species of aquatic invertebrate is to be tested. Products that are expected to have direct water application need two species tested, including one benthic and one planktonic species. Again, maximum hazard dosing is necessary. If no toxic or pathogenic effects are observed, then no further testing is warranted.

In addition to freshwater testing, estuarine and marine animal toxicity and pathogenicity testing are required when the end-use product is intended for direct application into estuarine or marine environments or is expected to enter this environment in significant concentrations due to the proposed use pattern or intrinsic mobility. Toxicity and pathogenicity are determined for one species of shrimp, preferably *Paleomonetes vulgaris* and one estuarine or marine fish species. Again maximum hazard dosing is utilized. If no toxic or pathogenic effects are observed then further testing is not required.

Nontarget plant testing may be required depending on the proposed use site, target organism and degree of anticipated exposure. The number of plant species tested depends on the host range of the MPCA and its similarity to known plant pathogens. For microbial pesticides that have aquatic uses or that may be expected to disseminate to, and survive in aquatic ecosystems, aquatic plants must be included within the testing regimen. As with plants, nontarget beneficial insect testing may also be required. The kinds and number of species to be tested depends on the host range of the MPCA and use sites.

OPP Organization   The mechanics of the Agency's review for field testing or registration involves the coordination of many people and offices. Once a submission has passed preliminary screens, the Registration Division routes it to the science support divisions for technical review, namely the Health Effects Division and the Environmental Fate and Effects Division. The Registration Division Product Managers who are responsible for coordinating the registration process and who are involved in final decisions regarding registration or field test approval of MPCAs are Product Manager 17 and Product Manager 21. The coordination of the scientific reviews is handled through the Science Analysis and Coordination Staff within the Environmental Fate and Effects Division. In addition to the Office of Pesticide Programs, the Office of General Counsel is often involved in assisting with legal issues associated with MPCA registrations and field testing. The approval of field testing or registrations ultimately rests with the Assistant Administrator for Pesticides and Toxic Substances. However, it is referred to this level only in special situations. These decisions are usually delegated to the Director of the Office of Pesticide Programs or the Director of the Registration Division.

FIG. 1.

EPA REGISTERED MICROBIAL PESTICIDES

Microorganism	Year Registered	Pest Controlled
Bacteria		
Bacillus popilliae/ *B. lentimorbus*	1948	Japanese Beetle larvae
B. thuringiensis "Berliner"	1961	Moth larvae
Agrobacterium radiobacter	1979	*A. tumefaciens* (crown gall disease)
B. thuringiensis israeliensis	1981	Mosquito larvae
B. thuringiensis aizawai	1981	Wax Moth larvae
Pseudomonas fluorescens	1988	*Phythium, Rhizoctonia*
B. thuringiensis San Diego	1988	coleopterans
B. thuringiensis tenebrionis	1988	coleopterans
B. thuringiensis EG2348	1989	gypsy moth
B. thuringiensis EG2371	1989	lepidopterans
B. thuringiensis EG2424	1990	lepidopterans/ coleopterans
Viruses		

Heliothis Nuclear Poly-
    hedrosis Virus (NPV)
	1975	Cotton Bollworm,
		    Budworm

Tussock Moth NPV	1976	Douglas Fir Tussock Moth larvae
Gypsy Moth NPV	1978	Gypsy Moth larvae
Pine Sawfly NPV	1983	Pine Sawfly larvae
Fungi		
Phytophthora palmivora	1981	Citrus Strangler Vine
Colletotrichum gloeosporioides	1982	Northern Joint Vetch
Trichoderma harzianum/ *Trichoderma polysporum*	1989	wood rot
Protoza		
Nosema locustae	1980	Grasshoppers

FIG. 2.

NON-TARGET ORGANISM TESTING

TIER I

Avian Oral

Wild Mammal

Freshwater Fish

Freshwater Aquatic Invertebrate

Estuarine and Marine Animal

Nontarget Plant

Nontarget Insect

Honeybee

TIER III

Terrestrial Wildlife & Aquatic Organism

Avian Chronic Pathogenicity and Reproduction

Aquatic Invertebrate Range

Fish Life Cycle Studies

Aquatic Ecosystem

Special Aquatic

Nontarget Plant

TIER IV

Simulated and Actual Field Tests

   (Birds, mammals, aquatic organisms, insects)

FIG.3.

TOXICOLOGY

TIER I

Acute Oral toxicity/pathogeniciy

Acute dermal toxicity

Acute pulmonary toxicity/pathogenicity

Acute intravenous toxicity/pathogenicity

Primary eye irritation

Hypersensitivity incidents

Cell culture with viral pest control-agents

TIER II

Acute toxicity

Subchronic toxicity/pathogenicity

TIER III

Reproductive and fertility effects

Oncogenicity

Immunodeficiency

Primate infectivity/pathogenicity

VI. ALTERNATIVES OTHER THAN ANTAGONISTS FOR BIOLOGICAL CONTROL.

In developing alternatives to synthetic fungicides, a variety of options are available to us.

Induction of Resistance of Avocado Fruits to *Colletotrichum gloeosporioides* Attack Using CO_2 Treatments

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Abstract

The concentration of the antifungal compound 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12, 15 diene, in the peel of avocado fruits, was modulated by storing fruits in controlled atmospheres containing high levels of CO_2. Such treatment resulted in two distinct periods of elevated antifungal diene level: the initial increase occurred immediately after removal from the CO_2 and the second increase appeared 2-3 days later, after a period of depression in the concentration of the antifungal compound. The degree and duration of the elevation affected the appearance of symptoms of *Colletotrichum gloeosporioides* and were dependent on the concentration and the length of time that CO_2 was applied. The effect of 30% CO_2 on diene levels was the same for O_2 concentrations of 0.75% or 16%. Under the latter conditions, the concentration of epicatechin was significantly enhanced, showing pattern similar to that of the concentration of the antifungal diene. We suggest that CO_2 elicit epicatechin production which affects the breakdown of the diene and consequently the inhibition of decay development.

Introduction

Postharvest decay caused by *Colletotrichum gloeosporioides* is the principal problem encountered during storage of avocado fruits (Prusky et al., 1982). The fungus attack the fruit during fruit growth but symptom of disease are observed during ripening and softening after harvest. Prusky et al. (1981) have described the presence of a preformed antifungal compound 1-acetoxy-2- hydroxy-4-oxo-heneicosa-12, 15-diene in the peel and flesh of unripe avocado fruits. This compound has been suggested as the basis of resistance to decay in unripe fruit (Prusky et al., 1983). The antifungal diene is considered to be a simple pre-infectional, static inhibitor (Stoessl, 1983) that decreased to subfungitoxic concentrations after harvest (Prusky et al., 1981). This decrease was suggested to be catalyzed by lipoxygenase and regulated by its inhibitor epicatechin (Prusky et al., 1981, 1983). Recent results, however, have indicated that the concentration of the preformed antifungal diene can be enhanced by challenge inoculation with *C. gloeosporioides* (Prusky et al., unpublished; Karni et al. (1989) also found that despite an initial drop in the concentration of the antifungal compound in freshly harvested fruits, there is subsequently a rapid increase in the concentration which eventually reaches the initial level; this suggests a rapid turnover of the compound.

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The use of controlled atmosphere is a possible way to inhibit decay development during storage (Kader, 1980). Spalding et al. (1975) reported that storage of avocado fruits under a controlled atmosphere of 2% O_2 + 10% CO_2 for 3-4 weeks at 7°C, prevented the development of anthracnose. Fungal growth was not affected by those conditions and it was suggested that the inhibition of decay development was the result of an effect on the fruit rather than on the fungus. Much of the research on controlled atmosphere has been empirical, dealing primarily with optimum levels of CO_2 or O_2 for a given commodity (Kader, 1980). The mode of action of elevated CO_2 on the physiology of fruits is still largely unknown, as is the basis for its effect on decay development.

The present work is part of a project dealing with modulation of the levels of the preformed antifungal diene as a novel way to increase resistance to postharvest diseases. Results suggest that CO_2 treatments can enhance the level of epicatechin, a phenol that inhibits the activity of the enzyme lipoxygenase (Prusky et al., 1985), resulting in the accumulation of the antifungal diene and delay of decay development.

Experimental

Avocado

General: Avocado fruits (*Persea americana* Miller var. *drymifolia* (Schlect and Cham.) (Blake) of cv. Fuerte were obtained from an orchard at Ayanot, Israel. A single isolate of *C. gloeosporioides* from decayed avocado fruits was used to inoculate fruits in all experiments (Prusky et al., 1982). Spot inoculation was carried out as described previously (Prusky et al., 1983). For analysis of the CO_2 treatment effect on decay development, fruits were inoculated upon completion of the 24-h treatment. Darkening of the peel of 5 mm diameter was considered a symptom of disease. Firmness of avocado fruits was determined by recording the force (kg) required to penetrate the fruit skin and flesh with a 5-mm-diameter, 4-mm-long conic probe Prusky et al. (1983).

Extraction and Quantitative Analysis of the Antifungal Diene and Epicatechin

The antifungal diene was extracted according to the modified method described by Prusky et al. (1990) and analyzed by HPLC, using 50-ul aliquots.

Epicatechin was extracted from 10 g of avocado peel according to the method described by Prusky et al., with modifications to shorten the purification process (10). The organic fraction was dissolved in 8 ml of 25% ethyl acetate in dichloromethane and a 2 ml aliquot was further purified by flash chromatography. A "Spe-ed Silica gel" column (Laboratory Impex Ltd., U.K.), of 1.2-cm-diameter and 6-ml capacity, was filled with 1.45 g of 40-63 um silica to a depth of 2.2 cm and equilibrated with 6 ml dichloromethane. Inactive material was eluted by 8 ml of 30% ethyl acetate in dichloromethane. The active fraction was then eluted by washing the column twice with 6 ml of ethyl acetate, after which it was dried under nitrogen. Epicatechin was determined by HPLC analysis of 10-ul aliquots taken from the concentrated epicatechin fraction dissolved in 400 ul of methanol:water (80:20, v/v).

Calculations of the antifungal diene and epicatechin concentrations were based on the comparison of the HPLC peak areas with those of the standards (Prusky et al., 1982). Average values of three different extractions are presented. Experiments were repeated at least twice.

CO$_2$ Treatment

Approximately 1-2 h after harvest, fruits were placed in 15 l sealed glass jars through which was passed a stream of air containing 11%, 16% or 30% CO$_2$ at flow rate of 100 ml min^{-1}. CO$_2$ and air were obtained from commercial high pressure cylinders in some experiments air was replaced by N2. The CO$_2$ concentrations were obtained by quantitating the flow of the pure CO$_2$ through needle valves to give the appropriate mixture of gases. The outlet of the jars passed to the outside of the storeroom, which was maintained at 20°C. Control fruits were stored in similar jars under a stream of air. Fruits were removed to air at different intervals and stored at 20°C. The antifungal diene and epicatechin concentrations were assessed on removal from the jars at daily intervals until the fruits were fully ripe.

CO$_2$ and O$_2$ concentrations were monitored by GC using a thermal conductivity detector. Poropak Q and molecular sieve column and He as the carrier gas.

Results and Discussion

Although the antifungal diene is considered to be a preformed compound, involved in static resistance (Stoessl, 1983) significant changes in its levels could be induced by fruit harvesting (Karni et al., 1989) and by challenge inoculation by *C. gloeosporioides* (Prusky et al., 1990). Elevated CO$_2$ concentrations above 1%, in a controlled atmosphere, were reported to retard fruit ripening and decay during storage, by reducing respiration and ethylene production rates, and slowing down all the compositional changes associated with ripening (Kader, 1980, 1985). In the present work the effect of CO$_2$ on the antifungal diene concentration was obvious in two main periods of enhancement (Figures 1 and 3). The initial increase of the antifungal compound level occurred immediately after the 24-h treatment followed by a decrease to subfungitoxic concentration (Karni et al., 1989) before the second burst of diene production occurred between the 4th and 6th days after harvest. The increase in concentration of the antifungal compound was dependent on the duration and the concentration of the CO$_2$ treatment. Treatments with increasing, CO$_2$ concentrations (Figure 1) (from 11% to 30%) or length of the CO$_2$ supply (Figure 3) (from 3 to 24 h), all enhanced the initial increase in concentration of the diene. The effect on symptom development was dependent on the duration of the second increase of the antifungal diene (Figure 2 and 4). When fruits were treated for 24 h at 30% CO$_2$, the duration of the second increase lasted for 3 days (Figure 1) and decay by *C. gloeosporioides* was significantly delayed (Figure 2). When the duration of the second increase was 1-2 days (30% CO$_2$ for 10 h and 16% CO$_2$ for 24 h Figures 1 and 3, respectively), decay development in treated fruits did not differ from in the controls. In some cases (11% CO$_2$ for 24 h or 30% CO$_2$ for 3 h), the second increase was not observed and decay development was enhanced (Figures 2 and 4

respectively). This enhancement of the decay development is the result of the early or prolonged decrease of the antifungal compound to subfungitoxic levels occurring after the initial increase in concentration. No effect on the initial or second increase of the antifungal diene was observed if the 24-h 30% CO_2 treatment was applied 3 days after harvest. This suggests that the preclimacteric period is the receptive stage for abiotic elicitors affecting decay development.

Most of the studies aimed at examining the effect of CO_2 in controlled atmosphere were conducted under low O_2 and consequently the specific effect of CO_2 could not be evaluated (1, 2). In avocado fruit treatment with 16% O_2 and 30% CO_2 induced a similar two-peak increase as 0.75% O_2 and 30% CO_2 suggesting that high CO_2 and not low O_2 specifically affects the antifungal diene increase (Figure 5).

Diene Metabolism and CO_2 Treatment

How is the antifungal diene metabolism affected by the CO_2 treatment? Metabolism of the antifungal diene occurred by lipoxygenase activity which increases during fruit ripening (Prusky et al., 1983). This increase is not due to changes in the enzyme protein content, but is related to a decrease in the level of the inhibitor of lipoxygenase-epicatechin (Karni et al., 1989), which was suggested as a primary factor affecting decay development (Prusky, 1988). Treatments with 30% CO_2 for 24 h enhanced epicatechin concentration following the same two- peak behaviour observed for the antifungal diene, and suggests that the primary effect of controlled atmosphere may be on epicatechin metabolism. In stored lettuce, a similar treatment, 15% CO_2 for 10 days, did not significantly change the phenol content but when CO_2 was replaced by air the phenolic content increased considerably after one day (Siriphanich and Kader, 1985). CO_2 stress enhanced lettuce phenylalanine ammonia Tyase (PAL) activity during storage by 70%, but one day after transferring the lettuce to air the activity of PAL had increased more than threefold. Siriphanich and Kader, (1985) suggested that high CO_2 disrupts the normal metabolic balance of the plant cell and at the same time PAL is induced as a natural defense mechanism. Also wounding and inoculation of plant tissue are known to cause an increase in the metabolic pathways which lead to an increase in phenolic compounds as a mechanism of defense against fungal infection (Rhodes and Wooltorton, (1978).

How does CO_2 affect the second increase in antifungal diene? It is still not clear if this increase is the result of (a) a metabolic process induced by the CO_2 or (b) a 2-3-day delay in the processes occurring regularly in untreated fruits. The rapid effect of CO_2 on the initial increase supports the second hypothesis: 7 h after the 3-h treatments, the concentration of the antifungal diene in the peel of treated fruits was fivefold that in the control (Figures 3). Furthermore, the second increase in epicatechin level and the close relation between its level and that of the antifungal diene (Karni et al., 1989), suggest that the effect of CO_2 treatment might be to delay regularly occurring processes. On the other hand, the direct relation between the duration of the 30% CO_2 treatment and the length of the second increase in

level of the antifungal diene, suggests the possibility that CO_2 can be metabolized by the fruit tissue (Pesis and Ben-Arie, 1986), resulting in enhanced phenol synthesis. The results suggest that levels of the antifungal compound are modulated by factors inhibiting its breakdown (Karni et al., 1989; Prusky et al., 1983 and Prusky et al., 1988). The increase in soluble phenolic epicatechin (Ke and Stalveit, 1989) after a 24-h CO_2 treatment represents the key factor for the inhibition of the lipoxygenase-catalyzed oxidation of the antifungal diene and its enhanced concentration.

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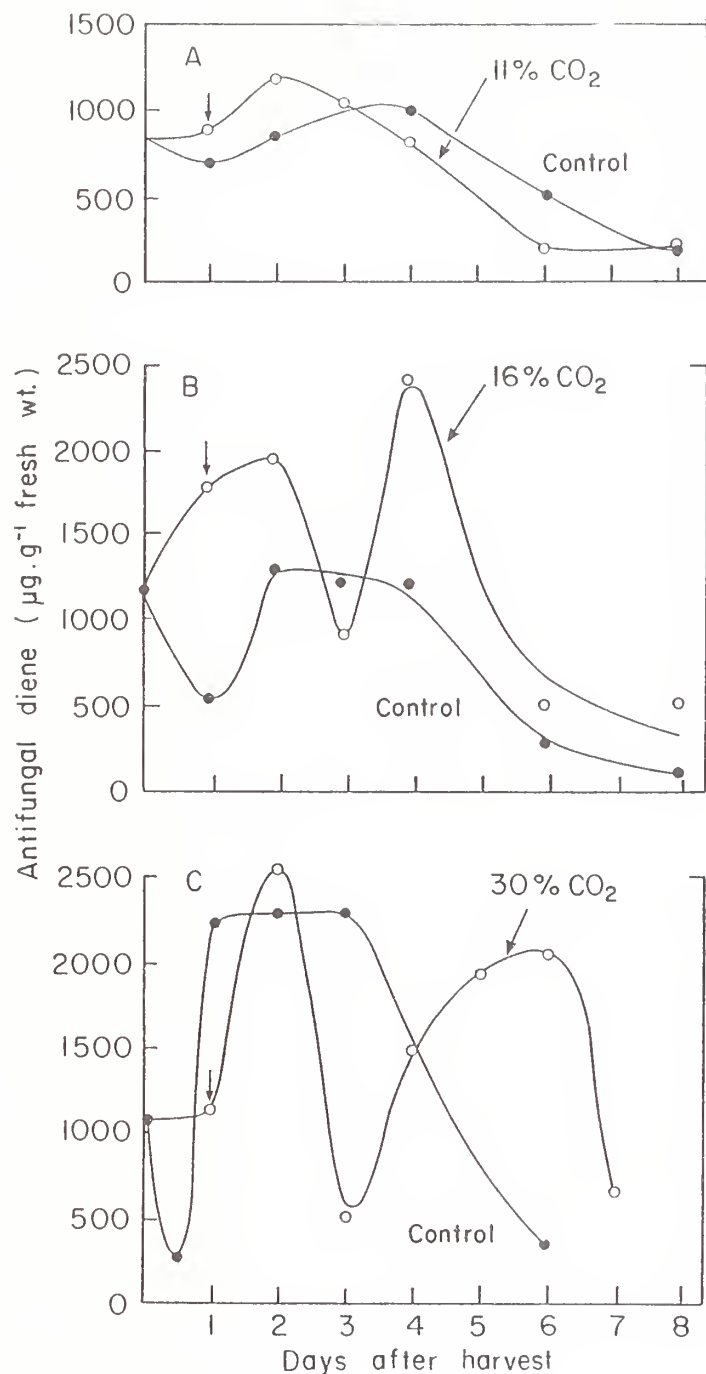
FIG. 1. Changes in the concentration of the antifungal diene in the peel of avocado fruits cv. Fuerte treated with different concentrations of CO_2. Freshly harvested fruits were sealed in flow-through jars, treated under a stream of CO_2 in air (100 ml min^{-1}) for 24 h and then transferred (arrow) to normal atmospheric conditions (0--0). A-11%, B-16% and C-30% CO_2. Control fruits were treated with air for the same period of time (•--•). Experiments were carried out at different periods after fruit set during the harvest season with a resulting difference in fruit maturity (7). The effect of the CO_2 treatment was determined by the increase in the level of the antifungal diene in each experiment. The mean of three extractions of the antifungal diene is shown. The SE ranged between 3% and 9% of the mean.
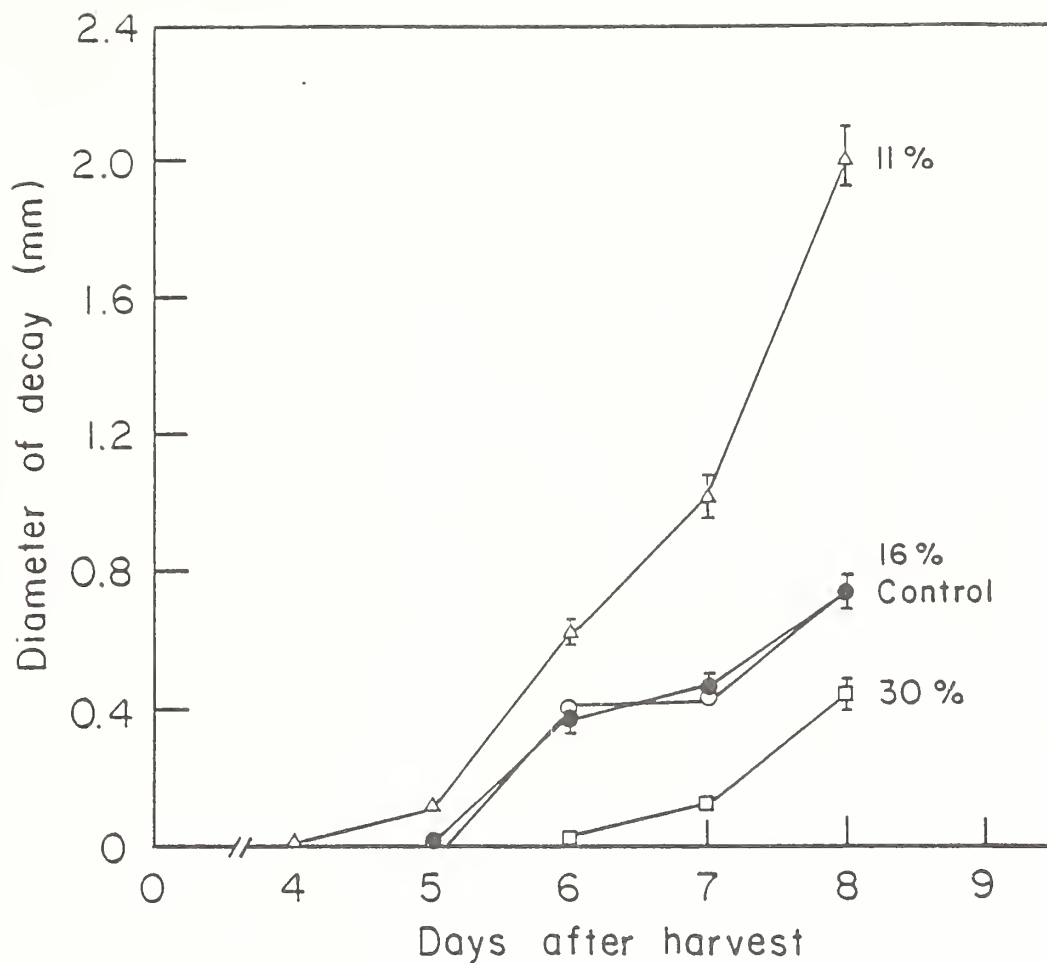
FIG.2. Decay development of *C. gloeosporioides* on avocado fruits cv. Fuerte treated with different concentrations of CO_2 as described in Fig. 1. •--•, control, Δ--Δ 11% CO_2, 0--0, 16% CO2 and □--□, 30% CO_2. After treatment the fruits were point-inoculated with a spore suspension of *C. gloeosporioides* (ca, 10^6 spores ml^{-1}). The fruits were incubated overnight at 25°C and then transferred back to 20°C until disease symptoms were visible. Bars indicate SE of the mean.
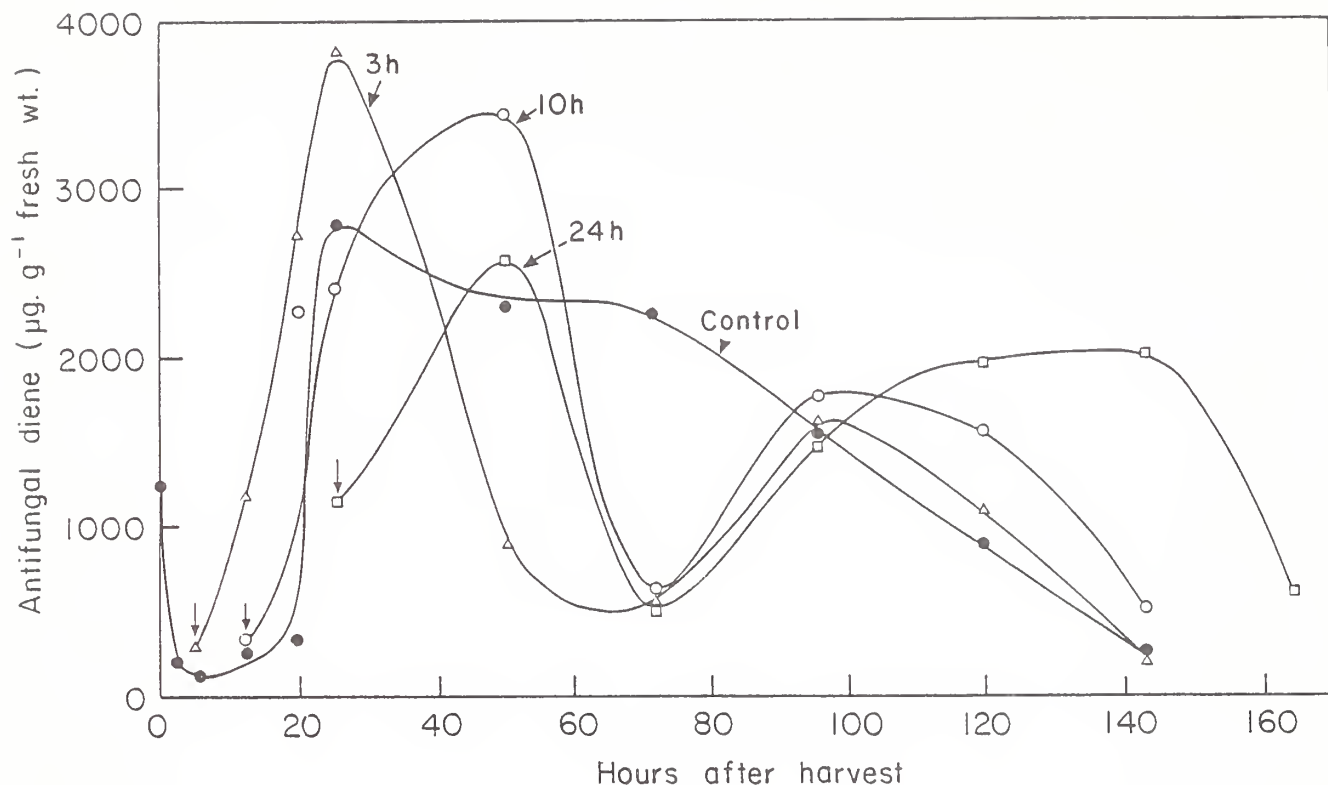
FIG. 3. Changes in the concentration of the antifungal diene in the peel of avocado fruits cv. Fuerte treated for different periods of time with 30% CO_2. Freshly harvested fruits were treated for △--△, 3 h; 0--0, 10 h; and □--□, 24 h under a stream of 30% CO_2 in air (100ml h^{-1}) and then transferred (arrow) to atmospheric conditions at 20°. The concentration of the gases during treatments was 30% CO_2 and 15% O_2. Control fruits (●--●) were treated with air under similar conditions for 24 h. The mean of three extractions of the antifungal diene is shown. The SE ranged between 3% and 9% of the mean.
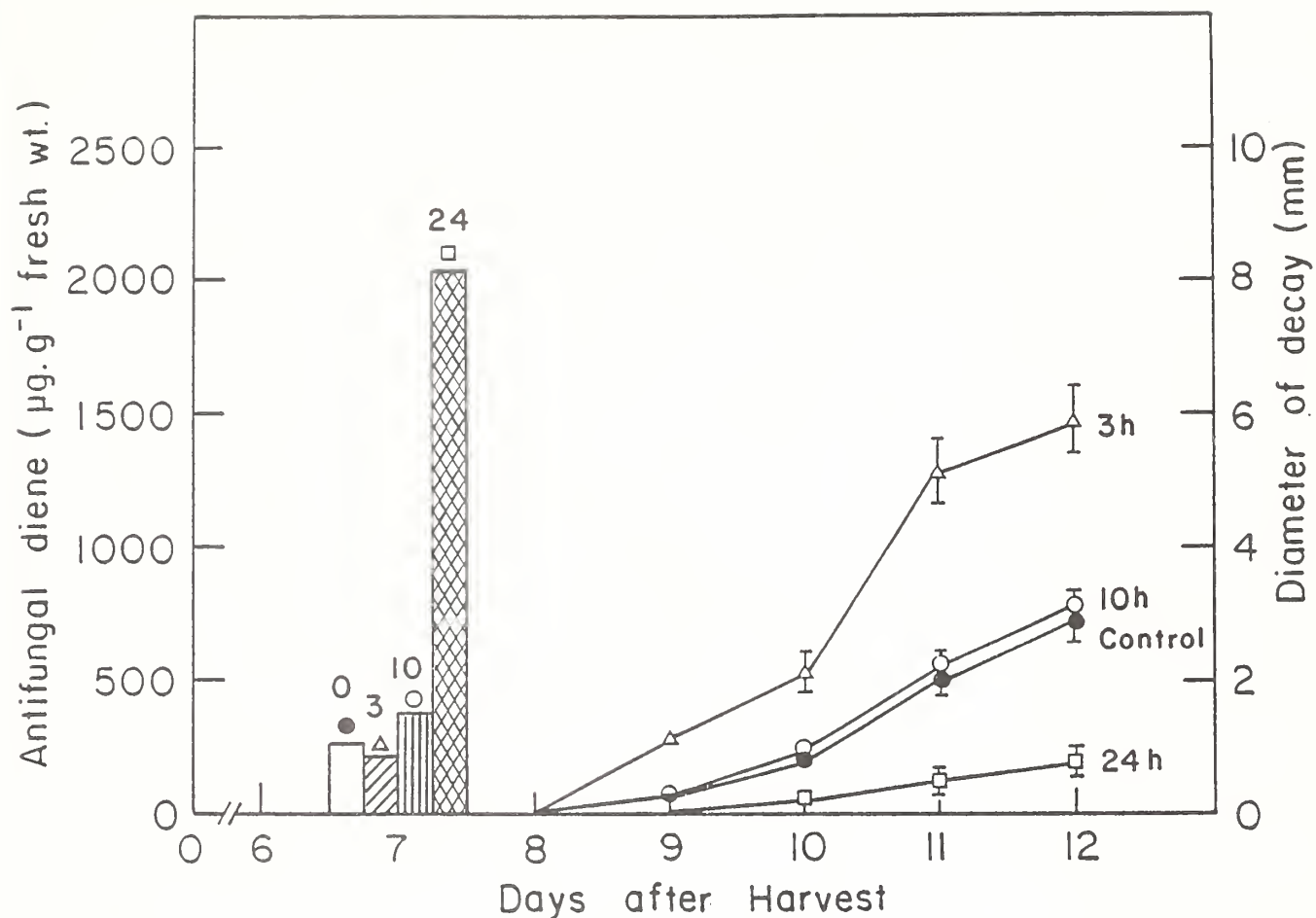
FIG. 4. Decay development of *C. gloeosporioides* on avocado fruits cv. Fuerte treated with 30% CO_2 in air at 20°C for different periods of time. Δ--Δ, 3 h; 0--0, 10 h; □ --□, 24 h; and •--•, untreated controls. Fruit inoculation was done as described in Fig. 2. The SE of the mean is indicated by bars. The concentration of the antifungal diene shown represents the mean of three extractions and was determined as described in the Materials and Method section.
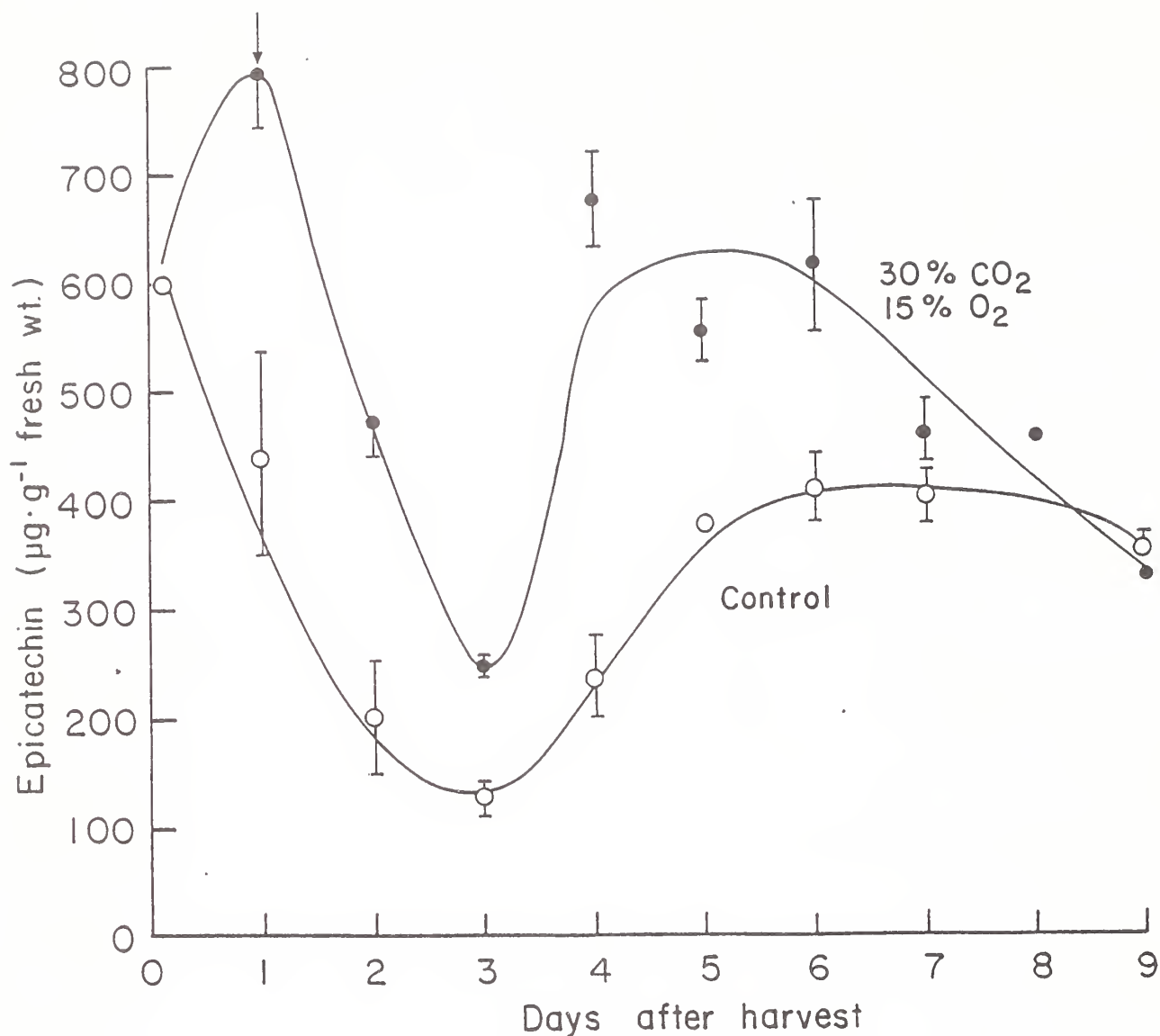
FIG. 5. Epicatechin concentration in the peel of avocado fruits cv. Fuerte treated with 30% CO_2 in air (100 ml h^{-1}) for 24 h at 20°C. Freshly harvested fruits were treated as in Fig. 1 and then transferred (arrow) to air (0--0). Control fruits (●--●) were treated with air under similar conditions for 24 h. The mean of three extractions of epicatechin from peel tissue is presented. The SE of the mean is indicated by bars.
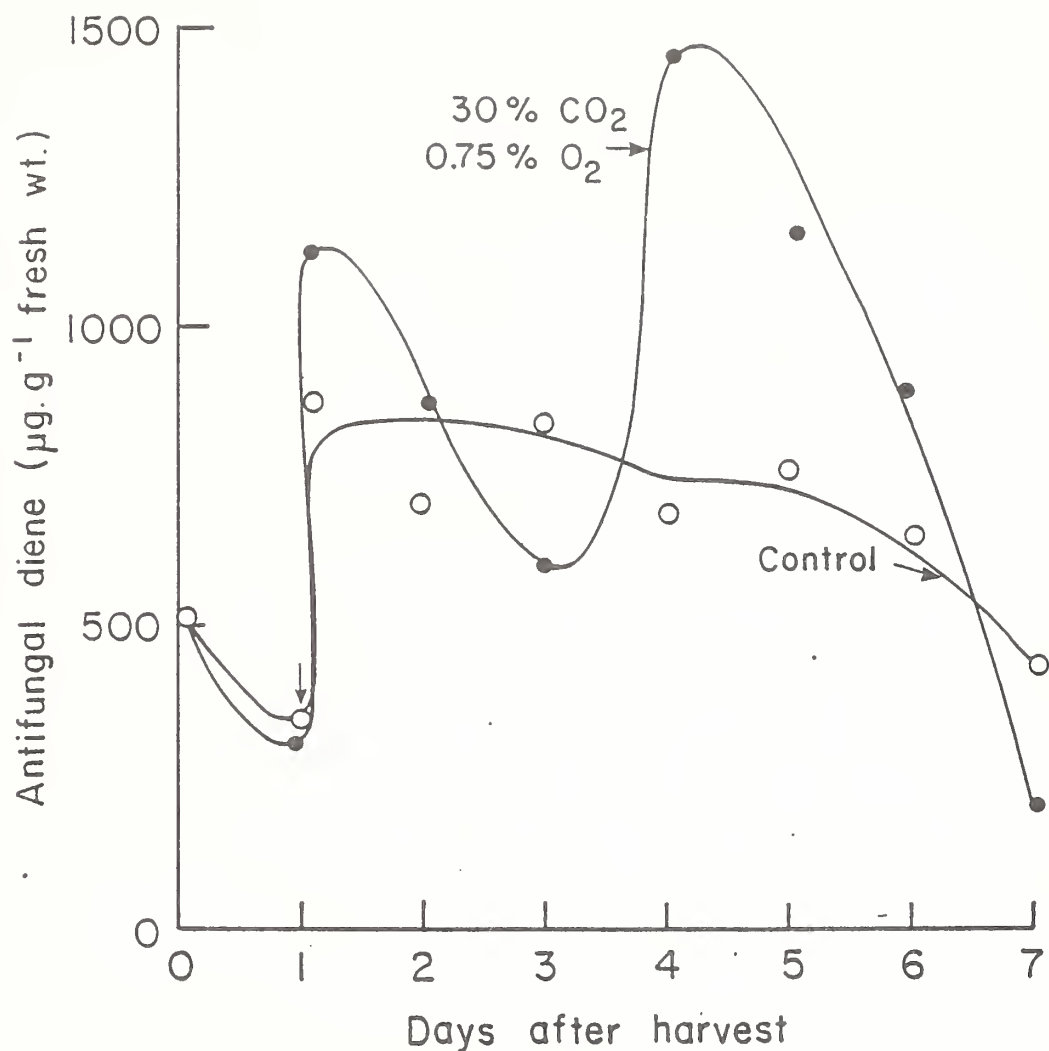
FIG.6. Changes in the concentration of the antifungal diene in the fruit peel of avocado cv. fuerte treated for 24 h by a stream of 30% CO_2 in N2 at 100 ml min^{-1} and then transferred (arrow) to atmospheric conditions (0--0). The concentration of the gases during application of the treatment was CO_2, 30%; O_2, 0.75%; and N2, 69.25%. Control fruits were treated with air during the same time ($\bullet$--$\bullet$). The mean of three extractions of diene from peel tissue is presented. The SE of the mean ranged between 3 and 9%.

The Use of Temperature for Postharvest Decay Control in Citrus Fruit

E. Cohen[1]

Abstract

Chemical fungicides are the principal means for controlling decay in commercial handling. However, biological and physical treatment that control fruit decay, which do not leave residue on the fruit, should be preferable. Among the postharvest treatments for control of fruit decay that have been evaluated, are higher and lower temperatures than the optimal recommended for storage. This, encouraged research on the application of hot treatment and possible storage at suboptimal temperature without causing chilling injury to cold sensitive fruits. Hot or cold treatments have greater effect than chemical fungicides, as they can penetrate the fruit surface and inhibit or control the pathogen.

Hot Treatment on Disease Control

The only way to eliminate establishment of fungi in perishable commodities is by immersing infected fruits and vegetables in hot water. For instance, *Phytophthora citrophthora* (R.E. Sm. & E,.H. Sm.) Leonian, the causal of brown rot in citrus fruit is controlled by immersing infected fruit in hot water. But, the period after infection related with the temperatures prevailing in the grove and the fruit temperature at the time of immersion are important on the effectiveness of this treatment: A certain correlation was observed between the effective treatment period and the incubation period. In most cases, when the incubation period was between 7 and 10 days, the treatment was effective when applied within 3 days after inoculation. The extreme case when the treatment was still effective on the seventh day from inoculation, the incubation period was found to be 19 days (Schiffmann-Nadel and Cohen, 1966).

During the citrus harvesting season in Israel (November till April) the average daily grove temperature ranged mostly from 12 to 15°C. Under these conditions hot water treatment at 48°C for 3 min dip was generally effective when applied within 2 or 3 days after inoculation. With daily average below 12°C, the effective treatment period was somewhat extended. On an exceptional occasion when the daily average temperature after infection ranged from 6 to 11°C, the treatment was still somewhat effective when given 7 days after infection. Both incubation period and effectiveness of the heat treatment are affected by grove temperatures after infection (Table 1). A longer incubation period, indicative of slower penetration of the fungus into the fruit, would imply a longer period of treatment effectiveness. The treatment is likely to be effective during the early stages of incubation, when penetration is still confined to the outer layers of the fruit (Barret and Fawcett, 1919; Fawcett, 1936; Klotz and DeWolfe, 1961; Schiffmann-Nadel, 1958, Schiffmann-Nadel and Cohen, 1966).

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In practice, the effectiveness of hot-water treatment depended to a considerable extent on the temperature of the fruit before the treatment. When the fruit temperature (peel and flesh) was between 20 to 25°C, the hot water immersion (48°C for 3 min dip) caused the flavedo-2-3 mm deep to exceed 45°C and the albedo-10 mm deep 40-41°C. But, when the fruit temperature was 18°C and below, the flavedo reached 43 to 45°C and the albedo was under 40°C, which is uneffective to control brown rot disease (Table 2).

The major problem with hot-water treatments, is that turgid freshly picked citrus fruits develop oleocellosis (injury due to peel oil release) if immersed after harvest in hot water. Therefore, it is necessary to hold the fruit for 1 day (Eckert and Brown, 1986) to 4 days if picked soon after rain (Pelser, 1974) in the packinghouse before immersion to allow the fruit to lose some peel moisture before exposure to hot water. This delay in hot treatment reduces its effectiveness as it prolongs period between infection and treatment. Even if the hot treatment does not cause oleocellosis, it has been observed that hot water- treated lemons and oranges tend to develop excessive secondary storage decays caused by *Penicillium italicum* Wehmer and *P. digitatum* Sacc. during storage (Harding and Savage, 1964; Smoot and Melvin, 1965). In these experiments based on large samples of lemons no rind oil was released by the hot water treatment and the fruit did not develop oil spotting or a scalded appearance. Possibly hot water lowered the resistance of the rind by decreasing cell vitality. These fruit were not artificially inoculated in order not to mislead (Harding and Savage, 1964).

Hot Treatment on Fungicides Effect
Hot treatment is used to enhance (accelerate) the effect of fungicides. In uninoculated grapefruit carefully picked, we found that reducing thiabendazole (TBZ) concentration from 4000 to 100 ppma.i. in water suspension applied by 3 min dip, did not cause significant increase in mold decay mainly caused by *P. digitatum*. However, reducing TBZ concentration increased chilling injury (CI) and mold decay subsequently develop on pitted CI injured fruit during 3 months storage at 5°C. When TBZ at 250 ppm was applied as hot treatment (48°C for 3 min dip), TBZ effectiveness to that was similar 4000 to 1000 ppm (Table 3). Heated benomyl and thiabendazole suspensions controlled rot caused by *P. expansum* Lk. ex Thom., when applied in both punctured and bruised apples, within 5 hours after wounding and inoculation. Heated chemical solutions controlled rot in bruised, but not punctured apples when applied 24 hours after wounding and inoculation (Spalding et al., 1969). Effect of heating on curing of injured fruit

High humidity and warm temperature condition enhanced flavedo wound healing processes, induced lignin formation and reduced decay incidence in superficially injured oranges (Brown, 1973); Ismail and Brown, 1975 and 1979). Free phenolic constituents increase more than 2-fold in injured peel of oranges after 48 hr at 30°C and 98% r.h. Phenylalanine ammonia-lyase (PAL) was a key enzyme in the healing and lignification processes of superficially injured citrus flavedo (Ismail and Brown, 1979), which reduces hypal penetration of *P. digitatum* into injured tissues. Ben-Yehoshua (1988) reported that curing for 48-72 hrs at 34-35°C, later than 48 hrs after harvest, is effective

in reducing decay without causing damage to Pummelo fruit. Shamouti oranges inoculated with *P. digitatum* and *P. italicum* and grapefruit inoculated with *P. citrophthora* after being cured for 3 days at 36°C developed only 11% green mold, and no blue mold or brown rot. However, the non-cured fruits developed 89% green mold, 61% blue mold and 5 to 22 brown spots per fruit, respectively (Barak and Weiss, 1986. Unpublished data) (Table 4).

Cold Storage on Decay Development

Storage at lower temperatures is considered to be the most effective method for preserving quality of most perishable commodities, because it retards aging, respiration, ripening, decay and other undesirable metabolic changes (Hardenburg et al., 1986). The direct effect of lower temperatures on the incidence of citrus diseases was well reviewed. For instance, grapefruit stored at 2°C no stem-end rot developed during 20 weeks of storage. Stem-end rot developing at temperatures between 4 to 10°C were caused by *Phomopsis citri* Fawc., *Alternaria citri* Ell. & Pierce and *Fusarium* spp. while those developing at temperatures above 10°C was caused mainly by *Diplodia natalensis* P. Evans (Schiffmann-Nadel et al., 1971). *P. citrophthora* in inoculated citrus fruit varieties showed the shortest incubation period of 3 to 5 days at temperatures between 20 and 32°C. The incubation period prolonged the lower temperatures and was 31 to 35 days at 6°C, the lowest temperature at which brown rot developed. At 4°C the fungus did not develop (Schiffmann-Nadel and Cohen, 1969). This temperature (4-5°C) is the minimum temperature for growth of many postharvest fungi in vitro (Facett, 1936; Schiffmann-Nadel, 1951).

Low Temperature on Chilling Injury Development

Prolonged storage of fruits and vegetables may cause chilling injury (CI), a damage that occurs at low temperature above the freezing point of the fruit tissue. Chilling injury results in a loss of quality, and a weakening of the tissue which renders the commodity very susceptible to decay by postharvest pathogens (Wang, 1982).

Among citrus fruit cultivars, the sensitivity to CI differs in peel pitting and subsequent rot development. Citrus fruits stored at 12°C, which does not cause CI, the incidence of rot was found to be relatively low- 5 to 10% after 12 weeks of storage. However, storage temperature of 6°C, pitting and subsequent rot developed on the pitted fruits were high- 15 to 70% (Chalutz et al., 1981). During prolonged storage of grapefruit at 2-8°C, most of the fruit pitted and rotted while still in cold storage. Shamouti orange in contrast to grapefruit, very little peel pitting were visible upon transfer of the fruit from cold storage and during shelf-life, however the incidence of mold rot increased. During storage of lemon fruits at 2°C showed 50% pitted and rotted fruits also an internal browning developed in the membrane and core of the fruits, the amounts of juice, total soluble solids and acid decreased than in fruit stored at the optimal temperature. This decrease could perhaps be related to some physiological disorders occurring in fruit during low temperatures storage (Cohen and Schiffmann-Nadel, 1978).

Prestorage Treatments to Alleviate CI and Decay

Different treatments prior to or during cold storage were applied for the alleviation of CI symptoms in citrus fruit. For instance, holding the fruit at ambient temperature with or without exposure to 40% carbon dioxide for 3 days "conditioning" (Hatton and Cubbedge, 1982, 1983; Purvis, 1984). The effect of delaying cold storage is to alter the peel stage of ripening at which the fruits enter cold storage. In general, ripening in the flesh of citrus fruits (non-climacteric) proceeds ripening in the rind. Reduction in transpiration from prestorage handling (Purvis, 1984), wrapping and seal-packaging with plastic film (Ben-Yehoshua et al., 1981; Cohen et al., 1990), wax coating or vegetable oils and vegetable oil-water immersion (McDonald, 1986; Aljuburi and Huff, 1984). The fungicides thiabendazole and benomyl have been reported to produce a variety of physiological effects in grapefruit resistance to CI (Schiffmann-Nadel et al., 1975).

Cold Storage with Intermittent Warming – New Method

Periodic interruption of chilling by periods of exposure to warm temperatures has been found to reduce or at least delay the onset of CI. Warming grapefruit during cold storage was found to reduce CI and decay (Davis and Hofmann, 1973); Eaks, 1965). Storing lemons at low temperature with intermittent warming (IW) in cycles of 21 days at 2°C following by 7 days at 13°C, enables lemons to be kept for 6 months and longer in storage in good marketable quality (Cohen et al., 1983). The IW during cold storage not only reduced pitting in the peel fruit surface and subsequent mold rot, but, prevented internal membranosis in the core segments, maintained the juice, total soluble solids, acidity, weight loss and respiration at a better level than in fruit stored at constant low temperature. This method is applied in commercial practice for lemons in Israel (Cohen, 1988). The benefit of short warming following long exposure periods to chilling temperatures may accrue from:

1. advanced ripening that occurs at the higher temperature.

2. repair of organelles, membranes and/or metabolic pathway before irreparable degenerative change occur.

3. release metabolism of potentially toxic compounds that may accumulate during chilling.

It is well known that citrus mold fungi are wound parasites and cause rots only when the fruit is injured. Shiffmann et al. (1980) supposed that the increase of mold rots in fruit during storage and shelf life could be related to the development of microscopic cracks (wounds) in the peel that enables the penetration of the fungus. Cohen et al. (1988) found that d-Limonene emanated from lemon and grapefruit stored at low temperature was about 100 times as great as that from fruit stored at optimal temperature. This indicates that d-Limonene, the main constituent in citrus peel oil glands (almost 90%) was released probably following chilling injuries developed in the fruit peel. Only in advanced stage of CI a visible macroscopic peel injures were formed, subsequently developed into pits, and were covered with mold. Further evidence was obtained by contamination (without wound) of these fruits with dry spores of *P. digitatum* led to infection of the fruits with mold rot. This proved that these fruits looked sound, but, were in fact suffering from microscopic injury on the peel.

A better understanding of the physiological responses in perishable commodities sensitive to CI stress would help us to design improved cultural practices based on storage at 1-2°C with IW, and develop simple, sure and effective technique to alleviate CI. This method is useful for retention of fruit color, extension of normal shelf-life, protection against microbial and fungal decay and fruits and vegetables free or with minimum chemical fungicides residue.

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Table 1. Effectiveness of hot treatment on Shamouti oranges infected with brown rot (%)[*].

	Days from infection to hot treatment		
	1	2	3
Control-untreated	100.0	100.0	100.0
3 min water dip at 45°C	1.6	20.2	38.6
3 min water dip at 48°C	0.0	0.9	4.5

[*]Calculated according to the average number of spots per fruit, compared to the control fruits 100%.

Table 2. Changes in peel fruit temperature following 3 min dip
in water at 48°C.

P E E L T E M P E R A T U R E °C		
Before treatment	After treatment	
	In flavedo 2-3 mm	In albedo 10 mm
25	above 45	41
20	above 45	40
18	45	39
13	44	37
10	43	35
6	43	34

Table 3. Effect of TBZ on mold decay control and on CI incidence in grapefruit[*] after 3 months' storage.

TBZ conc. (ppm)	At 11°C Decay (%)	Decay (%)	AT 5°C CI (%)	Decay on pitted fruit (%)
4000	0.33bc	0.33b	1.00c	0.00b
1000	0.00c	0.00b	7.67b	0.00b
250	0.67bc	1.67b	18.67a	0.33b
100	1.33b	2.00b	23.33a	2.00b
0	4.00a	16.67a	11.00b	16.67a
250/48°C	0.67bc	0.33b	6.00bc	0.00b

[*]Average from 3 carton, 30 fruit each. Within columns, means followed by a common letter do not differ significantly at P=0.05 according to Duncan's Multiple Range Test.

Table 4. Effect of curing (3 days at 36°C) on decay incidence in inoculated citrus fruits.

Treatment	Penicillium digitatum[*] (%)	Penicillium italicum[*] (%)	Phytophthora citrophthora[**] Ave. spots/10 fruits
Non-cured	89	61	5 – 22
Cured	11	0	0

[*]50 Shamouti oranges inoculated by puncturer 2 mm with 5×10^6 spore/ml, 24 hrs at 17°C and 85% r.h. Examination after 2 wks from inoculation.

[**]Grapefruit and Valencia oranges, spray inoculation with zoospore and sporangium, cured 3 days later and examined after 2 wks.

(Barak & Weiss, 1986. Unpublished data)

Ultraviolet Light Induced Resistance Against Postharvest Diseases in Vegetables and Fruits

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Abstract

Low hormetic doses of ultraviolet light (254nm UV-C) irradiation reduced postharvest rots of vegetables, pome, stone and citrus fruits. 'Walla Walla' onions, 'Jewel', 'Carver' and 'Georgia Jet' sweetpotatoes, 'Loring' and 'Elberta' peaches, 'Golden Delicious' apples, 'Marsh Seedless' grapefruits and 'Dancy' tangerines were irradiated with UV-C selected low doses then stored. 'Walla Walla' onions treated with doses of 1.3 to 19.6 x 10^4 erg/mm^2 of UV exhibited the greatest percentage of marketable onions and reduction in postharvest decay. The application of UV-C caused a reduction in storage rots which included soft rot (*Rhizopus stolonifer*) and Fusarium root rot (*Fusarium solani*) in sweetpotatoes. With the fruits, the type of rots reduced by UV-C irradiation were: Brown rot (*Monilinia fruicticola*) of peaches, Alternaria (*Alternaria* spp.) rot, brown rot (*Monilinia* spp.) and bacteria soft rot (*Erwinia* spp) of apples, and green mold rot (*Penicillium digitatum*) of grapefruits and tangerines, stem end rot (*Alternaria* spp.) and sour rot (*Geotrichum condidum*) of tangerines.

Introduction

The onset of postharvest decay in fruits and vegetables in forms of stem-end and surface rot is caused by a number of microorganisms (Snowdon, 1990). Postharvest storage rots cause serious economic loss of 25 to 50% worldwide. Pesticides, a major weapon for combating postharvest storage rots is often ineffective and pose hazards to the public's health and environment (Anon., 1987). As a result of these developments, researchers have sought to find new alternative to chemical pesticides for controlling postharvest diseases of vegetables and fruits (Lu et al., 1987; Scriven et al., 1988 and Wilson and Pusey, 1985).

A relatively recent concept of ultraviolet light (UV) hormesis (Luckey, 1981) was advanced by researchers who utilized UV-C light at low hormetic doses to reduce postharvest storage rot of onions (Lu et al., 1987) and sweetpotato (Stevens et al., 1990). Hormesis is defined as a stimulation of a beneficial plant response by low levels of inhibitors or stressors (Luckey, 1980). Since little work with UV-C application for controlling storage rots has been done, we sought to expand this new postharvest technology in a coordinated research

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effort to gain information on the phenomenon of UV-C induced resistance of storage rots of fruit and vegetables and evaluate some physiological and biochemical responses involved in induced resistance.

Experimental

Fresh 'Walla Walla' onions were obtained from Walla Walla, Washington, July 1985. 'Georgia Jet', 'Jewel' and 'Carver' sweet potatoes were harvested from fields at Tuskegee and Cullman, Alabama in 1985-1986. Sweet potato ('Jewel') storage roots were harvested in 1987 and stored for 6 months before UV-C treatment. 'Loring' and 'Elberta' peaches were harvested in June 1989 from Milstead, Alabama or Kearneysville, West Virginia in August 1989. 'Golden Delicious' yellow apples were shipped from the Appalachian USDA/ARS Fruit Research Station Kearneysville, WV two weeks after harvest in October 1989. Tangerines ('Dancy') were obtained from Whitmore Citrus Foundation Farm, USDA/ARS, Clearmont, Florida in December 1989. 'Marsh Seedless' grapefruits were harvested from Bet Dagan, Israel in 1989.

Ultraviolet Irradiation

All fruits and vegetables were irradiated with a General Electric germicidal low pressure vapor lamp (G30T8) with a nominal power output of 30 watts, an amperage of 0-36 amps and a maximum intensity perpendicular to the bare tube of 1.66 $mW/cm^2/sec$. The lamp had a tube diameter of 2.5 cm and a length of 88 cm and emitted wave energy at 254 nanometer wavelength.

Fruits were placed approximately 10 cm from the surface of the lamp and were individually rotated to expose four separate sides to the lamp. The fruits were also randomly moved to different positions in the UV-C field, which measured $1.2 mW/cm^2/sec$. All fruit and vegetable commodities were treated with dosage levels when indicated, with different profiles from 0 to $40 \times 10^4 erg/mm^2$. Except when indicated most vegetables and fruits were irradiated with UV-C within 48 hours after harvest.

Storage Conditions

After UV irradiation, all fruits and vegetables were placed in small lots in paper bags and stored under dark conditions. 'Walla Walla' onions were stored for 40 days at 20-25°C. All treated sweetpotato storage roots were kept in a storage house at 18 + 5°C for 1-2 months. 'Loring' and 'Elberta' peaches were stored for 10 and 20 days at 12 + 5°C, respectively. 'Golden Delicious' apples were stored for 30 days and 'Dancy' tangerines for 17 days before assessment for naturally infected diseases.

Nutrient Analysis

All nutrient analysis were performed as previously described (Lu et al., 1987). 'Jewel' sweet potato peeled roots were weighed, homogenized in a Waring blender and analyzed for proximate composition and total carotenoids. Thiamine and riboflavin in sweet potato were determined fluorometrically. The sweet potato sample for starch analysis was first homogenized and washed with 80% ethanol 2-3 times and was converted to glucose with a mixture of alpa amylase and amyloglucosidase (Sigma Chemical, St. Louis, MO). The hydrolysate was filtered by Whatman No. 1 filter paper, after

dilution an aliquot was taken for glucose analysis. Starch was determined by multiplying the amount of glucose (g) x 0.9. Ten roots were analyzed for each treatment. Peaches and apples that appeared healthy were randomly taken out for texture and percent soluble solids determination. The texture was determined by an Instron 1132 texture meter equipped with a Magness-Taylor's probe. The percent soluble solids was read with a refractometer. Ascorbic acid of sweet potato, tangerine and apples was determined by the titration method using 2.6-dichlorophenolindophenol.

Inoculation Study

'Marsh Seedless' grapefruits and 'Jewel' sweetpotato storage roots in some experiments were artificially inoculated with a pure culture. Grapefruits were wounded and inoculum suspension (10^4/ml conidia of *P. digatatum*) were inoculated into the fruits. Jewel sweet potatoes were inoculated with agar plugs from actively growing *F. solani*. These commodities were incubated at room temperature.

Storage Rot Examination

The fruits were taken out periodically for rot examination. Rotted fruits were kept separately until the end of the experiment. The causal organisms were isolated with appropriate media and identified (Snowdon, 1990).

Phenylalanine Ammonia-Lyase Extraction and Assay

Following the procedure of Koukol and Conn (1961), the sweet potato and apple disks and peels from grapefruits and tangerines were homogenized in cold 0.05 M sodium borate, pH 8.5 buffer. The homogenate was squeezed through cheese cloth and centrifuged at 14,500 x g for 30 minutes. The crude extract was assayed within a short period after homogenation.

The assay of phenyalanine ammonia-lyase activity (PAL) was based on determining the amount of cinnamic acid formed. The reaction mixture consisted of 40 mmoles of L-phenyalanine, 200 mmoles of sodium borate buffer, pH 8.8 and the enzyme solution in total volume of 5 ml. The reaction was arrested by the addition of 0.1 ml of 6N HCl. The acidified mixture was extracted with ethyl ether. The residue was dissolved in 0.05 M sodium hydroxide and the absorbance at 268 nm was measured.

Results and Discussion

The onions irradiated with 1.3 to 19.6 x 10^4ergs/mm^2 of UV-C exhibited the most noticeable reduction in postharvest rots and improvement in the marketable and storage life of 'Walla Walla' onions (data not shown). An examination of the spoiled onions showed that black mold (*Aspergillus niger*) and bacterial soft rot (*Erwinia* spp.) were the dominant pathogens. After 40 days, 25% of UV-C treated (7.5 x 10^4erg/mm^2) and 100% of the control onions rotted, respectively. However, the disease severity index was very low for the UV-C treated onions. The severity index was 2.5 and 0.2 for non-treated and UV-C treated onions, respectively (Table 1).

There was a hormetic relationship of the microbial load (propaglues forming unit/g tissue) of *A. niger* and the UV-C dosages. The optimum UV-C level of 3.6 x 10^4ergs/mm^2 suppressed *A. niger* by 777 times that of the microbial load of the control 34 days following the UV-C treatment (Figure 1).

Sweet Potato   UV-C treatment of sweet potato reduced the naturally occurring Fusarium (*Fusarium solani*) root rot and Rhizopus rot (*Rhizopus stolonifer*) that developed during storage in three different varieties (Table 2, Fig. 2). The effect was maintained throughout a storage period of 3 months, although in some varieties it gradually diminished with time. Composition of the root tissue was not affected by the treatment, except for a slight reduction in hydrolysis of starch to sugar and increased percent dry matter (Table 3).

'Jewel' sweet potatoes artificially inoculated with *Fusarium solani* following UV-C treatment showed a more significant reduction in lesion diameter and weight of rotted tissue than the control (data not shown). About 55 and 11% of inoculations failed to develop lesions in wounded UV-C treated and control storage roots, respectively, 10 days after inoculations (data not shown).

Apple   The results in two separate tests (Tables 4A and B) indicated that UV-C was effective in reducing the incidence of naturally infected total storage rots of 'Golden Delicious' apple. An intermediate UV-C dose of 7.5×10^4 ergs/mm^2 was the most effective in reducing total incidence of rots. Three types of rots identified were brown rot (*Monilinia* spp.), Alternaria rot (*Alternaria* spp.) and bacteria soft rot (*Erwinia* spp.). The prevalent rot was Alternaria rot. The optimum UV-C level which gave the best control of Alternaria rot was 4.8×10^4 ergs/mm^2. Lesion diameter of storage rots were significantly reduced by the UV-C light (Table 4A).

Exposure of apples to certain hormetic UV-C levels promoted increases in ascorbic acid content. A close inverse correlation between higher ascorbic acid content and increase resistance of Alternaria rot of apples occurred (data not shown).

Peaches   Brown rot of 'Loring' and 'Elberta' peaches was similarly reduced by exposure of the fruits to low dose UV-C treatment (Tables 5 and 6). Reduction of the incidence of rot and lesion diameter was evident (Table 5). As illustrated by the data in Table 6, UV-C treated peaches increased in firmness (texture) and reduced the percent soluble solids content.

Citrus   The application of UV-C was effective in controlling green mold rot (*Penicillium digitatum*), stem end rot (*Alternaria citri*), as well as sour rot (*Geotrichum candidum*) of 'Dancy' tangerines 17 days after irradiation. The optimum dosage levels for controlling sour rot, stem end rot were 0.84×10^4 to 20×10^4 ergs/mm^2. However, for green mold rot there were three UV-C optimum levels, showing almost a sinuate dose response at 1.3, 4.8, and 20×10^4 ergs/mm^2, while the 20×10^4 ergs/mm^2 treatments gave the best control (Table 7). The ascorbic acid content of UV-C irradiated tangerines were correlated with green mold incidence (data not shown).

'Marsh Seedless' grapefruits artificially inoculated with the green mold fungus following UV-C treatment, exhibited increased resistance to the diseases (Figure 4), maintaining the effect for several days. While the percentage of inoculated wounds slightly increased when the fruit was wounded and inoculated 72 hours after treatment, compared with shorter periods, the effect of the treatment in reducing rot diameter was still maintained when wounding and inoculation occurred 72 hours after UV-C treatment (Figure 4). The response of grapefruits to different UV-C treatments is shown in Table 8. As with the other commodities tested, an intermediate level of UV-C was most effective in reducing the infection. It should be noted that grapefruits treated early in the season, at the relatively high (and excessive) UV-C levels of 7.5 x 10^4ergs/mm^2 or higher, developed peel blemishes as a result of the treatment. However, as the season progressed, the fruit peel gradually became more resistant to the treatment and did not develop peel damage at high UV-C levels (data not shown).

An additional observation worth noting was the peculiar pattern of the decay development in UV-C treated fruits. On non-treated fruits, the fungus normally develops in both peel and pulp from the site of inoculation to other parts of the fruit, with abundant sporulation of the older mycelium on the surface of the fruit. In UV-C treated fruits, fungal development was mainly in the pulp, with less manifestation on the peel and with marked inhibition of sporulation.

In citrus peel tissue, UV-C treatment induced changes in protein patterns. Such changes were more readily detected in the membrane proteins than in the soluble proteins (data not shown). Further work is needed to determine additional changes, the identity of the proteins that undergo these changes, and their possible relevance to the phenomenon of UV-C induced resistance.

PAL Activity

Exposure of 'Jewel' sweetpotato, 'Golden Delicious' apples, 'Dancy' tangerines (Table 9) and 'Marsh Seedless' grapefruit (Figure 5) to certain hormetic UV-C levels promoted increase in PAL activity. In grapefruit peel (Figure 5), PAL activity increased within 24 hours after the treatment and remained high at 48 hours. In apples, sweetpotatoes and tangerine tissues, elevated PAL activity was detected after much longer periods following treatment (Table 10).

The application of UV-C light reduced development of storage rots in vegetables and fruits. Two possible explanations by which storage rots could conceivable be reduced in these UV-C irradiated commodities are the germicial effect on pathogen propagules on the surface of these commodities and induced resistance.

Sterilization or Induced Resistance?

UV-C sterilization of fruits and vegetables appeared attractive as a mean for reducing inoculum levels of postharvest storage rot pathogens. It is known that 1.1 x 10^4ergs/mm^2 of UV-C is sufficient to reduce the viability of R. *stolonifer in vitro* (Moy, 1983). However, since UV-C is not an ionizing radiation and does not penetrate plant tissue very deep (Luckey, 1980) the possibility of incipient infection resulting from

harvesting and postharvest handling indicate that the germicidal effect cannot totally explain the reduction in postharvest storage rots.

The rate of decay development around *F. solani* inoculum plugs on UV-C irradiated sweet potato roots progressed slowly as compared to nonirradiated roots. The reduction of *A. niger* PFU/g tissue in onion previously treated with UV-C and the inhibition of sporulation on *Penicillium digatatum* in grapefruit suggests that the UV-C treatment of these commodities induced resistance to postharvest pathogens. These observations suggest the presence of inhibitory substances in the tissues of these commodities, the biosynthesis of which, may be induced by UV-C.

Since the effect of UV-C hormesis on controlling storage rot development appeared to be a host response rather than a target effect on pathogens and is dose rate dependent (Luckey, 1980), suggests the possible presence of secondary biochemical products in which a relatively long-lived intermediary product may be active. Induction of PAL activity seems to be a common response in all treated tissues and may be related to the induced resistance phenomenon. Grapevines (Langcare and Pryce, 1977), pea (Hadwiger and Schwochau, 1989) and soybean seedlings (Bridge and Klarman) treated with UV-C radiation induced the formations of large quantities of phytoalexins.

Delayed Ripening

Also, results appeared to indicate that the ripening process of peaches has been delayed which might contribute to the increased resistance of the fruit. Hormetic UV-C light also increased ascorbic acid in fruit commodities. Ascorbic acid accumulates in resistance plants and suppresses disease symptom development in many host pathogen interactions (Vidhyasekarano, 1989). This may also explain the increased resistant to fruits.

Further work is needed to determine if phytoalexins, ethylene production or increased lignification in plant tissue in response to UV-C hormesis are involved in the increase in resistance of these commodities to postharvest diseases.

Advantage of UV Treatment

There are several advantages in using UV-C radiation to control postharvest storage rots in these commodities which include: a) no radioactivity or toxic accumulation of chemical residues; b) unlike gamma ray, UV-C does not cause softening of storage roots (Lu et al., 1987); c) UV-C is easier and safer to operate than ionizing radiation such as gamma rays and d) UV-C at selective low hormetic doses does not stimulate root rots.

Once fully elucidated, this promising new technology could be developed for use as a safe and effective method for the control of postharvest diseases and for extension of the shelf-life of fresh fruits and vegetables.

Acknowledgment

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Table 1. The effect of low-dose UV-C light on the disease incidence and severity index of naturally infected storage rots of 'Walla Walla' onions (1985).

Days after harvest	% Onions infected		Disease severity index	
	UV-C	control	UV-C	control
11	0	28	0	1.20
LSD 5%	.43	--	.04	--
26	24	78	0.1	1.30
LSD 5%	.86	--	.04	--
40	24	100	0.2	2.50
LSD 5%	.70	--	.09	--

Disease severity scale of 0 to 4; 0 = no infection, 1 = 0.5 to 25% infected, 2 = 25.5 to 50% infected, 3 = 50.5 to 75% infected and 4 = greater than 75% infected.

The UV-C dose was 7.5×10^4 ergs/mm^2 for 'Walla Walla' onions.

Table 2. Effect of low-dose UV-C treatment of decay
development in wounded sweetpotatoes stored at 18°C.

Cultivar/	Storage time (months)		
Treatment	1	2	3
	Incidence of rots (%)[*]		
'Jewel'			
Control	32a	88a	96a
UV	11b	19b	33b
'Carver'			
Control	28a	72a	88a
UV	9b	27b	36b
'Georgia Jet'			
Control	74a	91a	97a
UV	17b	42b	74ab

[*]Decay consisted mostly of Fusarium root rot and Rhizopus rot.

The UV-C dose at 254nm was 4.8×10^4 ergs/mm^2 for 'Jewel' and
'Carver' and 3.6×10^4 ergs/mm^2 for 'Georgia Jet.'

Means of each cultivar followed by the same letter in each
column do not differ significantly (P = 0.05) as determined by
Duncan's Multiple Range Test.

Table 3. Nutrient composition of UV-C treated 'Jewel' sweet
potatoes after two months of storage at 18°C.

Composition[*]	Unit	Non-treated	UV-C treated
Moisture	%	75.9a	75.1a
Protein	%	6.4a	6.8a
Fat	%	0.7a	0.6a
Ash	%	4.2a	4.5a
Starch	%	56.7a	63.5b
Ascorbic Acid	mg/100g	52.7a	51.9a
Thiamin	mg/100g	0.48a	0.44a
Riboflavin	mg/100g	0.24a	0.28a
Total carotenoids	mg/100g	62.90a	64.50a

[*]Expressed on dry weight basis. Mean of ten roots.

The UV-C dose at 254 nm was $4.8 \times 10^4 ergs/mm^2$.

Means of each nutrient followed by the same letter in each row
do not differ significantly at the 5% level.

Table 4A. Effect of UV-C treatments on the incidence of naturally infected storage rots of 'Golden Delicious' apples from Kearneysville, WV after 30 days storage at 24°C.

UV level $10^4/mm^2$	Total Incidence of rots (%)	Naturally infected storage rots					
		Brown rot		Alternaria rot		Bacterial soft rot	
		Incidence %	Lesion size(cm)	Incidence %	Lesion size(cm)	Incidence %	Lesion size(cm)
0	53.3	12.3	1.9	35.5	3.0	12.3	0.60
0.84	41.0	9.5	1.0	27.7	3.2	13.7	2.30
2.40	35.0	9.5	1.7	24.6	2.8	4.8	1.20
3.60	28.0	4.8	0.4	14.3	0.7	4.8	1.00
4.80	14.3	4.8	0.5	4.8	0.5	4.8	0.50
7.50	9.5	0	0	9.5	0.2	0	0
20.00	15.1	4.8	0.5	9.5	0.2	0	0
27.00	14.3	0	0	14.3	1.0	0	0
40.00	24.6	0	0	24.6	1.4	0	0
LSD 5%	3.4	6.3	1.1	3.4	0.9	6.4	1.31

Table 4B. Effect of low dose UV-C treatments of naturally infected storage rots of 'Golden Delicious' apples after 30 days storage at 24°C.

UV level (10^4 erg/mm^2)	Total incidence of rots %
0	85.7
1.9	42.9
3.6	57.1
7.5	14.2
11.0	28.6
LSD 5%	?

Table 5. Effect of low-dose UV-C treatments on brown rot development in 'Loring' peaches stored for 10 days at 12 ± 5 C.

UV-C levels 10^4erg/mm^2	Incidence of rot (%)	Lesion diameter (cm)
0	74	9.0
0.8	67	5.4
1.3	47	2.7
2.4	39	2.6
3.6	36	2.7
4.8	33	1.7
7.5	33	0.8
20.0	21	0.9
LSD 5%	0.23	0.03

Table 6. Effect of low-dose UV-C treatments on brown rot
development and ripening parameters of 'Elberta' peaches
stored for 20 days at 12±5°C.

UV-C levels 10^4erg/mm^2	Incidence of rots (%)	Total soluble solids (%)	Firmness (kg)
0	40	12.4	0.58
0.8	35	11.3	0.58
1.3	20	1.0	0.59
3.6	21	11.0	0.59
4.8	7	11.0	0.82
7.5	7	9.8	1.08
20.0	6	9.8	1.27
40.0	15	10.4	1.41
LSD 5%	0.23	0.81	0.20

Table 7. Effect of UV-C on the incidence of postharvest rots of 'Dancy' tangerines 17 days after treatment.

UV level 10^4erg/mm^2	Green[z] mold	Sour[y] rot	Stem and rot *Alternaria* spp.	Total rots
	Percent of naturally infected fruits in storage			
0	34.70	19.30	57.00	65.70
0.84	30.00	6.70	10.00	31.70
1.30	15.70	9.50	12.70	28.60
2.40	31.60	11.70	10.00	53.00
3.60	39.00	15.30	13.20	47.00
4.80	22.00	10.00	13.00	35.00
7.50	47.00	15.70	17.60	80.00
20.00	11.70	13.30	8.30	30.00
LSD 5%	3.50	2.17	0.78	2.31

[z]*Pencillium digitatum*

[y]*Geotrichum candidum*

'Dancy' tangerines were obtained from E. H. Whitmore Foundation Farm USDA/AR, Clermont FL.

Table 8. Effect of low-dose UV-C treatments on green mold
development in artificially infected 'Marsh Seedless'
grapefruits.

UV-C level 10^4erg/mm^2	Infection incidence[*] (%)
0	88
0.6	27
0.9	33
2.2	22
3.6	27
7.5	50
5.0	55
LSD 5%	

[*]Inoculations were done 24 hours after treatment and incidence
of infections after 5 days of incubation at 22°C.

Table 9. Induction of phenylalanine ammonia-lyase (PAL)
activity by UV-C treatment in 'Jewel' sweet potato, tangerine
and 'Golden Delicious' apple.

| | PAL activity (mmoles cinnamic acid/gDW/h) | | |
	Sweet potato	Tangerine	Apple
Control	15.9	44.0	3.8
UV treated (4.8×10^4ergs/mm^2)	38.0	95.0	7.0
LSD 5%	6.9	27.0	1.0

Assays were performed for sweet potato, tangerine and apple
88, 18 and 30 days, respectively, after the UV treatment.
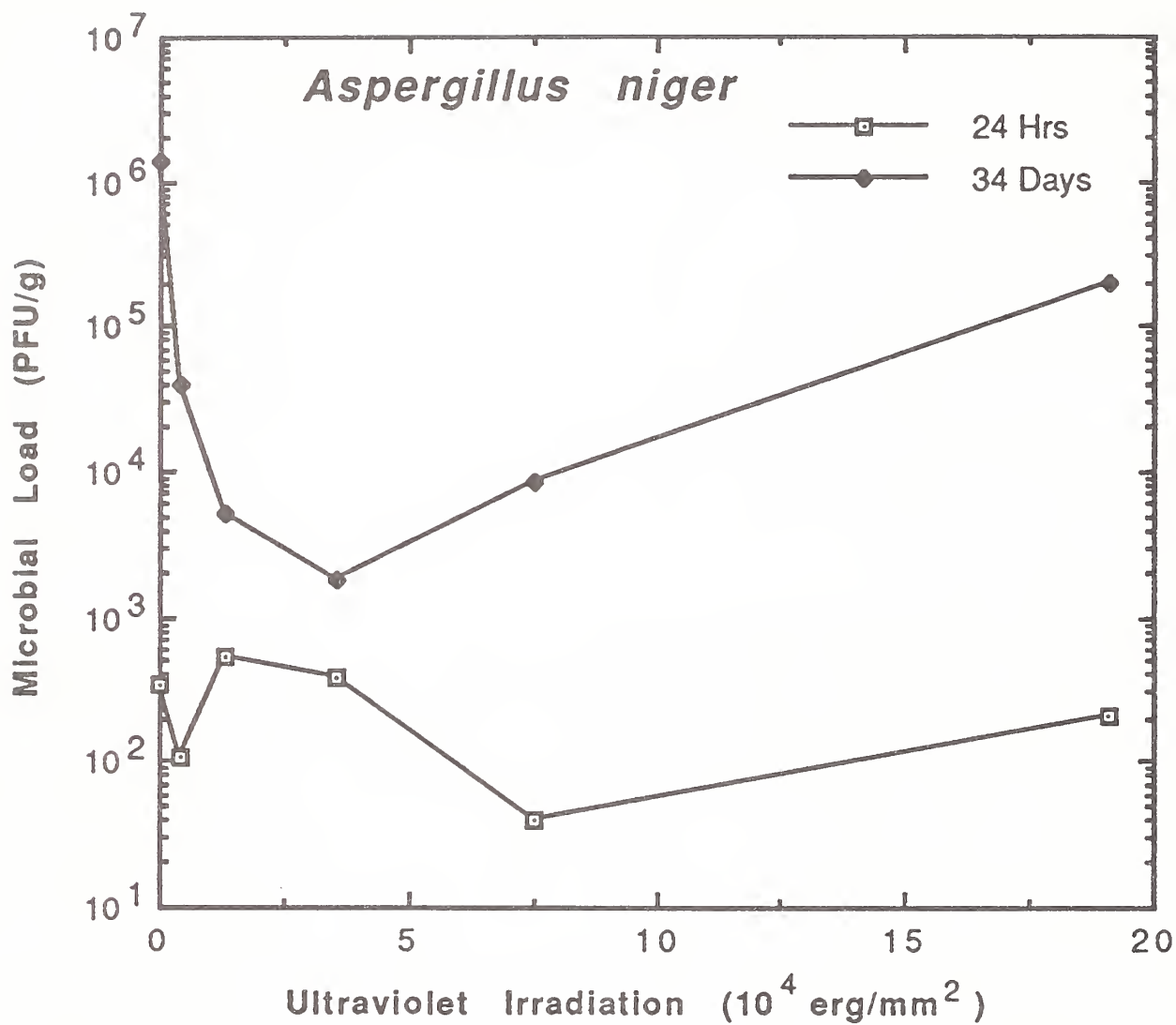
FIG. 1. Relationship between UV-C doses and microbial load
(PFU/g onion tissue) of *Aspergillus niger* occurring in 'Walla
Walla' onions.
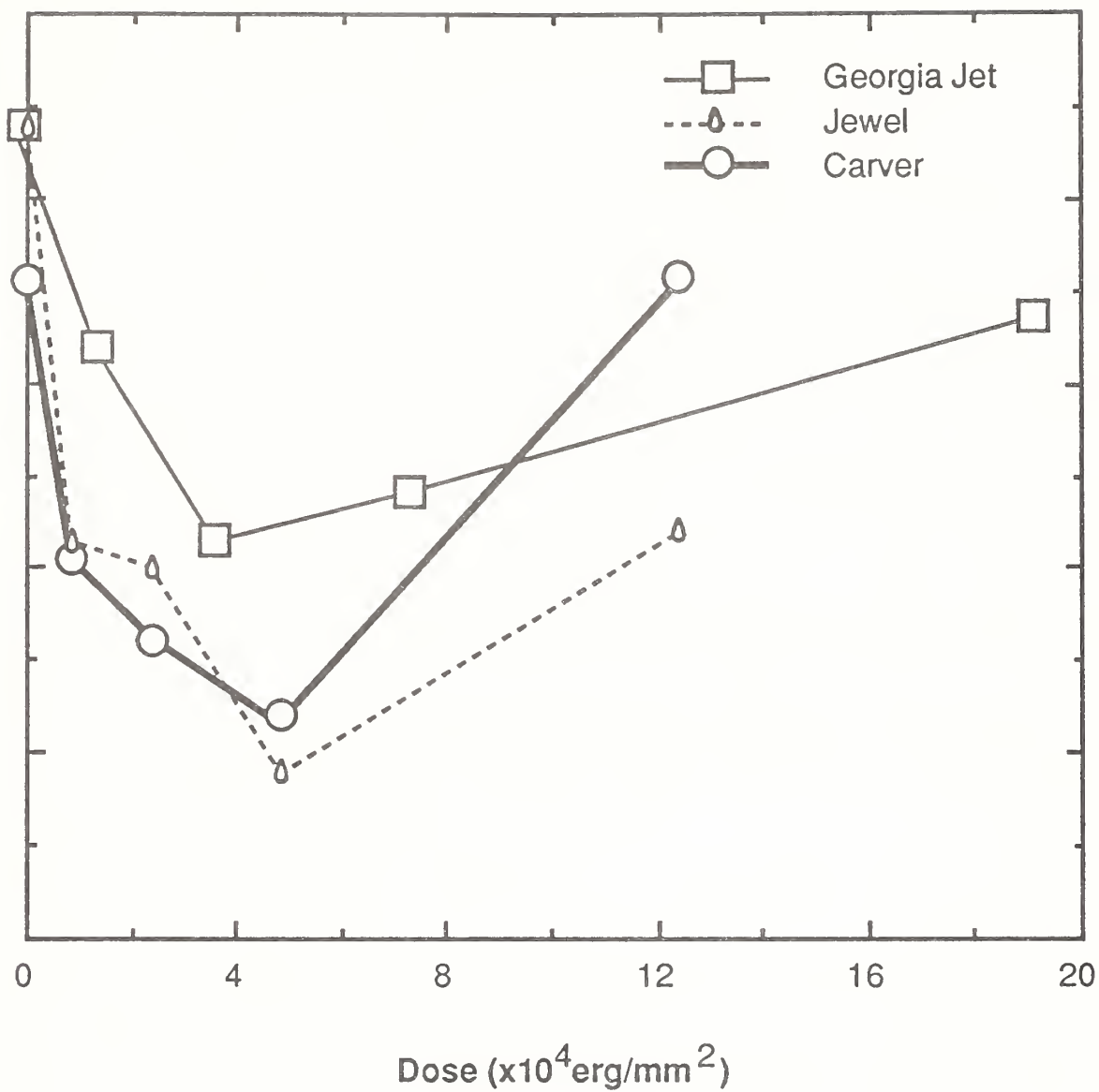
FIG. 2. Relationship between UV-C dosages and percent of
'Jewel' storage roots with naturally occurring sweetpotatoes
storage rots.
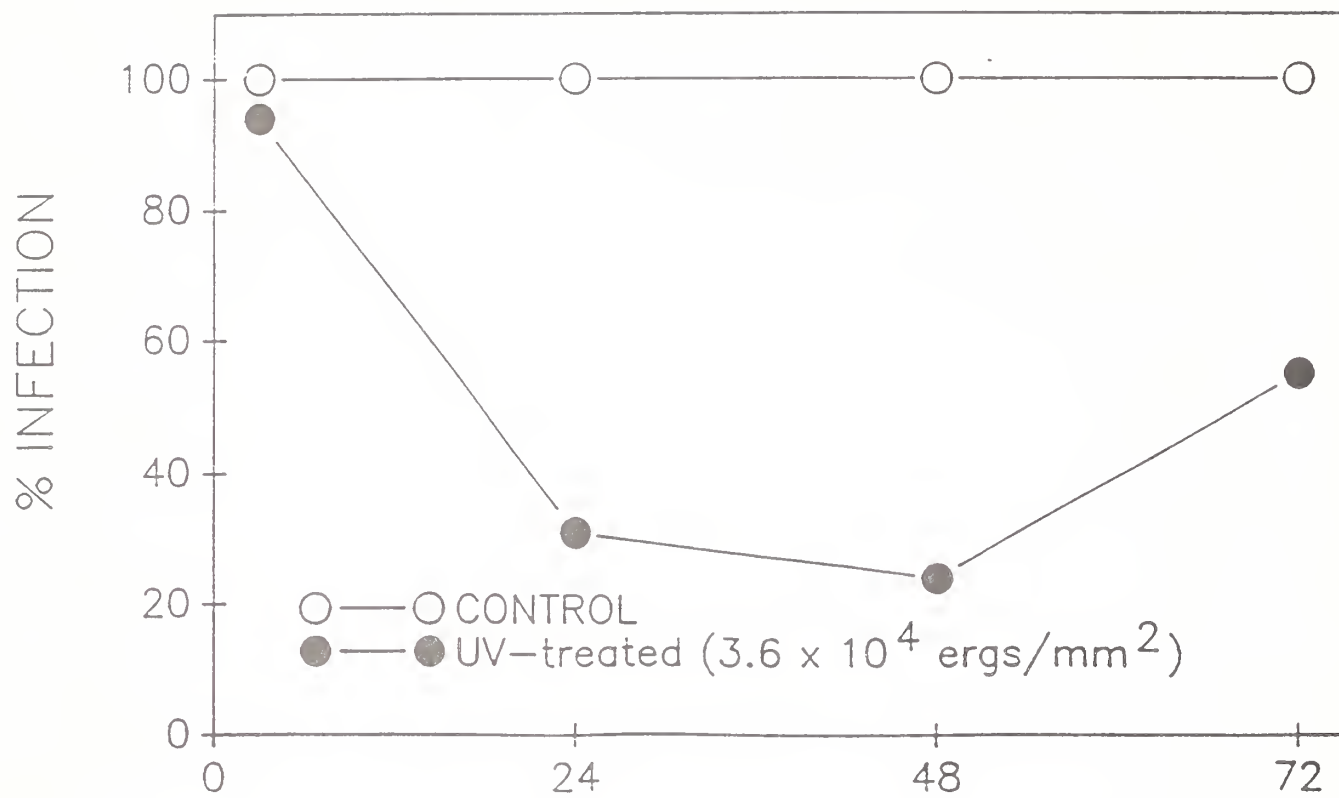
FIG. 3.   Induction of resistance in 'Marsh Seedless'
grapefruit to infection by the green mold fungus by low dose
UVC-treatment.
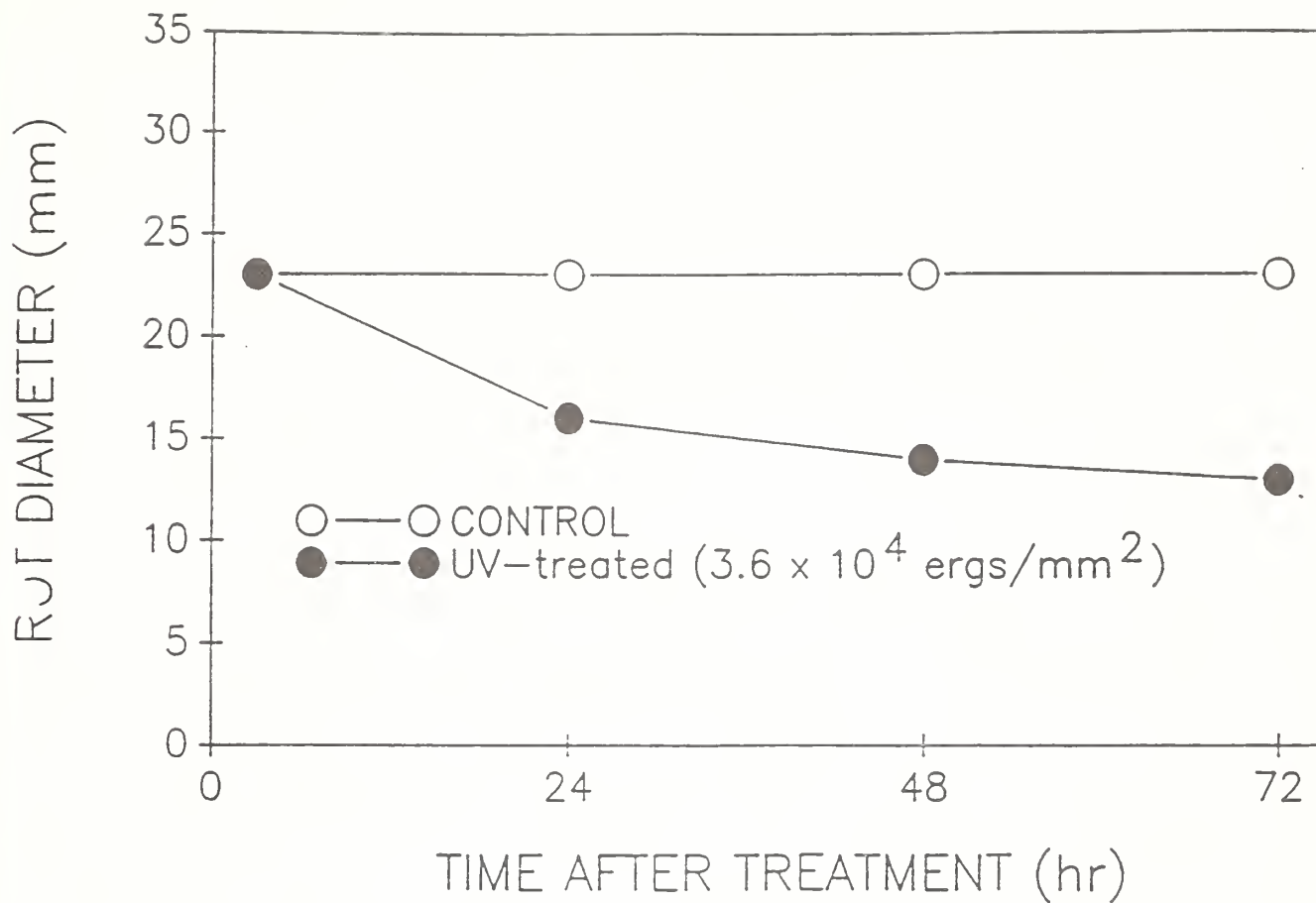
FIG. 4. Induction of resistance in 'Marsh Seedless' grapefruit to infection by the green mold fungus by low dose UV treatment.
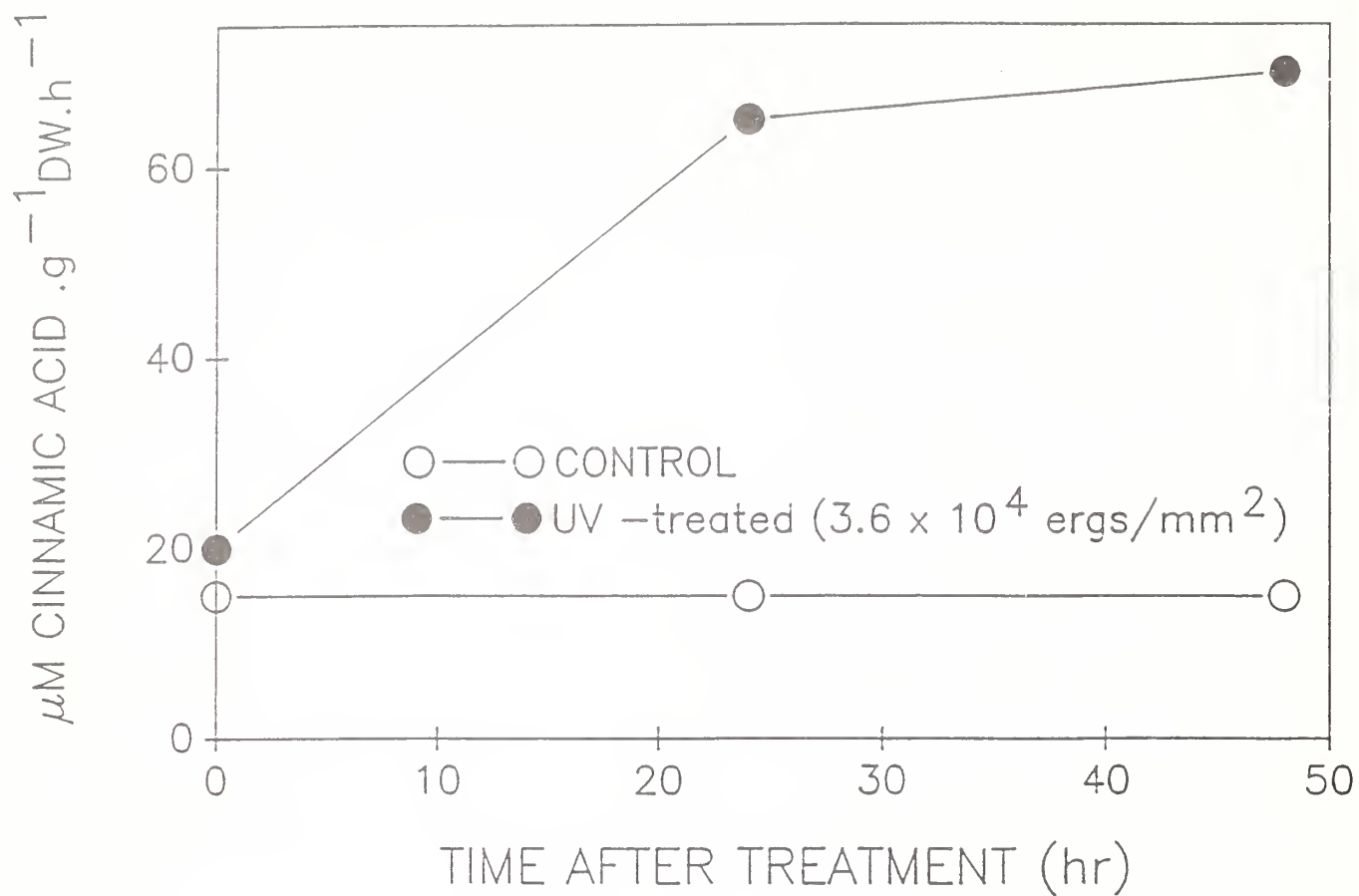
FIG. 5. Induction of PAL activity in 'Marsh Seedless'
grapefruit peel tissue following low dose UV-C treatment.

Natural Plant Compounds as Pesticides

J. A. Duke[1]

Abstract

Having constructed a database of 3000 bioactive compounds, I
estimated that 2000 of them already had pesticidal activities
reported. Rather than cover 2000 compounds in 20 minutes. I
will discuss some pesticidal plants growing in West Virginia,
emphasizing pennyroyal and mountain mint, and emphasizing
acaricidal and molluscicidal activity.

Introduction

For several years now, I have been constructing two databases,
in collaboration with the University of Maryland, from data in
the open literature. One database, the Father Nature's Farmacy
or FNF database, lists all the Generally Recognized as Safe
(GRAS) and many Generally Recognized as Food (GRAF) higher
plant species. For each species I have compiled the list of
compounds reported for that species, and, where available, the
quantities in parts per million (ppm), with a cryptic
indication of the source(s) of the data. The other,
Biologically Active Compounds or BAC database, lists the
biological activities for the compounds, and, where available,
the Effective Concentrations (EC's), Inhibitory Concentrations
(IC's), for various activities and occasionally the lethal
doses (LD-50's), again with cryptic references to the sources
for the data.

Methods and Results

Now, the FNF database treats about 600 species in about one
megabyte of a WordPerfect database. The BAC database treats
3000 compounds in about 150,000 bytes. In preparation for the
workshop, I combed the BAC database asterisking entries that
could be deemed pesticidal, e.g. antiseptic, bacteristat,
molluscicide, etc., but not necessarily flagging those
compounds termed cytotoxic in National Cancer Institute (NCI)
reports. About two-thirds of the entries now bear the asterisk
and can be moved to a strictly pesticidal file.

Discussion

Pesticidal Species I illustrate with color photos many pesticidal species that
grow or can be grown in West Virginia. For many, if not all of
these, there is a listing of the phytochemical constituents in
the FNF database. Here, I will tabulate only the entries for
pennyroyal (*Hedeoma pulegioides*) and mountain mint
(Pycnanthemum muticum) two aromatic West Virginia weeds that
may prove useful in our efforts to repel ticks. Although the
malodorous garlic (*Allium sativum*) has been reported to repel
both the dog-tick and the deertick genera, respectively the
carriers of Rocky Mountain Spotted Fever and Lyme Disease the
pennyroyal was not mentioned as a tick repellent by Grainge and

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Ahmed (1988). Garlic smells bad too and repels some people as well as some ticks. Pennyroyal smells good, rather like corn mint (*Mentha arvensis*), which also repels ticks and which contains up to 2500 ppm pulegone, one of the dominant aroma compounds reported in pennyroyal (See Table 1 below).

Pennyroyal

Numbers indicate the quantity of the individual compounds in ppm's. Ranges were often calculated by multiplying low percentages for essential oil by the lowest report of the percentage of the individual compound and the highest by the highest. Note that the highest pulegone figure is 27,600 ppm (equals up to 2.76% of the biomass). Pennyroyal is not a bulky plant, but it is the most frequently used natural insect repellent in rural Pendleton Co., W. Virginia, at least among my acquaintances there. Often growing in the same forest is the much bulkier mountain mint, whose chemical composition is tabulated below in Table 2.

A more advanced printout from the database shows not only the ppm's of most chemicals but the plant parts (SH=Shoots) and the source (BML=Lawrence, 1981). Note that mountain mint, which probably produces ten times the biomass of pennyroyal, also has a higher percentage of pulegone. Unlike the ephemeral annual, pennyroyal, mountain mint is a prolific perennial. I estimate that, under management, mountain mint could produce a conservative five MT biomass containing 1-4% pulegone, or 50-200 kilograms pulegone per hectars.

While it is difficult for me to believe that any of the named compounds are inert, I do not have reported activities for all of them in the BAC file. Some of the BAC's in various pennyroyal species are tabulated below. (SEE TABLE 3).

The asterisks, as mentioned, indicate that the compound has some pesticidal activity. In this case, only 2 of the 14 bioactives in pennyroyal did not have reported pesticidal activity. The numbers preceding the biological activities indicate the possible concentration of the compound in ppm's.

Table 1 and 2 are derived from the FNF database and may be searched mechanically. Table 3 is derived from the BAC database, which may also be searched mechanically.

Because of recent USDA interest, I also searched the BAC database for molluscicides. The results are shown in Table 4 below.

Molluscicides

Of the estimated 2000 pesticides in the BAC database, fewer than 2% were reported as molluscicides. The database can be searched mechanically for any keyword therein, and is proving very useful at addressing questions posed by our taxpaying public as well as by our USDA peers and superiors.

References

Grainge, M. and Ahmed, S. 1988. Handbook of Plants with Pesti-Control Properties. John Wiley & Sons, New York. 470 pp.

Lawrence, M.M. 1981. Essential Oils 1979-1980. Allured Publishing Corporation, Wheaton, ILL. 291 pp.

Table 1. Compounds Reported From American Pennyroyal.

ACETIC-ACID
BUTRIC-ACID
CARYOPHLLERNE 30-150
1, 8-CINEOLA 6-30
DECYLIN-ACID
DIOSMIN 10,000
DIPENTENE
EO 6,000-30,000
TRANS-BETA-FARNESENE TR
FORMIC-ACID
GERMACRENE-D 36-180
ALPHA-HUMULENE 30-150
ISOHEPTYLIC ACID
ISOMENTHONE 48-9, 300
LIMONENE 36-570
LINALYL-ACETATE 36-180
MENTHOFURAN 1
MENTHOL 6-30
(-) -MENTHONE 36-420
METHYLCYCLOHEXANONE
METHYL-SALICYLATE
MYRCENE 30-150
3-OCTANOL 18-90
1-OCTEN-3-OL 48-240
3-OCTYL-ACETATE 18-90
OCTYLIC-ACID
ALPHA-PINENE 24-210
BETA-PINENE 12-180
PIPERITONE 12-270
PIPERITENONE 1-1, 410
PULEGONE 3,678-27,600
SABINENE 18-90
SALINENE-ACID
TANNIN

Table 2. Compounds Reported from Mountain Mint.

TRANS-ALPHA-BERGAMOTENE 29,200 BML
BORNEOL 58-1, 350 SH BML
CAMPHENE 1-50 SH BML
CARYOPHLLENE 29-300 SH BML
1, 8-CINEOLE 1-50 SH BML
p-CYMENE SH BML
EO 29,000-50,000 SH BML
GERMACRENE-D 1-350 SH BML
ISOMENTHONE 29-2, 100 SH BML
CIS-ISOPULEGONE 29,750 SH BML
TRANS ISOPULEGONE 116,200 SH BML
LIMONENE 870900 SH BML
MENTHOL 87-450 SH BML
D-MENTHOL 4,350-7,500 SH BML
MENTHONE 2,320-11,300 SH BML
D-MENTHONE 2,320-4,000 SH BML
MYRCENE 29-200 SH BML
NEOISOMENTHOL 1-50 SH BML
NEOMENTHOL 29-100 SH BML
CIS-OCIMENE 1-50 SH BML
1-OCTEN-3-OL 29,900 SH BML
ALPHA-PINENE 29-200 SH BML
BETA-PINENE 29-150 SH BML
PIPERITONE 1-200 SH BML
PIPERITONENE 27-950 SH BML
TRANS-PIPERITOL 1-100 SH BML
PULRGONE 17,980-40,250 SH BML
SABINENE-50 SH BML

Table 3. Bioactive Compounds in *Hedeoma Pulegioides*.

*ACETIC-ACID: Antivaginitic; Bactericide (5,000 ppm); Osteolytic; Verrucolytic; LD50=3,310 (orl rat).

*CARYOPHYLLENE: 30-150 ppm. Insectifuge; Spasmolytic; Termitifuge.

*1,8-CINEOLE: 6-30 ppm. Anesthetic; Antibronchitic; Antilaryngitic; Antipharyngitic; Antirhinitic; Antiseptic; Antitussive; Choleretic; CNS-stimulant; Hepatotonic; Herbicide (IC50=3-180mM); Hypotensive; Insectifuge; LD50=2,480 (orl rat).

DIOSMIN: 10,000 ppm. Anti-capillary-fragility; Antihemorrhoidal; Antimetrorrhagic.

*FORMIC-ACID: Antiseptic; Antisyncopic; Astringent; Counterirritant. LD50=1,100 (orl mus). LD50-1,210 (orl rat).

*GERMACRENE-D: 36-180 ppm. Pheromonal.

*LIMONENE: 36-570 ppm. Anticancer; Cancer-preventive; insecticide; Insect-Repellant. LD50-4,600 (orl rat).

*MENTHOL: 6-30 ppm. Anesthetic; Antinflammatory; Antipruritic; CNS-depressant; Counter-irritant; Rubefacient; Vibriocide. LD50=3,180 (orl rat).

METHYL-SALICYLATE: Analgesic; Anti-inflammatory; Antipyretic; Antirheumatic. LD10=170 (orl hmn); LD50=887 (orl rat); LD50=1,110 (orl mus).

*MYRCENE: 30-150 ppm. Bactericide; Insectifuge; Spasmolytic.

*PINENE: 12,390 ppm. Antiseptic; Expectorant; Herbicide (IC50=30 mM): Insect-Repellant.

*PIPERITONE: 12-270 ppm. Antiasthmatic; Herbicide (IC50=30mM).

*PULEGONE: 3,678-27,600 PPM. Antipyretic; Avifuge; Herbicide (IC50=1.5 mM): Insecticide; Pulifugfe. LD50=150 (ipr mus).

SALICYLIC-ACID: Analgesic; Antidermatotic; Antieczemic; Antineuralgic; Antiperiodic; Antipodagric; Antipsoriac; Antipyretic; Antirheumatic; Antiseborrheic; Bactericide; Febrifuge; Fungicide; Keratolytic; Tineacide; Ucerogenic. LD10=450 (orl dog). LD50=891 (orl rat).

Table 4. Phytomolluscicides in BAC Database.

*ALLODESACETYLCONFERTIFLORIN Molluscicide (JNP 49: 133. 1986)

*ANACARDIC-ACID1 Antitumor; Bactericide; Molluscicide (<10 ppm);
Nematicide; Schistosomicide

*BALANITIN: Antifeedant; Antiseptic; Molluscicide (JNP 45:23.
1982)

BAYOGENIN (glycoside): Fungicide (7.5-25 ppm) 438/; Molluscicide
(7.5-25 ppm) 438/;

*BERGAPTEN: Antiapertif; Anticonvulsant; Antihistaminic; Anti-
inflammatory, Antipsoriac; Antitumor; Hypotensive; Insecticide
(FT: 1984): Molluscicide (FT: 1984); Spasmolytic;

*CANTALASAPONIN-2: Molluscicide; Schistosomocide

*CARDANOL: Molluscicide (ED=80>100)

*CARDOL Molluscicide (ED=7-15 ppm)

*CHALEPENSIN: Molluscicide

*CONFERTIFLORIN: Molluscicide (JNP 49: 133. 1986)

*CYTISINE: Antiinflammatory; Molluscicide; Psychoactive;
LD50=101 (orl mus)

*DAMNACATHIN: Molluscicide (10 ppm)

*DODONOSIDE: Molluscicide 438/

*HEDERAGENIN (glycoside): Fungicide (7.5-25 ppm) 438/;
Molluscicide (7.5-25 ppm) 438/;

*IMPERATORIN; Anticonvulsant; Antiinflammatory; Antileukodermic;
Hepatotoxic; Molluscicide; Antivitiligic; LD10=600 (par mus)

*ISOPIMPINELLIN: Antiappetant; Antifeedant 382/; Anti-
inflammatory (100 ppm); Diuretic (125 mg/kg); Insecticide;
Molluscicide; Mutagenic (FT 84)

*ISOTENULIN: Molluscicide

*LEMMATOXIN: Antifertility; Helicicide (LD90=1.5 mg/L:
Molluscicide (LD90=1.5 mg/L); Spermicide (JNP39: 424);

*LUPANINE: Hypoglycemic; Molluscicide (JNP 39: 444. 1976)

*MEDICAGENIC-ACID: Aphicide; Candidicide; Fungicide (7.5-25 ppm
438/;Molluscicide (7.5-25 ppm) 438/:

*2-METHYLANTHRAQUINONE; Antifeedant 438/; Molluscicide (10 ppm) (T35: 1985)

*7-METHYLJUGLONE: ALLELOCHEMIC (IC95-8 mM) 438/; ungicide; Molluscicide (5
PPM) (50: 279. 1984); =ermiticide 382/

*MUKAA IAL: Molluscicide 382/

*NAPTHAQUINONE: Antifeedant 438/; Fungicide; Molluscicide; Rubefacient

*OLEANOLIC-ACI 3-0GLUCOSI E: Molluscicide

*ORUWACIN: Molluscicide (1-3 ppm)

*ORUWAS: Molluscicide (10 ppm)

*PHEBALOSIN: Antitumor; Crustacicide (47 ppm); Fungicide; Molluscicide

*POLYGO IAL: Antifeedant 450/; Candidicide 452/; Helicicide; Molluscicide 382/; Piscicide

*PRIMULIC-ACI : Molluscicide

*SOLAMARGINE: Fungicide; Molluscicide

*SORAN I IOL-alpha-ACETATE: Molluscicide (10 ppm) (T#5: 1984)

*SPARTEINE: Antiinflammatory; Cathartic; Diuretic; Hypoglycemic (JNP39:444); Molluscicide; Oxytocic; L 10-30 (ivn rbt)

*TOMATINE: Antiinflammatory: Bactericide; Fungicide; Molluscicide; L 10=800 (orl rat)

*WARBURGANAL: Antifeedant (0.1-10) 382/; Candidicide (3-12) (JNP 45:22.1982); Cytotixic (10 ppb) 382/; Fungicide (3-100) 382/; Helicicide; Molluscicide (5-10) 382/; Piscicide; (JNP 45 & 50).

*XANTHOTOXIN: Antifeedant 382/; Antihistaminic; Antiinflammatory (20 mg/man/day); Antipsoriac; Antispasmodic; Antivitiligic (20 mg/man/day); Herbicide 438/; Insecticide 382/; Molluscicide; Spasmolytic (CRC).

(Users interested in a list of abbraviations may request the code to N)

What's Happening with Natural Compounds[1]

J. A. Duke[2]

Abstract

(QUOTE)   ...Many botanists, farmers, and gardeners have observed and
suggested many cases of allelopathy for over 2000
years...Controlled scientific experiments on this phenomenon
were not conducted until after the year 1900...Most of the
species suggested to have pronounced chemical effects on
themselves or other species have many demonstrated
subsequently to have such effects...Many, widely used in
medicine and...known to have powerful medicinal effects, have
pronounced allelopathic effects also. (Rice, 1984).

With that introductory comment from Rice (1984), Dean of
Allelopathy. I hope to justify my lingering interest in
medicinal plants, even though my USDA affiliation with
medicinal plants was terminated in 1982. At that time the
National Cancer Institute (NCI), with nearly 30 years and at
least that many million dollars down the drain, curbed their
plant screening program. After all this time and money, there
still wasn't a drug on the market that could point its finger
to the program. Worse, the major plant-derived anticancer
compounds at that time came from a plant, *Catharanthus roseus*,
that I'm told would not have tripped their cancer screen.
Small wonder Congress put the screws to the program. Poor
Rich Spjut with 2,000 pounds of plant material from Australia
and me, with 900 pounds of plant material from China (300
species). The program was over. That's how it was in '82.
Bob Perdue and I both predicted they'd be back into heavy duty
plant screening in less than a decade; Bob said five years.
Here is what happened.

Etoposide for   1984. Bristol-Myers gets approval for etoposide for cancer of
Cancer   the testicles. (Etoposide is the semisynthetic compound
derived from the mayapple, long ago studied by NCI's Jonathan
Hartwell, and long the source of a drug of choice for venereal
warts. Hartwell's studies were initiated because of reputed
folklore of the Penobscot Indians. Strangely, during a recent
trip to Penobscot country, I learned that the mayapple no
longer occurred in that part of the world).

1985. Weird book comes out with the following refrain:

> I'll venture to prognosticate
> Before my song is sung
> This herb will help eradicate
> Cancer of the lung

1986. Bristol-Myers announces approval of etoposide for small cell lung cancer.

Mayapple

1987. NCI launches new contracts with New York Botanical Garden, Missouri Botanical Garden, etc., getting back into heavy duty plant screening.

With USDA out of the medicinal plant business since 1982. I was still quite impressed by folklore panning out, making the mayapple the number two plant in the world against cancer. (Number 3 today is probably *Taxus brevifolia*, with promise for ovarian and lung cancer. According to Moerman (1986), Bella Koola and Quinault Indians used this species for lung ailments. Now, in 1990, the NCI looks to it as a possible cure for lung and ovarian cancer. It seems the Indians may have anticipated many of our important medicinal plants. Rice (1984) stated, talking about allelopathic rather than medicinal effects "...Most of the species suggested to have pronounced chemical effects...have such effects." That is empiricism at work.

If the Amerindians anticipated the anticancer potential of the mayapple. I thought it worthwhile to investigate more mayapple folklore. Moerman (1986) noted that the Menominee Indians sprinkled a decoction of the whole mayapple plant "on potato plants to kill potato bugs", a use reflected also in that weird book mentioned above (Duke, 1985 a):

(QUOTE)
> Further south the Cherokee
> Echoing Menominee
> Made a tea out of the roots
> To keep the bugs off tater shoots

If the Indians were right on the anticancer activity, couldn't they be right on the potato bugs? As a bad brown-thumb gardener, I had potatoes coming up in the same patch in my Howard County, Md. garden that had been heavily infested with potato beetles the year before. With mayapple abundant on my property, I pulled up several plants and tossed them casually between my potatoes as mulch. A day or two later, I realized that I really should do some *in vitro* work as well. When I went to collect some potato beetles to experiment with, there were none in my potato patch. Was it the mayapple, was it the year, or was it just fate? Since I wanted them, they weren't there! Though it is anecdotal, there were no potato beetles three days following my mulching lightly with mayapple. I was excited.

Enthusiastic, I gathered mayapple and took my story to Martin Jacobson, Dean of USDA natural product pesticides. He extracted the plant material and turned it over to Bill Cantelo with his memo of October 19, 1983. Some was extracted in ether, some in ethanol March 5, 1984, Cantelo sent a memo to Jacobson which I copy here:

(QUOTE)
> We have completed evaluating the extracts you provided on January 23, 1984. The procedures were the same as those described in my letter of November 17, 1984. We used a 1% concentration of the plant material and repeated each test four times. The results follows.

EXTRACT	% FEEDING REDUCTION	
	mean	(stan.dev.)
CH 331.1	73.14%	(21.38)
CH 331.2	28.16%	(59.56)
CH 331.4	45.04%	(25.02)
CH 331.5	2.16	(45.16)
CH 332.1	41.62	(35.57)
CH 332.2	23.68	(56.27)
CH 332.3	-22,20	(48.68)
CH 332.4	44.81	(38.93)

There was considerable variation in beetle response, which is why the standard deviations are so high. Increasing the number of replicates did not appear to improve the situation. With the exception of CH 332.3, which may be a feeding stimulant, all of the materials had an anti-feedant effect. (Underlines - J.A. Duke)

William W. Cantelo
Research Entomologist

Cantelo's memo of March 29 was still upbeat, though if you averaged the percent reductions and the standard deviation, the latter average was larger, 36.54 (47.30). That memo concluded: "Three of the fractions contained antifeedants, with 334.2 being most active."

Following fractionations and purifications, the antifeedant activity became more obscure. Field trials by Cantelo failed to corroborate my speculations. His memo of July 10 concluded: "The data do not show repellent activity." "So terminated the USDA investigations of the mayapple and the potato beetle." Still a believer, I recommend to anyone well endowed with mayapple to pull up a few plants, rhizome and all, and use them to mulch your potatoes before the potato bugs arrived. Keep two plots, treating one and not treating the other (as a control).

There's more to another verse than one might guess:

(QUOTE)
They couldn't know etoposide
Nor of it AIDS to homocide
Nor could they know the course it charts
For cancer of the private parts. (Duke, 1985a)

AIDS Knowing that even the aqueous extract has antiviral activity (herpes simplex, influenza A, and vaccinia viruses, acc. to Bedows and Hatfield, 1982), and having heard that the plant was used for suicide if not homocide, the author had no idea it might be useful in AIDS. Studying Kaposi's Sarcoma, Cole (1984) compared vinblastine with etoposide, reporting:

(QUOTE)
a response rate to vinblastine of approximately 35% to 40% with 25% of the treated patients developing opportunistic infections...A somewhat better response to therapy has been reported using etoposide and is associated with a lower incidence of opportunistic infections.

Antiviral Extract	In working up a paper on synergies, I found that mayapple produces, not one, but several related compounds with rather similar activities. From the antiviral aqueous extract, Bedows and Hatfield (1982) found podophyllotoxin most active at inhibiting the replication of the measles and herpes simplex type I virus; beta-peltatin and desoxypodophyllotoxin were marginally antiviral, while alpha-peltatin and picropodopyhllotoxin were inactive <u>at the levels tested</u>. That is how they interpreted their data. Let us look more closely! The aqueous extract reduced infectivity of herpes simplex by 93.9% at a level of 10/g/ml, podophyllotoxin reduced infectivity by 88.3% at 1 /M, desoxypodophyllotoxin by 83.8% at 1 /M, beta-peltatin by 78.3% at 1 /M, alpha-peltatin by 68.9% at 1 /M and picropodophyllotoxin by only 15.6% at 1 /M. From Table 1, it appears that the aqueous extract was, perhaps due to synergies, at least 4 to 5 times more potent proportionally than any of the individual compounds.
Synergies	At first glance, Table 1 might suggest that the isolated compounds are much more potent. The most common lignan, however, was alpha-peltatin at 0.4% of the aqueous extract residue (=4,000 ppm). That indicates that the extract above would contain only 40 ppb peltatin, and less than 40 ppb each of the other compounds, surely less than 160 ppb lignans. Yet that <160 ppb lignans is almost twice as strong as 414 ppb of its closest competitor, podophyllotoxin. There must be synergy with these lignans <u>or</u> there must be at least one more active ingredient of equal or greater antiherpetic activity in the aqueous extract. <u>THE WHOLE EXTRACT IS MORE EFFICACIOUS THAN THE SUM OF THE PARTS TABULATED!</u>.
Anti-cancer Activity	When I worked with the NCI, looking for cures for cancer, we were told that our odds improved if we selected anthelminthic species (29.3% active), piscicidal species (38.6% active), arrow- poison species (52.2% active), folkloric anticancer plants (52.4% active), then if we randomly selected a plant (18.9% active) (Spjut & Perdue, 1976). If antitumor activity can be sought by checking out pesticidal activities in plants, then we might conversely expect pesticidal activity in many antitumor plants. Table 2 lists some of the more important antitumor plants, listed in what I consider decreasing order of importance to the world war on cancer. Eleven out of 12 have reported pesticidal activity.

Recently the NCI invited me to cooperate with them on their knew cancer-preventive or designer-food program, which spawned already two workshops in 1990. One of those, the soybean workshop, ironically talked about the compounds usually regarded as antinutrients (for humans and other animals who eat soybean) as the most important cancer-preventive compounds in soybean. |
| **Soy Products** | On June 17, 1990 Mark Messina of NCI convened a workshop entitled <u>THE ROLE OF SOY PRODUCTS IN CANCER PREVENTION</u>. Triggered by epidemiologic data showing that oriental women were 1/8 as likely to get breast cancer as occidentals, scientists speculate that soybean may be the preventive. Steven Barnes and Kenneth Setchell championed the soybean estrogens, like genistein and daidzein, as possible |

cancer-preventives, functioning in the body much like the synthetic tamoxifen. Ann Kennedy championed the Bowman-Birk protease inhibitors. Ernst Graf championed phytic acid, while A.V. Rao championed saponins and sterols. Interestingly all these antinutrients are also the same antinutrients that serve as antifeedants, repellents or even insecticides. After the workshop, I saw a paper (Khanna et al., 1989) indicating that soybean seeds and roots contained ca 1,000 and 600 ppm rotenoids respectively.

Legumes

With the exception of the rotenoids, all or most of the chemopreventives mentioned above occur in many legumes, even in Hoxsey's red clover, *Trifolium pratense* (Duke, 1989). Soybean has no monopoly on the chemopreventives. Macrobiotic North Americans might want to turn to the groundnut. *Apios americana*, rather than the Latin American beans (*Phaseolus spp*) or the Chinese soybeans (*Glycine max*) for their chemopreventives. That soybeans is a $12 billion dollar US crop, more important economically than other legumes, may explain why it is receiving more chemopreventive attention.

Rotenoids

Bear rotenoids in mind as you contemplate *Dioscorea villosa*," wild yam, mother of the pill, changed us more than most herbs will." For a while *Dioscorea villosa* replaced mare's urine as the source of steroids for a blossoming contraceptive industry. Then we moved to Mexico's "barbasco", a tropical species of Dioscorea, which grew year-round instead of six months. After Mexico raised the price too frequently, the pharmaceutical firms resorted to soybean as starter material for steroids possibly using sitosterol, which has proven antitumor activity. If the soybean also contains 1,000 ppm rotenoids, as the Indian paper suggests, I can see another byproduct industry developing, soy pesticides. Can't you see the ads? SAVE THE ENVIRONMENT; USE NATURAL BIODEGRADABLE SOY ROTENOIDS INSTEAD OF PETROLEUM-DERIVED SYNTHETIC PESTICIDES!!! This may sound great to soybean societies and natural pesticide enthusiasts. There is a flip side. Heretofore, we have obtained our rotenoids from poor third world countries, short of hard currency. Some of our rotenoids were extracted from rain forest agroforestry scenarios, albeit not too renewably. Soybean rotenoids would damage the miniscule rotenone industries in Rain Forests.

Rain Forest Products

With gas prices spiralling as a result of the Middle Eastern crisis, there should be renewed interest in Rain Forest natural products to replace petrochemicals. Energy or "power" alcohol can be a byproduct of the extraction of these natural products. Rain forest oil palms could potentially fuel, renewably, some of the countries possessing them. Some of these palm oils (e.g. *Jessenia spp*) are probably as healthy as the soy oils, especially hydrogenated soy oils. But soy oil is not being promoted in Designer Foods.

Herbs

Under the able aegis of Dr. Herbert Pierson, there was a broader Designer Food Program Workshop March 19-21, 1990. "Cancer-preventive" or chemopreventive isothiocyanates and organosulfur compounds in garlic mustard, lignans and polyphenols in burdock, antiestrogenic lignans in flax, the antioxidant and antimutagenic isoflavone glabrene in licorice,

organosulfur compounds in garlic, limonene in citrus, anethol and apiole in various carrot relatives, antioxidant phenolics in rosemary, and capsaicin in peppers, were mentioned as possible chemopreventives. All these herbs have folk histories for curing or preventing cancers. Most of the possibly chemopreventive compounds are biocidal as well. Are most "cancer-curing" and "cancer-preventing" phytochemicals also pesticidal??? That's the loaded question I pose to this BARD (Binational (US/Israel) Agricultural Research and Development) symposium!

Growth Regulators

Buta and Kalinski (1988) were impressed with the plant growth regulators they found in some of the NCI's target species, especially camptothecin, in which they found a unique DNA-regulatory mechanism. They found also that extracts of Baccharis, Bersama, Cephalotaxus, Linum perenne (which contains lignans related to podophyllotoxin), Maytenus, Phyllanthus, Ricinus, Taxus et al. inhibited elongation of tobacco meristems at least 50% for one week. Maytansine inhibited growth in tobacco callus and rice seedling bioassays. Harringtonolide was one phytotoxic ingredient in Cephalotaxus. Trichothecenes from Baccharis inhibited growth of beans, corn and tobacco.

In Table 3, shown below I show that most of Grainge and Ahmed's broad-spectrum biocidal genera are also rather broad-spectrum medicines as well.

Pest Control

For some reason, Grainge and Ahmed (1988) dropped four species from their earlier top listing (Grainge and Ahmed, 1985); tomato, which had ca 28 pests controlled, prickly poppy which controlled ca 12 pests, garlic, which controlled ca 80 species, and "mata raton" (*Gliricidia spp*), controlling 13 species, as diverse as aphids, rats and ticks.

Native West Virginia Plants

Here in West Virginia, instead of talking about tropical plants like the annonas, castorbean, ginger, neem, ryanias, tuba roots, etc., I will discuss plants that are native here or grow here as cultivars. Berenbaum (1990) showed, that at 1 ppm, aristolochic acid (available from a serious West Virginia weed, the pipe vine, *Aristolochia durior* killed all her experimental mosquito larvae in less than 3 days. So did 5 ppm podophyllotoxin (from the same mayapple discussed earlier), and 10 ppm thymol (rather common compound partially responsible for the strong odor of some West Virginia *Monarda punctata*).

Once (Duke, 1986), I listed the top ten Amerindian medicinal plants, after a survey of too few people. All of the top ten occur in West Virginia.

Sweet Flag

The number one plant in that Amerindian survey was *Acorus calamus*, the sweet flag, ranked high in Table 2 also, having several biocidal compounds and activities: allelopathic, antifeedant, bactericidal, insecticidal, insect-repellant; in fact it long occupied some of our BARC scientist's time.

Bloodroot	#2, *Sanguinaria canadensis*, the bloodroot, also has reported allelopathic, bactericidal, and insecticidal properties, and was in several folk remedies for cancer, proving to have in-vitro antitumor activity.
The Docks	#3. *Rumex spp*, the docks, contain anthraquinones which have proven fungicidal activities. The plants are reported to have acaricidal, antifeedant and anti-insect properties.
The Coneflowers	#4, *Echinacea spp*, the coneflowers, some of which may be approaching extinction in the wild, contain the bactericide echinancin-B (Duke, 1986). Bactericidal, insecticidal and juvabional attributes are also reported (Grainge and Ahmed. 1988).
Sassafras	#5, *Sassafras albidum*, the well-known sassafras, has an antiseptic and pediculicidal essential oil, responsible for the great flavor in old fashioned root beer, now banned, perhaps unjustly, by the FDA. It has allelopathic, antibiotic, and insect-attractant activities (Grange and Ahmed, 1988).
Witch Hazel	#6, *Hamamelis virginiana*, the witch hazel, probably contains enough tannin, to be a minor repellant. But I find no pesticidal activity mentioned by Grainge and Ahmed, (1988) or Jacobson (1990).
Indian Tobacco	#7. *Lobelia inflata*, the Indian tobacco or pukeweed, was not cited as insecticidal by Jacobson (1990) but it was cited as insecticidal and poisonous by Grainge and Ahmed (1988). Tyler (1982) hints that it might even have homocidal potential.
Mayapple	#8, *Podophyllum peltatum*, the mayapple, was cited as antifeedant, insect-repellant, and rodent-repellant (Grainge and Ahmed (1988) and it also has viricidal properties. Amerindians were said to use the plant for suicidal purposes (Duke, 1985).
Wild Cherry	#9, *Prunus serotina*, the wild cherry, contains pesticidal HCN and benzaldehyde, enough to be poisonous to deer (hence "cervicidal"). It was not mentioned by Grainge and Ahmed (1988) or Jacobson.
The Willows	#10, *Salix spp*, the willows, contain chlorogenic acid which is an antifeedant compound. High concentrations of tannins and organic acids, catechins and compounds similar to quercitrin and salicin may impart some resistance to insect larval stages. (Jacobson, 1990) Grainge and Ahmed (1988) report pestfree willows, as well as antifeedant and antifungal activities.
	There is not 100% medicinal: pesticidal concordance with the top 10 eastern Amerindian medicinal plants, but 90% is pretty good. Nine of ten have some biocidal activity. I would wager that witchhazel liniment could repel if not kill a few organisms.

Datura stramonium	West Virginia's jimsonweed, *Datura stramonium*, has quite a repertoire as a pesticidal plant, reportedly effective against aphids, caterpillars, flax, fungi, leafhoppers, maggots, nematodes, rice-borers, and viruses (Grainge and Ahmed, 1988). Atropine, hyoscyamine and scopolamine (hyoscine) are possibly the active alkaloids.
Atropine	With the threat of chemical warfare on the Arabian Peninsula, sources of atropine assume a new significance. Iraq has nerve gases which may be used against their enemies. Atropine is an antidote to some of these nerve gases. Hence, West Virginia's jimsonweed (*Datura stramonium*) may receive more attention. (Atropine sulfate was listed at $10.00 - 11.00 per ounce and scopolamine hydrobromide at $36.00-46.50 per ounce, Chemical Marketing Reporter, May 28, 1990). Prices of atropine may rise while those of byproduct scopolamine may fall, until the Iraq trouble subsides. *Stramonium* is one trade name for jimson weed.
Belladonna	For several years, I got mysterious phone calls from pentagon types wanting to know if we grow belladonna (*Atropa belladonna*) in this country. Except for a few herbalists, there seem to be no overt growers here. It's cheaper to import some herbs, like belladonna, guar, psyllium, and stramonium, than it is to grow our own. The calls about belladonna were prompted by concerned strategists who wanted to insure that America had enough atropine, should we become involved in gas warfare. However, we can get all the atropine we need from the soybean fields around Jamestown, Virginia. Jimsonweed is one of the major weeds in our eastern soybean fields. Jimson is a corruption of Jamestown. The story goes that years ago (1676), John Smith was dispatched to Jamestown to quell the Bacon Rebellion. Some of his military men cooked up some jimsonweed, perhaps mistaking it for the also-dangerous poke salad (*Phytolacca americana*). They were made ill, but it doesn't sound too life-threatening in Robert Beverly's **History and Present State of Virginia (1705, 1855):**
(QUOTE)	...they turn'd natural Fools upon it for several Days. One would blow a Feather in the Air; another would dart Straws at it with much Fury; and another stark naked was sitting in a Corner, like a monkey grinning and making Mows at them; a Fourth would fondly kiss and paw his Companions, and snear in their Faces, with a Countenance more antik than any in a Dutch Droll. In this frantik Condition they were confined, lest they in their Folly should destroy themselves...Indeed they were not very cleanly, for they would have wallow'd in their own Excrements, if they had not been prevented. A Thousand such simple Tricks they play'd and after Eleven Days, return's themselves again, not remembring anything that had pass'd
Jimsonweed	Let us not take jimsonweed too lightly. Many fatalities are attributed to jimsonweed intoxication. Jimsonweed is but one of many sources of the very dangerous alkaloid atropine. Today, that alkaloid is strategically important. It may save some American lives on the Arabian Peninsula.

Those of you who read JAMA, the Journal of the American Medical Association, may have seen ads for SCOP, which seems to be a transdermal patch or scopolamine, another alkaloid derived from jimsonweed, and other members of the nightshade family. Scopolamine is also a dangerous alkaloid that is being promoted as an alternative to ginger or dramamine for seasickness and other types of vertigo (dizziness). One study, by an herbalist, showed that ginger, at ca 1,000 mg, was effective orally at preventing seasickness. Another study, possibly sponsored by the pharmaceutical industry, concluded that ginger was ineffective, and scopolamine effective. Regrettably, without an unbiased comparative study, we may never be absolutely sure which of these herbal alternatives is safer and more efficacious, ginger itself or scopolamine. We won't be sure whether they are better or worse than the conventional dramamine. Unless the government sponsors studies comparing new drugs (synthetic or natural), not only with placebo, but with the recognized best herbal alternatives, we may not get all the best medicines. Americans should expect the safest and best, be they holistic herbals, purified natural products, semisynthetics derived from natural products, or out-and-out synthetics. I want the best, whichever it is. The herbal alternative might be called an "orphan". America cannot always learn the best alternative under today's economic conditions, where, according to a recent Chemical Marketing Reporter, it costs $231 million to prove a drug safe and efficacious. Would you want to invest $231 to prove that non-patented carrots could prevent lung cancer, if you were making $100 million a year selling a patent-protected semisynthetic drug for the cure of lung cancer? Would you invest $231 million proving that jimsonweed cigarettes could alleviate asthma if you were making $100 million a year selling a proprietary synthetic for asthma?

Belladonna

Belladonna, like jimsonweed, can be useful or dangerous. Belladonna is reported to have antifeedant, antiviral, and insecticidal properties as well as insect-repellant properties. In an interesting article on synergism and antagonism in the pharmacology of alkaloidal plants, Izaddoost and Robinson (1987) make interesting comment on the belladonna which could apparently be extended to jimsonweed:

(QUOTE)

Among themselves, the alkaloids of this plant demonstrate synergistic pharmacological effects in their various activities. However, depending on the proportions of different alkaloids present within the plant, different types of response to treatments with plant extracts may be emphasized... Variability in pharmacological effects may be expected from whole plant extracts. Synergism and antagonism among the alkaloids means that spasmolytic activity of a total extract cannot be predicted accurately from knowledge of either the l-hyoscyamine content or the total alkaloid concentration.

When plant constituents other than alkaloid are considered, flavonoids appear to be synergistic with the alkaloids in spasmolytic action but antagonistic to the alkaloids in action on urine retention. Chlorogenic acid may be synergistic with the alkaloids in antihistaminic activity but antagonistic to alkaloids in the CNS... Widely varying effects would be expected when whole plant extract is administered. (Underlined letters capitalized by this reviewer).

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The authors note that tropanes act as antimuscarinic, antispasmodics, antihistaminic and also affect the CNS. Low doses may stimulate the CNS, while higher doses depress. Scopolamine has more effect on CNS than does atropine, while apoatropine is more active as an antispasmodic than atropine. Quercetin, kaempferol, and glucosidic flavones also occur in Atropa, if not Datura. These may exhibit antispasmodic and diuretic activities. Chlorogenic acid is a CNS-stimulant similar to caffeine, and is also antibacterial and antihistaminic.

Pokeweed

In West Virginia, the local gentry tend to think of pokeweed (*Phytolacca americana*) more as food (but dangerous and mitogenic) or medicine than as pesticide. Grainge and Ahmed (1988) list it as antifeedant, antifungal, insecticidal (against cockroaches) and viricidal. The Pokeweed Antiviral Proteins (PAP) are being studied as anticancer and antiAIDS agents (Bonness, 1990). These ribosomeinactivating proteins, popularly called RIP's (two from the leaves, one from the seed), "are potent toxins that kill most cells they are allowed to enter (bacterial cells excluded)". In a complex shearing of the ribosome, indispensable to the cell for manufacturing protein, the RIP prevents the cell from further protein synthesis. "Once the ribosomes are damaged, protein synthesis stops and the cell dies...A number of one-subunit RIPs (such as trichosanthin and pokeweed antiviral protein) have the uncanny ability to selectively act upon virally infected cells...These RIPs are selectively taken in by virus-infected cells." For more than a decade NIH has been investigating immunotoxins, toxic proteins like RIPs linked to antibodies, targeting them for carcinomas, leukemias and lymphomas.

Bonness (1990) recounts another novel approach, playing upon the Human Immunodeficiency Virus (HIV) affinity for a special molecule, CD4, produced on the surface of healthy cells. The RIP (usually castorbean's ricin) is attached to the CD4 molecule which still attracts HIV-infected cells, which bind to it (with fatal results to the HIV-infected cell) instead of a healthy cell.

Grainge and Ahmed (1988) do not list the American pokeweed as molluscicidal. Our Program Staff at Beltsville wants seed of an Ethiopian species, *Phytolacca dodecandra*, the endod, which shows promise as a molluscicide. Heretofore molluscicidal activity has been of interest to tropical countries suffering schistosomiasis. Ten percent of the population of Brazil, Latin America's most populous country, suffers from schistosomiasis, transmitted by an aquatic snail. In Adwa. Ethiopia, the incidence of schistosomiasis in 1-5 years old children dropped from 50% to 7% in five years when crushed endod berries were added to the river water (Adams et al., 1989). Further the endod produces a RIP called dodecandrin, which might also be investigated for medicinal applications.

Molluscicide

In the US, small mussels are now clogging up filters in American water works, so there is a monied interest in a good molluscicide. Endod is fungicidal, larvicidal, molluscicidal, spermicidal, and it is cheap and natural. Unfortunately, a preliminary survey of several species showed that our *Phytolacca americana* lacks the molluscicidal lemmatoxin and saponins found in *P. dodecandra*. (Parkhurst, 1974).

Bayogenin (14.9% in berries), hederogenin (8.9%, 2-hydroxyoleanolic-acid (6.5%), and oleanolic acid (66.2%), important in *P. dodecandra*, were not found in *P. americana, esculenta*, or *insularis*, all of which did contain alpha spinasterol, jaligonic-acid, esculentic acid and 10 other phytolaccosides. Phytolaccoside-E is the major saponin in *P. americana* and *P. esculenta*. There are probably a dozen saponins in *P. dodecandra*. The LD90 of endod saponin extracts against snails of import are ca 3 mg/L after 24 hours. The LD90 of the most active molluscicide, lemmatoxin, is 1.5 mg/L against *Biomphalaria glabrata* after 24 hours. According to Lemma (1983):

(QUOTE)

Recently, endod berries also have been discovered to possess potent spermicidal properties useful for birth control; aquatic insect larvicidal properties potentially useful in the control of mosquitoes and other water-breeding insects; trematodicidal properties for control of the larval stages of *Schistosoma* and *Fasciola* parasites; hirudinicidal properties for control of aquatic leeches; and fungicidal properties for the potential topical treatment of dermatophytes. Most of these studies are in experimental stages and need further support.

Water Pepper

Can West Virginia's *Polygonum hydropiperoides* repel the flies that gather around a new flesh wound in horses, as folklore suggests. A friend is checking out the rumored activity. This herb, the so-called water pepper, reportedly has allelopathic, antifeedant, insect repellant and insecticidal properties. My friend asked how she might recognize the right Polygonum. I told her to bit it (cautiously); if it bit back, it might contain the polygodial. The bite of the water pepper or smartweed is apparently due to polygodial, which has proven candidicidial, helicocidal and piscicidal activities, in addition to its noteworthy antifeedant activity. Polygodial is 2-8 times stronger and slightly more active than commercially available amphotericin B. It is a broad spectrum antibiotic, especially effective against yeast and filamentous microorganisms. It exhibits an even greater potential when combined with other antibiotics. For example, the activity of actinomycin-D against *Saccharomyces cerevisiae* was increased 16-fold in concert with polygodial. Kubo and Taniguchi (1988) speculate that the polygodial may act as an "advance scout", punching holes in the plasma membrane to gain entry for other antibiotics, which were less effective because of their inability to cross the membrane. The minimal inhibitory concentration of polygodial against *Saccharomyces cerevisiae* is 0.78 ppm, against *Candida utilis* 1.56 ppm.

Pennyroyal and Mountain Mint

Can West Virginia's pennyroyal, *Hedeoma pulegioides*, or mountain mint, *Pycnanthemum muticum*, repel ticks, carriers of the dread Lyme Disease? Both contain the aromatic compound, pulegone, which is reported to repel birds and fleas and which has rather striking herbicidal potential as well. Both of these plants are dominant members of the forest floor in parts of Pendleton Co., West Virginia, often to the exclusion of other species. With such a wide array of biological activities, pulegone might be predicated to have fungicidal activity as well, Lydon and Duke (1989) tabulated 25

phytotoxic terpenoids which in concentrations of 1.5-2,000 mM, inhibited plant growth. The pulegone was strongest, with an IC-50 (inhibitory concentrations of 50% seed germination) of only 1.5 mM. For a further discussion of these pulegone-bearing plants, see my NATURAL PLANT COMPOUNDS AS PESTICIDES in this volume.

I once bragged about not wanting to eat an apple if a bug did not want to eat it. I even bragged about eating the good sides of two bad apples to get away from a whole, but sprayed, flawless apple. But apples, like less palatable oak leaves, react when they are nibbled on. As Russell (1986) paraphrases, plants, like animals, have inducible disease-resistance mechanisms. If one leaf of a cucumber becomes infected, the whole cucumber plant develops increased resistance to further infection. If an oak-leaf caterpillar eats 10% of the leaf, the whole leaf becomes much more unpalatable. The plants produce antifungals and antifeedants, which could be harmful (or medicinal) to man. The disease- or bug- blemished organic apple may in fact have natural pesticides at levels ten times higher than the unaffected apple. That level of natural pesticide could easily be of more significance than traces of synthetic pesticides persisting on the apple.

Walnut

West Virginia's walnut, *Juglans nigra*, is conspicuous here at the Bavarian Inn. Though not in the top ten Amerindian medicinals, walnut has an active napthaquinone, juglone, which has certainly drawn praise as an herbicide. However, de Scisciolo et al. (1990) question the significance of the allelopathic nature of juglone under their field conditions. Nonetheless, juglone is reported to have antifeedant, bactericidal, fungicidal, and flea-repellant activity as well. The low LD-50 of 2.5 mg/kg in mice (Duke, 1985) would suggest that it could be used as a rodenticide as well. Though a natural toxin, it is not necessarily benign. Nature has produced a lot of serious toxins which can be harnessed as medicines and pesticides.

Summary

Concluding, I say a few things that may have long been obvious to you, but have only recently become apparent to me.

(1) Most compounds have several bioactivities. Often, the dosage determines whether these compounds will be active in experimental subjects. Minute doses may actually stimulate the immune system thru hormesis. Higher doses may be medicinal or bioregulatory. Still higher doses may be homocidal or pesticidal, if you choose to treat these as two rather than one category.

(2) Often a plant contains a suite of similar compounds with rather similar bioactivities. These often prove to be synergistic. In such cases, whole plant extracts might be both more economical and more effective than solitary isolates. Ames et al. state "Dozen of mammalian metabolites are commonly produced from any reasonably complex molecules." From this we might infer that if a plant has 10,000 complex molecules, there might be more than 100,000 metabolites in mammals who ingested the plant. If differences between individual herbs and herbivores are genetic, and genetics is based upon chemically different genes and reactions controlled

by these genes, there are about 5 billion chemically distinct humans whose chemical profiles vary with ecological, temporal (diurnal, lunar, annual at least) and psychological conditions to react differently to the metabolites resulting from the ingestion of foods coming from millions of individuals of hundreds of food species. Small wonder that pharmaceutical and pesticidal industries prefer to research pure compounds instead of highly variable whole-plant concoctions.

(3) Contrary to my previous unlearned expectations, semisynthetic derivatives of natural compounds are not necessarily more toxic to humans or experimental animals than the natural compound from which they were derived. In the first four cases I investigated, hoping to prove that synthetics were more dangerous, the converse proved true; more often than not, semisynthetic derivatives were less active than the natural toxin from which they were derived. Rationalizing, I suspect that easy modifications of the molecules might have already occurred in nature, being selected against, if they were less toxic. So, when the chemist makes the same semisynthetic modification in the lab, it proves less toxic in his experiments.

(4) Oregano contains 100,000 times more natural pesticides on a ppm basis, than synthetic pesticide residues, even more than the 10,000 Ames (1983) laments (Duke, 1990). I suspect that the 6 magnitude difference observed in oregano applies to most spices, whereas Ames' 5-magnitude difference applies to most foods normally consumed. Some people argue that the naturals are less harmful because we have co-evolved with them. The immune system is a huge complex of rapidly evolving cells, which, when healthy, are quick to learn to recognize a new alien, friend or foe, synthetic or natural.

(5) If industry is so adept at accidentally or intentionally removing fiber and nutrients from foods, might they just as well become adept at removing natural pesticides from the food chain, putting them into recyclable pesticide containers, leaving the synthetics like the relatively harmless Alar in the minds of man instead of the mouths of babes. If natural pesticides are more rapidly degradable than synthetics, as some scientists maintain, environmental and health benefits might accrue from using food- derived natural pesticides rather than synthetics. In another context Ames et al. (1990) state "Minimizing pollution is a separate issue, and is clearly desirable for reasons other than effects on public health."

(6) All plants contain phytobiocides (PBC's; that makes them sound dangerous, like PCB's). All plants contain medicinal compounds. All plants contain toxins and antitoxins, oxidants and antioxidants, nutrients and antinutrients. All plants contain vitamins, minerals etc. Until we have firm numerical data, the statement that a plant contains an acaricidal compound or a phytoalexin, is relatively meaningless. We need to know how much it contains and how much it takes to do the job. (See NATURAL PLANT COMPOUNDS AS PESTICIDES, this workshop proceedings). Since all plants contain shikimic acid and beta-sitosterol, e.g., it follows, ridiculously, that, if you define a biocidal or medicinal plant as one that contains a biocidal or medicinal compound, all plants are biocidal and medicinal, all plants are carcinogenic and anticarcinogenic.

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SINTHESIS

Sometimes methinks it pathetic
The bitter battle we witness
Twixt the nat'ral and synthetic
But I know which is the fittest!
Nat'ral is what the consumer prefers
But it's patently harder to patent
So the corp'rate crim'nal demurs,
Giving synthetics to the patient.

The same is true of biocides,
Natural is legendary:
But it's easily plagiarized
Synthetic's proprietary.

Those who say that nat'ral's worst
Should speak later, thinkin' first!
What would you prefer on your salad,
Vinegar or acetic acid?
Those who say that nat'ral's best
May have passed the acid test.
Mornin' glory seed may well be
Much gentler than the LSD

I'm sure that much more data exists
In Potter's little peachy pits.
I don't even know that anyone died
From this peachy form of cyanide.
Aspirin was termed safer back then,
Than nat'ral salicylic acid,
But NSAID's kill 10,000 men,
Regulators still remain placid.

Pyrethrums, Rotenones and Ryanias
Pesticides that Nature provided,
Probably sharing most of the dangers
Of synthetics, so often derided.
Insects develop tolerance to these
Just like they do to synthetics;
My organic friends, on hearing this,
Deem me one of the heretics.

Sitting there drinking their coffee and tea
They seem more thrifty than shifty,
They've forgotten how toxic caffeine can be,
At least judging its LD-50
I too consume a lot of caffeine,
Much more than I really should.
Alcohol, quinine and limonene;
Gin tonics still taste real good.

Never thought that I'd ever agree
With the numbers of Bruce Ames;
Herp Index, Ames test, verily,
Have earned him two Halls of Fames!
Then I did an oregano review,
Listing every nat'ral compound.
It may hold a surprise or two,
The numbers that I found.

Of synthetic residues, one ppm,
Persisted in samples in Canada,
Enough to make the chances dim,
For *Curvularia* and *Candida*.
Raticidal Richochet!
Alarmists quickly tooted!
The oregano we have today
is perilous polluted!

But nature has synthetic mimics,
Bringing botanists blood to a boil.
Do we count the allelochemics,
Tannin and essential oil?
If all nat'ral biocides he counted,
It might flatten a platitude,
The nat'rals outweigh the synthetics
Five orders of magnitude.

So whenever ye drink your herbal tea
At your factory, farm or your fountain,
If ye forget natr'l pesticides ye
Make molehill out of the mountain.
That many nat'ral pesticides are extant,
Should not be viewed as excuse;
We still should be quite vigilant
With synthetic residue.
Take the natural out of the food chain,
Put it into the pesticide can,
Let the synthetic remain
In the healthier mind of the man.
Instead of the Mouths of Babes
Evolution, wages of sin!
I reckon I'm over the hill!
If the pests don't do me in,
For sure the pesticide will. (annonpoet,1990)

Table 1. Antiherpetic Activity of Mayapple Extract and
Compounds (After Bedows and Hatfield, 1982).

	CONCENTRATION	HERPES-INFECTED CELLS (%)
CONTROL	------------	100
Aqueous extract	10 ppm	6.1
Podophyllotoxin	1 uM (=414 ppb)	11.7
Picropodophyllotox	1 uM (=414 ppb)	84.4
alpha-Peltatin	1 uM (=400 ppb)	31.1
beta-Peltatin	1 uM (=400 ppb)	21.7
Desoxypodophyll	1 uM (?414 ppb)	16.2

Table 2. Important Plants as Sources of Antitumor Activity
and their Biocidal Activity as Reported by Grainge and Ahmed
(1988).

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1. Catharanthus: Nematicide
2. Podophyllum: Antifeedant; Insect Repellant;
                  Rat Repellant
3. Taxus: Bactericide, Insecticide, Juvabional
4. Cephalotaxus: Insecticide
5. Camptotheca: Bactericide; Insecticide; Viricide
6. Phyllanthus: Insecticide; Juvabional
7. Pancratium:
8. Combretum: Antifeedant
9. Colchicum: Antifeedant; Insecticide; Juvabional
10. Tripterygium: Antifeedant; Insecticide
11. Maytenus: Insecticide
12. Physalis: Antifeedant; Insecticide

Table 3. Broad Spectrum Biocidal Genera With Their Major
Medicinal Activities.

Genus	Medicinal Entries Species (Genus)	Pests Controlled
1. Aconitum	14 (ca 175)	6
2. Acorus	150 (ca 175)	44
3. Ageratum	ca 50 (ca 50)	11
4. Aleurites	12 (ca 30)	12
5. Annona	25 (ca 200)	19
6. Arachis	16 (16)	25
7. Artabotrys	0 (2)	17
8. Azadirachta	ca 75 (ca 75)	126 (113)
9. Chrysanthemum	19 (ca 200)	66
10. Croton	ca 40 (ca 300)	11
11. Datura	ca 75 (ca 350)	18
12. Derris	13 (ca 50)	29
13. Haplophyton	2 (2)*	22
14. Justicia	20 (21) **	10
15. Madhuca	25 (ca 125)	19
16. Mammea	ca 45 (ca 50)	27
17. Melia	ca 120 (ca 130)	54
18. Mundelea	1 (11) (mostly biocidal entries)	25
19. Nicotiana	12 (ca 150)	12
20. Pachyrrhizus	17 (18) (mostly biocidal)	27
21. Piper	ca 75 (ca 750)	15
22. Pogostemon	4 (ca 60)	16
23. Pongamia	43 (44)	13
24. Quassia	34 (53)	15
25. Ricinus	ca 175 (ca 175)	34
26. Ryania	9 (12) (all biocidal)	18
27. Schoenocaulon	16 (18) (mostly biocidal)	40
28. Tagetes	ca 50 (ca 150)	25
29. Tephrosia	19 (175)	22
30. Tripterygium	0 (9) (mostly biocidal)	20
31. Veratrum	24 (ca 120)	16
32. Vitex	ca 60 (ca 220)	15
33. Zanthoxylum	10 (ca 260)	7
34. Zingiber	ca 150 (ca 220)	5

(After Grainge and Ahmed, 1988, and Duke and Wain, 1981).
Numbers following indicate the number of folk medicinal entries
in Duke and Wain (1981) for the first listed species in Grainge
and Ahmed, the parenthetical number, roughly the number of
entries for the genus in Duke and Wain. In the pest controlled
column, there is a count of the species or species groups listed
by Grainge and Ahmed. *= strictly biocidal entries, e.g.
pediculosis ** if treated as Adhatoda instead of Justicia;
otherwise 9 (ca 100) if Justicia, or 29 (ca 125 aggregating
both).

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