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BARD

FINAL REPORT

PROJECT NO. IS-2310-93

**Ecology, Population Dynamics and Genetic
Diversity of Epiphytic Yeast Antagonists of
Postharvest Diseases of Fruits**

S. Droby, S. Manulis, J.W. Eckert, R.K. Mehra

**הספריה המרכזית
למדעי החקלאות
בית דגן**

1999

Cover page-Scientific report

Date: April 17, 1999

BARD Project No. IS-2310-93

Title: *Ecology, Population Dynamics and Genetic Diversity of Epiphytic Yeast Antagonists of Postharvest Diseases of Fruits*

Investigators' Names
(Principal listed first)

Samir Droby

Shulamit Manulis

Joseph W. Eckert

Rajesh K. Mehra

Investigators' Institutions

ARO, The Volcani Center, Israel

ARO, The Volcani Center, Israel

University of California,
Riverside, CA, USAUniversity of California,
Riverside, CA, USA

Project Starting Date: October 15, 1994

Type of Report: 1st Annual _____ 2nd _____ Final X
Signature

Principal Investigator

DR. ELI PUTIEVSKY
HEAD OF A.R.O.

Signature
Institution's Authorizing
Official

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Ecology, Population Dynamics and Genetic Diversity of Epiphytic Yeast Antagonists of Postharvest Diseases of Fruits

Abstract

One of the emerging technologies is the use of microbial agents for the control of postharvest diseases of fruits and vegetables. A number of antagonistic microorganisms have been discovered which have the potential to effectively control postharvest diseases. Some of this technology has been patented and commercial products such as Aspire™ (Ecogen Corporation, Langhorne, PA, USA), Biosave 10™ and Biosave 11™ (Ecoscience Inc., Worcester, MA, USA) have been registered for commercial use. The principal investigator of this project was involved in developing the yeast-based biofungicide – Aspire™ and testing its efficacy under commercial conditions. This research project was initiated to fill the gap between the knowledge available on development and commercial implementation of yeast biocontrol agents and basic understanding of various aspects related to introducing yeast antagonists to fruit surfaces, along with verification of population genetics. The main objectives of this study were: study ecology, population dynamics and genetic diversity of the yeast antagonists *Candida guilliermondii*, *C. oleophila*, and *Debaryomyces hansenii*; and study the effect of preharvest application of the yeast antagonist *C. oleophila* naturally occurring epiphytic microbial population and on the development of postharvest diseases off citrus fruit during storage.

Our findings, which were detailed in several publications, have shown that an epiphytic yeast population of grapefruit able to grow under high osmotic conditions and a wide range of temperatures was isolated and characterized for its biocontrol activity against green mold decay caused by *Penicillium digitatum*. Techniques based on random amplified polymorphic DNA (RAPD) and arbitrary primed polymerase chain reaction (ap-PCR), as well as homologies between sequences of the rDNA internal transcribed spacers (ITS) and 5.8S gene, were used to characterize the composition of the yeast population and to determine the genetic relationship among predominant yeast species. Epiphytic yeasts exhibiting the highest biocontrol activity against *P. digitatum* on grapefruit were identified as *Candida guilliermondii*, *C. oleophila*, *C. sake*, and

Debaryomyces hansenii, while *C. guilliermondii* was the most predominant species. RAPD and ap-PCR analysis of the osmotolerant yeast population showed two different, major groups. The sequences of the ITS regions and the 5.8S gene of the yeast isolates, previously identified as belonging to different species, were found to be identical.

Following the need to develop a genetically marked strain of the yeast *C. oleophila*, to be used in population dynamics studies, a transformation system for the yeast was developed. Histidine auxotrophy of *C. oleophila* produced using ethyl methanesulfonate were transformed with plasmids containing *HIS3*, *HIS4* and *HIS5* genes from *Saccharomyces cerevisiae*. In one mutant histidin auxotrophy was complemented by the *HIS5* gene of *S. cerevisiae* indicating that the *HIS5* gene of *S. cerevisiae* is functionally homologous to the *HIS5* gene in *C. oleophila*. Southern blot analysis showed that the plasmid containing the *S. cerevisiae* *HIS5* gene was integrated at a different location every *C. oleophila* *HIS*⁺ transformant. There were no detectable physiological differences between *C. oleophila* strain I-182 and the transformants. The biological control ability of *C. oleophila* was not affected by the transformation. A genetically marked (with β -glucuronidase gene) transformant of *C. oleophila* colonized wounds on orange fruits and its population increased under field conditions.

Effect of preharvest application of the yeast *C. oleophila* on population dynamics of epiphytic microbial population on wounded and unwounded grapefruit surface in the orchard and after harvest was also studied. In addition, the effect of preharvest application of the yeast *C. oleophila* on the development of postharvest decay was evaluated. Population studies conducted in the orchard showed that in control, non-treated fruit, colonization of wounded and unwounded grapefruit surface by naturally occurring filamentous fungi did not vary through out the incubation period on the tree. On the other hand, colonization of intact and wounded fruit surface by naturally occurring yeasts was different. Yeasts colonized wounded surface rapidly and increased in numbers to about two orders of magnitude as compared to unwounded surface. On fruit treated with the yeast and kept on the tree, a different picture of fungal and yeast population had emerged. The detected fungal population on the yeast-treated intact surface was dramatically reduced and in treated wounds no fungi was detected. Yeast population on intact surface was relatively high immediately after the application of

Aspire™ and decreased to more than 70% of that detected initially. In wounds, yeast population increased from 2.5×10^4 to about 4×10^6 after 72 hours of incubation at 20C.

Results of tests conducted to evaluate the effect of preharvest application of Aspire™ on the development of postharvest decay indicated the validity of the approach.

Objectives of the Original Research Proposal

The following are the stated objectives of the original proposal:

1. Study the ecology, population dynamics and genetic diversity of the yeast antagonists *C. oleophila*, *P. guilliermondii*, and *D. hansenii* by the use of molecular techniques.
2. Study the effect of preharvest application of the yeast antagonist *C. oleophila* on the development of postharvest diseases of citrus fruit during storage.

Relevant Data, Methodology, Results and Discussion

As outlined in our two Annual Reports, the research in the Israeli laboratory was focused on: 1) characterization of the yeast population of citrus and determining their biocontrol activity; 2) development of a RAPD-PCR technique to be used in the characterization and population study; 3) Study genetic diversity of yeast antagonists by sequence divergence of the ITS1, ITS2 and 5.8S gene of the rDNA; 4) Study microbial population dynamics of citrus fruit in the orchard and after harvest as affected by the application of the yeast antagonist *C. oleophila*; 5) Evaluate the effect of preharvest application of yeast antagonists on the development of postharvest decay.

The research conducted in the American laboratory was primarily aimed at developing a genetically marked strain of the yeast *C. oleophila*. For that purpose a transformation system was developed and used to insert a marker gene into the yeast genome. The marked strain was successfully used in survival and population dynamics study.

Our findings has been summarized in three joint publication already accepted for publication in international journals and several abstracts of work presented in national and international congresses. In addition, other data, not yet published, is also included in this report.

The relevant methodology, data and discussion is divided into four major parts: (1) Characterization of the epiphytic yeast population of grapefruit capable of inhibiting the green mold decay caused by *P. digitatum*; (2) Development of DNA transformation of *Candida oleophila*; (3) Survival and population dynamics of *C. oleophila* and of genetically transformed strain of *C. oleophila* on orange fruit under field conditions and the effect of pre and postharvest application of the yeast preparation Aspire on epiphytic microbial population on the surface of grapefruit; (4) A method for studying the population dynamics of *C. oleophila* on orange in the grove using selective isolation medium and PCR technique. In each of these parts, the relevant publications are listed first followed by a brief summary of their content. The full manuscripts are enclosed at the end of the written text of the report.

1. Characterization of the Epiphytic Yeast population of Grapefruit capable of inhibiting the Green mold decay caused by *Penicillium digitatum*.

Detailed description and methodology related to this subject is discussed in the following publication:

Characterization of the Epiphytic Yeast population of Grapefruit capable of inhibiting the Green mold decay caused by *Penicillium digitatum* (Droby et al., 1999, Biological Control, in press)..

Our work in this field has determined the composition of the predominant epiphytic yeast antagonists present on grapefruit. Our approach was to characterize the epiphytic yeast population of grapefruit which is able to survive under diverse conditions. The criteria used were osmotolerance and growth at a wide range of temperatures as indicators of ability to colonize fruit surface under diverse climatic conditions prevailing

in the orchard and after harvest. According to our results, almost 37% of the total epiphytic yeast isolates obtained from grapefruit surfaces were able to grow at a wide range of temperatures and elevated osmotic conditions. Representatives of this group of yeasts were identified as *C. guilliermondii*, *C. sake*, and *Debaryomyces hansenii*. These strains were found to be the most promising antagonists of *P. digitatum* on grapefruit. RAPD and ap-PCR analysis of the osmotolerant yeast population included in this study showed two different amplification patterns, suggesting the presence of two yeast species, predominant on grapefruit surfaces.

The internal transcribed region that occurs between the nuclear small and large subunit of rDNA has been useful for distinguishing among closely related organisms. According to the similarity matrix analysis (constructed following sequencing of ITS1, ITS2 and 5.8S rDNA), one isolate of *C. oleophila* and two isolates of *D. hansenii* were genetically very similar to *C. guilliermondii*. Collectively, the findings of this work suggest that naturally occurring antagonistic yeast population, which are tolerant of extreme conditions, are likely to consist of a few different species with very high genetic similarity.

2. Development of genetically-marked strain of the yeast *Candida oleophila*

Detailed description and methodology related to this subject is discussed in the following publication:

Integrative DNA transformation of an environmental yeast *Candida oleophila* and its effect on biological control of green mold of citrus (Chand-Goyal et al., 1998, Current genetics)

Following the need to develop a genetically marked strain of the yeast *C. oleophila*, to be used in population dynamics studies, a transformation system for the yeast was developed. Histidine auxotrophy of *C. oleophila* produced using ethyl methanesulfonate were transformed with plasmids containing HIS3, HIS4 and HIS5 genes from *Saccharomyces cerevisiae*. In one mutant histidin auxotrophy was complemented by the HIS5 gene of *S. cerevisiae* indicating that the HIS5 gene of *S. cerevisiae* is functionally

homologous to the HIS5 gene in *C. oleophila*. Southern blot analysis showed that the plasmid containing the *S. cerevisiae* HIS5 gene was integrated at a different location every *C. oleophila* HIS⁺ transformant. There were no detectable physiological differences between *C. oleophila* strain I-182 and the transformants. The biological control ability of *C. oleophila* was not affected by the transformation. A genetically marked (with (-glucuronidase gene) transformant of *C. oleophila* colonized wounds on orange fruits and its population increased under field conditions.

3. Survival and population dynamics of *C. oleophila* and of genetically transformed strain of *C. oleophila* on orange fruit under field conditions

Detailed description and methodology related to this subject is discussed in the following publication:

A method for studying the population dynamics of *Candida oleophila* on oranges in the grove using a selective medium and PCR technique (Chand-Goyal et al., 1999, Microbiological Research, in press).

Populations of the biological control agent *C. oleophila* on fruit in an orange grove were monitored by plating fruit washes and peel homogenates on a selective medium, followed by PCR analysis of DNA from yeast colonies resembling *C. oleophila*. The *C. oleophila* transformant ADGus-10 formed colonies on selective medium that suppressed the development of 99% of the filamentous fungal and indigenous yeasts, *Aureobasidium* (a yeast-like fungus) and *Rhodotorula*. Only 6-8 indigenous yeasts/cm² fruit surface gave rise to colonies resembled *C. oleophila* on selective medium. The identity of the *C. oleophila* transformant colonies was verified by PCR analysis of yeast DNA. The presence of a 599-bp band in the PCR product primed by β -glucuronidase gene primers, confirmed the identity of *C. oleophila* transformant ADGus-10. Colony-forming units of *C. oleophila* were recovered efficiently from fortified washes of the fruit surface and from homogenates of wounds excised from fruit sprayed in the grove with *C. oleophila*.

The PCR technique for the amplification of a portion of the GUS gene, described here, combined with a selective medium that suppress most of the indigenous

microorganisms, provides an effective method for investigating the population dynamics of *C. oleophila* applied to oranges in groves for the biological control of fruit decay. Using the above described method, the population dynamics of *C. oleophila* survival on fruits can be studied under different field conditions on various cultivars of citrus.

4. Population dynamics of yeasts naturally colonizing wounded and unwounded surface of grapefruit in the grove and after harvest

Results from this work has not been published or submitted to publication yet. The results to be described are being prepared for publication. The following is a description of the methodology and the results:

a) Population study in the grove: Fruits from three different trees of grapefruits, in a nearby grove, were used. While on the tree, each fruit was superficially wounded at three locations using a sharp scalpel to make a uniform 1.5 cm in diameter wounds. Three fruits (from different trees) were picked at 0 time, 24, 48, 72, and 96 h after wounding and used to determine yeast and filamentous fungi population in wounded and intact fruit surface. Unwounded peel similar in its size to wounded area was cut from the same fruit.

Results presented in figures 1A and 1B showed that colonization of wounded and unwounded grapefruit surface by naturally occurring fungi did not vary through out the incubation period on the tree. On the other hand, colonization of intact and wounded fruit surface by naturally occurring yeasts was markedly different from that of unwounded surface. Yeasts colonized wounded surface rapidly and increased in numbers to about two orders of magnitude as compared to unwounded surface. On fruit treated with the yeast and kept on the tree, a different picture of fungal and yeast population had emerged. The detected fungal population on the yeast-treated intact surface was dramatically reduced and in treated wounds no fungi was detected. Yeast population on intact surface was relatively high immediately after the application of Aspire and decreased to more than 70% of that detected initially. In wounds, yeast population increased from 2.5×10^4 to about 4×10^6 after 72 hours of incubation at 20°C.

Fig. 1

Yeasts

Fungi

Yeasts

Fungi

b) Population study after harvest: This study was aimed at determining the colonization of wounded and non wounded grapefruit surface by the yeast *C. oleophila* after harvest and its effect on the natural epiphytic population of fungi and yeast. For that purpose, fruits were picked, wounded as described earlier (each fruit was wounded at three locations), sprayed until run-off with an aqueous cell suspension of the yeast at a final concentration of 10^8 cfu/ml and allowed to air dry and kept under moist conditions at 20°C. Untreated fruit was used as a control and kept under the same conditions. At different times after treatment (0 time, 24, 48, 72 and 96 h) population of yeasts and fungi was determined in wounded and non wounded fruit surface in yeast-treated and the control fruit (Figs. 2A & 2B).

5. Effect of preharvest application of yeast biocontrol agent on the development of postharvest decay on grapefruit

Results of tests conducted to evaluate the effect of preharvest application of Aspire™ on the development of postharvest decay indicated the validity of the approach. In this regard, we conducted two experiments in which the commercial yeast preparation Aspire™ (based on the yeast *C. oleophila*) was sprayed in the grove few hours (2–3 h) before harvest. In the first experiment, fruit was stored for 10 weeks at 11°C followed by 1 week of shelf life at 17°C and then assessed for the rate of decay. As shown in Table 1, the percentage of decay developed in fruit treated with the yeast preparation was 80% lower than that developed in the control (water-treated fruit). In the second experiment which was conducted early in the season, fruit was kept for 4 weeks at 17°C to allow development of decay. In control fruit (water treated) percentage of decay was only about 4% as compared to the yeast treatment in which 2% decay was observed (table 1).

Table 1: Effect of preharvest application of Aspire™ on the development of postharvest decay in grapefruit

Treatment	Exp. 1 Decay (%)	Exp. 2 Decay (%)
Water Control	10.4	4.2
Aspire™	3.8	2.0

Description of Cooperation

Close collaboration has characterized the work conducted throughout the research. The collaboration took the form of joint planning of the experiments, exchange of ideas, new information and materials as well as joint visits. The principal investigator (S. Droby) conducted two visits to the American laboratory, and in one of the visits he spent time to work together with the American team. These visits were used efficiently to jointly plan and direct the research in all its phases. In addition, all publications have been joint ones. The scientist of the collaborating groups directed their research towards the study of different objectives. The Israeli group focused on the development of PCR and other molecular biology techniques to be used to characterize the epiphytic yeast population on citrus fruit and studying population dynamics of epiphytic yeasts and filamentous fungi. The research conducted in the American laboratory was primarily aimed at developing a genetically marked strain of the yeast *C. oleophila*. For that purpose a transformation system was developed and used to insert a marker gene into the yeast genome. The marked strain was successfully used in survival and population dynamics study.

Evaluation of Research Achievements

The research conducted within this project deepened our knowledge and understanding of the ecology, genetics and population dynamics of epiphytic yeast population on citrus fruit. In addition, it has enabled the development of new research tools (RAPD and ap-PCR techniques) useful for studying survival and population dynamics of naturally and artificially introduced yeast used as biocontrol agents of postharvest diseases. It also have proven that our approach to apply yeast biocontrol agents in the grove, prior to harvest, for the control of postharvest diseases is valid and deserves some more attention in the future. A spin off of the results of this project is the development of a transformation system for the yeast *C. oleophila* which will be used in the future to

genetically improve biocontrol performance of the yeast by overexpressing of indigenous antifungal genes.

Conclusions

This research has achieved most of its objectives. The knowledge generated has deepened our understanding of various basic aspects on the ecology, genetics and population dynamics of epiphytic yeast population of citrus. No doubt, the research tools, and the information collected will enhance our ability to select better biocontrol agents and improve the implementation of the already commercialized yeast-based products used for the control of postharvest diseases of citrus fruit.

List of publications

1. publications in reviewed journals

Droby, S., Lischinsky, S., Cohen, L., Weiss, B., Chand-Goyal, T., Eckert, J.W. and Manulis, S. (1999). Characterization of an Epiphytic Yeast Population of Grapefruit Capable of Suppression of Green Mold Decay Caused by *Penicillium digitatum*. *Biological Control* (in press).

Chand-Goyal, T., Eckert, J.W., Droby, S., McCabe, L and Atkinson, K. (1998) A method for the population dynamics of *Candida oleophila* on oranges in the grove, using a selective isolation medium and PCR technique. *Microbiol. Res.* 153:1-0000.

Chand-Goyal, T., Eckert, J.W., Droby, S., Glickmann, E. and Atkinson, K. (1999). Integrative DNA transformation of an environmental yeast *Candida oleophila* and its effect on the biocontrol of green-mold of citrus. *Current Genetics* (in press)

Schena, L., Ippolito, A., Zahavi, T., Cohen, L., Nigro, F., and Droby, S. (1999). Genetic diversity and biocontrol activity of *Aureobazidium pullulans* isolates against postharvest rots. Postharvest Biology and Technology (submitted)

2. Other publications

Droby, S., Lischinsky, S., Cohen, L., Manulis, S., Mehra, R.K., and Eckert, J.W. (1995). Epiphytic yeasts of citrus fruit tolerant to extreme conditions are effective antagonists of green mold decay. *Phytopathology* 85: 1182 (abstr.).

Chand-Goyal, T., Eckert, J.W., Droby, S., and Atkinson, K. 1997. Development of an integrative DNA transformation system and studies on the expression of green fluorescent pigment (GFP) and beta-glucuronidase (GUS) genes in *Candida oleophila*. Phytopathology 87: S17 (abstract).