BARD

FINAL REPORT

PROJECT NO. IS-3271-01

**Ethanol for Postharvest Decay Control of Table
Grapes: Application and Mode of Action**

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Invited review papers				
Book chapters				
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Abstracts				
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Ozgur Karabulut (visiting scientist) Passport # TR-H 539471 TURKEY _____

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Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		3		3
Longer Visits (Sabbaticals)				

Description of Cooperation:

Cooperation was partly compromised due to limitations on US citizens to visit Israel, and problems in obtaining a visa to the US for the visiting scientist that was supposed to conduct the collaborative work. Nevertheless, 2 visits of the P.I. and one visit of the Israeli collaborating scientist, Dr. S. Lurie to the US, permitted better coordination of the research. One joint publication is in press and the P.I. and the Co-P.I. have agreed to publish a short review of the field covered by the research grant. _____

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				

Ethanol for postharvest decay control of table grapes: application and mode of action.

Original objectives: Dipping of table grapes in ethanol was determined to be an effective measure to control postharvest gray mold infection caused by *Botrytis cinerea*. Our objectives were to study the effects of ethanol on *B. cinerea* and table grapes and to conduct research that will facilitate the implementation of this treatment.

Background: *Botrytis cinerea* is known as the major pathogen of table grapes in cold storage. To date, the only commercial technology to control it relied on sulfur dioxide (SO₂) implemented by either fumigation of storage facilities or from slow release generator pads which are positioned directly over the fruits. This treatment is very effective but it has several drawbacks such as aftertaste, bleaching and hypersensitivity to humans which took it out of the GRAS list of compounds and warranted further seek for alternatives. Prior to this research ethanol was shown to control several pathogens in different commodities including table grapes and *B. cinerea*. Hence it seemed to be a simple and promising technology which could offer a true alternative for storage of table grapes. Further research was however required to answer some practical and theoretical questions which remained unanswered.

Major conclusions, solutions, achievements: In this research project we have shown convincingly that 30% ethanol is sufficient to prevent germination of *B. cinerea* and kill the spores. In a comparative study it was shown that *Alternaria alternata* is also rather sensitive but *Rhizopus stolonifer* and *Aspergillus niger* are less sensitive to ethanol. Consequently, ethanol protected the grapes from decay but did not have a significant effect on occurrence of mycotoxigenic *Aspergillus* species which are present on the surface of the berry. *B. cinerea* responded to ethanol or heat treatments by inducing sporulation and transient expression of the heat shock protein *HSP104*. Similar responses were not detected in grape berries. It was also shown that application of ethanol to berries did not induce subsequent resistance and actually the berries were slightly more susceptible to infection. The heat dose required to kill the spores was determined and it was proven that a combination of heat and ethanol allowed reduction of both the ethanol and heat dose. Ethanol and heat did not reduce the amount or appearance of the wax layers which are an essential component of the external protection of the berry. The ethanol and acetaldehyde content increased after treatment and during storage but the content was much lower than the natural ethanol content in other fruits. The efficacy of ethanol applied before harvest was similar to that of the biological control agent, *Metschnikowia fructicola*. Finally, the performance of ethanol could be improved synergistically by packaging the bunches in modified atmosphere films which prevent the accumulation of free water.

Implications, both scientific and agricultural: It was shown that the major mode of action of ethanol is mediated by its lethal effect on fungal inoculum. Because ethanol acts mainly on the cell membranes, it was possible to enhance its effect by lowering the concentration and elevating the temperature of the treatment. Another important development was the continuous protection of the treated bunches by modified atmosphere that can solve the problem of secondary or internal infection. From the practical standpoint, a variety of means were offered to enhance the effect of the treatment and to offer a viable alternative to SO₂ which could be instantly adopted by the industry with a special benefit to growers of organic grapes.

Achievements

Significance of main scientific achievements or innovations:

The main objective of the research was how to develop ethanol as a postharvest tool to control decay of table grapes. We first aimed at determining the exact dose of ethanol which will kill spores of *B. cinerea*. The lethal dose of ethanol was 30% and it was determined using 3 different techniques and on different strains (Lichter et al., 2003; Karabulut et al., 2004). Attempts to enhance sensitivity of the detection method using a strain that expressed the green fluorescent protein (GFP), proved of no advantage. We have also quantified the effect of temperature on survival of spores of *B. cinerea* (Lichter et al., 2003) whereas Gabler et al., (2004) compared the effect of ethanol and heat on survival of spores of *B. cinerea*, *A. alternate*, *R. stolonifer* and *A. niger*. The conclusion from this study was that the LD₅₀ values at 25°C were approximately 20, 25, 35 and 40% ethanol for the above mentioned fungi, and the LD₅₀ at 40°C were approximately half. These results demonstrate that there are significant differences between the fungi in their tolerance to ethanol and suggest that it is possible to reduce the effective concentration of ethanol by elevating the temperature of the treatment. One of the consequences of the reduced sensitivity to ethanol of *A. niger* and *R. stolonifer* is that ethanol can't be expected to be effective if grapes are to be stored at high temperature after harvest. This is because high temperature is conducive for development of *A. niger* and *R. stolonifer*. It should also be noted that ethanol was unable to reduce the inoculum density of *A. niger*, including the ochrotoxin A producer, *A. carbonarius*, during storage (Guzev et al., 2005). The effect of sub-lethal doses of ethanol and heat on *B. cinerea* was addressed by examination of the changes in the expression of the heat shock protein *HSP104* which is known in other systems to induce the tolerance to these stress agent. It was shown that the fungus transiently accumulated transcripts of this gene after heat and ethanol shock *in vitro* (Lichter et al., 2003). A similar response was not detected in mature 'Thompson Seedless' grapes and exposure of 'Emperor' grapes to ethanol and heat did not induce resistance to subsequent infection with *B. cinerea* (Gabler et al., 2005). These results suggest that ethanol acted primarily on the fungus rather than the berries. The natural content of ethanol and acetaldehyde in untreated grapes was relatively low and after treatment with ethanol and heat, the level of ethanol increased significantly but was still much lower than the natural ethanol content in oranges and comparable to the ethanol content of nectarines (Gabler et al., 2005). In addition, low

levels of ethanol and acetaldehyde could be detected in the head space of microperforated modified atmosphere packages, one week after treatment and cold storage, but thereafter their levels were below detection limit (Lichter et al., 2005). The levels of ethanol and acetaldehyde gradually accumulated in non-perforated films after 3 weeks of cold storage due to anaerobic respiration. These results suggest that the ethanol content within the berries treated by ethanol is not an issue which could prevent its implementation.

Agricultural and/or economic impacts of the research findings, if known.

During the progression of the research it became clear that ethanol could indeed reduce decay caused primarily by *B. cinerea* to a commercially acceptable level but in several experiments the level of control was below what is considered commercially acceptable. For example, it was reported that ethanol reduced natural decay from 34% to 10% in 'Superior' grapes stored for 40 d at 0°C (Lichter et al., 2005) or from more than 60 decayed berries/kg in 'Flame Seedless' to approximately 30 berries/kg (Karabulut et al., 2004). Assuming that ethanol killed all the external inoculum the decay after treatment could result from either development of latent internal infection during cold storage, or from secondary infection of the ethanol-treated grapes during storage. This variability in efficacy of ethanol did not ensure the level of confidence expected from a commercial application. Two major approaches were therefore tested to improve the efficacy of ethanol: 1. the combination of ethanol and heat (USA) or 2. modified atmosphere (Israel). The combination of heat and ethanol not only improved the efficacy of the treatment (Karabulut et al., 2004, Gabler et al., 2004, 2005) but also allows reducing the concentration of ethanol to below the threshold of 20% currently allowed by US authorities. This improved efficacy of the treatment still does not deal with the issue of secondary or latent infection. This problem was addressed by the complementary technology of MAP designed to avoid excess humidity (Lichter et al., 2005). The concept was that ethanol-treated grapes are protected from external decay in the first phase of storage and that latent infection is inhibited by the slow accumulation of CO₂ within the film. In this method the bunches are also protected during storage from secondary infection. The development of the complementary methods for the use of ethanol for grapes permits the industry to use any desired component of the treatment according to their specific demands. These tools are of primary value to organically grown grapes which can't use the SO₂ technologies.

However, recent developments in the European market may accelerate the rate of adoption of the ethanol technology by conventional growers.

Details of cooperation:

The research was conducted synergistically by taking two different approaches (heat and MAP) to enhance the effect of ethanol. The two visits of the IS-PI to the USA helped in coordinating the research. Unfortunately, due to the compromised safety in Israel during the program, and the difficulties in getting a VISA to the USA for Dr Karabulut, prevented implementation of better collaboration.

List of Publications:

- (Gabler, F. M., M. F. Mansour, J. L. Smilanick, and B. E. Mackey. 2004. Survival of spores of *Rhizopus stolonifer*, *Aspergillus niger*, *Botrytis cinerea* and *Alternaria alternata* after exposure to ethanol solutions at various temperatures. *Journal of Applied Microbiology* 96 (6):1354-1360.
- Gabler, F. M., J. L. Smilanick, J. M. Ghosop, and D. A. Margosan. 2005. Impact of postharvest hot water or ethanol treatment of table grapes on gray mold incidence, quality, and ethanol content. *Plant Disease* 89 (3):309-316.
- Guzev, L, A Danshin, T Zahavi, A Ovadia, and A Lichter. 2005. The effects of cold storage, sulfur dioxide and ethanol on species of black *Aspergillus* producing ochratoxin A. *International Journal of Food Microbiology* In Press.
- Karabulut, O. A., J. L. Smilanick, F. M. Gabler, M. Mansour, and S. Droby. 2003. Near-harvest applications of *Metschnikowia fructicola*, ethanol, and sodium bicarbonate to control postharvest diseases of grape in central California. *Plant Disease* 87 (11):1384-1389.
- Karabulut, O. A., F. M. Gabler, M. Mansour, and J. L. Smilanick. 2004. Postharvest ethanol and hot water treatments of table grapes to control gray mold. *Postharvest Biology and Technology* 34 (2):169-177.
- Karabulut, O. A., G Romanazzi, A Lichter, and J L Smilanick. 2005 .Postharvest ethanol and potassium sorbate treatments of table grapes to control gray mold .*Postharvest Biology and Technology* In Press.
- Lichter, A., H. W. Zhou, M. Vaknin, O. Dvir, Y. Zutchi, T. Kaplunov, and S. Lurie. 2003. Survival and responses of *Botrytis cinerea* after exposure to ethanol and heat. *Journal of Phytopathology* 151 (10):553-563.
- Lichter, A., Y. Zutahy, T. Kaplunov, N. Aharoni, and S. Lurie. 2005. The effect of ethanol dip and modified atmosphere on prevention of *Botrytis* rot of table grapes. *Hortechonology* 15 (2):284-291.

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1. The effect of ethanol on survival of *E. coli*
2. The effect of organic acids added to ethanol on spore germination
3. The effect of evaporated ethanol
4. The effect of heat treatment on spore tolerance to ethanol
5. The effect of ethanol and SO₂ on long storage of Red globe
6. Preference consumer survey for ethanol-treated grapes.

Unpublished data

1. Control of bacterial contamination by ethanol (Israel)

Bacterial contamination of agricultural produce is a matter for concern in the USA and it was questioned to what extent the ethanol technology can address this problem. Since natural infection is sporadic, 'Crimson' grapes were inoculated with *E. coli* strain ATCC 25922 followed by treatment with 20, 30, 50 and 70% of ethanol and for 30, 60 and 180 s.

Results indicate reduction of bacterial population by 2 to 3 from values around 10^4 cfu/g_{FW} and in some cases, complete elimination of all inoculated bacteria was observed. A 3 min dip was more effective than 1 min dip. This time frame is relevant to the dipping procedure of table grapes which lasts only 10 s because the drying time is much longer. Wounding the grapes caused a larger fraction of the bacteria to be protected from the treatment. In conclusion, it was found that ethanol can significantly reduce the amount of bacteria on the surface of the berry. Because bacterial pathological incidents are dependent on the amount of the ingested bacteria, this procedure can reduce or eliminate the risk of grapes being the source of human infection. A research note describing these results is in final preparation and is expected to be released for publication in the near future.

2. The effect of organic acids added to ethanol on *Botrytis* germination and grape decay (Israel)

Fumigation of grapes with acetic acid was shown to inhibit decay during storage (Scholberg et al., 1996). We tested the effect of acetic, propionic, sorbic and decanoic acid with and without ethanol at different sub-lethal concentrations. Results show that acetic acid and propionic acid up to 50 mM did not reduce spore germination in the presence of ethanol whereas decanoic acid was effective in combination with 25% ethanol. Sorbic acid at 1 or 10 mM reduced spore germination with a synergistic effect with ethanol. In one of the experiments conducted on 'Superior' grapes, 25% ethanol dip resulted in 30 decayed berries/kg whereas 25% ethanol and sorbic acid reduced decay to 9 berries/kg. These results offer another mean to enhance the effect of ethanol and they also served as the basis for the study of Karabulut et al., (2005).

3. The effect of evaporated ethanol (Israel)

A study by Chervin et al., (2005) indicated that ethanol applied onto paper sheets and packaged with 'Chasselas' grapes reduced decay significantly. We have tested this concept in combination with CO₂ in Xtend films or low level of SO₂ in polyethylene films. Our results confirm and elaborate this technique: evaporating ethanol can prevent decay but an over-dose causes browning of rachis. We also studied two different means of application of ethanol with different release kinetics. It is demonstrated that ethanol in the atmosphere of the grapes is converted to acetaldehyde and that ethanol induces and SO₂ inhibits the activity of Alcohol dehydrogenase and pyruvate decarboxylase. A manuscript describing this data is being prepared. However, further studies are required to bring this technology into applicability.

4. The effect of heat treatment on spore tolerance to ethanol (Israel)

Previous results demonstrated that either heat or ethanol increased the expression of Hsp104, a *B. cinerea* heat shock protein which was assumed to be involved in tolerance of yeasts to heat and ethanol. Heat shock was applied to mycelia developing on a Petri dish and spores collected from this plate were challenged with sub-lethal dose of heat or ethanol. Spore development was followed by spectral analysis of developing mycelia in tissue culture plates. Results with an Israeli isolate of *B. cinerea* were inconsistent. Further studies using monoascosporic strains of opposite mating types revealed that one of the parents was indifferent to a prior the heat shock whereas the other parent behaved as if heat shock induced tolerance to heat and ethanol. Isolates from a cross between the parents are further analyzed for segregation of the trait.

5. The effect of ethanol and SO₂ on long storage of 'Red Globe' grapes (Israel).

Prolonged storage of table grapes requires grapes of the highest quality and the best means of storage. Treatments such as ethanol can't supply a solution for storage duration beyond one to two month. It was shown that a pretreatment of 'Red Globe' grapes with half the dose of SO₂ combined with ethanol treatment after harvest gave 92% healthy bunches after 6 month of cold storage and 3 d at 20°C compared to 8% with SO₂ alone.

6. Preference consumer survey for ethanol-treated grapes (Israel).

A survey including 130 individuals was conducted to determine consumer preference for ethanol-treated grapes. Organoleptic and visual assessment of SO₂, ethanol and control grapes demonstrated no negative impact of ethanol compared to SO₂-treated grapes. Control grapes were negatively scored because of slight deterioration during 10 days at storage. These results demonstrate that taste and appearance are non-limiting factors for the ethanol-treated grapes.

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Results indicate reduction of bacterial population by 2 to 3 orders under specific conditions. For example, in one experiment dipping the bunch in 20% ethanol resulted in 5×10^3 cfu compared to 5.5×10^1 cfu in 30% ethanol and 0 cfu at 50% ethanol. This however, was not always the case because microscopic wounds caused internalization of bacteria into the flesh of the berry.

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